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DOCTORAL THESIS

Virgin olive oil: Potential of different omics approaches to authenticate its geographical and botanical origin

Presented by

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Aceite de oliva virgen: Potencial de diferentes aproximaciones ómicas para la autentificación de su origen geográfico y varietal

Memoria presentada por Aadil Bajoub
para optar al grado de Doctor Internacional en Química.

Granada, febrero de 2016

Aadil Bajoub

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El trabajo que se presenta en esta tesis doctoral, con el título **"Aceite de oliva virgen: Potencial de diferentes aproximaciones ómicas para la autentificación de su origen geográfico y varietal",** ha sido realizado bajo mi dirección y la de la Prof. Dra. Dña. Alegría Carrasco Pancorbo en los laboratorios que el grupo FQM-297 tiene en el Departamento de Química Analítica y, parcialmente, en el Centro de Proteómica y Metabolómica del Centro Médico de la Universidad de Leiden (LUMC) en Holanda, y reúne todos los requisitos legales, académicos y científicos para hacer que el doctoranda D. Aadil Bajoub pueda optar al grado de Doctor Internacional en Química.

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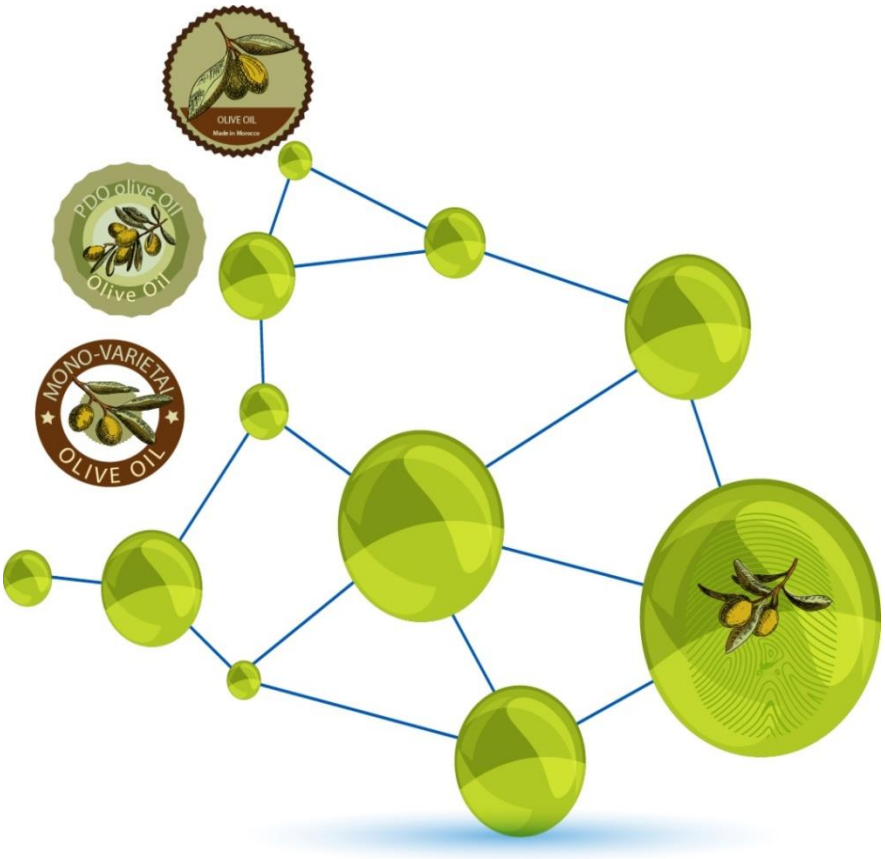

OBJECTIVES

Virgin olive oil is the main source of fat in the Mediterranean countries with origin that dates back to ancient times. Over the years, this foodstuff has acquired an extraordinary reputation as valuable nutritional and health-benefit product. Nowadays, scientific evidences associate moderate daily intake of virgin olive oil with increased life expectancy and a reduced risk of chronic and degenerative diseases, such as cardiovascular, atherosclerosis, cancer and diabetes. These protective effects have been attributed to the particular chemical composition of this product, which is especially rich on monounsaturated fatty acids and bioactive compounds, such as pigments, phenolic compounds, phytosterols and tocopherols. Capitalizing on the accumulated results regarding olive oil health-benefits properties, the use of health claims for this product is officially authorized since 2006 by the United States Food and Drug Administration and, since 2011, by the European Food Safety Authority. Consequently, virgin olive oil consumption is currently spreading all over the world, even in countries where it was not traditionally consumed, such as United States, Canada, Australia, Brazil, Japan and China, and indeed, the olive oil market has become increasingly globalized over the last decades.

The referred to internationalization of olive oil sector contributes to create market opportunities outside the Mediterranean region, where olive oil consumption level is relatively stable; it also entails important challenges related to strong competitiveness. Differentiation based on high quality standards, geographical and/or varietal origin claims is one of the most powerful competitive strategies that olive oil producers are currently adopting to face these dares. Thus, over the last years, there has been an increasing in the production of certified monovarietal and geographical origin-labeled olive oils in numerous olive growing regions. However, such products, which often are more expensive and show higher commercial value than the non-labeled ones, are targets for adulteration. That is reason explaining why, at the moment, there is a great need for accurate and promising analytical approaches able to restrain the authentication of the geographical and botanical origin of virgin olive oil. Within this context, the research in

this field is moving from classical methodologies to advanced analytical strategies in which omics approaches, especially metabolomics (chromatography-mass spectrometry based methodologies combined to chemometrics) plays a pivotal role.

Therefore, the main goal of the research conducted for the present Doctoral Thesis has been to evaluate the potential of different omics approaches to predict and authenticate the geographical and botanical origin of virgin olive oils, with special emphasis on oils samples coming from several Moroccan olive growing regions and different Moroccan and Mediterranean varieties cultivated in this country.

In order to achieve this main goal, the following specific objectives were contemplated:

- ✓ Making a careful experimental design and planning a highly standardized collection of representative olive oil samples, taking into account the main source of variability (intra-regional and intra-varietal variability) in such a way that obtained results will be conclusive and consistent with the dissertation purposes;
- ✓ Determination of quality and authenticity indices of Moroccan monovarietal virgin olive oil;
- ✓ Optimization and validation of different analytical methodologies based on the coupling of liquid or gas chromatography to mass spectrometry (or even the use of MS without previous chromatographic separation) to characterize the olive oil phenolic and volatile fractions;
- ✓ Applying the optimized methodologies to perform targeted, profiling and fingerprinting metabolomics studies on olive oil samples;
- ✓ To study the influence of crop season, geographical origin and cultivar on the determined quality parameters and identified compositional profiles;
- ✓ Developing robust and effective geographical and botanical authentication models by applying different chemometric techniques and packages to the obtained metabolomics data;
- ✓ Confirming the high potential of the developed chemometric models to solve geographical and botanical origin issues of olive oil-related by cross-validation and external validation;
- ✓ Building databases of quality, nutritional value and compositional parameters of Moroccan olive oil, whose main features had not been previously described;

- ✓ Proposing a methodology to assess the suitability of a specific region for setting up a geographical indication label, taking advantage of the different metabolomics approaches tested within the frame of this thesis.

OBJETIVOS

El aceite de oliva virgen, cuyo origen está datado en tiempos ancestrales, es la grasa que más se consume en los países de la Cuenca del Mediterráneo. A lo largo de los años, este alimento ha ido adquiriendo una extraordinaria reputación por sus características nutricionales y sus efectos beneficiosos para la salud. Hoy en día, existen evidencias científicas contrastadas que asocian el consumo de aceite de oliva virgen con un aumento de la esperanza de vida y una reducción del riesgo de padecimiento de enfermedades degenerativas, como afecciones cardiovasculares, arteriosclerosis, cancer y diabetes. Dichos efectos pueden ser atribuidos a la composición química de este producto, que es particularmente rica en ácidos grasos monosaturados y compuestos bioactivos, como tocoferoles, compuestos fenólicos, fitoesteroles, y pigmentos. Haciendo hincapié en las propiedades saludables que esta matrix exhibe, merece la pena mencionar que en 2006 se autorizaró el uso de declaraciones nutricionales y de propiedades saludables (*Health Claims*) por parte de la Administración de Medicamentos y Alimentos de los Estados Unidos (*FDA*) y, algo después, en 2011, también por parte de la Autoridad Europea de Seguridad Alimentaria (*EFSA*). Como consecuencia de todo lo expuesto, el consumo del aceite de oliva virgen se está extendiendo por todo el mundo, llegando incluso a países donde nunca se ha consumido de manera tradicional (Estados Unidos, Canadá, Australia, Brasil, Japón, China, etc.), lo que, lógicamente, provoca que el mercado sea cada vez más globalizado.

Esta internacionalización del sector posibilita que se creen interesantes oportunidades de mercado más allá de las fronteras de la Cuenca del Mediterráneo, generando ciertos desafíos en términos de competitividad. La diferenciación del producto basada en altos estándares de calidad y etiquetas que hagan alusión al origen geográfico o varietal del aceite, son algunas de las estrategias que los productores emplean para asegurar el éxito de su producto final. Estos productos "*premium*" son generalmente más caros, lo que los convierte en potencial objetivo de practicas fraudulentas. Por ello, es necesario disponer de potentes herramientas analíticas que sean capaces de autentificar el origen geográfico y botánico del aceite de oliva virgen. En este contexto, la evolución de las metodologías analíticas de uso en el ámbito oleícola es clara, desembocando en la última década, en el

empleo de estrategias ómicas, donde las técnicas cromatográficas acopladas a espectrometría de masas tienen un papel absolutamente protagonista (en particular, en el área de la metabolómica).

Considerando todo lo planteado, el objetivo principal de la presente tesis doctoral ha sido la evaluación del potencial de diferentes aproximaciones ómicas para predecir y autentificar el origen geográfico y botánico de aceites de oliva vírgenes. Se puso especial énfasis en el análisis de muestras de aceite provenientes de diferentes regiones productoras de Marruecos y en el de aceites de variedades de olivo mediterráneas que se cultivan en dicho país.

Para asegurar la consecución del objetivo global apenas formulado, era necesario contemplar algunos objetivos partiales:

- ✓ Llevar a cabo un diseño experimental y planificación global lo más adecuados possible, con una rigurosa selección de muestras representativas que considerase las principales fuentes de variabilidad (tanto intra-regional como intra-varietal), de modo que se pudiera asegurar la consistencia de los resultados y el alcance de conclusiones fiables que den respuesta a las hipótesis planteadas en esta tesis;
- ✓ Determinación de los índices de calidad y autenticidad del aceite de oliva marroquí;
- ✓ Optimización y validación de diferentes metodologías analíticas basadas en el acoplamiento de cromatografía líquida o de gases a espectrometría de masas (o incluso basadas en el uso de MS sin separación previa) para caracterizar los compuestos fenólicos y la fracción volátil de muestras de aceite de oliva virgen;
- ✓ Empleo de las metodologías desarrolladas en estudios metabolómicos (aproximaciones targeted, profiling o fingerprinting) sobre muestras de aceite de oliva;
- ✓ Estudio de la influencia de la campaña, origen geográfico y variedad del olivo en determinados parámetros de calidad y perfiles composicionales;
- ✓ Desarrollo de modelos robustos y fiables para autentificar el origen geográfico y botánico de los aceites analizados, basándonos en el empleo de técnicas quimiométricas.

- ✓ Evaluación (y confirmación) del poder predictivo de los modelos estadísticos desarrollados mediante validación-cruzada (cross-validation) y validación externa (external validation);
- ✓ Construcción de bases de datos que incluyan información de calidad, valor nutricional y parámetros composicionales del aceite de oliva marroquí, cuyas principales características no han sido previamente descritas;
- ✓ Proponer una metodología que, haciendo uso de algunas de las aproximaciones metabolómicas recogidas en esta tesis, sea capaz de asegurar la idoneidad de una región específica para establecer un sistema de calidad geográfica diferenciada y certificada.

SUMMARY/RESUMEN/RESUME

SUMMARY

Olive oil is a highly appreciated edible oil, considered as a very relevant component of the Mediterranean diet. Its potential health benefits have been widely described and its economic importance (in particular, in some areas of the Mediterranean Basin) is undeniable. The spread of this foodstuff all over the world, favored by the very good reputation of the Mediterranean diet, is making, to a certain extent, that new markets and consumers are getting used to this "Mediterranean's golden treasure", trade is intensifying, and international competitiveness is becoming fiercer than ever. Within the globalized olive oil market, the differentiation of diverse olive oils considering their geographical and botanical origins represents an attainable competitive strategy.

The results achieved within the frame of the doctoral thesis entitled "Virgin olive oil: Potential of different omics approaches to authenticate its geographical and botanical origin" are outlined herein.

The dissertation is divided into two main parts, namely: **Introduction: state-of-the art of the thesis topics (PART 1)** which represents a general overview of the subject after a deep study of the most relevant literature, and the **Experimental Part, Results and Discussion (PART 2)** which contains the experimental work carried out within this PhD, the achieved results, outstanding conclusions and future perspectives.

Therefore, **PART 1** starts by giving a comprehensive overview about the matrix under study, including a brief discussion about olive tree origin, diffusion, botany and morphological characteristics, the main structural features and compositional characteristics of olive fruit, as well as the different steps of olive oil processing and conservation. Afterwards, the concept of olive oil quality was introduced with especial emphasis on regulatory commercial quality, nutritional value and olive oil quality linked to geographical and/or varietal provenance. This part also deals with aspects regarding olive oil genuineness and authenticity, even succinctly. Then, the current status and major trends of international olive oil market were detailed, standing out the changes experienced during the last decades and the challenges that the market is currently facing. In the same subsection of the introductory part, the main undertaken strategies to

overcome these challenges or concerns were outlined, focusing on differentiating marketing strategies, in particular organic olive oil labels, monovarietal certification and geographical labeling schemes. After this, attention was paid to Moroccan olive oil sector, placing more emphasis on the strategic position adopted in this country in the current agricultural development policy, and the main limitations to its growth and progress. This discussion contributed to identify a knowledge gap, demonstrating the need of taking advantage of the modern and powerful omics approaches to characterize Moroccan olive oils and authenticate their geographical and botanical origins. This fact, from our point of view, supports the development of this thesis. The fourth subsection of the introduction is about omics approaches applied to olive oil characterization, properly addressing the different stages of a typical workflow and having a glance at the main applications of metabolomics.

The **PART 2** encompasses three principal sections:

- Section 1 entitled "***Authentication indices and geographical classification of North Moroccan virgin olive oils***" consists of two chapters. The first one (Chapter 1) is a review article in which a critical overview and comparative analysis of the main regulatory frameworks and standards currently applied to olive oil quality and authentication assessment was presented. After describing the regulated quality and purity criteria, special attention was paid to differences and similarities between the reviewed regulatory frameworks concerning the thresholds and the analytical methods employed for monitoring these parameters. Drawbacks and limitations of the official methods (used to evaluate olive oil quality and purity) were also addressed. Finally, the latest advances in analytical methods and chemometrics applied to olive oil authentication and geographical and botanical origin traceability were discussed. The second chapter of this section (Chapter 2) represents a first attempt to build a quality and compositional database of 'Picholine Marocaine' monovarietal olive oil produced in the main North Moroccan olive growing regions. Thus, over two consecutive crop seasons (2011 and 2012), 279 olive samples of 'Picholine Marocaine' cultivar were collected in 7 regions, and oils were extracted and analyzed considering all international olive council (IOC) regulated quality and purity criteria. The main findings of this work were the determination of variability ranges of quality and composition of the studied samples and the geo-location of olive growing areas which produced oils which showed a linolenic acid content higher than 1%

(which is the maximum value fixed by the IOC regulation). Furthermore, after an exploratory multivariate data analysis using principal component analysis (PCA), a geographical origin tracing model -combining the obtained data and stepwise linear discriminant analysis (s-LDA)- was built; good rates of correct classification and prediction were achieved and several geographical markers were identified.

- Section 2 entitled "***Metabolomics approaches for geographical and botanical origin authentication***" deals with the optimization, validation and application of different metabolomics approaches to a wide range of carefully selected olive oil samples with the aim of differentiating them on the basis of their geographical and botanical origin. This section includes 8 chapters. In the first one (Chapter 3), a liquid chromatography-mass spectrometry (LC-MS) based methodology was optimized and applied to exhaustively characterize the phenolic fraction of 156 olive oil samples from 'Picholine Marocaine' cultivar, grown in 7 Moroccan regions. 25 phenolic compounds belonging to different chemical families (flavonoids, lignans, phenolic acids, phenolic alcohols and secoiridoids) were identified and quantified. The effect of the geographic origin on the phenolic composition of the studied samples was highlighted and a general overview of data structure was given by applying PCA analysis. Then, by means of s-LDA, the potential of phenolic compounds to trace the geographical origin of studied oils was ascertained. Immediately after, Chapter 4 investigates the volatile fraction of North Moroccan monovarietal 'Picholine Marocaine' virgin olive oil, trying to evaluate its suitability for assessing the geographical origin of the selected samples. To this end, an analytical approach combining the data obtained by headspace solid-phase microextraction coupled to gas chromatography with flame ionization and MS detectors (HS-SPME/GC-FID-MS) with a chemometric tool (s-LDA) was used. A total of 40 volatile compounds, belonging to different chemical classes, were identified and quantified and the used strategy was remarkably efficient for the classification of the examined oils according to their geographical origin. In Chapter 5, two metabolic profiling approaches (LC-MS and GC-MS) were compared taking into account their potential to characterize the phenolic fraction and trace the botanical origin of virgin olive oils coming from five different cultivars. PCA and partial least squares-discriminant analysis (PLS-DA) were applied to build botanical origin authentication models and identify potential varietal markers. In Chapter 6, we compared the geographical discrimination power of several chemometric

methods (s-LDA, PCA, PLS-DA and soft independent modeling of class analogies (SIMCA)) applied to triacylglycerols (TAGs) fraction of virgin olive oil (determined by LC equipped with a refractive index detector). Satisfactory results were obtained both in terms of classification and prediction power, being s-LDA and PLS-DA those giving the best results. The main objective of research included in Chapter 7 was the comprehensive characterization of the phenolic fraction of 4 autochthonous Moroccan cultivars ('Picholine Marocaine', 'Dahbia', 'Haouzia' and 'Menara'), and 9 Mediterranean varieties recently introduced in Morocco ('Arbequina', 'Arbosana', 'Cornicabra', 'Frantoio', 'Hojiblanca', 'Koroneiki', 'Manzanilla', 'Picholine de Languedoc' and 'Picual'). The LC-MS analytical methodology developed in chapter 3 was used in this case. Once the phenolic fraction of 203 olive oil samples coming from the above-mentioned cultivars was examined, the effects of crop season and variety on the phenolic composition were explored and, afterwards, the data were subjected to PCA and s-LDA. Three botanical authentication models were established within this study: the first one was able to discriminate between 'Picholine Marocaine' olive oils and oils from the other 9 Mediterranean varieties investigated; the second one differentiated between autochthonous Moroccan cultivars; and the third model was constructed to discriminate between 'Dahbia', 'Haouzia' and 'Menara' oils and the 9 Mediterranean varieties. The subsequent chapter (Chapter 8) summarizes the results achieved by applying data fusion and chemometrics to the phenolic fingerprints revealed by LC with diode array (DAD) and fluorescence (FLD) detection. Fingerprints of 140 virgin olive oil samples processed from olive fruits of seven varieties, collected over three consecutive crop seasons (2011-2014), were recorded and PCA, PLS-DA, SIMCA and k-Nearest Neighbors (k-NN) were firstly applied to DAD and FLD chromatographic fingerprint data sets separately, and, secondly, to the data set resulting from the data fusion of both matrices. The obtained classification models were very sensitive and selective, showing considerably good recognition and prediction abilities. In Chapter 9, selected ion flow tube-mass spectrometry (SIFT-MS) in conjunction with chemometrics was evaluated as analytical strategy for the prediction of the origin of 130 Mediterranean geographical indications labeled olive oils, coming from Greece, Italy, Morocco and Spain. The headspace volatile fingerprints of precursors (H_3O^+, NO^+ and O_2^+) and product ions were collected (full scan mode on the range m/z 10-200 over 60 s) and, then, used to build different classification

models for geographical discrimination. Effective classification models were set regardless of the considered precursor ions. PCA was used for preliminary multivariate data analysis and PLS-DA was applied to build (for each data set) three different models (considering the three reagent ions) to classify samples according to the country of origin and/or regions (within the same country). The samples were satisfactorily classified bearing in mind their geographical origin, and it was even possible to distinguish those coming from the same country but from different regions.

The last chapter of this section (Chapter 10) deals with the development and validation of a new direct injection-based LC-MS method for the simple, sensitive, accurate, and rapid characterization of virgin olive oil phenolic compounds. Optimization of the most appropriate solvent for sample dilution, selection of the optimum oil/solvent ratio, and establishment of column cleaning strategy and maximum number of injections allowed (maintaining satisfactory analytical performance) were some of the most relevant steps to go through. After choosing the optimum conditions, the analytical parameters of the method were evaluated, considering detection and quantification limits, precision and trueness and checking possible matrix effect. The method was then applied to analyze the phenolic fraction in oils from 6 different botanical origins and PCA was applied in an attempt to distinguish the studied samples according to their varietal origin.

- Section 3 entitled "***A proposal for a methodological approach to the implementation of virgin olive oil geographical indication labels: An application to Meknès Moroccan region***" presents a strategy to evaluate the suitability of an olive growing area to acquire a geographical indication label for its virgin olive oil production. This methodology was tested on Meknès region (Northern-center Morocco) by analyzing the typicality of the virgin olive oil produced in this region, over various consecutive crop seasons. Within this section, the first contribution (Chapter 11) is a book chapter which tries to clarify the terminology used in the field of geographical indication labels and describes the main activities to be undertaken to evaluate the suitability of a given olive growing area to acquire a geographical indication label for its virgin olive oil production while ensuring optimal and efficient use of this label. The main findings of this study highlighted the need for the adequate definition of the territorial dimension of the studied region and the characterization of the typicality of its olive oil trough the determination of a set of properties of belonging (defining qualitative and compositional profiles) and distinction

(properties that make possible to differentiate, identify, and recognize the olive oil produced in the studied region when compared with others). The stress was placed on Meknès region, and the most relevant results obtained applying the described methodology were summarized. The second chapter (Chapter 12) constitutes a contribution to define the properties of belonging of Meknès virgin olive oil through the determination of its physico-chemical and sensorial quality compositional profiles. In total, over four consecutive crop seasons (from 2010 to 2013), 298 monovarietal 'Picholine Marocaine' olive oil samples, collected from selected olive oil mills within the Meknès region, were analyzed. Physicochemical quality indices, sensory attributes, total phenols, pigment and tocopherol contents and fatty acid composition were determined. The intra-regional and intra-annual variability were examined and the results showed that crop season exerted significant effect on the characteristics of the virgin olive oils produced in this region; however, these oils showed high physicochemical and sensory overall quality, as well as a homogeneous composition over the four considered years. Moreover, with the aim of defining the compositional profile of oils produced in Meknès region, and keeping in mind the importance of the phenolic compounds on the nutritional value and sensory quality of this matrix, a 3-years evaluation of the quali-quantitative composition of Meknès olive oil (in term of these compounds) was carried out. The main findings of this study are reported in Chapter 13, where the phenolic profile of 142 olive oil samples, collected over three consecutive crop seasons, was established. The crop season effect and the intra-regional variability were investigated. This study allowed us to define the phenolic profile of Meknès virgin olive oils. At the end of this section, Chapter 14 is found. It represents an investigation to define the distinction properties of Meknès olive oil, in comparison with other Moroccan labeled olive oils. The potential of phenolic profiles as discriminant features among commercial extra virgin olive oils from Meknès region and two other Moroccan origin-labeled regions was evaluated after applying a LC-MS analytical methodology and the subsequent chemometric treatment (to the obtained quantitative data) using s-LDA. The main distinctive features were stood out; high rate of both classification and prediction abilities were achieved by the created statistical models, and, finally, potential geographical markers were identified.

RESUMEN

El aceite de oliva es una grasa comestible muy apreciada y uno de los elementos más importantes de la dieta Mediterránea. Las ventajas saludables que se le atribuyen y su relevancia económica (particularmente, en ciertos países de la Cuenca Mediterránea) son innegables. Estos factores, unidos a la buena reputación de nuestra dieta, han provocado que nuevos mercados y los, hasta hace poco, inexpertos consumidores se vayan habituando a este "oro líquido", por lo que su comercio a nivel internacional se ha visto muy intensificado y la competencia en este campo, evidentemente, también. En la era global en la que nos hallamos, la diferenciación de productos (en este caso, por ejemplo, atendiendo al origen geográfico y botánico del aceite) es una de las estrategias comerciales que mayor garantía de éxito ofrece.

Los resultados que se han alcanzado en el marco de la tesis doctoral titulada: "Aceite de oliva virgen: potencial de diferentes aproximaciones ómicas para la autentificación de su origen geográfico y botánico" se intentan plasmar en esta sección de manera resumida.

La tesis se ha estructurado en dos partes principales: **Introducción: Estado el arte de las temáticas tratadas en esta tesis (PARTE 1),** que trata de ofrecer una visión general (obtenida tras un profundo estudio bibliográfico) y contextualizar el trabajo presentado; y la sección **Parte Experimental, Resultados y Discusión (PARTE 2)** que recoge todo el trabajo experimental llevado a cabo, los resultados más relevantes, conclusiones de mayor interés y perspectivas futuras.

Así, la **PARTE 1** comienza con una visión de conjunto acerca de la matriz objeto de estudio, los orígenes del olivo, su expansión hacia otros territorios, características morfológicas y botánicas, peculiaridades estructurales y composicionales de la aceituna, así como las diferentes etapas del proceso de elaboración del aceite de oliva y su conservación. Tras ello, se introduce el concepto de calidad de este alimento, haciendo especial hincapié en la calidad comercial, el valor nutricional y la calidad del aceite ligada a su proveniencia geográfica y/o varietal. Otra sección dentro de esta primera parte recoge algunas ideas, de manera bastante sucinta, acerca de la genuinidad y autenticidad del aceite de oliva. El estado actual del mercado internacional y las tendencias existentes se

recogen a continuación, resaltando, en cierta medida, los cambios sufridos en las últimas décadas y los retos actuales que debe afrontar. También se comentan las estrategias creadas para hacer frente a los mencionados desafíos, centrándonos en las estrategias de mercado, en particular, en el aceite ecológico, la certificación monovarietal y los sistemas de etiquetado de calidad geográfica. Poco a poco, la parte introductoria se va centrando en el contexto del sector oleícola en Marruecos, describiendo la posición estratégica que este país ha adoptado y sus mayores limitaciones para el crecimiento y evolución del sector. Esta discusión contribuye a evidenciar ciertas grietas estructurales o falta de conocimiento en algunos aspectos, demostrando la necesidad del empleo de modernas herramientas (aproximaciones ómicas) que ayuden a caracterizar el aceite marroquí y autentificar su origen geográfico/botánico. Además, desde nuestro punto de vista, también respalda el interés de la presente tesis doctoral. La cuarta subsección, dentro de la introducción, versa sobre aproximaciones ómicas aplicadas a la caracterización del aceite de oliva, intentando reflejar cuáles son las etapas del flujo de trabajo típico (y aspectos importantes de cada una de ellas) y recoger algunos ejemplos relevantes del ámbito de la metabolómica.

La **PARTE 2** se divide en tres secciones principales:

-La Sección 1 se titula **"*Índices de autenticidad y clasificación geográfica de los aceites de oliva del Norte de Marruecos*"** y consta de 2 capítulos. El primero de ellos (Capítulo 1) es un artículo de revisión en el que se plasma una visión global crítica y comparativa de los distintos marcos legislativos reguladores que velan por el aseguramiento de la calidad y la autentificación del aceite de oliva hoy en día. Tras describir los criterios de pureza y calidad regulados, se presta especial atención a las similitudes y diferencias existentes entre los distintos marcos legislativos en lo referente a los valores mínimos y máximos establecidos y los métodos de análisis empleados en la determinación de dichos parámetros. Las limitaciones de algunos de los métodos oficiales (para evaluar los parámetros de calidad y pureza) se mencionan, de igual modo, en este capítulo. Finalmente, se recogen los avances más novedosos en relación al empleo de herramientas analíticas y el uso de la quimiometría aplicados en el campo de la autentificación y trazabilidad del origen geográfico y varietal. El segundo capítulo de esta sección (Capítulo 2) representa el primer intento de construir una base de datos de calidad y composicional del aceite de oliva de la variedad 'Picholine Marocaine', que se produce en

las principales zonas oleícolas de Marruecos. Para ello, durante dos campañas consecutivas (2011 y 2012), se recogieron 279 muestras de aceituna de 7 regiones diferentes, y los aceites obtenidos se analizaron considerando todos los parámetros contemplados por el Comité Oleícola Internacional (COI) en relación a la calidad y pureza de esta matriz. De este modo, se pudieron determinar los rangos de variabilidad de la calidad y composición de las muestras estudiadas, así como explorar la geo-localización de áreas de cultivo del olivo cuyos aceites mostraban contenidos de ácido linolénico superiores al 1% (que es el límite establecido por el COI). Además, tras un primer estudio exploratorio de los datos mediante análisis de componentes principales (PCA), se construyó un modelo (aplicando *stepwise linear discriminant analysis (s-LDA)*) para establecer el origen geográfico, que mostró buenos índices de acierto en clasificación y predicción e identificó algunos potenciales marcadores geográficos.

-La Sección 2, por su parte, ha sido titulada "***Aproximaciones metabolómicas para autentificar el origen geográfico y botánico***" y se ocupa de la optimización, validación y aplicación de distintas aproximaciones metabolómicas para el análisis de un buen número de muestras de aceite de oliva cuidadosamente seleccionadas, con el objetivo de intentar distinguirlas atendiendo a su origen geográfico o a su variedad. Esta sección incluye 8 capítulos; en el primero de ellos (Capítulo 3), se desarrolló una metodología que empleaba cromatografía líquida acoplada a espectrometría de masas (LC-MS), que posteriormente fue aplicada al análisis de 156 muestras de aceite de oliva de la variedad 'Picholine Marocaine', provenientes de 7 zonas productoras marroquíes. Se determinaron 25 compuestos fenólicos pertenecientes a diferentes familias (flavonoides, lignanos, ácidos fenólicos, fenoles simples y secoiridoides). El efecto del origen geográfico sobre la composición fenólica de las muestras fue, lógicamente, evaluado. Primero se aplicó PCA para explorar la estructura de los datos y, en una segunda etapa del tratamiento estadístico, se utilizó s-LDA, para poner de manifiesto el potencial de los compuestos objeto de estudio para rastrear el origen geográfico de las muestras. En el Capítulo 4 se estudia la fracción volátil de aceites de oliva monovarietales del norte de Marruecos (de la variedad 'Picholine Marocaine'), intentando estimar su idoneidad para evaluar o asegurar el origen geográfico de las muestras seleccionadas. Para alcanzar el mencionado propósito, se utilizó microextracción en fase sólida sobre el espacio en cabeza para pre-concentrar la fracción volátil, que luego se inyectaba en un cromatógrafo de gases con detección por

ionización de llama y espectrometría de masas (HS-SPME/GC-FID-MS); los resultados obtenidos se estudiaban aplicando s-LDA. Un total de 40 compuestos pertenecientes a la fracción volátil del aceite fueron determinados y la estrategia usada demostró ser bastante eficaz, logrando una adecuada clasificación de los aceites en base a su proveniencia geográfica. En el Capítulo 5 se compara el potencial de dos potentes plataformas (LC-MS y GC-MS) para determinar el perfil metabólico de la fracción fenólica de aceites de oliva que provenían de 5 variedades distintas. Además de explotar el poder de identificación de ambas plataformas y caracterizar la fracción fenólica de las muestras de modo bastante profundo, se utilizaron herramientas estadísticas que permitieron trabajar con los perfiles cromatográficos completos y aplicar PCA y análisis discriminante-mínimos cuadrados parciales (PLS-DA) para construir modelos de autentificación varietal e identificar marcadores botánicos. En el Capítulo 6 se contrasta el poder discriminante (en base al origen geográfico) de diferentes herramientas quimiométricas (s-LDA, PCA, PLS-DA y modelado suave independiente por analogía de clases (SIMCA)), basadas, en este caso, en los datos de la fracción triglicerídica (TAG) de los aceites de oliva seleccionados. Para estudiar dicha fracción se utilizó LC con un detector de índice de refracción. Los resultados alcanzados fueron satisfactorios, tanto considerando el poder de clasificación como el de predicción de los modelos, siendo s-LDA y PLS-DA las herramientas que condujeron a mejores resultados. El principal objetivo del Capítulo 7 fue el estudio exhaustivo de la fracción fenólica de aceites de oliva obtenidos de aceitunas de 4 variedades autóctonas de Marruecos ('Picholine Marocaine', 'Dahbia', 'Haouzia' y 'Menara'), y 9 variedades mediterráneas que se han incorporado recientemente al panorama oleícola de dicho país ('Arbequina', 'Arbosana', 'Cornicabra', 'Frantoio', 'Hojiblanca', 'Koroneiki', 'Manzanilla', 'Picholine de Languedoc' y 'Picual'). La metodología LC-MS que se desarrolló en el capítulo 3, se utilizó para estudiar la fracción fenólica de 203 muestras de aceite de oliva de las variedades apenas mencionadas. El efecto de la campaña y la variedad en la composición de los perfiles fenólicos fue evaluado, siendo los datos sometidos, como en otras ocasiones, a PCA y s-LDA. Nos pareció pertinente construir tres modelos botánicos diferentes: el primero permitía discriminar entre aceites 'Picholine Marocaine' y el resto de las variedades "mediterráneas"; el segundo modelo fue útil para lograr la discriminación entre las variedades autóctonas del país; y el tercero fue construido para discriminar los aceites

'Dahbia', 'Haouzia' y 'Menara' de las 9 variedades recientemente adaptadas en Marruecos. El capítulo que le sigue (Capítulo 8) resume los resultados obtenidos tras aplicar una estrategia de "fusión de datos" (*data fusion*) y análisis multivariante a la huella dactilar fenólica alcanzada mediante LC con *diodo array* (DAD) y detección fluorescente (FLD). La huella dactilar de 140 aceites de oliva virgen obtenidos de aceitunas de 7 variedades, recogidas durante tres campañas consecutivas (2011-2014), se sometió a diferentes tratamientos quimiométricos. Primero, PCA, PLS-DA, SIMCA y *k-Nearest Neighbors* (k-NN) fueron aplicados a los datos DAD y FLD de modo independiente, y en una segunda fase del estudio, se aplicaron al conjunto de datos resultante de la fusión de ambas matrices. Los modelos de clasificación creados fueron bastante sensibles y selectivos, y mostraron una habilidad considerable en términos de reconocimiento y predicción. En el Capítulo 9 se utilizó una plataforma analítica bastante novedosa, *selected ion flow tube-mass spectrometry* (SIFT-MS), junto a herramientas quimiométricas, intentando predecir la proveniencia de 130 aceites con certificación de origen geográfico que se obtuvieron de Grecia, Italia, Marruecos y España. La fracción volátil de dichos aceites se examinó observando las señales de los iones precursores o gases reactivos (H_3O^+, NO^+ y O_2^+) y de los iones producto (en modo *full scan* en el rango *m/z* 10-200 durante 60 s). Con los datos recabados se construyeron los modelos estadísticos que perseguían la discriminación geográfica, y estos resultaron mostrar características satisfactorias independientemente del ión precursor que se considerase. Tras explorar la estructura de los datos, se utilizó PLS-DA para construir tres modelos diferentes (teniendo en cuenta los tres iones reactivos) para cada conjunto de datos en los que se subdividió el total de las muestras, persiguiendo poder clasificarlas de acuerdo al país de origen, e incluso región de procedencia (dentro de un mismo país).

El último capítulo de esta sección (Capítulo 10) describe el desarrollo y la validación de un nuevo método LC-MS donde se aplica la inyección directa del aceite de oliva virgen (tras una dilución) para la caracterización de su fracción fenólica, ofreciendo así una alternativa a todos los métodos que implican la extracción de los mismos. Durante la optimización se eligió el disolvente más apropiado para la dilución, la proporción aceite/disolvente, y se estableció la estrategia para limpiar la columna tras llevar a cabo un número de inyecciones consecutivas definido como máximo (manteniendo buenas prestaciones analíticas). Tras elegir las condiciones óptimas, se evaluaron los parámetros

analíticos, considerando los límites de detección y cuantificación, precisión y veracidad, y evaluando el posible efecto matriz. El método se aplicó entonces al análisis de aceites con 6 orígenes botánicos distintos y PCA se utilizó en un intento de discriminar entre las muestras seleccionadas.

-La Sección 3, titulada "***Propuesta de una aproximación metodológica para implementar sistemas de indicación geográfica certificada de aceite de oliva: Aplicación a la región marroquí de Meknès***" presenta una estrategia que permite evaluar la idoneidad de un área concreta para adquirir la etiqueta que acredite el reconocimiento del aceite de oliva virgen ahí producido bajo un sistema de indicación geográfica certificada (denominaciones de calidad diferenciada). La metodología planteada se ha aplicado a la región de Meknès (Norte-Centro Marruecos) analizando la tipicidad del aceite producido en este área durante varias campañas consecutivas. En esta misma sección, la primera de las contribuciones (Capítulo 11) es un capítulo de libro, que intenta clarificar la terminología usada en este ámbito de denominaciones de calidad diferenciada y describe las principales actividades que deben ser consideradas en una región específica para evaluar la viabilidad de la obtención de un etiquetado de este tipo, así como para asegurar que tras lograr dicho etiquetado, el sistema funcionará de manera satisfactoria. Una de las ideas más relevantes que se recoge en este capítulo es la necesidad de definir adecuadamente la dimensión territorial de cada área y caracterizar la tipicidad de su aceite a través de la determinación de una serie de propiedades de pertenencia (que definirán los perfiles cualitativos y composicionales) y de distinción (que permitirán diferenciar ese aceite de cualquier otro y reconocerlo cuando se compare). Como en el resto de esta disertación, el énfasis fue puesto sobre la región de Meknès. El segundo capítulo en esta última sección (Capítulo 12) constituye una contribución para definir las propiedades de pertenencia del aceite de oliva virgen de Meknès, determinando su perfil composicional, físico-químico y sensorial. Durante 4 campañas consecutivas, se analizaron un total de 298 muestras de la variedad 'Picholine Marocaine', obtenidas de almazaras seleccionadas dentro de la citada región. Los índices de calidad físico-química, los atributos sensoriales, y el contenido de fenoles totales, pigmentos, tocoferoles, y ácidos grasos fueron determinados. La variabilidad *intra*-regional e *intra*-anual fue examinada y los resultados mostraron que, como era esperable, el año de campaña ejercía un efecto significativo sobre las características de los aceites producidos; sin embargo, todos los

aceites sin excepción exhibieron una alta calidad global (índices de calidad físico-química y atributos sensoriales), así como una composición globalmente homogénea a lo largo de los 4 años considerados. Tras ello, y con objeto de definir el perfil composicional y nutricional de los aceites producidos en la región de Meknès (y teniendo en consideración la importancia de los compuestos fenólicos en relación al valor nutricional y las características sensoriales de esta matriz) se realizó un estudio de 3 años evaluando el perfil de los aceites en términos de los mencionados compuestos. Los resultados más destacados han sido incluidos en el **Capítulo 13**, donde el perfil fenólico de 142 muestras de aceite de oliva virgen, recogidas a los largo de tres campañas, fue caracterizado. El efecto del año de campaña y la variabilidad *intra*-regional fue estimado y pudo definirse el perfil polifenólico característico del aceite proveniente de esta región. Cerrando esta sección, el lector encontrará el **Capítulo 14**, donde se investigan las propiedades de distinción (definidas y discutidas en el capítulo 11) del aceite de Meknès y se compara dicho aceite con muestras de otros aceites de oliva vírgenes marroquíes (2 regiones) incluidos en sistemas de denominación de calidad diferenciada. El potencial, como variables discriminantes, del contenido individual de un buen número de compuestos fenólicos fue evaluado tras determinar los compuestos objeto de estudio mediante LC-MS y aplicar, posteriormente, s-LDA. Los modelos estadísticos obtuvieron excelentes porcentajes de éxito en clasificación y predicción; las variables que se apuntaron como más discriminantes fueron, lógicamente, estudiadas con mayor profundidad, identificando algunos potenciales marcadores geográficos.

RESUME

L'huile d'olive est une huile alimentaire fortement appréciée et considérée comme une composante essentielle du régime alimentaire méditerranéen. Ses bienfaits sur la santé humaine ont été largement décrits et son importance économique (particulièrement au niveau de certaines zones méditerranéennes) est indéniable. Impulsée par la bonne réputation du régime alimentaire méditerranéen, la consommation de cet aliment, appelé aujourd'hui, et à juste titre, "Or liquide Méditerranéen", est en augmentation continue partout dans le monde ce qui s'est traduit, inévitablement par l'émergence de nouveaux marchés et de nouveaux consommateurs. Le commerce s'intensifiant, la compétitivité internationale devient de plus en plus féroce. Dans ce climat de globalisation du marché oléicole, les différents types d'huile d'olive, de par l'origine géographique et variétale de l'arbre, constituent une stratégie de compétitivité raisonnable.

Les résultats obtenus dans le cadre de cette thèse de Doctorat intitulée "Huile d'olive vierge : potentiel de différentes approches en "omiques"» pour l'authentification de son origine géographique et variétale" sont exposés ici.

La rédaction de cette thèse est divisée en deux principales parties : Une Introduction qui traite de l'état de l'art des thématiques abordées dans le cadre de ce travail **(PARTIE 1)**, retraçant ainsi une vue générale de l'ensemble du sujet après une étude approfondie de la bibliographie la plus pertinente ; et une Partie Expérimentale, Résultats et Discussion **(PARTIE 2)** qui contient le travail expérimental effectué dans le cadre de ce doctorat, les résultats obtenus, ainsi que des conclusions et des perspectives à venir.

Ainsi, la **PARTIE 1** commence par une vue d'ensemble de la matrice de l'étude proposée, constituée d'une brève discussion de l'origine de l'olivier, de sa diffusion, sa botanique et caractéristiques morphologiques, les principales caractéristiques et la composition du fruit, ainsi que les différentes étapes de production et de conservation de l'huile d'olive. Est ensuite abordé le concept de qualité de l'huile d'olive en mettant l'accent sur la qualité réglementée, la valeur nutritionnelle, et la qualité liée à l'origine géographique et/ou variétale de la plante. Cette partie traite aussi, bien que brièvement, des aspects relatif à la pureté et l'authenticité de l'huile d'olive. L'état actuel et les tendances majeures de marché d'huile d'olive international sont par la suite détaillés, en soulignant les changements expérimentés durant les dernières décennies et les défis

auxquels le marché fait actuellement face. Dans cette même introduction, les principales stratégies adoptées pour surmonter ces défis ont été décrites, en se focalisant notamment sur les stratégies de marketing de différentiation, en l'occurrence des schémas de labellisation pour la production biologique d'huile d'olive, les certifications monovariétales et les indications géographiques. Une attention particulière a été ensuite accordée au cas du secteur oléicole marocain en mettant davantage l'accent sur l'importance stratégique de ce secteur dans l'actuelle stratégie de développement agricole dans ce pays, ainsi que les principales contraintes qui entravent son développement. De cette discussion ressort l'existence, à l'heure actuelle, d'une lacune majeure liée aux connaissances sur les principales caractéristiques qualitatives et compositionnelles de l'huile d'olive marocaine, démontrant ainsi le besoin de tirer profit des avantages que présentent les modernes et puissantes approches "omiques" pour la caractérisation de cette huile et l'authentification de son origines géographique et botanique. Ce fait, de notre point de vue, soutient le développement de cette thèse. La quatrième sous-partie de l'introduction décrit les approches "omiques" actuellement appliquées pour la caractérisation de l'huile d'olive, adressant concrètement les différentes étapes d'un flux de travail typique en ayant un regard sur les applications métabolomiques.

La **PARTIE 2** englobe trois principales sections :

- Section 1 : intitulée "***Indices d'authenticité et classification géographique des huiles d'olive vierges du Nord du Maroc***" consiste en deux chapitres. Le premier (Chapitre 1) est un article de synthèse qui présente une analyse globale, critique et comparative des principaux cadres réglementaires applicables actuellement pour le contrôle de la qualité et l'authenticité de l'huile d'olive. Après une description des critères de qualité et de pureté exigés par ces règlementations, une attention particulière a été prêtée aux différences et similarités entre les différentes normes passées en revue en ce qui concerne notamment les seuils fixés pour ces paramètres et les méthodes analytiques employées pour les déterminer. Les inconvénients et les limitations de ces méthodes ont également été soulevés. Enfin, les récentes avancées analytiques et chimiométriques appliquées à l'authentification d'huile d'olive et la traçabilité de son origine géographique et variétale ont été discutées. Le deuxième chapitre de cette section (Chapitre 2) représente une première tentative pour créer une base de données de la qualité et la composition des huiles d'olive monovariétales de la variété 'Picholine Marocaine' produites dans les principales zones oléicoles du Nord du Maroc. Ainsi, tout au long de deux campagnes oléicoles (2011 et 2012), 279 échantillons d'olives de la variété 'Picholine Marocaine' ont été collectés de 7 régions marocaines différentes, et les huiles ont été extraites et analysées en prenant en compte tous les critères de qualité et de pureté exigés par le Conseil Oléicole International (IOC). Les principales constatations issues de ce travail furent la détermination des intervalles de variation de la qualité

et de la composition des échantillons étudiés et la géolocalisation des sites oléicoles où les huiles d'olive présentent une teneur en acide linolénique supérieur à 1 % (valeur maximale fixée par la norme du IOC). En outre, après une analyse multivariée exploratoire utilisant l'analyse en composante principale (PCA), un modèle de traçabilité géographique, combinant les données obtenues et l'analyse discriminante linéaire en plusieurs étapes (s-LDA), a été construit ; de bons taux de classement correct et prédiction ont été obtenus et différents marqueurs géographiques ont été identifiés.

- Section 2 : intitulée "***Approches métabolomiques pour l'authentification géographique et variétale de l'huile d'olive***" traite l'optimisation, la validation et l'application de différentes approches métabolomiques à une vaste gamme d'échantillons d'huile d'olive soigneusement choisis dans l'objectif de les différentier sur la base de leur origine géographique et variétale. Cette section inclut 8 chapitres. Dans le premier (Chapitre 3), une méthode analytique basée sur le couplage de la chromatographie liquide à la spectrométrie de masse (LC-MS) a été optimisée et appliquée pour caractériser de manière exhaustive la fraction phénolique de 156 échantillons d'huile d'olive de la variété 'Picholine Marocaine' cultivée au niveau de 7 régions du Maroc. 25 composés phénoliques appartenant à différentes familles chimiques (flavonoïdes, lignanes, alcools et acides phénoliques et sécoïridoïdes) ont été identifiés et quantifiés. L'effet de l'origine géographique sur la composition phénolique des échantillons étudiés a été mis en évidence et une vue d'ensemble de la structure des données a été réalisée en appliquant la technique PCA. Ensuite, au moyen de l'analyse s-LDA, le potentiel de composés phénoliques pour tracer l'origine géographique d'huiles étudiées a été vérifié. Immédiatement après, le Chapitre 4 examine la fraction volatile des huiles d'olive monovariétales de la variété 'Picholine Marocaine' produites dans les principales zones oléicoles du Nord du Maroc, en essayant d'évaluer son aptitude à déterminer l'origine géographique des échantillons sélectionnés. À cette fin, une approche analytique combinant les données obtenues par micro-extraction sur phase solide par espace de tête couplée à la chromatographie en phase gazeuse avec détection par ionisation de flamme et MS (HS-SPME/GC-FID-MS), et un outil chimiométriques (s-LDA) a été utilisée. Un total de 40 composés volatiles, appartenant à différentes classes chimiques, ont été identifiés et quantifiés et la méthodologie utilisée a été remarquablement efficace pour la classification des huiles examinées selon leur origine géographique. Dans le Chapitre 5, deux approches de profilage métaboliques (LC-MS et GC-MS) ont été comparées prenant en compte leur capacité à caractériser la fraction phénolique et tracer l'origine variétale des huiles d'olive vierges provenant de cinq variétés différentes. La PCA et l'analyse discriminante par les moindres carrés partiels (PLS-DA) ont été appliquées pour construire des modèles d'authentification de l'origine variétale et identifier des marqueurs variétaux potentiels. Dans le Chapitre 6, nous avons comparé le

pouvoir discriminant par origine géographique de plusieurs méthodes chimiométriques (s-LDA, PCA, PLS-DA et la douce modélisation indépendante de l'analogie de la classe (SIMCA) appliquées à la fraction triglycéridique (TAGs) de l'huile d'olive vierge (déterminée par LC équipé d'un détecteur d'indice de réfraction). Des résultats satisfaisants ont été obtenus tant en termes de pouvoir de classification que de prédiction, s-LDA et PLS-DA étant celles qui ont donné les meilleurs résultats.

L'objectif principal de l'étude inclue dans le Chapitre 7 fut la caractérisation exhaustive de la fraction phénolique de de 203 échantillons d'huile d'olive provenant de 4 variétés cultivées autochtones marocaines (' Picholine Marocaine ', 'Dahbia', 'Haouzia' et 'Menara') et 9 variétés méditerranéennes récemment introduites au Maroc ('Arbequina', 'Arbosana', 'Cornicabra', 'Frantoio', 'Hojiblanca', 'Koroneiki', 'Manzanilla', ' Picholine du Languedoc ' et 'Picual'). La méthodologie analytique LC-MS développée dans le chapitre 3 a été utilisée dans ce cas. Une fois que la fraction phénolique des 203 échantillons d'huile d'olive des variétés sus- mentionnées a été examinée, les effets de la campagne de production et de la variété sur la composition phénolique ont été explorés et les données soumises à PCA et s-LDA. Trois modèles d'authentification de l'origine variétale ont été établis dans cette étude: le premier a permis de distinguer les huiles de la 'Picholine Marocaine' de celles des 9 variétés méditerranéennes examinées; le deuxième a différencié entre les variétés autochtones marocaines; et le troisième modèle a été construit pour distinguer les huiles de 'Dahbia', 'Haouzia' et 'Menara' de celles des 9 variétés méditerranéennes. Le chapitre suivant (Chapitre 8) récapitule les résultats obtenus à travers l'application d'une technique de fusion des données et la chimiométrie aux empreintes métabolomiques des composés phénoliques obtenus par LC avec deux détecteurs : l'un à barrette de diodes (DAD) et l'autre, un fluorimètre (FLD). Les empreintes métabolomiques de 140 échantillons d'huiles d'olive vierges extraites des fruits de sept variétés prélevés au cours de trois successives campagnes oléicoles (2011-2014), ont été enregistrées et PCA, PLS-DA, SIMCA et k plus proches voisins (k-NN) ont été appliqués, dans un premier lieu, aux deux bases de données constituées par les empreintes chromatographiques DAD et FLD séparément, et dans un deuxième lieu, à la base de données obtenue après la fusion des deux matrices de donnés de DAD et FLD. Les modèles de classification obtenus étaient très sensibles et sélectifs, montrant considérablement une bonne capacité de reconnaissance et de prédiction. Dans le Chapitre 9, la technique de tube d'écoulement d'ions sélectionnés spectrométrie de masse (SIFT-MS) en combinaison avec la chimiométrie a été évaluée comme une stratégie analytique pour la prédiction de l'origine de 130 échantillons d'huile d'olive méditerranéennes (Grèce, Italie, Maroc et Espagne) certifiées (ou en cours de certification) avec un label géographique. Les empreintes métabolomiques des produits de réaction des trois ions précurseurs H_3O^+, NO^+ et O_2^+ ont été collectées (en mode balayage

complet, dans l'intervalle m/z 10-200 pendant 60 s), et alors, utilisées pour construire différents modèles de discrimination géographique. Des modèles de classification efficaces ont été établis indépendamment de l'ion précurseur utilisé. La PCA a été utilisé pour l'analyse multivariée préliminaire des donnés et la PLS-DA a été appliquée pour construire (pour chaque ensemble de données) trois modèles différents (considérant les trois ions précurseurs) pour classer les échantillons selon le pays d'origine et/ou selon la région (dans le même pays). Les échantillons ont été classés d'une manière satisfaisante selon leur origine géographique et il a même été possible de distinguer ceux venant du même pays, mais de régions différentes.

Le dernier chapitre de cette section (Chapitre 10) traite le développement et la validation d'une nouvelle méthode LC-MS à base d'injection directe pour la caractérisation simple, sensible, précise et rapide des composés phénoliques de l'huile d'olive vierge. L'optimisation du type de solvant le plus approprié pour la dilution, la sélection du ratio optimal huile/dissolvant, et l'établissement d'une stratégie de nettoyage de la colonne et du nombre maximal d'injections permises (en maintenant une performance analytique satisfaisante) sont quelques étapes parmi les plus importantes qui ont été réalisées. Après le choix des conditions optimales, les paramètres analytiques de la méthode ont été évalués, considérant les limites de détection et de quantification, la précision et la justesse et vérifiant un effet matrice possible. La méthode a alors été appliquée pour analyser la fraction phénolique dans des huiles de 6 origines variétales différentes et la PCA a été appliquée dans une tentative de distinguer les échantillons étudiés selon leur origine variétale.

- Section 3 : intitulée "***Proposition d'une approche méthodologique pour la mise en place des labels géographiques de l'huile d'olive vierge: application à la région marocaine Meknès*** " présente une stratégie d'évaluation de l'opportunité d'une zone oléicole d'acquérir un label géographique pour sa production d'huile d'olive vierge. Cette méthodologie a été testée sur la région Meknès (Centre -Nord du Maroc) en analysant la typicité de l'huile d'olive vierge produite dans cette région, au cours de plusieurs campagnes oléicoles successives. Dans cette section, la première contribution (Chapitre 11) est un chapitre de livre dans lequel on a essayé d'élucider la terminologie utilisée dans le domaine des indications géographiques, et de décrire les principales activités à entreprendre pour l'évaluation de l'aptitude d'une région oléicole donnée pour acquérir un label géographique pour sa production d'huile d'olive vierge tout en assurant une utilisation convenable et efficiente de ce label. Les principales constatations de cette étude ont mis en évidence le besoin d'une définition adéquate de la dimension territoriale de la région concernée par l'étude et la caractérisation de de la typicité de son huile d'olive à travers la détermination des propriétés d'appartenance (définir les profiles qualitatifs et

compositionnels) et de distinction (propriétés qui rend possible la différenciation, l'identification et la reconnaissance de l'huile produite dans la zone de l'étude par rapport à d'autres produites en dehors de cette zone). L'accent a été mis sur la région de Meknès, et les résultats les plus pertinents obtenus en appliquant la méthodologie décrite ont été récapitulés. Le deuxième chapitre (Chapitre 12) constitue une contribution à définir les propriétés d'appartenance de l'huile d'olive vierge de Meknès à travers la détermination de sa qualité physico-chimique et sensorielle ainsi que son profil compositionnel. Au total, tout au long des quatre saisons consécutives (de 2010 à 2013), 298 échantillons d'huile d'olive monovariétal de la variété 'Picholine Marocaine' ont été prélevés au niveau d'unités de trituration présélectionnées dans la région de Meknès, et analysés. Les indices de qualité physico-chimique, les attributs sensoriels et la teneur totale en composés phénoliques, en pigments, en tocophérols et la composition en acides gras ont été déterminés. Les variabilités intra-régionale et interannuelle ont été examinées. Les résultats montrent que la campagne de production exerce un effet significatif sur les caractéristiques des huiles vierges produites au niveau de cette région; cependant, ces huiles montrent une qualité physico-chimique et sensorielle élevée ainsi qu'une composition homogène tout le long des quatre années considérées par l'étude. En outre, et toujours dans le but de définir le profil compositionnel des huiles produites dans la région Meknès tout en prenant en compte l'importance des composés phénoliques dans la définition de la valeur nutritive et la qualité sensorielle de cette matrice, une évaluation, sur trois années consécutives, de la composition quali-quantitative d'huile d'olive Meknès (en terme de ces composés) a été réalisée. Les conclusions principales de cette étude sont rapportées dans le Chapitre 13, où le profil phénolique de 142 échantillons d'huile d'olive, prélevés le long de trois campagnes oléicoles consécutives, a été établi. L'effet de la saison et la variabilité intra-régionale ont été examinés. Cette étude nous a permis de définir le profil phénolique des huiles d'olive de vierge de Meknès. À la fin de cette section, il y a le Chapitre 14. Il représente une tentative de définition des propriétés de distinction de l'huile d'olive Meknès, en comparaison avec d'autres huiles marocaines géographiquement labellisés. L'utilisation des profils phénoliques en tant que caractéristiques discriminantes entre des huiles d'olive extra vierges commerciales de la région Meknès et celles de deux autres régions marocaines certifiées avec une indication géographique a été évaluée par l'application d'une méthodologie analytique LC-MS et le traitement chimiométrique subséquent en utilisant s-LDA. Les principales caractéristiques distinctives ont été soulevées; des taux élevés de classification et de prédiction ont été obtenus en appliquant les modèles statistiques crées, et finalement des marqueurs géographiques ont été identifiés.

PART

1

Introduction: state-of-the art of the thesis topics

1. From olive tree to olive oil

1.1. Olive tree

1.1.1. Origin and diffusion

The olive tree (*Olea europaea* L.) is a long-lived evergreen tree that has been cultivated in the Mediterranean Basin for thousands of years [1,2]. Before its domestication, wild olive was endemic across the Mediterranean region, but particularly in the Middle East. Wild olive abundantly grows in thick forest, and is believed to be autochthonous to the Mediterranean Basin [3]. The origin and initial domestication of the Mediterranean olive tree are still debated. However, it is usually accepted that it has probably occurred in the Near East (the region currently located at the border between Southwestern Turkey and Northwestern Syria) approximately 6000 years ago. Indeed, it has been demonstrated both by the discovery of olive oil presses and by the presence of pollen grains, stones and wood remains [4]. From that moment, the know-how of olive tree cultivation and the olive fruit utilization spread across the whole Mediterranean Basin by Phoenicians, Greeks, Romans and other human civilizations (Figure 1) [5]; with the discovery of the new world in the fifteenth century, the olive tree was propagated in areas of the American continent with Mediterranean-like climate conditions.

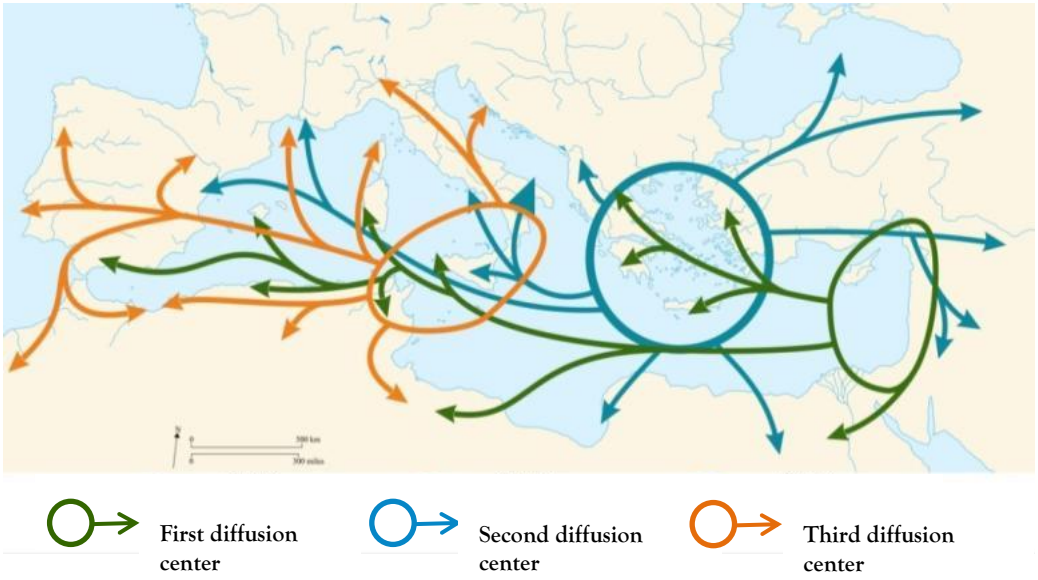

Figure1. Potential diffusion of the olive tree over the Mediterranean Basin [6].

Olive tree cultivation is, nowadays, distributed over all the five continents (Figure 2), although it prevails, especially, in the Mediterranean Basin, which represents 98% of the production area, estimated at 11 million ha [7]. 80% of the cultivated olive surface is located in Northern Mediterranean countries (Spain, Italy, Greece, Turkey), 17% in the Middle East (Jordan, Syria, Iraq, Iran) and in North Africa (Morocco, Algeria, Tunisia, Egypt), and only 2% is located in North (USA) and South America (Mexico, Argentina, Peru). Olive tree cultivation has been also recently initiated in India, Pakistan and China. However, the spread of olive tree cultivation remains geographically limited between 30° and 45° N, mainly due to climate conditions (olive tree's vulnerability to low temperature and extreme water stress) (Figure 2).

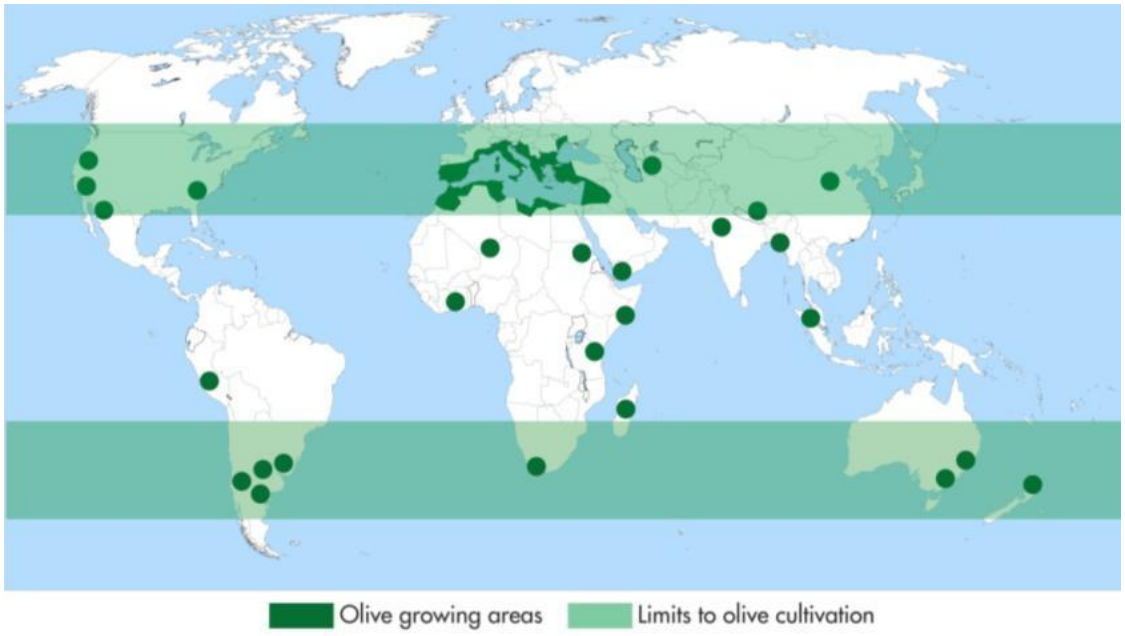

Figure 1. World distribution of *Olea europaea* L. producing areas [8].

1.1.2. Botany

Taxonomically olive tree belongs to the kingdom of green plants, subkingdom of vascular plants, class of *Magnoliopsida-Dicotyledons*, order of *Lamiales* and *Oleaceae* family. This family includes 29 genera, being *Fraxinus*, *Ligustrum*, *Syringa*, *Jasminum*, *Phillyrea* and *Olea* those with economic or aesthetic importance. The genus *Olea* consists of 35 species, which are distributed in Europe, Asia, Oceania and Africa, with only *Olea europaea* L. being cultivated [9]. Although controversial opinions can be found regarding the botanical classification of the olive tree, the division into two subspecies within the species *Olea europaea* L.: *Olea europaea* subs. *europaea*, or *sativa* (Hoffm. and Link) for

domestic olive tree; and *europaea* L. subs. *sylvestris* Miller, or oleaster for wild olive tree, is widely accepted. At present, around 2500 varieties are known; 250 of them are classified as commercial cultivars by the International Olive Council (IOC) [10]. General information about the main cultivated olive varieties all over the world can be found in the World catalogue of olive varieties edited by the IOC [11]. In Table 1 a list of the main cultivars in different olive growing countries is given, together with some information about their relative share on the country's overall olive-growing surface, according to Ilarioni and Proietti [12].

Table 1. List of main olive varieties cultivated in the nine major olive-producing countries (from Ilarioni and Proietti [12]).

Country	Total	Most cultivated cultivars in order of decreasing olive-growing surface area	Additional information
Spain	262	Picual, Cornicabra, Hojiblanca, Manzanilla de Sevilla, Arbequina, Morisca de Badajoz, Empeltre, Manzanilla, Cacereña, Lechín de Sevilla, Picudo, Lechín de Granada, Verdial de Badajoz, Morrut, Sevillenca, Villalonga, Castellana, Farga, Verdial de Huevar, Blanqueta, Gordal Sevillana, Verdial de Velez-Malaga, Aloreña, Changlot Real, Alfafara	96% of the total olive-growing area is represented by the mentioned varieties; the three cultivars at the top of the list account for 63%
Italy	538	Coratina, Ogliarola salentina, Cellina di Nardò, Carolea, Frantoio, Leccino, Ogliarola barese, Moraiolo, Bosana, Cima di Mola, Dolce di Rossano, Ogliarola messinese, Ottobratica, Sinopolese, Nocellara del Belice, Canino, Carboncella, Itrana, Moresca, Rotondella, Taggiasca, Tondina, Grossa di Gerace, Nocellara etnea	58% of the total olive-growing area
Greece	52	Koroneiki, Kalamata, Mastoidis	90% of the total olive-growing area
Tunisia	44	Chetoui, Chemali Sfax	95% of the total olive-growing area
Portugal	24	Galega Vulgar, Cobrançosa, Cordovil de Serpa	96% of the total olive-growing area
Turkey	80	Ayvalik, Memecik, Gemlik	75% of the total olive-growing area
Morocco	6	Picholine Marocaine	97% of the total olive-growing area
Syria	75	Zaity, Sorani, Doebli, Kaissy, Khodieri	90% of the total olive-growing area

1.1.3. Morphology

The cultivated olive tree is a long-living evergreen tree with grey-green leaves and small, white, fragrant flowers in the spring that produce a lot of pollen (Figure 3a, b, c and d). It can attain a mature height of up to 15 m and a spread of 9 m, however, under agricultural practices the height achieved after the pruning is, generally, of about 4-5 m. The root system is usually shallow, being the greatest proportion of roots growing at a soil depth of 60-70 cm, and the thick roots, in the top 20 cm [13]. In dry areas the root

system covers a section seven to eight times greater than the leaf area [14]. The life span of this tree is typically more than 500 years, but trees older than 2000 years have been registered.

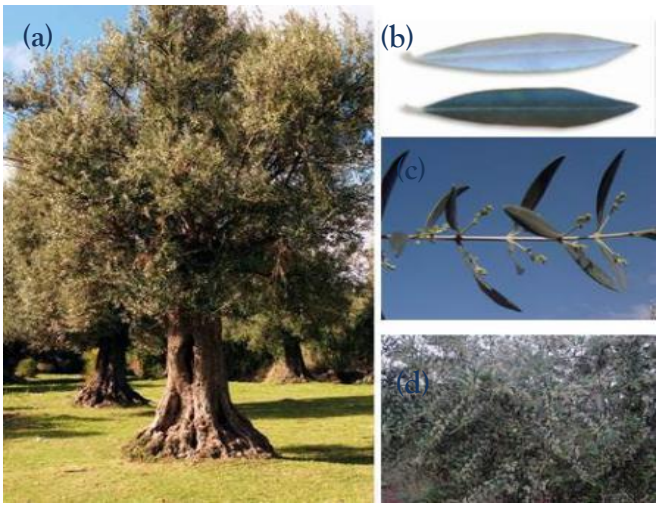

Figure 2. *Olea europaea* L. (a) tree; (b) leaves; (c) inflorescence before blooming; and (d) full bloom.

Leaves (Figure 3b) are thick, leathery, oppositely arranged, presenting stomata only on their abaxial surfaces, their skin is rich in tannins giving its grey-green appearance to the mature leaf, and they usually abscise in the spring when they are 2 or 3 years old. In the axil of each leaf inflorescences are born (Figure 3c), originated from buds of the current season's growth, and containing 15-30 flowers (each one), depending on both the prevailing conditions and the cultivar. In full bloom (Figure 3d), flowers are wind pollinated, and although most cultivars are self-incompatible, some are self-compatible.

The flowers are frequently hermaphroditic, but certain cultivars are male-sterile [15], while others are purely staminate. About 500,000 flowers can be present in a mature tree, however, it is widely accepted that only 1 or 2% of these flowers remain as developing fruit [16].

1.2. Fruit

The olive fruit (Figure 4) is a drupe, spherical or elliptic in shape, its size is variable (even on the same tree), and depends on cultivar, fruit load, pedoclimatic conditions and agronomical practices. It can weigh from 2-12 g, although some varieties may reach a weigh of20 g approximately. Anatomically, olive fruit consists of the following three

parts: (1) the skin, so-called epicarp (1.0-3.0% of the drupe weight), (2) the pulp or flesh, also named mesocarp (70-80% of the whole fruit), and (3) the stone, referred to as woody endocarp (18-22% of the olive weight), which contains the seed [17]. Average chemical composition of olive fruit is illustrated in Figure 4.

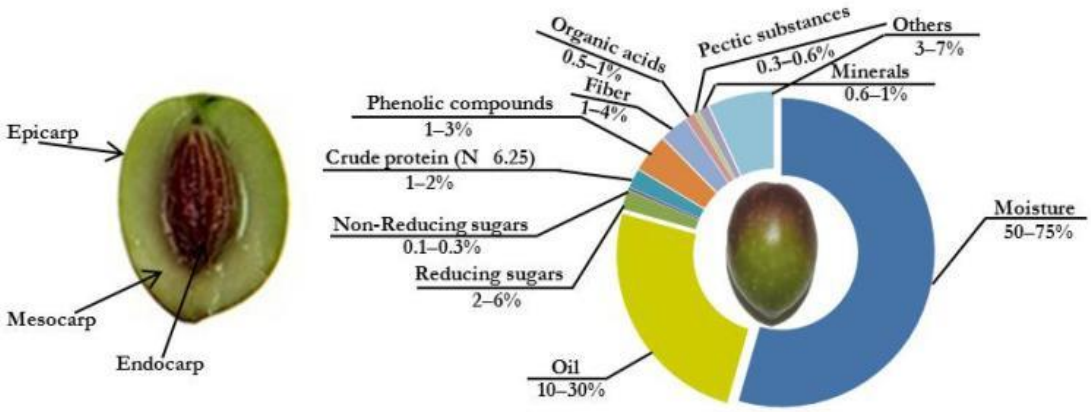

Figure 3. Morphological aspect and structural composition of olive fruit.

Olive fruit development and ripening go on for several months; over this period oil synthesis and accumulation take place. Therefore, during maturation, the olive fruit undergoes a color shift owing to a progressive decrease of total chlorophyll and carotenoids, followed by the appearance of anthocyanins and hydrophilic pigments, conferring the typical purple/black color to the mature drupes (Figure 5).

Figure 4. Changes in olive fruit color during maturation.

Furthermore, throughout ripening certain metabolic processes take place involving changes in the oil content and composition [14]. Such variations extensively affect the quality, stability and composition of the extracted oil. For this reason, the choice of the optimum harvesting stage (point at which an optimum balance between olive oil content and quality can be reached) is of extraordinary importance.

Within this context, several indices such as olive respiration, evolution of lipids in olive fruit and leaves, variation of the content and distribution of oil fatty acids, changes in the content of organic acids in leaves and fruits, ratio maleic/citric acid, and the skin color have been described [18]. The latter is currently the one suggested by the IOC for the determination of olive fruit ripening index. This parameter is based on the assessment of the color of 100 olives which are randomly drawn from 1 Kg of the sample, and the following formula is used:

$$\textbf{Ripening index} = \frac{(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + \cdots \ldots \ldots + (7 \times n_7)}{100}$$

Where n_0, n_1, n_2, ...n_7, are the number of olives belonging to each of the following eight categories:

0 = Olives the skin of which is a deep or dark green color;

1 = Olives the skin of which is a yellow or yellowish-green color;

2 = Olives the skin of which is a yellowish color with reddish spots;

3 = Olives the skin of which is a reddish or light violet color;

4 = Olives the skin of which is black and the flesh is still completely green;

5 = Olives the skin of which is black and the flesh is a violet color halfway through;

6 = Olives the skin of which is black and the flesh is a violet color almost right through the stone;

7 = Olives the skin of which is black and the flesh is completely dark.

1.3. Olive oil

1.3.1. Designations and definitions

Olive Oil is the oil obtained only from olive fruits, excluding oils obtained using solvents or re-esterification processes and any mixture with oils of other kinds [19]. The IOC, which is the world's international intergovernmental organization, amongst other things, recognizes several commercial categories of olive oil; each of them shows its particular qualities and market value (Figure 6). These designations, specified in the trade standards adopted by this entity, are grade names allowing to classify olive oils in various categories according to several physico-chemical and sensory quality criteria. The description of the characteristic and the production process of each one of these olive oil categories are detailed in chapter 1.

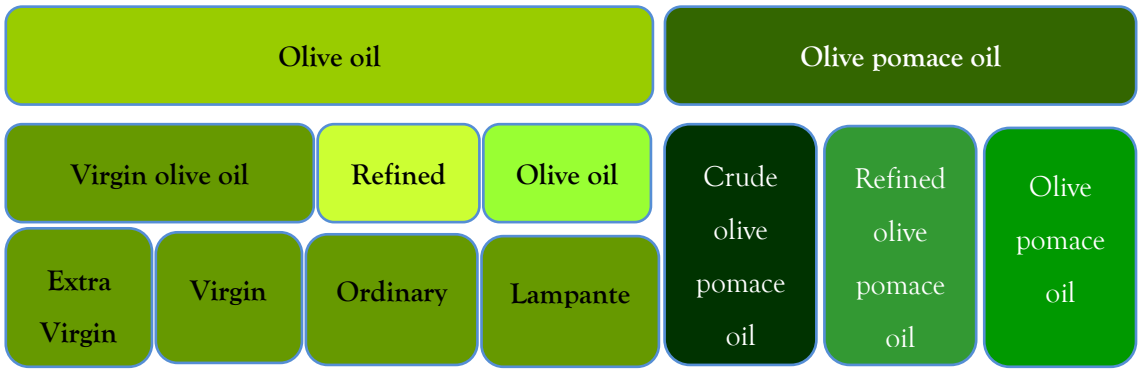

Figure 5. IOC classification of olive oil and olive pomace oil grades.

1.3.2. Olive oil processing

1.3.2.1. Fruits harvesting

Nowadays, various methods, ranging from the simple and traditional manual methods to the modern and sophisticated ones, are employed in the harvesting of olive oil fruits (Figure 7). Fruits harvesting in traditional olive orchards is still mainly performed by manual methods combined with the use of long poles or sticks (Figure 7a and b) or hand-held harvesting systems such as hand-held shakers or shaker combs (Figure 7c) to increase operating performance. When fruits are manually harvested, workers handpick the fruits into bags or let them fall into canvases or nets which are placed under the tree to collect the fallen fruits. Ladders are often used to reach the fruit located in the upper branches. Hand harvest is undoubtedly the best in terms of the quality of the produced

oil because the handpicked fruits are not damaged and relatively free from foreign matter such as soil, branches and leaves. The clear disadvantage is its cost as well as the fact that it is very labor intensive and time consuming. To overcome these limitations, the manual harvesting method is usually complemented with semi-automated machines (branch shakers and combing machines), and various mechanical harvesting technologies have been proposed and their efficiency has been investigated [20,21]. Currently available machines mainly comprise: (1) tree shakers machines operating by vibration on trunk (side-by-side trunk shakers (figure 7d)) or canopy contact (canopy-shaker (figure 7e)); and (2) straddle harvester machines (Colossus) used for harvesting olive high-density orchards (figure 7f).

Figure 6. Different pictures illustrating the main harvesting systems.

Once harvested, olive fruits are then put into aerated crates for transport from the field to the mill. The olives must be processed as quickly as possible, normally within 24 hours of picking, in order to keep down the oxidation and acidity.

1.3.2.2. Olive oil extraction

In the olive fruits the oil is produced in the mesocarp cells, and stored in a particular type of vacuole called lipo-vacuole (i.e., every cell contains an olive oil droplet). Thus, around 96-98% of the oil is concentrated in the pericarp part of the olive fruit, particularly in the mesocarp [22], as already stated. Hence, olive oil extraction refers to the process of separating the oil from the other phases in the olive paste (solid material and vegetative extract liquid) and this separation is should be done only by physical means [19]. This process includes various steps: paste preparation by means of breaking the fruit

structure (crushing), the liberation of the oil from the cells and the formation of solid and liquid phases (malaxation), the separation of solid and liquid phases, and, finally, the liquid phase separation. Below, a typical layout of the main tasks that are performed after harvesting the olives in order to extract olive oil is given (Figure 8). The most crucial steps of the three methods of olive oil extraction that are in use nowadays are going to be briefly described in the coming paragraphs.

Thus, the flow sheet of the olive oil mechanical extraction process (Figure 8) includes three essential steps: (1) preliminary operations (fruits reception and cleaning); (2) preparation of paste (crushing and malaxation); (3) separation of the oil from solid (pomace) and liquid phases (oil and wastewater) by pressing or horizontal centrifugation, and separation of the liquid phases (oil and wastewater) by decantation or vertical centrifugation.

- **Fruit cleaning**

To start, the harvested olives are poured from the crates into a vat and pushed through an opening to a conveyer belt for defoliating and washing. First, stems, twigs and leaves are removed by a defoliator through a powerful airflow generated by an exhaust fan. After that, the olives are washed in a current of water to remove any remaining earth, stones, and other debris. The water used for olive washing should be frequently changed during processing to prevent its charge or loading on earthy particles, which could release the responsible compounds for the "Earthy" sensory off-flavor into the oil [23].

- **Crushing**

After cleaning, the olive fruits are crushed. For grinding, either stone rollers (traditional system) or a metal tooth grinder or hammer mill (continuous system) can be used. Each one, logically, has its own advantages and disadvantages; discussing them in depth is perhaps beyond the scope of this introduction section.

- **Malaxation**

Malaxation (also mentioned as beating or kneading) is one of the most critical steps of the entire olive oil extraction process. Complex physical and biochemical phenomena occur during malaxation with great effects on the extraction yield and on the nutritional and sensory quality of the oil [24-26]. This operation consists on slowly churning or mixing

milled olives to enhance the effect of crushing, making the paste uniform and breaking up the oil/water emulsion, so that the droplets of oil join together to form larger drops.

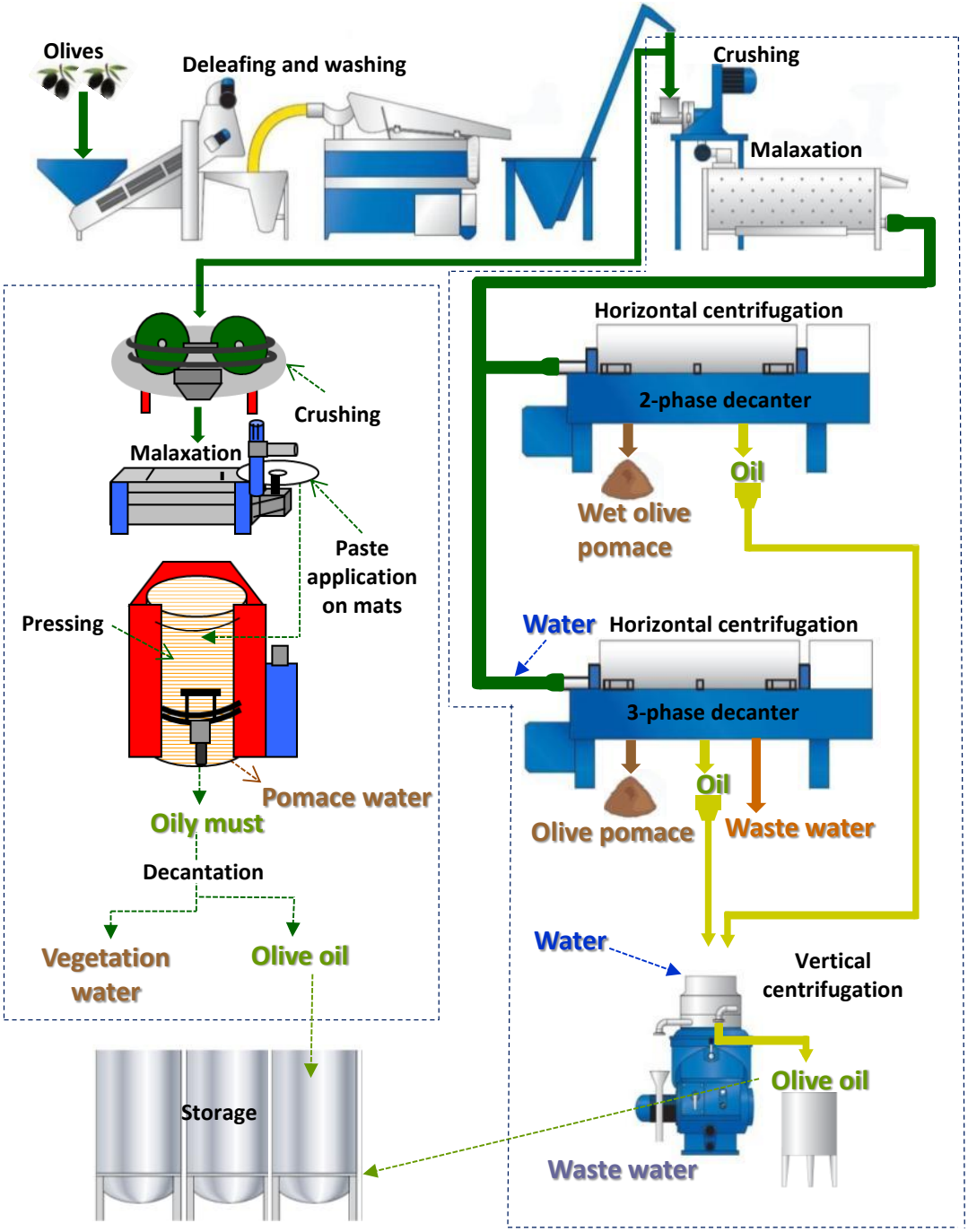

Figure 8. Main operations of the manufacturing process of virgin olive oil.

Cylindrical mixers with a vertical or horizontal shaft equipped with blades are used for this purpose. Malaxation is considered as one of the most crucial stages of the integral oil extraction process, being its temperature and duration very influential on the quality and

composition of the obtained oil [26]. In this regard, various authors suggest an optimal malaxation range of temperature/duration between 25 and 30 ∘C, over 20-50 minutes [27]. Furthermore, to protect olive paste against any oxidation over this beating stage (exposure of the oil paste to air results in oxidation of aglycons by the correponding enzymes catalyzing this reaction) some malaxation machines have been designed to work with an inert gas (nitrogen, and top-covered malaxers has been proposed as a way to control oxygen contact with the olive paste during this step).

- **Solid-liquid and liquid-liquid phase separation**

Olive paste derived from the milling and malaxing operations is a heterogeneous mixture of three phases: solid phase, consisting of organic semisolid components and the woody fragments from the pit shells; the aqueous phase vegetation water and water-soluble components; and the oil phase. The main objective of the remaining steps of the oil extraction process is the adequate and efficient separation of these three phases, trying to reach the highest possible recovery of the oily phase. To do so, two main systems are currently used: a traditional system based on mechanical separation by pressing the olive paste, and a continuous system where the separation is performed by centrifugation.

- **Traditional system (pressing)**

Pressing is the traditional or "classical" oldest method employed to separate olive pomace (solid phase) from the oily must (oil and water from the olives) (liquid phase). Mechanical pressing is performed in a hydraulic press. Thus, the resultant mash from olive millstones crushing is uniformly spread (usually of 2-3 cm thickness and often using an automatic paste distributor) around synthetic fiber draining mats which operate as filters. Mats were traditionally made of hemp or coconut fiber, but nowadays they are made from synthetic fibers, which are easier to clean and maintain; inox (stainless) disc diaphragms are placed among every five mats so that the charge is uniform for pressing. The mats are then threaded onto a hydraulic piston, forming a pile. Pressure is them applied on the mats, which allows compacting the solid phase of olive paste (pomace cake) and separating the oily must from the solid phase through drainage. Oil and water are further separated by natural decanting, into vats or settling pits that are linked together, taking advantage of the difference in density between the oil (0.915-0.918) and the residual water (1.015-1.086). However, this operation of decantation requires a large space and a long period of

time; over this interval of time the contact of the oil with the aqueous phase can cause fermentation and some sensory defects can be created.

- **Continuous systems (centrifugation)**

The main device for any continuous oil centrifuge extraction is the horizontal centrifugal decanter. Since the late 1960s, the process of olive oil extraction underwent a radical change with the introduction of decanter centrifugation for separating the insoluble solids from the liquid fraction (water and oil) of the olive paste, replacing the pressing [28,29]. The main advantages of the continuous system if compared with the traditional one are: increased working capacity, reduction of manual labor, smaller space requirement, automation of the industrial plants, improved process control and better quality oils (mainly due to the production capacity of this system allowing the reduction of the storage time of olives before processing), and the elimination of mat flavor. In contrast, it has higher cost than the traditional system. The decanter is a large-capacity horizontal centrifuge rotating at around 3000 rpm; the high centrifugal force created allows the phases to be readily separated according to their different densities (in the order of increasing density: the oil, the aqueous phase and the solid phase). Different generations of decanters have been designed and set up (in chronological order): two-phase, three-phase, water-saving three-phase, and variable decanters. However, two-phase and three-phase decanters remain, nowadays, themost widely ones used for olive oil production worldwide. These systems are principally differing in the process water requirements. Thus, when water is added with the paste to facilitate the extraction process, the system is called three-phase mode, whereas when oil is centrifuged without adding any water the process is described as two-phase system. The main steps included in both processes are summarized in Figure 8.

The three-phase system is a continuous process dating from 1970-1980, which has three exits for oil, water, and solids (Figure 10 a). The main drawbacks of this technology are the use of large quantities of warm water (10-30 L of added water per 100 kg of olive pastes) that results in the reduction of the olive oil content on some important compounds, such as phenols, and the production of great quantities of waste water that constitute an important environmental pollution problem (due to its high polluting load and its content on some compounds with biostatic and phytotoxic activity). A three-

phase decanter, at the end, generates three products: oil, pomace and wastewater. However, the obtained oil fraction still contains water droplets in emulsion and insoluble solids impurities in dispersion [30,31], so it has to passed through a vertical centrifuge (Figure 10c), where the oil separation and cleaning are performed. This vertical centrifuge is formed by a double truncoconical shaped bowl and a pool of plates inside that rotate at the same time at high speed (6000-7000 rpm) (Figure 10 c). The oil coming from the decanter, with the addition of an adjusted water flow (in order to facilitate the separation of the continuous water phase) are loaded on the top through a hollow axis which leads to a deflector, where they are partitioned. Then, they are introduced among the plates that work as elemental centrifuges separating the different phases [32]. In this manner, the obtained final product is clarified oil, while solid impurities and residual water are discharged as waste.

In contracts, the two-phase system was essentially introduced with the aim of eliminating the environmental problems caused by the three-phase method. This system was implemented in many places in Spain since the beginning of the ´90s, whilst the other olive oil producing countries started adopting this technology some time later (or are currently adopting it). The adoption of this system has significantly contributed to reduce the production of contaminant wastewater and the energy and water consumption. The working concept of the two-phase decanter is similar to that of a three-phase, except for the fact that no additional water is required for the separation process. Hence, this system only delivers two final streams: the oil and very wet olive pomace (also known as alperujo), including pomace and wastewater (Figure 10b). However, moist pomace is not appreciated by the industries of pomace oil extraction because requires prior dehydration, increasing the energy costs [33].

Finally, as for the oil obtained by the three-phase decanter, the separated oil is washed in a vertical centrifuge with lukewarm tap water, added to remove the remaining vegetation water from it.

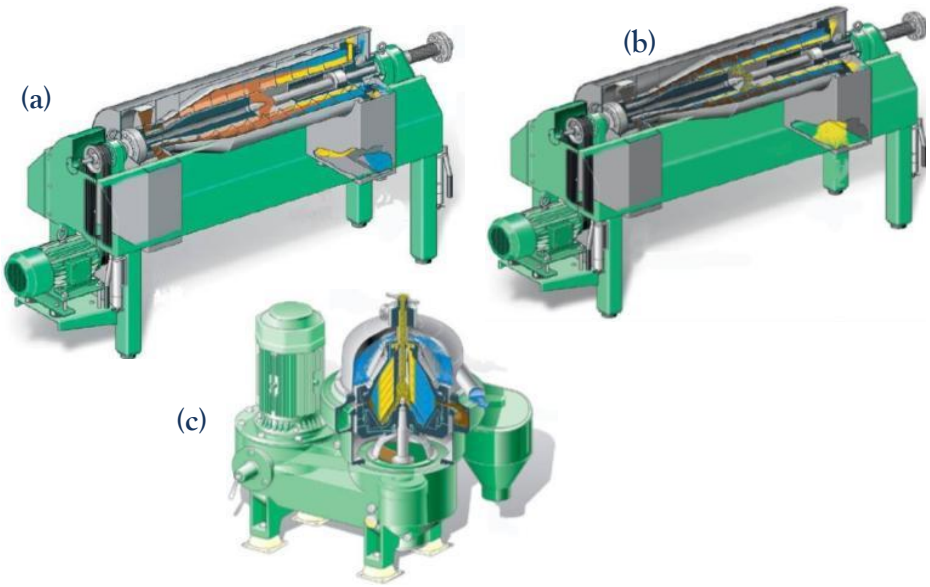

Figure 10. Illustrations of (a) three-phase decanter, (b) two-phase decanter and (c) vertical centrifuge. Reproduced from Gea Westfalia [34].

- **Filtration and storage**

After processing, the obtained olive oil dos not have to be filtered before storage if deposition of a residue is complete. Otherwise, it will develop some sediment in the bottom of the stainless steel tanks. Such a deposit continues to be at risk of enzymatic spoilage and, in the worst case scenario, of development of anaerobic micro-organisms with further deterioration and hygienic risk. Therefore, some olive oil producers prefer filtering using diatomaceous earth or cellulose fibers to remove possible suspended solids and moisture before storage. The latter is, indeed, a critical point to be taken into account in order to preserve to the greatest extent possible the flavor of freshly extracted olive oil and protect it from oxidation and deterioration. Certainly, the olive oil profile evolves during storage because of the simultaneous and drastic reduction of compounds from lipoxygenase pathways and the neo-formation of several volatile compounds responsible for some defects known as "rancid" and "muddy sediment" attributes [35]. Olive oil oxidation (which can even start during the oil processing) can be accelerated in the course of the storage step by exposition to air, heat, light and metals. So, to avoid these problems olive oil should be stored in stainless steel containers (prevention of exposure to light), with nitrogen blanketing (prevention of exposure to air (oxygen)), and kept at about 15-18°C to avoid oxidation (prevention of undue temperatures).

1.3.3. Olive oil quality attributes and authenticity

Over the last decades, olive oil quality and authenticity are subjects of current relevance because of increasing consumers' expectations (consumers have become very demanding about quality and show willingness to pay more for olive oils of outstanding quality), governmental regulations and expanding competitiveness in the olive oil market. Indeed, the worldwide popularity gained by olive oil as a natural nutritious food (with specific chemical composition and sensory characteristics, with nutritional and healthy significance) has contributed to increase the consumption of this product, and, at the same time, to expand its production and consumption outside of the traditional Mediterranean producing countries. This has led to the existence of innumerable olive oil brands in the olive oil market, showing different quality grades and composition properties. Accordingly, the olive oil customer's markets require, nowadays, greater transparency in the supply chain. Moreover, the assessment of quality and authenticity of the commercialized olive oils is becoming more and more important from the perspective of both the olive oil manufacturers and customers.

1.3.3.1. Olive oil quality

As any other food product, defining the overall concept of olive oil quality is a very difficult task, since there are different ways to address this subject. So, there is probably no single universal definition that adequately satisfies and coves all possible situations. However, olive oil quality can be described by (but not limited to) physicochemical and sensory quality indices (regulatory commercial quality), and nutritional value. Besides these intrinsic characteristics of the olive oil, the quality of this product can be established from a geographical and/or varietal perspectives, linking olive oil distinctive characteristics to a specific geographical area (concept of geographical indication systems) or to the use of a single olive variety (concept of monovarietal olive oils).

1.3.3.2. Olive oil regulatory commercial quality

The regulated quality and the standards set by IOC, European Commission, *Codex Alimentarius* and other regulation systems represents, perhaps, the easiest manner to define olive oil quality. An overview of all these regulatory systems will be given in chapter 1. To complete the reading about this topic, extended chapters on olive oil

regulatory commercial quality can be found in relevant publications edited by Boskou, and Harwood and Aparicio [36,37].

Briefly, in these regulations, a distinction among the different commercial grades of olive oil (extra virgin olive oil (EVOO), virgin olive oil (VOO), lampante virgin olive oil (LVOO) and ordinary virgin olive oil (considered only by IOC and Codex Alimentarius regulations)) is made on the basis of well-defined quality parameters: the content of free acidity (which measures the percentage of free fatty acids in the oil, expressed as oleic acid); the peroxide value (which allows evaluating the state of primary oil oxidation (expressed in milliequivalents of active oxygen by kg of oil)); and spectrophotometric values (or ultraviolet specific extinction coefficients), conventionally indicated as K_{232}, K_{270} and ΔK (that inform about the secondary oil oxidation state, and are measured in the UV region, at the wavelengths corresponding to the maximum absorption (about 232 and 270 nm) of secondary products formed in the autoxidation process). It should be noted, however, that in addition to these olive oil quality parameters adopted by all regulatory systems, there are other quality indices of no mandatory application, since no common decision has been made by consensus by all standardizing bodies. They involve pyropheophytins and 1,2-diacylglycerol, as indicators of olive oil freshness and the content on fatty acid alkyl esters, which can be considered as indicator of the health conditions of processed olive fruits.

Besides these physico-chemical parameters, olive oil regulatory commercial quality definition includes the determination of its organoleptic characteristics. Within this frame, a methodology for evaluating the sensory attributes of olive oil, known as the Panel Test method, was developed in the´80s by the IOC and later adopted by the European Commission (Regulation No. 640/2008) [38] and other standardizing bodies. This methodology, also known as the IOC test, aims to identify and evaluate the intensities of positive and negative sensory perceptions for the classification of various grades of the tested olive oil. To do so, the IOC method defines the main steps for the selection and training of tasters, the minimum number of tasters to be involved in the sensory analysis, basic olive oil vocabulary (including both negative attributes, such as fusty/muddy, musty-humid-earthy, winey-vinegary-acid-sour and rancid; and positive attributes mainly fruity, bitter and pungent), the tasting methodology to be followed,

profile sheet and characteristics and specificity of tasting glass and booth (Figure 11 a and b).

Figure 11. Tasting glass and booths of the test room for olive oil sensory analysis.

It should be also noted that the regulation systems of olive oil quality establish, for the above-mentioned quality parameters (both physico-chemical indices and sensory analysis), the official analytical methods to be used to determine their values as well as the legal thresholds for parameter, which allow the classification of tested olive oils into the different commercial grades. A detailed overview of these olive oil quality parameters, the official methods for their analytical determination, and their threshold limits according to the most relevant regulatory legislations are reported in chapter 1 of the current doctoral thesis.

1.3.3.3. Olive oil quality from nutritional and healthy perspectives

Olive oil has been reported as part of Mediterranean population nutrition since ancient times. Its consumption is closely associated with human health benefits to the point of being considered by ancient Greeks as an "elixir of youth and health" [39]. Nowadays, this foodstuff constitutes the main fat source in the well-known Mediterranean diet. It presents broad ranges of nutritional and therapeutic values, which are obviously ascribed to its composition. Table 3 shows the nutritional composition of 100 g of olive oil, according to the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference.

Table 2. Nutritional composition of 100 g of olive oil according to the USDA database [40].

Nutrient	Unit	Value per 100 g
Energy	kcal	884
Minerals		
Calcium, Ca		1
Iron, Fe		0.56
Magnesium, Mg		0
Phosphorus, P	mg	0
Potassium, K		1
Sodium, Na		2
Zinc, Zn		0
Vitamins		
Vitamin E (alpha-tocopherol)	mg	14.35
Vitamin K (phylloquinone)	µg	60.2
Lipids		
Fatty acids, total saturated		13.808
Fatty acids, total monounsaturated	g	72.961
Fatty acids, total polyunsaturated		10.523
Cholesterol	mg	0

In terms of dietary lipids, olive oil provides lower level of saturated fatty acids (SAFA), high amount of monounsaturated fatty acids (MUFA) (mainly oleic, 55-83 %), a appropriate quantity of essential polyunsaturated fatty acids (PUFA) and a relatively low n-6 PUFA/n-3 PUFA ratio (omega-6/omega-3 fatty acids) [36].

Furthermore, despite basic nutritional consumer's requirements that olive oil consumption can fulfill, its outstanding richness on highly bioactive compounds, such as phenolic compounds, triterpenic derivatives, sterols (mainly β-sitosterol), tocopherols and pigments, among others, provides additional health benefits which lead to consider this foodstuff as an accurate example of functional food [41].

However, it is noteworthy that the relative proportion (as well as the absolute concentration) of the different olive oil compounds are rather variable due to the influence of intrinsic factors (olive cultivar and olive fruits ripening stage) [42-44] and extrinsic factors, such as environmental growth conditions [45-47], agronomical practices [14,48] and olive oil extraction, filtration and conservation technological factors [49-53]. Detailed information about the main constituents of olive oil will be given and deeply discussed throughout the different chapters of this thesis.

As far as the olive health-benefits are concerned, it is possible to say that numerous epidemiological and clinical studies associate the regular consumption of olive oil within the Mediterranean diet with the reduction of the risk of suffering several chronic diseases [54,55]. Figure 12 exemplifies the main health benefits potentially linked to olive oil consumption.

Within the frame of its healthy properties evaluation, over the past decades, to main research lines have been proposed. In one of them, the stress has been placed on evaluating and proving its biological properties (including, among others, antioxidant, antimicrobial, anti-inflammatory, chemopreventive and anti-cancer characteristics) and bioavailability of several of its bioactive compounds, in particular, phenolic components [56-58]. In the second one, a number of epidemiological studies and clinical trials (such as EUROLIVE [59,60], PREDIMED [61] and European Prospective Investigation into Cancer and Nutrition (EPICN) [62]) have suggested that individuals with a regular intake of olive oil have lower risk of suffering some coronary diseases. Further detailed information about this matter can be found in very interesting manuscripts, which give a deep insight into recent clinical studies, showing the effects of dietary olive oil intake on the human health [63-66]. Likewise, very stimulating information about the main bioactive compounds which naturally occur in olive oil and their healthyeffects are well detailed in various relevant publications edited by Boskou, Quiles et al., Preedy and Watson, and Aparicio and Harwood [37,67-70].

1.3.3.4. Olive oil quality linked to geographical and/or varietal provenance

Olive oils produced in various olive growing regions can be distinguished by quality attributes and compositional characteristics. These "typical" features can be of interest to consumers, since they are presumably linked to the olive variety from which the product is elaborated (monovarietal olive oils) and/or to their specific geographical origin or particular "know-how" applied for olive oil production in these regions (olive oils with geographical indications). The main justifications behind this quality concept have to do with the fact that olive oil quality, as well as its compositional characteristics, are widely defined by a highly complex and interactive system, formed by climate, soil and management practices, that greatly influence olive tree development. The effects of

technological practices for olive oil extraction and conservation have to be also considered together with the already mentioned factors.

Figure 12. Principal healthy effects attributed to olive oil consumption.

Indeed, it is well established that there is a high diversity of olive cultivars from which oil can be produced all over the world, and each one of them brings a unique composition and peculiar flavor characteristics. Because of its wide range of adaptability to various pedoclimatic conditions, olive tree is nowadays cultivated in very contrasting conditions, what is reflected in the high variability of the quality and composition of the obtained oils. Many aspects related to the effect of variety and geographical origin factors (environmental conditions and agronomic and technological practices used for olive fruit production and oil extraction) have been intensely investigated, with the ultimate goal of proving the distinctive features of olive oils coming from different varieties and/or geographical areas. As a result, a plethora of manuscripts are available emphasizing the impact of all the aforementioned factors on the main characteristics of the olive oil [25,71-77].

1.3.3.5. Olive oil genuineness and authenticity

The disparity between the price of olive oil and other vegetable oils is one of the most influential factors that make olive oil, and, in particular, EVOO category, a preferred target for fraudulent practices that have lately become a serious issue. In recent years, olive oil authenticity control agencies have faced an increasing number of cases of olive oil samples which were questionable; in other words, samples likely adulterated or not authentic. In the olive oil sector, typical adulteration practices are: addition of olive oil of low quality grades and/or olive pomace oils to EVOO, adulteration of EVOO by the addition of other vegetable oils, and mislabeling practices concerning the declaration of origin and the variety used for oil production [78]. Consequently, fighting against olive oil adulteration is, nowadays, a topic of prominent importance within the olive oil sector.

Form a regulatory point of view, a variety of physical and chemical tests have been developed and officially adopted by different olive oil regulatory bodies, such as IOC, European Commission and Codex Alimentarius, for checking the identity and genuineness of olive oil, as stated before. These regulated authenticity indices are limits set for fatty acids composition and geometrical isomers, palmitic acid in the 2-position, ΔECN42, sterols and total sterols composition, erythrodiol plus uvaol (%), waxes (mg/kg), saturated acids in the 2-position of the triglycerides (mg/kg) and sum of trans-linoleic and trans-linolenic isomers (%). All these quality and purity criteria should be fulfilled for a specific category of olive oil. Furthermore, the analysis of these specific olive oil authenticity marker compounds -which are indicative of a certain property of this product- must be limited to the use of official analytical methods, measured accurately and, subsequently, compared to the thresholds fixed by the regulatory system. A more detailed description of these parameters, the method to determine them, as well as the limits fixed by the main olive oil regulatory bodies will be given in chapter 1.

2. The international olive oil market: current status and major trends

Olive oil is a Mediterranean iconic product with a great environmental, social and economic importance in most of the countries of this part of the world. More than 800 million olive trees, approximately, are currently grown over the world, of which greater than 90% are grown for oil production and the rest for table olives [13]. The countries surrounding the Mediterranean Sea have consistently been the largest olive oil

producers, consumers, importers and exporters (Figure 13); however, over the last two decades, the world olive oil sector has experienced a clear trend toward internationalization, pointing out to expanding broadening or expansion of olive oil consumption in other countries, such as United Sates (USA), Canada, Australia, Brazil, Japan and China, where this commodity was not traditionally consumed. This increase in olive oil consumption and trade recently enhanced the expansion of olive orchard planting outside the Mediterranean region, particularly in countries such as Argentina, Australia, Chile, China, India, Japan, Mexico, New Zealand, South Africa and USA (especially in Californian State). Overall, olive oil is currently produced in about 47 countries all over the world [7].

2.1. The current status of international olive oil sector: world olive oil production, consumption and trade

A general overview of production, consumption, imports and exports of the olive oil sector during the crop season 2013/2014 is given in Figure 13. It is easy to deduce from this illustrative picture that, during the crop season 2013/2014, about 3.25 million tons of olive oil were produced worldwide, being 93.5% of them originating from Mediterranean area. Within the Mediterranean countries, those belonging to European Union (EU) supplied about three-quarters of global production. This group of countries is led by Spain, which has the highest production (about 1.78 million tons, representing 54.8% of global production), followed by Italy (463.7 thousand, equivalent to 14.3% of global production), Greece (132 thousand tons, corresponding to 4.1% of total production) and Portugal (91.6 thousand tons, representing about 2.8% of global outcome). Almost one-quarter of global olive oil was produced in non-EU Mediterranean countries, mainly in Syria (180 thousand tons), Turkey (135 thousand tons), Morocco (130 thousand tons) and Tunisia (70 thousand tons). However, olive oil production in these countries often shows a pronounced year-to-year fluctuation, due to interannual climatic conditions variations. For instance, according historical statistical data from IOC, outside the EU, Tunisia -except for a limited number of crop seasons- has always been the largest olive oil producer country (between 70 thousand tons obtained in 2013/2014 and 220 thousand tons produced in both 2005/2006 and 2012/2013) [79]. Finally, the non-Mediterranean countries, particularly Argentina, Australia, and Chile, accounted for a small share of total world production.

As far as olive oil world consumption is concerned, IOC historical statistical data [79] suggest that the apparent olive oil consumption shows some similar trajectories to world production, but with less noticeable fluctuations, being the major olive oil consuming countries the major olive oil producing ones. In this regard, during the crop season 2013/2014 (Figure 14b), most olive oil globally produced (about 70.7% of the total amount) was consumed within the Mediterranean area. EU is the world's largest olive oil consumer, accounting for 56.3% of global olive oil consumption, where Italy (21%), Spain (17.1%), Greece (5%) and France (4%) are the main EU olive oil consuming countries. Additionally, it should be emphasized that, when considering the annual per capita consumption of olive oil in the EU countries, in 2013/2014, Greece led the ranking with 16.3 kg, followed by Spain (10.4 kg), Italy (9.2 kg) and Portugal (7.1 kg) [80]. Furthermore, non-EU Mediterranean countries consumed about 14.4%, amongst which 5.5% was consumed by Syria, 3.9% in Morocco, and 3.4% in Turkey. It can also be noticed from Figure 13b that some countries that are not traditional olive oil consumers are currently accounting for an important percentage of global olive oil consumption; they are USA (9.8%), Brazil (2.4%) and Japan (1.8%).

With regard to the international trading of olive oil, the export data (Figure 13c and Figure14) show that the main producing countries are also the main exporting ones.

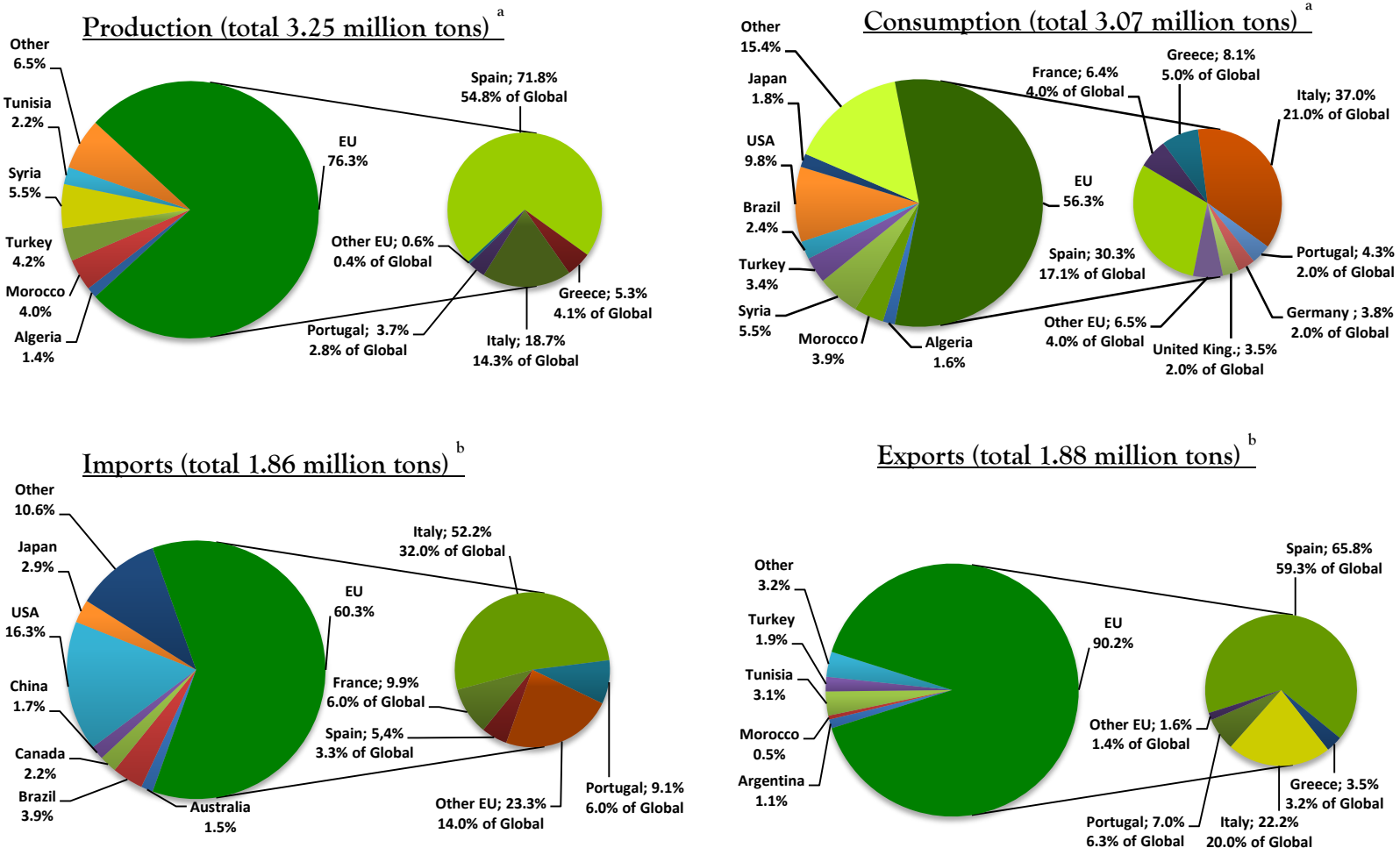

Figure 13. Global olive oil market statistics. Crop year 2013/2014. (a): source IOC [79] ; (b) source IOC and European Commission [79,81].

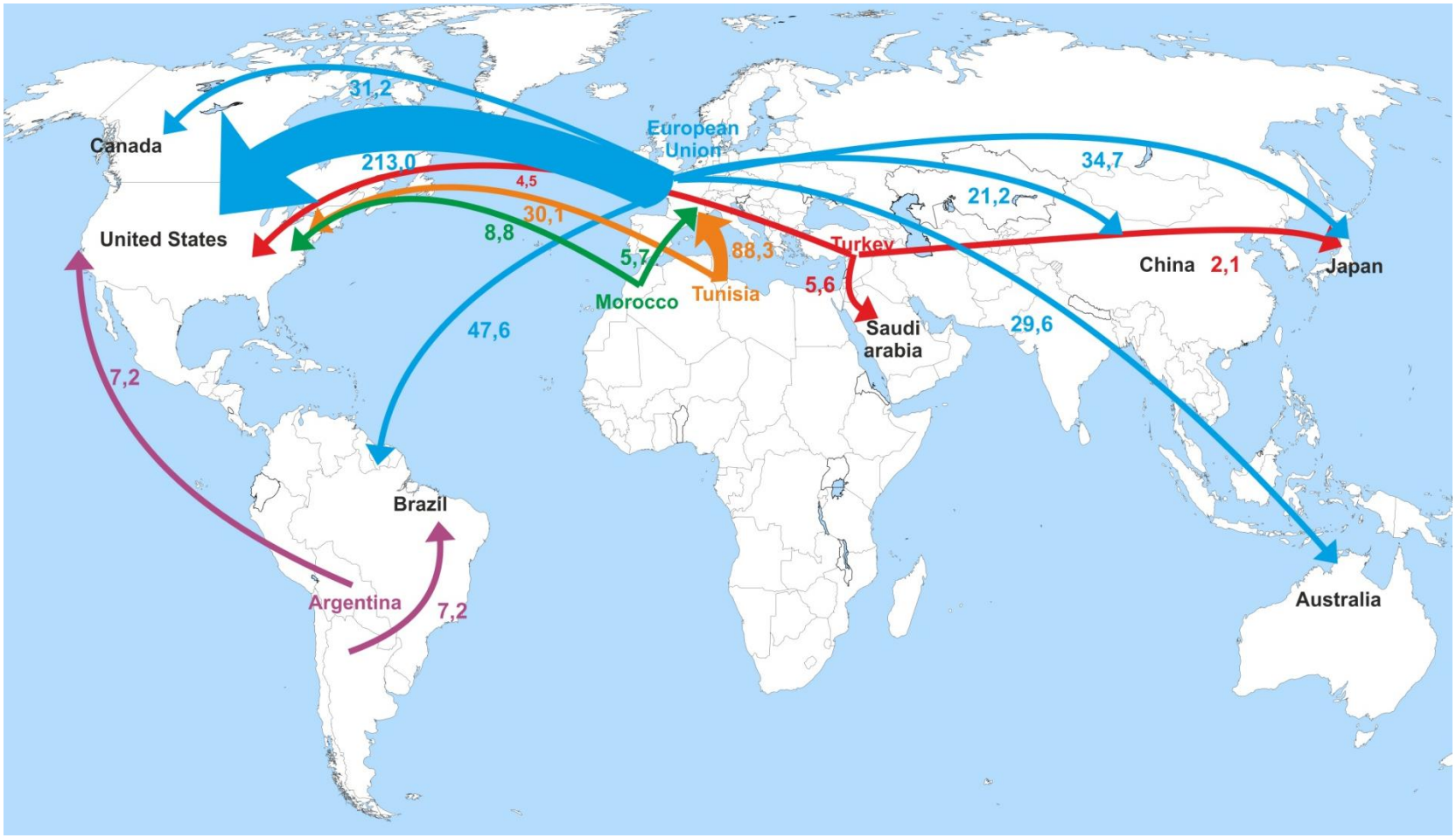

Figure 14. Olive oil major global trade flows, 2008-2012. Reproduced from United States International Trade Commission [82].

Thus, during the crop season 2013/2014, EU countries provided more than 90% of global olive oil exports (most of them were within-EU trade), comprising exports from Spain (68.8%) and Italy (22.2%). Non-EU Mediterranean countries also contribute to the international trading of olive oil, particularly, Tunisia, which is the third largest global olive oil exporter.

Concerning olive oil imports, once again, EU was the main olive oil importer (taking into account intra-EU imports), covering 60.3% of global imports, mainly, made by Italy (52.2%). This country imports large amounts from Spain.

If the EU is considered as one market and intra-EU trade is ignored, USA can be considered as the world's largest market for imports of olive oil. Indeed, this market has currently become the biggest one outside the Mediterranean area. During the last 20 years, imports have increased more than five times and they are now surpassing 200 thousand tons. Other emerging olive oil importers countries can be seen in Figure 14.

In the light of this brief description of the current status of the global olive oil market, it can be concluded that there is a fast, growing and significant internationalization of the supply and demand. Indeed, the international olive oil market has gone through a rapid change over the last decades, passing from being a rather localized Mediterranean market to turn into an extensive market competing in the global marketplaces. Within this context, the gradual increase of worldwide olive oil production and consumption outside its "natural" area, the Mediterranean region, is one of the important factors driving market growth. However, although olive oil market internationalization can be, to a certain extent, advantageous as it contributes to create market opportunities outside the Mediterranean region where olive oil consumption levels are relatively stable [83], it also entails important challenges that should be taken very much into consideration when draft any national or international production and commercialization strategies to gain new markets and achieve sustained competitive advantages. Most significant challenges and current marketing strategies adopted to deal with olive oil market's globalization are summarized in the following paragraphs.

2.2. Challenges and differentiating trends in olive oil market

In the described scenario of olive oil market globalization, some of the greatest challenges that this sector is currently facing can be cited as following:

- Even though the olive oil trade was globally rising at a rate of about 4% annually, over the period 1990-2013, this sector is currently suffering a profitability decline due to lower prices in recent years, resulting from oversupply and a strong power imbalance in the sector [84].
- A significant intensification of horizontal and vertical competition along the olive oil's marketing chain, where few producing countries are relevant players in different segments of the world olive oil market. In this concern, the leading position of EU in the global olive oil market has a decisive impact on international olive oil pricing [85].
- Price fluctuation: fluctuating weather patterns, in producing countries, have been clearly reflected in price variations in the past years. Thus, forsome years with low olive oil production, high prices are achieved. In the traditional olive oil countries, price raise (up to certain levels) can only causes a small, temporary drop in consumption, while in emerging olive oil consuming countries, an increase of the price could led to a long-run shift into consumption of other types of oils and/or lower quality and cheaper categories of olive oils [86].
- High level of vertical integration in production and processing, and an increasing concentration in the retail sector with a strong power of the distribution chain. Indeed, over the course of the last years, important olive oil producing companies, large packaging groups and retailer labels have emerged into the global olive oil value chain becoming relevant. For example, in Spain -the leading olive oil producing country- a few large groups nearly control the olive oil market; the five main cooperatives in the production of olive oil (Hojiblanca, Oleoestepa, Jaencoop, Tierras Altas and Olivar de Segura) dominate about 37.2% of market share. Furthermore, DCoop Group (very large, second-degree cooperative) is the result of merging many cooperatives (Hojiblanca, Tierras Altas, among others) is the largest oil producer in the world, with a market share of 24.4% [87]. This fact obviously hinder the competitiveness of other olive oil producing countries in the international market, especially, because the production levels of many of these countries are not comparable to the high olive oil volumes managed by these companies.

- Heterogeneous regulatory frameworks regarding olive oil quality and authenticity control. Indeed, different international (Codex Alimentarius, EU, IOC standards) and national (USA, Australia, California, etc.) currently exist; this fact has caused a very lively debate about the risk/difficult that they can represent for the harmonization of the international olive oil trade standards. Moreover, all these standards contribute to the coexistence of confusing product denominations for the consumers and can produce conflict of interest between different chain agents.
- Demand is stable in most of the Mediterranean olive oil producing countries. The increased consumption of olive oil is, nowadays, driven by the demand from countries outside the Mediterranean Basin. This has caused a change in the aimed excellence of the supplied olive oil categories in favor of the EVOO and VOO grades and oils with differentiating signs [88]. Consumers from these countries generally perceive the olive oil as an expensive high-quality gourmet oil, associated with beneficial health effects and evoking positive feelings. Some recent studies have also shown that an important proportion of these consumers are still confused about olive oil benefits and terminology [89]. It means that is still necessary to undertake profound efforts to communicate about olive oil and its attributes and to promote them amongst consumers in the olive oil emerging consumers' counties.

Accordingly, in response to these challenges and concerns, numerous initiatives were (and still are) undertaken at both national and international levels.

A remarkable effort is being made by different components of olive oil supply chain aiming at the promotion of research and innovation, the improvement of the yield and quality of olive oil, and the development of new technological processes that could result in an adaptation of the supply to the market´s demands and the consumers' needs. Moreover, multitude of actions are undertaken to promote olive oil quality and enhance its consumption in both traditional and emerging olive oil consumer's countries Generic promotional activities are also annually launched by the IOC into both traditional and emerging olive oil markets. Furthermore, in the main olive producing countries, various promotional activities (congress, exhibitions, gastronomy or tasting events…) are annually carry out to advertise quality, flavors attributes and the healthy features of olive

oil to consumers (adult and young age (schools) people), professional gastronomy chefs, artisans, HORECA channel (hotel, restaurant and catering companies) users, ect.). Within this context, some olive promoting centers and inter-professional organizations, such as the Spanish Interprofessional organization of olive oil, are making a very interesting and valuable promotional work with the intention of disseminate the olive oil culture among diverse type of consumers.

Besides, to promote olive oil as healthy product, or in other words, as a functional food, multidisciplinary scientific studies, carried out by research groups which include, among others, nutritionists, epidemiologists, hospital clinicians, primary care doctors, and university researchers, are commonly encouraged and financed by the main producing countries. In 2004, the US Food and Drug Administration (FDA) stated the admissibility of specific health claim related to the daily consumption of two tablespoons (23 g approx..) of olive oil and a reduction of coronary heart disease risk: "Limited and not conclusive scientific evidence suggests that eating about two tablespoons (23 g) of olive oil daily may reduce the risk of coronary heart disease due to the monounsaturated fat in olive oil" [90]. Most recently, European Food Safety Authority (EFSA) approved a health claim stating that the dietary intake of olive oil phenolic compounds could be able to prevent low density lipoprotein (LDL) oxidation. The exact wording of the claim is olive oil polyphenols contribute to the protection of blood lipids from oxidative stress. The EU restricts the use of this claim to olive oils which contains at least 5 mg of hydroxytyrosol and its derivatives per 20 g of olive oil [91]. Lastly, the recent recognition of United Nations Educational, Scientific and Cultural Organization (UNESCO)(which has declared the Mediterranean diet as 'the intangible cultural heritage of humanity') offers promising perspectives for the olive oil consumption, as it constitutes the main source of fat in the Mediterranean diet [92].

Changing the subject, to overcome the intensive global competition, various marketing strategies are nowadays adopted within the olive oil sector. Among them, those based on the creation of added-value products through differentiation and high quality outputs for the competitive positioning on both traditional and emerging markets are, nowadays, arousing great interest (in particular for medium and small olive oil companies, because they enable such firms to partly escape direct confrontation with big companies that have higher producing, processing and trading power) [84]. Basically, these differentiation

strategies guide to a kind of market segmentation, where medium and small olive oil firms move away from commodity market into more lucrative niche markets through the differentiation of their oils from others, demonstrating the uniqueness of their products. Today, several differentiating trends are common in the world olive oil market, including those based on olive oil internal attributes (olive oil commercialized under monovarietal label certification), those taking advantage of the renewed interest of the consumers in organic food (olive oils commercialized under organic label), and those that linked olive oil to a specific geographical origin or territory (olive oils commercialized under geographical indications labels).

Typically, olive oils labeled as "Monovarietal" or “Single Varietal" are elaborated using only one variety, in order to achieve unique flavor and certain specific characteristics directly related to this variety. To understand the essence and definition of the concept of monovarietal olive oils as a marketing differentiation strategy, it is necessary to recall that olive oil’s composition and organoleptic characteristics are widely correlated to the cultivar from which it is elaborated. With this perspective, tremendous efforts have been made to catalogue and characterize the compositional and sensory profile of monovarietal olive oils elaborated from the main olive varieties cultivated in each one of the olive oil producing countries. The case of Italian and Spanish monovarietal olive oils can be mentioned, since they have been deeply studied and their main characteristics have been summarized in various books, such as those edited by Barranco, and Hernández [93,94] (for Spanish olive oils), and by Muzzalupo, and Poljuha and Sladonja [95,96] (for Italian olive oils).

Figure 15 gives illustrative examples of the sensory profiles of the main olive varieties cultivated in Italy (‘Coratina’ and ‘Frantoio’ olive oils), Greece (‘Koroneiki’ olive oil), and Spain (‘Arbequina’, ‘Hojiblanca’ and ‘Picual’ olive oils). As can be seen in the figure, the organoleptic profiles of the monovarietal olive oil obtained from these varieties are very distinctive.

The second dimension of the differentiating trends that are currently characterizing the olive oil sector is the use of geographical indications. Theyy are defined by the Trade-Related Aspects of Intellectual Property Rights (TRIPS) Agreement, in 1994, as: "*indications which identify a good as originating in the territory of a member, or a region or locality*

in that territory, where a given quality, reputation or other characteristic of the good is essentially attributable to its geographical origin" [98]. In other words, and applying it to olive oil, these tools are mostly protected geographical names linking quality, notoriety, reputation or other characteristics of olive oil to the natural environment where production takes place, and to human factors such as the know-how of producers. Alongside economic profits, these tools procure interesting social and environmental benefits and, for this reason, they are currently considered as models of rural sustainable development and preservation of the cultural heritage; they are definitely much more than simple trademarks [99].

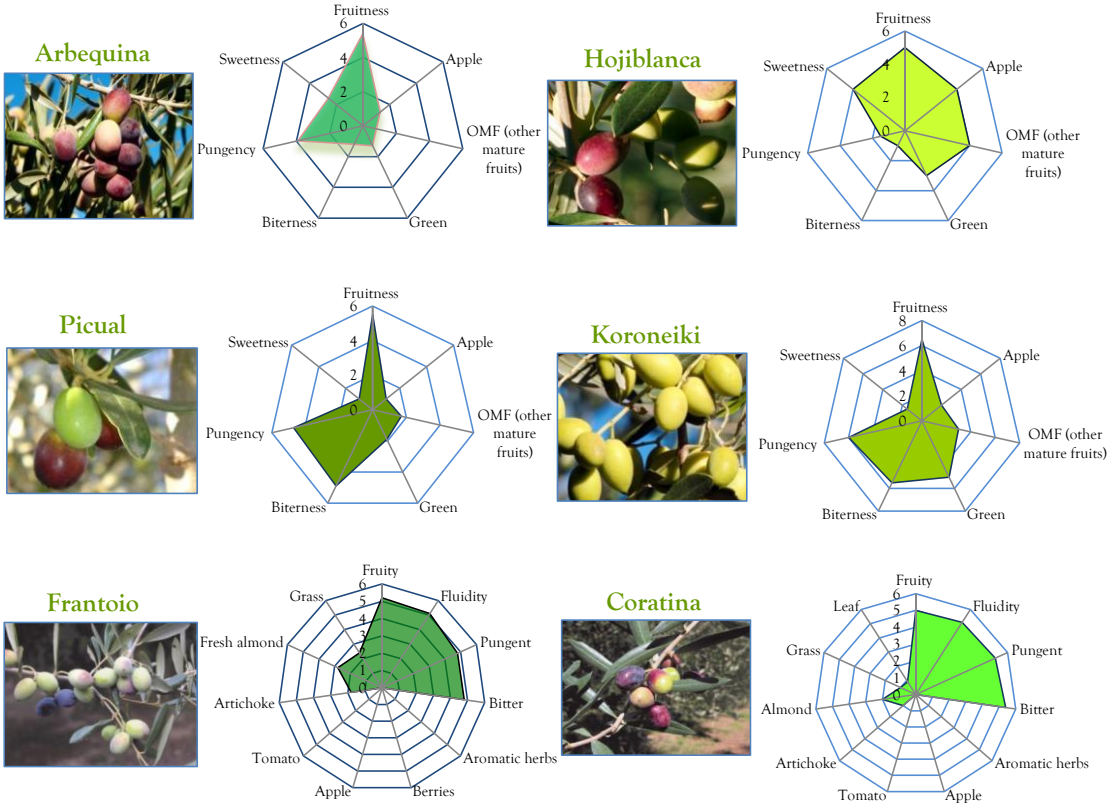

Figure 15. Sensory profiles of some monovarietal olive oils. Source: 'Frantoio' and 'Coratina' profiles reproduced from Poljuha and Sladonja [95], while the remaining profiles were reproduced from Monteleone and Langstaff [97].

Labeling olive oil with geographical indications has legal requirements in almost all the olive oil producing countries where the use of these quality-guaranteed tools is permitted. Indeed, specific legal frameworks have been progressively established by a large number of olive oil countries (at both national and regional levels) to regulate the

use of geographical indications. They are at the moment regulated by a wide range of concepts which include, among other, laws against unfair competition, the assurance of consumer´s protection and specific regulations for the protection of geographical indications. This is the case, for example, of the EU quality schemes, which identify geographical indications and traditional specialties with the purpose of promoting and protecting names of quality foodstuffs; this is regulated by Commission Regulation (EC) No 510/2006, concerning the protection of geographical indications and designations of origin for agricultural products and foodstuffs [100].In this framework, as it happens with other food product, three geographical origin-based quality schemes are commonly used to recognize, promote and protect names of quality olive oil. They are: protected designation of origin (PDO), protected geographical indication (PGI) and traditional speciality guaranteed (TSG) (Figure 16).

As a matter of fact, for an olive oil to be registered under the PDO scheme, it should exhibit qualities or characteristics due *exclusively or essentially* to a delimited geographical environment in which its production takes place, including natural and human factors. Furthermore, every production stage, processing and preparation of the protected olive oil must occur within the defined geographic area.

Figure 16. Official labels for PDOs, PGIs, TSG and organic products in European Union.

In addition, for an olive oil to be qualified as PGI, a certain level of its quality, reputation or other characteristics must be attributed to the delimited geographic area where it was produced. In other words, the link with the geographical area does not need to be "*essential or exclusive*", but it has to be sufficient to assure that the features or the reputation of the olive oil is "attributable" to its geographical origin. Moreover, it is absolutely required that, at least, one of the stages of olive oil production, processing or

preparation takes place in the delimited geographical area. Therefore, compared to PDO scheme, the link with the geographical origin in the case of PGI is less strong or robust.

The last quality scheme, TSG, denotes traditional character, either in the composition or means of production. In other words, TSG is defined by two distinct and highly related elements: olive oil specificity and its traditional nature. Within this context, this tool refers with a traditional name, to an olive oil with well-known specific characteristics that are not necessarily linked to its geographical origin, but are related to the use of local know-how, such as an ancient practice of olive production or oil processing based on a tradition.

If we now focus on the current situation concerning the use of geographical indications in the olive oil sector (Figure 17), it can be said that since 1975, where a PDO was for the first time recognized in Spain for "Les Garrigues" olive oil (and later certified by the EU in 1996), an impressive expansion of these quality-guaranteed tools has been observed. Thus, in 2015, about 123 olive growing regions registered their olive oil production under PDOs (108) and PGIs (15) schemes. EU olive oil producing countries have obviously taken the lead in identifying and recognizing various olive oils under these designations; about 114 PDOs and PGIs, most of which are located in Italy (43), Greece (29), and Spain (28). Outside EU zone, Morocco (2 PDOs and 3 PGIs) and Turkey (4 PDOs) are the only olive producing countries which have registered geographical indications for some of their olive growing areas.

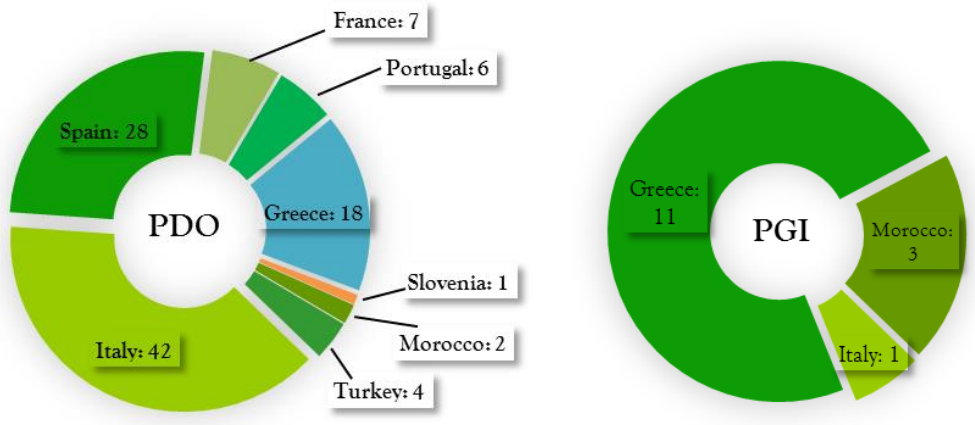

Figure 17. Current status of the use of official labels for PDOs and PGIs olive oil certification all over the world. Figure elaborated using the available information from different sources [101-105].

Furthermore, within the EU zone, sales volumes are concentrated in Spain (44% of the total EU volume, coming from about 696,147 ha), while in France PDOs enjoy the largest share of national olive-oil production (19%) [106]. In economic terms, the production value of the olive oil marketed using PDOs-PGIs was, on average, 215 M€ per year from 2006 to 2008, and 203 M€ in 2010 [107].

Notwithstanding this importance, it should be noted that geographical indications themselves do not provide an effective competitive advantage at national and international levels, especially if the product is not promoted through an effective marketing strategy. That means that a proper communication and promotional planning represents a strategic role for valorizing the olive oil produced under geographical indications schemes; what has to be made throughout highlighting the uniqueness of their quality attributes and compositional characteristics, as well as the local know-how and the historical richness of their geographical origins. However, promotional actions are often neglected or not applied because of financial feasibility constraints. This is the reason explaining why the price of olive oils produced under geographical schemes varies in a wide range (for example, in Italy, the price of one liter of olive oils produced under PDO certification can vary between a minimum of 2.3€ and a maximum of 30.7€ (Figure 18).

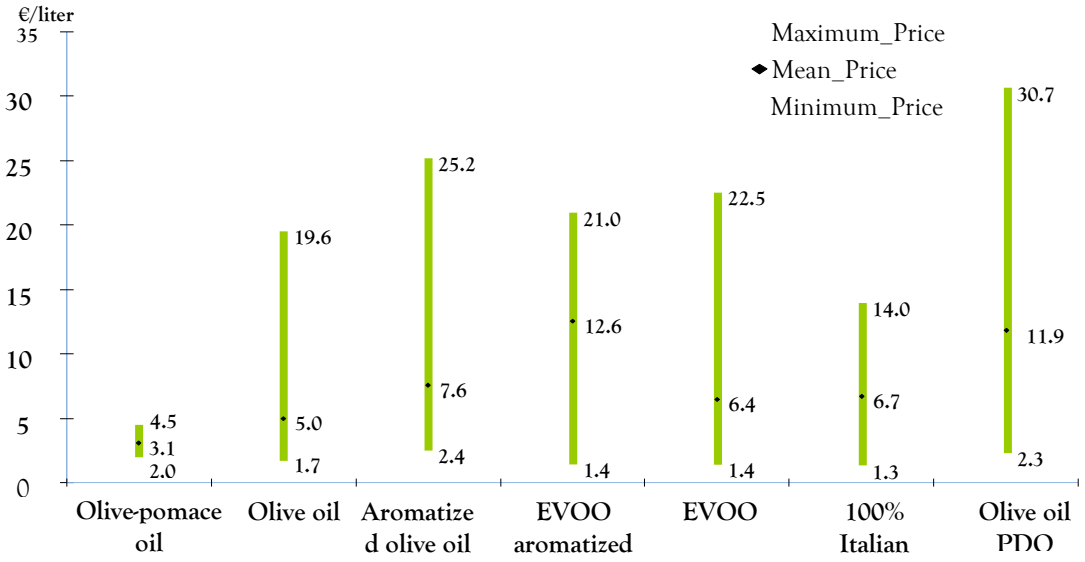

Figure 18. Variation of olive oil prices in Italy according to commercial category and labels. Reproduced from ISMEA [108].

As already mentioned, another relevant marketing differentiating tool commonly used in the olive oil sector is the label "Organic Olive Oil". Indeed, over the past decades, even though organic olive growing still only accounts for a relatively small share of olive oil production is expanding in numerous olive oil producing countries, propelled by an increasing environmental concern, governmental technical and financial support, and also stimulated by the increasing consumers´ "green attitude". Premium price for organic olive oil is also relevant. Thus, organic olive oil production is no longer limited to those farmers to whom organic production is a holistic life-style, and has not be to be sold through specialist outlets; it has reached the mainstream of the olive oil supply chain as an appropriate opportunity to satisfy a niche market at premium prices.

Like any other organic foodstuff, the organic olive oil market is highly dependent on the consumers' trust in the system of certification and guarantee of the original products. In almost all countries where organic labeling is permitted, there are production standards to be applied and strict certification procedures often controlled by a legally constituted organism or authority. Giving this as pretext, "organic" label is mainly considered a claim on the production process rather than a claim on the product itself. For instance, it can be cited the example of EU, where organic olive oil production is regulated by the Council Regulation (EC) No 834/2007 of 28 June 2007 on organic production and labeling of organic products, repealing Regulation (EEC) No 2092/91 and the subsequent amendments [109]. In recent times EU has established equivalence arrangements with various non-EU countries for the import-export of organic products, which widely contribute to enhancing organic olive oil trade worldwide.

According to Swiss Research Institute of Organic Agriculture-International Foundation for Organic Agriculture (FiBL-IFOAM) data, in 2013, worldwide cultivated surfaces with organic olive growing exceeded 611,000 ha, almost doubled since 2004 (Figure 21). This represents 5.55% of the world's olive growing area (about 11 million hectares according to IOC data) [110].

Figure 19. Evolution of the organic olive growing area worldwide over the period 2004-2013. Reproduced from FiBL-IFOAM [110].

Furthermore, at a worldwide level, the organic olive growing sector is widely dominated by European countries (Figure 20). About 68% of the world's organic olive growing area is located in EU. Italy is the country in the world in which organic olive growing is most spread (175,946 ha, 2013), followed by Spain (168,830 ha, 2013) and Greece (44,948 ha, 2013). Outside EU zone, Tunisia shows the largest organic olive growing with 124123 ha in 2013.

In terms of organic olive oil production, based on the estimation made by French olive and olive oil interprofessional organization (AFIDOL), Spain (55000 tons) Italy (45000 tons) and Tunisia (28000 tons) are leading the world's organic olive oil market [106].

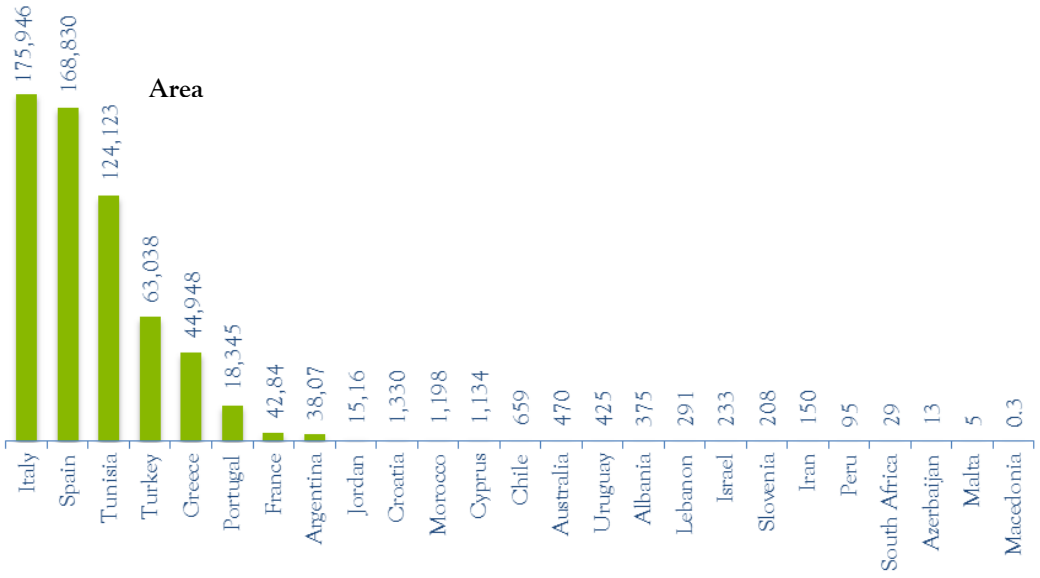

Figure 20. Distribution of the worldwide organic olive growing area (ha) per countries in 2013. Reproduced from FiBL-IFOAM [110].

3. Focus on Moroccan olive oil supply chain

Morocco has traditionally been a land of olive tree cultivation and olive oil production. The beginnings of olive farming in Morocco date back to the first millennium B.C., as evidenced by historical and archeological investigations carried out at Phoenician and Roman sites in Morocco (Lixus and Volubilis) [4]. The archeological and ethnographic objects and utensils found out in these sites attest the importance of olive tree and olive oil in the everyday life and people's art living throughout the history of Morocco. Therefore, over time, this crop has become an essential component of the agricultural landscape of Morocco; indeed, its environmental, social and economic importance in various regions of this country is undeniable. This crop represents over 50% of the surface occupied by fruit trees in Morocco, its cultivation mobilizes an intense agricultural activity with more than 11 million working days per year, and it covers 16% of Morocco's consumption requirements in edible vegetable oils and contributes up to 5% of upstream agricultural gross domestic product [111]. For all these reasons, olive oil sector is, at the moment, arousing unprecedented interest, to such an extent, that is considered as one of the main pillars of agricultural sustainable development of various Moroccan regions. In 2008 the new Moroccan strategy for agricultural sustainable development, called "The Green Morocco Plan" (GMP), was launched; since then, major restructuration and modernization programs of the Moroccan olive sector have been

adopted, being essentially based on investment and value chain organization as keys to success. Within this program, tools such as infrastructure development, planting of new olive trees, rehabilitation of old olive groves, organization of olive farmers in cooperatives and providing them modern olive oil processing and storage units, training and assistance to olive farmers in enhancing the productivity of their orchards and the quality of their olive oils, are being used. The long-term objective behind this strategy is to double Moroccan olive growing area (from 648,110 ha in 2008 to 1,300,000 ha in 2020) and to multiply olive oil production by nearly 4 times (from 75,000 tons in 2008 to 330,000 tons in 2020) [112].

3.1. Production and trade profile: general characteristics and main statistical findings

Owing to its adaptability to highly adverse soil and climatic conditions, olive tree is currently grown, under very contrasting ecological conditions, throughout the country, except for the Atlantic coastal areas. The total olive growing area in Morocco, in 2015, was 1,020,000 ha [7], corresponding to about 9% of the world's olive growing area (estimated in about11 million ha according to IOC); the olive production in 2013/2014 was over 1.5 million tons. The main Moroccan olive producing regions in 2013 are listed in Figure 21.

Thus, as can be seen, olive tree is grown in many regions across Morocco, but approximately 54% of the Moroccan olive growing area is concentered in northern regions (Meknès, Fès, Taza-Taounate-Al Hoceima and Tanger-Tétouan), where approximately 50% of Moroccan olive fruits are produced. It should be also emphasized that, practically, in all Moroccan olive gowning zones, 70 to 80% approx. of olive production is processed into olive oil. The olive orchards productivity per hectare widely varies between regions (an even within the same region), mainly according to the farming practices used and pedoclimatic conditions of the region.

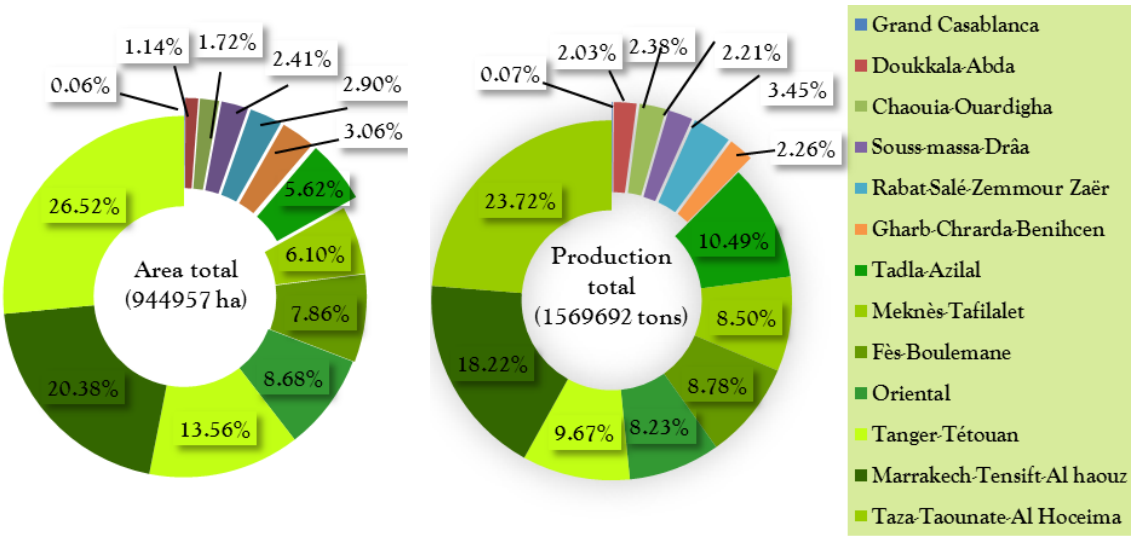

Figure 21. Moroccan olive producing regions (area and production in 2013/2014). Elaborated from data of Moroccan Ministry of Agriculture and Marine Fisheries (MAPM) [113].

Figure 22 illustrates the evolution of olive growing area and olive oil production in Morocco between 2002/2003 and 2014/2015. What can be observed is that, when considering the area evolution, two distinct periods can be outstanding: the first one between 2002/2003 and 2007/2008, corresponding to the period before adopting the GMP strategy, showing a relatively low growth rate of 8.7%; and the second period, 7 years post GMP adoption, between 2007/2008 and 2014/2015, for which the expansion of Moroccan olive growing area was remarkably strong, displaying a growth rate of 57.4%.

Furthermore, under the GMP reforms, olive oil production in Morocco has consistently increased from 85,000 tons in 2007/2008 to 130,000 tons in 2013/2014. However, as clearly shown by Figure 22, the Moroccan olive oil production shows noteworthy year to year fluctuations. Variations in weather conditions and alternate-bearing habit of the cultivated olive varieties are the main determinant factors of olive oil production annually in Morocco. The principal olive variety in this country is 'Picholine Marocaine', which represents 90% to 96% share in production, depending on the olive growing region. This typical dual-purpose olive variety (used as table olive and in oil production) is especially appreciated for its high adaptability to the different pedoclimatic conditions across Morocco, as well as presenting interesting agronomic traits, oil quality and compositional characteristics [114].

However, in contrast, this variety shows a strongly alternate bearing (produces heavy crops every other year; i.e. a biennial bearing) [115], and it is very susceptible to the main olive fungal diseases, particularly, those caused by *Spilocaea oleagina*, *Verticillium dahliae* and *Fusarium solani* [116], which explains the obtained low per hectare yields in various Moroccan olive growing regions and the year to year fluctuations in the overall Moroccan olive oil production.

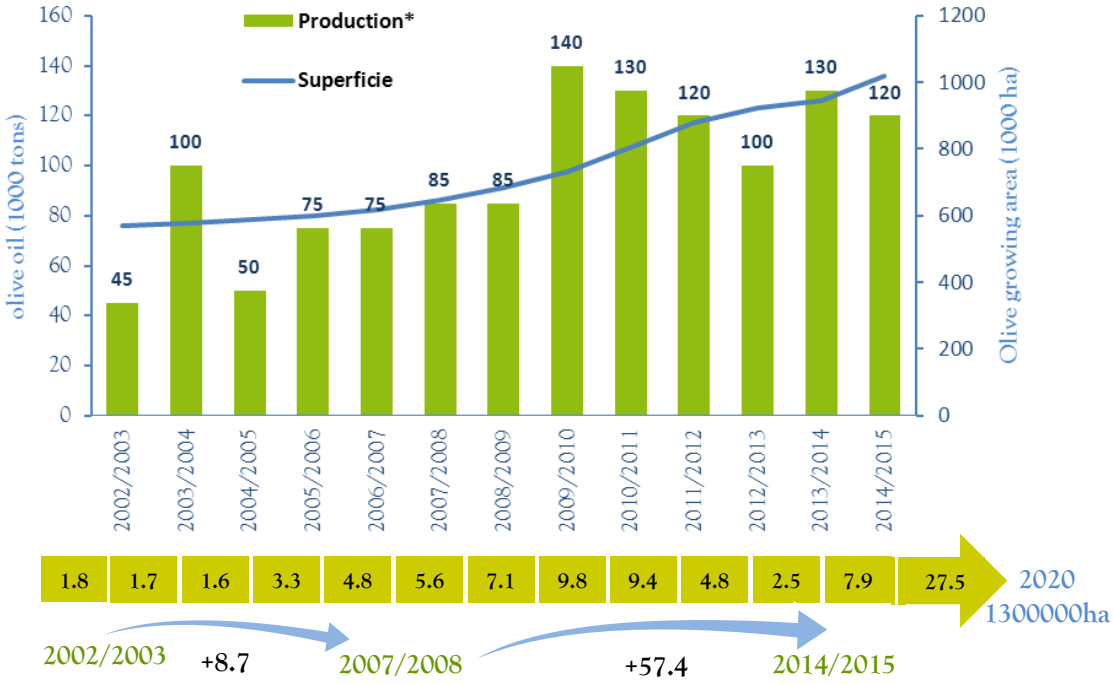

Figure 22. Moroccan olive growing area and olive oil production evolution. Elaborated from data of MAPM [117] and IOC [79]. * 2014/2015 production was an estimation.

Furthermore, other drawback of this variety is its high extent phenotypic and genetic heterogeneity, nowadays recorded in almost all the Moroccan olive growing regions [114], suggesting that the variety commonly denominated 'Picholine Marocaine' masks or includes several denominations of local varieties ('Bouchouika', 'Meslala', 'Noukal', 'Berri Meslal', ect.) with very distinct morphological and genetic features, as well as oil composition characteristics. Some of the local genotypes identified in North Moroccan regions are presented in Figure 23. In an agricultural context, this fact can logically complicate olive orchard management, when fertilization, irrigation and other

agricultural practice have to be applied, accordingly to the cultivar demands and its agronomic characteristics.

Other cultivated varieties in Morocco, in small proportions and varying according to olive growing regions, are 'Houzia', 'Menara', 'Meslala', and 'Dahbia', obtained via clonal selection from 'Picholine Marocaine', and other Mediterranean olive varieties, recently introduced in Morocco, such as 'Picholine de Languedoc', 'Picual' , 'Arbequina' and 'Frantoio'.

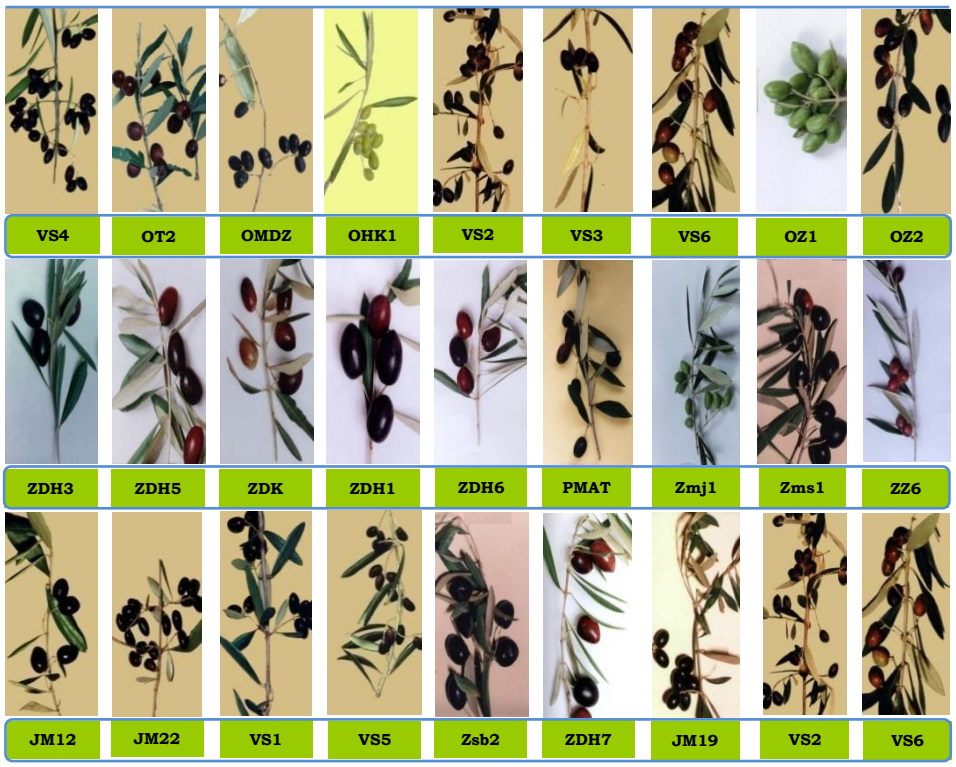

Figure 23. Phenotypic diversity of the cultivar 'Picholine Marocaine'.

It is expected that, in the future, the proportion of 'Houzia' and 'Menara' in the overall Moroccan olive growing area will increase, because of the current interest in using these varieties for planting new olive orchards. The planted variety largely determines the type of olive growing systems adopted in Morocco. The traditional rain-fed systems with olive tree density of 100 trees per hectare, and even lower, are the most common in almost all Moroccan regions. This can be explained considering the agronomical characteristics of the predominant 'Picholine Marocaine' variety in Morocco (it is a vigorous variety that can properly tolerate drought). Other olive growing systems such as traditional irrigated, intensive irrigated and super-intensive irrigated can be also found in Morocco, in

particular, in the case of olive orchards planted using varieties introduced from various Mediterranean countries.

As far as the Moroccan olive oil manufacturing sector is concerned, what can be highlighted is that on the basis of technological progress, Moroccan olive oil mills may be broadly grouped into two distinct categories [112]:

- Traditional small mills called "Maâsras", encompassing over 16,000 traditional grinding units based on the classical pressing system for oil extraction. They are generally characterized by a low technological level and processing capacity. These "Maâsras" approximately produce 29% of Moroccan olive oil, and, recently, many of them have adopted modern crushing methods for the millstones or are separating water from the oil using vertical centrifuge;
- Modern milling units, including over 288 units using advanced technology for olive oil extraction (two and three phase extraction systems) and many of them are vertically integrated to comprise milling, bottling and commercialization. Olives for these producers can be sourced directly from the firm's own trees (through contracts with multiple olive producers). These plants are located in Fès, Meknès and Marrakech regions.

Considering the Moroccan olive oil trade, most of the olive oil produced in Morocco is traditionally sold domestically, because of its price in domestic market (generally between 3 and 4.5 euros/liter). This can explain why Moroccan olive oil export is still low (10,000 to 15,000 tons/year) [117], even if the production level is rising. However, thanks to supportive government policies (within the GMP strategy), there is a trend of increasing export through private traders and specialized firms. The EU has traditionally been Morocco's largest export market for olive oil. However, under a free trade agreement, Moroccan olive oil exports to the US market have increased over the last years. Most of Morocco's olive oil exports are in bulk, either to the EU or to USA, even if recently some Moroccan olive oil producers have established premium olive oil brands, with sensational packaging layout and export high-paying gourmet or niche market rather than traditional mass markets.

3.2. Challenges and perspectives

Besides the utmost importance of olive oil sector in Moroccan economy and the numerous initiatives started within the GMP strategy for its upgrading and modernization, there are still existing constraints which slow down the development of this sector. This is particularly true at four levels:

- The first is related to the income from olive oil sales, which remains very low in various Moroccan regions, even if it widely depends on adopted olive growing system. Indeed, although various olive oil producers from other Mediterranean countries put forward low production costs as a competitive advantage for Moroccan olive oil sector, the low productivity of Moroccan olive orchards (in terms of olive fruits: between 0.5 and 1.5 t/ha in rainfed areas and 1.5 and 3 t/ha in irrigated areas) compared to the yield per hectare obtained in other Mediterranean countries, causes that the average production costs in Morocco are generally higher than in other major producing countries. This globally reduces the Moroccan olive oil's competitiveness. Furthermore, interannual irregularity of production constitutes a serious problem for both income of farmers (in particular, small farmers) and for Moroccan olive oil exporters, risking their customer's loyalty. To overcome this impediment, the adoption of good and efficient olive orchard management practices is absolutely mandatory to increase olive productivity and reduce irregularity of production from one year to another;
- The second handicap is the low per capita consumption of olive oil in Morocco, estimated to be annually around 3 kg,, remaining quite low in comparison to other olive oil producer countries. Indeed, although most olive oil produced in Morocco is domestically consumed, this foodstuff accounts for only a small share of Morocco's overall consumption of edible oils. The edible oil consumed in Morocco is mostly imported. Thus, tremendous efforts have to be made for promoting olive oil consumption in this country;
- The third limitation is the quality reputation of Moroccan olive oil; the idea or perception that most of the olive oil produced in Morocco is of too low quality must be fought with. In fact, even though the quality of Moroccan olive oil produced by traditional methods still requires further improvements (oil sold domestically), it should not be denied that great advances have been achieved over

the last years regarding production of olive oil of high quality, to the point that many of Moroccan olive oil producers have won multiple awards at international olive oil competitions. Accordingly, in addition to the different actions undertaken to support olive oil producers to improve the final quality of their products, there is a need to promote Moroccan olive oil quality at both national and international levels. Within this context, the MAPM has recently drawn up a strategy to support the competitiveness of Moroccan olive oil through enhancing the quality labels so-called "Distinctive Signs of Origin and Quality (SDOQ)" which cover PDO, PGI and TSG certifications as well as organic olive oil. Specific laws (law No. 25-06 (published in 2006) related to distinctive signs of origin and quality and law N°39-12 of organic production of agricultural and aquatic products (published in 2013)) were adopted to regulate the acquisition of such labeling schemes. In fact, these schemes claim to be an assurance to consumers (in Morocco and internationally) through improving companies reputation, inspection, certification or specific product control. Thus, since the adoption of these regulations, the productions of five Moroccan regions were labeled (3 PGI and 2 PDO) (Figure 19) and others are about getting the label. Also, around 1198 hectares are actually certified for producing Moroccan organic olive oil (Figure 20);

- The research & development (R&D) efforts going into supporting Moroccan olive oil sector development are still insufficient. A renewal of Moroccan olive growing system and sustainable development of its olive oil sector, in our opinion, cannot be accomplished by only adopting new production techniques, organizational solutions and promotional actions (although there are mandatory); it is also necessary to examine the knowledge status in this field in Morocco (which unfortunately is quite low) and go for R&D projects and scientific researches, that could drive and support sustainable development of this sector in Morocco. Within this context, there is no doubt that tremendous efforts have been made for characterizing the genetic diversity of 'Picholine Marocaine' and other local varieties; indeed, since 2011, a very wide number of scientific papers published about Moroccan olive sector fall within this scope. However, with regard to olive-growing management practices, oil extraction technology along

with an evaluation of their effects on Moroccan olive oil quality and composition, limited amount of research works can be found in literature. Basically, there is a knowledge gap in some matters, such as: (1) determination of quality and purity criteria of Moroccan olive oil: this fact could obviously have negative repercussions on the commercialization of Moroccan olive oil, exposing it to risks of rejection for failing to comply with IOC regulation. (2) Characterization of nutritional quality of Moroccan olive oil: this issue is of paramount importance, as the scientific advances in this area will not only led to a better understanding of Moroccan olive oil composition, but also will allow Moroccan olive oil companies to market their products with specific health claims. To the best of our knowledge, until 2011, no work addressing this topic could be found. (3) The establishment of analytical approaches to control possible Moroccan olive oil adulteration: the price of olive oil (both in national and international market) can lead to adulteration attempts trying to increase economic profits, so, the development of fast, efficient and low cost analytical methodologies to authenticate olive oil could be of great help to Moroccan authenticity control agencies as alternative to the highly empirical, time consuming and costly standardized procedures). And (4) the development of methodological approaches to prove distinctiveness of Moroccan olive oils and trace their geographical and botanical origin: these approaches are of great importance for Moroccan olive oil produced under geographical indications schemes and/or certified as monovarietal olive oils.

The need of adopting approaches dealing with these scientific topics applied to Moroccan olive oil is quite evident. From our point of view, new high-throughput omics technologies together with advanced data analysis strategies could provide unprecedented opportunities to characterize olive oil. Some relevant aspects related to omics approaches used in food analysis are going to be considered in the next section.

4. Omics approaches applied to olive oil analysis: from data acquisition to biomarkers discovery

Over the last decades, progresses in olive oil analytical methods allowed scientists to demonstrate the role of this foodstuff in human health, and not to simply hypothesize it. Results from recent scientific studies have established a credible basis for considering this vegetable oil as a functional food. As a result, researchers in olive oil analysis are pushing to move from classical methodologies to more advanced, robust, efficient, sensitive, and cost-effective analytical methodologies focused, among others, on the analysis of the main compounds from this complex matrix, their bioavailability and bioactivity and their possible effects on human health, as well as on ensuring the quality, safety, and traceability of this product in compliance with legislative regulatory systems and consumers' expectations. The number of studies derived from these trends is impressive, with valuable contributions and interaction between multidisciplinary groups with members coming from different areas of expertise, in particular nutrition, medicine, pharmacology, biotechnology, analytical chemistry, etc. Subsequently, there is an increasing attention around the use of the so-called "omics technologies" to investigate topics of relevance in olive oil science, that some time ago could be considered out of reach. In any case, there are some difficulties to be faced, mainly derived, among other factors, from olive oil matrix complexity, the large number of different nutrients and bioactive compounds contained in this matrix, their very different concentration levels, and the numerous targets with different affinities and specificities that they might have.

To contextualize, let´s start with the word "omics", it is derived from the Greek suffix "*ome*" (meaning all, every, whole, or complete) and refers to the comprehensive analysis of all the biological components of a given system, and often consists of systems biology approaches to assimilate tremendous amounts of data generated by omics technologies [118]. Within this frame, technologies that measure some characteristics of a large family of cellular and molecular components, such as genes, proteins, or small metabolites, have been named by appending the suffix -omics. These technologies encompass an increasingly wide variety of fields, which now range from genomics (analysis of the complete genome in order to understand the function of single genes), transcriptomics

(analysis of gene expression) and proteomics (the systematic study of proteins to provide a comprehensive view of the structure, function and regulation of biological systems) to metabolomics (comprehensive characterization of small molecules in the biological system investigated) [119]. Starting from these four categories a variety of omics sub-disciplines (epigenomics, lipidomics, interactomics, metallomics, diseasomics, etc.) has emerged.

Applied to olive oil field, omics technologies have provided important outcomes regarding several different issues. Figure 24 tries to illustrate how 'omics-based' approaches can contribute to olive oil authentication.

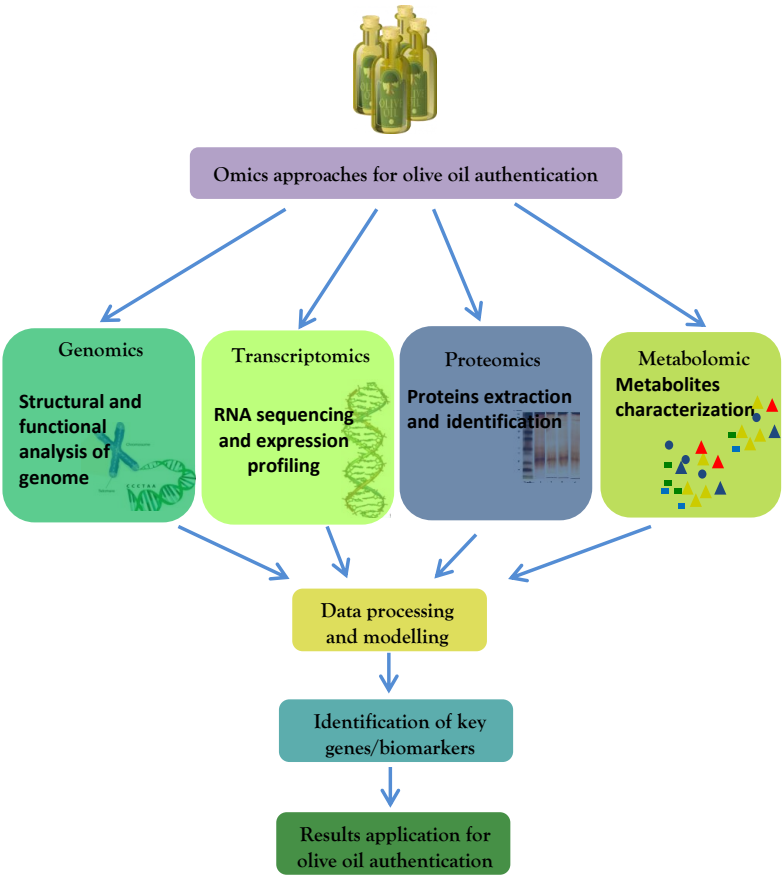

Figure 24. 'Omics-based' approaches in olive oil analysis field.

Obviously, our purpose is not listing or summarizing all relevant applications. Nevertheless, some illustrative examples can be cited. As far as genomics is concerned, olive oil DNA sequencing is progressing very quickly and different genetic markers have been discovered and are increasingly being applied to solve traceability and provenance issues [120]. Moreover, employing global gene expression profiling to understand the biological effects of olive oil-derived fatty acid ethoxylates on ageing skin [121] could

represent an example for transcriptomics; the same happens with exploring *in vivo* transcriptomic response of genes related to cardiovascular risk after a dietary intake of Mediterranean diet supplemented by VOO [122]. Interesting examples can be mentioned for proteomics too, such as the characterization of olive oil health benefits properties using various proteomic approaches [123] and the detection of olive oil adulteration and discovery of peptides markers [124,125]; and last but not least, metabolomics is also an in-vogue field which is becoming fundamental in a wide range of olive oil areas of expertise. Far from the aim of an exhaustive description of the abundant literature dedicated to the applications of omics in olive oil analysis, the following paragraphs will be restricted to explore the metabolomics approaches that have been used to carry out the experimental part of this doctoral thesis.

4.1. Metabolomics

As already stated, metabolomics is nowadays attracting a huge interest from different fields of research. Its application to olive oil analysis is not an exception, as a great variety of different methods and approaches are being constantly developed with the aim of solving challenging analytical problems in this field. Indeed, considered from a physico-chemical perspective, olive oil is a complex metabolome, consisting of numerous chemically and functionally distinct metabolites that widely vary in concentration (even throughout several orders of magnitude). This makes the comprehensive analysis of the entire olive oil metabome composition very difficult. Consequently, analytical technologies with high specificity and powerful qualitative and quantitative capabilities are in great demand to investigate the whole or a specific part of this matrix.

Metabolomics, defined as the analysis of a metabolome (the full set of endogenous or exogenous low molecular weight entities of approximately <1500 Da (metabolites), and the small pathway motifs that are present in a biological system (cell, biofluid, tissue, organ, organism, etc.) [126], can play a relevant role in the investigation of olive oil. Indeed, as the successor (or final step) of genomics, transcriptomics and proteomics, metabolomics shifts the view of biology from genes, to proteins, and to metabolites, and is, therefore, the best possible representation of the olive oil phenotype, so, for instance, it could provide in-depth insights into the substances that interact with human organism upon olive oil consumption.

Typically, the metabolomics approaches developed and applied to an olive oil study can be grouped, according to increasing levels of exploration of the olive oil metabolome, into four major groups:

- ***Targeted metabolite analysis***: refers to the detection and precise quantification of specific metabolites or a few preselected metabolites. To perform targeted analysis, one must know the structure of the target metabolite and have an analytical method developed to properly measure its concentration in the sample.
- ***Metabolite profiling:*** analysis is focused on a specific group of metabolites and requires the identification and quantification of a set of known and related metabolites (because of their structural or functional similarity or their common belonging to a metabolic particular pathway). This type of metabolomics is important to evaluate the behavior of a specific group of compounds in a sample under certain conditions; it typically requires a higher level of selective metabolite extraction and purification. A derivation of this term is global metabolic profiling, which allows detection of a broad range of metabolites by using a single analytical platform or a combination of complementary analytical platforms to obtain a comprehensive profile of the metabolome.
- ***Metabolic fingerprinting:*** corresponds to a rapid and high-throughput screening of the metabolic composition with the primary aim of sample comparison and discrimination analysis. Generally no attempt is initially made to identify the detected metabolites.
- ***Metabolomics:*** analysis geared towards providing an essentially unbiased and comprehensive (qualitative and quantitative) overview of the metabolites of the system under study. It would mean that non-biased identification and quantification of all metabolites in a biological system. Sample preparation must not exclude metabolites, and selectivity and sensitivity of the analytical technique must be high.

To date, there is no universal workflow described for all metabolomic studies. However, regardless of the applied approach, a typical metabolomic experiment (Figure 25) consists of a sequence of steps including: **(i)** design of the experiment, **(ii)** sample preparation, **(iii)** sample analysis, **(iv)** data treatment and **(v)** biological interpretation of the data.

Nevertheless, not every step is always needed. Only the starting study design, the detection and data analysis are essential steps in all abovementioned metabolomics approaches.

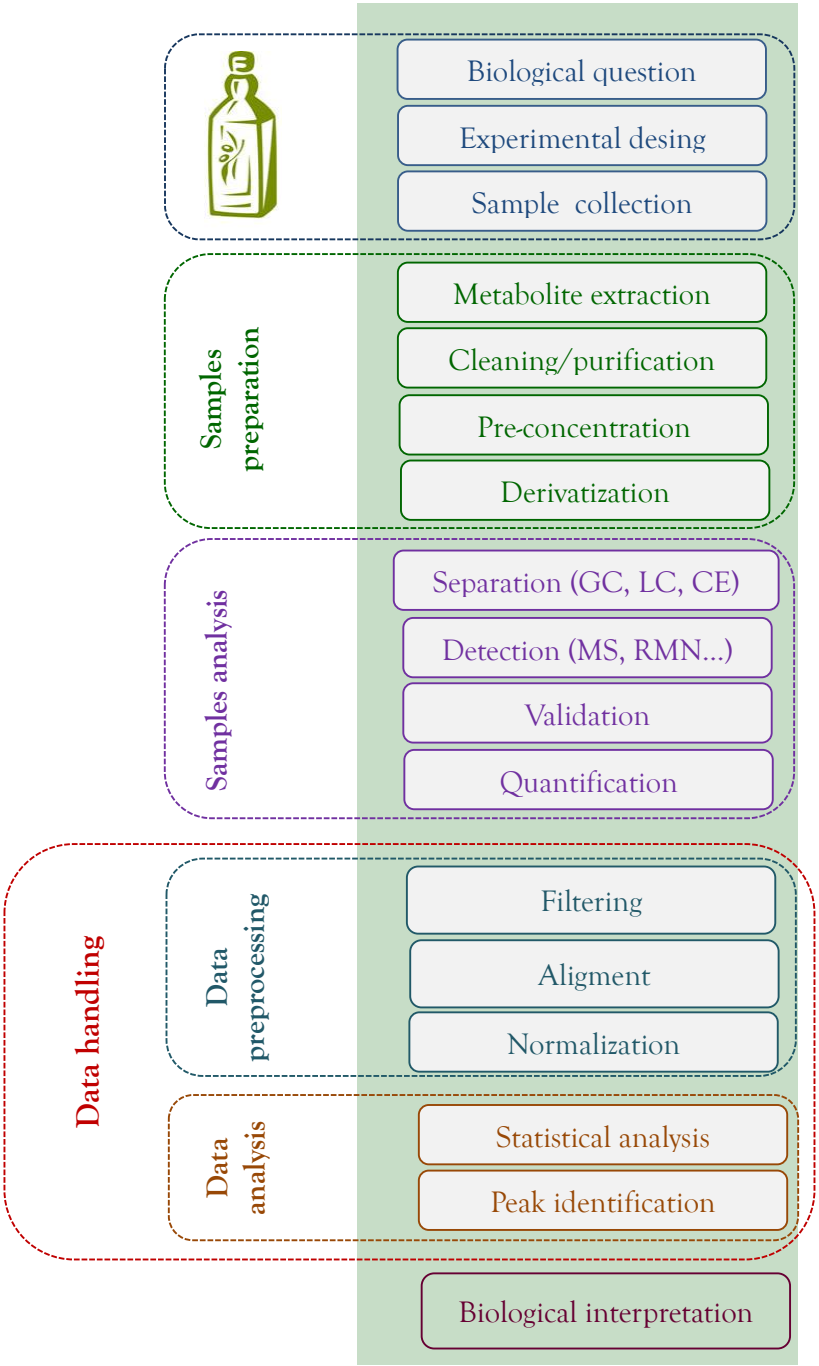

Figure 25. An outline of general workflow of the main steps involved in conventional olive oil metabolomics analysis.

4.1.1. Workflow in olive oil metabolomics studies

A careful selection of the most suitable metabolomic approach, guided by the study global purpose, is required; from the time that experimental planning commences, in order to achieve successful metabolomics experiments. A broad diversity of sampling methods, sample preparation techniques as well as analytical approaches have been reported. For this reason, understanding how to proceed in each step of the metabolomic workflow (Figure 25) is extremely important. In other words, each step needs to be clearly defined in order to ensure the accuracy of the information generated at the time of the experiment and also for the reviewing or the reuse of the raw metabolomics data at a later stage.

- ***Experimental design***

A metabolomics study should begin with asking the right biological question. Once the problem that requires an answer is fully understood, a suitable working hypothesis can be generated and the study purpose(s) are properly defined. Upon defining the study aims, the most suitable metabolomic approach can be decided and, then, an appropriate experimental design can be formulated to test the working hypothesis. The pertinent design of the study is critical to guarantee that robust scientific conclusions will be reached. By definition, experimental design is a procedure aimed at planning experiments in the most efficient way to obtain data that describe the variability associated with the investigated parameters [127].

The experimental design efficiency is mainly related to its ability to provide the appropriate foresight to plan a study to assure that the variation related to the biological observations are significantly greater than process variation - the variation introduced by performing the study. Without it, large experimental datasets can be acquired, providing no relevance to the biological objectives or leading to data what are not robust and can conduct to false observations and biological conclusions [128]. Therefore, good planning of experiments is of critical importance to warrant the reproducibility of the metabolomics analysis and to obtain high-quality data that are relevant to the problem one wants to assess.

- ***Samples selection and collection***

From our point of view, the most important decision in designing a metabolomics experiment is the samples choice. The appropriate choice of samples is crucial and widely depends on the desired research outcomes and, obviously, on the availability of the samples [129]. In the case of olive oil, there is a large variety of potential samples that can be selected depending on the study purpose. For instance, samples could be provided by producers (samples collected from vertical centrifuge, the storage containers, etc.), acquired from trademarks (commercial packaged olive oil bottles) or prepared by researchers under controlled conditions.

Another very important aspect is that the sampling conditions are very essential in terms of representativeness of the analyzed sample. In other words, the sample has to show a high degree of similarity to the total entity to be studied in order to understand the natural variation within a population. However, it is worth noting that metabolic composition of olive oil samples can widely vary inter-individual and even intra-individual. This depends on both intrinsic factors (genetic) and extrinsic factors (environmental, processing techniques…); only in specific examples, where the genetic and environmental variability is controlled to a significantly higher level (e.g., olive oil obtained from fruits samples from olive trees cultivated in the same experimental field, or in studies collecting multiple time-point samples from the same subjects), certain variability can be controlled. In all the other cases, the various factors inducing metabolomic composition variability in a particular olive oil sample set must be taken into consideration when designing the sampling protocol and, even, when interpreting the obtained results.

Regarding the number of samples examined within a study, in general, the larger the sample collection, the more credible are the results obtained [130]. Samples replications (at least 3 replications) will further eliminate the experimental operation error and probably lead to obtain statistically significant data [131].

Moreover, immediate inhibition of enzymatic activities and changes in the olive oil metabolic composition is required, generally by appropriate storage in dark glass bottles, excluding any head space volume (or by any other needed strategy). Depending on the intended analyses, and the storage duration, olive oil samples can be stored by freezing,

with or without liquid nitrogen, stored in the fridge at 4°C or at the ambient temperature in darkness [132-137].

- ***Samples preparation***

Sample preparation is another important stage in any olive oil metabolomics workflow. It involves the extraction of the compounds into a compatible format with the analytical platform to be used. In cases of metabolites present in low-abundant concentrations, sample preparation can include pre-concentration steps. The complete coverage is always a difficult task, due to the presence in this matrix of a wide array of compounds at different concentrations with varied polarities. This, together with other factors, makes a single, high-throughput extraction procedure nearly incapable to extract the whole range of olive oil metabolites. Based on the aim of the research and the metabolomic approach adopted (targeted or untargeted), many extraction protocols (widely differing in terms of their degree of selectivity, speed, and convenience) have been published, offering different advantages, but showing some limitations. For untargeted approaches, the preparation of the samples should be unselective, simple, fast and reproducible to assure the detection of a high number of metabolites. In this type of study, sample preparation is sometimes omitted (going for a direct injection of the sample). Otherwise, for targeted analysis, the sample preparation should be selective for the metabolites under evaluation and reproducible, and it should allow the incorporation of other steps (clean-up, pre-concentration, etc.), if necessary. Some examples can be cited: for olive oil volatile compounds extraction techniques such as dynamic headspace, solid phase microextraction, direct thermal desorption, simultaneous distillation-extraction, headspace sorptive extraction, and supercritical fluid extraction have been reported; whereas for phenolic compounds, the extraction procedures were traditionally based on liquid-liquid extraction, however, other extraction techniques such as pressurized-liquid extraction, liquid-phase microextraction, solid phase extraction, solid phase microextraction, ultrasound-assisted, microwave-assisted and supercritical fluid extractions have been lately applied. Detailed and exhaustive information about the most used sample preparation techniques in olive oil targeted analyses can be found in Aparicio and Harwood [37].

- ***Samples analysis***

Considering the complexity of the olive oil metabolome, the choice of the analytical method is often a critical step. Although various kinds of analytical techniques can be used for olive oil metabolomics studies, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the most common ones. Before continuing, it must be pointed out that no single analytical technique covers the entire spectrum of the olive oil metabolome; therefore, several complementary analytical platforms should be employed to improve metabolite coverage and identification power. The choice of the analytical technique depends on not only the physicochemical properties of the target compounds, but also on their expected concentrations in the olive oil matrix and on the adopted metabolomics approach.

The wide use of NMR-based methodologies in olive oil metabolomics studies can be mainly explained because it is fast, non-destructive (the sample can be recovered for further analyses) and it provides a high-throughput method that requires minimal sample preparation [138,139]. The analysis of the metabolic fingerprint of olive oil does not usually require extraction or other pre-treatment procedures. The samples or pre-concentrated samples are directly subjected to NMR analysis (just with the addition of a proper solvent), even if in some specific cases, such as the analysis of phenolic compounds, an extraction procedure could be applied [78]. The NMR applications in olive oil analysis employ ^{1}H, ^{13}C and ^{31}P protons for profiling a broad range of metabolites, such as fatty acids, triacylglycerols, mono- and diglycerides, phospholipid and phenolic compounds [78,140-142]. Also, authentication, geographical and varietal discrimination are among the main aims assessed by NMR methodologies dealing with olive oil analysis[37]. However, despite the importance of this technique in olive oil metabolomics, it mainly remains efficient for the detection of highly abundant polar metabolites. So, as an alternative to overcome the major drawback of NMR, its low sensitivity, MS-based approaches can be explored.

Compared to NMR-based metabolomics, MS-based methodologies (used alone or in combination with a previous separation technique) are superior in terms of having higher sensitivity, higher operability of instruments and better separation ability of the metabolites, since MS can work in combination with chromatography [143,144]. Among

hyphenated techniques, gas chromatography coupled with mass spectrometry (GC-MS), liquid chromatography coupled with mass spectrometry (LC-MS), ultra-high performance liquid chromatography coupled with mass spectrometry (UPLC-MS), and capillary electrophoresis coupled with mass spectrometry (CE-MS) cover a wide range of applications in olive oil metabolome analysis [145].

Since MS-based techniques were those primarily used (apart from some others) in the methodological development of most of experimental chapters carried out within this dissertation, the following paragraphs will focus on giving a brief description these analytical platforms.

- ***Mass spectrometry***

MS is a sensitive analytical technique which is able to quantify known analytes and to identify unknown molecules at very low level. The core of a MS experiment is the ionization process. Any sample or standard mixture to be analyzed must be ionized first and subsequently transferred into the gas phase before its components can be separated according to their different mass to charge (m/z) ratios by the mass analyzer. A mass spectrometer is formed by the following components: a sample introduction device (direct probe inlet or liquid interface), a source to produce ions, one or several mass analyzers, a detector to measure the abundance of ions, and a system for data treatment. Since the mass analyzer and the detector (and many of the ion sources) require low pressure for operation, the instrument also needs a pumping system to operate under high vacuum or at low pressure, so that the charged particles do not deviate from their trajectories due to collision with residual gas and, thus, never reach the detector.

There are a number of different methods commonly used for ionizing compounds; these include, among other, electron impact (EI), chemical ionization (CI), electrospray ionization (ESI), matrix-assisted laser desorption ionization (MALDI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) (Figure 28). MALDI, CI and ESI are classified as 'soft' ionization techniques, since, in principle, they do not excessively fragment the target compounds. Ionization with ESI is suitable for a broad range of compounds, and charges (and polarities, as it will be seen in following paragraphs). EI is a 'hard' ionization technique (operating at 70 eV) that produces large fragmentation of the molecule under study, but these ion fragmentation

patterns are highly distinctive and reproducible and, therefore, are useful for determining the identities of the metabolites in the sample though the use of established molecular fragmentation libraries. Most of the time it is the type of sample that dictates the best ionization technique to be applied and the type of information that scientists can derive from [146]. Table 3 gives a concise description of the most commonly used ion sources which have been applied for olive oil analysis, while Figure 26 illustrates a scheme of the molecular weights ranges and polarities covered by the different ionization systems.

Table 3. Ionization techniques commonly used in olive oil analysis by MS-based approaches. Adapted from Hurtado-Fernández [147].

Ion source	Description
APCI	A corona discharge needle provides ionization of the solvent molecules, which are in excess. The plasma so formed, contains both solvent and oxygen and nitrogen radical ions, which react with traces of water to form $H3O^+$ and OH^-, as well as other ions. These undergo ion/molecule reactions with the analyte to form $[M+H]^+$ and $[M-H]^-$ species.
APPI	The analyte is sprayed into the source in a solvent frequently containing a dopant molecule. The spray is irradiated by a powerful UV source that forms excited species which undergo secondary ion/radical molecule reactions with the analytes to cause ionization.
ESI	The eluent of the capillary or chromatographic column is sprayed at atmospheric pressure from a fine needle held at a high voltage. With the addition of heat and drying inert gases, the solvent is evaporated and a series of fine charged droplets is formed. Then, the coulombic forces between ions of similar charge on the surface exceed the surface tension of the liquid and spawn ever smaller droplets. The successive desolvation processes provide transfer of ions from the liquid to the gas phase and their introduction into the MS vacuum.
CI	Chemical reagent gases are introduced into a source, in large excess over metabolite molecules, and the reagent gas molecules are ionized by electron bombardment. Ion or charge transfer induces ionization of metabolites. The product ion is an even-electron ion with low internal energy and undergoes little fragmentation.
EI	Bombardment of the molecules by high-energy electrons creates positively charged ions. It is characterized by a heavy fragmentation highly reproducible.

Once the ions have been produced, they need to be separated according to their masses (mass/charge ratio). The ions are transported to a mass analyzer, which sorts them by their m/z ratio by means of the application of appropriate electromagnetic fields. The main characteristics of a mass analyzer are the upper mass limit, the transmission and the resolution. The upper limit determines the highest value of the m/z that can be measured. The transmission is the number of ions reaching the detector compared to the number of ions produced by the source. Finally, the resolving power is the ability to yield distinct signals from two ions with a small mass difference [146].

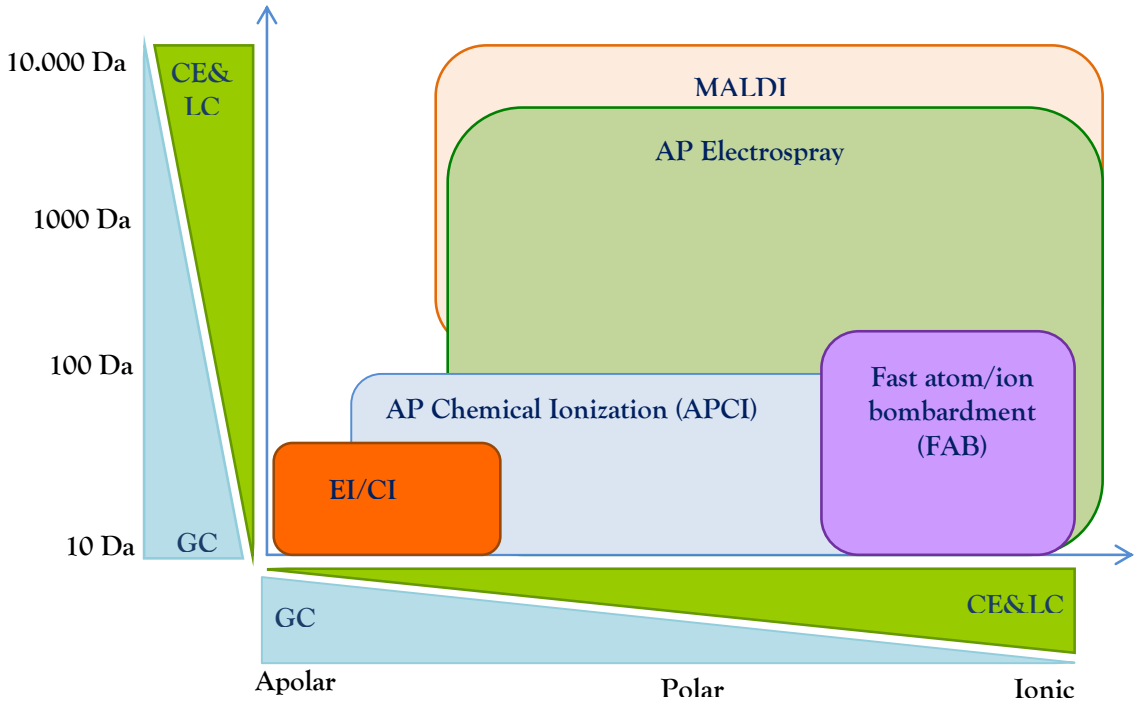

Figure 26. Range of molecular weight and polarity covered by some of the most used ionization sources.

There are many types of mass analyzers [148], using either static or dynamic fields, or magnetic or electric fields. Some of the common types are quadrupoles (single and triple), three-dimensional ion traps (3DIT), linear ion traps (LTQ), time of flight (TOF), quadrupole time of flight (QqTOF), and different types of orbitrap mass analyzers. Schemes of the most used ones are outlined in Figure 27.

- ***Gas chromatography coupled to mass spectrometry (GC-MS)***

GC-MS is one of the most extensively used analytical techniques in olive oil metabolomics studies. It is utilized to analyze qualitatively and/or quantitatively a wide range of volatile and/or derivatized non-volatile olive oil metabolites with high analytical reproducibility. This technique combines the high separation efficiency and resolution of capillary GC with the spectral information and high sensitivity of mass-selective detection provided by MS. The main drawback of GC-MS is the handling of the sample prior to analysis, which aims to generate extracts compatible with the GC technique. Indeed, a major pre-requisite for GC-MS analysis is the analyte volatility and thermal stability.

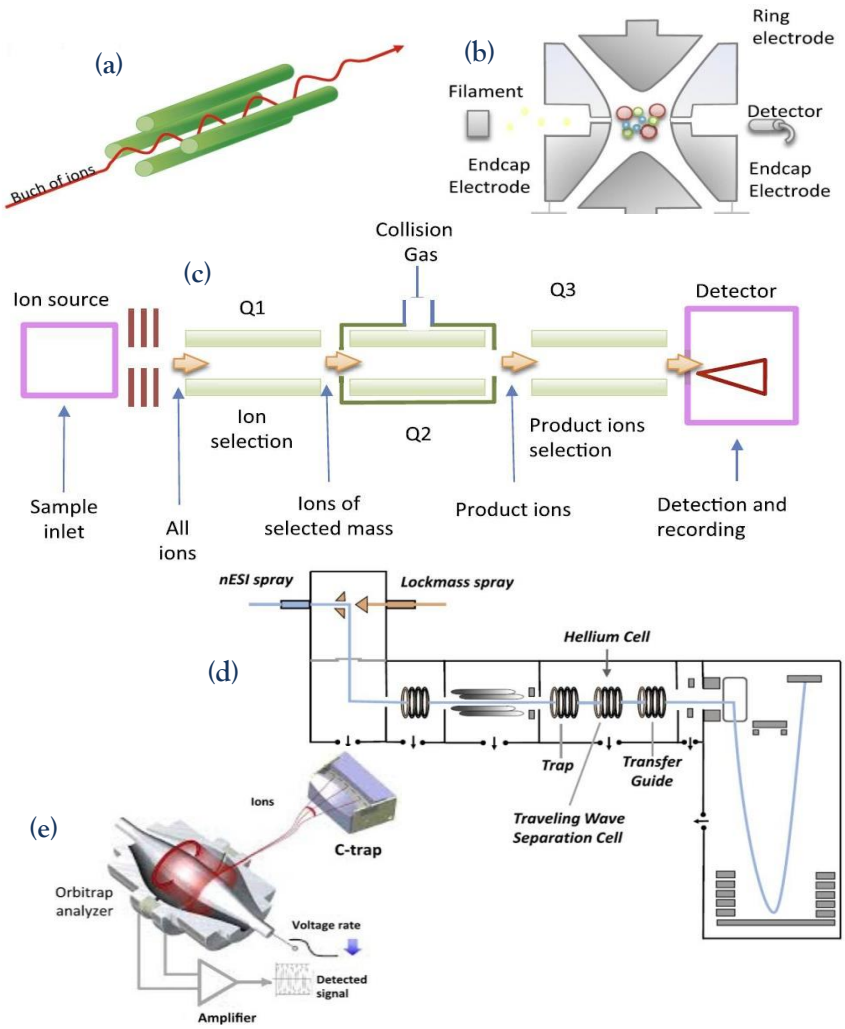

Figure 27. Scheme of several mass analyzers (a) Quadrupole, (b) 3D ion trap, (c) triple quadrupole, (d) quadrupole time-of-flight and (e) Orbitrap. Reproduced from Picó [146].

A few olive oil metabolites (especially aroma compounds) meet these requirements, whereas numerous metabolites (e.g., fatty acids, esters, hydrocarbons, and phenolic compounds) must be made volatile through chemical derivatization prior to GC-MS analysis. Sometimes the derivatization also improves the stability of thermally labile compounds. Therefore, in accordance with the functional group and the type of the mass spectrometric ionization technique, the proper derivatization reagent must be selected from a large number of commercially available reactive substances or mixtures. In general, for olive oil GC analysis, the most typical derivatization procedures utilize silylation and alkylation reactions, and a broad range of reagents with different properties [37].

In silylation (particularly effective with alcohols, phenols and carboxylic acids), the active hydrogens from acids, alcohols, thiols, amines and other groups from the molecules are modified with an inert trimethylsilyl (TMS) group; that means that silylation also

increases the molar mass of the compounds. The most common silylation reagents are bistrimethylsilyltrifluoroacetamide (BSTFA) or N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS). Both BSTFA and MSTFA are sufficiently volatile to provide little interference with early-eluting peaks, and TMCS is used as a catalyst to increase the TMS donor potential. The derivatives obtained are generally less polar, more volatile, and more thermally stable. Also, they have better MS properties, producing more favorable diagnostic fragmentation patterns for use in structure elucidations [149]. The disadvantage of silylation is its sensitivity to water, which requires that reactions must be carried out under anhydrous conditions and the derivatized samples must be stored in a dry environment to prevent degradation.

Alkylation represents the replacement of active hydrogen by an aliphatic or aliphatic-aromatic (e.g., benzyl) group in a process referred to as esterification. The principal chromatographic use of this reaction in olive oil analysis is the conversion of fatty acid into methyl esters (methylation in a methanolic medium with acid or alkaline catalysis) that produce better chromatograms than the fatty acids [37]. In general, the products of alkylation are less polar than the starting materials, because active hydrogen has been replaced by an alkyl group. The alkyl esters formed offer excellent stability and can be isolated and stored for extended periods, if necessary.

For GC separation, analytes have to be transferred into the gas phase, what is typically achieved by a hot split/splitless injector or by a programmed temperature vaporizer. With the latter, the sample can be injected into a cold injector, which is then rapidly heated using a defined temperature program. Analytes are evaporated according to their vapour pressure, reducing temperature stress for thermally labile compounds. Hot injection in the split/splitless mode is easy to handle and segregation of analytes and matrix is achieved, as non-volatile matrix components remain in the injection port. To prevent sample overload, the amount of sample transferred into the column can be controlled by the split ratio. The GC-column stationary phase determines the mode of separation; non-polar stationary phases separate analytes predominantly according to their boiling point, while polar stationary phases separate the analytes according to their polarity. Nowadays, GC separation is commonly performed using capillary columns.

Coupling of GC and MS is typically accomplished by means of EI interface. In an initial step, radical cations are generated from gas-phase molecules. Subsequent fragmentation reactions, losses of neutral groups and rearrangement of secondary ions occur because the electron energies far exceed the ionization energies of organic molecules. This yields predictable ion patterns, which can be interpreted. Moreover, EI is highly reproducible and, therefore, huge commercial spectral libraries such as NIST 11 Mass Spectral Library, have been built for the routine identification of analytes. However, structural isomers often show similar EI spectra, thus precluding unambiguous compound identification in the absence of additional information such as differences in chromatographic retention. Further, both the still incomplete coverage of natural metabolites in spectral libraries and the frequent absence of molecular ions in EI mass spectra continue to constitute the major bottlenecks in the identification of group-discriminating features. Therefore, soft ionization techniques that preserve the quasi-molecular ions (such as chemical ionization (CI) and atmospheric pressure chemical ionization (APCI)) have become outstanding in recent years.

- ***Liquid chromatography coupled to mass spectrometry (LC-MS)***

LC-MS offers a very powerful technology that provides high specificity and sensitivity for the analysis of a very large range of olive oil metabolites. LC is a more universal separation technique that can be tailored for the targeted analysis of specific metabolite groups or utilized in a broader non-targeted manner. It also offers the added benefit of analyte recovery by fraction collection and/or concentration. Furthermore, this technique does not require any derivatization step, what makes the sample pre-treatment less complicated and time-saving in comparison to GC-MS technique.

LC is recognized as one of the best ways of separation and analysis for multicomponent mixtures with molecular weights from 50 to several millions (simple molecules, macromolecules, ions, and even viruses). Furthermore, it allows us to separate mixtures of different classes of compounds, including all kinds of isomers (structural, geometrical, *cis* and *trans*, optical, etc).

The selectivity of any LC system is strictly dependent on chemistry of the stationary phase of the chromatographic column. In general, in olive oil LC-MS based metabolomics studies, the separation of the metabolites might be obtained with the use of reversed-

phase (RP) columns and electrospray ionization (ESI), both in positive and negative modes to provide better metabolome's coverage. Consequently, ESI is the most regularly used ionization technique in olive oil LC-MS metabolomics studies, although APCI is also used to a lesser extent [150]. Since the gradient RP-LC separation is intended for medium or low polarity compounds, it does not provide proper retention of hydrophilic metabolites, such as amino acids or carbohydrates. To overcome this limitation, different column chemistries, such as hydrophilic interaction liquid chromatography (HILIC) columns or weak-ion exchange columns were developed. In addition, LC column dimensions (usually 4.6 mm × 150 mm) or particle sizes (usually 3-5 μ m) logically affect the sensitivity and separation power of this technique.

However, a major disadvantage of LC-MS in metabolomic profiling (if compared with GC-MS) is the lack of transferable LC-MS libraries for metabolite identifications. The mass-spectral variability between LC-MS systems in terms of the relative ion abundances associated with adduct formation, in-source fragmentation, tandem mass spectra fragment ions, and the lack of LC retention indices that compensate for instrumental and experimental variations hinder the comparison of LC-MS data between laboratories. Thus, several research groups have constructed custom, own in-house LC-MS or LC-MS/MS libraries for automated metabolite identifications in metabolite-profiling experiments [151].

- ***Ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS)***

This subsection could be probably presented together with previous one, not making distinction, since, nowadays, quite often both types of chromatography are many times referred to by the term "LC-MS".

The arrival of the UHPLC technology into the market represented an improved and modernized version of LC technique. It provides chromatograms with high resolution and, possibly, high retention-time reproducibility, which may improve the separating ability of LC-MS. By employing columns that are packed with fully porous sub-2-µm particles and systems that can withstand pressures of up to 1200 bar, UHPLC has been demonstrated to be the most flexible, promising strategy for decreasing separation times and/or increasing resolution. Indeed, compared to conventional LC columns, the

reduction of the particle size to sub-2 µm theoretically increases both the throughput, what is often useful for metabolic fingerprinting purposes, ; and the chromatographic resolution, which is required for metabolic screening methodologies [152,153]. In combination with MS, this technique gives higher peak capacity, improved resolution and increased sensitivity compared to conventional LC. Consequently, during the past decade, UHPLC-MS platform has proved its worth as a powerful analytical tool for metabolomics studies of olive oil [154-156].

- ***Capillary electrophoresis coupled to mass spectrometry (CE-MS)***

CE is a miniaturized form of electrophoresis, where the separation is usually performed in buffer-filled capillaries made of fused silica. This material has excellent thermal, optical and electrical properties and an outer coating of polyimide makes the fused silica flexible and mechanically stable. This technique is reported for its high-resolution separation of a diverse range of chemical compounds, even if is particularly suitable for the separation of polar and charged compounds. It is a powerful technique with respect to separation efficiency and the amount of sample required for analysis is small. Furthermore, the cost is, in general, lower than in LC, due to very low organic solvent consumption, the small amount of reagents needed, and the use of capillaries instead of more expensive LC columns. The major drawbacks of CE are the poor concentration sensitivity due to the limited amount of sample volume that can be introduced into the capillary, the low absorption path-length (if UV detection is used), and its low reproducibility. In combination with electrospray ionization (ESI)-MS, lower limits of detection (LODs) can be obtained. CE combined with ESI-MS seems to have very promising characteristics as a complementary technique for metabolomic studies [157].

CE can work by using different electrophoretic modes, being capillary zone electrophoresis (CZE) the most common one. Other methods are based on the same principle but that differ in the selection of electrolyte system: capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP) capillary gel electrophoresis (CGE) and capillary electrochromatography (CEC). An overview of the main analytical approaches developed for olive oil characterization using CE and CE-MS platforms can be found in Monasterio et al. [158].

- ***Data treatment***

Due to the large amounts of information produced by the abovementioned analytical platforms, data treatment is an important challenge of any metabolomics olive oil studies. Software and computational tools, therefore, play an essential role in metabolomics data processing, analysis and interpretation. The data treatment is, generally, divided into two steps: data pre-processing and data analysis. The data pre-processing step consists into transform the raw data into a suitable format (extracted data) for the subsequent data analysis steps (multivariate data analysis and metabolites identification). The pre-processing steps usually needed in untargeted metabolomics studies are as follows: baseline correction, alignment, binning, normalization and scaling. Data treatment in targeted analyses is more straightforward than in untargeted analyses and data-processing is typically limited to matrix data construction, centering and/or normalization. In untargeted analysis, the way the signals or chromatograms should be handled depends on the technique and the instrument used. Pre-processing of raw NMR data is often incorporated in machine-vendor software and it practically includes all the above-mentioned pre-processing steps. In the case of MS-based techniques, a typical raw data file contains all the information collected as a series of full scan mass spectra acquired in successive time points (a scan event typically last 2-20 ms). Hence a data file can be considered as a cube constructed by joining a large number of planes: each plane represents a 2D full scan mass spectrum that is defined by mass (m/z) and signal intensity at the two axes. Once format arrangements have been performed, other typical pre-processing tools, including signal alignment, baseline correction, peak feature filtering, and noise reduction, are further applied to improve the data quality. Commercially available (such as SIEVE (Thermo Fisher Scientific), MarkerLynx (Waters) and MassProfiler (Agilent)) and freely (such as mzMine and XCMS) software packages exist to carry out this data processing pipeline. The task for these tools is to detect all the peaks within a mass spectrometric/chromatographic run and produce a two-dimensional data matrix: a peak table where all samples and all peaks along with their signal intensities are reported. This is done by compressing the three dimensionality of data to two dimensions, by combining the mass with the retention time information into a feature identity [159].

Once pre-processing is finished, the next step in the data treatment is multivariate statistical analysis. The approaches used (also called chemometrics) can be divided in two main categories: supervised and unsupervised chemometric data analysis[160]. The goal of unsupervised data analysis is to obtain an overview of the data without any prior knowledge and trying to identify any underlying trends in the dataset. Examples of unsupervised approaches are principal component analysis (PCA), hierarchical clustering analysis (HCA) and k-means. Typically, the preferred unsupervised method is PCA, which concentrates the information of samples and variables into the so-called scores and loadings, respectively. This technique is often used as a starting point in the data analysis process. Supervised techniques assume that some structure exists in the data and requires assigning of the samples to pre-specified subgroups using latent variables to build classification rules which are later used for allocating new and unknown samples to the most probable subgroup [161]. The supervised techniques use the mathematical algorithms to maximize the separation between different classes, and information on the analytes contributed to such classifications. The power of supervised pattern recognition methods lies in the model construction, which consists of the following steps: selection of the training set, feature or variable selection, derivation of a classification rule, and the validation of the classification rule [162]. There are three major differences between supervised pattern recognition algorithms [163,164]. There is a first distinction between methods focusing on discrimination, such as linear discriminant analysis (LDA), and those which place more emphasis on similarity within a class, for example soft independent modeling of class analogy (SIMCA). The second difference concerns linear and non-linear methods, such as neural methods. The third distinction divides the parametric and non-parametric computations. In the parametric techniques such as LDA, statistical parameters of the normal distribution of samples are used in the decision rules.

Overall, according to the pursued objective these chemometric data analysis can be divided into two main categories [165]:

- ***The discriminant-classification techniques:*** also known as two-class or multiclass classifiers. They use information about a characteristic that represents a difference among samples, so that a classification can be proposed. New samples can be ascribed to one or another group according to the proposed model. Among this family of methods may be mentioned LDA, k-Nearest Neighbor (k-NN), Partial

Least Squares Discriminant Analysis (PLS-DA), support vector machines (SVM), SIMCA, and different variants of Neural Networks, such as Artificial Neural Networks (ANN).

- ***The regression techniques*** that are used in multivariate calibration to establish the relation between two matrices by means of a mathematical model. Each matrix contains different information about the same group of objects (samples). Most often, the first matrix contains information obtained by means of a method to be replaced, and the second matrix contains information obtained by an alternative method. The most popular multivariate regression techniques include Multilinear Regression (MLR), Principal Component Regression (PCR) and Partial Least Squares (PLS). There are some other techniques derived from PLS or MLR, such as, Genetic Algorithms Partial Least Squares (GA-PLS), Moving Window Partial Least Squares (MW-PLS) and Successive Projections Algorithm Multiple Linear Regression (SPA-MLR).

Obviously, the final step of the metabolomic workflow should be the biological interpretation of the obtained results and biomarkers identification keeping in mind the aim of the study.

4.1.2. Application fields

Metabolomics has been an application-driven science with broad range of applications in various olive oil analytical areas. Among this applications, we canmention: studies of quality, composition, safety and authenticity [166,167] and geographical and botanical origin traceability [37,168-170]. Detailed information about current and emerging applications of Metabolomics in the field of olive oil authentication and botanical and geographical traceability are available in chapter 1 of this thesis.

Besides, as part of the researches activities undertaken within the present thesis, two book chapters were redacted to address the current state-of-the art concerning the use of metabolomics approaches in the analytical filed of olive oil. Thus, the first chapter was about the potential of analytical techniques combined to chemometrics to discriminate PDO's VOOs according to their geographical origin. This chapter has been published in the book: "El sector de elaboración de aceite de oliva: un estudio multidisciplinar" edited by Hernández [171]. The second chapter was dealing with the current state-of-art of the use

of UHPLC-MS in olive oil metabolomics studies. This chapter has been published in the book: Ultra Performance Liquid Chromatography Mass Spectrometry: Evaluation and Applications in Food Analysis, edited by Naushad, and Rizwan Khan, 2014 under the title "UHPLC-MS in Virgin Olive Oil Analysis: An Evolution toward the Rationalization and Speed of Analytical Methods" [172].

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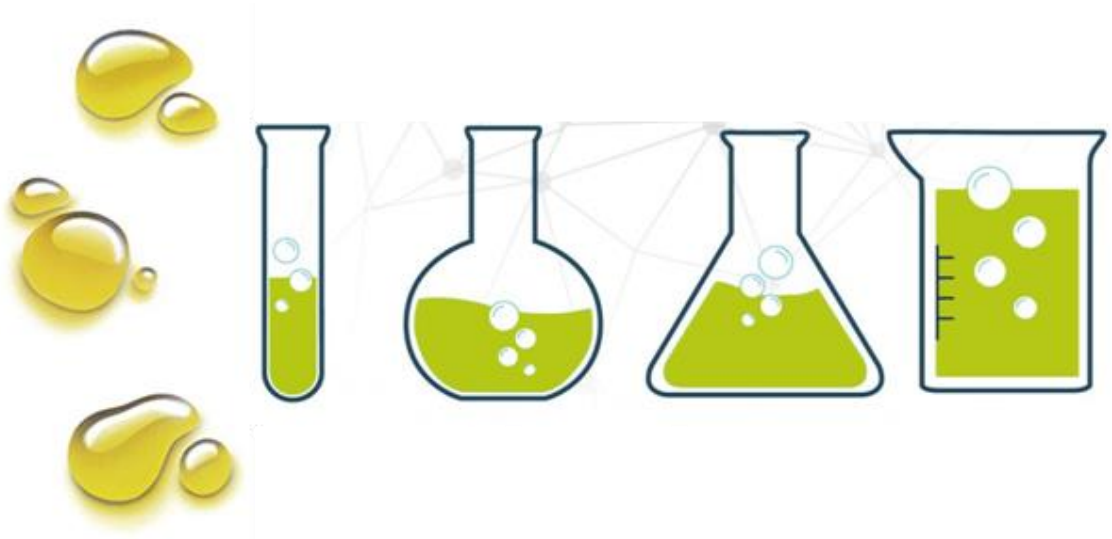
PART

2

Experimental Part, Results and Discussion



SECTION

1

"Authentication indices and geographical classification of North Moroccan virgin olive oils"

Chapter 1

Olive Oil Authentication: A Comparative Analysis of Regulatory Frameworks with Especial Emphasis on Quality and Authenticity Indices, and Recent Analytical Techniques Developed for Their Assessment. A Review

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Critical Reviews in Food Science and Nutrition *(Under evaluation)*

Abstract

Over the last decades, olive oil quality and authenticity control has become an issue of great importance to consumers, suppliers, retailers, and regulators in both traditional and emerging olive oil producing countries, mainly due to the increasing worldwide popularity and the trade globalization of this product. Thus, in order to ensure olive oil authentication, various national and international laws and regulations have been adopted, although some of them are actually causing an enormous debate about the risk that they can represent for the harmonization of international olive oil trade standards.

Within this context, this review was designed to provide a critical overview and comparative analysis of selected regulatory frameworks for olive oil authentication, with special emphasis on the quality and purity criteria considered by these regulation systems, their thresholds and the analytical methods employed for monitoring them. To complete the general overview, recent analytical advances to overcome drawbacks and limitations of the official methods to evaluate olive oil quality and to determine possible adulterations were reviewed. Furthermore, the most recent trends on analytical approaches to assess the olive oil geographical and botanical origin traceability were also examined.

Keywords: olive oil; authentication; trade standards; regulatory frameworks; analytical advances.

INTRODUCTION

Olive oil is an economically important product in most of the Mediterranean countries, where its production has longstanding historical roots. Interest in this product has recently been accentuated to a larger extent, both inside and outside the Mediterranean region, by various studies that have focused on demonstrating its human health beneficial effects and its wide culinary applications (Boskou, 2011). Large amounts of olive oil are globally consumed every year; indeed, over 2.27 million tons are estimated to be consumed from the olive crop of 2015-2016 (International Olive Council (IOC), 2015). It is also important to highlight that the worldwide olive oil consumption has steadily risen, achieving an average annual growth rate of 2.7% between 1991 and 2012 (IOC, 2014).

In parallel to this quantitative expansion of olive oil consumption, there has been an intensification of the consumer interest in high-quality oil and some labeled olive oil categories, such as organic olive oil and oils with certified geographic indications or declared as monovarietal (Di Vita et al., 2013). Keeping in mind that consumers are willing to pay higher prices for these categories of olive oil, the price achieved in the market for these products is often remarkably high, which makes them prone to suffer adulteration and mislabeling practices (Garcia et al., 2013). For this reason, olive oil authentication issues are, actually, topics of prominent importance, not only for consumers, but also for suppliers, retailers, regulatory agencies, and administrative authorities.

In a broad sense, the concept of "authentication" refers to the control of different kinds of fraudulent practices, including adulteration, mislabeling, and misleading origin, among others (Aparicio et al., 2013a; Gallina Toschi and others 2013). Indeed, as illustrated by Figure 1, because of the large number of olive oil categories that can be produced, extra virgin olive oil (EVOO), for been the olive oil top grade, is more susceptible of adulteration practices, being the most common one the addition of other olive oils of lower commercial value and/or seed oils, such as sunflower, soybean and hazelnut oils (De Oliveira and Catharino, 2015). Furthermore, the guarantee of olive oil authentic and reliable geographical and botanical origins is another subject of concern for the olive oil sector (Dias et al., 2014).

Traditional strategies to control olive oil adulteration and guarantee its quality are relied on the analytical determination of various quality and purity parameters in the evaluated material and the subsequent comparison of the obtained value(s) with those established as thresholds by the standard regulations. In this context, olive oil authentication is governed by specific regulations that define standards and criteria for classifying it, and give a comprehensive description of the analytical methods for assessing its quality and testing its authenticity. Nowadays, extensive regulatory frameworks have been laid down by different national and international organizations, such as: United States Department of Agriculture (USDA) standards (USDA, 2010), Californian State regulations (California Department of Food and Agriculture, 2014), European Commission standards (EEC, 1991), Codex Alimentarius regulations (Codex, 1991) and IOC standards (IOC, 2013).

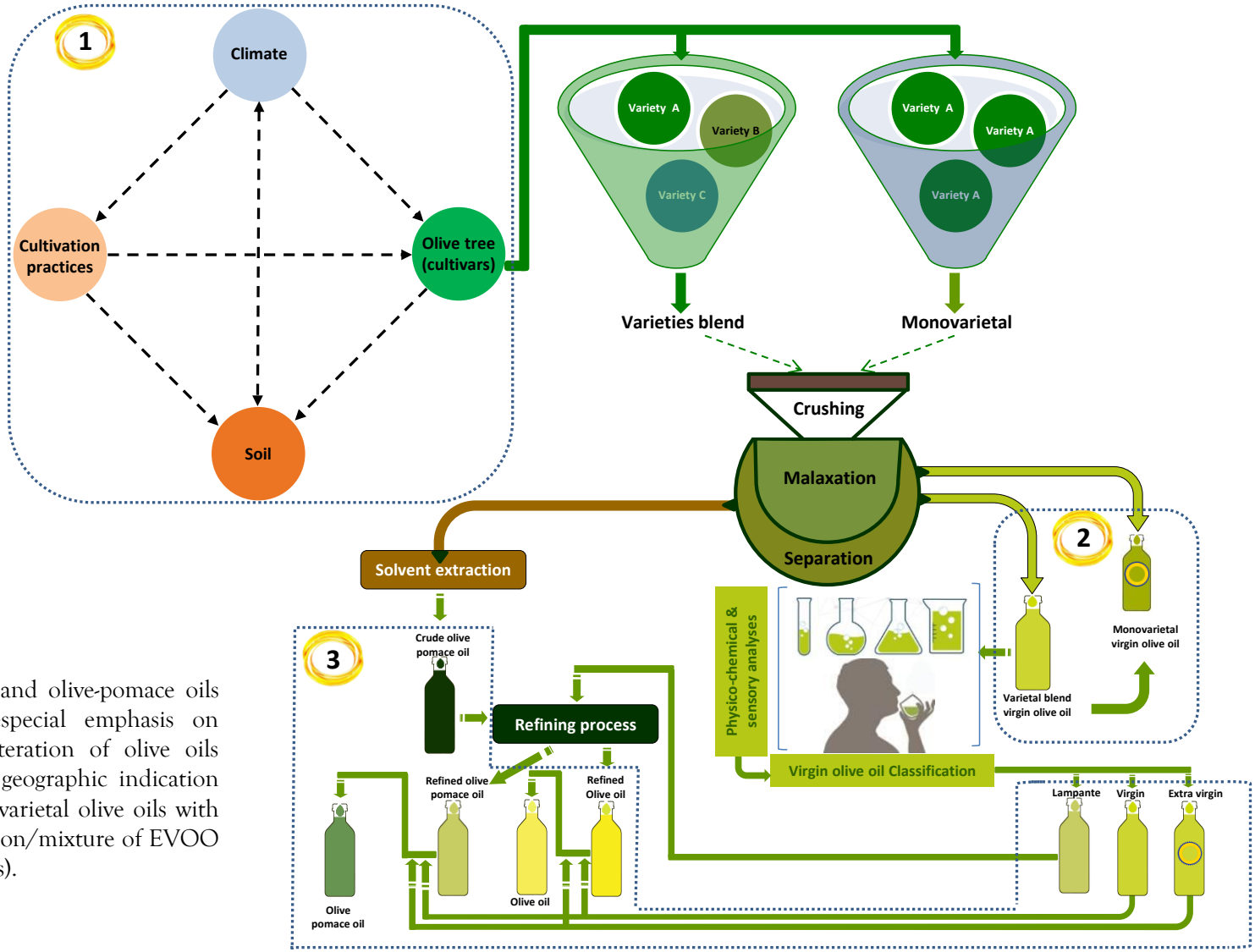

Figure 1. Flow diagram of olive oils and olive-pomace oils categories production steps, with especial emphasis on possible adulteration types ((1) adulteration of olive oils produced on specific territory/under geographic indication certification; (2) adulteration of monovarietal olive oils with varieties blend olive oils; and (3) addition/mixture of EVOO by other olive oils and olive-pomace oils).

Those from IOC have always been the most widely used for oliveoil standards grading all over the world, since they are drawn up and updated on the basis of IOC olive oil records and databases of the countries which are members of this council, which covers the vast majority of the global olive oil production. Nevertheless, although considerable efforts are dedicated to continuously update and amend IOC regulations in order to make them evolve at the same rhythm as the constant analytical innovations as well as the sophisticated fraudulent practices, currently, there is a very active global debate about olive oil standards setting and the effectiveness of official analytical methods (Aparicio et al., 2013b).

The starting point for establishing threshold values of olive oil quality and purity criteria is to know in depth the regular and usual olive oil physico-chemical characteristics and composition; in other words, it is necessary to verify what the "normal values" are. However, it is important to be aware about the fact that these properties can greatly vary in oils coming from the same country (even more if they come from different countries), depending on various factors such as variety, pedoclimatic conditions, ripening, extraction system and storage conditions, among others (Dabbou et al., 2010). In this sense, considering the fact that not all IOC countries members have developed proper and comprehensive databases for their olive oil, and the spreading of olive tree (*Olea europeae* L.) cultivation and oil production outside the Mediterranean region (the historical region of cultivation of *Olea europeae* L.), some studies have reported that certain IOC regulation limits cannot be fulfilled by some olive oils produced in various regions or countries (Ceci and Carelli, 2007; Bajoub et al., 2015). Consequently, some olive producing countries are requesting the revision of certain limits fixed by the IOC standards. Moreover, the above-mentioned debate also includes olive oil quality criteria, as some emerging olive oil producing countries, especially Californian State, suggests to modify the threshold for some parameters, such as free fatty acids (FFAs) and peroxide values (PV) (California Department of Food and Agriculture, 2014), whereas other countries, like Australia, New Zealand, and Californian State consider in their standards the measurement of new quality parameters, such as pyropheophytins (PPPs) and the 1,2-diacylglycerol (1,2-DAG) as indicators of olive oil freshness (Standards Australia, 2011).

When the official analytical methods for olive oil authenticity assessment are considered, it is necessary to face a number of challenges that can be broadly categorized into three key areas. The first is associated with the characteristics of conventional analytical methods used for the official control of olive oil quality and authenticity. Indeed, most of these methods are highly empirical, time consuming, require the use of organic solvents, generate wastes and their accuracy is strongly dependent on reproducing very literally the operating instructions of the standardized procedure (Dais and Hatzakis, 2013). The second is associated with some limitations that the conventional methods for olive oil adulteration control exhibit, such as their inability to identify the nature of the adulterant agent, their ineffectiveness at low adulteration levels, as well as their difficulties in the detection of some adulterants such as hazelnut oil, which present great similarities to olive oil regarding the triacylglycerols (TAGs) and fatty acid composition (Zabaras, 2010). The third challenge is linked to the lack of a standardized workflow, which would allow monitoring olive oils labeled with a declaration of production within a specific region (geographic indications) or certified as monovarietal olive oils.

In order to overcome the aforementioned limitations, researchers in olive oil authentication field are continuously working for the development of more robust, efficient, sensitive, rapid and cost-effective analytical methodologies to guarantee the quality, authenticity, and geographic and botanic origins traceability of this valuable matrix, promoting the recent technological progress in the analytical field.

Thus, in view of all the stated above, the present review paper aims to give an overview on the current state-of-the-art of the most relevant regulatory standards for olive oil authentication, highlighting their differences and discussing their effectiveness, limitations, and the future perspectives of the analytical methods used to carry out the official controls. The paper is structured in two main parts: in the first one, the principal quality and authenticity indices -required for officially assessing the quality of olive oil and performing its adulteration control- are introduced and their legal thresholds are made explicit and discussed, comparing the values established by the most relevant national and international olive oil authentication legislations. The regulations reviewed herein, were selected on the basis of the importance of the contribution of the countries adopting these systems to olive oil worldwide trade. Furthermore, in this part of the

paper, official analytical methods used for the determination of these parameters are outlined. The second part of this review focuses, however, on recent developments and applications of modern instrumental analytical techniques to ensure olive oil quality and authenticity, as well as the trends and advances on olive oil geographical and botanical traceability.

OLIVE OIL REGULATORY FRAMEWORKS: A COMPARATIVE ANALYSIS

The international olive oil market can be considered, as one of the most worldwide regulated markets, in particular because of the existence, for a long time, of international standards (IOC and Codex standards), and European standards (EEC (No 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis and subsequent modifications)) which regulate the European Union olive oil sector that represents more than 76% and 69% of olive oil production and consumption respectively (IOC, 2014). However, the globalization of this sector, the emergence of new olive oil producing countries outside the Mediterranean area, and the rise of olive oil consumption in non-traditional olive oil markets, are among the factors that recently stimulated the interest in setting national standard regulations in some of these new producing countries. Some of these regulations are the following: the "United States standards for grades of olive oil and olive-pomace oil" adopted by the USDA in 2010 (USDA, 2010); the "Olive oils and olive-pomace oils Australian standards" adopted by Australian government in 2011 (Standards Australia, 2011), and, most recently, the "Grade and labeling standards for olive oil, refined-olive oil and olive-pomace oil" approved on 2014 by the Department of Food and Agriculture of the State of California (California Department of Food and Agriculture, 2014). However, from the beginning, the emergence of these regulatory standards is prompting a lively debate about their utility, the risk that can represent for the harmonization of the international olive oil trade standards and the need to consolidate efforts to bring major coherence and clarity of olive oil grading and authentication.

Nevertheless, in spite of the differences that can be observed between the above-mentioned olive oil regulations, their basic form remains quite similar. It consists of a

description of olive oil grades, and a list of quality and purity criteria, highlighting their threshold values. Furthermore, references for food additives, contaminants, hygiene, and methods of sampling and analysis can be found in these legislations.

Olive oil legal designations and grades

In general, the above-mentioned olive oil regulatory standards gather the various types of oils that can be obtained through olive fruits extraction, on two main categories:

-**Olive oil**: representing the oil obtained solely from the fruit of the olive tree and excludes oils obtained using solvents or mixture of other type oils. It includes two main types of oils: virgin olive oils (also called "natural olive oils" in Australian regulations) which correspond to those oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration; and oils obtained from virgin olive oils by refining methods.

-**Olive-pomace oils**: comprising oils obtained by treating olive pomace (the solid by-product remaining after the mechanical extraction of olive oil) with solvents or other physical treatments, excluding the oils obtained by synthetic processes or by re-esterification processes and mixture with oils of other kinds.

Each one of these categories includes various oil grades, classified according to specific quality criteria fixed by each one of the previously mentioned olive oil regulatory standards (Table 1). Thus, the category of virgin olive oils is divided in two sub-categories, the first one including those oils fitting for direct consumption, which are: EVOO, virgin olive oil (VOO) and ordinary virgin olive oil (OVOO); and the second one constituted by lampante virgin olive oils (LVOO), also called lampante olive oil, that is not fitting for direct consumption but gives rise, after a refining procedure, to refined olive oil (ROO), and olive oil (OO) (consisting of a blend of refined olive oil and virgin olive oils). Furthermore, within the context of these legislations, the category of olive-pomace oils is divided in three grades: crude olive-pomace oil (COPO); refined olive-pomace oil (ROPO) and olive-pomace oil (OPO). Figure 1 illustrates the way to obtain the oils belonging to each one of the mentioned categories. However, despite the

similarities among the considered legislations regarding olive oil terminology of nomenclature, some differences can be revealed. In particular, the OVOO category is just considered by the IOC and Codex legislations (indeed, regarding EU Regulation, the ordinary virgin olive oil category has been deleted since 2001 with the regulation EU 1513/2001); Codex does not consider the LVOO and COPO categories; the LVOO category is denominated "crude olive oil" in the regulation standards adopted by the Department of Food and Agriculture of the State of California, and both this standard legislation and the Australian Standards use the terms crude olive-pomace oil, refined olive-pomace blend, and refined olive-pomace oil for designating the three categories of olive-pomace oils. Therefore, the existence of the described heterogeneous terminology will likely cause certain confusion.

Olive oil quality criteria

The comprehensive official quality control of olive oil requires both diverse analytical determinations and a sensory evaluation; the analytical determination of a considerable number of physico-chemical parameters considered as indicator of hydrolytic modification, oxidation, and freshness status of olive oil has to be carried out, and furthermore, the evaluation of its sensory quality by a panel test recognized by the standardizing body is also needed. Table 1 summarizes the most frequently required physico-chemical and sensory quality indices, as well as their threshold limits according to the regulatory legislations considered in this paper. They mainly include:

- **Content of free fatty acids (FFAs):** these compounds are the product of TAGs hydrolytic degradation that can occur, during olive oil manufacturing process and storage, due to the action of enzymes (lipase) naturally present in the olive fruit and/or caused by enzymes produced by micro-organisms which grow on the fruit (De Oliveira et al., 2010). Olive oils obtained from healthy fruits, regardless of the cultivar, processed just after harvesting, often show very low FFAs content. Official method for the determination of FFAs content (International Organization of Standardization (ISO) 660 (ISO, 2009a) and American Oil chemists Society (AOCS) Cd 3d-63 (AOCS, 1999)) is based on acid/base titration using potassium hydroxide with phenolphthalein as an indicator, and the results are reported as percentage of oleic acid.

All considered regulatory olive oil legislations establish an upper limit for distinguishing olive oil commercial categories according to FAAs content. However, some differences can be observed considering these limits (Table 1).

Table 1. Comparative analysis of the threshold values of physico-chemical (Free fatty acid content (FFAs in % of oleic acid), Peroxide value (PV expressed in meq O2/kg oil), Ultraviolet specific extinction coefficients (K_{232}, K_{270} and ΔK), Free fatty acids ethyl esters content (FAEEs in mg/kg), Pyropheophytins content (PPPs in %) and1,2-diacylglycerol (1,2- DAG in %)) and sensory (median of olive oil fruitiness (MeF) and defects (MeD)) quality parameters fixed by the different reviewed olive oil regulatory systems.

				Physico-chemical quality parameters								Sensory evaluation	
				FFAs	PV	K_{232}	K_{270}	Δ K	FAEEs	PPPs	1,2-DAG	MeD	MeF
Olive oils	Virgin olive oils	EVOO	IOC	≤ 0.8	≤ 20.0	≤ 2.50	≤ 0.22	≤ /0.01/	≤ 30	N/C		0	> 0.0
			Codex						N/C				
			EU						≤ 30				
			USDA						N/C	≤ 17	≥ 35		
			AUS										
			CAF	≤ 0.5	≤ 15.0	≤ 2.40							
		VOO	IOC	≤ 2.0	≤ 20.0	≤ 2.60	≤ 0.25	≤ /0.01/	N/A	N/C		0 < Me ≤ 3.5	> 0.0
			Codex									0 < Me ≤ 2.5	
			EU						N/A			0 < Me ≤ 3.5	
			USDA						N/C	N/A		0 < Me ≤ 2.5	
			AUS										
			CAF	≤ 1.0									
		OVOO	IOC	≤ 3.3	≤ 20.0	N/A	≤ 0.30	≤ /0.01/	N/A	N/C		3.5 <Med ≤ 6.0	N/A
			Codex						N/C			2.5 < Me ≤ 6.0	
		LVOO	IOC	> 3.3	N/A				N/A	N/C		Me > 6.0	N/A
			EU	> 2.0								Me > 3.5	
			USDA						N/C			Me > 2.5	
			AUS		> 20.0	>2.60	>0.25	>/0.01/ ≤ /0.01/		N/A		Me > 2.5	
			CAF	> 1.0									
	ROO		IOC	≤ 0.3	≤ 5.0	N/A	≤ 1.10	≤ /0.16/	N/A	N/C		N/A	N/A
			Codex						N/C				
			EU						N/A				
			USDA						N/C	N/A		≤ 2.5	
			AUS										
			CAF										
	OO		IOC	≤ 1.0	≤ 15.0	N/A	≤ 0.90	≤ /0.15/	N/A	N/C		N/A	N/A
			Codex						N/C				
			EU						N/A				
			USDA						N/C	N/A		≤ 2.5	> 0.0
			AUS										
			CAF	≤ 0.8									
Olive-pomace oils	OPO		IOC	≤ 1.0	≤ 15.0	N/A	≤ 1.70	≤ /0.18/	N/A	N/C		N/A	N/A
			Codex						N/C				
			EU						N/A				
			USDA						N/C	N/A		≤ 2.5	> 0.0
			AUS										
			CAF	≤ 0.8									N/A
	ROPO		IOC	≤ 0.3	≤ 5.0	N/A	≤ 2.0	≤ /0.20/	N/A	N/C		N/A	N/A
			Codex						N/C				
			EU						N/A				
			USDA						N/C	N/A		≤ 2.5	
			AUS										
			CAF										

N.B:

- In all the tables presented in this paper the used abbreviations are listed in the Abbreviations section at the end of the paper.

- COPO has not been included in this table because no limit, for none of the quality parameters, was fixed by the regulatory systems reviewed in this study.

Indeed, as far as virgin olive oils category is concerned, the Californian regulation indicates lower FAAs content limits for defining the different grades belonging to this category. Thus, while 0.8% and 2.0% are the limits fixed by IOC, EU, Codex, USDA and Australian standards for EVOO and VOO grades, respectively, the Californian regulation establishes 0.5% and 1.0%, respectively, as the upper limits for defining the same categories. Other differences that can be emphasized are that while EU, USDA and Australian standards classify virgin olive oils with FAAs content upper the limit of 2.0% as LVOO, this limit is much lower in Californian regulation which considers the oils with FFAs content upper 1.0% as LVOO. IOC regulation, however, fixed a higher upper limit (>3.3%) to classify a virgin olive oil as LVOO. IOC and Codex standards are the only examples of regulations which set the limit in 3.3%, so the oils with FFAs values below (or equal to) that value will be considered as OVOO. With regard to the remaining olive oils and olive-pomace oils categories, the reviewed regulation systems require the same FFAs content threshold (≤ 0.3%) for both ROO and ROPO grades, whereas the Californian regulation indicates lower FFAs content limit for defining the OO and OPO (≤ 0.8%), comparatively to the other reviewed regulation systems that establish an upper limit of 1.0%.

- **Peroxide value (PV):** is an indicator of the primary oxidation status of the olive oil, which can be calculated by measuring the concentration of hydroperoxides, which constitute the first compounds to be formed in the degradation process of the olive oil unsaturated FAs. These compounds are not stable; their value increases, reaches a maximum and then decreases because of their further degradation into secondary oxidation products (such as ketones, aldehydes and conjugated dienes) (Mariotti, 2014). The official method for the determination of PV ((ISO 3960 (ISO, 2007) or AOCS Cd 8b-90 (AOCS, 2003)) is based on the iodometric titration of iodine liberated from potassium iodide after reacting with the peroxides present in the oil samples. Results are expressed as milliequivalent of active oxygen per kilogram of olive oil (meq O_2/kg oil). In general, PV upper limit established for olive oil grading are the same on all the standard legislations considered in the current study, with the exception of the limits established by Californian legislation for the EVOO (being 15 O_2/kg the upper limit required by this legislation, whereas the other legislations are a bit more permissive, fixing this limit in 20 O_2/kg), and by Australian and Californian legislations for the

LVOO (being 20 O_2/kg the lower limit set by these legislations, while this parameter is not contemplated for LVOO in the other regulations).

- **Ultraviolet specific extinction coefficients:** conventionally indicated by K_{232} and K_{272} and obtained by the spectrophotometric measurements, in the ultraviolet, of extinctions of the olive oil sample diluted in cyclohexane at the wavelengths corresponding to the maximum absorption of the conjugated dienes and trienes, respectively, at about 232 and 270 nm (ISO 3656 (ISO, 2011) or AOCS Ch 5-91 (AOCS, 1991a)). Besides, the absorption around 270 nm could also be caused by substances formed during earth treatment in the refining process. In addition to these parameters, ΔK value is often calculated according to the following equation: $\Delta K = K_{max} - [1/2(K_{max}+4 + K_{max}-4)]$ where K_{max} is the specific extinction at the wavelength for maximum absorption at 270 nm. The maximum allowed values of K_{232}, K_{270} and ΔK for the different grades of olive oils and olive-pomace oils are included in Table 1. Some differences among the considered regulatory systems can be found. Thus, for EVOO, the maximum permitted values of K_{232}, K_{270} (2.5 and 0.22, respectively) and ΔK (≤ /0.01/) are the same for all the legislative standards. Likewise, when the VOO category is considered, all the reviewed legislative standards require the same threshold values of K_{232}, K_{270} (2.6 and 0.25, respectively) and ΔK (≤ /0.01/). Moreover, in the case of LVOO, only Californian and Australian legislations fix a limit for K_{232}, K_{270} and ΔK (being >2.60, >0.25 for K_{232}, K_{270}, respectively, in both legislations, whereas for ΔK the value of /0.01/ is the upper limit in the Californian legislation and the down limit in Australian regulation. In addition, for OO, ROO and OPO grades, no limit is fixed for K_{232}, being the limits for K_{270} and ΔK the same for all the legislative systems taken into account (Table 1).

- **Content of fatty acid alkyl esters (FAAEs):** this quality criterion has been recently adopted by IOC and EU for the assessment of EVOO quality. However, so far, it is not considered by the other olive oil regulatory standards. FAAE compounds result from the esterification of free fatty acids with low molecular weight alcohols (mainly methanol and ethanol) yielding methyl and ethyl esters (Pérez-Camino et al., 2002; Boggia et al., 2014). The olive oil content in terms of these compounds was related to the health conditions of processed olive fruits. Indeed, damaged olive fruits were reported to be susceptible to undergo a hydrolytic process (lipolysis of TAGs with

liberation of FFAs) and fermentative degradations (pectin demethylation and sugar fermentation), which create appropriate conditions for the synthesis of FAAEs (Biedermann et al., 2008). Furthermore, olive fruits storage before processing was reported to be a factor that increases the formation of these compounds. Other factors, such as inappropriate practices during oil extraction, catalyze the esterification reaction which increases the amount of these compounds in the obtained oils. In addition to their role as a quality parameter, FAAEs content has been reported as a relevant tool for detecting EVOO adulteration with low quality virgin olive oils, that have undergone a mild deodorization treatment conducted at a moderate temperature (≤ 100 °C), which remove volatile compounds that are responsible for their undesirable sensory attributes (Pérez-Camino et al., 2008). With regard to the analytical determination of FAAEs, the official method (COI/T.20/Doc.No 28 (IOC, 2010a)) requires a preliminary separation of these compounds from the oil by means of a classical column chromatography, using silica gel as adsorbent, with hexane and ethyl ether as eluents; then, the solvents are evaporated by a rotary evaporator, and finally, the fraction containing the methyl and ethyl esters is diluted with *n*-heptane or *iso*-octane and analyzed by a gas chromatography (GC) system for further identification and quantification purposes. IOC and EU regulate both the content of fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs). A legal limit of 75 mg/kg for the sum of FAME and FAEE, or superior than 75 mg/kg and inferior than or equal to 150 mg/kg for the sum of FAME and FAEE (if the ratio of FAEE/FAME is below 1.5) was fixed for oils produced over the crop season 2012/2013 (EU Commission Regulation No 61/2011). However, from the crop season of 2013/2014, only FAEE content is considered, with a maximum value of 40, 35 and 30 mg/kg for oils produced during 2013/2014, 2014/2015 and 2015/2016, respectively (EU Commission Implementing Regulation No 1348/2013). After the crop season of 2016 the maximum value of FAEEs for the EVOO grade is going to be lower than 30 mg/kg (EU Commission Delegated Regulation 2015/1830).

- **Content of pyropheophytins (PPPs):** determination of PPPs content is only required by Australian and Californian standards for EVOO freshness evaluation. PPPs are formed during olive oil extraction and storage, due to the degradation of chlorophyll pigments (pheophytinization and a certain degree of allomerization). Chlorophyll breaks down to pheophytin *a*, then converts to pyropheophytins *a* as a result of the loss of the

carbomethoxy group at carbon 13 (C13) (Aparicio-Ruiz et al., 2010; Guillaume et al., 2014). The generated amount of pyropheophytins *a* remains small, but their content in olive oil increases during the storage depending on various factors, such as olive fruits variety, ripeness and seasonal conditions (Gallardo-Guerrero et al., 2005). For this reason, the PPPs *a* content in terms of the ratio of pyropheophytin *a* divided by total pheophytins*a* -which is independent of these factors (Aparicio et al., 2013b)- was considered as a freshness parameter of EVOO. The standard method for the determination of PPPs content in olive oil (ISO 29841 (ISO, 2009b)) involves their separation using a miniaturized column chromatography on silica gel and chromatographic analysis using a reverse phase liquid chromatography with a photometric or fluorescence detector. In both Australian and Californian standards, as can be seen in Table 1, a legal limit of 17% of PPPs *a* is set for classifying a virgin olive oil as EVOO.

- **1,2-diacylglycerols (1,2-DAGs):** this is a quality and freshness parameter just considered by Australian and Californian standards for grading olive oil as EVOO. The estimation of this parameter is made by calculating the mass fraction ratio between 1,2-DAG and the sum of 1,2-DAGs and 1,3-DAGs. DAGs are present in virgin olive oils in low amounts (between 1% and 3%) as intermediate products of the biosynthesis of TAGs (1,2-DAGs) or as products of enzymatic or chemical hydrolysis of TAGs (1,3-DAGs) (Pérez-Camino et al., 2001). During storage, the 1,2-DAGs undergo isomerization, yielding 1,3-DAG, that are thermodynamically more stable. Consequently, assessing the amounts of these isomeric forms could be informative about the age and the freshness of virgin olive oils. Currently, the official method for the determination of this quality criterion (ISO 29822 (ISO, 2009c)) includes the separation of these isomeric forms on a silica gel chromatography column, derivatization (sylilation) and GC analysis. To classify a virgin olive oil as an EVOO, both Australian and Californian standards have set as minimum level the value of 35% for the ratio between 1,2-DAG and the sum of 1,2-DAG and 1,3-DAG (Table 1).

- **Sensory quality:** pleasant sensory characteristics of olive oil are one of the main reasons for the acceptability and preference of consumers of this foodstuff. In addition, the cultivation of various olive tree varieties in different pedoclimatic conditions and the use of diverse agronomical and technological techniques for olive extraction and

production are the main factors behind the existence, in the olive oil market, of a myriad of olive oils with very distinctive flavor characteristics. The official method for the sensory evaluation of olive oil (COI/T.20/Doc. No 15 (IOC, 2007)) consists on a panel test method applied by a fully selected and trained taste panel recognized by the regulatory body. The method determines the category of olive oil according to the detection and intensity of sensory positive and negative attributes in the analyzed oil. Fruitiness, bitterness and pungency are sensory positive attributes determined by the panelists; whereas fusty-muddy, mustiness-humidity, winey-vinegary, frostbitten olives and rancid constitute the main defects. The panelists provide an intensity scale of each attribute, and then the median values of olive oil fruitiness (MeF) and of the most perceived defect (MeD) are calculated. Finally, each grade of olive oil is defined according to the obtained results (Table 1). Thus, when no negative attributes are detected, and the MeF is superior to zero, all the regulatory standards classify the virgin olive oil as EVOO. However, some differences exist among these regulatory systems regarding the sensory evaluation of the other categories. Indeed, in the case of VOO grade, IOC and EU standards fix a maximum value of MeD of 3.5; whereas the other standards trades demand lower values (lower than or equal to 2.5). USDA, Australian and Californian standards classify all olive oils with a MeD superior to 2.5 as LVOO (also, in the case of USDA regulation, an olive oil is classified as LVOO when MeD is less than or equal to 2.5 and the MeF is equal to 0). EU standards, however, consider a higher value (3.5) (or when MeD is less than or equal to 3.5 and the MeF is equal to 0), and the IOC standards threshold is much higher for the LVOO category (value of MeD superior to 6 is established). Both IOC and Codex are the only ones defining olive oils with MeD value between 3.5 and 6 (or when MeD is less than or equal to 3.5 and the MeF is equal to 0), and between 2.5 and 6 (or when MeD is less than or equal to 2.5 and the MeF is equal to 0), respectively, as OVOO. For the other categories, mainly ROO, OO, OPO, and ROPO, the Australian and Californian standards are the only ones which set a limit value of MeD (2.5) for defining these grades. However, for the COPO category, neither regulatory trade limits of MeD nor MeF have been established.

Olive oil purity criteria

In accordance with the regulations concerning olive oil authentication, the olive oil genuineness is defined by values with the lowest and/or highest limits for the content of the selected purity criteria specified by these legislations. Such criteria are related to the amount of diverse groups of chemical compounds in olive oil. In contrast to quality criteria for which some parameters are not considered by all the reviewed olive oil standards trades, the contemplated purity criteria are the same for every legislation (even if the fixed thresholds show some differences). Eight purity criteria are considered; the limits for each parameter in different grades of olive oils and olive-pomace oils are given in Table 2 and 3, respectively.

- **Fatty acid composition** (%)**:** fatty acids (FAs) are the main constituents of olive oil forming part of TAGs molecules.Olive oil is characterized by the predominance of monounsaturated (in particular, oleic acid), the low percentage of saturated and a very low percentage of polyunsaturated FAs. According to the official methods, these compounds are evaluated by means of the analysis of methyl esters of FAs using GC with flame ionization detector (FID) (preparation of methyl esters in accordance with AOCS Ce 2-66 (AOCS, 2009) or ISO 5509 (ISO, 2000) or COI/T.20/Doc.24 (IOC, 2001a), and analysis of these compounds by GC-FID according to ISO 5508 (ISO, 1990) or AOCS Ch 2-91 (AOCS, 1991b)).

The limits of variability of the content of olive FAs of olive oils and olive-pomace oils, expressed as percentage of total FAs, as set by the different reviewed regulations are reported in Tables 2 and 3. From these Tables, it can be seen that such limits are consistent for each category of olive oils and olive-pomace oils with the exception of behenic acid for which the upper limit is a bit higher for olive-pomace oils category (0.3%) in comparison with olive oils categories (0.2%). The first remarkably observation that can be revealed when analyzing data from Tables 2 and 3 is that in contrast to Australian, IOC, Codex, EU, and USDA regulations that include the content of 13 FAs as purity criteria, in Californian legislation only the determination of the content of 6 FAs (myristic, heptadecenoic, stearic, arachidic, behinic and lignoceric) is mandatory. Besides, as shown in these Tables, the percentages of some FAs (palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2) and stearic (C18:0)) are expected to

vary within a quite large range, whilst the other FAs (myristic (C14:0), heptadecanoic (C17:0), heptadecenoic (C17:1), linolenic (C18:3), arachidic (C20:0), eicosenoic (C20:1), behenic (C22:0) and lignoceric (C24:0)) are found at lower levels than 1.5% and only their upper limits are established. In addition, with regard to the value of the limits set by these regulations (Tables 2 and 3), IOC and EU standards establish the same limits for all regulated FAs, whereas some differences can be observed with the other regulation standards.

Thus, with the exception of C17:0, C18:0, C20:0, C22:0 and C24:0 FAs for which the same upper limits are fixed by all the regulations considered by the current study, the thresholds of the other regulated FAs show some differences. The disparity can be illustrated, for instance, with the case of linolenic fatty acid; there no limit according to the Codex and Californian regulations; however, IOC and EU regulations establish a limit of 1%, whereas USA and AUS standards set a higher limit (1.5%).

- **Fatty acids *trans*isomers content** (%)**:** the normal arrangement of double bonds in unsaturated FAs in olive oil is *cis* configuration. The presence of *trans* isomers of oleic (trans C18:1), linoleic and linolenic acids (C18:2T+C18:3T), in percentages exceeding the established limits (Tables 2 and 3), can indicate adulteration of virgin olive oils with hydrogenated seed oils, ROO and OPO, among others (Aparicio et al., 2013b). These compounds are determined in accordance with COI/T.20/DOC. No 17 (IOC, 2001b), or ISO 15304 (ISO, 2002) or AOCS Ce 1f-96 (AOCS, 1996a). Concerning the upper limits fixed for these parameters, all the reviewed regulations consider the same values.

- **Difference between actual and theoretical content of triacylglycerols (ΔECN42):** in contrast to many seed oils, the chemical composition of olive oil shows an abundance of TAGs with equivalent carbon number (ECN) 44, 46, 48, and 50, whereas TAGs with ECN40 and ECN42 are absent or found at trace amounts (Angerosa et al., 2006). Therefore, the determination of the difference between the experimental values of TAGs ECN42 obtained by HPLC with refractive index detector and the theoretical value (ECN 42 theoretical) calculated from the fatty acid compositionby GC-FID is used to detect blends of virgin olive oils with unsaturated oils. This parameter is determined according to COI/T.20/Doc. No. 20 (IOC, 2010b) or AOCS Ce 5b-89 (AOCS, 1989).

All the reviewed regulations establish the same values as upper limit (Tables 2 and 3) for each one of the olive oils and olive-pomace oils grades defined by these regulations.

- **Sterols:** these compounds constitute the major proportion of olive oil unsaponifiable fraction. The determination of olive oil sterol total content as well as its individual composition (content of cholesterol, brassicasterol, campesterol, stigmasterol,Δ7-stigmastenol and apparent β-sitosterol (the sum of contents of Δ5,23 and Δ5,24 stigmastadienols, chlerosterol, β-sitosterol, sitostanol, and Δ5-avenasterol), is required by some trade standards to detect possible adulteration of olive oil with foreign oils (Youseff et al., 2014). Official methods for the analysis of sterols in olive oil (COI/T.20/Doc. No. 10 (IOC, 2001c), or ISO 12228 (ISO, 1999a) or AOCS Ch 6-91 (AOCS, 1991c)) involve several steps. First, olive oil saponification is required for the separation of saponifiable and unsaponifiable fractions, then separation by thin-layer chromatography on silica gel plates and derivatization of the sterols have to be carried out. The sterols as trimethylsilyl derivatives are identified and quantified, afterwards, by means of a capillary GC-FID platform.

As can be seen in Table 2, concerning the total sterols content in olive oil categories, Californian regulation is the only one that establishes no limit for grading olive oils into EVOO, VOO and LVOO categories, while the other regulations set a minimal value of 1000 mg/1000 g. Regarding the RVOO and OO categories, all the considered regulations set a minimal value of 1000 mg/1000 g. In the case of pomace oils, all the reviewed regulations fix the same minimal values (Table 3). Besides, when the individual sterols content are considered, some differences can be found for both olive oils and olive-pomace oils categories. Within this context, as noticed for FAs composition, Californian standards is the only one requiring the determination of a restricted number of sterol compounds (specifically two compounds: brassicasterol and stigmasterol) (Tables 2 and 3).

Table 2. Comparative analysis of the threshold values of purity criteria established for olive oils grades by the different reviewed olive oil regulatory systems.

		Olive oils grades																	
		EVOO+ VOO						OVOO		LVOO					RVOO+OO					
		IOC	EU	Codex	USDA	AUS	CAF	IOC	Codex	IOC	EU	USDA	AUS	CAF	IOC	EU	Codex	USDA	AUS	CAF
Fattyacidscomposition (%)	**Myristic**	≤ 0.03		≤ 0.05				≤ 0.03	≤ 0.05	≤ 0.03		≤ 0.05			≤ 0.03		≤ 0.05			
	Palmitic	7.50 - 20.0				7.0 - 20.0	N/C	7.50 - 20.00		7.50 - 20.0			7.0 - 20.0	N/C	7.50 - 20.0				7.0 - 20.0	N/C
	Palmitoleic	0.30 - 3.50						0.30 - 3.50		0.30 - 3.50					0.30 - 3.50					
	Heptadecanoic	≤ 0.30						≤ 0.30		≤ 0.30					≤ 0.30					
	Heptadecenoic	≤ 0.30				≤ 0.40	N/C	≤ 0.30		≤ 0.30			≤ 0.40	N/C	≤ 0.30				≤ 0.40	N/C
	Stearic	0.50 - 5.0						0.50 - 5.00		0.50 - 5.0					0.50 - 5.0					
	Oleic	55.0 - 83.0				53.0 - 85.0	N/C	55.00 - 83.00		55.0 - 83.0			53.0 - 85.0	N/C	55.0 - 83.0				53.0 - 85.0	N/C
	Linoleic	3.5 - 21.0	2.5 - 21.0	3.5 - 21.0		2.50 - 22.0	N/C	3.50 - 21.00		3.5 - 21.0	2.5 - 21.0	3.5 - 21.0	2.50 - 22.0	N/C	3.5 - 21.0	2.5 - 21.0	3.5 - 21.0		2.50 - 22.0	N/C
	Linolenic	≤ 1.00		N/C	≤ 1.50	≤ 1.50		≤ 1.00	N/C	≤ 1.00		≤ 1.50			≤ 1.00		N/C	≤ 1.50	≤ 1.50	
	Arachidic	≤ 0.60						≤ 0.60		≤ 0.60					≤ 0.60					
	Eicosenoic	≤ 0.40				≤ 0.50	N/C	≤ 0.40		≤ 0.40			≤ 0.50	N/C	≤ 0.40				≤ 0.50	N/C
	Behenic	≤ 0.20						≤ 0.20		≤ 0.20					≤ 0.20					
	Lignoceric	≤ 0.20						≤ 0.20		≤ 0.20					≤ 0.20					
	C18:1 T (%)	≤ 0.05						≤ 0.05		≤ 0.1					≤ 0.20					
	C18:2 T+C18:3 T(%)	≤ 0.05						≤ 0.05		≤ 0.1					≤ 0.30					
	ΔECN42	≤/0.2/						≤/0.2/		≤/0.3/					≤/0.3/					
Sterolscomposition (%)	**Cholesterol**	≤ 0.5					N/C	≤ 0.5		≤ 0.5				N/C	≤ 0.5					N/C
	Brassicasterol	≤ 0.1																		
	Campesterol (camp)	≤ 4.5	≤ 4.0		≤ 4.5	≤ 4.8	N/C	≤ 4.0		≤ 4.0	≤ 4.5		≤ 4.8	N/C	≤ 4.0			≤ 4.5	≤ 4.8	N/C
	Stigmasterol	< camp				≤ 1.9		< camp		N/A					< camp				≤ 1.9	
	Delta-7-stigmastenol	≤ 0.5					N/C	≤ 0.5		≤ 0.5				N/C	≤ 0.5					N/C
	Apparent β-sitosterol	≥93.0				≥92.5		≥93.0		≥93.0					≥93.0				≥92.5	
Total sterol content (g/1000g)		≥1000					N/A	≥1000		≥1000				N/A	≥1000					
	Erythrodiol+Uvaol	≤ 4.5					N/A	≤ 4.5		≤ 4.5				N/A	≤ 4.5					N/A
	Wax	≤ 150		≤ 250				≤ 250		≤300					≤350					
	Aliphaticalcohols(mg/kg)	An olive oil is classified as LVOO when the wax content is between 300 and 350 mg/kg, if the total aliphatic alcohol content is < 350 mg/kg or the erythrodiol + uvaol content is < 3.5%.																		
	Stigmastadienes	≤ 0.05		≤ 0.15		≤ 0.10		≤ 0.1	≤ 0.15	≤ 0.50					N/A					
2P (%)	**If C:16:0 ≤ 14.0%**	≤ 0.9		≤ 1.5	≤ 0.9	≤ 1.5	N/A	≤ 0.9	≤ 1.5	≤ 0.9			≤ 1.5	N/A	≤ 0.9		≤ 1.8	N/A	≤ 1.8	
	IfC:16:0 > 14.0%	≤ 1.0			≤ 1.0			≤ 1.0		≤ 1.1					≤ 1.0*					

*: ≤ 1.1 for RVOO grade in EU regulation.

Table 3. Comparative analysis of the threshold values of purity criteria established for olive-pomace oils grades by the different reviewed olive oil regulatory systems.

		Olive pomace oils																	
		OPO						ROPO						COPO					
		IOC	EU	Codex	USDA	AUS	CAF	IOC	EU	Codex	USDA	AUS	CAF	IOC	EU	USDA	AUS	CAF
Fattyacidscomposition	Myristic	≤ 0.03		≤ 0.05				≤ 0.03		≤ 0.05				≤ 0.03		≤ 0.05		
	Palmitic	7.50 - 20.0				7.0 - 20.0	N/C	7.50 - 20.0				7.0 - 20.0	N/C	7.50 - 20.0			7.0 - 20.0	N/C
	Palmitoleic	0.30 - 3.50						0.30 - 3.50						0.30 - 3.50				
	Heptadecanoic	≤ 0.30						≤ 0.30						≤ 0.30				
	Heptadecenoic	≤ 0.30				≤ 0.40	N/C	≤ 0.30				≤ 0.40	N/C	≤ 0.30			≤ 0.40	N/C
	Stearic	0.50 - 5.0						0.50 - 5.0						0.50 - 5.0				
	Oleic	55.0 - 83.0				53.0 - 85.0		55.0 - 83.0				53.0 - 85.0		55.0 - 83.0			53.0 - 85.0	
	Linoleic	3.5 - 21.0	2.5-21.0	3.5 - 21.0		2.5 - 22.0	N/C	3.5 - 21.0	2.5-21.0	3.5 - 21.0		2.5 - 22.0	N/C	3.5 - 21.0	2.5 - 21.0	3.5 - 21.0	2.5 - 22.0	N/C
	Linolenic	≤ 1.00		N/C		≤ 1.50		≤ 1.00		N/A		≤ 1.50		≤ 1.00		≤ 1.50		
	Arachidic	≤ 0.60						≤ 0.60						≤ 0.60				
	Eicosenoic	≤ 0.40				≤ 0.50	N/C	≤ 0.40				≤ 0.50	N/C	≤ 0.40			≤ 0.50	N/C
	Behenic	≤ 0.30						≤ 0.30						≤ 0.30				
	Lignoceric	≤ 0.20						≤ 0.20						≤ 0.20				
	C18:1 T (%)	≤ 0.40						≤ 0.40						≤ 0.20				
	C18:2 T+C18:3 T(%)	≤ 0.35						≤ 0.35						≤ 0.10				
	ΔECN42 (%)	≤/0.5/						≤/0.5/						≤/0.6/				
Sterolscomposition (%)	Cholesterol	≤ 0.5					N/C	≤ 0.5					N/C	≤ 0.5				N/C
	Brassicasterol	≤ 0.2				≤ 0.1		≤ 0.2				≤ 0.1		≤ 0.2			≤ 0.1	
	Campesterol (camp)	≤ 4.0			≤ 4.5	≤ 4.8	N/C	≤ 4.0			≤ 4.5	≤ 4.8	N/C	≤ 4.0		≤ 4.5	≤ 4.8	N/C
	Stigmasterol	< camp				≤ 1.9		< camp				≤ 1.9		N/A			≤ 1.9	
	Delta-7-stigmastenol[b]	≤ 0.5					N/C	≤ 0.5					N/C	≤ 0.5				N/C
	Apparent β-sitosterol	≥93.0				≥92.5		≥93.0				≥92.5		≥93.0			≥92.5	
Total sterol content (g/1000g)		≥1600						≥1800						≥2500				
	Erythrodiol+Uvaol (%)	> 4.5		N/A		> 4.5	N/A	> 4.5		N/A		> 4.5	N/A	> 4.5				N/A
	Wax (mg/kg)	>350						>350						>350				
	Aliphaticalcohols (mg/kg)	If the total aliphatic alcohol content is > 350 mg/kg, the erythrodiol+uvaol content is > 3.5% and the oil shows a wax content between 300 mg/kg and 350 mg/kg, olive oil is considered as COPO.																
	Stigmastadienes (%)	N/A						N/A						N/A				
	2P (%)	≤ 1.2		≤ 2.2	≤ 1.2	≤ 2.2	N/A	≤ 1.4		≤ 2.2	≤ 1.4	≤ 2.2	N/A	≤ 1.4			≤ 2.2	N/A

Thus, IOC, EU and Codex regulations consider the same values for all the regulated compounds; however, USDA, Californian and Australian standards show some differences as can be observed in the tables. For example, Codex and EU regulation fix an upper limit of 4.0% for the campesterol content, and a decision tree is proposed to verify the authenticity of oils having contents between 4 and 4.5%. However, IOC and USDA regulations fix the maximum content for the campesterol on 4.5% (even though it requires the authentication of the oils showing content between 4 and 4.5%); Australian regulation allows higher content for this compound, 4.8%; and no limit is established by Californian regulation.

- **Triterpene dialcohols (sum of erythrodiol and uvaol):** they are also part of the unsaponifiable fraction of olive oil and their determination is usually carried out together with the sterol fraction. These compounds are mainly found in the fruit skin, so that they are detected at higher concentrations in pomace that undergoes solvent extraction (Habib et al., 2015). For this reason, percentage of erythrodiol and uvaol in relation to those of sterols is considered as a suitable authenticity index to detect possible fraudulent admixtures of virgin olive oils with olive-pomace oils. As shown by Tables 2 and 3, all the reviewed regulations fix a value of 4.5% as the maximum content for virgin olive oils, ROO and OO on erythrodiol and uvaol, except for Californian regulation which establish no limit. In the case of olive-pomace oils, both Codex and Californian regulations establish no limit for grading tested oils, whereas the remaining standards legislations fix the same value (> 4.5%).

- **Wax esters:** they are a group of esters of FAs and long-chain aliphatic alcohols accumulated in the skin of olive fruits and, therefore, they are found in considerably higher amounts in olive-pomace oils than virgin olive oils (Tena et al., 2015). Hence, wax content is used to detect virgin olive oils adulteration with olive-pomace oils. Furthermore, this parameter can be used as a quality parameter, considering total aliphatic alcohols content and/or the sum of erythrodiol and uvaol. In the unsaponifiable fraction of olive oils, three classes of waxy compounds can be detected: waxes with chain lengths lower than 40 (C36 and C38), others as C40 and C42, and waxes with 44 or more carbon atoms (C44 and C46). The official methods for the determination of wax content (COI/T.20/Doc. No. 18 (IOC, 2003a) or AOCS Ch 8-02

(AOCS, 2002)) are based ontheir separation from the olive oil unsaponifiable fraction by silica gel chromatography and analysis by capillary GC-FID. The waxes content is expressed as the sum of C40, C42, C44 and C46 waxes; however, in the case of EVOO and VOO, IOC and EU just consider the C42, C44 and C46 waxes and establish a maximum value of 150 mg/kg (Table 2). For the other categories, similar values are established by all the reviewed regulations.

- **Total aliphatic alcohols content:**these compounds are mostly located in the stone of olive fruits, therefore they are found at significantly higher concentration levels in olive-pomace oils than in virgin olive oils (Gandul-Rojas and Mínguez-Mosquera, 2006). The main aliphatic alcohols components detected in the unsaponifiable fraction of virgin olive oils are docosanol (C22), tetracosanol (C24), hexacosanol (C26) and octacosanol (C28). Other aliphatic alcohols, such as tricosanol (C23), pentacosanol (C25) and heptacosanol (C27) are present in low amounts. The standard methodology for the determination of aliphatic alcohols (COI/T.20/Doc. No. 26 (IOC, 2003b)) includes their separation from the oil unsaponifiable fraction by chromatography on a basic silica gel plate and their analysis and quantification by using GC-FID with a capillary column. The total content of these compounds (expressed by the sum of the concentrations of individual aliphatic alcohols), in combination with other purity parameters (erythrodiol and uvaol, and wax content) is used to distinguish the presence of LVOO and olive-pomace oils in virgin olive oils. All the reviewed standards regulations consider an olive oil as LVOO when the wax content is between 300 and 350 mg/kg, if the total aliphatic alcohol content is < 350 mg/kg or the erythrodiol + uvaol content is < 3.5%. In contrast, if the total aliphatic alcohol content is > 350 mg/kg, the erythrodiol+uvaol content is > 3.5% and the oil shows a wax content between 300 mg/kg and 350 mg/kg, the sample will be considered as COPO.

- **Stigmastadienes:** these compounds are formed in olive oils during the refining process as a consequence of the acid catalyzed sterol dehydration reaction in the course of bleaching process, or during the deodorization process, promoted by high temperatures (Crews et al., 2014). Among these compounds, stigmasta-3,5-diene originated from the dehydration of β-sitosterol is the most abundant. Therefore, its determination in olive oils (COI/T.20/Doc. No. 11 (IOC, 2001d), or ISO 15778-1(ISO, 1999b) or AOCS Cd 26-96 (AOCS, 1996b)) by means of preparative chromatography

and the subsequent analysis by GC-FID is an important indicator of the presence of refined oils in virgin olive oils, even at very low concentrations (Cert et al., 1994; Angerosa et al., 2006). Some differences can be found regarding the limits set by the reviewed standards regulations in the case of EVOO and VOO categories (Table 2). Indeed, while IOC and EU regulation establish an upper limit of 0.05 ppm, the other regulations allow a higher content (0.15 for Codex and USA regulations and 0.10 for Australian and Californian standards). For the LVOO category, an upper limit of 0.50 ppm of stigmasta-3,5-diene has been fixed by all the reviewed regulations. For the remaining olive oils and olive-pomace oils, the determination of this parameter is not required by any of the reviewed regulations (Table 2 and 3).

- **2-glyceryl monopalmitate (2P):** this parameter characterizes the percentage of palmitic acid at the 2-position of TAGs by means of 2P evaluation. In virgin olive oils only about 2% of the amount of the palmitic acid present is bonded on position 2 of TAGs compounds, whilst in oils artificially esterified the bonding with glycerol occurs in a random manner and significantly increase that percentage (Boskou, 2015). Therefore, the determination of virgin olive oil content on 2P is used for the detection of admixtures with esterified oils. The concentration of 2P is determined in accordance with COI/T.20/Doc. No. 23 (IOC, 2006) or ISO 12872 (ISO, 2010), after hydrolysis of TAGs by enzymatic digestion with pancreatic lipase which only hydrolyzes the ester bonds in positions 1 and 3, leaving intact the bond in position 2 of glycerol. The method also implies the separation by silica gel chromatography, silanization and the analysis with capillary GC-FID. Limits adopted by IOC, EU and USDA regulations are the same for the olive oils and olive-pomace oils categories, with the exception of RVOO and OO categories, for which no limits are established by USDA regulation. In these standards, the upper limit (%) of 2P is assigned according the oil content on palmitic acid. In contrast, in the other reviewed regulations (Codex, Australian and Californian), the content on palmitic acids is not considered, and higher content of 2P is allowed (Tables 2 and 3). As can be also seen from these tables, in the case of Californian standards, the content of 2P is only regulated for oils from the categories RVOO and OO.

RECENT PROGRESS AND TRENDS IN OLIVE OIL AUTHENTICATION

Given the drawbacks and limitations that some of the official analytical methods used for the authentication of virgin olive oils show regarding some aspects, a number of alternative analytical methods and techniques have been suggested over the past decade.

Advances in analytical methods to determine olive oil quality indices

Considering the physicochemical olive oil quality criteria, for the determination of FFAs, several spectroscopic methods, including Near-infrared (NIR) (Marquez et al., 2005; Cayuela et al., 2009), Visible/NearInfrared (Vis/NIR) (Cayuela Sánchez et al., 2013; García Martín 2015), Fourier transform infrared (FT-IR) (Bendini et al., 2007) and Fourier transform-Raman (FT-Raman) (Muik et al., 2003) have been proposed for the determination of olive oil acidity reaching significantly good results. Furthermore, others analytical techniques based on flow injection analysis (FIA) in automated systems (Bonastre et al., 2004), electrochemical methods using electrical impedance spectroscopy detector (Grossi et al., 2014), enzymatic methods (Ben Rejeb and Gargouri, 2011) and capillary electrophoresis (CE) (Balesteros et al., 2007) have been proposed for the determination of virgin olive oil FFAs. Further details about the recent analytical methods proposed for the determination of olive oil FFAs content can be found in De Oliveira et al. (2010).

Besides, some papers have focused on the development of analytical methods for the PV evaluation in virgin olive oils, based on either direct or indirect measurement of hydroperoxides. They include the development and application of a direct parallel flow injection multichannel spectrophotometric method (Thomaidis et al., 2000), the use of electrochemical sensors (Adhoum and Monser, 2008), the application of electrical conductivity methods (Yang et al., 2014), the use of chemiluminescent methods (Tsiaka et al., 2013), the application of stepwise orthogonalization of predictors to mid-infrared (MIR) spectra (Pizarro et al., 2013a), the use of NIR spectroscopy (Inarejos-García et al., 2012), the utilization of an on-line system based on hyperspectral information (Martínez Gila et al., 2015)and an opto-electronic system (Grossi et al., 2015).

As far as the olive oil content on FAAEs is concerned, to date, several analytical methods have been developed and applied for the determination of FAMEs and FAEEs in virgin olive oils. A solid phase extraction (SPE) using silica cartridges was first

proposed by Perez-Camino et al. (2008), trying to achieve an efficient extraction protocol. Another analytical approach combining the FT-IR screening of olive oil and partial least-squares (PLS) analysis has been also applied to determine these compounds (Valli et al., 2013). Furthermore, a rapid procedure based on the screening of FAAEs in virgin olive oils using time-domain reflectometry (TDR) and PLS analysis was developed and applied with noticeable success (Berardinelli et al., 2013). An approach based on direct thermo-desorbed and cryo-focalised in the cooled injector of a gas chromatography coupled to electron impact mass spectrometry (GC-EI MS) can be also mentioned; the authors used principal component analysis (PCA) data treatment (Boggia et al., 2014).

Even though the illustrated examples of methods for the determination of the physicochemical olive oil quality criteria offer some advantages when compared to the conventional analytical methods used by official regulations (they represent, in general, simple, efficient and non-destructive methodologies), they also exhibit certain limitations, such as, requiring expensive instrumentation, the need of frequent calibration, the fact that most of the proposed methods have been validated only on small sample-sets, and the circumstance that the different procedures must be separately calibrated for different types of virgin olive oils. For these reasons, each one of these alternative methodologies should be adapt taking into account necessity, cost, accessibility, analysis time (number of samples analyzed per hour), sample preparation requirements (with or without previous treatment) and sensitivity, among other features.

As far as the olive sensory quality evaluation is concerned, various instrumental techniques have been proposed, mainly based on establishing the link or association between virgin olive oil's volatile compounds composition and its sensory attributes (positive and/or negative). It is nowadays well-known that numerous volatile compounds (with diverse molecular weight, chemical nature, odour thresholds, and probably present in olive oil at very low amounts) are distinctive to the aroma, and hence, to the sensory quality of olive oil (Kalua et al., 2007). However, owing to the complex chemical composition of the volatile fraction of virgin olive oils and the fact that most of the volatile components are present in this matrix at very low amounts, there is a need for highly sensitive analytical methods for the characterization and quantification of these compounds. Therefore, a great number of analytical strategies including chemical pre-

concentration, separation and detection techniques have been developed and applied to the olive oil aroma characterization (Gomes da Silva et al., 2012).

Table 4. Representative examples of recent analytical methodologies proposed for olive oil quality parameters determination.

Analytical techniques	Instrumental measurements	Data treatment	Main findings	References
Vibrationalspectroscopictechniques	NIR	PLS	Real-time evaluation of olive oil FFAs with a standard error of prediction of 0.999.	Marquezet al.,2005
		PLS	Development of chemometric models with good predictive potential for olive oil FFA determination by directly measuring the fruit.	Cayuela et al., 2009
		PLS	Predictive models with good performance for the determination of PV, sensory attributes and others olive oil analytical parameters and compounds.	Inarejos-García et al., 2013
	Vis/NIR	PLS	Development, with good performances, of predictive models for the determination of FFAs, PV, and conjugated dienes.	Cayuela et al., 2013
		PLS, MCUVE and SPA	Improving accuracy of Vis/NIR chemometric models for olive oil FFA determination by using longer path lengths and wavelengths long of the visible.	García Martín 2015
	ATR-FTIR	PLS	Development of predictive models with very good performances for rapid and accurate measurements of FFA and PV.	Bendini et al., 2007
	FT-Raman	PLS	Development of chemometric models with very good predictive ability for rapid and accurate measurement of olive and olive oil FFA.	Muiket al.,2003
	FT-MIR	PLS, OLS	Rapid determination of olive oil PV. Results were in agreement with results obtained with the official standard method.	Pizarro et al., 2013a
	FT-IR	PLS	The developed chemometric models allowed the determination FFAES with an accuracy similar to the reference official standard method.	Valliet al.,2013
Chromatographictechniques	GC-FID	ANOVA	Solid-phase microextraction is an efficient alternative method for FAAEs preparation.	Perez-Camino et al.,2008
	GC-EI MS	PCA and PBr	Discrimination between EVOO and low quality olive oils based on FAAEs direct determination and multivariate data analysis.	Boggiaet al., 2014
Electrophoretictechniques	CE	ANOVA	Ethanolic extraction of the long-chain free fatty acids followed by electrokinetic chromatography quantitative determination of oil FFA.	Balesteroset al.,2007
Electro-chemicalmethods	Electricalimpedancespectroscopy	NLR	FFA determination with a good accuracy applying electrical conductance to olive oil emulsion with hydro-alcoholic solution.	Grossi et al.,2014
	Electrochemical sensor	ANOVA	The proposed method was successfully applied to measurement of olive oil PV with an excellent agreement with results obtained with the official standard procedure.	Adhoum and Monser, 2008
	Determination of changes in the electrical conductivity value	ANOVA and LR	Determination of PV value with accuracy similar to the reference official standard methods throughoutevaluating changes in the electrical conductivity values of the aqueous phase during the reaction of potassium iodide with the hydroperoxides presented in oil samples.	Yang et al.,2014
	Opto-electronicsystem	PLS	Real time Determination of PV value with good accuracy.	Grossi et al.,2015
	TDR	PLS	Predictive models for rapid determination of FAAES in olive oil samples with good accuracy in respect to official method.	Berardinelliet al., 2013
Flowinjection analysis	FIA in combination with a distributed expert system	ANOVA	On-line rapid and accurate chemical quality control of olive oil (FFA, PV, K_{232} and K_{272}).	Bonastreet al., 2004

Table 4. (continued)

Analytical techniques	Instrumental measurements	Data treatment	Main findings	References
Enzymatic methods	Enzymatic reacion- UV spectrophotometry determination	ANOVA	Determination of FFA with good accuracy by enzymatic oxidation of free polyunsaturated fatty acids present in oil samples with lipoxygenase and spectrophotometric detection of the hydroperoxy-fatty acids produced at 234nm.	Ben Rejeb and Gargouri, 2011
Chemilumin-escence Spectroscopy	Chemiluminescent reaction of alkaline luminol and the hydroperoxides of oil, catalyzed by Fe(III) using 1-propanol as the reaction solvent	ANOVA and LR	Determination of PV value with excellent agreement with results obtained with the official standard procedure.	Tsiakaet al.,2013
Hyperspectralimagingtechniques	Hyperspectralimagesensorsr	GA, LASSO, SPA and MLR	Real time determination of PV value and FFA with good accuracy.	Martínez Gila et al.,2015
Biosensors	Electronic tongue	LDA	The used electronic tongue showed satisfactory correct prediction capability of the overall intensity sensory perception of EVOO.	Velosoet al., 2016
	Electronic Nose sensors equipped with 10 Metal Oxide Semiconductor (MOS) sensors	PCA and LR	The method employed showed good reliability and discriminating power among olive oils of different quality (EVOO and LVOO) and among different intensities of fruity and rancid attributes. Results were in good agreement with those obtained by the official panel test method.	Apetrei and Apetrei, 2014

The combination of these analytical methods with multivariate data analysis techniques has proved to be useful for the sensory classification of virgin olive oils. However, it is also necessary to explain that in many papers that try to correlate the information about the volatile fraction composition with negative and positive attributes to classify virgin olive oils, the description of this complex relationship is tentative, because no information is often given about the odour threshold and activity of the identified compounds. Furthermore, in some instances, the aroma attributes should not be associated to a single compound, since they can result from the interaction of very similar odorants (in terms of aroma and structural terms) present in olive oil at low concentrations (even below their sensory threshold), but, in certain cases, exerting a concerted action (Angerosa et al., 2004). This challenging situation has opened up the way to the application of new olfaction instrumentation, in particular GC-olfactory (GC-O), and chemical sensor technologies (electronic tongue and noses), combined with multivariate data processing methods, which have been used with considerable success to classify olive oil according to their sensory quality (Sinelli et al., 2010; Escuderos et al., 2011; Savarese et al., 2013; Veloso et al., 2015).

Advances in analytical methods to detect olive oil adulteration

The rapid and reliable detection of adulteration (with a proper degree of sensitivity and selectivity) is a very challenging issue in the field of virgin olive oil authentication. Indeed, the tedious and, sometimes, time-consuming procedures of the conventional analytical methods approaches need to be improved or replaced by faster and precise techniques. In this sense, during the last decade, numerous analytical procedures (including sample preparation, analysis data acquisition and processing) have been developed and proposed for the adulteration control of virgin olive oil (Table 5). They have garnered general acceptance as powerful methods, offering some advantages such as high separation efficiency and resolution, rapid analysis and minimal consumption of reagents and samples, which make them attractive alternatives to the conventional analytical methods used, so far, for virgin olive oil adulteration control. In this section, we have considered different method categories, being the most relevant the following ones:

-Vibrational spectroscopic techniques: vibrational spectroscopic techniques based on both infrared and mid-infrared absorptions (FT-IR, FT-MIR, NIR and MIR) and Raman scattering, have demonstrated their great potential as promising tools to uncover olive oil adulteration over the last years; they offer important advantages over the conventional analytical methods used in this area, in particular, regarding the volume of consumed reagents, rapid measurements and fast data acquisition, relatively low cost, samples handling and their non-destructive nature (analysis is performed directly on intact samples or with only minimal sample preparation), etc. Table 5 shows a selected number of applications of vibrational-spectroscopy-based methods for virgin olive oils adulteration control. As can be observed, these applications can be roughly divided into two broad categories.

The first is mainly related to alternative applications to conventional methods for the determination of some purity criteria. The second category deals with the rapid adulteration detection, identification of the type of adulterant and the quantification of this adulteration. In both cases, given the nature of the data sets obtained (generation of typical spectra of analyzed samples), chemometric techniques are usually required to

develop predicting models that correlate the complex spectra to the level of a compound, class, or parameter to be predicted.

Belonging to the first category, vibrational spectroscopy such as NIR (Galtier et al., 2007), MIR (Dupuy et al., 2010), FT-NIR (Azizian et al., 2015), Attenuated Total Reflection Fourier Transfer Infrared (ATR-FTIR) (Maggio et al., 2009) and Raman scattering (Korifi et al., 2011) have been favorably applied to monitor the content of FAs, *trans* FAs and TAGs in virgin olive oil samples (Table 5). In these studies, the small estimation errors achieved throughout the application of chemometrics to the spectral data demonstrated the quality of the developed models and the suitability of these techniques to the determination of these purity criteria of olive oil.

Furthermore, with regard to the second category of applications of vibrational spectroscopy to virgin olive oil adulteration control, a large number of studies have been published over the last years about NIR (Christy et al., 2004; Kasemsumran et al., 2005), Vis/NIR (Mignani et al., 2011), MIR (Gurdeniz and Ozen, 2009), FT-IR (Lerma-García et al., 2010; Rohman et al., 2015), ATR-FTIR (De la Mata et al., 2012; Aftab et al., 2014), Raman (Lopez-Diez et al., 2003; Zou et al., 2009; Zhang et al., 2011), Vis-Raman (El-Abassy et al., 2009) and FT-Raman (Heise et al., 2005) spectroscopy. These techniques have been employed to develop rapid and simple methods to detect adulteration and to determine the nature and quantity of adulterant in the olive oil samples under study. The application of statistical data evaluation allowed establishing approaches with high capability and great potential to detect EVOO's adulteration and identify the adulterants´ nature (Table 5).

Table 5. Representative examples of recent analytical methodologies proposed for olive oil adulteration control (alternative to conventional method to determine purity criteria (category 1); rapid adulteration detection/quantification and identification of the adulterant oils (category 2)) and botanical and geographical origins assessment.

Analytical techniques	Instrumental measurement	Adulteration control		Origin authentication		Data treatment	Main findings	Reference
		Category 1	Category 2	Geographic	Botanic			
Vibrational spectroscopic techniques	NIR		***		***	PLS-DA	Rapid and efficient quantification of the main FA and TAGs on French PDO olive oils and their discrimination according to geographic origin.	Galtieret al., 2007
		***		***		PCA and PLS	Successful discrimination and quantification of olive oil adulteration with vegetable oils.	Christy et al.,2004
		***		***		PCA and PLS	Detection and quantification of olive oil adulteration with vegetable oils.	Christy et al.,2004
		***		***		PLS and PLS-DA	Discrimination, detection and quantification of olive oil adulteration with vegetable oils.	Kasemsumran et al.,2005
	Vis/NIR	***		***		PCA and PLS-DA	Quantification and identification of EVOO adulteration with lower-grade olive and olive pomace oils.	Mignani et al.,2011
		***	***		***	GA-PLS	Rapid and efficient classification of studied virgin olive oils according to theirgeographic origin.	Lin et al.,2012
	FT-NIR			***		PLS	Prediction of EVOO fatty acid composition with satisfactory accuracy and identification of the kind and amount of adulterant vegetable oils in EVOO.	Azizian et al.,2015
	MIR	***		***		PCA, PLS and PLS-DA	Detection and quantification of olive oil adulteration with different edible oils.	Gurdeniz and Ozen, 2009
	NIR and MIR		***		***	PLS, PLS-DA and H-PLS	Quantitative analysis of FA and TAGs compounds, and geographical classification of studied oils.	Dupuy et al.,2010
	ATR-FTIR		***			PLS	Quantification of the main olive oil FA. Results were in agreement of those obtained using official method.	Maggio et al.,2009
		***		***		HCA, PCA, PLS and PLS-DA	Fast and accurate discrimination between olive oils from different grades and varieties and blended olive oils by different edible oils.	De la Mata et al.,2012
		***	***		***	PCA, LDA, PLS-DA	Successfuldiscrimination between Ligurian (Italy) and non-Ligurian olive oils.	Hennessy et al.,2009
	SB-ATR-FTIR	***		***		PLS and PCS	EVOO adulteration with RVOO detection and quantification.	Aftab et al., 2014
	FT-IR	***		***		LDA and MLR	Detection and quantification of EVOO adulteration with different edible oils at concentration less than 5%.	Lerma-García et al.,2010
		***		***		LDA, PCr and PLS	Quantification of pumpkin seed oil blended into EVOO.	Rohman et al.,2015
	FT-Raman	***		***		PLS	Efficient chemometric models to the detection of olive oil adulteration by sunflower oil.	Heise et al.,2005
	Raman	***		***		PCA and GA-PLS	Rapid and accurate detection and quantitative assessment of the olive oil adulteration with hazelnut oils.	Lopez-Diez et al.,2003
		***		***		***	Rapid discrimination between various grades of olive oil and seed oils and detection of olive oil adulteration with at least 5% of edible oils.	Zou et al., 2009
		***		***		SVM	Rapid detection and quantitative evaluation of adulterated olive oil with vegetable oils.	Zhang et al.,2011
	Vis/Raman	***		***		PCA and PLS	Fast monitoring and detection of EVOO adulteration with sunflower oil.	El-Abassy et al.,2009

Table 5. (continued)

Analytical techniques	Instrumental measurement	Adulteration control		Origin authentication		Data treatment	Main findings	Reference
		Category 1	Category 2	Geographic	Botanic			
Mass spectrometry	ESI MS	***		***		PLS-DA	Rapid accurate discrimination between EVOO and OVO grades and the detection of adulteration of EVOO with vegetable oils.	Alves et al., 2013
	ESI MS and APPI MS	***		***		PCA, LDA	Both methods seem to be rapid procedures with high discrimination power allowing detection of olive oil adulteration,especially by hazelnut oils.	Gómez-Ariza et al.,2006
	MALDI-TOF MS	***		***		***	Characterization of the polar components in hazelnut and EVOO and identification of some of them as EVOO adulteration markers.	Calvano et al., 2010
		***		***		***	Identification of some phospholipids compounds as markers of EVOO adulteration with hazelnut oil.	Calvano et al., 2012
			***			ANOVA	Fast and reliable characterization of TAGs and FA profiles in virgin olive oils without any derivatization.	Chapagain and Wiesman, (2012)
	DART-TOF MS	***		***		LDA	Rapid and easily differentiation of EVOO, OPO and OO grades and detection of EVOO adulteration with hazelnut oil based on TAGs profiles.	Vaclavik et al.,2009
	HS-Qp-MS	***		***		CA, PLS, PCA, PCR and MLR	Reliable analytical procedure to detect olive oil adulteration with hazelnut oil.	Peña et al., 2005
	HS-Qp-MS	***		***		LDA	Rapid and reliable method to differentiate the adulterated olive oil (with sunflower oil and OPO) from the non-adulterated olive oils.	Lorenzo et al.,2002
Chromatography	GC-FID		***			LR	Improved method simplifying the preparation step for rapid and reliable determination of sterol, uvaol, and erythrodiol using SPE.	Mathison and Holstege, 2013
			***			ANOVA	Improved method simplifying the preparation step for rapid and accurate evaluationof olive oil waxy fraction using SPE.	Pérez-Camino et al.,2012
		***	***		***	ANOVA and LDA	Geographical classification of Extremadura Spanish olive oils according to their geographic origin based on FA composition.	Martínez et al.,2014
		***	***	***		PCA and LDA	Successful assessment of botanical origin of monovarietal Turkish virgin olive oils by sterols and triterpene diols profiling.	Lukić et al.,2013
		***	***		***	PCA and SIMCA	Reliable and efficient discrimination between Italian ad non Italian virgin olive oil based on sterolic fraction profiling and chemometrics.	Giacalone et al.,2015
	HPLC-APCI MS		***			***	Quali-quantitative evaluation of the main sterols and triterpenic dialcohols from the unsaponifiable fraction of VOO, ROO, OPO and COPO.	Cañabate-Díaz et al.,2007
			***			***	Separation and identification of 15 sterols and 2 triterpenic dialcohols from the unsaponifiable fraction without using thin-layer chromatography.	Carretero et al.,2008
			***			PCA	Characterization of TAGs and FA profiles of olive oil and others vegetable oils and detection of olive oil adulteration with sunflower oil.	Holčapek and Lísa, 2009

Table 5. (continued)

Analytical techniques	Instrumental measurement	Adulteration control		Origin authentication		Data treatment	Main findings	Reference
		Category 1	Category 2	Geographic	Botanic			
Chromatography	HPLC-APCI MS/MS		***			PCA, CA	Characterization and quantification of the main sterolic and triterpenic dialcohols compounds occurring in virgin olive oil without previous derivatization.	Zarrouk et al.,2010
			***			PCA	Characterization of EVOO TAGs profiles and detection of adulteration with soybean oil.	Fasciotti and Pereira Netto, 2010
	HPLC-UV	***		***		PCA, HCA, PLS-DA	Fingerprinting of the sterolic fraction of virgin and pomace olive oils and some vegetable oils.	Bagur-González et al.,2015
		***	***			PCA and HCA	Using TAGs profiles to trace the geographical and botanical origin of Turkish olive oils.	Gökçebağ et al.,2013
	HPLC-UV & HPLC-FLD	***	***	***		PCA and HCA	Botanical classification of monovarietal virgin olive oils by pigments compounds profiling.	Cichelli and Pertesana, 2004
	HPLC-CAD	***		***		iPLS and PLS	TAGs fingerprinting for the detection and quantification of EVOO adulteration with others edible vegetable oils.	De La Mata-Espinosa et al., 2011
Chromatography	GC-MS	***	***			ANOVA and LDA	Correct geographical and botanical classification of Western Greek monovarietal virgin olive oil based on volatile compounds profiling.	Pouliarekou et al.,2011
		***		***		GA-PLS, PLS and SIMCA	Reliable identification and quantification of EVOO adulteration with others vegetable oils.	Ruiz-Samblás et al.,2012
	HPLC-ESI MS	***	***		***	ANOVA, PCA and LDA	Geographical classification of North Moroccan monovarietal virgin olive oils by phenolic compounds profiling.	Bajoub et al.,2014
	GC-FID and HPLC with different detectors	***	***		***	ANOVA, PCA and LDA	Geographical classification of North Moroccan monovarietal virgin olive oils combining their composition (major and minor compounds) and chemometrics.	Bajoub et al.,2015
	UHPLC-APCI MS	***	***	***		LDA	Rapid fingerprinting method based on chemometric and sterols profiles allowing correct botanical classification of studied oils.	Lerma-García et al.,2011
NMR	1H NMR	***	***		***	ANOVA, PCA, CA and NCM	Correct classification of studied virgin olive oils according to their geographic origin.	Longobardi et al., 2012

Table 5. (continued)

Analytical techniques	Instrumental measurement	Adulteration control		Origin authentication		Data treatment	Main findings	Reference
		Category 1	Category 2	Geographic	Botanic			
Other techniques	**DNA-based techniques**	***		***		***	Efficient and rapid identification of olive oil adulteration with vegetable oils.	Vietina and others 2013
		***	***			PCA	The efficiency of RAPD, ISSR, and SSR molecular markers combined with chemometrics was tested and validated as geographical and botanical origin tracing approach.	Martins-Lopes et al.,2008
	Electronic nose	***		***		PCA and PLS	Very good results were obtained en terms of the detection of EVOO adulteration by rapeseed and sunflower oils, as well as predicting the percentage of adulteration.	Mildner-Szkudlarz and Jeleń, 2010
	Electronic nose	***	***	***		LDA	Rapid and efficient fingerprinting approach for the classification of virgin olive oils according to their varietal origin.	Dias et al.,2014
	Voltammetric E-tongue	***		***		ANOVA, PCA, PLS and PLS-DA	Detection and identification of EVOO adulterationwith sunflower, soybean and corn oils at concentrations lower than 10%.	Apetrei and Apetrei, 2014
	Thermal techniques: DSC		***			PLS	Rapid and efficient characterization and quantification of the main FA occurring in different olive oils grades, OPO and three seed oils (canola, sunflower and hazelnut oils).	Cerretani et al.,2011
		***		***		***	Rapid and efficient detection of EVOO adulteration with sunflower oil.	Wetten et al.,2015
	Isotopic techniques	***		***		***	$^{13}C/^{12}C$ isotope ratio measurements have been successfullyused for the detection of olive oil adulteration with pomace oil.	Angerosa et al.,1997
	Electrophoretic techniques: CE-MS/MS	***		***		***	Very sensitive method for the simultaneous determination of six non-protein amino acids in vegetable oils (soybean, sunflower and corn oils) and olive oil samples and their use as novel markers for the detection of adulterations in olive oil with the cited vegetables oils.	Sánchez-Hernández et al.,2011

- **Nuclear magnetic resonance (NMR) spectroscopy:** NMR spectroscopy techniques (^{1}H, ^{13}C, ^{31}P), have been extensively utilized in virgin olive oil adulteration control, both for quantitative analysis of some purity criteria and for targeted or untargeted fingerprinting approaches. These analytical approaches (considering the methodology development, advances and applications in the field of olive oil adulteration) have been comprehensively described in various interesting critical review papers (Mannina and Sobolev, 2011; Dais and Hatzakis, 2013). The authors basically showed that NMR spectroscopy techniques and the subsequent use of suitable chemometric techniques seem to be a simple, fast and powerful approach for the quantitative analysis of olive oils TAGs, *trans* and *cis* FAs and sterols. In the mentioned very interesting papers, the authors also highlight that NMR can apply fingerprinting approaches allowing the detection of adulterant low price olive oils or vegetable oils in EVOOs. The detection limit and the high discriminative capability of the models developed using NMR and chemometric treatments suggest their use as plausible alternative to the official methods.

- **Mass spectrometry:** various MS methodologies have been established (not requiring prior separation) to be applied for virgin olive oils adulteration control. When no chromatographic or electrophoretic previous separation is coupled to MS, an overall mass spectrum of all the sample's compounds may be obtained in a short analysis time. In the studies employing direct infusion MS in this field, electrospray ionization (ESI), atmospheric pressure photoionization ion sources (APPI), and matrix-assisted laser desorption/ionization (MALDI) have been used for the detection and identification of the most common EVOO's adulterant vegetable oils, in particular hazelnut oil. Within this context, Goodacre et al. (2002)used a direct infusion ESI-MS approach combined with chemometric treatments (PCA and cluster analysis (CA)); the results were very promising and showed that the obtained spectra generated very interesting information and allowed a good discrimination between EVOO and adulterated oils without the need of any chromatographic separation. Using a similar approach, more recently, Alves et al. (2013) demonstrated the suitability of combining the spectral information achieved by ESI-MS with a chemometric data analysis using partial least squares discriminant analysis (PLS-DA) for discriminating EVOO from others vegetable oils commonly used as adulterants, particularly OVOO, corn, sunflower, soybean and canola

oils. Most lately, besides applying PLS-DA to ESI-MS data, PLS treatment was used to build predictive models for the detection of EVOO adulteration with four adulterant oils (soybean, corn, sunflower and canola) (Alves et al., 2014). Furthermore, Gómez-Ariza et al., (2006) described, in a comparative study, the potential of ESI-MS and APPI-MS for the control of EVOO adulteration with hazelnut oil; both methods seemed to be optimum to virgin olive oil adulteration detection in short time (approximately 1 min per sample). Alternatively, approaches coupling FIA to time-of-flight mass spectrometry (TOF MS) equipped with a MALDI source have been described, and their capability to detect EVOO adulteration with hazelnut oil has been evaluated (Calvano et al., 2010; Calvano et al., 2012).Chapagain and Wiesman (2009) also proposed a worthy example, where a fingerprinting method based on MALDI-TOF MS was applied as reliable and fast strategy for the effective determination of FAs and TAGs composition in virgin olive oil samples without any required derivatization. The application of chemometric treatments to obtained data, allowed the authors to achieve the correct discrimination of the studied virgin olive oils from others common adulterant vegetable oils. It is also meritorious to be mentioned an analytical methodology based on direct analysis in real time coupled to high-resolution time-of-flight (DART-TOF MS) and linear discriminant analysis (LDA) as chemometric approach for the data treatment that was developed and successfully applied to differentiate non-adulterated EVOO from those adulterated with OPO, OO, and hazelnut oil (Vaclavik et al., 2009). Some other examples concerning the use of MS as an appropriate tool for the EVOO adulteration control can be illustrated, for instance, the potential of combining headspace-mass spectrometry (HS-MS) and chemometric analysis to detect adulteration of olive oil samples with different levels of hazelnut oil (Peña et al., 2005), sunflower and OPO (Lorenzo et al., 2002) has been tested.

Even though these fingerprinting MS approaches represent attractive analytical alternatives to olive oil adulteration control, especially for their minimal requirements of sample preparation, no need of chemical derivatization or chromatographic separation, short analysis time, and their environmentally friendly nature, they obviously show some drawbacks too. The major disadvantage of MS based-techniques is that they are one of the most expensive analytical techniques to be used (both in terms of the initial investment and the subsequent maintenance costs).

- Chromatographic techniques: outstanding advances have been made to fulfil the goal of improving the current official analytical methods (based on chromatographic techniques (both HPLC and GC)) in terms of sample preparation, instrumental analysis, data processing and interpretation for the efficient control of olive oil adulteration. Some examples exemplifying these improvements are given in Table 5. Work has been mainly made regarding sample preparation and selecting powerful detection systems. Indeed, methods using SPE prior separation of free and esterified sterols (Mathison and Holstege, 2013), and wax (Pérez-Camino et al., 2012) have been proposed. The main advantages of using SPE are: relatively short preparation time, reduced solvent and sample consumption, and the possibility of handling several samples simultaneously. As mentioned above, the use of potent detection systems such as MS, coupled to chromatographic separation techniques for the structural and quantitative analysis of some VOO purity criteria has represent one of the growing areas. In this sense, Cañabate-Díaz et al. (2007) proposed for the first time the analysis of sterols and triterpenic dialcohols from the unsaponifiable fraction of virgin olive oils, ROO, OPO and COPO using a HPLC-APCI MS analytical platform, obtaining a proper separation of cholesterol, stigmasterol, β-sitosterol, sitostanol, campesterol, erythrodiol and uvaol. The same analytical platform was used to develop a rapid and effective method for the characterization of sterols and triterpenic dialcohols from the unsaponifiable fraction of virgin olive oils (Segura-Carretero et al., 2008; Zarrouk et al., 2010), allowing the structural characterization and the quantification of the main sterolic andtriterpenic dialcohols compounds occurring in virgin olive oil shortening substantially the sample preparation procedure. By using similar approaches, some other authors chose HPLC-APCI MS for the detailed characterization of TAGs profiles of virgin olive oil and others vegetable oils (Holčapek and Lísa, 2009); moreover, when PCA was applied for the treatment of the obtained data, adulterated virgin olive oil with sunflower oil could be detected even at a very low levels (1%). In like manner, a direct injection HPLC-APCI MS/MS method was proposed for the characterization of EVOO's TAGs profiles, showing the potential of the developed methodology when it is combined to PCA, for the detection of EVOO's adulteration with soybean oil (Fasciotti and Pereira Netto, 2010). Furthermore, over the last years, various fast and reliable approaches based on fingerprinting methods (in either targeted or untargeted mode using chromatographic

techniques, lonely or in conjunction with MS detectors, and combined to chemometrics) were found to be valuable to provide a solution to EVOO adulteration control. Among these approaches, it is possible to mention as example the use of sterols profile determined by liquid chromatography with ultraviolet absorption detection and chemometrics (PCA, hierarchical cluster analysis (HCA), and PLS-DA) to build discriminative models which exhibited good predictive capability allowing the correct classification of virgin olive oils and other vegetable edible oils (Bagur-González et al., 2015); the use of TAGs profile, as determined by GC-MS, combined with chemometrics (genetic algorithm partial least squares (GA-PLS), PLS andSoft independent modeling of class analogies (SIMCA)) for the identification and quantification of EVOO adulteration with others vegetable oils (sunflower, corn, seeds, sesame and soya) (Ruiz-Samblás et al., 2012); or the quantification of virgin olive oil in blends with other vegetable oils using a targeted fingerprinting approach combining the TAGs profile determined by HPLC coupled to a Charged Aerosol Detector (CAD) and chemometrics (interval PLS (iPLS) and PLS) (De La Mata-Espinosa et al., 2011). Some other examples regarding this topic and the adulteration control of virgin olive oil using chemometrics and chromatographic methods of TAGs profile can be found in an interesting review authored by Bosque-Sendra et al., 2012.

Although the afore-mentioned studies provide evidence that advances in chromatographic techniques appear to solve some drawbacks of the conventional methods used for olive oils adulteration control, the exhaustive overview of these studies also reveals some weak points that make difficult their adoption as alternative methods in official measurement of VOO authenticity. Some of them are: the fact that most of the studies used a limited number of samples; the analyzed oils are usually coming from restricted geographical areas and belong to few varieties; and the use of MS detectors, which increases the overall method costs.

- **Other analytical approaches:** a number of important analytical methods have been developed and suggested for virgin olive oils adulteration control purposes using other emerging analytical techniques (Table 5). The following are some pertinent examples:

- Various genetic and deoxyribonucleic acid (DNA) based techniques have been proposed as useful procedures for the qualitative and quantitative determination of adulterant vegetable oils and other lower-price virgin olive oils and olive-pomace oils in EVOO (Rabiei and Enferadi 2012; Ben-Ayed et al., 2013; Vietina et al., 2013). The employment of these techniques seem to provide some advantages, such as increased specificity and sensitivity, high durability of DNA molecules, as well as the fact that they are independent from environmental conditions (compared to other authenticity compounds) and show a reliable performance with highly steady processed samples. Diverse molecular markers have been typically used for VOO adulteration control, mainly simple sequence repeats (SSRs), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR) and single nucleotide polymorphisms (SNP) (Ben-Ayed et al., 2013; Ou et al., 2015). Various authors showed, however, that the reliability and reproducibility of these methods are widely conditioned by the quality of the DNA extracted from studied oils. For this reason tremendous effort has been done to develop reliable and effective DNA extraction methods (Raieta et al., 2015) and to extend the use of polymerase chain reaction (PCR) to amplify the extracted microsatellite markers (Wu et al., 2011; Vietina et al., 2013).

- Electronic nose andelectronic tongue technologies, in combination with chemometrics, have been successfully applied for the detection of adulteration of EVOO with different kinds of olive oils, pomace oils and/or vegetable oils (Mildner-Szkudlarz and Jeleń 2010; Apetrei and Apetrei 2014).

- Thermal techniques: thermal properties (measured both in cooling and heating regimes) of EVOO have been reported to widely correlate with its chemical composition (Chiavaro et al., 2007). In this context, olive oil FAs composition was successfully determined using an approach that combine differential scanning calorimetry (DSC) and PLS regression (Cerretani et al., 2011). Furthermore, thermo-analytical techniques, in particular DSC, have been suggested as a valuable tool to fight against olive oil adulteration. Thus, Ferrari et al. (2007), and Van Wetten et al. (2015) described DSC methods to authenticate olive oils and other edible oils; Chiavaro et al. (2008a) developed a technique based on DSC to differentiate olive oils of five distinct

commercial categories, and to detect adulteration of EVOO with refined hazelnut oil (Chiavaro et al., 2008b) and/or high oleic sunflower oil (Chiavaro et al., 2009).

- Isotopic techniques: although the reported applications in this category are, to date, limited, these techniques have shown great potential for virgin olive oil adulteration control. Thus, methods such as: stable isotope ratio analysis and $^{13}C/^{12}C$ measured using elemental analyzer-isotopic ratio coupled to MS, or determined by a gas chromatography-combustion-isotopic ratio MS (GC/C-IRMS) have demonstrated to be useful for detecting the adulteration of olive oil with olive-pomace oils or with other vegetable oils (Angerosa et al., 1997; Spangenberg, 1998)

- Electrophoretic techniques: analytical methods based on capillary electrophoresis coupled to MS or ultraviolet have demonstrated to be valuable and helpful tools in olive oil adulteration control (Sánchez-Hernández et al., 2011; Monasterio et al., 2013).

TRENDS AND ADVANCES IN ANALYTICAL APPROACHES TO TRACE THE GEOGRAPHICAL AND BOTANICAL ORIGIN OF VIRGIN OLIVE OILS

As stated in the first section, the geographical and botanical origin authentication of virgin olive oil remain not legally regulated by official analytical methods. However, over the last years, and due to the increasing interest of consumer to labeled geographic origin and monovarietal virgin olive oils, tremendous efforts have been devoted to the development of robust and reliable analytical strategies to verify their declared botanical and geographical origin. In this sense, differentiation of virgin olive oil according to geographic origin and/or variety has been performed by using mainly three strategies: targeted analyses, the use of profiling approaches and the utilization of more untargeted ones. The first one is based on identifying various olive oil´s compounds, determining their content, and correlating the obtained data with the geographical and/or botanical origins. The second one includes the qualitative and/or quantitative determination of a larger set of olive oil compounds that are related, considering their chemical nature and/or biosynthesis pathway. The third strategy implies the use of fingerprinting approaches using a chemometric screening of the whole sample fingerprint in order to identify key markers that differentiate the geographic and/or cultivars of interest. Regardless of the approach applied, chemometric models built by using different

multivariate data analysis have been used to get a correct classification of the sample´s botanical and/or geographical origin. Relevant literature examples have been summarized in Table 5. Indeed, several studies have been undertaken to develop chemometric models for classifying olive oils according to their geographical and/or botanical origins, based on a range of chemical compounds such as TAGs (Gökçebağ et al., 2013), FAs (Diraman et al., 2010; Diraman et al., 2011; Martínez et al., 2014), phenolic compounds (Alkan et al., 2012; Bajoub et al., 2014), pigments (Cichelli and Pertesana, 2004), sterols(Lukić et al., 2013; Giacalone et al., 2015) and volatile compounds (Pouliarekou et al., 2011). Besides, in some studies dealing with the olive oil geographical and botanical origin verification, data from several major and minor compounds have been combined into one model, in order to exploit the different information provided by each type of compounds (Yorulmaz et al., 2014; Bajoub et al., 2015). As far as fingerprinting approaches are concerned, we find stimulating examples based on separative and MS techniques (Lerma-García et al., 2011; Riccio et al., 2011), vibrationalspectrometric techniques (Hennessy et al., 2009; Lin et al., 2012), NMR (Petrakis et al., 2008; Longobardi et al., 2012), DNA-based techniques (Martins-Lopes et al., 2008), electronicnose and electronictongues techniques (Dias et al., 2014), etc. In all the mentioned instances, the fingerprints were treated by different chemometric techniques and allowed the authentication of geographic and/or varietal provenance of studied oils. Another tactic which is becoming quite popular is the simultaneous use of data from different analytical techniques (the so-called data-fusion), since the information provided by each of them might be different and complementary (Karabagias et al., 2013; Pizarro et al., 2013b). Undoubtedly, it can be concluded that important work has been done over the last years in the field of virgin olive oil geographic and botanical origin assessment. However, in spite of these advances, this issue is still far from being completely resolved. Certainly, universal analytical methods for the determination of the geographical/botanical origin of virgin olive oil do not really exist mainly due to the restricted character of most of the studies carried out in this field (in terms of analyzed samples, studied varieties and considered geographical areas). The latter mentioned items represent a kind of barrier or obstacle which complicate the acceptance of the discriminative models proposed by these studies and impede that those methodologies become more widespread. To face this situation there

is a need for further plurianual and large studies with higher number of samples collected from the main olive-growing areas over the world, representing the main varieties cultivated worldwide. Such studies could lead to build a large database that would make possible the geographical and botanical traceability of the most representative olive oils around the world.

CONCLUSIONS

The worldwide proliferation of olive oil quality and authenticity standards regulations, driven predominantly by the trade globalization of this product and the emergence of new producing and consuming countries outside the Mediterranean region, has stimulated the discussion and debate about the harmonization of olive oil standards and trade regulations, which should take into account the natural variation of olive oil composition due to environmental conditions and agro-technological practices. Furthermore, limitations of the conventional analytical methods used for olive oil quality and authenticity control, have generated a need for newer, fast, cost-effective and reliable analytical approaches. However, although various more modern analytical techniques used in combination with chemometric analyses have improved the efficiency and effectiveness of conventional methods and cover some of their gaps and limitations, from the studies reviewed herewith two major deficiencies can be identified: (a) most of the proposed methods were developed using a limited number of olive oil samples coming from restricted botanic origins and geographic areas, which appear to limit their use on a wider scale; and (b) some of the reviewed methods, even if their authentication potential was proved, are very costly, and, therefore, cannot be useful for routine analysis in many laboratories. For this reasons, it is assumed that they will not become an alternative to conventional methods in a short-term scenario. Hence, it seems necessary to conceive further developments which should aim at improving the representativeness of the studied samples to the main olive-growing areas and cultivated varieties together with the employment of cost-effective analytical techniques.

ABBREVIATIONS

1,2-diacylglycerol, **1,2-DAG**; 2-glyceryl monopalmitate, **2P**; American Oil Chemists' Society, **AOCS**; amplified fragment length polymorphism, **AFLP**;atmospheric pressure photoionization ion sources, **APPI**; attenuated total reflection Fourier transfer infrared, **ATR-FTIR**; capillary electrophoresis, **CE**; Australian Standards, **AUS**; cluster analysis, **CA**; charged aerosol detector,**CAD**; Californian Standards, **CAF**; Codex Alimentarius, **Codex**;crude olive-pomace oil, **COPO**; deoxyribonucleic acid, **DNA**; diacylglycerols, **DAG**; difference between actual and theoretical content of triacylglycerols, **ΔECN42**; differential scanning calorimetry,**DSC**; direct analysis in real time, **DART**;electrospray ionization, **ESI**;equivalent carbon number, **ECN**; European Commission, **EC**; European Union, **EU**; extra virgin olive oil, **EVOO**; fatty acid alkyl esters, **FAAEs**; fatty acids ethyl esters, **FAEEs**;fatty acids methyl esters, **FAMEs**; fatty acids, **FAs**; flame ionization detector,**FID**; Flowinjection analysis,**FIA**;Fourier transform infrared, **FT-IR**;Fourier transform-mid-infrared, **FT-MIR**;Fourier transform-Raman, **FT-Raman**; free fatty acids, **FFAs**; gas chromatography coupled to electron impact mass spectrometry, **GC-EI MS**;GC-olfactory, **GC-O**;genetic algorithm partial least squares,**GA-PLS**; headspace-mass spectrometry,**HS-MS**;hierarchical cluster analysis, **HCA**; hierarchical partial least-squares, **H-PLS**;high pressure liquid chromatography,**HPLC**;International Olive Council, **IOC**; International Organization of Standardization, **ISO**; inter-simple sequence repeats,**ISSR**;interval partial least-squares,**iPLS**; lampante virgin olive oils, **LVOO**; least absolute shrinkage and selection operator, **LASSO**; linear discriminant analysis, **LDA**; linear regression, **LR**; mass spectrometry, **MS**;matrix-assisted laser desorption/ionization,**MALDI**; median of olive oil defects, **MeD**; median of olive oil fruitiness, **MeF**; mid-infrared,**MIR**; montecarlo uninformative variable elimination, **MCUVE**; multiple linear regression, **MLR**; not applicable, **N/A**; not considered, **N/C**; near-infrared,**NIR**;nuclear magnetic resonance,**NMR**; olive oil, **OO**; olive-pomace oil, **OPO**; one way analysis of variance, **ANOVA**; ordinary virgin olive oil, **OVOO**; partial least squares discriminant analysis,**PLS-DA**;partial least-squares,**PLS**; passing-bablok regression, **PBr**; peroxide values, **PV**; polymerase chain reaction,**PCR**; principal component analysis, **PCA**;principal component spectra diagnostic, **PCS**; principle component regression, **PCR**; pyropheophytins, **PPPs**; random amplified polymorphic DNA,**RAPD**;

refined olive oil, **ROO**; refined olive-pomace oil, **ROPO**; select-ordinary least squares, **OLS**; simple sequence repeats,**SSRs**; single nucleotide polymorphisms, **SNP**; soft independent modeling of class analogies,**SIMCA**; solid phase extraction,**SPE**; successive projections algorithm, **SPA**; support vector machine, **SVM**;time-domain reflectometry, **TDR**;time-of-flight mass spectrometry, **TOF MS**;triacylglycerols, **TAGs**; ultraviolet specific extinction coefficients, $\mathbf{K_{232}}$ **and** $\mathbf{K_{272;}}$Ultraviolet, **UV**; United States Department of Agriculture, **USDA**; virgin olive oil, **VOO**; Visible/near infrared, **Vis/NIR**; Visible/Raman, **Vis-Raman**.

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Chapter

2

Quality and chemical profiles of monovarietal north Moroccan olive oils from 'Picholine Marocaine' cultivar: registration database development and geographical discrimination

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Abstract

Current knowledge of the quality and composition of Moroccan olive oil is still incomplete and no consistent database compiling its properties is available. This study was carried out to achieve a comprehensive characterisation of north Moroccan olive oils. Thus, 279 olives samples of *"Picholine Marocaine"* cultivar grown in 7 Moroccan regions were collected, and oils extracted over two consecutive crop seasons (2011 and 2012) and analysed (considering physicochemical quality parameters and purity criteria). Results indicated that all the studied samples showed values fulfilling the established limits by the International Olive Council (IOC) standards, with the exception of 32 samples having linolenic acid content higher than 1%, which is the maximum value fixed by the IOC regulation. Furthermore, the usefulness of the evaluated parameters for tracing the geographical origin of the studied samples was tested by using canonical discriminant analysis. A good rate of correct classification and prediction was achieved.

Keywords: virgin olive oil; 'Picholine Marocaine'; physicochemical quality parameters; purity criteria; geographical origin.

1 Introduction

The olive tree (*Olea europea* L.) is a perennial, long-lived and evergreen tree, which is probably among the oldest domesticated trees in the Mediterranean region (Zohary & Spiegel-Roy, 1975). About 90% of the olive fruit world production is used for obtaining olive oil; according to the International Olive Council (IOC), in 2013, the olive oil world production reached 2,425,000 tonnes (IOC, 2013b), placing olive tree as the sixth most relevant oil crop in the world.

Besides the significant role that olive oil production plays in several Mediterranean countries´ economy, its many virtues in both gastronomy and health are remarkably promoting the scientific and commercial attention lately given to this natural juice of olive fruit (Bendini et al., 2007; López-Miranda et al., 2010). Thus, as a result of the high nutritional value and the unique flavour characteristics of olive oil, the latter is a price premium product if compared to other vegetable oils. For this reason, attempts to adulterate this commodity with less expensive oils, such as seed oils and olive oils of

lower quality, have become a serious concern from both commercial and health perspectives (Garcia, Martins, & Cabrita, 2013).

Different analytical methodologies have been developed and proposed over the last years to evaluate VOO quality, as well as assuring its authenticity (Ben-Ayed, Kamoun-Grati, & Rebai, 2013; Frankel, 2010). However, both its commercial classification and genuineness evaluation remain legally defined by trade standards compiled in the European Commission Regulations (EC, 1991), the Codex Alimentarius Norms (Codex, 1991), and the IOC Trade Standards (IOC, 2012). The latters are recognized by most of the olive oil producers and marketers all over the world, since they are drawn up and updated on basis of IOC olive oil records and databases of the countries that are members of this Council, which are the major suppliers of olive oil in the global market. These trade standards establish physicochemical and organoleptic tests aimed at differentiating between each grade and checking product authenticity, regulating, in this way, the effective control of the purity and quality of the olive oils that are sold, trying to avoid, therefore, unfair competition.

In Morocco, olive cultivation, besides being a very ancient agricultural practice dating from the Roman era (Lenoir & Akerraz, 1984), represents nowadays a crucial socio-economic pillar, since it yields an important source of income (contributes to 5% of upstream agricultural gross domestic product and accounts for 15% of Moroccan agricultural foodstuff export) and employment (100,000 full time employees) (IOC, 2013a). In terms of surface, since the adoption of the strategy of the "Green Morocco Plan", new olive tree plantations, as well as the intensification and rehabilitation of traditional olive orchards, have taken place resulting in a smooth and gradual increase of the surface area covered by olive tree, passing from 763,000 ha in 2007 to 933,475 ha in 2013 (MAPM, 2013). Likewise, new farming practices and olive oil processing techniques have been embraced over the last years, promoting the production of high quality olive oil. Morocco is the world's 6th largest producer; over the last four years this country has produced an annually average of about 90,000 tonnes of olive oil (IOC, 2013a).

However, one of the major challenges that Moroccan olive oil sector is currently facing is ensuring compliance with the IOC authentication and quality standards. Indeed, even if

Morocco is an IOC member, the lack of a proper and comprehensive database which could reflect possible quality and compositional peculiarities of its olive oils, makes that IOC regulations do not consider Moroccan olive oil distinctive features. This fact can obviously have negative repercussions into the commercialisation of this commodity, exposing it to risks of rejection for falling to comply with IOC requirements.

In this regard, it is very important to highlight that there are 3 main factors explaining the qualitative and compositional variations that Moroccan olive oils can show: the diversity of edafoclimatic conditions of Moroccan olive growing areas (olive tree cover a wide range of soil types and contrasting regional climates with annual precipitations ranging from 800 to 1,000 mm in the North to less than 200 mm in the South); the dominance of the standard cultivar "*Picholine Marocaine*", which consist of several distinct local genotypes (Ouazzani, Villemur, & Lumaret, 1996); and regional variations related to the degree of specialization in olive cultivation, as well as adopted farming and processing techniques. Consequently, it is very necessary to perform quality and compositional characterisation studies of the olive oil produced in the main Moroccan olive-growing areas.

Keeping all this in mind, the global aim of the current study includes several objectives. The first one was to contribute to the creation of a comprehensive Moroccan olive oil database by carrying out a deep characterisation of quality and purity criteria of olive oil samples collected from the main north Moroccan olive-growing areas. The second goal was to achieve, within the studied regions, the geographical identification of olive growing areas where the produced oils do not fulfil the upper established limits by the IOC standards for some purity parameters. The third objective was to attain the geographical discrimination of the studied samples based on their physicochemical quality and composition parameters and using a multivariate data analysis approach.

2 Materials and methods

Taking into account our purpose of contributing to the establishment of a compositional database of Moroccan olive oil, both the sampling representativeness and the use of the official methods for the analysis of the collected samples were crucial.

2.1 Sampling design

Seven north Moroccan regions were selected in the current study: Chefchaouane, Fès, Meknès, Ouazzane, Sefrou, Taounate, and Taza (Figure 1a). For each one of these regions, the work started by the location and delimitation of homogenous olive growing blocks. Information including soil types, topographic and climate characteristics, and practiced olive farming and olive oil processing techniques were collected, using databases of local agricultural authorities and national and local weather stations. Afterwards, data compilation and treatment were conducted using Microsoft® Access™ 2010 (Microsoft Corp, and Redmond, WA, USA)and mapped with ArcGIS® (ArcMap™ 9.3, ESRI®, Redlands, CA, USA) Figure 1b represents the obtained delimitation maps. A total of 29 homogenous olive growing blocks were identified. Then, in each block perimeter, representative olive orchards were selected and geo-referenced by global positioning system (GPS).

2.2 Olive sampling and oil extraction

279 olive fruit samples from the main Moroccan cultivar "*Picholine Marocaine*" were collected over two consecutive crop seasons (2011 and 2012). Only fresh and healthy olive fruits were hand-harvested from olive trees randomly selected at each one of the chosen olive orchards from the 29 homogeneous olive growing blocks, as explained above. Olive fruits were collected between November and January, placed in rectangular plastic crates (approximately one sample was of about 35 kg of olives) and immediately transported to the laboratory. A sample of 100 olive fruits was randomly taken from every plastic crates to calculate the ripening index (RI), according to the method developed by the Agronomic Station of Mengíbar (Jaén, Spain). This method define the RI as a function of fruit colour in both skin and pulp (Uceda & Frías, 1975). Oil extraction was performed at laboratory scale by an Oliomio laboratory mill (Oliomio, Italy), following a previously described procedure (Bajoub, Carrasco-Pancorbo, Ajal, Ouazzani, & Fernández-Gutiérrez, 2014). Afterwards, olive oil samples were filtered and stored in dark glass bottles (250 mL), excluding any head space volume, in order to assure the proper conservation of the olive oil against oxidation, and kept in the freezer at −18 °C until the analysis.

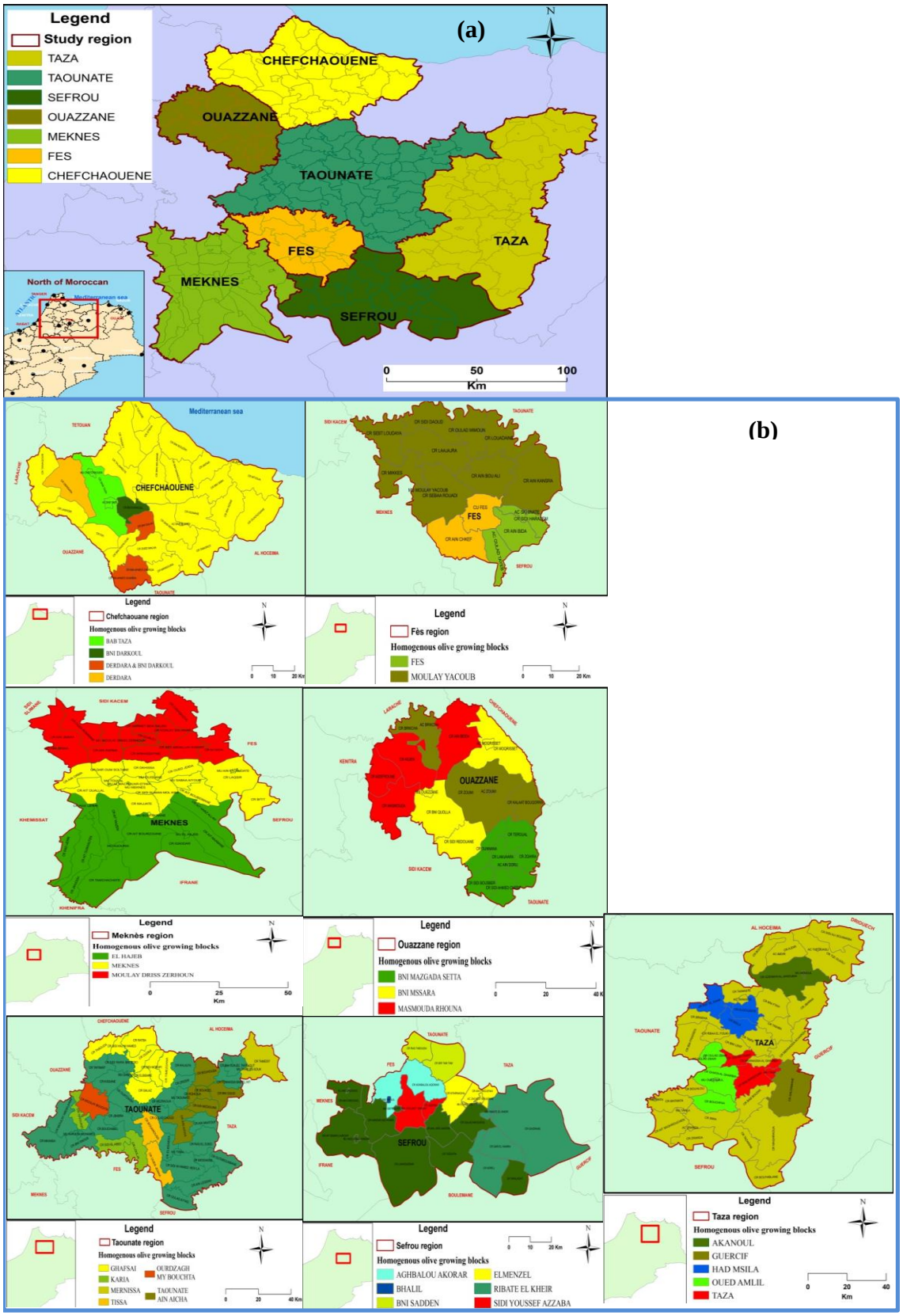

Fig. 1. Geographical localisation of (a) the studied north Moroccan areas under study and (b) homogenous olive growing blocks.

2.3 Quality criteria

Free acidity, expressed as percentage of oleic acid (%), peroxide value expressed as milliequivalents of active oxygen per kilogram of oil (meq O_2/kg), K_{232} and K_{270} extinction coefficients calculated from absorption at 232 and 270 nm, respectively, and fatty acid methyl and ethyl esters were measured using the analytical methods described in the European Union standard methods Regulations 2568/91 and the subsequent amendments (EC, 1991). Every determination was carried out in triplicate.

2.4 Purity criteria

All the studied parameters were determined by the Official Agricultural and Food Quality Control Laboratory of Granada, Atarfe (Spain), which is a recognized laboratory by the IOC.

The following purity criteria were analysed as described in Annexes V and VI of Regulation EEC/2568/91 of the European Union Commission and the subsequent amendments (EC, 1991).

- Fatty acids composition and trans fatty acids content

Fatty acids methyl esters (FAMEs) were prepared, and then analysed using a Varian CP3800 gas chromatography (GC) system equipped with a Flame Ionization Detector (FID) (Walnut Creek, CA, USA) and running on a Varian CP 9103 FactorFour™ VF-624MS (60 m × 0.25 mm id, 1.4 µm film thickness) capillary column. The carrier gas was helium at a flow rate of 1 mL/min. The temperatures of the injector and detector were set at 250 °C and the oven temperature was 210 °C. The injection volume was 1 µL. The individual FAMEs and trans FAMEs were identified through a comparison of their retention times versus pure standards analysed under the same conditions. The percentage for each fatty acid was expressed as relative percent of total area.

- Difference between Actual and Theoretical Content of triacylglycerols (ΔECN42)

The theoretical value of triacylglycerols with equivalent carbon number 42 (ECN42 theoretical) was calculated from the fatty acid composition. The ECN42 actual value was determined by liquid chromatography using a Waters Alliance 2690 liquid chromatograph (LC) with a refractive index detector (Waters, Milford, MA), equipped with a reversed-phase Superspher® RP-18 column (length 250 mm,4.6 mm id, 4 mm

particle size, Merck). The ECN42 triacylglycerols analysis, identification and quantification, as well as the ΔECN42 calculation were identical to those reported by the official method.

- Composition and content of sterols and erythrodiol and uvaol

5 g of olive oil sample, with added α-cholestanol (used as internal standard), were saponified with potassium hydroxide in an ethanolic solution. Then, the unsaponifiable fraction was extracted with diethyl ether, purified with water and dried over sodium sulphate. The diethyl ether was evaporated under vacuum and nitrogen and the residue was re-dissolved in chloroform. Sterol and triterpene alcohols fractions were separated from the extract by thin layer chromatography on silica gel plates, then transformed into trimethylsilyl ethers and the mixture was analysed by gas chromatography using Varian 450-GC con detector FID and a Varian CP 8751 wcot fused silica capillary column (30 m length×0.25 mm i.d) coated with CP-Sil 8 CB (0.25 mm DF). Sterols and triterpene alcohols were identified based on their relative retention times with respect to the internal standard, according to the standardized reference method. The concentrations of identified compounds were expressed as proportions (%) of total sterols. Apparent β-sitosterol was calculated, according to the reference method, as the sum of Δ5,23−stigmastadienol, clerosterol, β-sitosterol, sitostanol, Δ5−avenasterol, and Δ5,24-stigmastadienol. The erythrodiol and uvaol content was calculated, in percentage, as the ratio between the sum of the areas of erythrodiol + uvaol and the sum of sterols + erythrodiol + uvaol.

- Waxes content

A silica gel chromatography was used for the wax separation from oil samples. 500 mg of olive oil samples with 0.1 mL of lauryl arachidate (Aldrich, Madrid, Spain) (used as internal standard) were placed into a silica column previously prepared and conditioned. Then, the waxy fraction was eluted with hexane-diethyl ether (99:1 v/v), evaporated in a rotary evaporator at room temperature and the residue was re-dissolved in heptane. 1 μL of the waxy fraction was finally injected and analysed using the same equipment mentioned above for sterols and triterpenic dialcohols. Both the experimental conditions and the data treatment were carried out keeping in mind the standardized reference method.

- Stigmastadienes content

20.0 ± 0.1 g of oil sample with 1 mL of the standard solution of cholesta-3,5-diene were saponified with potassium hydroxide in ethanolic solution. Then, the unsaponifiable fraction was extracted with diethyl ether, purified with water and dried over sodium sulphate. The diethyl ether was evaporated and the residue was re-dissolved in hexane. Separation was carried out on a silica gel chromatography, the eluted steroidal hydrocarbon fraction was evaporated and dissolved in 0.2 ml of hexane. All the GC conditions, the stigmasta-3,5-diene peak identification and the quantitative analysis were carried out considering the recommendations of the official method.

- Total aliphatic alcohols content

Aliphatic alcohols were determined by saponifying 5 g of oil sample, with 1-eicosanol added as internal standard, under the same conditions mentioned above for the determination of sterols and triterpene dialcohols. The alcohol fraction was separated from the unsaponifiable matter by chromatography on a basic silica gel plate. The alcohols recovered from the silica gel were turned into trimethylsilyl ethers and analysed by capillary column gas chromatography using the same equipment as mentioned above within the sterols and triterpene dialcohols section. Chromatographic conditions, peaks identification and quantitative analysis were those described by the standardized reference method. Seven aliphatic alcohols were identified: decosanol, tricosanol, tetracosanol, pentacosanol, hexacosanol, heptacosanol and octacosanol, and the contents of each one were calculated and expressed in mg/1000 g of olive oil. However, since the IOC trade standards only require information about the total aliphatic alcohols content (expressed by the sum of the content of individual aliphatic alcohols compounds) in combination with other parameters (erythrodiol, uvaol and wax content) to detect fraudulent addition of olive pomace oil to VOO, within this study, only the total content of aliphatic alcohols was considered and included into the results for carrying out the statistical analysis.

2.5 Data Analysis

2.5.1 Univariate analysis

Statistical analysis of data regarding quality and chemical composition of the north Moroccan olive oils was performed by SPSS statistical package software (SPSS for

Windows, Version 20, SPSS Inc., and Chicago, USA). One-way analysis of variance (ANOVA) was used to evaluate the influence of geographic origin on all the analysed parameters and the significance of differences at 5% ($p<0.05$) level between mean values was determined using Tukey's test.

2.5.2 Multivariate analysis

Canonical discriminant analysis (CDA) was performed using Microsoft® Excel 2013/XLSTAT© (Version 2013.2, Addinsoft, Inc., Brooklyn, NY, USA). This tool makes easier the class separation by maximising the ratio between-class variance to the within-class variance or the ratio of the overall variance to the within-class variance. The CDA analysis was performed by setting p-in and p-out values at 0.05 and 0.1, respectively, with the same weighing of each group to choose effective variables and to decrease the number of variables for classification functions. Forward stepwise regression was selected, and p-in and p-out values were set so that the highest classification accuracy could be obtained when each factor was moved. Furthermore, the prediction ability was examined, by a leave-out cross-validation method.

2.5.3 Maps construction

To obtain a proper characterisation of the overall Moroccan olive oils´ composition, a very important step was identifying specific areas within the studied regions that show, for some the studied parameters, contents exceeding the upper limits established by the IOC regulation.Thus, ArcGIS® (ArcMap™ 9.3, ESRI®, Redlands, CA, USA)was used for mapping the spatial distribution of the obtained values for a particular parameter; in each one of the studied regions geo-reference coordination of the sampling site was used.

3 Results and discussions

3.1 Olives ripening index and physicochemical quality indices

The mean ± standard deviation values of ripening index and regulated quality criteria of olive oil samples extracted from fruits of "*Picholine Marocaine*" cultivar population grown in the 7 areas under study are given in Table 1.As can be seen, average values of ripening index were found between 3.66 (Meknès) and 4.08 (Sefrou).

Table 1. Mean values and standard deviations (S.D.) for the ripening index (RI), the regulated physicochemical quality parameters (Free fatty acids (FFAs), Peroxide value (PV), extinction coefficients K_{270} and K_{232}), and fatty acid methyl (FAMEs) and ethyl esters (FAEEs) in the olive oil samples under study.

		Chefchaouane (n=10)	Fès (n=41)	Mèknes (n=69)	Ouazzane (n=37)	Sefrou (n=39)	Taounate (n=49)	Taza (n=34)
RI	Mean ± S.D.	3.74^{a}±0.73	3.98^{a}±0.88	3.66^{a}±1.24	3.92^{a}±1.08	4.08^{a}±0.97	3.69^{a}±1.09	4.01^{a}±1.31
FA (%)		0.39^{a}±0.20	0.37^{a}±0.12	0.28^{a}±0.16	0.34^{a}±0.15	0.38^{a}±0.20	0.35^{a}±0.16	0.39^{a}±0.20
PV (meqO2/kg)		5.38^{a}±1.59	4.27^{b}±0.72	4.54^{b}±1.03	4.41^{b}±1.09	4.09^{b}±0.59	4.55^{b}±0.83	4.40^{b}±0.70
K232		1.40^{a}±0.48	1.25^{abc}±0.48	1.15^{abc}±0.68	1.61^{ad}±0.32	1.52^{abd}±0.56	1.20^{ab}±0.48	1.53^{abd}±0.45
K270		0.15^{a}±0.03	0.12^{a}±0.04	0.11^{a}±0.04	0.07^{b}±0.03	0.12^{a}±0.05	0.11^{a}±0.09	0.11^{a}±0.06
FAEEs		35.69^{ab}±33.76	16.99^{a}±7.77	39.19^{a}±72.41	37.41^{ah}±47.57	77.16^{b}±87.03	20.93^{a}±21.68	43.93^{ab}±70.55
FAMEs		21.89^{ab}±14.66	18.18^{a}±8.90	21.12^{a}±24.20	22.70^{a}±21.99	40.97^{b}±41.55	16.24^{a}±8.30	25.72^{ab}±30.83
∑ FAEEs+FAMEs		57.58^{ab}±48.20	34.75^{a}±15.83	60.30^{a}±96.40	60.11^{a}±69.32	118.13^{b}±127.48	37.18^{a}±29.04	69.65^{ab}±99.37
Categories	Extra-virgin (n= 241)	8	40	60	32	27	46	28
	Virgin (n=38)	2	1	9	5	12	3	6

-Significant differences within the same line are indicated with different lowercase letters (comparison between studied regions, $p < 0.05$). These differences were checked considering all the samples selected from each area.

-In this Table, Table 2 and Table 3 the mean values are those calculated for all the samples coming from the same area, therefore, SD gives to the reader only an idea about the variability of the olive oils in terms of composition, and obviously not about the repeatability of the analytical methods used.

The maturation stage of collected olive fruits samples is a very important parameter, since it affects olive oil quality, stability and composition (Rotondi et al., 2004; Salvador, Aranda, & Fregapane, 2001). Results of the ANOVA test applied to ripening index data revealed that no statistically significant differences can be found among the studied areas considering this parameter. Thus, the effect of the maturity stage on the other studied parameters can be dismissed and therefore, only the geographical origin of the processed olive fruits was considered as variation factor.

As far as the physicochemical quality criteria are concerned, and keeping in mind the limits set by the IOC regulation (IOC, 2012), most of the studied samples (93.55% of the total of 279 studied samples) were falling into the "Extra-virgin" olive oil category, whereas the rest (6.45%) were classified as "Virgin" olive oils. This result was expected, since the raw material was carefully selected, picked and processed. As highlighted in Table 1, average values of free fatty acids content, expressed as percentage of oleic acid, ranged from 0.28 up to 0.39%; peroxide value was found between 4.09 and 5.38 meq O_2/kg, whereas K_{232} and K_{270} ranged from 1.15 up to 1.61 and from 0.07 up to 0.15, respectively. Furthermore, the content of fatty acid alkyl esters (FAAE) and fatty acid methyl esters (FAME), as recently adopted parameters for the assessment of extra virgin olive oil, were determined. They are useful indicators for determining the quality of olives and the oil produced from them, as they are direct indicators of degradation. Indeed, they result from the degradation and fermentation processes of low quality processed olive fruits (Biedermann, Bongartz, Mariani, & Grob, 2008). During the period in which the current study was carried out, the legal limit of this parameter for the Extra VOO was established as sum of FAME and FAEE ≤75 mg/kg or between 75 and 150 mg/kg if the FAEE/FAME ratio is <1.5 (IOC, 2012). In terms of average values, the sum of FAME and FAEE was between 34.75 and 118.13 mg/kg, for Fès and Sefrou, respectively. Table 1 also shows that when ANOVA test is applied to the physicochemical quality criteria data, statistically significant differences were observed among the studied samples according to their geographic area of origin, except for free fatty acids.

3.2 Purity criteria

3.2.1 Fatty acids profile

Table 2 includes the compositional data of the north Moroccan olive oil samples under study regarding fatty acids composition (%), amounts of fatty acids groups (%), and ratios having nutritional interest. Thirteen fatty acids were identified and classified in three groups: monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA). MUFA was the most abundant group of fatty acids in all the analysed samples regardless of the area of origin, being the highest average content found in oils from Sefrou with 77.30%, and the lowest in Ouazzane olive oils (75.10%). Within this group, the oleic acid (C18:1) remains the most abundant, showing a mean amount ranging between 73.97% (in oils from Ouazzane) and 76.24% (in oils from Sefrou). Palmitoleic acid (C16:1) was the second MUFA in terms of abundance. Its level varied ranging from 0.64 to 0.78% in oils from Chefchaouane and Meknès, respectively. Other determined monounsaturated fatty acids in decreasing order of abundance were: eicosanoic acid (C20:1) with an average content between 0.34% (in oils from Meknès and Sefrou) and 0.36% (in Fès olive oil samples), and heptadecenoic acid (C17:1) (between 0.06 and 0.09% in samples from Sefrou and Chefchaouane, respectively). PUFA were found between 10.42% (mean concentration) in oils samples from Chefchaouane region, and 12.42%, in oils from Fès. Two main fatty acids composed this group: linoleic acid (C18:2), found in a mean concentration which varied between 9.53% in oils from Chefchaouane and 11.48% in oils from Fès; and linolenic acid (C18:3), with a mean concentration fluctuating from 0.84% in samples from Meknès and 0.94% in oils from Fès, Ouazzane and Taounate. SFA was the third group of fatty acids found in north Moroccan olive oil samples; in this case, mean concentrations had values between 11.92% and 12.81% in Sefrou and Ouazzane olive oil samples, respectively. Within this group, palmitic acid (C16:0) was the most prevalent compound (with average concentrations comprised in the range from 8.77 to 9.92% in oils from Sefrou and Meknès, respectively). Stearic acid (C18:0) was, in order of relative abundance, the second analyte from the SFA, with mean concentrations varying between 2.27 in Meknès oils and 2.67% in samples coming from Sefrou. Other four saturated fatty acids were also found, at very low concentration levels: arachidic (C20:0), margaric (C17:0), behenic acid (C22:0), and lignoceric acid (C24:0), with mean

concentration ranges of 0.31-0.35%, 0.04-0.06%, 0.07-0.09%, and 0.03-0.04%, respectively.

Table 2. Composition of north Moroccan olive oil samples regarding fatty acid (%), trans fatty acids content (%) and maximum difference between the actual and theoretical ECN 42 triacylglycerol content (%).

		Chefchaouane (n=10)		Fès (n=41)		Mèknes (n=69)		Ouazzane (n=37)		Sefrou (n=39)		Taounate (n=49)		Taza (n=34)	
	IOC regul.	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
C14:0	≤0.03	0.02^{a} ±0.00	0.01–0.02	0.02^{a} ±0.00	0.01–0.03	0.02^{a} ±0.02	0.01–0.21	0.02^{a} ±0.01	0.01–0.03	0.02^{a} ±0.00	0.01–0.02	0.02^{a} ±0.00	0.01–0.03	0.02^{a} ±0.00	0.01–0.03
C16:0	7.50-20.00	9.30^{abc} ±1.18	8.46–12.38	9.15^{ac} ±0.72	8.00–10.70	9.92^{bc} ±1.56	7.51–16.50	9.83^{c} ±0.99	8.27–11.16	8.77^{a} ±0.44	8.06–9.70	9.37^{abc} ±0.81	7.55–12.28	9.06^{a} ±0.99	7.55–11.96
C16:1	0.30-3.50	0.64^{a} ±0.05	0.54–0.72	0.67^{a} ±0.08	0.48–0.86	0.78^{b} ±0.28	0.56–2.08	0.70^{ab} ±0.06	0.54–0.82	0.65^{a} ±0.06	0.57–0.79	0.68^{a} ±0.09	0.55–0.91	0.67^{a} ±0.10	0.53–0.99
C17:0	≤0.30	0.06^{a} ±0.04	0.04–0.13	0.04^{b} ±0.00	0.04–0.05	0.05^{c} ±0.02	0.04–0.12	0.04^{bc} ±0.01	0.04–0.05	0.04^{bc} ±0.00	0.04–0.05	0.04^{bc} ±0.02	0.04–0.16	0.04^{bc} ±0.00	0.04–0.05
C17:1	≤0.30	0.09^{a} ±0.04	0.06–0.17	0.08^{a} ±0.08	0.05–0.59	0.08^{a} ±0.04	0.05–0.23	0.07^{a} ±0.01	0.05–0.08	0.06^{a} ±0.01	0.06–0.08	0.06^{a} ±0.02	0.05–0.21	0.06^{a} ±0.01	0.05–0.10
C18:0	0.50-5.00	2.48^{abc} ±0.44	1.75–3.17	2.39^{ab} ±0.24	1.95–3.13	2.27^{b} ±0.37	1.69–4.05	2.45^{ab} ±0.25	2.00–3.01	2.67^{c} ±0.22	2.04–3.04	2.43^{ab} ±0.28	1.82–3.15	2.57^{ac} ±0.31	2.08–3.35
C18:1	55.00-83.00	76.16^{abc} ±2.94	70.05–79.42	74.47^{b} ±2.43	68.66–77.99	75.23^{abc} ±2.38	65.96–80.19	73.97^{b} ±2.19	70.43–78.10	76.24^{c} ±1.66	71.9–78.94	74.82^{ab} ±1.77	70.72–78.69	76.05^{ac} ±2.04	69.13–79.08
C18:2	3.50-21.00	9.53^{abcdf} ±2.26	7.39–13.76	11.48^{bd} ±2.16	8.27–15.91	9.98^{cf} ±1.81	3.38–13.49	11.15^{d} ±1.94	7.49–15.17	9.91^{acdf} ±1.59	7.28–13.86	10.85^{abcd} ±1.57	7.63–15.06	9.55^{f} ±2.03	1.96–13.73
C18:3	≤1.00	0.89^{abc} ±0.13	0.73–1.10	0.94^{b} ±0.09	0.78–1.15	0.84^{c} ±0.10	0.53–0.98	0.94^{b} ±0.06	0.80–1.11	0.88^{c} ±0.05	0.79–0.97	0.94^{ab} ±0.11	0.78–1.23	0.89^{abc} ±0.11	0.75–1.24
C20:0	≤0.60	0.35^{a} ^{c}±0.04	0.3–0.42	0.31^{b} ±0.02	0.28–0.35	0.31^{b} ±0.03	0.26–0.39	0.34^{c} ±0.02	0.30–0.38	0.32^{ab} ±0.02	0.28–0.38	0.32^{ab} ±0.02	0.26–0.42	0.33^{ac} ±0.03	0.27–0.43
C20:1	≤0.40	0.35^{ab} ±0.03	0.33–0.42	0.36^{a} ±0.03	0.32–0.40	0.34^{b} ±0.02	0.28–0.39	0.35^{a} ±0.03	0.32–0.41	0.34^{ab} ±0.01	0.32–0.37	0.35^{ab} ±0.03	0.31–0.4	0.35^{ab} ±0.02	0.31–0.39
C22:0	≤0.20	0.09^{a} ±0.02	0.07–0.11	0.09^{a} ±0.12	0.06–0.83	0.08^{a} ±0.02	0.07–0.14	0.08^{a} ±0.00	0.07–0.09	0.07^{a} ±0.01	0.06–0.09	0.08^{a} ±0.01	0.06–0.12	0.08^{a} ±0.01	0.06–0.12
C24:0	≤0.20	0.04^{ac} ±0.01	0.03–0.06	0.03^{b} ±0.00	0.02–0.04	0.04^{ac} ±0.01	0.02–0.06	0.04^{c} ±0.01	0.03–0.05	0.03^{bd} ±0.00	0.02–0.04	0.04^{ab} ±0.01	0.02–0.05	0.04^{ab} ±0.01	0.02–0.07

Table 2. (continued)

		Chefchaouan (n=10)		Fès (n=41)		Mèknes (n=69)		Ouazzane (n=37)		Sefrou (n=39)		Taounate (n=49)		Taza (n=34)	
	IOC regul.	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
trans C18:1	≤0.05	0.01^{ab} ±0.00	0.01–0.02	0.01^{b} ±0.00	0.01–0.02	0.01^{a} ±0.00	0.01–0.02	0.01^{a} ±0.00	0.01–0.02	0.01^{a} ±0.00	0.01–0.01	0.01^{b} ±0.00	0.01–0.02	0.01^{a} ±0.00	0.01–0.01
trans C18:2T +C18:3T	≤0.05	0.02^{abd} ±0.01	0.01–0.03	0.02^{d} ±0.00	0.02–0.03	0.02^{ad} ±0.00	0.01–0.04	0.02^{ad} ±0.00	0.01–0.03	0.02^{b} ±0.00	0.01–0.02	0.02^{c} ±0.00	0.02–0.03	0.02^{ab} ±0.00	0.02–0.03
SAFA		12.34^{abc} ±0.98	11.47–14.65	12.04^{a} ±0.66	11.14–13.82	12.69^{b} ±1.42	11.15–18.84	12.81^{c} ±0.96	11.55–14.17	11.92^{a} ±0.37	11.17–13.06	12.30^{abc} ±0.71	10.88–14.74	12.13^{abc} ±0.91	10.92–14.70
MUFA		77.24^{abc} ±2.90	71.16–80.40	75.58^{ab} ±2.40	69.87–79.03	76.43^{c} ±2.17	68.38–81.60	75.10^{b} ±2.15	71.61–79.14	77.30^{c} ±1.64	72.96–80.06	75.92^{ab} ±1.73	71.88–79.62	77.14^{ac} ±1.99	70.39–80.05
PUFA		10.42^{acd} ±2.36	8.13–14.86	12.42^{bc} ±2.19	9.17–16.98	10.82^{ad} ±1.82	4.31–14.27	12.09^{c} ±1.99	8.35–16.28	10.79^{ad} ±1.60	8.1–14.76	11.79^{abc} ±1.60	8.42–16.16	10.44^{d} ±2.06	2.75–14.91
MUFA/PUFA		7.76^{ab} ±1.75	4.94–9.89	6.30^{a} ±1.28	4.11–8.59	7.34^{ab} ±1.85	4.96–18.94	6.41^{a} ±1.27	4.40–9.47	7.34^{ab} ±1.22	4.94–9.88	6.57^{a} ±1.04	4.45–9.36	8.00^{b} ±3.65	4.72–27.40
O/P		8.30^{abc} ±1.03	5.66–9.07	8.19^{ac} ±0.75	6.48–9.52	7.74^{ab} ±1.05	4.06–9.63	7.60^{b} ±0.84	6.47–9.36	8.71^{c} ±0.50	7.73–9.58	8.05^{ab} ±0.74	5.96–10.42	8.50^{ac} ±10	5.78–10.11
O/L		8.40^{ab} ±1.95	5.25–10.74	6.75^{a} ±1.47	4.32–9.38	7.91^{ab} ±2.37	5.06–23.73	6.87^{a} ±1.45	4.64–10.41	7.91^{ab} ±1.41	5.19–10.85	7.06^{a} ±1.19	4.70–10.18	8.88^{b} ±5.35	5.03–38.03
ΔECN42	≤0.20	0.00^{a} ±0.00	0.00–0.00	0.05^{b} ±0.07	0.00–0.20	0.04^{b} ±0.05	0.00–0.10	0.03^{b} ±0.05	0.00–0.20	0.02 ±0.04	0.00–0.10	0.02^{b} ±0.05	0.00–0.20	0.01^{b} ±0.04	0.00–0.10

-Significant differences in the same line are indicated with different lowercase letters (comparison between the studied regions, $p < 0.05$).

Bearing in mind their importance as indicative parameters of the oxidation stability and nutritive value of olive oil (Aparicio et al., 1999; Beltrán et al., 2004; Haddada et al., 2007), ratios between oleic and linoleic acids (O/L), oleic and palmitic acids (O/L), and MUFA/PUFA were evaluated in the studied samples. Olive oil samples coming from Taza showed the highest values of both O/L and MUFA/PUFA ratios (8.88 and 8.00%, respectively), whilst oils from Fès had the lowest values for both ratios (6.75 and 6.30%, respectively). The highest value of O/P ratio was found in Sefrou olive oils (8.71%), while in contrast, the lowest level was found in Ouazzane oils (7.60%).

Furthermore, the global variability of fatty acid composition of the studied olive oil samples was evaluated. To achieve this purpose, we first characterised the range of obtained values for each identified fatty acids; and then, the influence of the geographic origin on this variability was evaluated. Considering the limits established by the IOC regulation (IOC, 2012), the fatty acid levels found in the analysed samples covered the normal ranges considered by these standards for VOO, with the exception of 32 samples (2 from Chefchaouane, 8 from Fès, 5 from Ouazzane, 11 from Taounate and 6 from Taza). For these samples, linolenic acid content exceeded the upper limit of 1.00%. The geolocation of the olive growing areas where olive oil linolenic acid content exceeded this limit is depicted in Figure 2. These results, are in good agreement with those previously obtained for olive oils from the South-center of Morocco (El Antari et al., 2003; Houlali et al., 2014), even if linolenic acid values for oils from these regions were much higher than those obtained in the current work. Furthermore, the existence of monovarietal VOOs with fatty acids levels exceeding the upper limits established by the IOC regulation has been previously reported for other monovarietal olive oils, as in the case of French VOOs with high level of linolenic acid (Ollivier, Artaud, Pinatel, Durbec, & Guérère, 2003); and Tunisian VOOs, in particular, the Chemlali VOOs (Dabbou et al., 2011; Guerfel, Baccouri, Boujnah, & Zarrouk, 2008; Kotti et al., 2008), where the amount of palmitic acid surpassed the upper limit of 20% fixed by the IOC regulation.

The importance of linolenic acid in plants is well established; it participates forming the building blocks of lipid molecules, contributes to the structure of cell membranes and hormones, provides cells with energy, etc. (Weber, 2002). Regarding its levels in olive oil, even if Uceda (2009) reported that the cultivar is the main source of variability for the major fatty acids in VOO, Beltrán et al. (2004) indicated that both ripening index

and crop season temperature have a considerable influence on the content of linolenic acid.

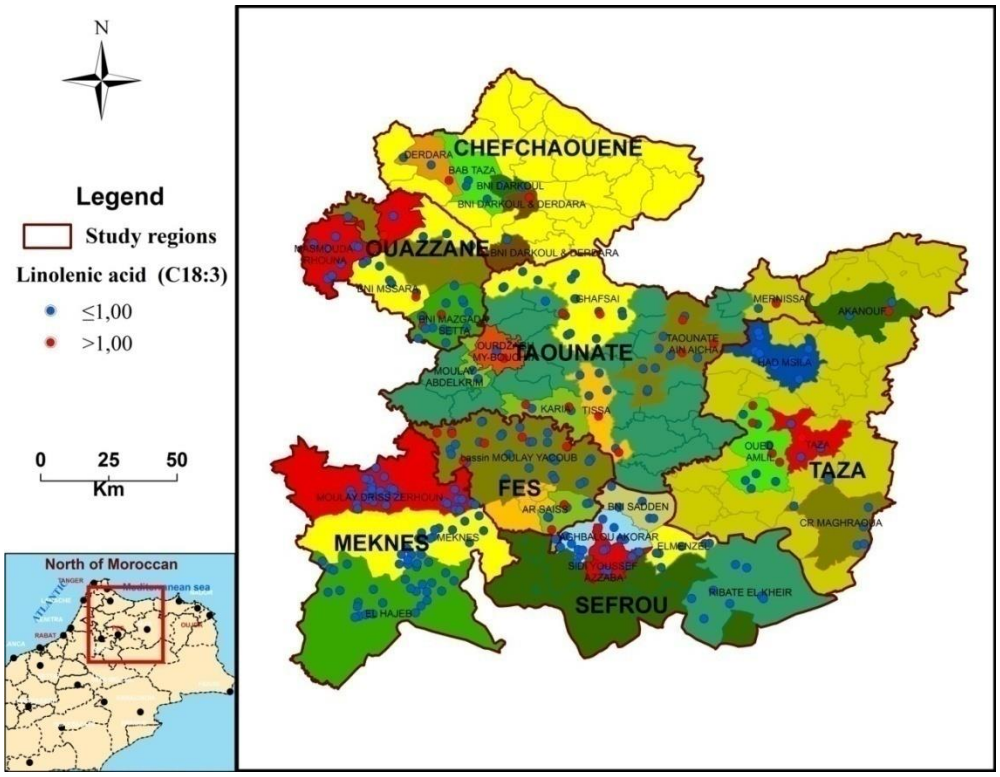

Fig. 2. Linolenic acid geo-location map showing the geographical positioning of olive growing areas with a linolenic acid content upper the limit of 1.00%.

According to these authors, the air temperature recorded during oil biosynthesis could affect the amount of polyunsaturated fatty acids (in particular, linoleic and linolenic acids) by means of the regulation of desaturase enzymes activities. Therefore, the high level of linolenic acid in north Moroccan olive oil samples can be related to drought and temperature stress that characterise these olive growing areas during the oil biosynthesis period. However, in order to prove this hypothesis, it is necessary to conduct rigorous, comprehensive and plurianual studies to characterise the response of "*Picholine Marocaine*" cultivar to stress conditions.

The application of the ANOVA test to the individual fatty acids data, as well as fatty acids groups and ratios results, showed that apart from heptadecenoic and myristic acids, all the others exhibited statistically significant differences according to the geographic area of origin.

3.2.2 Fatty acids trans isomers content

Table 2 summarize the results obtained for the trans C18:1 and C18:2T+C18:3T content in the studied oils. At can be seen form this Table, these compounds were detected in trace amounts in all of the analysed samples and not a single value exceeded the fixed limit by the IOC regulation. Furthermore, by applying the ANOVA test, some statistically significant differences were detected among the analysed samples according their geographic origin.

3.2.3 Difference between Actual and Theoretical Content of triacylglycerols (ΔECN42)

The addition of other edible vegetable oils to olive oil modifies TAG distribution, fact which turns TAGs into a powerful tool for adulteration detection purposes (Ruiz-Samblás, Arrebola-Pascual, Tres, van Ruth, & Cuadros-Rodríguez, 2013)Some examples illustrating this are the detection of vegetable oils particularly rich in terms of linoleic acid, such as sunflower and colza; presence of some high oleic vegetable oils such as hazelnut; or detection of high oleic sunflower and olive pomace oils (Angerosa, Campestre, & Giansante, 2006). Table 2 includes the ranges and average values of ΔECN42 of the olive oil samples under study, as well as the result of ANOVA test applied to these data. Despite the statistically significant differences observed among the studied regions with regard to this parameter, all the obtained values were in conformity with IOC regulation.

3.2.4 Sterols composition

Sterols and triterpene diols are the major constituents of VOO unsaponifiable fraction. Besides their importance as bioactive compounds exhibiting a wide range of health beneficial activities (Hidalgo, Mercedes León, & Zamora, 2009), these compounds are considered as a very useful tool for authenticating the genuineness of VOO and detecting fraudulent admixtures with lower value vegetable oils, such as olive-pomace oil, refined olive oil and some seed oils (Monfreda, Gobbi, & Grippa, 2012; Vichi, Pizzale, Toffano, Bortolomeazzi, & Conte, 2001). Results from the quantitative analysis of sterols and triterpene diols in north Moroccan olive oil samples are summarized in Table 3. All the analysed oil samples fulfilled the established limit by the IOC regulation (IOC, 2012): total sterols levels were higher than the minimum required of 1000 mg/kg; cholesterol and campesterol percentages were below the established limits of 0.5% and

4.0%, respectively; the percentages of stigmasterol were lower than those of campesterol, and the apparent β-sitosterol content was higher than the legal minimum of 93%. As shown in Table 3, both the total sterols content and the amounts of individual sterols varied significantly according to the production area. Fès (1977.00 mg/kg) and Chefchaouane (1794.60 mg/kg) had, respectively, the highest and lowest mean of total sterols content among the north Moroccan regions examined within this study. Besides, in all the evaluated oils, β-sitosterol was the most abundant phytosterol, being found within the range 84.87-87.20% of the total sterols (range demarcated by olive oil samples from Sefrou and Ouazzane, respectively), followed by Δ5-avenasterol, with a mean value (%) ranging between 6.23 and 8.58% in oils from Ouazzane and Taza, respectively; and campesterol, for which the highest mean value was observed in Ouazzane olive oils samples (3.11%) andthe lowest one (2.84%) was found in Taza olive oils. Furthermore, small amounts of cholesterol, 24-methylenecholesterol, clerosterol, campestanol, sitostanol, Δ5,24-stigmastadienol, Δ7-stigmastenol, and Δ7-avenasterol were also found in all the analysed olive oil samples.

When the influence of the geographical origin was considered, statistically significant differences were observed in both total content and the amounts of individual sterols, except for brassicasterol and sitostanol.

3.2.5 Triterpene dialcohols

The determination of the sum of erythrodiol and uvaol is considered as a suitable authenticity index to detect possible fraudulent admixtures of VOO with olive-pomace oil (Angerosa et al., 2006). Table 3 shows the levels of these two analytes found in the evaluated north Moroccan olive oil samples. As can be seen in this Table, in all the checked samples the sum of erythrodiol and uvaol was below the upper legal limit of 4.5% established by the IOC regulation (IOC, 2012). Furthermore, by evaluating the effect of geographical area on the sum of erythrodiol and uvaol content, it can be observed that this parameter was influenced; it showed the highest mean values for Taza olive oils (1.69%), and the lowest (1.37%) for Meknès samples.

Table 3. Sterol, triterpenic dialcohols, 2-glyceryl monopalmitate (%), aliphatic alcohols, stigmastadienes and wax composition of the olive oil samples studied.

	Chefchaouane (n=10)		Fès (n=41)		Meknès (n=69)		Ouazzane (n=37)		Sefrou (n=39)		Taounate (n=49)		Taza (n=34)	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
Cholesterol (%)	0.12^{ab} ±0.08	0.06–0.31	0.10^{ab} ±0.04	0.05–0.18	0.11^{b} ±0.05	0.04–0.33	0.07^{c} ±0.02	0.03–0.15	0.08^{ac} ±0.04	0.03–0.24	0.08^{ac} ±0.04	0.04–0.23	0.08^{ac} ±0.04	0.04–0.24
Brassicasterol (%)	0 ±0.00	0.00–0.00	0 ±0.00	0.00–0.00	0 ±0.00	0.00–0.01	0 ±0.00	0.00–0.01	0 ±0.01	0.00–0.07	0 ±0.01	0.00–0.03	0 ±0.00	0.00–0.02
24–Methylene-cholesterol (%)	0.12^{a} ±0.06	0.01–0.19	0.19^{a} ±0.05	0.09–0.31	0.17^{a} ±0.05	0.06–0.33	0.12^{a} ±0.04	0.05–0.19	0.63^{a} ±2.77	0.08–17.00	0.16^{a} ±0.05	0.07–0.26	0.18^{a} ±0.07	0.07–0.33
Campesterol (%)	2.89^{ab} ±0.33	2.60–3.73	2.87^{a} ±0.19	2.59–3.40	2.89^{a} ±0.24	2.54–3.61	3.11^{b} ±0.38	2.64–3.66	3.00^{ab} ±0.22	2.66–3.75	2.87^{a} ±0.23	2.61–3.84	2.84^{a} ±0.22	2.52–3.40
Campestanol (%)	0.03^{acd} ±0.01	0.02–0.05	0.04^{abcd} ±0.01	0.02–0.08	0.05^{b} ±0.02	0.01–0.09	0.03^{c} ±0.01	0.01–0.06	0.04^{acd} ±0.02	0.01–0.07	0.04^{d} ±0.01	0.01–0.07	0.03^{ac} ±0.01	0.01–0.07
Stigmasterol (%)	0.95^{abc} ±0.29	0.62–1.43	0.71^{b} ±0.22	0.09–1.35	0.79^{c} ±0.34	0.33–2.13	0.81^{abc} ±0.19	0.50–1.42	0.99^{a} ±0.46	0.00–1.83	0.88^{abc} ±0.28	0.57–1.89	0.96^{ac} ±0.38	0.47–1.88
Δ7–Campesterol (%)	0.04^{ab} ±0.02	0.01–0.08	0.04^{a} ±0.04	0.01–0.29	0.05^{a} ±0.02	0.00–0.15	0.04^{a} ±0.02	0.00–0.09	0.07^{b} ±0.07	0.02–0.36	0.04^{a} ±0.03	0.02–0.19	0.04^{a} ±0.02	0.01–0.13
Δ5,23–stigmastadienol (%)	0.11^{a} ±0.05	0.04–0.24	0.10^{a} ±0.05	0.01–0.28	0.10^{a} ±0.08	0.00–0.28	0.10^{a} ±0.05	0.00–0.19	0.20^{b} ±0.17	0.00–0.56	0.14^{a} ±0.05	0.08–0.27	0.14^{a} ±0.07	0.06–0.46
Clerosterol (%)	0.90^{abc} ±0.08	0.78–1.04	0.90^{ac} ±0.05	0.78–1.00	0.95^{b} ±0.09	0.70–1.21	0.92^{ab} ±0.04	0.82–0.98	0.84^{c} ±0.17	0.48–1.07	0.94^{ab} ±0.07	0.80–1.22	0.89^{ac} ±0.08	0.58–1.00
β-sitosterol (%)	86.48^{ab} ±1.67	84.36–89.22	85.38^{a} ±1.69	81.79–89.31	85.35^{a} ±2.01	78.23–90.08	87.20^{b} ±1.5	83.51–90.18	84.87^{a} ±1.84	81.19–88.09	86.14^{ab} ±2.05	81.17–89.84	84.96^{a} ±2.95	78.85–88.92
Sitostanol (%)	0.20^{a} ±0.07	0.13–0.37	0.15^{a} ±0.13	0.09–0.93	0.20^{a} ±0.11	0.09–0.94	0.20^{a} ±0.04	0.12–0.31	0.17^{a} ±0.06	0.11–0.43	0.17^{a} ±0.04	0.11–0.33	0.16^{a} ±0.03	0.09–0.23
Δ5–avenasterol (%)	7.02^{ab} ±1.70	4.67–9.22	8.36^{a} ±1.58	4.86–11.83	8.24^{a} ±1.76	4.10–13.97	6.23^{b} ±1.60	3.86–9.85	8.45^{a} ±1.75	5.29–11.77	7.37^{ab} ±1.80	4.43–10.99	8.58^{a} ±2.98	4.72–15.02
Δ5,24-stigmastadienol (%)	0.55^{ab} ±0.06	0.42–0.64	0.57^{ab} ±0.07	0.44–0.71	0.55^{ab} ±0.07	0.31–0.75	0.58^{a} ±0.10	0.47–1.01	0.56^{ab} ±0.06	0.41–0.71	0.52^{b} ±0.07	0.38–0.77	0.59^{a} ±0.11	0.41–0.87
Δ7-stigmastenol (%)	0.19^{ab} ±0.03	0.14–0.23	0.17^{b} ±0.03	0.11–0.23	0.20^{ab} ±0.04	0.14–0.33	0.22^{a} ±0.09	0.14–0.73	0.20^{ab} ±0.04	0.16–0.37	0.22^{a} ±0.10	0.14–0.85	0.21^{ab} ±0.07	0.13–0.41
Δ7-Avenasterol (%)	0.38^{abc} ±0.06	0.31–0.47	0.38^{bc} ±0.03	0.30–0.44	0.37^{c} ±0.05	0.26–0.49	0.36^{abc} ±0.05	0.28–0.51	0.35^{ac} ±0.03	0.31–0.45	0.34^{ac} ±0.05	0.24–0.44	0.35^{ac} ±0.04	0.25–0.41

Table 3. (continued)

	Chefchaouane (n=10)		Fès (n=41)		Meknès (n=69)		Ouazzane (n=37)		Sefrou (n=39)		Taounate (n=49)		Taza (n=34)	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
Apparent β–sitosterol (%)	95.26^{ab} ±0.31	94.74–95.65	95.47^{a} ±0.30	94.71–95.95	95.37^{a} ±0.42	94.26–96	95.23^{ab} ±0.45	94.19–96.01	95.09^{b} ±0.46	94.11–95.81	95.35^{ab} ±0.49	92.89–95.94	95.32^{ab} ±0.39	94.24–95.91
Total sterol (mg/kg)	1794.60^{ab} ±269.01	1396.77–2387.19	1977.00^{b} ±175.38	1479.07–2278.85	1956.52^{ab} ±287.21	1227.22–3235.15	1957.69^{ab} ±264.63	1651.40–2592.34	1844.70^{a} ±162.20	1497.15–2098.12	1856.66^{ab} ±199.66	1347.76–2432.47	1880.59^{ab} ±250.54	1411.86–2490.16
Erythrodiol and uvaol (%)	1.64^{ab} ±0.39	1.07–2.30	1.39^{a} ±0.39	0.79–2.47	1.37^{a} ±0.41	0.63–2.55	1.38^{a} ±0.22	0.86–1.95	1.55^{ab} ±0.25	1.08–2.03	1.42^{a} ±0.34	0.63–2.53	1.69^{b} ±0.53	0.94–2.98
2–glyceryl monopalmitate (%)	0.43^{ab} ±0.05	0.40–0.50	0.41^{a} ±0.04	0.30–0.50	0.44^{a} ±0.08	0.30–0.80	0.48^{b} ±0.06	0.40–0.60	0.41^{a} ±0.04	0.30–0.50	0.44^{ab} ±0.06	0.20–0.50	0.42^{a} ±0.05	0.30–0.50
Aliphatic alcohols (mg/kg)	35.50^{ab} ±11.34	19.90–58.70	36.93^{a} ±11.02	23.20–80.70	52.57^{b} ±43.77	19.50–29.40	35.05^{a} ±8.41	22.40–61.50	49.34^{ab} ±19.17	20.90–107.60	39.22^{ab} ±10.80	16.50–64.50	38.97^{ab} ±10.25	22.20–70.60
Stigmastadienes (mg/kg)	0.01^{a} ±0.00	0.00–0.01	0.01^{a} ±0.01	0.00–0.05	0.01^{a} ±0.02	0.00–0.04	0.01^{a} ±0.01	0.00–0.04	0.01^{a} ±0.01	0.00–0.03	0.01^{a} ±0.00	0.00–0.02	0.01^{a} ±0.00	0.00–0.03
C40 (mg/kg)	15.16^{a} ±5.94	9.63–29.49	16.51^{a} ±3.67	11.31–26.43	20.08^{a} ±15.24	4.46–110.19	19.93^{a} ±7.69	10.08–45.73	18.12^{a} ±6.15	8.49–34.87	20.82^{a} ±6.40	10.32–43.52	17.75^{a} ±6.06	9.63–41.83
C42 (mg/kg)	13.96^{ab} ±2.81	10.52–20.47	11.98^{b} ±2.49	8.79–18.57	13.84^{ab} ±5.25	6.53–37.18	15.84^{a} ±3.99	11.66–27.61	13.82^{ab} ±4.12	7.78–26.54	14.53^{ab} ±3.67	9.78–23.15	14.34^{ab} ±3.72	8.53–24.11
C44 (mg/kg)	4.72^{ab} ±1.28	2.71–6.69	3.97^{b} ±1.44	2.22–7.07	5.32^{a} ±1.93	2.35–12.91	5.74^{a} ±1.46	3.16–10.21	4.88^{ab} ±1.50	2.35–9.23	5.02^{ab} ±1.79	1.99–9.98	5.03^{ab} ±1.71	3.20–10.90
C46 (mg/kg)	5.81^{abc} ±4.20	1.41–11.19	6.32^{bc} ±2.69	1.00–10.06	8.57^{d} ±1.33	5.08–11.26	7.50^{acd} ±3.10	1.70–11.08	8.19^{ad} ±1.25	5.62–10.53	5.79^{b} ±3.14	1.38–10.08	9.00^{d} ±2.34	2.66–14.95
Wax (mg/kg)	39.65^{ab} ±10.71	26.66–65.02	38.78^{b} ±7.52	26.80–58.65	47.81^{a} ±21.81	21.18–165.78	49.01^{a} ±13.14	32.80–89.37	44.86^{ab} ±12.30	25.08–81.10	46.37^{ab} ±10.70	28.75–78.18	46.13^{ab} ±11.95	26.82–83.05

-Significant differences among columns for the same parameter are indicated with different lowercase letters (comparison between studied regions, $p < 0.05$).

3.2.6 Total aliphatic alcohols content

Aliphatic alcohols are mostly located inside the olive stone and are partially extracted by mechanical processes, as a consequence, they are contained in solvent extracted oils in higher amounts than in mechanically extracted VOO (Aparicio, Conte, & Fiebig, 2013). Therefore, the concentration of these compounds, in combination with other parameters (erythrodiol, uvaol and wax content) is applied as an effective and reliable means to detect fraudulent addition of olive pomace oil to VOO. The mean values of the total aliphatic alcohols content in the studied north Moroccan olive oil samples were ranged from 35.05 to 52.57 mg/kg (Table 3). The maximum average value was observed for Meknès olive oils, whilst the minimum was found for oils from Ouazzane. Furthermore, the effect of geographic origin on the total aliphatic alcohols content was observed to be significant ($p<0.05$).

3.2.7 Wax

The average concentration for the individual wax esters (C40, C42, C44 and C46) and for the total wax esters evaluated in the studied samples is also included in Table 3. All the analysed samples showed a total wax ester concentration below the legal established limit by the IOC legislation (IOC, 2012) for edible VOOs (C40–C46 waxes 150.00 mg/kg). Furthermore, there were statistical significant differences among the analysed samples in terms of total wax content and the amounts of individual wax, with the exception of the parameter C40.

Fès olive oils exhibited the lowest mean value of 38.78 mg/kg and Ouazzane olive oils showed the highest one of 49.01 mg/kg. Characterising the waxy fraction is of utmost importance, since together with the amount of erythrodiol and uvaol, can be used as a criterion of both quality and purity of VOO (Angerosa et al., 2006).

3.2.8 Stigmastadienes

Stigmastadienes, and particularly stigmasta-3,5-diene, are an important indicator of the presence of refined oils in VOO -even at very low concentrations- because they are formed during the refining process throughout the acid catalysed sterol dehydration reaction during bleaching process (Cert, Lanzn, Carelli, Albi, & Amelotti, 1994), or during the deodorization process, promoted by high temperatures (Verleyen et al.,

2002). As can be observed in Table 3, the mean content of stigmasta-3,5-diene in the analysed samples was lower than 0.05 ppm, the level set by the IOC regulation (IOC, 2012) for VOO and Extra VOO categories . Furthermore, when the content of this compound was statistically checked in all the dataset, no statistical significant differences among the samples under study were observed according to their geographic origin.

3.2.9 2-glyceryl monopalmitate

This parameter is used to determine whether the oil has been re-esterified by synthetic means or by addition of animal fat. Additional information is gained by determining which fatty acids are located at the 2-position (middle) of the triglyceride, since high levels of saturated fatty acids at the 2-position prove an adulteration with interesterified oil. The results about the content of 2-glyceryl monopalmitate in the studied samples are given in Table 3. As can be seen, this content differed among the 7 geographical origins selected. Thus, olive oil samples from Ouazzane exhibited the highest mean amount of this compound (0.48%), whereas the lowest levels were detected in oils from Fès and Sefrou (0.41%). All the obtained values were below the maximum limit (0.9% when C16:0 ≤ 14.0%; and 1.0% when C16:0 >14.0%) fixed by the IOC regulation.

3.3 Chemometric analysis

To achieve the classification of the studied olive oil samples according to their geographical origin, all the obtained data (a matrix data composed of 55 analytical parameters corresponding to physicochemical quality criteria, purity parameters and evaluated ratios for 279 samples) were submitted, without any previous variable reduction step, to a classification test using a stepwise canonical discriminant analysis. This approach allows extracting the variables with the highest discriminant ability between the geographical regions studied, as well as the evaluation of the contribution of these variables to discrimination model. Those variables that cannot discriminate among the studied samples are, in contrast, deleted from the discriminant function. Afterwards, the prediction ability of the built model was estimated, by a leave-out cross-validation method. The Classification parameters (eigenvalues, canonical correlations, Wilk's lambda and Chi-square coefficients) are included in Table 4a (supplementary material). As can be seen from this Table, six discriminant functions were obtained. The

first four discriminant functions contain 96.49% of the total discriminating power in this model.

Table 4a supplem. Within-groups correlations between discriminating variables and standardized canonical discriminant functions for north Moroccan VOO origin discrimination.

	F1	F2	F3	F4	F5	F6
Eigenvalue	74.23	49.26	40.49	9.35	4.39	1.9
% of variance	41.32	27.42	22.54	5.2	2.44	1.1
% Cumulative	41.32	68.75	91.28	96.5	98.9	100
Canonical correlation	0.99	0.99	0.99	0.95	0.9	0.8

Lambda de Wilks				
Test of function (s)	**Wilks' Lamda**	**Chi-square**	**df**	**Sig**
1 to 6	0	4357.5	192	0
2 to 6	0	3253.6	155	0
3 to 6	0	2252.77	120	0
4 to 6	0.01	1300.93	87	0
5 to 6	0.06	703.85	56	0

Canonical correlation coefficients were quite high, showing values between 0.81 and 0.99, and low values of Wilk's lamda (between 0.00 and 0.06) were obtained, which indicates that the group's mean discriminant scores differ. The sig value was 0.00, and it indicates a high significant difference between the group's centroids. Furthermore, 31 analytical parameters were selected as discriminating variables: Free fatty acids, K_{270}, O/P, Δ5,23-stigmastadienol, campestanol, Δ7-stigmastenol, Δ5,24-stigmastadienol, apparent β-sitosterol, Δ5-avenasterol, stigmastadienes, 2-glyceryl monopalmitate, C40 and C44, that mainly contributed to the first discriminant function; peroxide value, K_{232}, C16:1, C18:0, C18:1, C18:3, C20:1, Δ7-campesterol, cholesterol, β-sitosterol, erythrodiol and uvaol, Δ7-avenasterol, aliphatic alcohol content, C42 and C46 were mainly associated with the second discriminant function; C17:0, C20:0, trans C18:1 and 24-methylene-cholesterol had a main influence into the third discriminant function; C16:0, trans C18:2T+C18:3T, campestanol and Δ7-stigmastenol were mainly contributing to the fourth discriminant function; C14:0, total sterol and stigmasterol were primarily associated with the fifth discriminant function; and finally, C24:0, brassicasterol and clerosterol, that were especially related to the sixth discriminant function.

Table 4b supplem. Matrix classification and cross validation of north Moroccan VOOs according to their geographic origin**.**

		Predicted group membreship								
		Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza	Total	% correct
Original group	Chefchaouane	10	0	0	0	0	0	0	10	100.00%
	Fès	0	41	0	0	0	0	0	41	100.00%
	Meknès	0	0	69	0	0	0	0	69	100.00%
	Ouazzane	0	0	0	37	0	0	0	37	100.00%
	Sefrou	0	0	0	0	39	0	0	39	100.00%
	Taounate	0	0	0	0	0	49	0	49	100.00%
	Taza	0	0	0	0	0	0	34	34	100.00%
	Total	10	41	69	37	39	49	34	279	100.00%
	Cross validated									
		Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza	Total	% correct
Original group	Chefchaouane	10	0	0	0	0	0	0	10	100.00%
	Fès	0	41	0	0	0	0	0	41	100.00%
	Meknès	0	3	63	0	3	0	0	69	91.30%
	Ouazzane	0	0	0	37	0	0	0	37	100.00%
	Sefrou	0	0	0	0	39	0	0	39	100.00%
	Taounate	3	0	0	1	0	45	0	49	91.83%
	Taza	0	0	0	0	0	0	34	34	100.00%
	Total	13	44	63	38	42	45	34	279	96.41%

The plot of the studied samples on the canonical plane formed by the first two functions obtained by the stepwise canonical discriminant procedure (Figure 3) clearly displayed differences among the studied regions. Thus, groups of olive oil samples from Chefchaouane, Fès, Ouazzane, Taounate and Taza are well separated; whereas in olive oil samples coming from Meknès and Sefrou regions it is possible to observe a slight overlapping (three samples from Meknès region are positioned among the samples from the Sefrou area).

As far as the classification ability is concerned, the stepwise canonical discriminant model showed, in the cross-validation, recognition and prediction abilities of 100 and 96.41%, respectively (Table 4b, supplementary material). Among the 279 samples studied, only 10 samples were not correctly classified. Six Meknès samples were classified as belonging to Fès zone (three samples) and Sefrou zone (three samples), and four

samples from Taounate were classified as belonging to Chefchaouane (three samples) and Ouazzane (one sample).

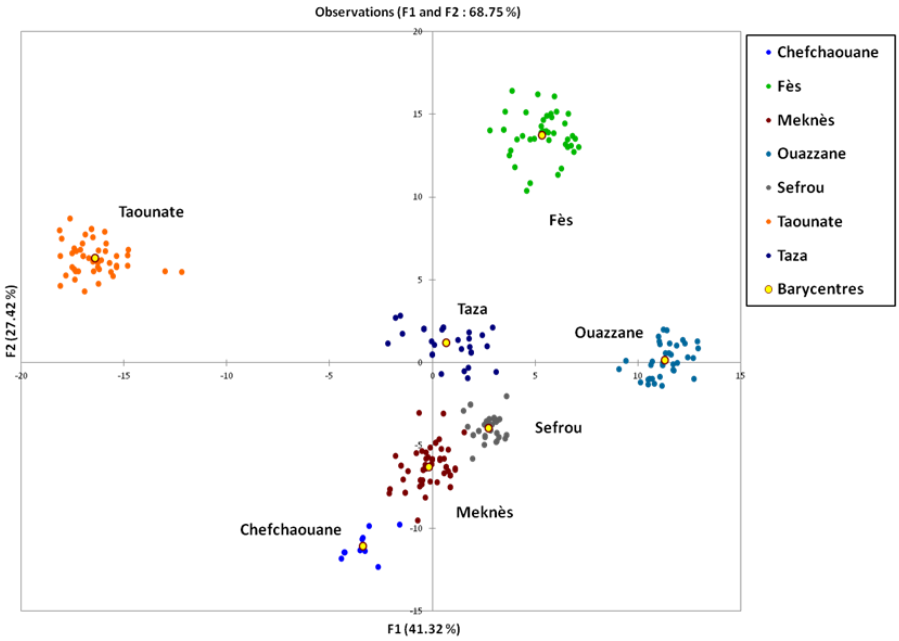

Fig. 3 Scores of the 279 VOO samples on the map defined by the first two principal components.

4 Conclusions

The characterisation of north Moroccan olive oils based on their physicochemical quality parameters and purity criteria was achieved within this study and a database compiling all the obtained results was built. This work also enable us to geolocate, within the studied regions, some olive growing area that showed higher levels of linolenic acid than those defined by the IOC regulation. With regard to linolenic acid, it is important to stand out that these results suggest that continuing this work with the comprehensive characterisation of a greater number of VOOs from the identified olive growing areas is crucial to establish the real value of this fatty acid in north Moroccan VOOs, allowing Moroccan authorities applying for the revision and adjustment of commercial criteria and current IOC regulations. Furthermore, by processing all the evaluated parameters using a stepwise canonical discriminant analysis, a very good geographical discrimination rate was obtained.

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SECTION

2

"Metabolomics approaches for geographical and botanical origin authentication"

Chapter 3

Potential of LC-MS phenolic profiling combined with multivariate analysis as an approach for the determination of the geographical origin of North Moroccan virgin olive oils

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Abstract

The applicability of two different platforms (LC-ESI-TOF MS and LC-ESI-IT MS) as powerful tools for the characterization and subsequent quantification of the phenolic compounds present in north Moroccan virgin olive oils was assessed in this study. 156 olives samples of "Picholine Marocaine" cultivar grown in 7 Moroccan regions were collected and olive oils extracted. The phenolic profiles of these olive oils were studied using a resolutive chromatographic method coupled to ESI-TOF MS (for initial characterization purposes) and coupled to ESI-IT MS (for further identification and quantification). 25 phenolic compounds belonging to different chemical families were identified and quantified. Secoiridoids were the most abundant phenols in all the samples, followed by lignans, phenolic alcohols, and flavonoids, respectively. For testing the ability of phenolic profiles for tracing the geographical origin of the investigated oils, multivariate analysis tools were used, getting a good rate of correct classification and prediction by using a cross validation procedure.

Keywords: Virgin olive oil; Liquid chromatography-Mass spectrometry; Phenolic compounds; Multivariate analysis; Geographical origin.

1. Introduction

Olive growing and virgin olive oil (VOO) production have played an indisputable socio-economic role throughout the Mediterranean region history. The use of olive oil nowadays extends beyond the Mediterranean region and its significance, linked to its many virtues is undeniable. Beyond its nutritional values and specific sensory properties that distinguish it from other edible vegetal oils, VOO has enjoyed, over the last decades, an increasing worldwide popularity due to the beneficial effects on human health attributed to several of its compounds. Indeed, there is a growing evidence of the healthy bioactive properties of some of the minor components of VOO, such as vitamin E, pigments, phytosterols and phenolic compounds (López-Miranda et al., 2010). Among its several minor constituents, phenolic compounds, are attracting considerable attention because they contribute to the stability of VOO against auto-oxidation (Gallina-Toschi et al., 2005; Tsimidou, 1998; Tura et al., 2007), they influence on the organoleptic characteristics of VOO by conferring bitterness, pungency and astringency

(Apparicio & Morales 1998; Bendini et al., 2007 ; Servili et al., 2009), and they are also considered as components with an important health promoting properties (Boskou et al., 2006; Covas et al., 2006). Different epidemiological and clinical studies carried out to assess the implications of VOO phenolic compounds in prevention and treatment against many diseases, have shown a broad spectrum of bioactive properties that characterize these compounds, including anti-inflammatory (Cicerale et al., 2012; Lucas et al., 2011), antioxidant (Carrasco-Pancorbo et al., 2005; Morelló et al., 2005) and antimicrobial properties (Bisignano et al., 1999; Medina et al., 2006), among others. Indeed, VOO phenolic compounds have shown beneficial effects on diseases associated with oxidative damage, such as coronary heart disease and cancer (Covas et al., 2006; López-Miranda et al., 2010; Owen et al., 2000).

The phenolic fraction of VOO consists of a heterogeneous mixture of compounds belonging to several families with varying chemical structures. At least 36 structurally distinct phenolic compounds have been identified in this matrix. These compounds belong to five main classes: (i) phenolic acids (hydroxybenzoic and hydroxycinnamic acids, such as caffeic, vanillic, syringic, *p*-coumaric, etc.), (ii) simple phenols (hydroxytyrosol and tyrosol), (iii) flavonoids (luteolin and apigenin), (iv) secoiridoids (including oleuropein and ligstroside derivatives), and (v) lignans (such as pinoresinol and 1-acetoxypinoresinol) (Carrasco-Pancorbo et al., 2007; Tuck et al., 2002).

The qualitative and quantitative phenolic composition of VOO is strongly affected by the agronomic factors and technological conditions of production. They include those factors related to the geographic area, such as climate and soil (Nieves Criado et al., 2004), the cultivar (Gómez-Alonso et al., 2002, Tura et al., 2007), the olive cultivation practices (Gimeno et al., 2002; Servili et al., 2007) and the olive oil-making procedures (extraction system and the conditions of processing, filtration and storage) (Angerosa et al., 2001; Ranalli et al., 2005). All this can explain the recent tendency to examine the possibility of using VOO phenolic profiles, characterized by various analytical methods, as geographical markers for VOO discrimination (Bakhouche et al., 2013; Lerma-García et al., 2009; Ouni et al., 2011; Taamalli et al., 2012).

The development of methodologies for the identification and quantification of phenols in VOO has been discussed extensively in literature; separative techniques (LC, GC or,

in some applications, CE) coupled to different detectors have been used, being LC-MS one of the most popular coupling nowadays. LC-MS combines relatively short analysis times and high separation efficiency for the analysis of VOO phenolic compounds (Bendini et al., 2007 ; García-Villalba et al., 2010). Besides, the application of multivariate statistical techniques for the treatment of the chromatographic data opens up great expectations for the assessment of VOO phenolic fraction. The combination of innovative LC-MS methodologies together with statistical approaches stand out as an appropriate tool for the traceability and classification according to geographical origin of VOO considering its phenolic composition (Bakhouche et al., 2013; Lerma-García et al., 2009; Ouni et al., 2011; Taamalli et al., 2012).

In Morocco, the olive tree cultivation represents a significant land use with important environmental, social and economic considerations. With an olive crop area of 840.000 ha (more than half of the total fruit-tree area occurring in the country) and an annual average olive oil production of about 90.000 tones, Morocco is the world's 6th largest producer of olive oil (International Olive Oil Council, 2012). It is important to highlight that over 96 percent of olive oil in Morocco is produced from only one cultivar, commonly referred as a "*Population variety*" called "*Picholine Marocaine*" (MAPM, 2012), which includes several distinct genotypes locally cultivated (Esadiki et al., 2006; Ouazzani et al., 1996). Thus, this genetic diversity of the cultivar "*Picholine Marocaine*" combined with variable pedoclimatic conditions that characterize the Moroccan olive growing regions, could represent, from our point of view, an important source of variability of phenolic compounds content in VOO produced in diverse Moroccan zones.

In the current study, two different platforms were used to determine the phenolic fraction of 156 VOO samples extracted from olives samples collected from 7 different north Moroccan regions. In a first step, LC-ESI-TOF MS was used to characterize the phenolic profiles and, afterwards, LC-ESI-IT MS was used to analyze the whole sample set and carry out the quantification of the phenolic compounds of interest. Besides, through the application of a stepwise canonical discriminant analysis to the chromatographic data obtained, we have developed a method with great potential to achieve the geographical discrimination of north Moroccan VOOs. To the best of our knowledge, this is the first study carried out for the identification and quantification of

the phenolic compounds of Moroccan VOO and their use for the geographical classification.

2. Materials and methods

2.1. Sample collection

156 VOO samples were obtained by collecting olives from seven different North Moroccan regions: Chefchaouane, Fes, Meknès, Ouazzane, Sefrou, Taounate, and Taza. These regions were chosen based on the importance of their olive crop area and their production of olive oil. Indeed, they are the main olive growing areas of the North of the country. The location and data related to olive crop area and olive oil production of these regions are given as supplementary material.

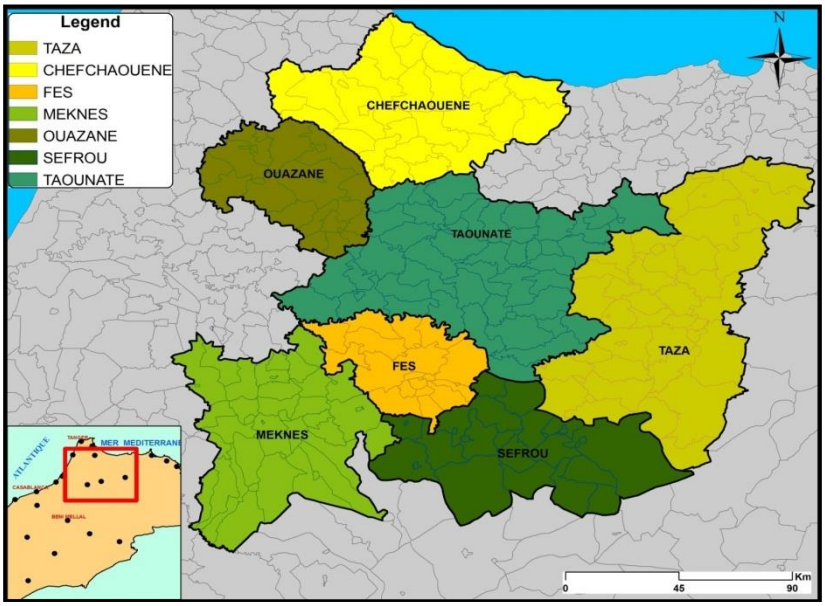

Fig. 1 (supplementary info). *Geographical localization of the studied north Moroccan areas under study and olive oil production statistic data.*

All olive samples collected were from the cultivar Picholine Marocaine. The fruit of this variety is medium to large; with a fat yield of about 18-27% of fresh weight and the oil is characterized by a high content of total phenols and a good stability (Uceda, 2009). The olives were picked by hand from the selected trees, and only not damaged, fresh and healthy fruits were selected. Olives were collected during the harvesting period 2011-2012, between November and January, placed in rectangular plastic crates (approximately one sample is 35 Kg of olives) and immediately transported to the laboratory. An homogeneous sample of 100 olive fruits from every plastic crates was

taken to calculate the ripening index (RI), parameter which was determined according to the method developed by the Agronomic Station of Mengíbar (Jaén), defining the RI as a function of fruit color in both skin and pulp (Uceda & Frias, 1975).

An Oliomio laboratory mill was used to extract the olive oil samples. Fruits were processed as follows: olives were washed to eliminate any foreign material and poured into the receiving hopper, where a screw feeds the crusher that is equipped with fix hole grid and groove knives impeller at a temperature of 25-27ºC. The paste produced falls into the malaxing part; malaxing was carried out for 45 min at 28-30 ºC. The resulting olive paste was decanted at temperature of 23-27ºC without the addition of water. Finally, olive oil was centrifuged, filtered and stored in dark glass bottles (250 mL) at - 18 ºC, excluding any head space volume in order to assure the proper conservation of the olive oil against oxidation until chemical analyses were performed.

2.2. Analytical official indices

Free acidity, peroxide value, and UV spectrophotometric indices (K_{232}, K_{270}, and ΔK) were evaluated according to the official methods described in Regulation EC 2568/91 of the Commission of the European Union (and its further modifications). All parameters were determined in triplicate for each sample.

2.3. Analysis of Phenolic Compounds

2.3.1. Chemicals and reagents

All reagents were of analytical grade and used as received. Methanol and *n*-hexane of HPLC grade used for the extraction of the phenolic compounds from the olive oil samples were supplied from Panreac (Barcelona, Spain). Acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Panreac (Barcelona, Spain) were used for preparing mobile phases. Doubly deionized water with a conductivity of 18.2 MΩ was obtained by using a Milli-Q-system (Millipore, Bedford, MA, USA). Standards of hydroxytyrosol, tyrosol, luteolin, apigenin, *p*-coumaric, quinic, ferulic and caffeic acids, as well as 3,4-dihydroxyphenylacetic acid (DOPAC) (IS), were purchased by Sigma-Aldrich (St. Louis, MO, USA) and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein was purchased from Extrasynthese (Lyon, France). Stock solutions at concentration of 500 mg/L for each phenol were first prepared by dissolving the appropriate amount of the compound in methanol and then serially diluted to working

concentrations (within the range 0.5-250 mg/L). All solutions were stored in dark flask at -20 ºC.

2.3.2. Phenolic compounds extraction

A liquid-liquid extraction method was used for the isolation of phenolic compounds from olive oil samples. 4.0 ± 0.001 g of olive oil were weighed in a test tube with a screw cap and 0.05 mL of internal standard solution was added. The solvent was evaporated using nitrogen, and phenolic compounds were extracted three times, adding every time 2 mL of *n*-hexane and 4 mL of methanol/water (60/40); the extract was centrifuged at 3500 rpm for 6 minutes. The supernatants were collected and the resulting solution was evaporated to dryness using a rotary evaporator under reduced pressure and a temperature of 30 °C. Finally, the residue was redissolved in 2 mL of methanol and filtered through a 0.45 µL membranes (Millipore) filter.

When the phenolic fractions of the 156 olive oil samples were extracted, we prepared some quality control (QC) samples. A mixture of the extracts from the samples under study belonging to the same geographical area (mixing an equivalent volume of each extract) was used with two purposes: to characterize the phenolic profiles of each mixture by LC-ESI-TOF MS in the initial screening and as a quality control sample, re-injecting them every 20 analyses within a sequence to assure the proper performance of the system. A QC sample prepared by mixing equivalent volumes of each extract of the 156 under study was prepared too. QC samples were injected after the optimization studies to corroborate the chosen conditions of analysis.

2.3.3. Chromatographic separation

An Agilent 1260-LC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler and a diode-array detector (DAD) was used. Phenolic compounds were separated by using a Zorbax C18 analytical column (4.6 x 150 mm, 1.8 µm particle size) protected by a guard cartridge of the same packing, operating at room temperature and a flow rate of 0.8 mL/min. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B), and the solvent gradient changed according to the following conditions: 0 to 10 min, 5 % B; 10 to 12 min, 30% B; 12 to 17 min, 38% B; 17 to 20 min, 50% B; 20 to 23 min, 95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-

equilibrated for 2.5 min. A volume of 10 µL of the methanolic extracts of olive oil or standard mix was injected in each case. The compounds separated were monitored in sequence first with DAD (240 and 280 nm), and then with a mass spectrometry detector.

2.3.4. ESI-TOF MS and ESI-Ion Trap MS conditions

As mentioned before, LC-ESI-TOF MS was used in a first stage of this research to characterize the samples under study, identifying as many phenolic compounds as possible in the profiles. Several samples belonging to each zone and QC mixtures were analyzed by this platform. Peak identification was done by comparing both migration time and MS spectral data (accurate MS spectra and MS/MS spectra (in auto MS/MS mode)) obtained from olive oil samples and standards (commercial standards or isolated compounds by HPLC); we also used the information previously reported (García-Villalba et al., 2010) and the information provides by the mass spectrometer with time of flight and ion trap analyzers. After the characterization of the profiles, all the samples were analyzed by LC-ESI-IT MS, getting quantitative data very useful for further statistical treatment.

The LC system was firstly coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer was operated in negative ionization mode and acquired data in the mass range from m/z 50 to 800 with a spectra rate of 1 Hz. The capillary was set at +4000V, the End Plate offset at -500V, the Nebulizer Gas at 2 Bar and the Dry Gas at 9 L/min at 250°C. The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonik), which provides a list of possible elemental formulas by using the SmartFormula™ Editor tool. External mass spectrometer calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA). The standard solution was injected at the beginning of the run, and all the spectra were calibrated prior to phenolic compounds identification.

The quantification of the phenolic fraction of all samples, as stated above, was conducted using a Bruker Daltonics Esquire 2000™ ion trap (Bruker Daltonik, Bremen,

Germany). The ion trap MS was operated in negative ion mode and the capillary voltage was set at +3200 V. The ion trap scanned at 50-800 *m/z* range, and the optimum values of the ESI-MS parameters were: drying gas temperature, 300°C; drying gas flow, 9 L/min; and nebulizing gas pressure, 30 psi. Instrument control, data acquisition and processing were carried out using the software Data Analysis 4.0 (Bruker Daltonik).

The polarity of ESI and all the detection parameters of both IT and TOF mass spectrometers were optimized using the height of the MS signal for the compounds included in our standard mix and some other compounds found in the olive oil QC extracts. For both mass spectrometer detectors, in order to achieve stable electrospray, a flow divisor 1:4 was used, reducing the flow rate used in the LC method to 0.2 mL/min, which is low enough to avoid the introduction of humidity in the system.

Linearity of the MS response was verified with solutions containing 10 standards at 7 different concentration levels over the range from the quantification limit to 250 mg/L. Each point of the calibration graph corresponded to the mean value from three independent injections. The limits of detection (LODs) and quantification (LOQs) of the individual analytes in standard solutions were calculated according to the IUPAC recommendations (Thompson et al. 2002). Furthermore, the precision of the analytical procedure was expressed as the relative standard deviation (RSD). The intra- and inter-day repeatabilities in the peak areas were determined as the RSD obtained for 5 and 10 consecutive injections, of each standard at a selected concentration value, carried out within the same day and on four different days, respectively.

2.4. Data Analysis

2.4.1. Univariate analysis

Statistical analysis of chemical composition of the north Moroccan olive oils was performed by SPSS statistical package software (SPSS for Windows, Version 20, SPSS Inc., and Chicago, USA). Analysis of variance was applied to all variables studied. The mean values obtained were compared by one-way ANOVA, using Tukey's test.

2.4.2. Multivariate analysis

Principal component analysis (PCA) and canonical discriminant analysis (CDA) were performed using Microsoft® Excel 2013/XLSTAT© (Version 2013.2, Addinsoft, Inc.,

Brooklyn, NY, USA). PCA as an exploratory data analysis method was used to visualize any possible grouping of samples according to the geographical origins, whereas, CDA was used for the geographical discrimination. This technique makes easier the class separation by maximizing the ratio of the between-class variance to the within-class variance or the ratio of the overall variance to the within-class variance. The CDA analysis was performed by setting p-in and p-out values at 0.05 with the same weighing of each group to choose effective variables and to decrease the number of variables for classification functions. Forward stepwise regression was selected, and p-in and p-out values were set so that the highest classification accuracy could be obtained when each factor was moved. Furthermore, the prediction ability was examined, by the leave-out cross-validation method.

3. Results and discussions

3.1. Olives ripening index and classical quality parameters values of olive oils samples

Table 1 shows the mean values for the ripening index and the regulated physicochemical quality indices as determined, respectively, in olive fruits samples and olive oils obtained through their extraction.

As far as ripening degree is concerned, small differences can be observed among studied zones, varying within a range between a mean values of 3.55 ± 0.97 (zone of Meknès) and 4.59 ± 0.76 (zone of Taza). Because of this low variation, we can dismiss the effect of the maturity stage on the differences found in the phenolic fractions of VOO from the diverse studied zones.

Furthermore, taking into account the limits set by the Commission of the European Community for the olive oil classification (Commission Regulation (ECC) 2568/91 (and its further modifications)), it is possible to say that all the olive oils under study showed low values for the regulated physicochemical parameters evaluated (free acidity given as % of oleic acid, peroxide value expressed in milliequivalents of active oxygen per kilogramme of oil (meq O_2/kg), and K_{270} and K_{232} extinction coefficients, respectively). 84.62% of them were falling within the "Extra-virgin" category, and the rest (15.38%) were within the "Virgin" category. The high quality of the raw material (health olive fruits selected and picked carefully) and good processing conditions are certainly the main factors that explain the high physicochemical quality obtained.

Table 1. Mean values of ripening index **(RI)** and classical quality indices **(FA**, % acidity; **PI,** peroxide value/index, K_{232}, K_{270}) of "*Picholine Marocaine*" olive oil samples from North Moroccan regions

Geographical origin	Ripening index & quality indices	Mean± S.D.	Category		
			Virgen-extra	Virgen	Lampante
Chefchaouan (n=15)	RI	4.45 ± 0.62	15	0	0
	FA (%)	0.51 ± 0.13			
	PI (meqO2/kg)	4.77 ± 0.81			
	K232	1.77 ± 0.39			
	K270	0.15 ± 0.03			
Fès (n=21)	RI	3.86 ± 0.85	21	0	0
	FA (%)	0.35 ± 0.10			
	PI (meqO2/kg)	2.43 ± 0.73			
	K232	0.84 ± 0.04			
	K270	0.08 ± 0.00			
Meknès (n=36)	RI	3.55 ± 0.97	31	5	0
	FA (%)	0.35 ± 0.44			
	PI (meqO2/kg)	3.19 ± 0.74			
	K232	0.88 ± 0.42			
	K270	0.13 ± 0.04			
Ouazzane (n=18)	RI	3.75 ± 0.56	13	5	0
	FA (%)	0.40 ± 0.24			
	PI (meqO2/kg)	3.24 ± 0.25			
	K232	1.62 ± 0.26			
	K270	0.04 ± 0.02			
Sefrou (n=19)	RI	4.62 ± 0.78	10	9	0
	FA (%)	0.63 ± 0.59			
	PI (meqO2/kg)	2.41 ± 0.43			
	K232	1.76 ± 0.36			
	K270	0.14 ± 0.05			
Taounate (n= 32)	RI	4.15 ± 0.84	29	3	0
	FA (%)	0.36 ± 0.27			
	PI (meqO2/kg)	2.99 ± 0.53			
	K232	0.99 ± 0.41			
	K270	0.09 ± 0.12			
Taza (n=15)	RI	4.59 ± 0.76	13	2	0
	FA (%)	0.47 ± 0.46			
	PI (meqO2/kg)	3.03 ± 2.14			
	K232	1.70 ± 0.31			
	K270	0.08 ± 0.04			
	Total		132	24	0

3.2. Identification and quantification of phenolic compounds

3.2.1. Identification of the phenolic compounds present in the samples under study

As reported in material and methods section, different strategies (analysis of standards, DAD and TOF MS data, and information previously reported) were used to carry out the identification of the compounds found in the phenolic profiles.

Table 1 (supplementary information). Main phenolic compounds identified in the olive oil extracts under study by LC-ESI-TOF MS including: retention time, internal source collision induced dissociation (ISCID) fragments, m/z experimental and calculated, molecular formula, error and sigma.

Compounds	Retention time (min)	Fragments	m/z experimental	m/z calculated	Molecular Formula	Error (ppm)	mSigma
Quinic acid	2.0		1.910.567	1.910.570	$C_7H_{11}O_6$	1.6	8.5
Hydroxytyrosol	6.8	123	1.530.559	1.530.557	$C_8H_{10}O_3$	-0.9	2.0
Tyrosol	8.5		1.370.607	1.370.608	$C_8H_9O_2$	0.7	3.3
p-coumaric acid	11.6		1.630.406	1.630.401	$C_9H_8O_3$	-3.1	9.1
Elenolic acid	14.1	139	2.410.713	2.410.718	$C_{11}H_{13}O_6$	2.0	1.2
Decarboxymethyl oleuropein aglycone	15.0	183	3.191.190	3.191.187	$C_{17}H_{19}O_6$	-1.0	3.1
Syringaresinol	16.1		4.171.537	4.171.555	$C_{22}H_{25}O_8$	4.3	1.1
Luteolin	16.4		2.850.407	2.850.405	$C_{15}H_{19}O_6$	-0.8	6.1
Pinoresinol	17.2		3.571.347	3.571.344	$C_{20}H_{21}O_6$	-0.9	1.7
Acetoxypinoresinol	17.9		4.151.391	4.151.398	$C_{22}H_{24}O_8$	1.7	1.3
Methyl decarboxymethyl oleuropein aglycone	18.0		3.331.357	3.331.344	$C_{18}H_{21}O_6$	-3.9	5.2
Decarboxymehtyl Ligstroside aglycone	18.3	183, 291	3.031.239	3.031.238	$C_{17}H_{20}O_5$	-0.2	8.7
Apigenin	19.4		2.690.452	2.690.455	$C_{15}H_{19}O_5$	1.4	2.1
Oleuropein aglycone	21.5	345,307,275	3.771.253	3.771.242	$C_{19}H_{21}O_8$	-2.8	8.3
Isomer 1	14.5						
Isomer 2	15.2						
Isomer 3	16.8						
Isomer 4	18.0						
Isomer 5	19.5						
Isomer 6	22.1						
Methyl oleuropein aglycone	23.4	345,275	3.911.405	3.911.398	$C_{20}H_{23}O_8$	-1.8	7.1
Ligstroside aglycone	23.6	291,241	3.611.303	3.611.293	$C_{19}H_{21}O_7$	-2.8	1.2
Isomer 1	16.9						
Isomer 2	21.3						
Isomer 3	22.5						

Table 1 (supplementary material) summarizes the information about the phenolic compounds identified, in *Picholine Marocaine* VOO samples, with their retention time, experimental and theoretical *m/z*, molecular formula, mass error and SigmaFit quality value.

Five main phenolic groups can be clearly distinguished: **simple phenols** (hydroxytyrosol and tyrosol), **lignans** ((+)-pinoresinol, (+)-1-acetoxypinoresinol and syringaresinol), **flavonoids** (luteolin and apigenin), various **secoiridoids** (particularly, oleuropein aglycone, ligstroside aglycone, and 6 and 3 isomers of each one, respectively, decarboxymethyl oleuropein aglycone, methyl oleuropein aglycone, methyl decarboxymethyl oleuropein aglycone, decarboxymethyl ligstroside aglycone and elenolic acid), and a **phenolic acid** (such as *p*-coumaric acid). Apart from them, another polar compound was found too, quinic acid. In total, 16 compounds (two of them with different isomeric forms (oleuropein aglycone and ligstroside aglycone) have been successfully identified.

Typical Base Peak Chromatograms (BPC) of VOO extracts of samples belonging to each studied zone, achieved by using the HPLC-ESI-TOF MS method described above, are shown in Figure 2 (supplementary material), to illustrate the variability of the profiles. Figure 3 (supplementary material) shows a profile (BPC) of a quality control sample where all the compounds are identified with a number; the extracted ion chromatograms (EICs) of the representative phenolic compounds identified are also shown.

3.2.2. Analytical parameters of the LC-ESI-IT MS method and quantitative results

Table 2 shows the analytical parameters which enabled to evaluate the method performance: repeatability (% RSD), limits of detection (LOD) and quantification (LOQ), linearity, calibration curves and regression coefficient (r^2). Linear regression analysis using the least-squares method was used to evaluate the MS responses of each analyte as a function of its concentration. The linearity of the method was studied by injecting standard solutions in the range from 0.5 to 250 mg/L. The responses fitted well to a straight line with r^2 values between 0.985 for luteolin and 0.997 for tyrosol, respectively. Detection limits were found between 0.008 µg mL^{-1} for both hydroxytyrosol

and p-coumaric acid, and 0.047 μg mL^{-1} for oleuropein. The RSD values for repeatability did not exceed 4.88 % for intra-day assays and 6.62 % for inter-day assays.

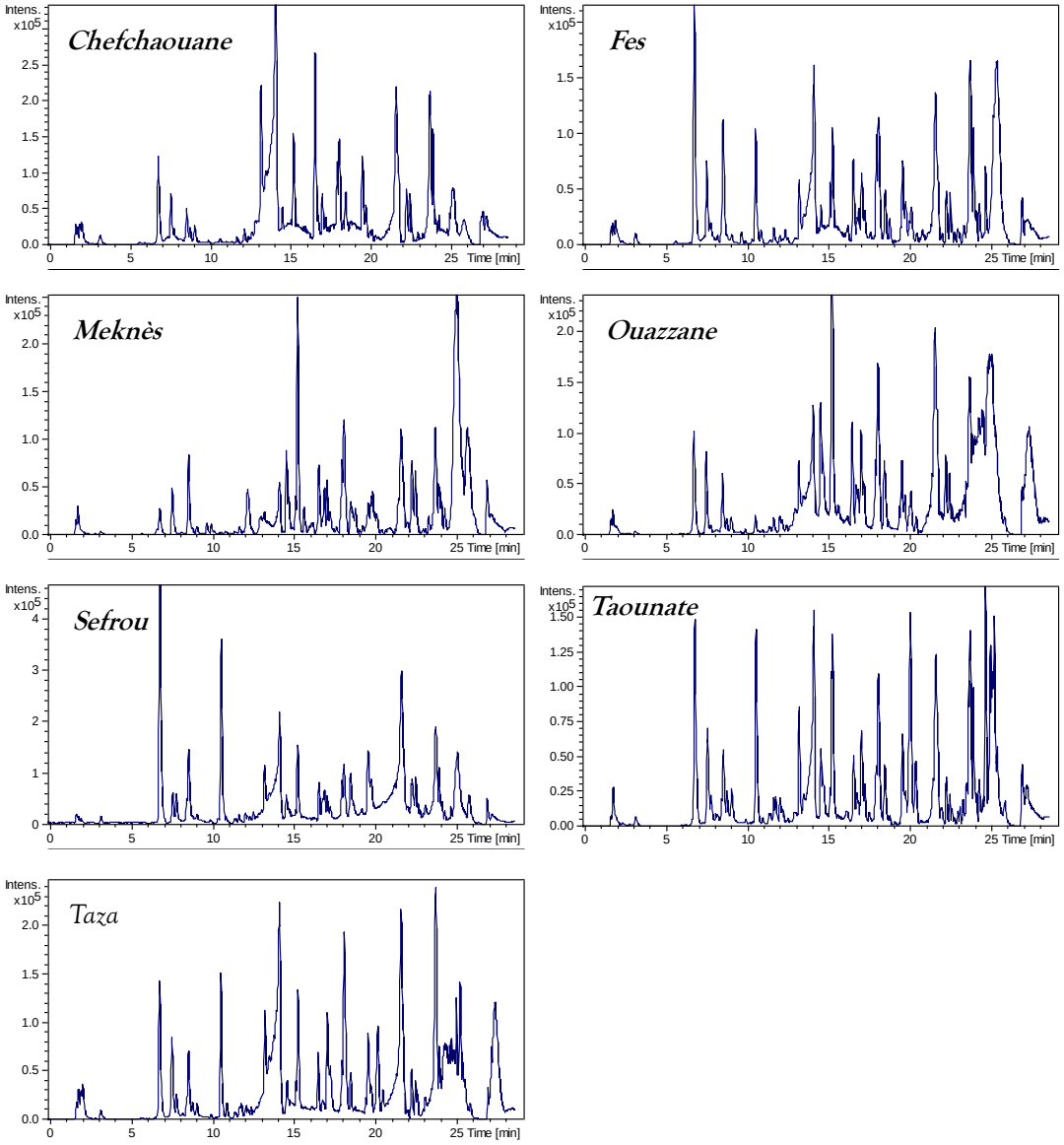

Fig. 2 (supplementary info). BPCs of VOO extracts of samples belonging to the 7 areas under study achieved by using HPLC-ESI-TOF MS. Each BPC is shown at its own scale.

Table 2. Analytical parameters of the developed LC-ESI-IT MS method: relative standard deviation (RSD%) inter- and intraday, limit of detection (LOD) and quantification (LOQ), linearity, calibration curves and r^2

Compound	RSD_{inter} (%)	RSD_{intra} (%)	LOD (µg/mL)	LOQ (µg/mL)	Linearity	Calibration curves	r^2
Quinic acid	6.62	4.76	0.028	0.094	LOQ-115	y = 54 694.896x - 271 459.268	0.993
Hydroxytyrosol	1.36	2.05	0.008	0.027	LOQ-50	y = 95 959.908x + 222 901.107	0.982
Tyrosol	1.45	4.59	0.025	0.085	LOQ-50	y = 55 482.516x + 158 562.409	0.997
p-Coumaric acid	1.64	1.83	0.008	0.027	LOQ-25	y = 92 202.519x + 105 393.426	0.991
Pinoresinol	4.68	2.39	0.012	0.039	LOQ-50	y = 88 672.611x + 272 528.634	0.995
Ferulic acid	5.97	4.88	0.013	0.045	LOQ-25	y = 109 384.253x + 119 760.025	0.992
Cafeic acid	5.13	1.19	0.009	0.032	LOQ-25	y = 136 232.543x + 325 292.701	0.995
Oleuropein	3.02	1.77	0.047	0.157	LOQ-100	y = 16 542.616x + 51 259.338	0.994
Luteolin	3.14	1.19	0.010	0.034	LOQ-25	y = 197 602.362x + 241 750.354	0.985
Apigenin	1.42	0.75	0.010	0.032	LOQ-25	y = 256 210.407x + 295 838.628	0.993

Once that the analytical performance of the LC-ESI-IT MS method was evaluated, it was successfully applied to quantify the phenolic compounds in 156 VOO samples from 7 different north Moroccan regions. The phenolic compounds concentration was determined using the area of each individual compound (two replicates) and by interpolation in the corresponding calibration curve. Results are reported in Table 3 which shows the mean values of concentrations for each phenolic compound at each region.

Moreover, the table includes the total content (mg/kg) for each chemical family of phenols in each area. It can be observed that the results showed no qualitative drastic differences among VOO samples from the 7 geographical studied regions, since all the compounds are present to a greater or lesser extend in all the samples. However, significant quantitative differences were found in a wide number of phenolic compounds according to the geographical origin of the samples

Secoiridoids were by far the most abundant group of phenolic compounds in all the analyzed samples regardless of the zone of origin. As reported by different authors, these compounds are widely associated with the sensory properties of VOO as they are the main contributors to its bitterness and pungency (Bendini et al., 2007).

The higher mean concentration of secoiridoids was observed in VOOs from Taounate with 814.01 mg kg^{-1}, whereas the lowest mean concentration was detected in Sefrou VOO samples with 438.4 mg kg^{-1}. Furthermore, the effect of the geographical origin on

the quantitative content of secoiridoids can be clearly highlighted when the variation of the concentration of the predominant compounds of this group (in relation with the 7 zones under the study) is observed. EA remain the most abundant complex phenol in VOO of the zones of Chefchaouane (135.39 mg kg^{-1}), Fes (97.83 mg kg^{-1}) and Sefrou (104.77 mg kg^{-1}), whilst in VOOs of the zones of Ouazzane, Taounate and Taza, the oleuropein aglycone´s concentration is the highest with mean values of 85.75 mg kg^{-1}, 134.15 mg kg^{-1} and 104.11 mg kg^{-1}, respectively. Finally, the zone of Meknès is distinguished among the other zones by the abundance of isomer 5 of the Ol Agl with an average concentration of 74.55 mg kg^{-1}.

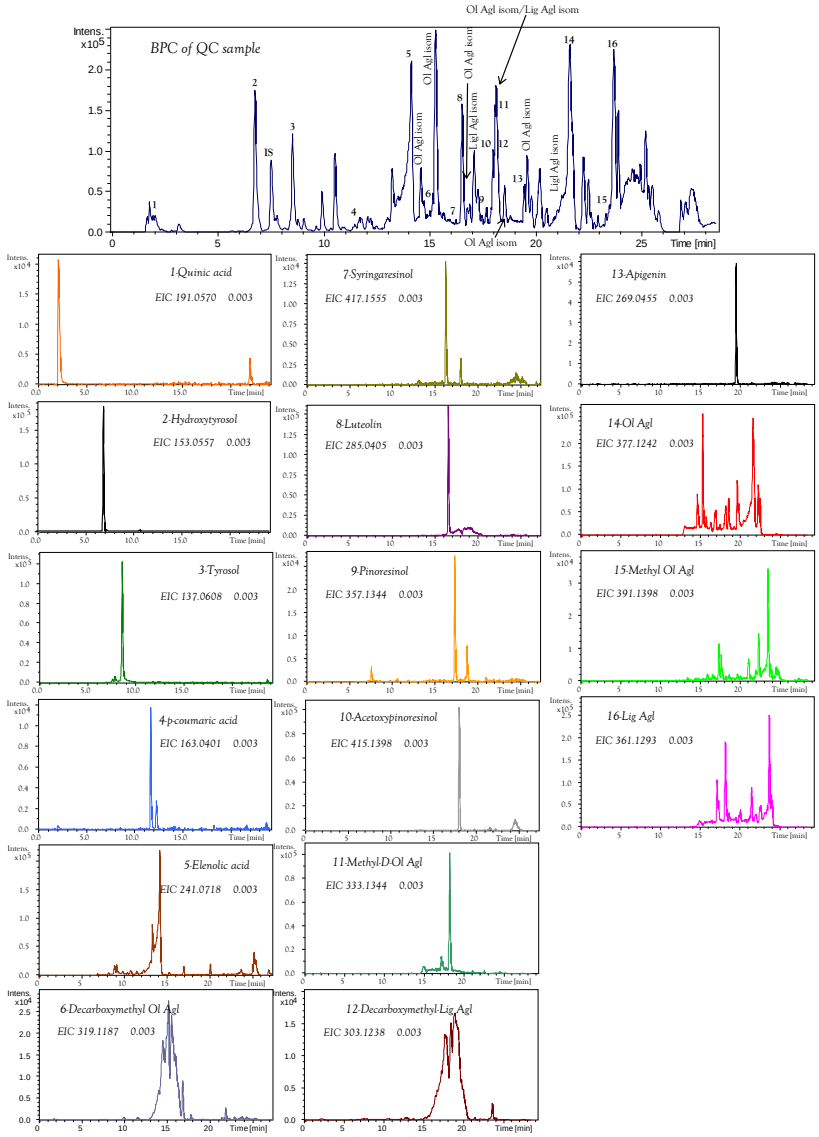

Fig.3 (supplementary info). BPC of a quality control sample, indicating with a number all the determined compounds and EICs ± 0.003 of each analyte. Data obtained by HPLC-ESI-TOF MS.

Table 3. Phenolic compounds composition and content (mg/kg) as achieved by HPLC-ESI-Ion Trap MS for 156 north Moroccan VOO samples grouped according to their zone of origin.

Origin	CHF	FES	MKN	OZN	SFR	TNT	TAZA
Sample no.	15	21	36	18	19	32	15
Phenolic compounds	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Quinic acid	$1.11^{bc} \pm 0.11$	$0.41^{a} \pm 0.06$	$1.97^{a} \pm 0.15$	$1.05^{bc} \pm 0.09$	$1.25^{bc} \pm 0.13$	$0.76^{ac} \pm 0.06$	$0.78^{bc} \pm 0.17$
Hyty	$5.86^{cd} \pm 0.30$	$1.54^{a} \pm 0.09$	$4.02^{b} \pm 0.18$	$3.47^{b} \pm 0.19$	$6.65^{d} \pm 0.37$	$5.10^{c} \pm 0.22$	$5.10^{c} \pm 0.34$
Ty	$4.77^{ac} \pm 0.50$	$4.96^{ac} \pm 0.36$	$5.87^{ac} \pm 0.29$	$4.84^{ac} \pm 0.25$	$6.32^{ab} \pm 0.37$	$7.46^{b} \pm 0.30$	$4.43^{c} \pm 0.47$
p-Cou	$0.32^{g} \pm 0.02$	$0.27^{a} \pm 0.01$	$0.28^{b} \pm 0.02$	$0.28^{f} \pm 0.03$	$0.24^{cd} \pm 0.02$	$0.37^{abc} \pm 0.03$	$0.24^{ce} \pm 0.03$
EA	$135.39^{d} \pm 9.19$	$97.83^{ac} \pm 1.27$	$72.54^{b} \pm 2.90$	$71.14^{bc} \pm 3.06$	$104.77^{a} \pm 5.31$	$82.31^{c} \pm 3.16$	$94.30^{ac} \pm 6.26$
DOA	$42.60^{ab} \pm 4.46$	$28.06^{ac} \pm 1.61$	$29.73^{b} \pm 1.62$	$33.73^{d} \pm 2.28$	$8.45^{abcd} \pm 0.99$	$51.55^{c} \pm 1.73$	$61.37^{abd} \pm 3.10$
Syr	$0.23^{ace} \pm 0.10$	$0.95^{e} \pm 1.26$	$1.92^{a} \pm 2.04$	$1.35^{a} \pm 1.32$	$0.43^{ade} \pm 0.16$	$0.92^{ae} \pm 1.33$	$0.29^{abe} \pm 0.17$
Pin	$0.51^{bc} \pm 0.22$	$1.53^{bc} \pm 1.93$	$2.58^{bc} \pm 3.88$	$2.47^{ac} \pm 2.91$	$0.93^{c} \pm 0.07$	$1.33^{c} \pm 1.80$	$0.57^{b} \pm 0.14$
Ac Pin	$1.39^{ab} \pm 0.61$	$5.97^{bc} \pm 8.19$	$5.09^{a} \pm 7.09$	$4.92^{ab} \pm 7.19$	$3.86^{ab} \pm 1.08$	$5.65^{ab} \pm 8.81$	$1.68^{abc} \pm 0.79$
D-Lig Agl	$73.71^{abc} \pm 7.44$	$42.92^{abcd} \pm 2.11$	$22.15^{b} \pm 1.63$	$23.82^{a} \pm 1.33$	$22.91^{c} \pm 1.28$	$20.89^{d} \pm 0.96$	$37.89^{bcd} \pm 1.34$
Ol Agl	$99.24^{d} \pm 3.62$	$76.70^{bd} \pm 1.23$	$19.18^{abcd} \pm 0.96$	$85.75^{c} \pm 6.43$	$84.25^{a} \pm 4.92$	$134.15^{abcd} \pm 2.70$	$104.11^{bc} \pm 4.62$
Ol Agl IS1	$10.76^{cde} \pm 0.59$	$28.17^{bcd} \pm 1.67$	$28.78^{abc} \pm 2.40$	$65.98^{abcde} \pm 2.51$	$6.57^{bde} \pm 0.94$	$30.05^{abce} \pm 2.49$	$15.59^{ae} \pm 2.05$
Ol Agl IS2	$28.38^{bc} \pm 4.96$	$20.64^{a} \pm 0.82$	$14.81^{c} \pm 1.19$	$69.97^{abc} \pm 4.02$	$14.04^{b} \pm 2.05$	$98.13^{abc} \pm 3.41$	$35.37^{abc} \pm 4.90$
Ol Agl IS3	$11.26^{b} \pm 0.53$	$17.16^{abd} \pm 0.86$	$14.80^{bcd} \pm 1.58$	$26.56^{bcde} \pm 2.35$	$11.18^{bd} \pm 1.20$	$37.66^{be} \pm 1.80$	$14.54^{bde} \pm 1.86$
Ol Agl IS4	$16.08^{e} \pm 1.17$	$14.66^{ad} \pm 1.65$	$27.37^{a} \pm 1.67$	$25.60^{a} \pm 2.73$	$17.01^{b} \pm 1.46$	$57.40^{abce} \pm 2.51$	$21.38^{c} \pm 2.42$
Ol Agl IS5	$36.22^{e} \pm 5.46$	$26.87^{a} \pm 1.62$	$74.55^{abcde} \pm 3.17$	$34.01^{d} \pm 2.98$	$30.79^{b} \pm 2.56$	$52.65^{abde} \pm 2.65$	$40.63^{c} \pm 3.79$
Ol Agl IS6	$34.17^{e} \pm 2.23$	$35.92^{a} \pm 1.59$	$37.81^{b} \pm 1.90$	$35.01^{d} \pm 2.10$	$21.14^{abde} \pm 2.34$	$57.61^{abcde} \pm 1.93$	$27.48^{bc} \pm 2.99$

Table 3. (continued)

Origin	CHF	FES	MKN	OZN	SFR	TNT	TAZA
Sample no.	**15**	**21**	**36**	**18**	**19**	**32**	**15**
Phenolic compounds	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**
Lut	$4.35^{abcd} \pm 0.46$	$1.41^{a} \pm 0.05$	$2.02^{b} \pm 0.09$	$2.12^{d} \pm 0.19$	$0.83^{bcd} \pm 0.09$	$1.67^{c} \pm 0.11$	$1.00^{bd} \pm 0.11$
Methyl D-Ol Agl	$17.12^{g} \pm 1.40$	$12.22^{a} \pm 1.09$	$20.44^{ab} \pm 1.03$	$26.72^{abdfg} \pm 1.89$	$13.54^{bcd} \pm 1.12$	$21.52^{ac} \pm 1.25$	$21.27^{ade} \pm 2.11$
Lig Agl	$72.33^{bc} \pm 1.50$	$60.59^{abc} \pm 1.44$	$23.76^{abd} \pm 0.85$	$61.70b^{cd} \pm 5.01$	$18.72^{cd} \pm 1.94$	$32.71^{cd} \pm 2.22$	$65.83^{bc} \pm 8.09$
Lig Agl IS1	$20.43^{ab} \pm 0.86$	$38.35^{ad} \pm 1.09$	$48.81^{bd} \pm 1.64$	$51.79^{abcd} \pm 3.34$	$15.06^{bcd} \pm 1.58$	$29.56^{cd} \pm 1.91$	$37.23^{bcd} \pm 3.56$
Lig Agl IS2	$15.91^{d} \pm 0.90$	$15.44^{a} \pm 1.00$	$13.47^{be} \pm 0.61$	$47.81^{abcde} \pm 2.94$	$13.56^{c} \pm 1.29$	$22.99^{abc} \pm 0.81$	$19.95^{bce} \pm 1.93$
Lig Agl IS3	$15.90^{e} \pm 0.68$	$11.59^{ab} \pm 0.63$	$22.92^{abce} \pm 0.89$	$17.71^{c} \pm 1.41$	$48.61^{ace} \pm 3.00$	$79.50^{abce} \pm 3.60$	$24.70^{ab} \pm 4.60$
Apig	$2.10^{f} \pm 0.26$	$0.18^{af} \pm 0.01$	$0.38^{bf} \pm 0.03$	$0.45^{cf} \pm 0.10$	$0.03^{bf} \pm 0.01$	$0.25^{cf} \pm 0.03$	$0.11^{df} \pm 0.05$
Methyl Ol Agl	$7.04^{a} \pm 1.09$	$2.38^{acd} \pm 0.16$	$4.16^{b} \pm 0.21$	$4.83^{e} \pm 0.53$	$7.81^{abd} \pm 0.88$	$5.33^{c} \pm 0.96$	$7.30^{ab} \pm 0.74$
∑Phenolic alcohols	10.63	6.5	9.89	8.31	12.96	12.55	9.53
∑Flavonoids	6.45	1.59	2.4	2.57	0.86	1.92	1.11
∑Lignans	2.27	8.45	8.92	7.93	5.22	7.9	2.54
∑Secoiridoids	636.56	529.21	475.28	682.84	438.4	814.01	628.25

Abbreviations used within the table (alphabetical order): **Ac Pin**, (+)-1-acetoxypinoresinol; **Apig**, apigenin; **CHF,** Chefchaouane; **D-Lig Agl,** decarboxymethyl ligstroside aglycone; **DOA,** decarboxymethyl oleuropein aglycone; **EA**, elenolic acid; **Hyty**, hydroxytyrosol; **IS,** Isomer; **Lig Agl**, ligstroside aglycon; **Lut**, luteolin; **Methy D-Ol Agl**, methyl D-oleuropein aglycone; **MKN,** Meknès; **Methyl Ol Agl**, methyl oleuropein aglycone; **Ol Agl**, oleuropein aglycon; **OZN,** Ouazzane; **Pin**, (+)-pinoresinol; **p-Cou,** *p*-coumaric acid ; **SFR,** Sefrou; **Syr,** syringaresinol; **TNT**, Taounate; **Ty**, tyrosol.

-Values are shown as Mean ± SD. Significant differences within the same row are shown by different letters from [a] to [f] ($P < 0.05$).

-Quinic acid, Hyty, Ty, Pin, Lut, Apig, and p-Cou were quantified in terms of their commercial pure standards. Secoiridoids were quantified in terms of Oleuropein. Lignans were quantified in terms of Pin.

In addition, marked variations have been observed in the amounts of the other determined secoiridoids; three main compounds were identified at a relatively high concentrations: Lig Agl, with a mean concentration ranged from 18.72 mg kg^{1} in samples from Sefrou to 72.33 mg kg^{-1} in VOO from Chefchaouane; D-Lig Agl, whose lowest concentration was detected in oils from Taounate (20.89 mg kg^{-1}) and highest concentration was recorded in VOO from Chefchaouane (73.71mg kg^{-1}); and DOA with amounts ranged from 8.45 mg kg^{-1} in oils from Sefrou, to 61.37 mg kg^{-1} in oils from Taza.

These variations of the content of secoiridoids depending on the area of olive production were also observed when the isomers of Ol Agl and Lig Agl were studied. In the first case, the most abundant isomers found in the analyzed oils were isomers 2 and 5, present in concentration range varying between 14.04 mg kg^{-1} (Sefrou) and 98.13 mg kg^{-1} (Taounate) for the first one, and between 26.87 mg kg^{-1}(Fes) and 74.55 mg kg^{-1}(Meknès) for the second one. When the attention is paid to Lig Agl isomers, except for samples from both Taounate and Sefrou areas in which the isomer 3 is the predominant secoiridoid in the profiles, the isomer 1 remains the prevailing complex phenol in the other VOO samples with a mean concentration ranging between 11.59 mg kg^{-1} and 79.50 mg kg^{-1}, found, respectively, in oils from Fes and Sefrou. The other secoiridoids determined, Methyl D-Ol Agl and Methyl-Ol Agl, were detected at lower concentrations, the content of the first one ranged from 12.22 mg kg^{-1} in oils from Fes, to 26.72 mg kg^{-1} in oils from Ouazzane; whilst the mean amount of the second compound varied between 2.38 mg $kg^{-1}$and 7.81 mg kg^{-1}, quantified, respectively, in oils from Fes and Sefrou.

Beyond the dominance of secoiridoids in the phenolic fraction of north Moroccan VOO, which is reported also for the majority of olive tree cultivars (Bakhouche et al., 2013; Lerma-García et al., 2009; Ouni et al., 2011; Taamalli et al., 2012), the amounts of secoiridoids present in the *Picholine Marocaine* VOO show some features that distinguish it from other varieties. Indeed, several authors by using HPLC-MS for the characterization of the phenolic fraction of Spanish VOOs (Bakhouche et al., 2013; García Villaba et al., 2010), and Tunisian VOOs (Ouni et al., 2011; Taamalli et al., 2012), have observed that in these VOOs, the DOA content is higher than EA content, being the amounts of Ol Agl and Lig Agl low. In the case of north Moroccan VOOs the

contrary situation is observed, fact which could be tested to be used as marker for the botanical discrimination of *Picholine Marocaine* among other olive cultivars.

Lignans composed another group of phenolic compounds of considerable abundance in north Moroccan VOOs, with a total amount that vary between 6.27 mg kg^{-1} (Chefchaouane) and 46.15 mg kg^{-1} (Meknès). Within this group, three compounds have been determined: Ac Pin from 4.34 mg kg^{1} (Chefchaouane) to 31.66 mg kg^{-1} (Fès); pinoresinol with amounts found between 1.05 mg kg^{-1} in oils from Chefchaouane to 8.73 mg kg^{-1} in oils from Meknès; and Syr for which the highest mean concentration was observed in Meknès oils (5.43 mg kg^{-1}) and the lowest in oils from Chefchaouane (0.88 mg kg^{-1}).

Simple phenols represented by Hyty and Ty, are the third group in terms of abundance in north Moroccan VOOs. Mean concentration of Hyty varied between 1.54 mg kg^{-1} in oils from Fès, and 6.65 mg kg^{-1} in oils from Sefrou; whereas, values of mean amounts of Ty ranged from 4.43 mg kg^{-1} (Taza) to 7.46 mg kg^{-1} (Taounate). The concentration of Ty was higher than that of Hyty in VOOs from 4 of the studied zones (Fès, Meknès, Ouazzane and Taounate).

As far as flavonoids are concerned, two main compounds have been found, Lut and Apig. Lut, which is the most abundant flavonoid occurring in the analyzed VOOs, was found in concentration ranging from 0.83 mg kg^{-1} (Sefrou) to 4.35 mg kg^{-1} (Chefchaouane). Apig was detected in lower levels, fluctuating between 0.03mg kg^{-1} in oils from Sefrou and 2.10 mg kg^{-1} in oils from Chefchaouane.

Finally, phenolic acids, particularly *p*-Cou acid was also found. As observed in all other chemical families, the mean concentrations of phenolic acid (considering *p*-Cou) varied according to the geographical zone of origin too, with concentrations oscillating between 0.24 mg kg^{-1} and 0.37 mg kg^{-1}.

An analysis of variance (ANOVA), performed on obtained data, highlighted statistically significant differences ($p < 0.05$) in the values of 17 compounds and 9 isomers among the VOOs produced from olives grown in seven different geographical areas of the North of Morocco. Despite these differences, examination of the results obtained applying a Post-Hoc test (Tukey's) showed that there was not any compound that could classify correctly the VOO samples according to geographical origin, because the mean

values for each variable were not significantly different ($p > 0.05$) for all the seven studied regions at the same time (Table 3). Therefore, a multivariate approach was evaluated.

3.3. Multivariate analysis

Principal component analysis was applied to a matrix composed by 25 analytical parameters for 156 samples, in order to provide an overview of the capacity of phenolic compounds to discriminate VOO samples from different north Moroccan regions and to find the discriminating power of these variables. After applying PCA, six PCs were extracted because their eigenvalues were higher than 1, and they explained 71.88 % of the total variance. In Figure 4 (supplementary material) scatter plots of PC1 *vs.* PC2 (describing 39.57 % of the sample variability) and PC2 *vs.* PC3 (describing 32.28 % of the sample variability) are reported. All variables (phenolic compounds) were relevant for building the PCA model; however, only modest visual clustering is apparent in both scatter plots.

In particular, Meknès and Chefchaouane samples appeared distinguishable from the others. VOO samples from Taounate could not be separated from those coming from Sefrou; and similar result was found for samples from Fès and Ouazzane.

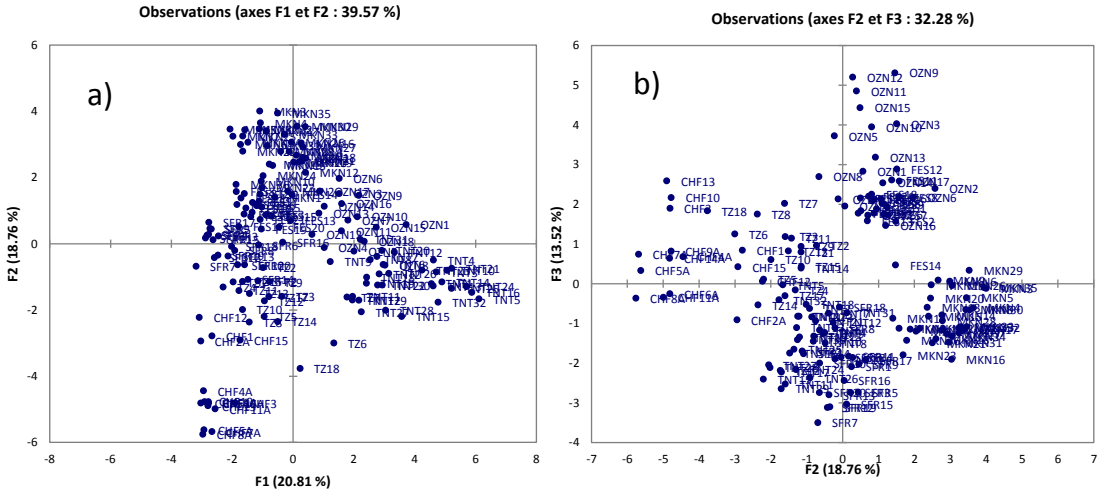

Fig. 4 (supplementary info). a) Scores of the 156 VOO samples on the map defined by the first two principal components. b) Scores of the 156 VOO samples on the plan of the second and third principal components.

Afterwards, a stepwise canonical discriminant analysis was applied to raw data without any variable reduction step. In this approach, variables which can discriminate VOO samples according to their geographical origin are introduced into the discriminant function; those variables that cannot discriminate among the studied samples are, in contrast, deleted from the discriminant function (Figure 5 (supplementary material)).

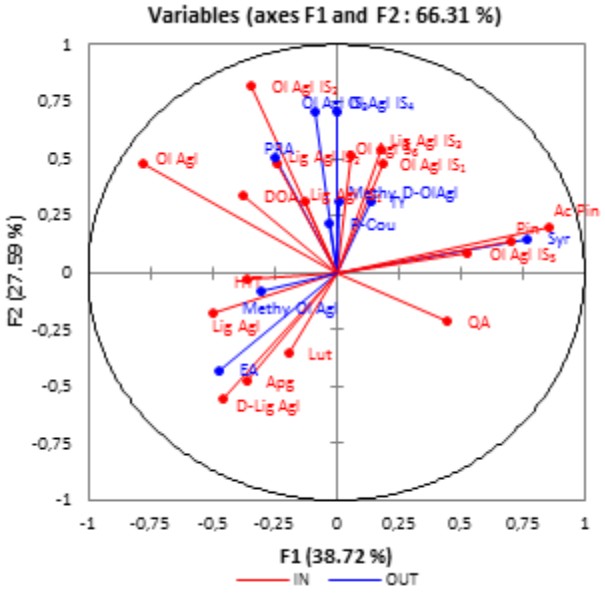

Fig. 5 (supplementary info). Stepwise variable selection.

The prediction ability was estimated, by the leave-out cross-validation method. As can be seen in Figure 5 (supplementary material), seventeen phenolic compounds (those in red) were selected as discriminating variables: eleven secoiridoids (Ol Agl and its isomers 1, 2, 5 and 6; Lig Agl and its isomers 1, 2 and 3; DOA; and D-Lig Agl); one simple phenol (Hyty); two lignans (Pin and Ac Pin); the two determined flavonoids (Lut and Apig); and quinic acid. Six discriminant functions were obtained. Table 4 shows their eigenvalues and measures of importance (canonical correlation, Wilk's lambda and Chi square coefficients).

The first four discriminant functions contain 90.53 % of the total discriminating power in this model. Canonical correlation coefficient, which is a measure of the degree of relatedness between the groups and the function, are rather higher showing values between 0.979 and 0.888, respectively, for the first and the fourth discriminant function. Indeed, Wilk's Lamda indicates the proportion of the total variance in the discriminant scores not explained by differences among groups. A low lambda value

(near 0) indicates that the group's mean discriminant scores differ. The sig ($p < 0.001$) is for the Chi-square test, which indicates a highly significant difference between the group's centroids.

Discriminating variables Ac Pin, Pin and Ol Agl contribute mainly to the first discriminant function; Ol Agl isomers 2 and 4 contribute mainly to the second discriminant function; the third discriminant function is mainly associated to Ol Agl isomer1, Lig Agl and its isomers 1 and 3, and Hyty; whereas the fourth discriminant function is mainly associated to the Ol Agl isomer 6. The other discriminate variables contribute to the two remainder functions.

Table 4. Within-groups correlations between discriminating variables and standardized canonical discriminant functions for north Moroccan VOO origin discrimination.

	F1	F2	F3	F4	F5	F6
Eigenvalue	22.571	16.086	10.393	3.73	3.12	2.4
% of variance	38.718	27.595	17.828	6.39	5.36	4.1
% Cumulative	38.718	66.313	84.141	90.5	95.9	100
Canonical correlation	0.979	0.97	0.955	0.89	0.87	0.8

Lambda de Wilks				
Test of function (s)	**Wilks' Lamda**	**Chi-square**	**df**	**Sig**
1 to 6	0	1801.36	90	0
2 to 6	0	1346.738	70	0
3 to 6	0.002	939.247	52	0
4 to 6	0.018	585.42	36	0
5 to 6	0.078	369.443	22	0

From the stepwise discriminant analysis, the group centroid was also generated for all the seven studied zones. A group centroid is the mean discriminant score for each zone. When group centroids and individual cases were plotted on the plane determined by F1 *vs.* F2; F2 *vs.* F3 and F3 *vs.* F4 (Figure 1a, 1b and 1c), the studied zones were quite distinct and their centroids were well separated.

Thus, VOOs of Chefchaouane, Meknès and Taza are well separated in the plane determined by F1 *vs.* F2, whilst VOO samples from Fès, Ouazzane, Sefrou and Taounate are well separated in the plane determined by F2 *vs.* F3.

The discriminant function used in the present study describes the optimal separation among the VOO samples coming from the main olive tree variety of the north of Morocco using the phenolic profiles, and also shows that there are significant variations between them and it is substantiated by classification accuracy of functions provided in Table 5.

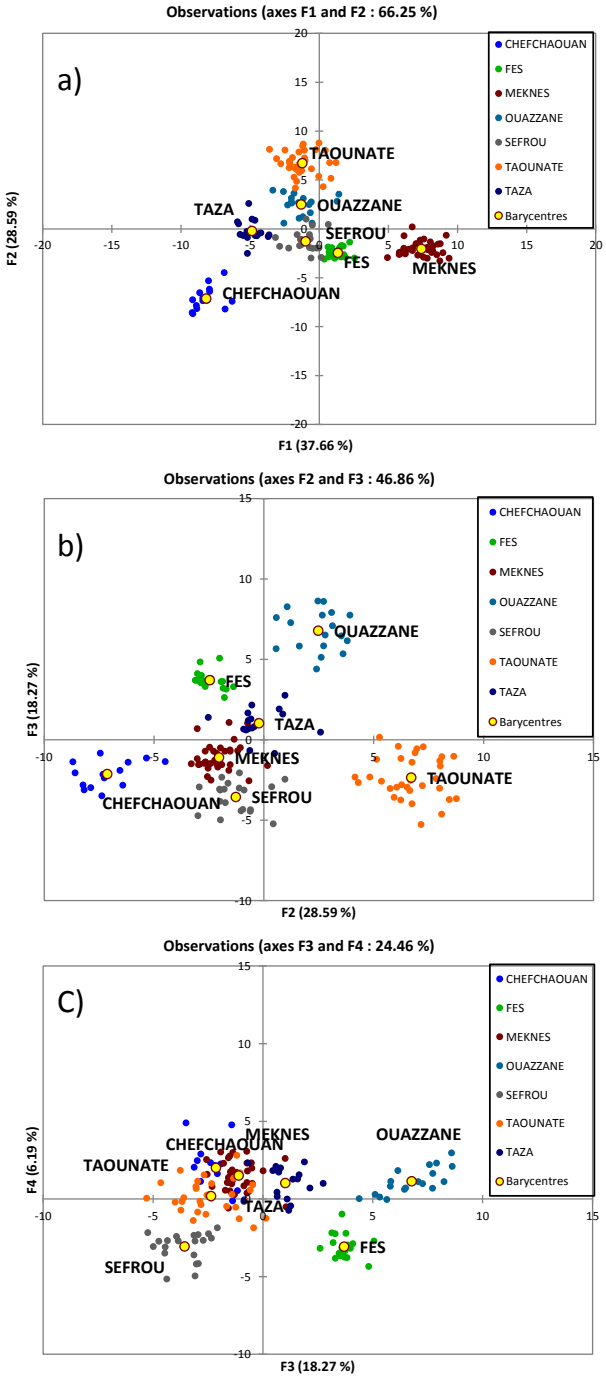

Fig. 1. Discriminant analysis similarity map obtained for the geographical classification of North Moroccan VOOs determined by: a) the two first discriminant functions; b) the 2nd and 3rd discriminant functions; c) discriminate factors 3 and 4.

Table 5. Matrix classification and cross validation of north Moroccan VOOs according to their geographic origin.

		Predicted group membreship								
		Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza	Total	% correct
Original group	Chefchaouane	15	0	0	0	0	0	0	15	100.00%
	Fès	0	21	0	0	0	0	0	21	100.00%
	Meknès	0	0	36	0	0	0	0	36	100.00%
	Ouazzane	0	0	0	18	0	0	0	18	100.00%
	Sefrou	0	0	0	0	19	0	0	19	100.00%
	Taounate	0	0	0	0	0	32	0	32	100.00%
	Taza	0	0	0	0	0	0	15	15	100.00%
	Total	15	21	36	18	19	32	15	156	100.00%
	Cross validated									
		Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza	Total	% correct
Original group	Chefchaouane	13	0	0	0	0	2	0	15	86.67%
	Fès	0	18	0	0	3	0	0	21	100.00%
	Meknès	0	0	33	0	0	3	0	36	91.67%
	Ouazzane	0	0	0	18	0	0	0	18	100.00%
	Sefrou	0	0	0	0	19	0	0	19	100.00%
	Taounate	0	0	0	0	0	32	0	32	100.00%
	Taza	1	0	0	0	0	0	14	15	93.34%
	Total	14	18	33	18	22	37	14	156	94.23%

Hence the original grouped cases correctly classified were 100 %. The leaving-one-out cross-validation procedure used to evaluate the classification performance showed a prediction ability of 94.23 % for the seven zones. Among 156 samples analyzed, only 9 samples were incorrectly classified. Three Meknès samples were classified as Taounate, three Fes samples as Sefrou, two Chefchaouane samples as Taounate and 1 Taza sample was classified as belonging to Chefchaouane zone.

4. Conclusions

In this study, an approach based on the combination of phenolic compounds profiling and multivariate analysis allowed obtaining a promising model for geographical origin classification and prediction of VOOs of seven north Moroccan regions (the most productive areas of the country).

This is the first time in which an exhaustive characterization of the phenolic composition of Moroccan oils is carried out, getting quantitative information about 25

phenolic compounds of 156 VOO samples of 7 different north Moroccan zones; this fact has allowed assessing the composition of *Picholine* Marocaine *cv.* in terms of phenolic compounds. Moreover, these data together with the use of multivariate analysis (stepwise canonical discriminant analysis) represent an appropriate tool to achieve the unbiased discrimination among VOOs from different north Moroccan zones.

Although this contribution represents the first application of this methodology for the geographical discrimination of a Moroccan VOOs, the results are quite promising. However, a more extensive set of experiments with a greater number of VOOs from other Moroccan olive cultivation zones is needed in order to establish the real value of what has been observed in this study. With this prospect it should be possible to have an analytical tool to assure the traceability of the product to its provenance and to differentiate VOOs from different areas, contributing in the development of protected designation of origin.

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Chapter 4

First comprehensive characterization of volatile profile of North Moroccan olive oils: a geographic discriminant approach

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Abstract

Herewith we have evaluated the variability of the composition in terms of volatile compounds of monovarietal "Picholine Marocaine" olive oils and checked the possible influence of their geographical origin. For this purpose, 92 olive samples were collected during the harvesting period 2012/2013 from 7 north Moroccan regions, and the analysis of the volatile profiles of the obtained oils was performed by using headspace solid-phase micro-extraction coupled to gas chromatography with flame ionization and mass spectrometry detectors (HS-SPME/GC-FID-MS). A total of 40 volatile compounds belonging to different chemical classes were identified and quantified. Significant differences in the concentration levels of volatile constituents from oils of different geographical origins were found. Furthermore, for testing the ability of the identified volatile compounds for the geographical origin discrimination of the investigated oils, a stepwise linear discriminant analysis (s-LDA) was applied. Results revealed a very satisfactory classification of the studied oils according their geographic origin.

Keywords: virgin olive oil; Picholine Marocaine; volatile compounds; geographical origin; gas chromatography.

1 Introduction

Virgin olive oil (VOO) is a highly appreciated vegetable oil all over the world. In fact, besides been the main source of fats in Mediterranean countries where olive oil production is gathered, according to data from the International Olive Council (IOC), both the olive oil production and consumption are growing considerably in areas outside the Mediterranean region, mainly in the United States, Australia, Brazil, Japan and China (IOC, 2014). In both traditional and new olive oil consumer countries, VOO is consumed as a healthy and nutritious food with peculiar and delicate flavour.

Flavour is one of the most important attributes of VOO, and it is an important factor determining its acceptability and preference by consumers (Aparicio, Morales, & García-González, 2012; Kalua et al., 2007). The flavour perception of VOO results from a complex sensory interactions between taste and aroma compounds, and olfactory and taste human receptors (Bendini & Valli, 2012). Therefore, the characterization of VOO

aroma and taste profiles has become an important issue over the last years. Within this context, the aroma profiles of various VOOs have been extensively studied (Angerosa, Basti, & Vito, 1999; Kaftan & Elmaci, 2011), and numerous volatile compounds, including aldehydes, alcohols, esters, ketones and terpenes, etc..., have been identified and described as the main aroma compounds occurring in VOO (Gomes, Freitas, Cabrita, & Garcia, 2012).

Volatile compounds are considered as potential indicators of olive oil sensory quality. Both positive and negative olive oil sensory attributes can be associated with volatile substances. In this regard, it has been stated that while endogenous enzymes such as acyl hydrolase, lipoxygenase, hydroperoxide lyase, alcohol dehydrogenase, and alcohol acyltransferase, are responsible (through the lipoxygenase pathway (LOX)) for the biosynthesis of volatile compounds so-called "green odorants" related to the perception known as "Green Odour" notes in olive oil, chemical oxidation and exogenous enzymes usually derived from microbial activity are associated with sensory defects (Angerosa et al., 2004; Angerosa, Mostallino, Basti, & Raffaella, 2000; Morales, Luna, & Aparicio, 2005).

Besides, volatile compounds have been found to play a main role in pattern recognition and classification studies, and are recognised as relevant VOO geographical and botanical markers (Cajka et al., 2010; Haddada et al., 2007; Luna, Morales, & Aparicio, 2006; Pizarro, Rodríguez-Tecedor, Pérez-del-Notario, & González-Sáiz, 2011; Pouliarekou et al., 2011). As a matter of fact, even if the composition and concentration of volatile compounds in olive oil are regulated by the enzymatic store which is genetically determined, they remain strongly affected by botanic origin (cultivar) as well as geographic origin factors such as climate, soil type, ripeness of the processed fruits and processing method (in particular, the malaxation time and temperature) (Aparicio & Morales, 1998; Brkić Bubola, Koprivnjak, & Sladonja, 2012; Kiralan et al., 2012; Sánchez-Ortiz, Romero-Segura, Sanz, & Pérez, 2012; Servili et al., 2008).

In Morocco, olive tree (*Olea europaea* L.) cultivation is a long and millenary tradition (Lenoir & Akerraz, 1984). Its favourable pedoclimatic conditions and the high adaptability of its main olive standard variety *"Picholine marocaine"* to different bioclimatic stages (Essadki, Ouazzani, Lumaret, & Moumni, 2006) contribute to the production of

VOO with high quality. Therefore, the olive oil sector plays a very important role in the agricultural development policy pursued by Moroccan government over the last years. This strategy set (so called "Green Morocco Plan") includes, among others, olive area extension, improvement of farming practices and olive oil processing, storage and bottling techniques and olive oil chain value restructuration (MAPM, Moroccan Ministry of Agriculture and Marine Fisheries, 2013). In addition, to increase profitability and competitiveness of Moroccan VOOs in a growing and globalized olive oil market, the adopted development plan promotes and supports the establishment of geographical designations.

Even though the Moroccan legislative framework for the establishment and protection of geographical designations was adopted in 2008, there is still a lot of work to be done to develop methodological approaches to prove distinctiveness of Moroccan VOOs according to their geographical origin. Very few works can be cited to illustrate this point (Bajoub, Carrasco-Pancorbo, Ajal, Ouazzani, & Fernández-Gutiérrez, 2014; Haddi et al., 2011, 2013; Houlali et al., 2014), but they open up great expectations for testing the suitability of some olive oil compounds to be used as geographical markers of Moroccan VOOs.

The research presented herein constitutes a part of a pluriannual research program carried out by our group, aimed at evaluating the potential of using various olive oil compounds and multivariate analysis to discriminate north Moroccan virgin olive oils according to their geographical origin. It was designed to, firstly, determine the aroma profile of olive oils obtained from olive fruits of the standard variety *"Picholine marocaine"* grown in seven north Moroccan regions and, secondly, to evaluate the ability of the identified volatile compounds to trace the geographical origin of investigated olive oil samples. To the best of our knowledge, this is the first detailed and comprehensive characterization study of volatile compounds (evaluating their use for the geographical classification too) of Moroccan VOOs. Only a recent work characterizing the effect of storage on volatile compounds of eastern Moroccan olive oils (Tanouti, Serghini-Caid, Sindic, Wathelet, & Elamrani, 2012) can be found in the scientific literature.

2 Materials and methods

2.1 Sampling

A total of 92 *"Picholine marocaine" cv.* homogeneous olive fruits samples were collected, over the crop season 2012/2013, from seven north Moroccan olive growing areas: Chefchaouane, Fès, Meknès, Ouazzane, Sefrou, Taounate, and Taza. Only fresh and healthy olive fruits were hand-harvested, at breast height and circumference of olive trees randomly selected in representative olive orchards of the studied regions. Selected olive trees were mature trees (more than 30 year-old), spaced at 10 m × 10 m and conducted under non-irrigation conditions, which corresponds to the typical traditional olive orchards profile dominant in Morocco. The olive fruit samples (approximately one sample is 35 kg of olives) were collected from early November until mid-January, placed in rectangular plastic crates and immediately transported to the laboratory. At each sampling date, ripeness stage of collected olive fruits was determined according to the method proposed by the Agronomic Station of Mengíbar (Jaén) (Uceda & Frías, 1975), relying on assessment of the extent of the colour of olive fruit epidermis and mesocarp. Oil extraction was performed using an Oliomio laboratory mill (Oliomio, Italy) within 24 h from harvest. This system reproduces at laboratory scale the industrial process: olives were washed to eliminate any foreign material and poured into the receiving hopper, where a screw feeds the crusher that is equipped with fix hole grid and groove knives impeller at a temperature of 25-27 °C. The paste produced falls into the malaxing part; malaxing was carried out for 45 min at 28-30 °C. The resulting olive paste was decanted at temperature of 23-27 °C without the addition of water and finally, olive oil was centrifuged. The obtained oils were filtered and stored in amber glass bottles at 4 °C in darkness without headspace until analysis.

2.2 Physico-chemical quality criteria

Free fatty acids (expressed as percentage of oleic acid (%)), peroxide value (expressed as milliequivalents of active oxygen per kilogram of oil (meq O_2/kg)), and K_{232} and K_{270} extinction coefficients (calculated from absorption at 232 and 270 nm, respectively) were measured in accordance with the European Official Methods described in the European Union Standard Methods Regulations 2568/91 and the subsequent amendments (EC, European Community Commission, 1991). All parameters were determined in triplicate for each sample.

2.3 Volatile compounds analysis

Volatile compounds in north Moroccan olive oil samples were analyzed, in duplicate, by HS-SPME. The conditions associated with this extraction procedure were selected taking into account the results reported in previous research involving the analysis of VOO's volatile compounds (Cavalli, Fernandez, Lizzani-Cuvelier, & Loiseau, 2003; Sánchez-Ortiz et al., 2012; Vichi, Castellote, Pizzale, et al., 2003). Sections from 2.3.1 to 2.3.4 describe the chemical and reagents, sample preparation protocol, and the methodology used for the analysis, as well asthe approach to identify and quantify the volatile compounds.

2.3.1 Chemicals and reagents

Chemical standards of (E)-2-pentenal, (E)-hex-2-en-1-yl acetate, (E)-hex-2-enal, (E)-hex-2-enol, (E)-hex-3-enol, (E)-pent-2-en-1-ol, (Z)-3-hexen-1-ol acetate, (Z)-hex-2-enol, (Z)-hex-3-enol, (Z)-pent-2-en-1-ol, 1-pentanol, 2-methyl-butanal, 3-methyl-butanol, acetic acid, ethyl acetate, ethyl hexanoate, hexan-1-ol, hexanal, methyl acetate, methyl hexanoate, nonanal, ocimene, pent-1-en-3-ol, pentan-2-one, and pentanal were purchased from Sigma Aldrich (St. Louis, MO, USA). All standards were of high-purity grade. Volatile compounds standard stock solutions were first prepared by dissolving the appropriate amount of these compounds in redeodorized high-oleic sunflower oil and then diluted to working concentrations. All solutions were stored in dark flask at -20 °C.

2.3.2 Headspace solid phase microextraction (HS-SPME) procedure

Olive oil samples were tempered at room temperature. Then, 1.000 ± 0.001 g of each oil sample were weighted and placed in a 10 mL glass vial obtained from Agilent Technologies (Palo Alto, CA, USA), tightly capped with polytetrafluoroethylene (PTFE) septum, and left for 10 min at 40 °C on a heating magnetic platform agitation to allow the equilibration of the volatile compounds in the headspace. After reaching the equilibration, volatile substances were adsorbed using a Supelco fibre holder (Bellefonte, PA, USA) and a 50/30 μm divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS) fibre, preconditioned according to manufacturer's instructions. An exposition of 50 min with continued heating (40 °C) and agitation was applied for the adsorption of the volatile compounds existing in the headspace. Desorption of volatile compounds trapped in the SPME fibre was performed directly into the gas chromatograph (GC) injector port equipped with a 0.75mm i.d. inlet liner for 1 min at 250°C and operated

in splitless mode. The type of fibre and the extraction conditions of volatile compounds were selected according to the previously published results by Vichi et al., 2003.

2.3.3 GC-FID and GC-Q MS analyses

Two different GC platforms were used within the current study. Chromatographic analyses were performed using an Agilent HP 6890 GC instrument with a flame ionization detector (FID), and equipped with a SupelcoWax® 10 capillary column (30 m x 0.25 mm, 0,25 µm, Sigma-Aldrich Co. LLC). Thermal desorption of volatile compounds trapped in the SPME fibre was conducted directly into the GC injector. Helium was used as carrier gas at a flow rate of 0.9 ml/min, and the desorption temperature program included the following settings: the injector and detector temperature 250 °C; column held for 5 min at 40 °C and then ramped at 4 °C/min to 200 °C and held for 5 min. The temperature and time were automatically controlled by a Combipal (CTC Analytics AG, Zwingen, Switzerland) using the software Workstation version 5.5.2 (Varian, Walnut Creek, CA).

A gas chromatography-quadrupole mass spectrometry system (GC-Q-MS) was also used and operated at identical chromatographic conditions than the GC-FID platform. It was a Thermo Scientific ISQ single quadrupole GC-MS system (Waltham, MA). The mass spectrometer operated in the electron impact mode (EI) at 70 eV scanning within the range 29-250 *m/z* in a 7 scans/s, in full scan acquisition mode. The temperature of the ion source and the transfer line were 170 and 280°C, respectively. Chromatograms and mass spectra were recorded and processed using the Thermo Xcalibur software (Thermo Fisher Scientific, San Jose, California, USA).

2.3.4 Identification and quantification of volatile compounds

The identification of volatile compounds was achieved by comparing their MS spectra, MS fragmentation patterns and retention time with those of the pure standards analyzed under the same conditions. Table 1-supplementary material shows the calibration curves, squared adjusted regression coefficient (R2) and m/z Identification ions (Ii) corresponding to the volatile compounds used for quantitative purposes. The unknown peaks were tentatively identified by comparing their MS spectra with reference spectra of compounds of different libraries (resemblance percentage above 80%). Search was mainly

made in the following GC-MS libraries: NIST 2011 EI Mass Spectral Library, and Wiley Registry™ 2007 Edition Mass Spectral Library.

Table 1 supplementary. Calibration curves, squared adjusted regression coefficient (R2) and m/z Identification ions (Ii) corresponding to the volatile compounds used in the method for quantitative purposes.

Volatile compounds	Calibration curve*	R^2	Ii
Pentanal	y = 44293x + 45240	0.998	44/58/41
Pentan-2-one	y = 87931x + 42594	0.993	55/84
Hexanal	y = 19183x + 1844	0.999	44/56/41
(E)-2-pentenal	y = 68569x + 23930	0.991	55/84/83
Pent-1-en-3-ol	y = 32220x + 29448	0.969	57/41/43
(E)-hex-2-enal	y = 60844x + 48898	0.991	41/39/55
Ocimene	y = 15584x - 384	0.999	93/121/79
1-pentanol	y = 51416x + 6392	0.99	42/55/70
(Z)-3-hexen-1-ol acetate	y = 24757x + 5261	0.998	43/67/82
(E)-pent-2-en-1-ol	y = 28644x + 9398	0.987	57/41/68
(Z)-pent-2-en-1-ol	y = 50794x + 15244	0.995	57/41/68
(E)-hex-2-en-1-yl acetate	y = 16925x + 2677	0.998	43/67/82
Hexan-1-ol	y = 33581x + 10592	0.992	43/56/69
(E)-hex-3-enol	y = 23162x + 13578	0.983	41/67/82
(Z)-hex-3-enol	y = 43813x + 9730	0.955	41/67/82
(E)-hex-2-enol	y = 31695x + 16675	0.99	41/67/82
(Z)-hex-2-enol	y=33581x+10592	0.992	57/41/82
3-methyl-butanol	y = 56334x + 16977	0.994	44/43/58
2-methyl-butanal	y = 28536x + 11202	0.9924	44/43/58
Nonanal	y = 12034x - 2702.5	0.9954	43/57/70
Methyl acetate	y = 23424x - 4528.5	0.9944	43/74
Ethyl acetate	y = 63616x + 73072	0.9999	43/70/84
Methyl hexanoate	y = 68323x + 36036	0.991	74/87/43
Ethyl hexanoate	y = 33691x + 3054.5	0.9965	88/43/99
Acetic acid	y = 10437x - 11385	0.9994	43/45/60

** The response factors used to quantify volatile compounds with no available pure standards were: (E)-2-pentenal for (Z)-2-pentenal; Pent-1-en-3-ol for 1-penten-3-ol; (E)-hex-2-enal for (E)-hex-3-enal, (Z)-hex-3-enal and (Z)-hex-2-enal; Ocimene for penten dimers (3-ethyl-1,5-octadiene IS1-IS7) and limonene; 2-methyl-butanal for 3-methyl-butanal and (E)-hex-2-en-1-yl acetate for hexyl acetate.*

Quantitative data of the identified volatile compounds were obtained using their integrated peak area from the Workstation V5.5.2 software (Varian, Walnut Creek, CA),

and interpolating the value on the corresponding calibration curve according to the validated method by Vichi et al. 2003 for the analysis of virgin olive oil volatiles (Table 1 supplementary material). The concentration of those compounds for which the pure standard was not available was calculated by using the calibration curves of another compound with a similar chemical structure, according to the formula and chemical properties.

2.4 Statistical analysis

One-way analysis of variance (ANOVA) was applied on variables studied to detect any significant differences between samples. Before applying ANOVA, Shapiro-Wilk's and Levene's tests were used to test the normality and homogeneity of variances respectively. Tukey's test was then carried out to detect significant differences ($p < 0.05$) between mean values. The SPSS statistical package software (SPSS for Windows, Version 20, SPSS Inc., and Chicago, USA) was used to carry out the univariate statistical analysis. Furthermore, multivariate analysis was performed on the data set composed by the concentration values (mg/kg) of the identified volatile compounds (variables) for the 92 investigated oil samples (objects) under study. First of all, a Principal component analysis (PCA), as an exploratory data analysis method, was used to visualise any possible grouping of samples according to the geographical origins. Then, a stepwise linear discriminant analysis (s-LDA) was applied for the geographical discrimination. LDA is, actually, one of the most widely used classification techniques. This method makes easier the class separation by maximising the ratio of the between-class variance to the within-class variance or the ratio of the overall variance to the within-class variance. The analysis was performed by setting p-in and p-out values at 0.05 and 0.10, respectively, with the same weighing of each group to choose effective variables and to decrease the number of variables for classification functions. Then, the probabilities of correct classification were estimated using leave-out cross-validation method.

Multivariate analysis was performed using a demo version of Microsoft® Excel 2013/XLSTAT© (Version 2013.2, Addinsoft, Inc., Brooklyn, NY, USA).

3 Results and discussions

3.1 Olives ripening index and physicochemical quality criteria values

The ripening index (RI), free fatty acids (FFAs), peroxide value (PV), and K232 and K270 values for the north Moroccan olive oil samples studied are given in Table 1. K_{270} values for the north Moroccan olive oil samples studied are given in Table 1.

Table 1. Mean values and standard deviations (SD) of ripening index (RI) and the regulated physicochemical quality parameters (Free fatty acids (FFAs), Peroxide value (PV) and extinction coefficients K270 and K 232) in the north Moroccan olive oil samples under study.

	IR	FA (%)	PV (meqO2/kg)	K232	K270	Categories
	Mean± S.D.					Virgin-extra
Chefchaouane (n=5)	3.31^{a}±0.43	0.27^{ab}±0.10	4.40^{a}±0.42	1.54^{ab}±0.16	0.15^{a}±0.04	5
Fès (n=17)	3.33^{a}±0.44	0.31^{a}±0.08	4.39^{a}±0.66	1.15^{b}±0.42	0.12^{b}±0.03	17
Meknès (n=21)	3.25^{a}±0.61	0.20^{b}±0.07	3.99^{a}±0.43	1.60^{a}±0.25	0.13^{a}±0.03	21
Ouazzane (n=8)	3.25^{a}±0.48	0.30^{ab}±0.13	3.83^{a}±0.70	1.60^{a}±0.17	0.12^{ab}±0.02	8
Sefrou (n=16)	3.43^{a}±0.77	0.31^{a}±0.12	4.18^{a}±0.47	1.62^{a}±0.22	0.13^{a}±0.03	16
Taounate (n=13)	3.16^{a}±0.47	0.31^{a}±0.12	4.07^{a}±0.60	1.40^{ab}±0.29	0.11^{ab}±0.03	13
Taza (n=12)	3.54^{a}±0.65	0.24^{ab}±0.05	4.21^{a}±0.62	1.32^{ab}±0.38	0.11^{ab}±0.04	12
						Total = 92

- Significant differences in the same row are indicated with different superscript letters (comparison among regions, $p< 0.05$).

From a physiological point of view, olive fruit ripening is a complex process which implies a large number of physiological and metabolic variations taking place (Conde, Delrot, & Geros, 2008; Dağdelen, Tümen, Ozcan, & Dündar, 2013). All of these changes can affect to a great extend olive oil quality, stability and composition (Baccouri et al., 2008) . Thus, to avoid possible effects of this parameter on the volatile fraction of the studied oils, olive fruits samples were carefully taken at very similar maturation stages in all the regions considered in this study. Therefore, as can be seen in Table 1, the ripening index was practically unaffected by the olive cultivation region, showing values within the range from 3.16 (Taounate) to 3.54 (Taza).

As far as quality criteria are concerned, in all the analyzed samples, these parameters were within the established range for "Extra Virgin" (EC, 1991) (Table 1). Indeed, in all the samples, the mean value of free fatty acids content was lower than the upper limit of 0.8% established for Extra Virgin olive oil category, ranging from 0.20 up to 0.31%. Besides, the overall ranges observed for the mean values of peroxide value

(from 3.83 up to 4.40 meq O_2/kg), K_{232} (from 1.15 up to 1.62) and K_{270} peroxide (from 0.11 up to 0.15) were lower than 20 meq O_2/kg, 2.50 and 0.22, respectively, which are the legal limits established for Extra Virgin olive oil category (EC, 1991).

3.2 Analysis of volatile compounds

All the volatiles compounds determined in this study were previously identified as important VOO aroma compounds and investigated for their sensory significance and their odour thresholds were determined (Kesen, Kelebek, Sen, Ulas, & Selli, 2013; Luna et al., 2006; Morales, Rios, & Aparicio, 1997; Peres et al., 2013). As reported by the cited authors, some volatile compounds identified in our samples were found to contribute to the green odour notes characteristic of aroma of high quality VOO, whereas other compounds were reported as responsible of unpleasant aroma odours. Thus, the identified volatile compounds were classified into two categories: on-flavour and off-flavour compounds.

Figure 1 displays the total amount (mg/kg) and the general composition (%) of the volatile fraction determined in north Moroccan VOOs.

As shown in this Figure, concentrations of the total volatile compounds varied within the range 29.41 to 40.41 mg/kg. VOOs from Ouazzane region showed the highest amounts of aroma compounds, whilst Sefrou VOOs had the lowest one. Furthermore, 75.92 to 86.98% of this total amounts were represented by on-flavor compounds. The latter category involves different classes of chemical compounds, including aldehydes (18.65-33.80%), alcohols (15.11-21.83%), esters (1.96-3.46%), seven isomeric unsaturated hydrocarbons known as pentene dimers (14.06-31.86%), and ketones (1.19-2.97%). Furthermore, others minor on-flavour compounds (limonene and ocimene) were found in the analyzed oils within the range of 3.34-8.68%. The occurrence of each one of these chemical families varied amongst the studied samples according to their geographical origin, suggesting possible effect of this factor on the volatile fraction of north Moroccan VOOs (Figure 1). Furthermore, when the global composition of volatile fraction of the samples under study was compared, considering their geographical origin, the major difference observed was that the most abundant

chemical class was the aldehydes family except for olive oil samples from Fès, Meknès and Sefrou regions, where hydrocarbons (pentene dimers) were predominant.

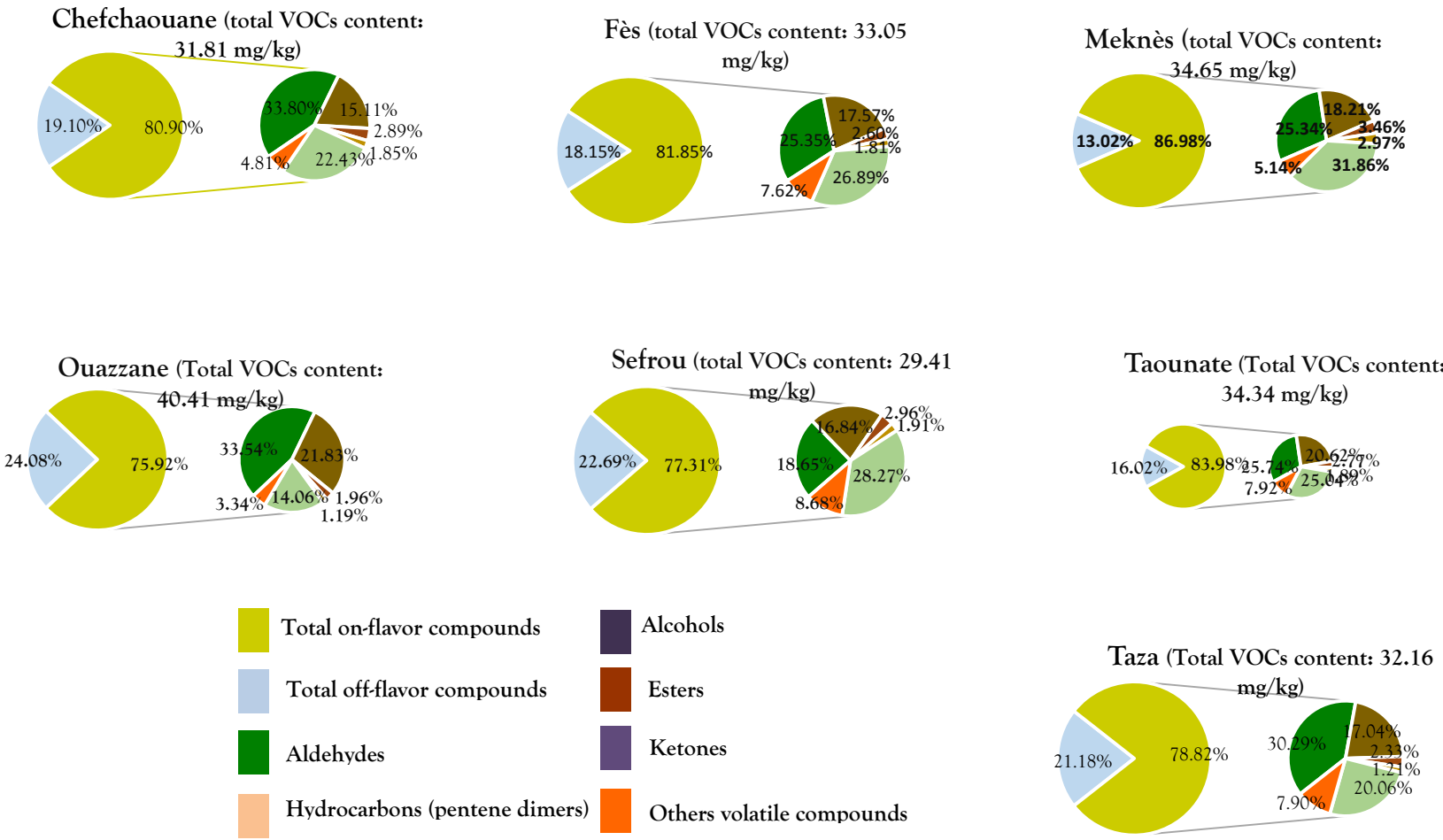

Figure 1: Total volatile compounds content (mg/kg) in studied olive oil samples and contribution (%) of different chemical groups of volatile compounds to this total amount according to the region of provenance.

3.2.1 On-flavour volatile compounds

The products of the lipoxygenase pathway, including C6 and C5 volatile compounds were the most abundant on-flavour volatile compounds detected in the studied samples regardless of their geographical origin (Table 2 and 3). These compounds are considered as the most characteristic volatile compounds in high quality VOOs (Angerosa et al., 2004; Vichi, Pizzale, Conte, Buxaderas, & López-Tamames, 2003). Other on-flavour compounds present at relatively low concentrations were limonene (0.06-0.27 mg/kg) and ocimene (1.46-2.65 mg/kg).

In all the analyzed oils (with the exception of the olive oils from Meknès region that showed high content of pentene dimers compounds), C6 compounds, including aldehydes, alcohols and esters, were the major LOX aroma components (Table 2 and 3). Indeed, as shown in Table 2, the total content of C6 compounds in north Moroccan VOOs varied between 10.34 and 22.53 mg/kg, in oils from Sefrou and Ouazzane regions, respectively. Among the C6 compounds group, aldehydes were the most abundant. They accounted for 52.69%, 58.20%, 58.53%, 60.19%, 61.63%, 62.29%, and 66.60% of the whole C6 fractions of Sefrou, Fes, Ouazzane, Taounate, Taza, and Meknès, respectively. It is also noteworthy that among the five C6 aldehydes compounds found in the studied oils, (E)-hex-2-enal was by far the most abundant one, showing mean concentrations values in the range from 3.58 to 12.04 mg/kg. The other C6 aldehydes identified in analyzed oils were hexanal (0.67-1.52 mg/kg), (E)-hex-3-enal (0.21-0.74 mg/kg), (Z)-hex-3-enal (0.16-1.00 mg/kg), and (Z)-hex-2-enal (0.39-0.62 mg/kg). In addition, five alcohol compounds were detected in the evaluated samples.

Table 2. C6 lipoxygenase pathway (LOX) volatile compounds (VOCs) composition and content (mean values and standard deviations (SD) in mg/kg) for the studied olive oil samples.

			Chefchaouane	Fès	Mèknes	Ouazzane	Sefrou	Taounate	Taza
C6 LOX VOCs	Hexanal	Mean ± S.D.	0.96^{abc}±0.41	1.12^{ac}±0.41	1.52^{bc}±0.46	0.67^{a}±0.41	0.71^{a}±0.30	1.28^{c}±0.28	0.71^{a}±0.40
	(E)-hex-3-enal		0.53^{abd}±0.26	0.33^{ac}±0.13	0.51^{b}±0.13	0.21^{c}±0.06	0.26^{c}±0.07	0.74^{d}±0.27	0.33^{ac}±0.19
	(Z)-hex-3-enal		0.65^{ab}±0.47	0.47^{a}±0.31	1.00^{b}±0.45	0.16^{a}±0.16	0.20^{a}±0.16	0.52^{a}±0.27	0.17^{a}±0.16
	(Z)-hex-2-enal		0.49^{ab}±0.16	0.46^{a}±0.13	0.62^{b}±0.19	0.39^{a}±0.09	0.61^{b}±0.15	0.55^{ab}±0.17	0.41^{a}±0.13
	(E)-hex-2-enal		8.04^{abc}±3.27	5.90^{ac}±3.09	4.97^{ac}±2.85	12.04^{b}±5.86	3.58^{c}±1.02	5.64^{ac}±2.20	8.04^{ab}±3.75
	∑ C_6 aldehydes		10.67^{ab}±2.45	8.28^{ac}±2.62	8.62^{a}±2.60	13.47^{b}±5.27	5.36^{c}±0.91	8.74^{a}±1.93	9.67^{ab}±3.70
	Hexan-1-ol		0.99^{a}±0.79	1.34^{ab}±0.98	0.78^{a}±0.56	2.23^{b}±0.90	1.25^{a}±0.57	0.82^{a}±0.39	1.28^{ab}±0.61
	(E)-hex-3-enol		0.06^{a}±0.03	0.06^{a}±0.02	0.07^{a}±0.06	0.07^{a}±0.04	0.05^{a}±0.01	0.09^{a}±0.05	0.04^{a}±0.01
	(Z)-hex-3-enol		2.23^{a}±0.76	2.22^{a}±0.71	2.63^{a}±1.94	3.03^{a}±2.56	1.90^{a}±0.68	3.19^{a}±1.73	1.83^{a}±0.82
	(E)-hex-2-enol		0.94^{ac}±0.70	1.41^{be}±1.49	0.42^{c}±0.22	2.81^{d}±1.44	0.77^{ce}±0.70	0.57^{ace}±0.20	1.47^{ace}±1.29
	(Z)-hex-2-enol		0.20^{abcd}±0.09	0.18^{abcd}±0.06	0.30^{b}±0.07	0.13^{a}±0.04	0.15^{c}±0.06	0.24^{d}±0.07	0.12^{a}±0.05
	∑ C_6 alcohols		4.43^{ab}±1.52	5.21^{a}±2.70	4.20^{a}±2.57	8.27^{b}±2.59	4.11^{a}±1.76	4.92^{a}±2.25	4.73^{a}±2.02
	Hexyl acetate		0.05^{ab}±0.03	0.05^{ab}±0.02	0.05^{b}±0.02	0.07^{abc}±0.02	0.11^{c}±0.05	0.07^{abc}±0.05	0.09^{ac}±0.03
	(Z)-3-hexen-1-ol acetate		0.84^{ab}±0.32	0.78^{a}±0.23	1.13^{b}±0.57	0.67^{a}±0.18	0.73^{a}±0.18	0.85^{ab}±0.19	0.59^{a}±0.21
	(E)-hex-2-en-1-yl acetate		0.03^{ab}±0.01	0.03^{a}±0.01	0.02^{a}±0.01	0.04^{a}±0.02	0.04^{a}±0.01	0.03^{a}±0.01	0.07^{b}±0.06
	∑ C_6 esters		0.92^{ab}±0.29	0.86^{a}±0.21	1.20^{b}±0.57	0.79^{a}±0.16	0.87^{a}±0.20	0.95^{ab}±0.19	0.75^{a}±0.23
	Total C_6 LOX VOCs		16.02^{a}±3.67	14.35^{a}±4.50	14.02^{a}±3.34	22.53^{b}±5.65	10.34^{c}±2.11	14.61^{a}±2.09	15.15^{a}±3.97
	% C_6 aldehydes/Total C6 LOX VOCs		66.60±1.77	58.20±8.86	62.29±12.05	58.53±13.85	52.69±8.32	60.19±11.7	61.63±15.69
	% C_6 alcohols/Total C6 LOX VOCs		27.05±3.78	34.95±10.3	29.1±11.49	37.49±13.31	38.69±8.73	33.06±12.31	33.22±15.68
	% C_6 esters /Total C6 LOX VOCs		6.35±3.35	6.85±3.18	8.61±2.70	3.98±2.29	8.62±2.26	6.75±2.05	5.16±1.66
	% C_6 compounds /Total LOX VOCs		65.66±11.75	57.59±9.11	49.13±7.69	75.65±11.97	51.10±5.87	56.00±5.84	66.00±5.62

- *In Tables 2, 3 and 4 significant differences in the same row are indicated with different superscript letters (comparison among regions, $p< 0.05$).*

Among them, (Z)-hex-3-enol was the most abundant one with a mean value concentration ranging between 1.83 and 3.19 mg/kg. The others C6 alcohols found in studied oils were hexan-1-ol (0.78-2.23 mg/kg), (E)-hex-3-enol (0.04-0.09 mg/kg), (E)-hex-2-enol (0.42-2.81 mg/kg), and (Z)-hex-2-enol (0.12-0.30 mg/kg).

Other C6 compounds found at relatively low concentrations were esters. This chemical class is reported to contribute to the fruity and sweet aroma of olive oil (Salas, 2004). The detected amounts of esters could be arranged in the following order: Taza (0.75 mg/kg) < Ouazzane (0.79 mg/kg) < Fes (0.86 mg/kg) < Sefrou (0.87 mg/kg) < Chefchaouane (0.92 mg/kg) < Taounate (0.95 mg/kg) < Meknès (1.20 mg/kg). This chemical class was composed by three compounds; (Z)-hex-3-enol acetate was the most abundant one, with a mean value ranging between 0.59 and 1.13 mg/kg, followed by hexyl acetate (0.11-0.05mg/kg), and (E)-hex-2-enol acetate (0.11-0.05 mg/kg).

On the other hand, Table 3 shows the composition and concentration levels of the C5 compounds. Amounts of total C5 volatile compounds followed this order: Taza (7.66 mg/kg) < Chefchaouane (8.20 mg/kg) < Sefrou (9.83 mg/kg) < Fes (10.18 mg/kg) < Taounate (11.51 mg/kg) < Meknès (14.344 mg/kg). The detection of C5 compounds indicate the presence of an additional branch of the LOX pathway when the substrate is linolenic acid (Angerosa et al., 2000).

In Table 3, it can be clearly observed that the most abundant substances within the C5 chemical class were hydrocarbons, group compose by seven isomeric unsaturated hydrocarbons of 3-ethyl-1,5-octadiene isomers, known as pentene dimers. In the evaluated samples, the total concentration levels of these compounds were within the range from 5.68 to 11.04 mg/kg, representing between 75.57 and 87.17% of the total amount of C5 volatile compounds fraction. Among the seven pentene dimers isomers identified, the isomer 6 was the most abundant one. Beyond the dominance of pentene dimers compounds in the C5 volatile compounds fraction of north Moroccan VOOs, others C5 volatile compounds found in the headspace of the selected oils were aldehydes (0.07-0.16 mg/kg), alcohols (0.38-2.16 mg/kg) and ketones (0.39-1.03 mg/kg).

3.2.2 Off-flavour volatile compounds

Table 4 includes the off-flavour compounds identified in the studied north Moroccan VOOs.

Table 3. C5 lipoxygenase pathway (LOX) other on-flavour volatile compounds (VOCs) composition and content (mean values and standard deviations (SD) in mg/kg) of the studied olive oil samples.

			Chefchaouane	Fès	Mèknes	Ouazzane	Sefrou	Taounate	Taza
C5 LOX VOCs	1-pentanol	Mean ± S.D.	0.02^{ab}±0.00	0.03^{ab}±0.01	0.03^{ab}±0.03	0.04^{ab}±0.02	0.05^{b}±0.03	0.02^{a}±0.01	0.02^{a}±0.01
	1-penten-3-ol		0.28^{a}±0.11	0.49^{a}±0.40	1.98^{b}±0.74	0.45^{a}±0.35	0.70^{a}±0.34	2.08^{b}±1.02	0.66^{a}±0.47
	(E)-pent-2-en-1-ol		0.05^{abc}±0.01	0.06^{ac}±0.01	0.08^{bc}±0.03	0.05^{ac}±0.01	0.07^{c}±0.02	0.05^{a}±0.01	0.05^{a}±0.01
	(Z)-pent-2-en-1-ol		0.02^{ab}±0.01	0.02^{ab}±0.01	0.02^{a}±0.01	0.01^{b}±0.00	0.02^{a}±0.01	0.02^{ab}±0.01	0.02^{ab}±0.01
	∑ C_5 alcohols		0.38^{a}±0.12	0.60^{a}±0.41	2.11^{b}±0.72	0.55^{a}±0.36	0.84^{a}±0.32	2.16^{b}±1.02	0.75^{a}±0.47
	Pentanal		0.01^{abc}±0.00	0.02^{abc}±0.01	0.03^{b}±0.02	0.03^{abc}±0.01	0.04^{c}±0.01	0.02^{abc}±0.01	0.01^{a}±0.00
	(Z)-2-pentenal		0.03^{ac}±0.01	0.02^{ac}±0.01	0.05^{b}±0.01	0.01^{c}±0.01	0.02^{ac}±0.01	0.03^{a}±0.00	0.02^{ac}±0.00
	(E)-2-pentenal		0.05^{a}±0.01	0.05^{a}±0.01	0.08^{c}±0.03	0.04^{a}±0.01	0.05^{a}±0.01	0.05^{a}±0.01	0.04^{a}±0.01
	∑ C_5 aldehydes		0.09^{ac}±0.02	0.10^{ac}±0.02	0.16^{b}±0.05	0.08^{ac}±0.01	0.12^{c}±0.02	0.10^{ac}±0.01	0.07^{a}±0.01
	pent-1-en-3-ol		0.50^{ac}±0.22	0.49^{ac}±0.21	0.88^{b}±0.34	0.33^{ac}±0.15	0.36^{ac}±0.20	0.58^{ac}±0.14	0.30^{a}±0.14
	pentan-2-one		0.10^{ab}±0.02	0.11^{ab}±0.03	0.16^{ab}±0.16	0.15^{ab}±0.04	0.20^{b}±0.07	0.07^{a}±0.02	0.09^{a}±0.03
	∑ C_5 ketones		0.59^{a}±0.21	0.60^{a}±0.19	1.03^{b}±0.39	0.48^{a}±0.15	0.56^{a}±0.19	0.65^{a}±0.14	0.39^{a}±0.14
	3-ethyl-1,5-octadiene IS1		0.39^{acd}±0.15	0.53^{ad}±0.11	0.71^{c}±0.16	0.40^{a}±0.17	0.67^{bcd}±0.06	0.59^{cd}±0.16	0.38^{a}±0.11
	3-ethyl-1,5-octadiene IS2		0.29^{ac}±0.12	0.33^{ac}±0.08	0.54^{b}±0.17	0.24^{ac}±0.10	0.36^{c}±0.07	0.34^{ac}±0.08	0.21^{a}±0.13
	3-ethyl-1,5-octadiene IS3		1.70^{ab}±0.77	1.81^{a}±0.60	2.53^{b}±0.74	1.16^{a}±0.69	1.58^{a}±0.55	1.94^{ab}±0.39	1.35^{a}±0.57
	3-ethyl-1,5-octadiene IS4		0.29^{ac}±0.15	0.28^{ac}±0.13	0.76^{b}±0.37	0.14^{ac}±0.08	0.41^{c}±0.30	0.15^{a}±0.04	0.15^{a}±0.03
	3-ethyl-1,5-octadiene IS5		0.70^{abc}±0.31	0.75^{abc}±0.24	0.96^{bc}±0.27	0.46^{a}±0.28	0.53^{a}±0.20	0.82^{c}±0.19	0.54^{ac}±0.26
	3-ethyl-1,5-octadiene IS6		3.27^{abc}±0.54	4.49^{b}±1.89	4.81^{c}±1.09	2.68^{a}±0.61	3.60^{ab}±1.32	4.13^{abc}±0.63	3.30^{ab}±0.74
	3-ethyl-1,5-octadiene IS7		0.51^{a}±0.04	0.70^{a}±0.26	0.73^{a}±0.12	0.60^{a}±0.14	1.16^{b}±0.45	0.63^{a}±0.05	0.64^{a}±0.29
	∑ C_5 hydrocarbons (penten dimers)		7.14^{acde}±2.05	8.89^{ad}±1.93	11.04^{b}±1.39	5.68^{ce}±1.83	8.31^{d}±1.40	8.60^{d}±1.00	6.45^{e}±1.54
	Total C_5 LOX VOCs		8.20^{abd}±2.37	10.18^{bec}±2.08	14.34^{d}±2.14	6.80^{abe}±2.33	9.83±1.70	11.51^{e}±2.05	7.66^{ad}±1.93
	% C_5alcohols/Total C5 LOX VOCs		4.60±0.61	5.83±3.17	14.45±3.91	7.53±2.21	8.41±2.27	17.93±5.87	9.58±4.46
	% C_5 aldehydes/Total C5 LOX VOCs		1.17±0.19	0.95±0.17	1.10±0.3	1.29±0.23	1.19±0.27	0.84±0.11	0.94±0.16
	% C_5 ketones/Total C5 LOX VOCs		7.10±0.89	6.04±1.79	7.15±2.26	7.23±0.44	5.67±1.77	5.66±0.57	4.98±0.92
	% C_5hydrocarobs/Total C5 LOX VOCs		87.13±1.50	87.17±3.77	77.30±2.95	83.95±1.98	84.73±3.74	75.57±5.70	84.50±4.72
	% C_5 compounds /Total C5 LOX VOCs		34.34±11.75	42.41±9.11	50.87±7.69	24.35±11.97	48.90±5.87	44.00±5.84	34.00±5.62
Other on-flavour VOCs	Limonene		0.07^{a}±0.07	0.07^{a}±0.04	0.06^{a}±0.05	0.15^{ab}±0.08	0.27^{b}±0.27	0.07^{a}±0.03	0.13^{ab}±0.07
	Ocimene		1.46^{a}±1.23	2.45^{a}±1.82	1.72^{a}±1.39	1.19^{a}±0.68	2.28^{a}±1.00	2.65^{a}±2.07	2.41^{a}±1.80
	Total other VOCs		1.53^{a}±1.27	2.52^{a}±1.83	1.78^{a}±1.40	1.35^{a}±0.69	2.55^{a}±1.10	2.72^{a}±2.07	2.54^{a}±1.83

Table 4. Off-flavour volatile compounds (VOCs) composition and content (mean values and standard deviations (SD) in mg/kg) of the olive oil samples studied.

			Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza
Off-flavour VOCs	3-methyl-1-butanol	Mean ± S.D.	0.02^{ad}±0.01	0.04^{a}±0.01	0.06^{bc}±0.03	0.02^{ad}±0.00	0.06^{b}±0.01	0.06^{c}±0.01	0.02^{d}±0.00
	∑ alcohols		0.02^{ad}±0.01	0.04^{a}±0.01	0.06^{bc}±0.03	0.02^{ad}±0.00	0.06^{b}±0.01	0.06^{c}±0.01	0.02^{d}±0.00
	2-methyl-butanal		0.01^{a}±0.00	0.02^{ac}±0.01	0.03^{ac}±0.01	0.03^{ac}±0.01	0.07^{b}±0.04	0.03^{ac}±0.01	0.04^{c}±0.01
	3-methyl-butanal		0.01^{a}±0.00	0.01^{a}±0.01	0.01^{a}±0.01	0.01^{a}±0.00	0.05^{b}±0.03	0.02^{a}±0.01	0.02^{a}±0.01
	nonanal		0.25^{ab}±0.13	0.25^{a}±0.11	0.24^{a}±0.06	0.27^{ab}±0.08	0.36^{b}±0.12	0.25^{ab}±0.10	0.23^{a}±0.07
	∑ aldehydes		0.27^{a}±0.13	0.29^{a}±0.11	0.29^{a}±0.06	0.31^{a}±0.08	0.48^{b}±0.16	0.30^{a}±0.10	0.30^{a}±0.08
	Methyl acetate		0.11^{a}±0.03	0.10^{a}±0.04	0.11^{a}±0.04	0.21^{b}±0.10	0.18^{b}±0.04	0.09^{a}±0.03	0.10^{a}±0.05
	Ethyl acetate		0.27^{cbd}±0.22	0.22^{cd}±0.12	0.13^{d}±0.08	0.63^{a}±0.41	0.46^{ab}±0.22	0.18^{cd}±0.06	0.35^{ch}±0.25
	Methyl hexanoate		0.01^{a}±0.00	0.01^{a}±0.00	0.01^{a}±0.00	0.01^{a}±0.00	0.01^{a}±0.00	0.02^{b}±0.00	0.01^{a}±0.00
	Ethyl hexanoate		2.75^{abc}±0.75	3.63^{ac}±0.92	2.86^{ab}±1.17	2.41^{b}±0.50	2.73^{ab}±0.63	3.95^{c}±0.93	3.37^{abc}±1.09
	∑ esters		3.14^{ab}±0.75	3.96^{ab}±0.94	3.11^{b}±1.17	3.26^{ab}±0.54	3.39^{ab}±0.81	4.24^{a}±0.97	3.83^{ab}±1.07
	Acetic acid		2.64^{ab}±2.39	1.72^{a}±1.01	1.06^{a}±1.27	6.15^{b}±4.48	2.75^{a}±2.06	0.90^{a}±0.38	2.66^{a}±4.43
	∑ carboxylic acids		2.64^{ab}±2.39	1.72^{a}±1.01	1.06^{a}±1.27	6.15^{b}±4.48	2.75^{a}±2.06	0.90^{a}±0.38	2.66^{a}±4.43
	Total off-flavour VOCs		6.08^{ab}±2.66	6.00^{ab}±1.85	4.51^{a}±2.28	9.73^{b}±4.87	6.67^{ab}±2.86	5.50^{a}±1.27	6.81^{ab}±4.55
	Hexanal/Nonanal		5.06±3.44	5.92±3.70	7.14±3.88	3.17±2.93	2.63±2.25	5.92±2.83	3.87±3.22
	% alcohols/Total off-flavour VOCs		0.47±0.25	0.64±0.22	1.90±1.61	0.29±0.14	1.00±0.51	1.13±0.41	0.29±0.13
	% aldehydes/Total off-flavour VOCs		4.17±0.50	4.16±1.34	6.26±2.69	3.09±1.08	5.80±1.5	4.79±2.05	4.07±1.88
	% esters/Total off-flavour VOCs		57.47±22.74	67.42±8.65	71.19±11.24	38.91±15.36	56.17±14.27	77.24±4.17	65.18±18.35
	% carboxylic acids/Total off-flavour VOCs		37.59±23.07	27.15±8.46	19.27±12.39	57.20±16.4	35.23±15.72	15.92±4.39	29.18±19.22

They cover different chemical classes, alcohols, aldehydes, carboxylic acids and esters. The total amount of this group was between 4.41 and 9.67 mg/kg. Furthermore, it was noted that esters were quantitatively found at higher concentration (3.11-4.24 mg/kg), accounting for 38.91 to 77.24 % of the total amount of off-flavour compounds fraction (Table 4).Ethyl hexanoate was the dominating ester in every case, with a mean concentration value fluctuating between 2.41 and 3.95 mg/kg. Other off-flavour ester compounds found were ethyl acetate (0.13-0.63 mg/kg), methyl acetate (0.09-0.21 mg/kg) and methyl hexanoate (0.01-0.02 mg/kg).

The carboxylic acids group, including one compound, acetic acid, was the second most abundant class. Its concentration values varied from 0.90 to 6.15 mg/kg, representing for 16.25-57.61% of the total amount of the off-flavour compounds fraction.

With regard to aldehydes class, nonanal was the aldehydic compound found at the highest concentration levels in the evaluated oils (0.23-0.36 mg/kg), whereas 2-Methyl-butanal and 3-Methyl-butanal were found at very low amounts, between 0.01-0.07 mg/kg and 0.01-0.05 mg/kg, respectively. Various studies have pointed at nonanal as a primary indicator of rancidity (Vichi, Pizzale, Conte, Buxaderas, & López-Tamames, 2003); the ratio of hexanal/nonanal has been also proposed as marker of oxidation incitement in VOOs (Kiritsakis, 1998; Morales et al., 1997). As can be observed in Table 4, the mean value of this ratio was upper the limit of 2 for all the regions, varying between 2.63 (Sefrou) and 7.14 (Meknès).

Another chemical class of off-flavour compounds was alcohols. In the studied oil samples only one alcohol (3-Methyl-1-butanol) was found, at relatively low concentration levels (0.02-0.06 mg/kg).

3.3 Statistical data analysis

In this study, the variability of the aroma fraction composition in north Moroccan VOOs was assessed by applying the ANOVA test to each identified volatile component as well as to the total content of each characterized compounds group by considering the geographical origin as factor (Table 2, 3 and 4). Accordingly, statistically significant differences ($p < 0.05$) in the values of 37 identified volatile compounds were highlighted. Only (E)-hex-3-enol, (Z)-hex-3-enol and ocimene compounds, as well as the total amount

of other minor on-flavor VOCs have not shown statistically significant differences among the studied samples according to their geographical provenance.

When statistical significant differences among the studied samples are examined, we can observe that the content values of some of the identified volatile compounds were characteristic for oils from certain regions. Indeed, the volatile composition of Meknès VOOs was rather different in comparison with the other analyzed oils in terms of the content of 5 C5 volatile compounds: (Z)-2-pentenal, (E)-2-pentenal, pent-1-en-3-ol, and 3-ethyl-1,5-octadiene IS2 (Table 3); Meknès VOOs exhibited the highest content of these compounds. Likewise, among the studied VOOs samples, oils from Sefrou region were distinguished by high content of 3-ethyl-1,5-octadiene IS7, 2-methyl-butanal and 3-methyl-butanal (Table 3 and 4). The same can be said for Ouazzane and Sefrou oil samples, as they were different from the others considering the content of methyl acetate (Table 4). Methyl hexanoate was the volatile compound distinguishing the samples from Taounate region from the other studied oils (Table 4).

On the other hand, a multivariate approach was used to test the suitability of the volatile fraction for tracing the geographical provenance of the studied oils. Thus, as a first step, the data matrix obtained by using the concentration values of the detected volatile compounds in the 92 north Moroccan oil samples was subjected to PCA, in order to explore the global effect of the geographical origin on the volatile fraction. Figure 1 (supplementary material) shows scores of samples plotted on the two first principal components (describing 45.86% of the sample variability). As can be seen in this Figure, an overlapping of the samples coming from the different areas was obtained, and no grouping of VOOs according to the geographical origin could be achieved, except for Sefrou VOO samples that appeared distinguishable from the other studied oils.

Moreover, a supervised s-LDA approach was applied to the complete data set, without applying any variable reduction. In the application of LDA, variable selection was performed by leave-out cross-validation analysis to discard redundant information and to select only those variables that actually contributed to increase of classification ability.

The results showed that six statistically significant discriminant functions are formed with the eigenvalues 69.33, 26.05, 14.08, 6.69, 3.69, and 2.09. The first five discriminant functions (F1, F2, F3, F4, and F5) account for 56.86%, 21.36%, 11.55%, 5.49%, and 3.03%, respectively. Furthermore, the most discriminating variables are selected on the basis of a Fisher's test. Thus, 21 variables revealed high discrimination ability: pentan-2-one, pentanal, pent-1-en-3-ol, 3-ethyl-1,5-octadiene IS3, (Z)-hex-3-enal, 1-penten-3-ol, and hexyl acetate contribute mainly to the first discriminant function; hexanal, (Z)-3-hexen-1-ol, acetate, (E)-hex-3-enol, (Z)-3-hexen-1-ol, and (Z)-hex-2-enol were mainly correlated to the second discriminant function; ethyl acetate, 2-methyl-butanal, and 3-methyl-butanal mainly contributed to the third discriminant function; the fourth discriminant function was mostly associated to (E)-hex-3-enal and ethyl hexanoate; whereas the fifth discriminant function was mainly associated to 3-ethyl-1,5-octadiene IS2 and 1-pentanol; and finally, (Z)-2-pentenal and (E)-pent-2-en-1-ol were principally correlated with the sixth discriminant function.

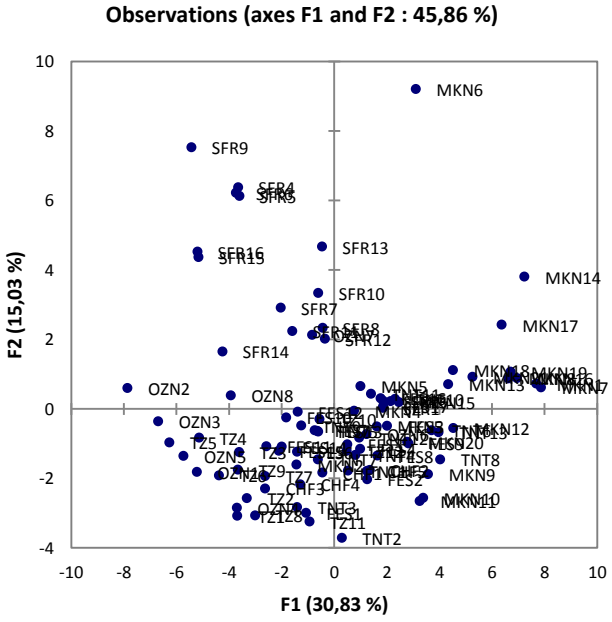

Figure 1-supplementary material: Score plot for the first two principal components of the studied north Moroccan olive oil samples.

In Figure 2, it can be observed that Chefchaouane, Fès and Taounate VOOs were clearly separated from all other regions in the plane determined by F1 *vs.* F2, whilst VOO samples from Chefchaouane, Meknès, Sefrou and Taza were properly separated in the plane determined by F2 *vs.* F3. Finally, VOOs samples from Ouazzane region were

separated from all other region in the plane determined by F4 *vs.* F5. Furthermore, as can be seen in Table 2 (supplementary material) the overall correct classification rate was of 100% for the original and 93.48% for the cross validation method, being both results very satisfactory.

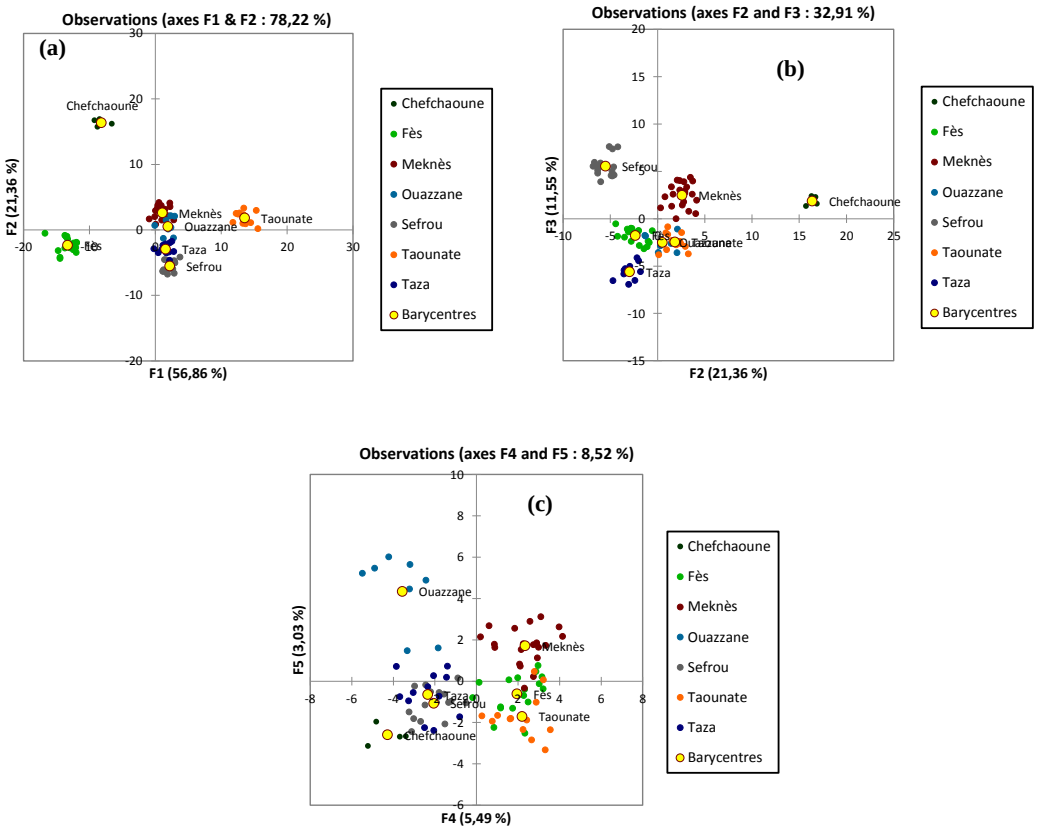

Figure 2: Discriminant analysis similarity map obtained for the geographical classification of North Moroccan VOOs determined by the discriminant functions F1, F2, F3, F4 and F5

Correct classification rate (100%) was obtained for VOO samples from Chefchaouane, Fès and Taounate, followed by oil samples from Taza (correct classification 91.67%), Meknès (correct classification 90.48%) and Ouazzane (correct classification 62.50%) (Table 2, supplementary material).

Table 2a supplementary. Within-groups correlations between discriminating variables and standardized canonical discriminant functions for north Moroccan VOO origin discrimination.

	F1	F2	F3	F4	F5	F6
Eigenvalue	69.33	26.05	14.08	6.7	3.69	2.09
% of variance	56.86	21.36	11.55	5.49	3.03	1.71
% Cumulative	56.86	78.22	89.77	95.26	98.29	100
Canonical correlation	0.99	0.98	0.97	0.93	0.89	0.82

Table 2b supplementary. Matrix classification and cross validation of north Moroccan VOOs according to their geographic origin.

		Predicted group membreship								
		Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza	Total	% correct
Original group	Chefchaouane	5	0	0	0	0	0	0	5	100.00%
	Fès	0	17	0	0	0	0	0	17	100.00%
	Meknès	0	0	21	0	0	0	0	21	100.00%
	Ouazzane	0	0	0	8	0	0	0	8	100.00%
	Sefrou	0	0	0	0	16	0	0	16	100.00%
	Taounate	0	0	0	0	0	13	0	13	100.00%
	Taza	0	0	0	0	0	0	12	12	100.00%
	Total	5	17	21	8	16	13	12	92	100.00%
		Cross validated								
		Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza	Total	% correct
Original group	Chefchaouane	5	0	0	0	0	0	0	5	100.00%
	Fès	0	17	0	0	0	0	0	17	100.00%
	Meknès	1	0	19	1	0	0	0	21	90.48%
	Ouazzane	0	0	1	5	1	0	1	8	62.50%
	Sefrou	0	0	0	0	16	0	0	16	100.00%
	Taounate	0	0	0	0	0	13	0	13	100.00%
	Taza	0	0	0	1	0	0	11	12	91.67%
	Total	6	17	20	7	17	13	12	92	93.48%

4 Conclusions

The results obtained in the current study provide a way for further research aimed at finding markers of the geographic origin of Moroccan VOOs. Indeed, this first comprehensive characterization of the volatile profiles of *"Picholine marocaine"* monovarietal VOOs produced in the main olive growing areas of North Morocco. We have provided quantitative information about 40 volatile compounds identified in the

investigated oils and demonstrate that, even if no qualitative differences were detected, geographic origin has a marked influence on the content of 37 compounds among identified volatile compounds.

Another important finding of this study was the high percentage that pentene dimers compounds represent among the C5 volatile fraction of north Moroccan VOOs. Nevertheless, considering the lack of data about the volatile composition of Moroccan VOOs in literature, it was not possible to compare our results with others previously obtained. A reliable and pluriannual characterization of the volatile profile of Moroccan VOOs should be carried out to verify whether the high content of pentene dimers isomers could be considered as a varietal marker of *"Picholine marocaine"* olive oils.

Although we have clearly demonstrated that combining data of volatile compounds composition determined by HS-SPME-GC-FID/MS technique and multivariate statistical analysis seems to be a very promising approach for geographical classification of north Moroccan VOOs, further validation studies are required to confirm these findings.

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Chapter 5

Comparing two metabolic profiling approaches (liquid chromatography and gas chromatography coupled to mass spectrometry) for extra-virgin olive oil phenolic compounds analysis: A botanical classification perspective

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Abstract

Over the last decades, the phenolic compounds from virgin olive oil (VOO) have become the subject of intensive research because of their biological activities and their influence on some of the most relevant attributes of this interesting matrix. Developing metabolic profiling approaches to determine them in monovarietal virgin olive oils could help to gain a deeper insight into olive oil phenolic compounds composition as well as to promote their use for botanical origin tracing purposes. To this end, two approaches were comparatively investigated (LC-ESI-TOF MS and GC-APCI-TOF MS) to evaluate their capacity to properly classify 25 olive oil samples belonging to five different varieties (Arbequina, Cornicabra, Hojiblanca, Frantoio and Picual), using the entire chromatographic phenolic profiles combined to chemometrics (principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA)). The application of PCA to LC-MS and GC-MS data showed the natural clustering of the samples, seeing that 2 varieties were dominating the models (Arbequina and Frantoio), suppressing any possible discrimination among the other cultivars. Afterwards, PLS-DA was used to build four different efficient predictive models for varietal classification of the samples under study. The varietal markers pointed out by each platform were compared. In general, with the exception of one GC-MS model, all exhibited proper quality parameters. The models constructed by using the LC-MS data demonstrated superior classification ability.

Keywords: monovarietal extra-virgin olive oils; phenolic compounds; liquid chromatography-electrospray ionisation-time of flight mass spectrometry; gas chromatography-atmospheric pressure chemical ionisation-time of flight mass spectrometry; varietal origin; multivariate analysis.

1. Introduction

Nowadays, the health benefits of VOO are not a hypothesis or a working concept anymore, but a fact supported by epidemiological and clinical evidences. Indeed, it has been shown that VOO consumption may reduce the risk of many diseases associated with the oxidative damage, such as coronary heart diseases and cancer [1,2]. Those effects strongly depend on VOO's composition and, in particular, on its richness in

terms of various minor compounds, including, among other, phenolic compounds, tocopherols, sterols and pigments [3]. Phenolic compounds are attracting considerable attention over the last decades. The qualitative and quantitative composition of VOO hydrophilic phenols is strongly affected by intrinsic (e.g. cultivated variety [4]), and extrinsic factors (e.g. climate, soil and geography of the olive growing area [5], harvesting time [6], and differences regarding the processing and storage conditions [7]). The variety plays the most crucial role among these factors, conditioning not only the VOO composition peculiarities, but also its sensorial properties [8]. For this reason, the production of monovarietal VOOs from certain cultivars has increased over the last few years as a response for the demand for unique high-quality olive oils with exceptional and singular sensory attributes [9], which could be an appropriate differentiation marketing strategy to facilitate the commercialisation of this product.

In Spain, the world's leading olive oil producer, amongst more than about 250 cultivated varieties, Picual, Hojiblanca, Cornicabra, and Arbequina are the most widely used cultivars for VOO production [10,11]. Frantoio -an Italian variety which was introduced in Spain mainly because of its resistance to *Verticillium dahliae* Kleb [12]- is one of the most relevant cultivars in Italy and is getting a lot of attention nowadays also in Spain. The monovarietal VOOs obtained from theses varieties are characterised by flavour and sensory features that allow distinguishing them from other VOOs [13,14].

It is known that sensory properties of VOO are resulting from complex interactions between taste and VOO's flavour compounds (phenolic and volatile compounds), and olfactory and taste human receptors [15]. Moreover, the phenolic compounds, apart from playing an important role in the overall taste properties of VOO, due to their contribution to the bitterness and pungency of this foodstuff, they are arousing a lot of interest because of their biological activities and their contribution to VOO oxidative stability [16].

Bearing in mind the importance of these compounds, it is easy to understand that there is an extensive literature concerning the detection and quantification of polyphenols in VOO. Traditional and non-specific analytical methods (paper, thin-layer and column chromatography, ultraviolet (UV) spectroscopy) were applied to phenolic compounds analysis from the very early days, but the practical value of those studies

remained limited [17]. Later on, the need to profile and identify individual phenolic compounds caused the replacement of the traditional methods by high-resolution gas chromatography (GC), capillary electrophoresis (CE) and, in particular, high performance liquid chromatography (HPLC). GC has been used coupled to different detectors (mainly flame ionisation detector (FID) [18] or mass spectrometry (MS) [19]). Different CE methodologies with UV [20] and MS as detection systems [21] have been also proposed. As far as HPLC is concerned, it is possible to find research works where photodiode array (DAD) [22], fluorescence [23], electrochemical [24], biosensors [25] and MS [26,27] detectors were used. More recently, improvements regarding chromatographic performance were achieved by the introduction of rapid-resolution LC (RRLC) [28] and ultra high-performance LC (UHPLC) [29].

Using powerful chromatographic techniques or mass spectrometry approaches and focusing on phenolic compounds, several studies have been reported concerning VOOs classification according to their cultivar and/or geographic origin [30,31]. However, to the best of our knowledge, so far, there has been no systematic cross-platform comparison where the analysis of phenolic compounds from extra-VOOs (together with the use of chemometric approaches) was used for achieving a correct classification of the samples under study based on the olive variety, identifying potential markers.

Considering all the above, and in light of the great demand for accurate, reliable and robust analytical methodologies in order to identify and authenticate the botanical origin of monovarietal VOOs, the aim of this study was to carry out a systematic comparison of the potential of two diverse metabolic profiling approaches (LC-ESI-TOF MS and GC-APCI-TOF MS) combined with statistical tools to achieve the varietal discrimination of extra-VOOs coming from 5 cultivars. The comparison was mainly done considering the discrimination power of the models built by using the LC-MS and GC-MS data, their usefulness regarding the identification of possible varietal markers, and the similarity/complementarity of the metabolites eventually identified as markers by each platform.

2. Materials and methods

The overall workflow followed to apply the metabolic profiling approaches used within the current study is shown in the scheme included in Fig. 1.

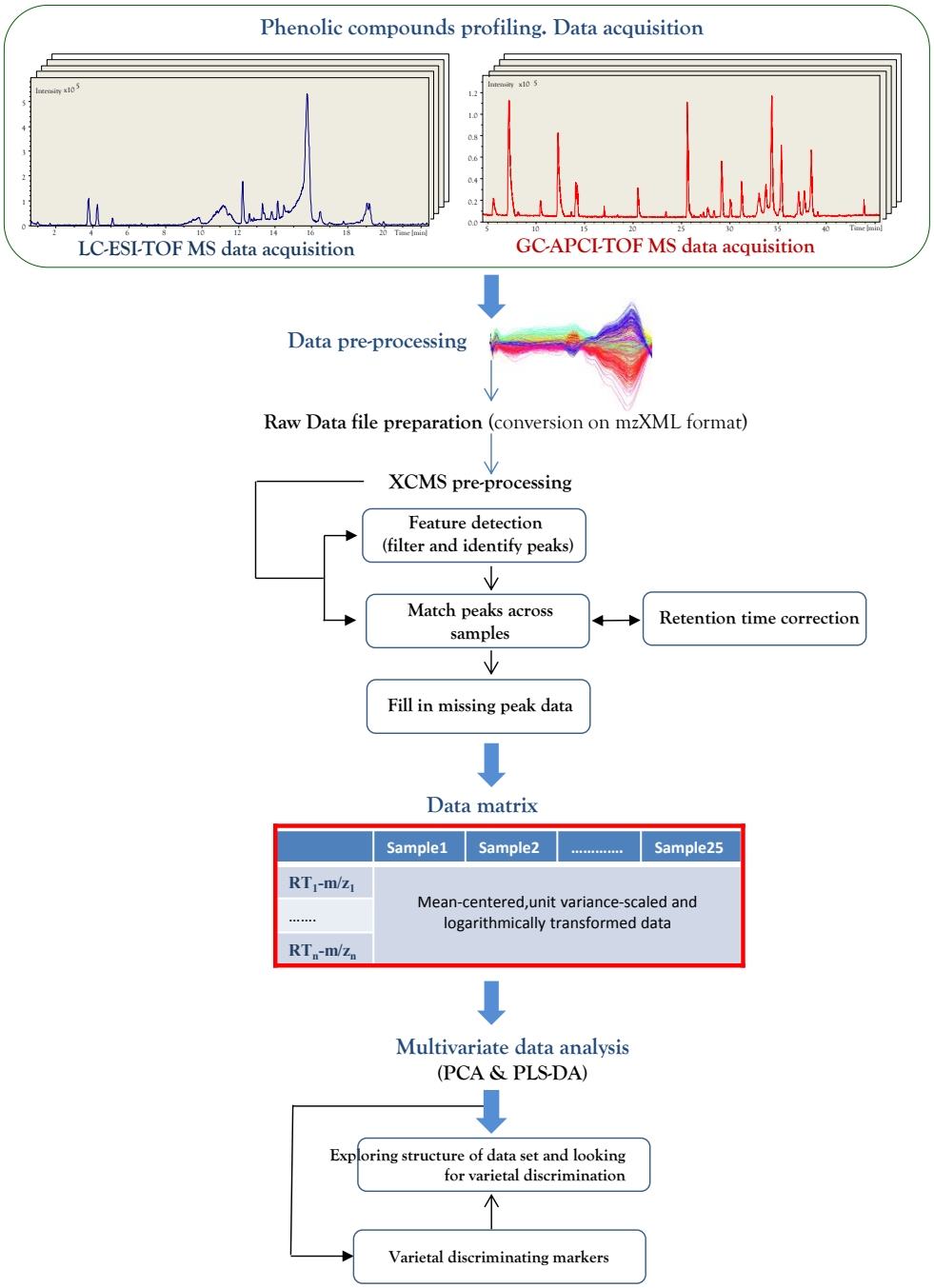

Fig. 1. Study Workflow scheme, including data acquisition, pre-processing and multivariate data analysis.

2.1. Virgin olive oil samples

A total of 25 extra-VOO samples coming from 5 different varieties (Arbequina, Cornicabra, Hojiblanca, Picual and Frantoio) were analysed. The samples were acquired from different trademarks (Arbequina (Valderrama, Castillo de Canena, Romanico,

Vea, and Oleorum Verd); Cornicabra (Arzuaga, Hipercor, and Sierra de Nambroca); Hojiblanca (Coosur, Columela, Carbonell, Selección, Auchan); Picual (Carbonell, Coosur, Oleo Cazorla, Castillo de Tabernas and Señorío de Segura); and Frantoio (Arturo Archibusacci, and Alce Nero). To complete the selection, and in order to have the same number of samples coming from each variety, 2 Cornicabra and 3 Frantoio extra-VOO samples were received from the experimental field of the Agronomic Station of Mengíbar (Jaén, Spain).

2.2. Analysis of phenolic compounds

2.2.1. Chemicals and reagents

All reagents used in this study were of analytical grade and used as received. Sinapinic, gentisic, 4-hydroxyphenylacetic, vanillic, caffeic, gallic, *trans*-cinnamic, protocatechuic, and *p*-coumaric acids, vanillin and hydroxytyrosol were purchased from Sigma-Aldrich (St. Louis, MO, USA); syringic, *m*-coumaric, 4-hydroxybezoic, homovanillic, and ferulic acids, taxifolin and tyrosol were from Fluka (Buchs, Switzerland), as well as 3,4-dihydroxyphenylacetic acid (DOPAC), which as used as internal standard (IS). Luteolin and apigenin were from Extrasynthèse (Genay, France); and pinoresinol was purchased from Arbo Nova (Turku, Finland). Stock solutions at concentration of 200 mg/L for each phenol were first prepared by dissolving the appropriate amount of the compound in methanol and then serially diluted to working concentrations. The IS was used with quality control purposes (mainly to evaluate the repeatability of the extraction system and the chromatographic methods); the other standards were basically used for quality control purposes as well, since they were injected every 4 analyses to check the evolution of the analytical systems and to detect possible shifts of retention time or slight reduction of the MS signals. They were also used for defining the linear dynamic range of both methods, making sure that the concentration of the injected extracts was within the proper range. Since secoiridoids are not available as commercial standards, we isolated them by semi-preparative HPLC [32] for qualitative purposes.

Acetonitrile, methanol, and *n*-hexane were from Sigma-Aldrich (St. Louis, MO, USA) and acetic acid from Merck (Darmstadt, Germany). Deionised and organic-

eliminated water was from the water purifier system (USFELGA from Purelab Plus, Ransbach-Baumbach, Germany). N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane from Pierce (Oud-Beyerland, The Netherlands) was used as derivatisation reagent, being taken from freshly opened 1 mL bottles.

2.2.2. Phenolic compounds extraction procedure

Phenolic compounds were isolated from the extra-VOO samples using solid phase extraction with Diol-cartridges according to the procedure previously described by Gómez-Caravaca *et al.*, [33]. Briefly, the cartridge was placed in a vacuum elution apparatus and pre-conditioned passing 10 mL of methanol and then 10 mL of *n*-hexane. 60 g of VOO were thoroughly mixed with 60 mL of hexane and carefully loaded onto the pre-conditioned column, leaving the sample on the solid phase. After a wash with hexane (15 mL) to remove the non-polar fraction of the oil, the sample was eluted with methanol (40 mL). The eluents were evaporated to dryness under reduced pressure in a rotary evaporator at 35 ºC. The dried residue was then dissolved in 2 mL of methanol. The phenolic extracts were diluted (1:10, v:v) with methanol before their chromatographic separation.

2.2.3. LC-ESI-TOF MS analyses

An Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump and a DAD was used for the chromatographic separation. Phenolic compounds were separated by using a Zorbax C18 analytical column (150 x 4.6 mm, 1.8 µm particle size) protected by a guard cartridge of the same packing, operating at 30 ºC and a flow rate of 1.5 mL/min. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B), and the solvent gradient changed according to the following conditions: 0 to 10 min, 5-30% B; 10 to 12 min, 30-33% B; 12 to 17 min, 33-38% B; 17 to 20 min, 38-50% B; 20 to 23 min, 50-95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 2 min. A volume of 10 µL of the 1:10 diluted methanolic extracts of olive oil was injected. The separated compounds were monitored in sequence first with DAD (240 and 280 nm) and, then, with a mass spectrometry detector (although only MS data were statistically treated).

The RRLC system was coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). However, as the flow rate used in the RRLC method was too high for achieving a stable electrospray ionisation (ESI), it was necessary to use a flow divisor 1:6. In that way, the flow delivered into the mass spectrometer was reduced to approx. 0.21 mL/min, low enough to avoid the introduction of humidity in the system. According to this inflow the ESI parameters were chosen: nebuliser pressure was set at 2 bar, dry gas flow at 9 L/min and dry gas temperature at 190 ºC. Other parameters of the mass spectrometer were similar to those previously optimised by our group [28], acquiring spectra in the range of 50-800 m/z in the negative mode. External mass spectrometer calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA). The calibration solution (sodium formate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 v/v) was injected at the beginning of the run, and all the spectra were calibrated prior to phenolic compounds identification. SmartFormula™ tool within DataAnalysis 4.0 (Bruker Daltonik) was used for the calculation of elemental composition of compounds; it lists and rates possible molecular formulas consistent with the accurate mass measurement and the true isotopic pattern (TIP). If the given mass accuracy leads to multiple possible formulas, the TIP adds a second dimension to the analysis, using the masses and intensities of each isotope for doing a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma Value). The smaller the sigma value and the error, the better the fit, therefore for routine screening an error of 5 ppm and a threshold sigma value of 0.05 are generally considered as appropriate.

2.2.4. Derivatisation reaction and GC-MS conditions

A speed vacuum concentrator was used for drying the proper volume of the standard mixture (depending of the desired final concentration level) and 50 µL of the VOO extracts to complete dryness. Then, 50 µL of the derivatisation agent (BSTFA plus 1% TMCS) were added to the dried sample. The solution was vortexed for 1 min and the trimethylsilylation reaction was performed at room temperature for 30 min. A

minimum of 30 min of equilibration time was required before the sample injection. The stability of BSTFA-derivatised samples, kept at ambient temperature (20-25 °C), was determined periodically by injecting replicate preparations of the processed samples consecutively for up to 48 h. Peak areas were chosen as parameter for stability evaluation.

The GC analyses were performed on an Agilent 7890A (Agilent, Palo Alto, USA) gas chromatograph equipped with an HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm) with helium as carrier gas and the injector temperature was set 250 °C. An aliquot of the derivatised samples (1 μL) was applied by splitless injection with a programmable CTC PAL multipurpose-sampler (CTC Analytics AG, Zwingen, Switzerland). Injection programs included sequential washing steps of the 10 μL syringe with *n*-hexane before and after the injection, and a sample pumping step for removal of small air bubbles.

A temperature gradient was applied for the GC-MS phenolic compounds analysis: the column temperature was initially kept at 160 °C for 5 min, from 160 °C to 188 °C at 3 ° C/min keeping that value for 1 min, from 188 °C to 241 °C at 15 °C/min, keeping that value for 1 min, from 241 °C to 282 °C at 2 °C/min, from 282 °C to 310 °C at 5 °C/min and the temperature value of 310 °C was held for 5 min. A constant flow rate of 1.0 mL/min was used. Using the described chromatographic conditions, the analysis time was about 50 min.

The GC system was coupled to an ultra-high resolution time of flight mass spectrometer maXis (UHR TOF MS, maXis, Bruker Daltonik, Bremen, Germany) using a multipurpose source equipped with a GC transfer line. The parameters of the APCI interface and all the parameters of maXis MS detector were optimised using the area of the MS signal of the phenolic compounds. The GC transfer line to the mass spectrometer was kept at 300 °C. The APCI source and MS were operated in positive mode. The pressure of the nebuliser gas (nitrogen) was set to 2 bars and temperature and flow rate of the dry gas (nitrogen) were 250°C and 5.0 L/min, respectively. The APCI vaporiser temperature was 450°C and the voltage of the corona discharge needle was 2000 nA. The analyser worked in scan mode with a mass range from 50 to 1000 at spectra rate of 1 Hz.

The instrument was externally calibrated using an APCI calibration tune mix. In addition an internal calibration using cyclic-siloxanes -a typical background in GC-MS- was used. The SmartFormula™ tool of DataAnalysis 4.0 (Bruker Daltonik) was used for the calculation of elemental composition of compounds.

2.4. Data pre-processing and multivariate data analysis

The LC-MS and GC-MS data files were exported as mzXML files and aligned by using in-house developed alignment algorithm msalign2 tool (http://www.ms-utils.org/msalign2/) [34]; peak picking was performed using XCMS package with "centWave" method (The Scripps Research Institute, La Jolla,USA). The XCMS used parameters were the default settings except for bandwidth (bw parameter) for grouping of features, which was set to 10.

The generated LC-MS and GC-MS data matrices were separately imported to SIMCA-P 13.0 software package (Umetrics, Umeå, Sweden). The data were mean centered, unit variance-scaled and Log-transformed prior to statistical analysis. Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were used for data analysis. The PLS-DA models were validated and their overfitting degree was cheeked using a 200 permutations test. Besides, the goodness of fit and predictive ability for the PLS-DA models were evaluated using several quantitative parameters, such as R2X, R2Y, and Q2. R2Y and Q2 were particularly relevant; R2Y is the percentage of the variation of the dependent variable explained by the model and Q2 is a measure of the predictive ability of the cross-validated model. These parameters range between 0 and 1; values approaching 1 indicating perfect fit of the model. Apart from these parameters, values of F and *p* -which are the parameters of the cross-validated ANOVA- were given as well. Furthermore, to pick out potential discriminating varietal markers, the corresponding scatter plots (S-plot) were generated for each PLS-DA model. The S-Plot is a kind of loading profile that visualises the influences of variables, combining two vectors *p* and *p*(corr), where *p* represents the modelled covariance (X-axis), and *p(corr)* means the modelled correlation for variables with respect to the component (Y-axis). The variables showing the highest *p* and *p(corr)* values are considered as the most relevant metabolites for achieving the discrimination between samples. Variable importance in

the projection (VIP) value was obviously calculated too; the VIP is a weighted sum of squares of the PLS loading weights taking into account the amount of explained Y-variation in each dimension.

3. Results and discussions

3.1. Chromatographic profiling and phenolic compounds identification

In the current study, LC-ESI-TOF MS and GC-APCI-TOF MS have been used to compare monovarietal VOO samples based on their phenolic profile, trying to discriminate among them. Five examples of the base peak chromatograms (BPCs) of the phenolic extracts from the varieties under study (one representative of each cultivar), for each platform, are shown in Fig. 2. Finding the identity of the detected metabolites in the profiles was, in principle, not a priority, since we were using the whole chromatographic profiles for the data treatment.

However, the advantage of using instruments such as the ones selected in this work is that TOF spectra (together with our previous knowledge and published reports) facilitate a lot the identification of a considerable number of metabolites. Table 1 and 2 include, for LC-MS and GC-MS, respectively, retention times, experimental and calculated mass to charge ratio (*m/z*), molecular formula, error (deviation between the measured mass and theoretical mass), sigma value, fragment ions, and the identities of the individual detected phenolic compounds. Fig. 2a displays the LC-MS BPCs, reporting about 30 compounds (we only numbered those appearing in all the samples under study, although some other features were detected), which can be grouped in four different chemical families: simple phenols, including hydroxytyrosol (HYTY), HYTY-acetate, oxidised-HYTY and tyrosol (TY); lignans represented by pinoresinol (Pin), acetoxypinoresinol (Ac Pin) and syringaresinol; flavonoids, with luteolin (Lut) and apigenin (Apig) as the most important metabolites of this family; and secoiridoids, with more than 20 compounds belonging to this chemical class. As far as secoiridoids are concerned, the following compounds were found within the profiles: dialdehydic form of decarboxymethyl elenolic acid (D-EA), desoxy EA, hydroxy EA, EA, hydroxy decarboxymethyl-oleuropein aglycon (hydroxy-D-Ol Agl), hydroxy D-ligstroside aglycon

(hydroxy-D-Lig Agl), 10-Hydroxy Ol Agl, D-Lig Agl, methyl D-Ol Agl, Methyl Ol Agl, Ol Agl (plus five isomers) and Lig Agl (with three different isomers).

In general, the profiles for samples coming from different varieties were quite similar from the qualitative point of view. Even though, as stated before, the used approaches did not have absolute quantitative purposes, the relative area values achieved by LC-MS and GC-MS profiles could allow the comparison of the extra-VOOs under study (in terms of relative areas), establishing those which showed the highest/lowest area values (and therefore, the highest/lowest concentration levels, if absolute quantification would be carried out. Although the analytical figures of merit are not included herewith, we obviously built calibration curves (to assure that all the analyses were made within the linear dynamic range) and a proper validation was carried out (to verify that the repeatability and reproducibility values were good enough).

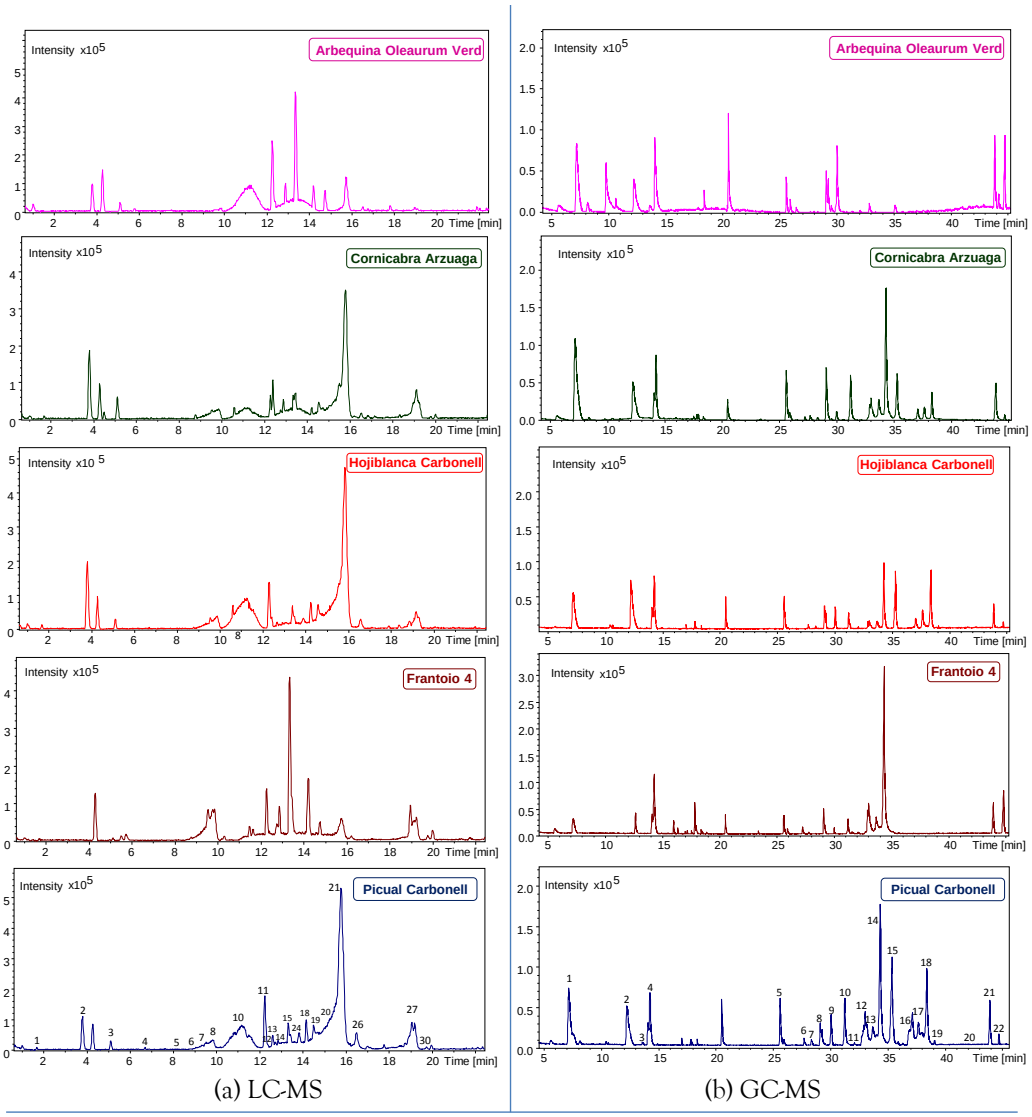

Fig. 2. Phenolic compounds profiles (Base peaks chromatograms (BPCs)) of five examples of monovarietal virgin olive oils from the cultivars under study. (a) BPCs obtained using LC-MS, and (b) BPCs obtained by GC-MS. Identification peak numbers (shown in the LC and GC profiles of the extracts from Picual) correspond with those numbers of Tables 1 and 2. In LC-MS, some secoiridoid-related compounds are not numbered in order to facilitate the visual inspection of the profile

Table 1. Phenolic compounds (or related analytes) found in the extracts of the analysed monovarietal VOOs, identified by LC-ESI-TOF MS.

	Compounds	Retention time (min)	*m/z* experimental	*m/z* calculated	Molecular formula	Error (ppm)	mSigma	Detected in-source fragments
1	Oxidised hydroxytyrosol	1.675	151.0399	151.0401	C8H8O3	0.9	7	
2	Hydroxytyrosol	3.799	153.0559	153.0557	C8H10O3	-1.4	4.8	123.0445
3	Tyrosol	5.103	137.0609	137.0608	C8H10O2	-0.9	4.1	
4	Decarboxymethyl elenolic acid	6.675	183.0672	183.0663	C9H12O4	-4.8	24	139.0769
5	Desoxy elenolic acid	8.163	225.0761	225.0768	C11H14O5	3.1	9.7	
6	Hydroxy elenolic acid	8.715	257.067	257.0667	C11H14O7	-1.3	17.3	
7	Hydroxytyrosol acetate	9.016	195.0666	195.0663	C10H12O4	-1.9	17.8	139.0087/241.0724
8	Elenolic acid	9.836	241.0717	241.0718	C11H14O6	0.3	17.5	139.0111
9	Hydroxy decarboxymethyl oleuropein aglycone	10.555	335.1146	335.1136	C17H20O7	-2.4	15.7	199.0615 / 257.0664
10	Decarboxymethyl oleuropein aglycon	11.14	319.1187	319.1187	C17H20O6	0.1	2.6	183.0662
11	Luteolin	12.21	285.0404	285.0405	C15H10O6	0.1	3.6	
12	Syringaresinol	12.294	417.1544	417.1555	C22H26O8	2.6	11.9	
13	Hydroxy decarboxymethyl ligstroside aglycone	12.328	319.1175	319.116	C17H20O6	-4.8	10.1	199.0606
14	Pinoresinol	12.829	357.1347	357.1344	C20H22O6	-1.1	19.6	
15	Acetoxypinoresinol	13.298	415.1394	415.1398	C22H24O8	1.2	19.9	
16	10-Hydroxy oleuropein aglycone	13.314	393.1199	393.1191	C19H22O9	-2.1	36.2	

Table 1. (continued)

	Compounds	Retention time (min)	*m/z* experimental	*m/z* calculated	Molecular formula	Error (ppm)	mSigma	Detected in-source fragments
17	Decarboxymethyl ligstroside aglycone	13.548	303.1225	303.1238	C17H20O5	4.3	25.9	183.0682
18	Apigenin	14.134	269.0449	269.0455	C15H10O5	2.3	19	
19	Methyl decarboxymethyl oleuropein aglycone	15.469	333.1342	333.1344	C18H22O6	0.5	23.7	
20	Methyl oleuropein aglycone	18.716	391.1399	391.1398	C20H24O8	0	22.1	
21	Oleuropein aglycone	15.739	377.1245	377.1242	C19H22O8	-0.8	6.9	345.1012 / 307.0846 / 275.0894 / 241.0716
22	Oleuropein aglycone (isomer 1)	11.407	377.1244	377.1242	C19H22O8	-0.5	35	345.0978/ 307.0844 / 275.0873/ 241.0742
23	Oleuropein aglycone (isomer 2)	12.578	377.1249	377.1242	C19H22O8	-1.8	19.4	345.1003 / 307.0825 / 275.0905 / 241.0730
24	Oleuropein aglycone (isomer 3)	13.799	377.1244	377.1242	C19H22O8	-0.9	12.1	345.1006 / 307.0846 / 275.0904 / 241.0714
25	Oleuropein aglycone (isomer 4)	14.518	377.1242	377.1242	C19H22O8	0	8.2	345.0997 / 307.0849 / 275.0920 / 241.0743
26	Oleuropein aglycone (isomer 5)	16.458	377.1226	377.1242	C19H22O8	4.2	5.3	345.1022 / 307.0835 / 275.0921 / 241.0744
27	Ligstroside aglycone	19.017	361.1291	361.1293	C19H22O7	0.5	10.8	213.0779/333.1369 / 291.0891
28	Ligstroside aglycone (isomer 1)	12.729	361.1302	361.1293	C19H22O7	-2.5	13.4	213.0779/333.1369 / 291.0899
29	Ligstroside aglycone (isomer 2)	13.415	361.128	361.1293	C19H22O7	3.4	13.2	213.0782/333.1380 / 291.0891
30	Ligstroside aglycone (isomer 3)	19.703	361.1266	361.1293	C19H22O7	7.4	12.1	213.0779/333.1349 / 291.0906

Table 2. Phenolic compounds (or related analytes) found in the extracts of the analysed monovarietal VOOs, identified by GC-APCI-TOF MS.

	Compounds	Retention time (min)	*m/z* experimental	*m/z* calculated	Molecular formula	Error (ppm)	mSigma value	Fragments
1	Tyrosol-2H+2TMS	7.212	282.1467	282.1466	C14H26O2Si2	-0.4	74.6	**193.1047**, 109.0943
2	Hydroxyyrosol-3H+3TMS	12.254	370.1815	370.181	C17H34O3Si3	-1.3	53.6	**281.1407**, 193.0709,121.0683
3	Protocatechuic acid-3H+3TMS+H	13.818	**371.1536**	371.1525	C16H31O4Si3	-3	11.2	281.1050, 355.1221, 209.0627
4	Elenolic acid-H+1TMS+H	14.254	315.1269	315.1258	C14H23O6Si	-3.4	32.6	**225.0774**, 267.1258, 283.1035, 139.0423
5	Decarboxymethyl ligstroside aglycone -H+1TMS+H	25.598	377.1787	377.1779	C20H29O5Si	-2.1	139.1	**193.1045**, 359.2882
6	Ligstroside aglycone-2H+2TMS+H isomer 1	27.663	507.224	507.2229	C25H39O7Si2	-2.2	32.3	**193.1052**, 475.1989
7	Ligstroside aglycone-2H+2TMS+H isomer 2	28.336	507.2252	507.2229	C25H39O7Si2	1.7	21.4	**193.1053**, 475.1990
8	Decarboxymethyl oleuropein aglycone-2H+2TMS+H	29.212	465.2139	465.2123	C23H37O6Si2	-3.5	78.1	**281.1402**, 209.0999, 411.3310, 129.0713
9	Ligstroside aglycone-2H+2TMS+H isomer 3	31.193	507.224	507.2229	C25H39O7Si2	-2.2	21.3	**193.1052**, 475.1989
10	Hydroxy decarboxymethyl-ligstroside aglycone-2H+2TMS+H isomer 1	31.294	537.2517	537.2518	C26H45O6Si3	0.2	54.2	**281.1404**, 193.0979, 447.1931
11	Hydroxy decarboxymethyl-ligstroside aglycone-2H+2TMS+H ismer 2	32.016	537.2517	537.2518	C26H45O6Si3	0.2	24.6	**281.1402**, 193.0988, 447.1921
12	Hydroxy decarboxymethyl-Oleuropein aglycone-3H+3TMS+H	32.89	553.2486	553.2468	C26H44O7Si3	-3.3	44.5	**193.1037**, 281.1400
13	Ligstroside aglycone-2H+2TMS+H	33.599	507.2232	507.2229	C25H39O7Si2	-0.6	12.6	193.1048
14	Oleuropein aglycone-2H+2TMS+H isomer 1	34.269	507.2252	507.2229	C25H39O7Si2	-4.5	20.7	**193.1053**, 475.1990
15	Oleuropein aglycone-2H+2TMS+H	35.228	523.2204	523.2178	C25H39O8Si2	-5.1	17.4	**281.1409**, 209.1001
16	Oleuropein aglycone-3H+3TMS+H isomer 2	36.976	595.256	595.2573	C28H47O8Si3	2.2	62.5	**281.1411**, 209.0999, 563.2309
17	Oleuropein aglycone-3H+3TMS+H isomer 3	37.595	595.2565	595.2573	C28H47O8Si3	1.3	42.6	**281.1401**, 209.0996, 563.2319
18	Oleuropein aglycone-3H+3TMS+H isomer 4	38.254	595.2574	595.2573	C28H47O8Si3	-0.1	29.8	**281.1399**, 209.1087, 563.2305
19	Apigenin-3H+3TMS+H	39.213	**487.1807**	487.1787	C24H35O5Si3	-4.1	10.9	415.1407, 192.9638
20	Luteolin-4H+4TMS+H	42.927	**575.2151**	575.2131	C27H43O6Si4	-3.4	20.9	503.1742, 281.0744
21	Pinoresinol-2H+2TMS+H	43.784	503.2297	503.228	C26H39O6Si2	-3.4	77.4	**485.2215**, 414.1709, 247.1195
22	Acetoxypinoresinol-2H+2TMS	44.572	561.2317	561.2334	C28H41O8Si2	3.1	85	**305.1225**, 501.2157, 275.1116, 483.2046

The most intense m/z signals for each compound are highlighted in bold letters.

Thus, among the identified compounds in the LC-MS profiles, secoiridoids were by far the most abundant group of phenols (in terms of area) in all the analysed samples, regardless of the variety. In particular, Ol Agl was the main secoiridoidic compound found in all the selected monovarietal VOOs. Cornicabra showed the highest total area values of complex phenols, being also the richest in terms of Ol Agl. The relative abundance of secoiridoids in the selected cultivars decreased in the following order: Cornicabra > Picual > Hojiblanca > Frantoio > Arbequina. As far as simple phenols is concerned, we can stand out that Frantoio was the variety which showed the lowest relative abundance in terms of these compounds, followed by Hojiblanca, Arbequina, Picual and Cornicabra (in increasing order of relative abundance). When flavonoids were observed, Arbequina resulted to be the variety with the highest area means, whilst Frantoio exhibited the lowest relative levels. Lignans composed another relevant group of phenolic compounds; their relative average area per variety decreased as follows: Arbequina > Picual > Cornicabra > Hojiblanca > Frantoio.

As described above, the chemical characterisation of the phenolic fraction of the extra-VOOs under study was also carried out by using a relatively new platform with a proved potential in the field of food metabolomics, i.e. GC-APCI-TOF MS. Sample preparation, in this case, included a protocol to create the trimethylsilyl derivatives; the BPCs of the derivatised extracts (with BSTFA + 1% TMCS) are shown in Fig. 2b. The mentioned reagent was selected because of its fast reactivity with compounds containing active hydrogens (hydroxyl groups), its high volatility, low thermal degradation and good solubility. Using the GC-APCI-MS method, the analysis time was about 50 min and chromatograms showing high efficiency and good separation of a noteworthy number of phenolic compounds were obtained (Fig. 2b). The chromatograms of the silylated phenolic compounds contained no peaks of underivatised compounds, indicating 100% efficiency of the silylation. 22 phenolic compounds belonging to the same categories as mentioned before (plus phenolic acids) were found in all the 25 evaluated samples (see Table 2), however, the total number of identified phenols was considerably higher when including 3 phenolic acids or related compounds (p-coumaric acid, ferulic acid and vanillin), and some other derivatives from simple phenols and secoiridoids found in some samples. The identification in this platform was not as straightforward and intuitive as in LC-MS and it required a combination of prior knowledge, commercially

available standards and semi-preparative HPLC isolated standards [32], supported by the intrinsic qualities of the UHR-TOF mass analyser.

The comparison of the relative areas led to the same results as in LC-MS, confirming the same differences pointed out after analysing the LC-MS chromatograms of these oils. From our point of view, it is quite interesting the fact that the content of Ol Agl- and Lig Agl-derivatives in the extra-VOOs could be quickly estimated from GC-APCI data just by using two extracted ion chromatograms (EICs), 281.1411 and 193.1049, respectively. In this regards, as can be seen in Fig.3, the screening of only two *m/z* signals appears to be of great utility for estimating the oleuropein- and ligstroside-analogues amount in the studied monovarietal oils.

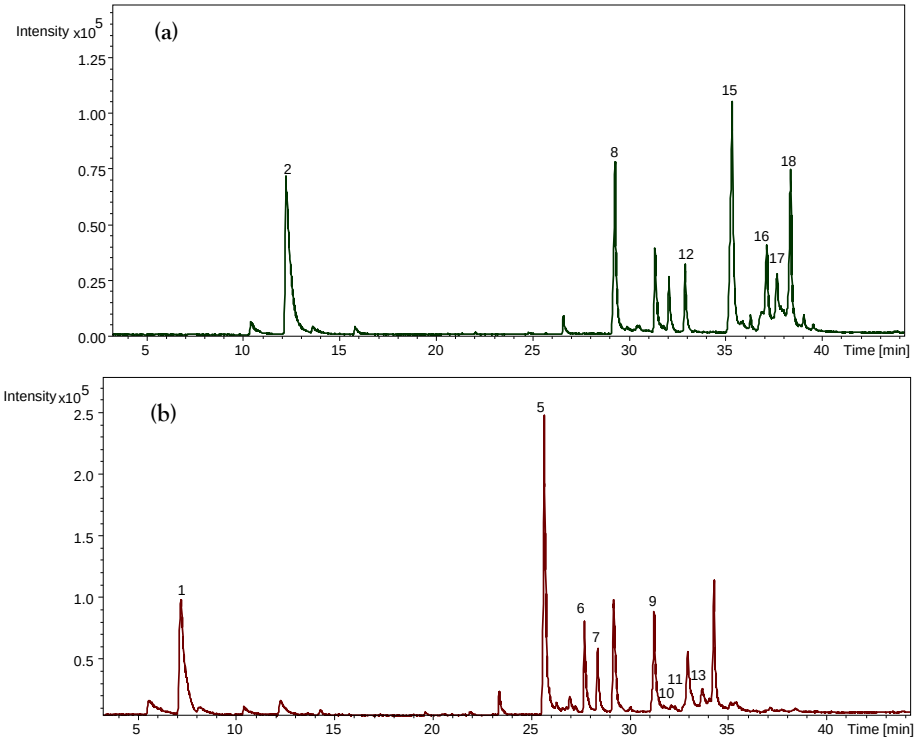

Fig. 3. Extracted ion chromatograms (EICs) of the MS signals (a) *m/z* 281.1411 and (b) *m/z* 193.1049 obtained in GC-MS (taking an extract of Arbequina extra-VOO as example), which facilitate the study of oleuropein aglycone- and ligstroside aglycone-analogues, respectively.

Furthermore, if the chromatograms obtained using LC-MS and GC-MS (Fig. 2) are compared, it is possible to claim that a proper separation and comprehensive characterisation of extra-VOO phenolic compounds can be achieved using both techniques. GC-MS is somewhat more adequate than the LC-MS coupling for the determination of flavonoids and lignans, even though they are properly detected by LC-

MS too; however in GC they are eluting at the end of the run without any other compound in close proximity which could produce ionisation suppression phenomena.

3.2. Multivariate data analysis and varietal marker identification

Although a visual inspection to compare and distinguish the LC-MS and GC-MS chromatograms obtained for samples with different varietal origins can be done, it is not very advisable, since the process is tedious, rough, subjective and non-quantitative. In addition, minor differences between very closely related compounds might be missed if this approach is selected. Moreover, if a traditional quantitative approach is used, the subsequent analysis will be eventually made by just considering the determined compounds and ignoring unknown analytes which could turn into important varietal markers. These drawbacks are overcome by multivariate analysis, which was employed as a more holistic approach to analyse the LC and GC profiles.

Thus, raw LC-MS and GC-MS data, from replicate samples, were pre-treated as described above and separately submitted to multivariate data. Hence, in a first step, after raw data pre-processing, auto-scaling and logarithmic transformation, PCA was applied to the whole data sets (LC-MS and GC-MS, separately), including both replicates (methodological, but not instrumental) of each sample (Fig. 4). As expected, considering the repeatability inter-day (expressed in terms %RSD) for LC-MS and GC-MS (lower than 10.13% and 7.08%, respectively), the corresponding replicates were laying very close on the PC plane.

The PCA score plots obtained using the entire LC-MS data set are displayed in a two-dimensional plot using the first two principal components, which covered 27.0% and 19.0% of the variance, respectively (Fig. 4a). The third principal component of the PCA covered 12% of the variance for LC data set. On the basis of the PCA scores plots, samples from Arbequina and Frantoio cultivars seem to dominate the PCA model suppressing any possible discrimination among samples obtained from the other varieties. Therefore, we decided to build an additional PCA model, considering separately Hojiblanca, Picual and Cornicabra (not including Arbequina and Frantoio), trying to evaluate data structure. The obtained PCA score plot is shown in Fig. 4b and, compared to the first one, exhibited slightly better discrimination capability among these three cultivars, even if they were no completely segregated from each other.

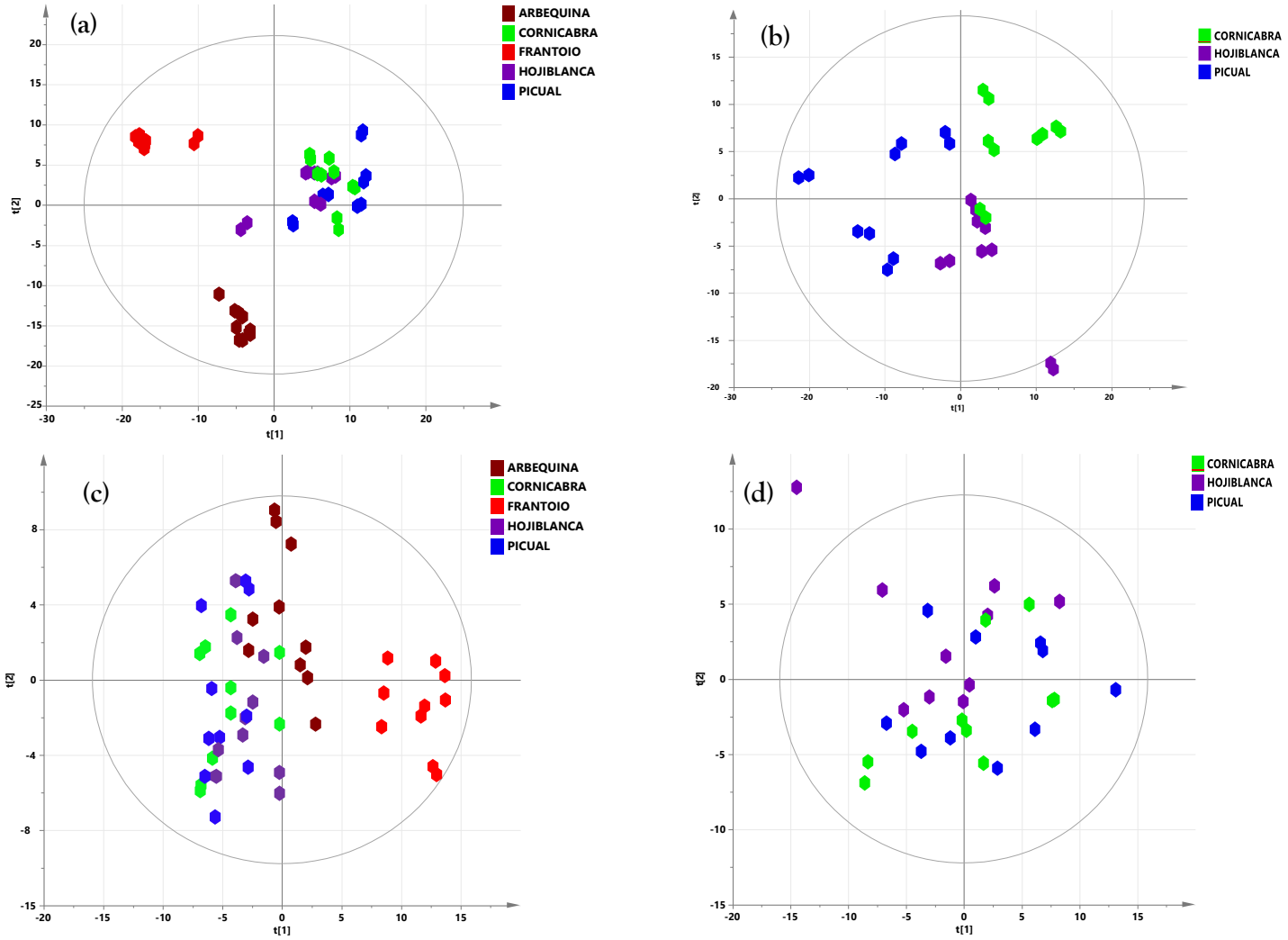

Fig. 4. Score plot panels for the first two principal components of PCA models built on LC-MS data (a, b), and GC-MS data (c, d).

Following the same strategy, PCA was applied for GC-MS data. When PCA was applied to the entire GC-MS data set (including samples from the five cultivars considered), the score plots (Fig. 4c) consistently distinguish Frantoio samples from the other monovarietal VOOs, being Cornicabra, Picual, Hojiblanca and even Arbequina olive oils defining an unique group. The first three components explained 30%, 20% and 10% of the total variance, respectively, for GC-MS data set. As previously done with LC-MS data, PCA was recalculated after removing data of Arbequina and Frantoio extra-VOOs. As can be observed from the PCA scores plot (Fig. 4d), the applied statistical analysis was not able to separate the studied samples according to their varietal origin. Consequently, we can conclude that applying PCA to the LC-MS data set composed by Cornicabra, Hojiblanca and Picual samples showed higher discriminating power than that obtained using GC-MS data of the same cultivars.

Once the natural clustering of the studied samples was visualised and examined by applying PCA, the subsequent step was to build supervised varietal discrimination models using PLS-DA. The results obtained with the initial PCAs, conditioned how to face this stage of the study and the different classes to be considered in each case (contemplating the variety as class characteristic). Therefore, the potential of different models was evaluated and the pointed classifiers were identified for each one. The four tested models (for LC-MS and GC-MS, respectively) were the following: a two-class PLS-DA model was built using Arbequina and Frantoio VOO samples; another two-class PLS-DA model was built using Arbequina (class 1) and Cornicabra, Hojiblanca and Picual VOO samples (class 2); Frantoio samples were taken as class 1 in another model where Cornicabra, Hojiblanca and Picual VOO samples composed class 2; and finally, a three-class PLS-DA model was made taking into account the phenolic extracts from Cornicabra, Hojiblanca and Picual. In the rest of the section, we will have a look at each one in detail.

As stated, a two-class PLS-DA model was firstly built using Arbequina and Frantoio extra-VOO samples. Fig. 5a and 5b represent the cross-validated score plots of the PLS-DA models for LC-MS and GC-MS, respectively. Both models revealed a good modelling performance, showing the following statistical parameters: R2X 0.561, R2Y 0.995, Q2 0.995, F =197.549, p=8.88475e-013 for LC-MS, and R2X 0.46, R2Y 0.983, Q2 0.925, F 39.764, p=8.13931e-008 for GC-MS.

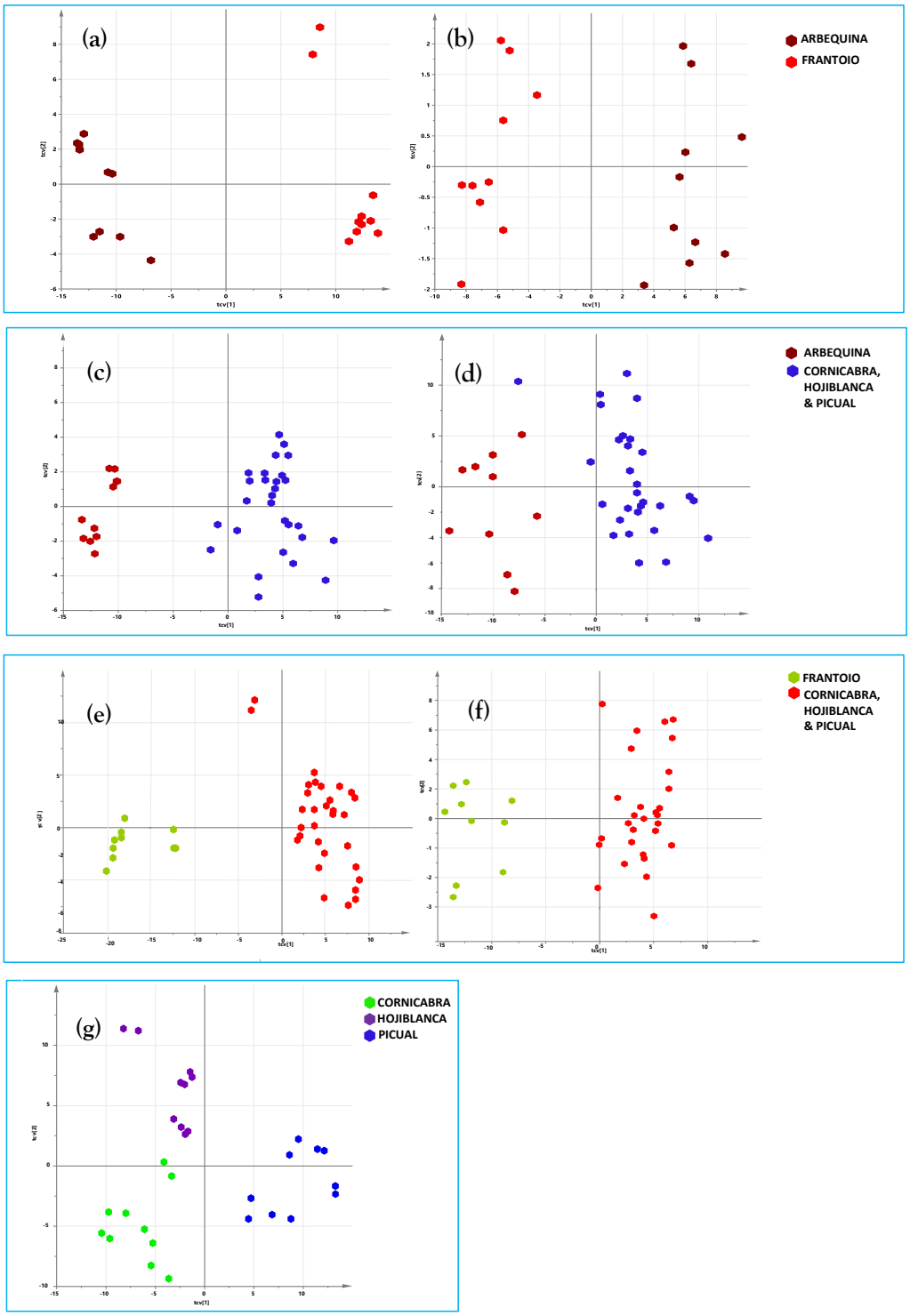

Fig. 5. Cross-validated score plots of the different PLS-DA models built on LC-MS data (a, c, e, g) and GC-MS data (b, d, f). Statistical parameters of each model were the following:

LC (**a**: R2X 0.561, R2Y 0.995, Q2 0.995, F =197.549, p=8.88475e-013; **c**: R2X 0.563, R2Y 0.99, Q2 0.953, F=47.8986, p=2.53582e-015; **e**: R2X 0.541, R2Y 0.987, Q2 0.965, F=137.24, p=6.96656e-022; and **g**: R2X 0.62, R2Y 0.983, Q2 0.94, F=13.5118, p=1.20281e-011);

GC (**b**: R2X 0.46, R2Y 0.983, Q2 0.925, F 39.764, p=8.13931e-008; **d**: R2X 0.439, R2Y 0.896, Q2 0.819 F=36.8021, p=6.55659e-012, and **f**: R2X 0.455, R2Y 0.957, Q2 0.903 F=65.5538, p=1.67209e-015).

In both cases, the separation between the two classes was more than evident (being obviously the cultivar the main source of variability in the data), fact which is not actually very commendable. Much more interesting was to have a look at the features, most influential for the model, which can be selected on the basis of the S-plots (Fig. 1a, b supplementary) and VIP scores. Chromatographic mass features showing VIP values higher than 1.0 were located and ranked according to their retention time. Afterwards, the compounds identification was carried out by comparing the selected mass feature's retention time, *m/z* signal and fragmentation-patterns with the information included in Tables 1 and 2. Table 3 includes a list of varietal discriminating phenolic compounds with VIP values above 1.0 (for all the tested models).

Table 3. Variable importance in projection (VIP) values of potential varietal discriminating variables identified by GC-MS and LC-MS for the four evaluated models. (we just include those with VIP value higher than 1.0)

	PLS-DA (M1)[a]		PLS-DA (M2)[b]		PLS-DA (M3)[c]		PLS-DA (M4)[d]	
	VIP		VIP		VIP		VIP	
Discriminating variables	GC-MS	LC-MS	GC-MS	LC-MS	GC-MS	LC-MS	GC-MS	LC-MS
Hydroxytyrosol	1.52	1.2	1.3	1.14	1.49	1.5	no valid model	1.19
Tyrosol	****	****	1.28	1.24	****	1.4		1.22
Decarboxymethyl elenolic acid	****	1.29	****	****	****	1.69		****
Desoxy elenolic acid	****	****	****	****	****	****		1.7
Elenolic acid	1.48	1.36	****	****	1.4	1.64		
Hydroxy decarboxymethyl oleuropein aglycone	****	1.46	****	****	****	1.65		1.07
Decarboxymethyl oleuropein aglycone	1.39	1.47	****	1.53	1.14	1.52		1.43
Syringaresinol	****	1.44	****	****	****	1.58		1.44
Hydroxy decarboxymethyl ligstroside aglycone	****	1.46	****	****	****	1.65		1.07
Pinoresinol	1.42	1.44	1.22	1.76	****	1.6		1.77
Acetoxypinoresinol	1.29	1.46	1.67	1.71	1.5	1.63		****
Apigenin	****	1.47	****	1.59	****	****		1.51
Oleuropein aglycone	****	1.47	1.45	1.62	1.22	1.64		1.62
Oleuropein aglycone (isomer 1)	****	****	****	****	****	1.51		****
Oleuropein aglycone (isomer 3)	****	1.48	1.51	****	****	****		****
Oleuropein aglycone (isomer 4)	****	****	1.5	1.54	****	****		****
Ligstroside aglycone	1.53	1.47	1.44	****	****	1.51		1.55
Ligstroside aglycone (isomer 1)	****	****	****	1.56	****	1.4		****
Ligstroside aglycone (isomer 3)	1.51	1.48	***	1.67	****	****		****

7 analytes were pointed out as relevant for GC-MS, whilst 14 resulted to be more influential when LC-MS data were used. HYTY, EA, D-Ol Agl, Pin, Ac Pin, Lig Agl and one of its isomers were influential in both models. Apart from these features, H-D-Ol Agl, Apig, Ol Agl (and its isomer 3), hydroxy-D-Lig Agl, syringaresinol, and D-EA had a considerable importance for LC-MS. The fact that lignans composition (Pin and Ac Pin) of Arbequina oils is very characteristic is well-known [35-37]; moreover, other authors have reported higher contents of Apig, HYTY and D-Ol Agl in olive oils form Arbequina in contrast with other Frantoio olive oils [38].

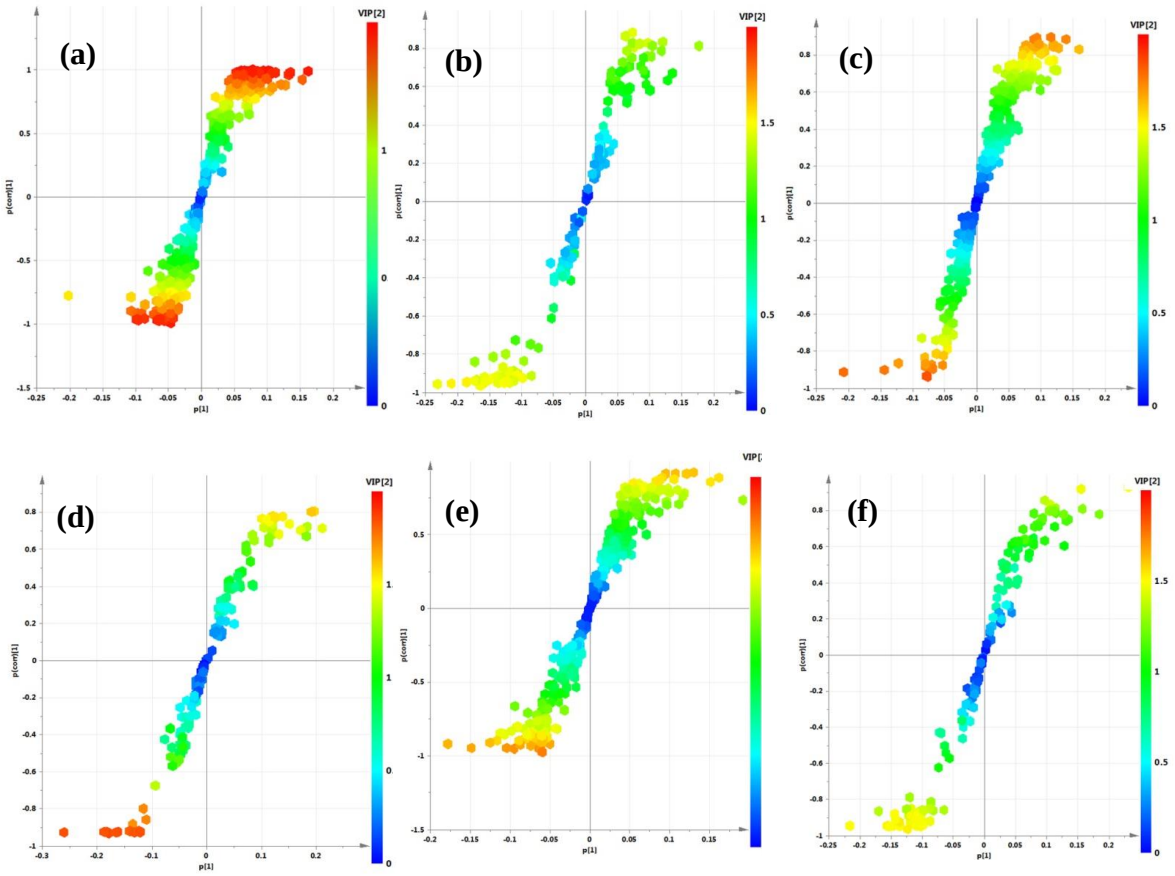

Fig. 1. Supplementary material. Scatter-plot (S-plot) obtained for orthogonal PLS-DA models for LC-MS data (a, c, e) and GC-MS data (b, d, f).

Later on, in a second step, PLS-DA models were constructed in a similar manner (using separately GC-MS and LC-MS data sets) including Arbequina as class identifier 1, and Cornicabra, Hojiblanca, and Picual as class 2. The predictive scores plot for the first two latent variables of PLS-DA models, for LC-MS data and GC-MS data, are shown

in Fig. 5c and d, respectively. Both models led to achieve a proper differentiation between the two groups, indicating that the phenolic profiles of Arbequina VOO's are quite specific and distinguishable from those of Cornicabra, Hojiblanca and Picual VOO's. The following statistical parameters were obtained: R2X 0.563, R2Y 0.99, Q2 0.953, F=47.8986, p=2.53582e-015 for LC-MS data, and R2X 0.439, R2Y 0.896, Q2 0.819 F=36.8021, p=6.55659e-012 for GC-MS data; results which indicate the excellent quality of the models. Moreover, an overview of the S-plots (Fig. 1c and d, supplementary material) and the examination of the features with VIP values ≥ 1.0 showed that 10 (for LC-MS) and 8 (for GC-MS) variables were responsible for the separation among the samples (Table 3). HYTY, TY, Pin, Ac Pin, Ol Agl and Ol Agl (isomer 4) were identified as relevant compounds for the models achieved by using the data coming from the two platforms; apart from that, two other secoiridoids (Ol Agl isomer 3 and Lig Agl) were influential in the GC model and D-Ol Agl, Apig and two isomers of Lig Agl showed an outstanding role for the LC model. Some of these identified varietal discriminant phenolic compounds have been previously reported by other authors, who observed significant quantitative differences in terms of these compounds between Arbequina VOOs and monovarietal oils from the other three varieties considered herein [36,39,40].

Afterwards, a third PLS-DA model was tested, considering Frantoio samples (class 1) and Cornicabra, Hojiblanca, and Picual extra-VOOs (class 2). Fig. 5e and f depict the discriminant predictive scores plot for the achieved LC-MS and GC-MS models. These models showed overall high-quality parameters: R2X 0.541, R2Y 0.987, Q2 0.965, F=137.24, p=6.96656e-022 for LC-MS and R2X 0.455, R2Y 0.957, Q2 0.903 F=65.5538, p=1.67209e-015 for the GC-MS platform. The simple visual inspection of the graphs shows how Frantoio samples are clustered far away from the rest of the oils, which are in a relative close proximity among them. Table 3 summarises all discriminative features with considerably high VIP values (the S-plots are shown in Fig. 1e and f, supplementary material), which were, for this model number 3, 14 for LC-MS and 5 for the GC-MS coupling. HYTY, EA, D-Ol Agl, Ac Pin and Ol Agl were common relevant analytes for both models; moreover, TY, syringaresinol and Pin and 6 secoiridoids-related compounds were influential for the LC model.

As explained before, the final step in our workflow was building two more models (one using the LC-MS profiles and the other with GC-MS data), trying to identify phenolic features which could help differentiating extra-VOOs from the cultivars Cornicabra, Hojiblanca and Picual. The results revealed that the model for GC was not a valid one, not fulfilling the quality requirements (R2X 0.554, R2Y 0.846 Q2 0.567, F=0.728997, p=0.888921) (the graphic is not shown). However, a different situation was experienced for LC-MS (Fig. 5g), where an appropriate model was created (R2X 0.62, R2Y 0.983, Q2 0.94, F=13.5118, p=1.20281e-011) and the samples belonging to different varieties could be correctly classified. Based on the VIP values, 11 phenolic compounds were ranked as responsible for the separation among extra-VOO samples coming from Cornicabra, Hojiblanca and Picual *cv*. (Table 3), being the most relevant: Pin (VIP = 1.77), desoxy EA (VIP = 1.70), Ol Agl (VIP = 1.62), Lig Agl (VIP = 1.55), and Apig (VIP = 1.51). Besides these compounds, HYTY, TY, H-D-Ol Agl, D-Ol Agl, syringaresinol and hydroxy-D-Lig Agl were also important features for the model, but their VIP values were below 1.5. Paying attention to the 5 most meaningful features within this model, Cornicabra´s Pin areas were much higher than Picual´s and Hojiblanca´s; meanwhile Desoxy EA was found at higher relative concentration levels in Picual (followed by Hojiblanca and Cornicabra extra-VOOs). Cornicabra was the richest in terms of Ol Agl and Picual, in terms of Lig Agl. Finally, it is important to stand out that Apig was particularly abundant (considering the relative areas) in Hojiblanca oils, showing medium levels in Picual samples and very low amounts for Cornicabra phenolic extracts.

Bearing in mind all the information about possible varietal markers coming from the different LC-MS models, it is possible to say that Ac Pin and isomer 3 of Lig Agl could be considered as Arbequina markers. For Frantoio, the relative levels of some phenolic compounds could help to distinguish olive oils from this *cv*. from others; we can highlight as potential markers for this variety: EA, D-EA. The achieved models in LC-MS pointed at Pin, Ol Agl and HYTY as very useful features to distinguish among all the evaluated varieties. As far as GC-MS markers are concerned, Pin and Lig Agl resulted to be essential for Arbequina oils, and EA and D-Ol Agl for Frantoio samples. Since the GC PLS-DA model number 4 was not good enough, no features can be mentioned as important for an overall classification; however, what can be addressed on

the basis of the GC results is that HYTY was useful both for discriminate between Frantoio and Arbequina samples, as well as to separate them from the rest.

4. Conclusions

Two MS approaches were applied herewith together with different statistical treatments to evaluate both the classification power and the capabilities of LC-ESI-TOF MS and GC-APCI-TOF MS to identify potential varietal markers for extra-VOOs obtained from Arbequina, Picual, Cornicabra, Hojiblanca and Frantoio *cv*. In general, the LC-MS method provided a shorter total analysis time with simpler sample preparation and a more straightforward identification for the phenolic compounds under study, whilst GC-MS gave better separation efficiency and showed some advantages for secoiridoids (and related compounds) determination. Frantoio and Arbequina dominated the PCA models, fact which conditioned the further steps of the study. Different PLS-DA predictive models were successfully constructed showing good discriminant ability and excellent predictability to assure the varietal origin of the studied samples (with the exception of the GC-MS PLS-DA model for Hojiblanca, Cornicabra and Picual).

Even though the number of samples selected was not considerably high, the achievements of this study are, from our point of view, very interesting and compelling, since it has been confirmed (by both platforms) that EA is a Frantoio marker, lignans and Lig Agl (two isomers) are Arbequina characteristic features, and HYTY and Ol Agl are very outstanding metabolites when the 5 varieties are considered.

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Chapter 6

A first approach towards the development of geographical origin tracing models for North Moroccan olive oils based on triacylglycerols profiles

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Abstract

The triacylglycerols composition of monovarietal Picholine Marocaine olive oils, produced in seven of the most productive areas in northern Morocco, has been established for the first time, starting a register of typicality and geographical identity of Moroccan olive oils. 279 olive samples were collected over two consecutive crop seasons (2011/2012 (n=140) and 2012/2013 (n=139)), the analysis of the triacylglycerol profiles of the obtained oils was performed using high performance liquid chromatography, the influence of the region of provenance was investigated, and chemometric data analysis (including principal components analysis, linear discriminant analysis, partial least squares-discriminant analysis and soft independent modeling of class analogies) was used to differentiate the studied samples according to their geographical origin. 21 triacylglycerols were characterized and the variability observed among the studied samples could be related to the production area. The combination of triacylglycerols composition with chemometrics provided a powerful tool to verify the geographical origin of north Moroccan olive oils.

Keywords: virgin olive oil; Picholine Marocaine; triacylglycerols composition; geographical origin; chemometrics.

Practical applications: Moroccan legislations established Geographic Indication labels for adding value to typical products. However, the general procedure to control the geographical authenticity of these protected products remains based on the traceability of their production and there is a lack of appropriate and reliable tools for their geographical unambiguous authentication and control. In this work, geographical authentication models for classifying olive oil samples (based on triacylglycerols composition) have been developed and evaluated, achieving adequate classification and prediction rates for oils coming from the main north Moroccan olive growing areas. The results obtained herein, could represent a significant contribution to the control of the geographical authenticity of Moroccan olive oils.

Abbreviations used

1,2-dilinoleoyl-3-oleoylrac-glycerol, OLL; 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol, PLL; 1,2-dioleoyl-3-palmitoyl-racglycerol, POO; 1,2-dioleoyl-3-stearoyl-rac-glycerol, SOO; 1,2-dipalmitoyl-3-oleoyl-rac-glycerol, PPO; 1-palmitoyl-2-oleoyl-3-linoleoylglycerol, POL; analysis of variance, ANOVA; carbon number, CN; equivalent carbon number, ECN; free fatty acids, FFAs; high performance liquid chromatography, HPLC; International Olive Council, IOC; linear discriminant analysis, LDA; partial least squares-discriminant analysis, PLS-DA; peroxide value, PV; principal components analysis, PCA; principal components, PCs; protected designation of origin, PDO; protected geographic indication, PGI; soft independent modeling of class analogies, SIMCA; standard deviation, SD; triacylglycerols, TAGs; trilinolein, LLL; trilinolenin, LnLnLn; triolein, OOO; tripalmitin, PPP; tripalmitolein, PoPoPo; tristearin, SSS; virgin olive oil, VOO.

1 Introduction

Virgin olive oil (VOO) is without question one of the most popular and widely produced and consumed vegetable oils in the Mediterranean area. Indeed, global olive oil production in 2014 was about 2,390,000 t, and more than 98% of this production came from the Mediterranean region, being the main olive oil producing countries Spain (44.83%), Italy (12.64%), Greece (12.54%), Tunisia (10.87%), Turkey (7.94%), and Morocco (4.60%) [1]. In the latter one, around 48.22% of olive oil production is located in northern regions, where Taounate, Ouazzane, Meknès, Fès, Sefrou, Chefchaouane and Taza are the most important [2]. In these areas, the olive oil sector plays a dynamic socio-economic development role due to the loyalty of the local farmers to olive trees cultivation and the presence of important olive oil processing companies; as a matter of fact the olive oil sector is the engine of job growth in the economy of these regions. Moreover, some of these areas are highly esteemed for their ancestral olive oil production history and the excellent quality of their olive oils [3]. Consequently, one of the most prominent research topics in the olive oil field is nowadays to establish a reliable system to distinguish olive oils produced in these regions, in order to increase their commercial value and competitiveness in both national and international olive oil markets.

Considering the regulations of some other countries to promote and protect names of quality agricultural products and foodstuffs, support the reputation of the regional foods,

and encourage rural and agricultural activity, Morocco has recently developed a system to provide legal protection to regional foods, through the Protected Geographic Indication (PGI) and Protected Designation of Origin (PDO) labels [4]. The main purpose of this legislation framework is to provide a fair and reliable tool for differentiating local products and, therefore, being useful to ensure the control of their safety and quality as well as protecting them against fraud and imitation. However, as for all other legislations with regard to geographic designations set down by other countries, there is only a very generic description of the discriminant characteristics that the product must present, and no statement on how checking the expected typicality. To overcome these limitations, over the last few years, some analytical approaches have been developed and proposed for the geographical discrimination of Moroccan foodstuffs [5], being some of them successfully applied to the geographical discrimination of Moroccan VOOs [6-8].

Indeed, over the last years, the chemical composition of olive oil has been widely used to identify its geographical origin, fact which is actually not surprising, since many authors have demonstrated that the olive oil composition is widely affected by the pedoclimatic conditions of the geographical area in which was produced [9-21]. Certainly, characterizing major and minor components of olive oil for geographical classification purposes has been satisfactorily carried out on samples coming from diverse olive oil producing countries, mainly Spain [22-24], Italy [25-27], Greece [28, 29], Tunisia [30, 31], etc. In all the mentioned studies, different approaches combining profiling or fingerprinting of olive oil samples with the appropriate chemometric tools have been tested for the geographical classification of the studied oils, as well as the identification of markers that could facilitate this discrimination. Numerous examples could be found where, for instance, data about phenols, volatile compounds, fatty acids and sterols together with statistics were used to classify olive oils. Within the context of this paper and focusing on triacylglycerols (TAGs), it is possible to mention that TAGs composition (alone or considering other olive oil compounds profiles too) has been successfully used for achieving the authentication of the geographical origin of olive oil and is gaining wider acceptance [32, 33].

Even though there is an important number of publications regarding the analysis of TAG composition of olive oil and its use for geographical classification purposes, the

information concerning the characterization of these compounds in Moroccan olive oils is, unfortunately, relatively scarce [34, 35]. Indeed, to the best of our knowledge, there is no previous report in regard to north Moroccan olive oils.

Therefore, within this study a first evaluation of the quali-quantitative TAG composition of olive oil samples coming from the main north Moroccan olive growing areas is presented. In addition, principal components analysis (PCA), two classification methods (linear discriminant analysis (LDA) and partial least squares-discriminant analysis (PLS-DA)), and a modeling classification technique (soft independent modeling of class analogies (SIMCA)) were used to test the ability of TAGs profiles for the geographical discrimination of the selected samples. The obtained results were compared with the aim to identify the classification model that could exhibit the best classification and prediction abilities.

2 Materials and methods

2.1 Olive fruits sampling and oil extraction

The study was carried out on monovarietal olive oils from the main Moroccan cultivar denominated Picholine Marocaine. Thus, over two consecutive crop seasons (2011/2012 and 2012/2013), during the period between November and January, olive samples (approximately one sample is 35 kg of olives) were carefully hand harvested from olive trees randomly selected in representative olive orchards located in seven north Moroccan regions. Figure 1 represents the geographical localization and map's altitude of these regions. As can be seen from this Figure, the sampling regions considered within this study show very contrast latitude conditions with important intra and inter region's variability. Furthermore, apart from being the most important olive growing areas of the north of Morocco, these regions were chosen because they represent a high variability of pedoclimatic conditions. Doing it so, the obtained results could inform about the TAG composition of monovarietal Picholine Marocaine olive oils that can be produced under the most commonly found pedoclimatic conditions encountered in the North of Morocco. Thus, this study was conducted in the following regions: **Chefchaouane** (olive growing area: 52,250 ha; climatic conditions: annual mean minimum temperatures: 9 °C, annual mean maximum temperatures: 22.3 °C; annual mean rainfall: 800 mm; soil classes: fluvisols, regosols, lithosols, vertisols, rendzinas, yermosols, xerosols, kastanozems,

chernozems, phaezems, cambisols, luvisols, acrisols, gleysols, planosols, solontchaks and solonetz);

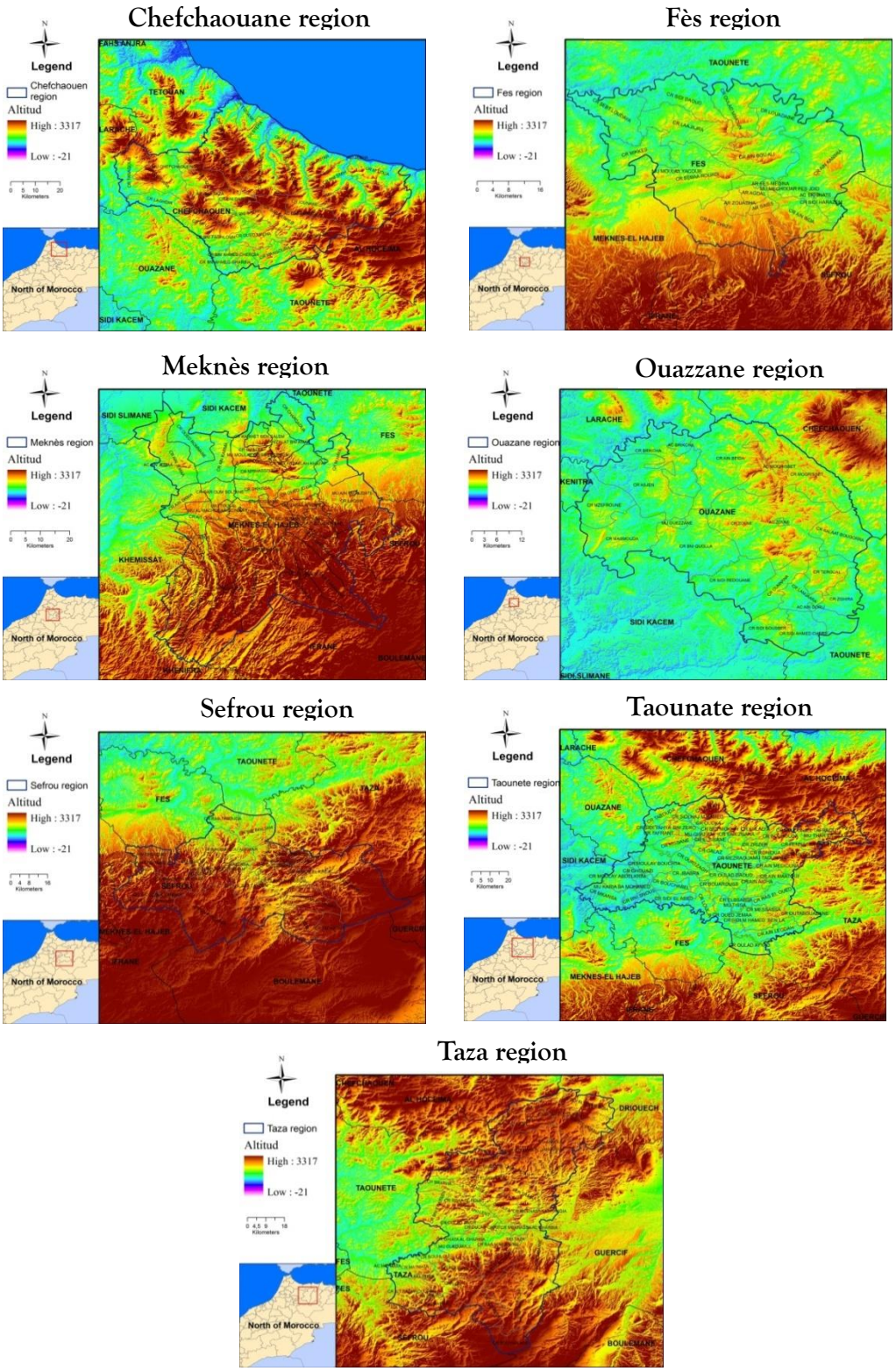

Figure 1. North Moroccan areas under study geographic location and altitude maps.

Fès (olive growing area: 36,930 ha; climatic conditions: annual mean minimum temperatures: 11.3 °C , annual mean maximum temperatures: 26.5 °C ; annual mean rainfall: 475 mm; soil classes: fluvisols, regosols, lithosols, rendzinas, yermosols, xerosols, vertisols, kastanozems, chernozems, phaezems, gleysols, planosols, luvisols and acrisols); **Meknès** (olive growing area: 43,000 ha; climatic conditions: annual mean minimum temperatures: 11 °C, annual mean maximum temperatures: 23 °C; annual mean rainfall: 400 to 600 mm; soil classes: fluvisols, regosols, lithosols, rendzinas, yermosols, xerosols, vertisols, kastanozems, chernozems, phaezems, luvisols and acrisols); **Ouazzane** (olive growing area: 40,885 ha; climatic conditions: annual mean minimum temperatures: 17.4 °C, annual mean maximum temperatures: 25.6 °C; annual mean rainfall: 676 mm; soil classes: fluvisols, regosols, lithosols, vertisols, rendzinas, yermosols, xerosols, cambisols, luvisols, acrisols, gleysols and planosols); **Sefrou** (olive growing area: 27,800 ha; climatic conditions: annual mean minimum temperatures: 10.6 °C, annual mean maximum temperatures: 25.6 °C; annual mean rainfall: 460 to 642 mm; soil classes: fluvisols, regosols, lithosols, vertisols, rendzinas, yermosols, xerosols, inceptisols, mollisols, Kastanozems, chernozems, phaezems, luvisols and acrisols); **Taounate** (olive growing area: 140,000 ha; climatic conditions: annual mean minimum temperatures: 14.6 °C, annual mean maximum temperatures: 24.8 °C; annual mean rainfall: 590 mm; soil classes: fluvisols, regosols, lithosols, vertisols, rendzinas, yermosols, xerosols, cambisols, luvisols, acrisols, gleysols and planosols); **Taza** (olive growing area: 90,000 ha; climatic conditions: annual mean minimum temperatures: 13.2 °C, annual mean maximum temperatures: 24.7 °C; annual mean rainfall: 580 mm; soil classes: fluvisols, regosols, lithosols, vertisols, rendzinas, yermosols, xerosols, kastanozems, chernozems, phaezems, luvisols and acrisols).

In all the regions considered by this work, the selected olive trees were more than 30 year-old; spaced at 10 m × 10 m, conducted under rainfed conditions and receiving limited amounts of fertilization (mainly organic manure and sometimes urea as chemical fertilizer) which corresponds to the typical traditional extensive system dominant in these regions and in general way in Morocco.

Thus, a total of 279 olive fruits samples were collected, from which 140 were sampled during 2011/2012 and 139 during 2012/2013. Among these samples 10 were from Chefchaouane, 41 from Fès, 69 from Meknès, 37 from Ouazzane, 39 from Sefrou, 49 from Taounate, and 34 from Taza. The samples were harvested at the spotted stage of

maturity (maturity index around 3.5–4.0, determined according to the method developed by the Agronomic Station of Mengíbar (Jaén, Spain) [36]), and were put into ventilated rectangular plastic crates and, immediately, transferred to the laboratory. Oil extraction was performed at laboratory scale by an Oliomio laboratory mill, SPREMOLIVA C30 Oliomio (Oliomio, Italy) with a work capacity of 30 kg/h, and equipped with a knife crusher, a horizontal malaxator and a two phase decanter. Olives were washed to eliminate any foreign material and, then, poured into the receiving hopper, where a screw feeds the crusher that is equipped with fix hole grid and groove knives impeller at a temperature of 25-27 °C. The paste produced falls into the malaxing part; malaxing was carried out for 45 min at 28-30 °C. The resulting olive paste was kept at 23-27 °C without the addition of water. Afterwards, olive oil samples were filtered and stored in dark glass bottles (250 mL), excluding any head space volume, in order to assure the proper conservation of the olive oil against oxidation, and kept into the freezer at −18 °C until the analysis. Samples were frozen to avoid any deterioration of their quality and composition until the analysis.

2.2 Determination of oil quality parameters

Free fatty acids (expressed as percentage of oleic acid (%)), peroxide value (given as milliequivalents of active oxygen per kilogram of oil (meq O_2/kg)), and UV absorption characteristics (K_{232} and K_{270}) were determined according to the analytical methods described in the European Union Commission Regulations EEC/2568/91 and the subsequent amendments [37]. For each sample, all the parameters were determined in triplicate.

2.3 Triacylglycerols composition

2.3.1 Standards and reagents

Standards of trilinolein (LLL), triolein (OOO), tripalmitin (PPP), tristearin (SSS), trilinolenin (LnLnLn), tripalmitolein (PoPoPo), 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol (PLL), 1,2-dilinoleoyl-3-oleoylrac-glycerol (OLL), 1,2-dipalmitoyl-3-oleoyl-rac-glycerol (PPO), 1,2-dioleoyl-3-stearoyl-rac-glycerol (SOO), 1-palmitoyl-2-oleoyl-3-linoleoylglycerol (POL), and 1,2-dioleoyl-3-palmitoyl-racglycerol (POO), with a purity of, at least, 98% were purchased from Sigma (St. Louis, MO, USA). Within this paper, TAG names are abbreviated by means of three letters corresponding to the fatty acid bound to the glycerol

backbone. In alphabetic order: L, linoleic acid (C18:2); Ln, linolenic acid (C18:3); O, oleic acid (C18:1); P, palmitic acid (C16:0); Po, palmitoleic acid (C16:1); and S, stearic acid (C18:0).

All the solvents used for TAG determination were high performance liquid chromatography (HPLC) grade and used without further purification. Chloroform and *n*-hexane were provided by Panreac (Barcelona, Spain), acetonitrile and diethyl ether were purchased from Lab-Scan (Dublin, Ireland), and acetone was obtained from Prolabo (Barcelona, Spain). The solvents used for determining spectrophotometric indices, free acidity, and peroxide value were of spectrophotometric grade.

2.3.2 Solid-phase extraction

A 0.2 g aliquot of each olive oil sample were weighed into a proper tube and diluted with 0.5 mL of *n*-hexane. Silica-gel cartridges of 1000 mg/8 mL reservoir volume, were connected to a vacuum manifold (Visaprep DL, Supelco), fitted with a small vacuum pump, and conditioned with 10 mL of *n*-hexane before the application of oil solution. The TAG fraction was then extracted with subsequent elution using mixtures of 10 mL of *n*-hexane-diethyl ether (87:13, v/v). Subsequently, the eluent solution was evaporated to dryness under reduced pressure at room temperature. The resulting dry residue was dissolved in 2 mL of acetone and filtered through a 0.22 μm membrane filters (Millipore) before the chromatographic analysis.

2.3.3 High performance liquid chromatography analysis

The identification and quantification of TAG compounds were performed using an Waters Alliance 2690 separation module (Waters, Milford, MA, USA) equipped with a degasser, quaternary pump, column oven, autosampler and a Waters 2414 refractive index detector. Separation was achieved on a Lichrospher 100 RP-18 (250 mm × 4 mm id; 5 μm particle size; Merck, Germany) column. The temperature of the column was set at 35 °C. The injection volume was 10 µL, and the mobile phase, at a constant flow rate of 1 mL/min over 47 min, consisted of acetonitrile/acetone (52:48, v/v). The molecular structure of each individual TAG was described by means of the calculation of its equivalent carbon number (ECN) defined as ECN = CN − 2*n*, where CN is the number of carbons of the fatty chains, and *n* is the total number of the double bonds present in

the three fatty acids bound to the glycerol backbone. Triacylglycerols identification was carried out by comparison with the standards analyzed under the same chromatographic conditions, by using reference chromatograms corresponding to olive oil analyzed with acetonitrile/acetone (52:48, v/v) as mobile phase and by comparison with previously published results [33, 35].

2.4 Data Analysis

2.4.1 Univariate analysis

The results of olive oil quality criteria and TAG composition of the studied samples are presented as means ± standard deviation. Significant differences between means were determined by an analysis of variance applying a Tukey's test. The geographical origin of the studied samples was taken as the independent variable, whereas TAG compounds and physicochemical quality criteria values were considered as the dependent variables. Differences were considered statistically significant when $p < 0.05$ (the probability was greater than 95%). The univariate statistical analysis was performed using SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

2.4.2 Multivariate analysis

In a first step, PCA was applied. This procedure provides a visualization of the data in a reduced dimension plot retaining the maximum amount of variability. Orthogonal axes are extracts according to the successive directions of maximum variance of data: the principal components or eigenvectors. Thus, by examining dimensional plots, in the spaces defined by the principal components in which samples and variables are visualized (scores plot and loadings plot, respectively), we can explore the existence of a natural clustering of the studied samples and evaluate variables relevance and correlations.

Subsequently, the Kennard-Stone algorithm [38] was used in order to separate the samples in two groups: a training group (188 samples) and a test group (91 samples), so that samples coming from all regions were obviously included in both sets. Such division allows a sufficient number of samples in the training set and a representative number of members among the test set. Variables were then auto-scaled and normalized. Then, three different statistical pattern recognition techniques (two classification techniques: LDA and PLS-DA) and one class-modeling method (SIMCA) were used to discriminate and

classify the evaluated olive oil samples according to their geographical origin and detect potential geographical markers.

LDA is a linear and parametric classification method based on maximizing the variance between classes and minimizing it within classes. In this study, LDA was used in the stepwise mode and the probability values of F_{in} and F_{out} were 0.05 and 0.10, respectively. The reliability of the classification was studied in terms of recognition ability (percentage of olive oil samples correctly classified) and prediction ability (percentage of olive oil samples correctly classified by using the cross validation and external validation methods).

PLS-DA is a discriminant classification technique based on partial least squares regression that builds latent variables, which are a combination of original variables. The optimal number of latent variables was estimated, in this case, using a cross-validation.

As far as SIMCA is concerned, it builds a mathematical model of each class, called disjointed class models or SIMCA boxes, by applying PCA to each class individually. A sample is accepted by the specific class if its distance to the model is not significantly different from the class residual standard deviation. SIMCA was applied, considering a 95% confidence level, to define the class space and an weighted enlarged distance [39].

The models achieved by PLS-DA and SIMCA were evaluated in terms of sensitivity (percentage of samples belonging to the class which are correctly identified by the model), specificity (the percentage of samples foreign to the class which are classified as foreign) and accuracy (percentage of correctly predicted samples divided by the number of samples in the class).

For the multivariate data analysis, Microsoft® Excel 2013/XLSTAT© (Version 2013.2, Addinsoft, Inc., Brooklyn, NY, USA) was used to perform LDA analysis, whereas PCA, PLS-DA and SIMCA analyses were performed on MATLAB 2012a platform (The MathWorks, Inc. Natick, MA, USA) with PLS toolbox (version 6.71, Eigenvector Research, Inc, USA).

3 Results and discussion

3.1 Physicochemical quality criteria

Table 1 shows the mean and standard deviation of the physicochemical quality criteria values obtained for the different olive oil samples grouped by region of provenance.

Table 1. Mean values and standard deviations (SD) regarding the quality criteria (Free Fatty Acids (FFAs), Peroxide Value (PV), Extinction Coefficients K_{270} and K_{232}) of the studied olive oil samples.

Geographical origin	Physicochemical quality indices	Mean± S.D.	Minimum	Maximum	Category	
					Extra-virgin	Virgin
Chefchaouane (2011: n=5; 2012: n=5; Total: n=10)	FA (%)	0.39a±0.20	0.17	0.72	8	2
	PV (meq O_2/kg)	5.38a±1.59	3.50	8.32		
	K_{232}	1.40a±0.48	0.31	2.30		
	K_{270}	0.15a±0.03	0.09	0.20		
Fès (2011: n=21; 2012: n=20; Total: n=41)	FA (%)	0.37a±0.12	0.20	0.68	40	1
	PV (meq O_2/kg)	4.27b±0.72	3.00	6.29		
	K_{232}	1.25abc±0.48	0.21	2.01		
	K_{270}	0.12a±0.04	0.05	0.21		
Meknès (2011: n=35; 2012: n=34; Total: n=69)	FA (%)	0.28a±0.16	0.11	0.94	60	9
	PV (meq O_2/kg)	4.54b±1.03	3.00	7.00		
	K_{232}	1.15abc±0.68	0.28	2.43		
	K_{270}	0.11a±0.04	0.08	0.21		
Ouazzane (2011: n=17; 2012: n=20; Total: n=37)	FA (%)	0.34a±0.15	0.14	0.85	32	5
	PV (meq O_2/kg)	4.41b±1.09	2.47	6.95		
	K_{232}	1.61ad±0.32	0.96	2.43		
	K_{270}	0.07b±0.03	0.05	0.11		
Sefrou (2011: n=17; 2012: n=22; Total: n=39)	FA (%)	0.38a±0.20	0.15	0.98	27	12
	PV (meq O_2/kg)	4.09b±0.59	3.00	5.62		
	K_{232}	1.52abd±0.56	0.21	2.52		
	K_{270}	0.12a±0.05	0.08	0.26		
Taounate (2011: n=20; 2012: n=29; Total: n=49)	FA (%)	0.35a±0.16	0.15	0.88	46	3
	PV (meq O_2/kg)	4.55b±0.83	3.00	6.08		
	K_{232}	1.20ab±0.48	0.21	2.29		
	K_{270}	0.11a±0.09	0.05	0.23		
Taza (2011: n=16; 2012: n=18; Total: n=34)	FA (%)	0.39a±0.20	0.20	0.98	28	6
	PV (meq O_2/kg)	4.40b±0.70	3.00	5.38		
	K_{232}	1.53abd±0.45	0.33	2.37		
	K_{270}	0.11a±0.06	0.05	0.24		
				Total	241	38

-Significant differences are indicated with different lowercase letters (comparison between studied regions, $p < 0.05$). The comparison of the letters of the one way ANOVA has to be made considering the values for the same parameter for different geographical regions.

-In the current table and Table 2 the mean values are those calculated for all the samples coming from the same area, therefore, SD gives to the reader only an idea about the variability of the olive oils in terms of composition, and obviously not about the repeatability of the analytical methods used.

Since the olive samples used in this study were healthy, not damaged and carefully selected, picked and processed, the obtained oils showed high physicochemical quality (taking into account free fatty acids, peroxide value and UV absorption characteristics), as can be observed in the table. Indeed, keeping in mind the limits set by the International Olive Council (IOC) regulation [40], from a physicochemical quality point of view, among the total of 279 analyzed samples, 261 were falling into the "Extra-virgin" olive oil category (which correspond to 93.55% of analyzed oils), whereas the rest (6.45%) were classified as "Virgin" olive oils. Mean values of free fatty acid content, expressed as percentage of oleic acid, ranged from 0.28 up to 0.39%; peroxide values were found between 4.09 and 5.38 meq O_2/kg; and K_{232} and K_{270} ranged from 1.15 up to 1.61 and from 0.07 up to 0.15, respectively.

3.2 Triacylglycerols profiling

The mean concentration and standard deviation (mean ± SD (%)) for the TAGs identified in the studied north Moroccan olive oil samples are listed in Table 2. As can be seen in this table, the TAG profiles for the samples coming from different regions were quite similar from the qualitative point of view. A total of 18 peaks were identified in the profiles, among which 15 represented one TAG and 3 corresponded to two TAG compounds. The identified TAG compounds include four ECN42 compounds (LLL, OLLn+PoLL, PLLn), four ECN44 (OLL, OOLn, PLL and POLn), six ECN46 (OOL+PPLn, PoOO, PoOP and SLL+PLO), five ECN48 compounds (PLP, OOO, SOL, POP and POO), and two ECN50 compounds (SOO and POS).

When the total concentration of ECN groups is considered, the compounds classified within the ECN48 group were the most abundant in the studied oil samples, mainly due to the contribution of OOO and POO; they were found between 58.22 and 67.68%, in olive oils produced in Fès and Sefrou, respectively. The second group in terms of abundance was ECN46, ranging between 20.76% and 27.00% in oils from Chefchaouane and Fès, respectively; it was followed by ECN50 group and/or total ECN44, depending on the observed geographical area. As far as ECN50 family is concerned, the total values were found within the range 5.13 (Ouazzane)-6.29% (Taounate).

Table 2. Quantitative results (Mean ± Standard Deviation, %) for TAGs in the studied samples considering the provenance region.

			Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza
ECN42	LLL	Mean ± SD	0.05a±0.02	0.41b±0.14	0.14a±0.06	0.28c±0.13	0.11a±0.03	0.25c±0.09	0.14a±0.06
	OLLn +PoLL		0.35a±0.13	0.52b±0.17	0.37a±0.09	0.50b±0.03	0.37a±0.06	0.38a±0.07	0.48b±0.09
	PLLn		0.07acd±0.03	0.11b±0.03	0.08c±0.03	0.10b±0.01	0.06d±0.01	0.08c±0.02	0.07acd±0.02
	Total ECN 42		0.58ade±0.29	1.04b±0.30	0.59ad±0.14	0.88c±0.16	0.54d±0.09	0.71e±0.15	0.70ae±0.16
ECN44	OLL		2.96ade±0.88	4.80b±0.88	2.41cd±0.71	3.45a±0.91	2.08c±0.51	3.52a±0.75	2.90e±0.63
	OOLn		1.58ab±0.21	1.79a±0.26	1.68a±0.32	1.77a±0.04	1.76a±0.06	1.47b±0.17	2.13c±0.19
	PLL		0.46a±0.27	0.90b±0.54	0.48a±0.14	0.85b±0.14	0.45a±0.1	0.46a±0.07	0.76b±0.17
	POLn		0.63ab±0.13	0.66a±0.16	0.66a±0.14	0.66ab±0.10	0.58b±0.03	0.43c±0.08	0.76d±0.11
	Total ECN 44		5.03ade±1.64	8.16b±1.61	5.24ad±0.99	6.73c±0.72	4.87d±0.64	5.88e±0.83	6.54ec±0.80
ECN46	OOL+PPLn		14.23ace±3.79	19.03b±1.19	14.91ce±1.68	16.87dfg±1.49	15.01eg±1.58	17.72f±1.42	16.07g±1.34
	PoOO		1.19abde±0.38	1.28abe±0.22	1.17ad±0.14	1.12acd±0.18	0.91f±0.12	1.31ae±0.14	1.01acf±0.16
	SLL+PLO		4.62acd±1.43	5.85e±1.00	5.41ae±1.26	6.58b±0.46	4.71cd±0.46	4.57d±0.49	5.41ae±0.67
	PoOP		0.72abcde±0.35	0.84abc±0.50	0.96b±0.42	0.67c±0.30	0.42de±0.06	1.01b±0.20	0.37e±0.24
	Total ECN 46		20.76ace±4.84	27.00b±1.64	22.45c±2.85	25.24d±1.65	21.05e±1.93	24.61d±1.94	22.86ac±1.89
ECN48	PLP		0.44ad±0.05	0.38b±0.04	0.42a±0.04	0.38c±0.01	0.45d±0.02	0.44d±0.02	0.40a±0.03
	OOO		45.62ac±4.71	39.33b±4.11	43.20ac±4.00	39.62b±0.83	46.67ad±2.03	45.33ad±2.54	41.91c±2.77
	SOL		0.55ace±0.08	0.47b±0.06	0.57ce±0.06	0.55ac±0.05	0.52ae±0.03	0.43d±0.03	0.55e±0.04
	POP		2.63abde±0.84	2.25b±0.64	2.94ade±0.76	2.89de±0.57	2.42ab±0.20	1.67c±0.35	2.94d±0.50
	POO		18.37ace±2.81	15.79b±1.86	19.33ce±1.90	18.54ace±1.73	17.61ae±0.84	14.64d±0.97	18.58e±1.28
	Total ECN 48		67.61ac±6.42	58.22b±2.69	66.46a±3.58	61.99d±2.48	67.68a±2.77	62.51cd±2.79	64.39c±2.72
ECN50	SOO		5.12ac±1.30	4.81ad±0.82	4.47a±0.63	4.28b±0.24	5.04ad±0.55	5.53c±0.81	4.66abd±0.62
	POS		0.79abcd±0.09	0.75ad±0.08	0.79abcd±0.11	0.85bc±0.1	0.82c±0.07	0.76d±0.14	0.78acd±0.09
	Total ECN 50		5.91abc±1.35	5.56ac±0.85	5.26c±0.70	5.13c±0.27	5.86ab±0.61	6.29b±0.90	5.44ac±0.68

Significant differences within the same line are indicated with different lowercase letters (comparison between studied regions, $p < 0.05$).

ECN44 total values fluctuated from 4.87 to 8.16% of the total TAG composition, being Sefrou oils the samples with the lowest mean concentration, whilst Fès samples showed the highest one. The compounds included in ECN42 group were found at low levels, ranging from 0.54 to 1.04% in oil samples produced in Sefrou and Fès, respectively.

After having a look at the results by groups, we studied the individual results. The predominant one was OOO (39.33-46.67%), followed by POO (14.64-19.33%), OOL+PPLn (14.23-19.03%), SLL+PLO (4.57-6.58%), SOO (4.28-5.53%), OLL (2.08-4.80%), POP (1.67-2.94%), OOLn (1.47-2.13%), PoOO (0.91-1.31%), and PoOP (0.37-1.01%). The other eight TAGs (LLL, OLLn+PoLL, PLLn, PLL, POLn, PLP, SOL and POS) had mean concentration values below 1% (Table 2).

Keeping in mind the possible influence of the other factors of olive oil composition variation (mainly the stage of maturity of the processed olives), the results of TAGs composition obtained in this work for monovarietal Picholine Marocaine north Moroccan olive oils, could be compared with those obtained for oils from other Moroccan regions or other Mediterranean countries, focusing on the content of some characteristic TAG compounds, such as LLL, OOO and POO.When our results are compared with the values obtained for oils from the same variety produced in other Moroccan regions, it is possible to highlight that, in general terms, north Moroccan olive oils show OOO mean concentration values higher than those previously published for olive oils produced in south-center Moroccan regions (OOO (33.22-39.06%) [41] and lower than those levels reported for olive oils produced in eastern zones OOO (47.54-58.30%) [35]. The results obtained for TAG composition of north Moroccan monovarietal olive oils remain comparable to those obtained for French olive oils (LLL(0.06-0.31%), OOO (32.63-54.59%) and POO (17.22-21.72%) [33]; Italian monovarietal olive oils (LLL (0.04-0.10%) and OOO (28.60-48.25%)) [42]; Tunisian monovarietal olive oils (LLL (0.18-1.38%), OOO (21.70-39.37%) and POO (18.84-22.01%) [43], and Turkish monovarietal olive oils (LLL (0.04-51%), OOO (24.72-48.68%) and POO (21.09-28.86%) [44].

One-way ANOVA and Tukey's HSD post hoc test involving as a factor the geographical origin of studied samples were carried out to detect significant differences. Before applying ANOVA, normality and homogeneity of variances tests were applied, by using

Shapiro-Wilk test and Levene's test, respectively. The ANOVA results are reported in Table 2. As can be seen from this table, significant differences ($p < 0.05$) were found among the seven geographic zones considering all the evaluated parameters (ECN group total content and the amounts of individual TAGs in the studied samples).

3.3 Classification of olive oils according to geographical origin

3.3.1 Principal components analysis

The results on TAGs individual content and the total amounts of ECN42, ECN44, ECN46, ECN48 and ECN50 for the 279 samples were used to build the matrix for the PCA analysis. PCA was utilized as an unsupervised multivariate analysis method in order to get a general overview of the ability of the whole set of TAG variables for grouping the studied north Moroccan olive oil samples. The scores plots obtained for the first four PCs, which explained the 89.99% of the total variance in the raw data (PC1 = 46.09%, PC2 = 29.38%, PC3 = 8.35% and PC4 = 6.17%) are shown in Figure 2A, 2B and 2C.

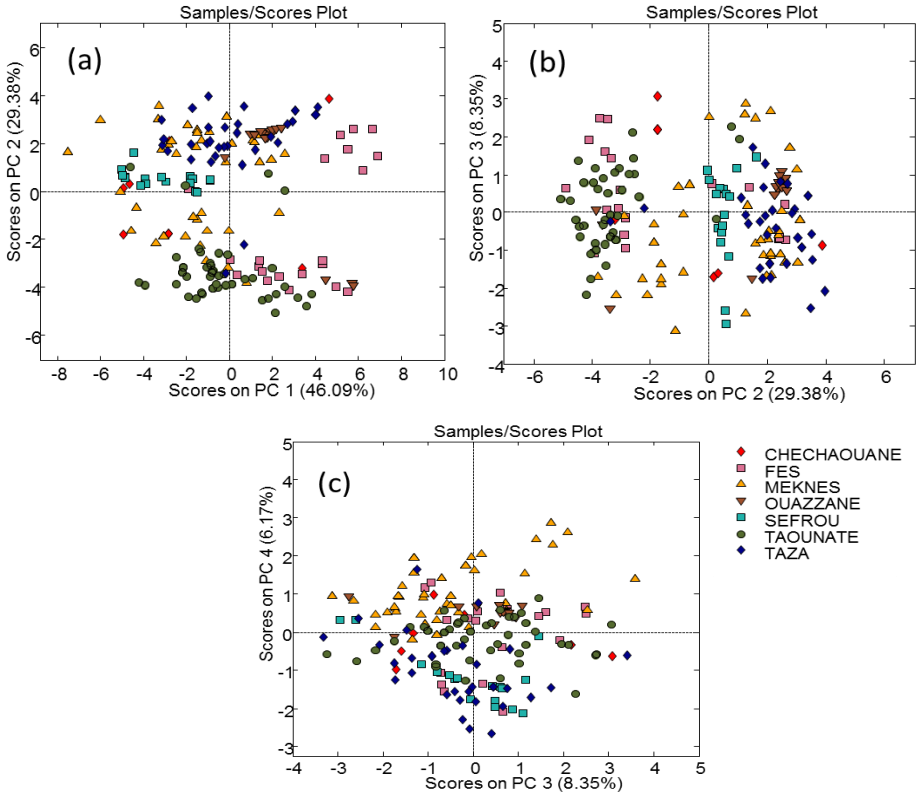

Figure 2. Scores plot for the 279 olive oil samples on the map defined by the first two principal components (Fig 2a), second and third principal components (Fig 2b), and third and fourth ones (Fig 2c).

As can be seen in Fig 2A (when the score plot for PC1 and PC2 is considered), there is a quite clear overlapping between the studied groups, except for two clusters, formed by some samples from Taounate and Sefrou regions which are slightly separated from the rest (even if their separation among samples from other regions was not complete). This result, therefore, demonstrated that it was required the use of other discriminant analysis approaches to attempt the geographical classification of studied samples. In the coming sections, the different used methods will be described and discussed, and the most relevant results presented.

3.3.2 Linear discriminant analysis

LDA was applied to the content of the TAG compounds listed in Table 2 and the total contents of ECN42, ECN44, ECN46, ECN48 and ECN50. However, given the fact that LDA technique requires the data matrices for each class to have a high ratio between the number of samples and the number of variables [45], within this study we decided to apply a stepwise procedure with leave-one-out cross-validation as variables reduction technique, because of the relatively low number of samples for Chefchaouane class (if compared with the other areas). For doing it, the technique was applied using the Wilks' lambda method with the usual probabilities of F (0.05) for a variable to be included and F (0.10) to be removed from the model, and led to remove five variables: PoOO, PLP, OOO, SOL and total ECN50. A LDA model was then built achieving very satisfactory statistical parameters. Results showed that six statistically significant discriminant functions were formed (Wilk's Lamda = 0.000, X2 = 569.210, df = 102, $p = 0.000 < 0.05$ for the first function, Wilk's Lamda = 0.003, X2 = 1047.065, df = 80, $p = 0.000 < 0.05$ for the second function, Wilk's Lamda = 0.017, X2 = 708.646, df = 60, $p = 0.000 < 0.05$ for the third function, Wilk's Lamda = 0.103, X2 = 398.560, df = 42, $p = 0.000 < 0.05$ for the fourth function, Wilk's Lamda = 0.317, X2 = 200.898, df = 26, $p = 0.000 < 0.05$ for the fifth function, and Wilk's Lamda = 0.665, X2 = 71.43, df = 12, $p = 0.000 < 0.05$ for the sixth function). It was found that the first three canonical discriminant functions explained more than the 85% of the total variance (function 1 explained the 36.51% of the total variance; function 2 explained 27.50%, and the 21.04% of the total variance was explained by function 3). Besides, by evaluating the standardized canonical discriminant function coefficients (indicated between brackets in absolute values), the variables

pointed out as the most relevant for the geographic origin discrimination of the studied samples were: OLL (0.564), OOL+PPLn (0.481) and POO (0.520) for the first discriminant function; LLL (0.606), total ECN46 (0.447) and total ECN48 (0.410) for the second discriminant function; and OOLn (0.413) and PLL (0.214) for the third one.

Table 3. Stepwise Linear Discriminant (S-LDA) classification results for the North Moroccan VOOs under study considering the triacylglycerol data.

	Calibration recognition ability								
	Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza	Total	% correct
Chefchaouane	7	0	0	0	0	0	0	7	100.00%
Fès	0	28	0	0	0	0	0	28	100.00%
Meknès	0	0	43	1	2	0	0	46	93.48%
Ouazzane	0	0	0	25	0	0	0	25	100.00%
Sefrou	0	0	0	0	26	0	0	26	100.00%
Taounate	0	1	0	0	0	32	0	33	96.97%
Taza	0	0	0	0	0	0	23	23	100.00%
Total	7	29	43	26	28	32	23	188	97.87%
	Cross-validation								
	Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza	Total	% correct
Chefchaouane	6	0	0	0	0	1	0	7	85.71%
Fès	0	27	0	0	0	1	0	28	96.43%
Meknès	0	0	43	1	2	0	0	46	93.48%
Ouazzane	0	0	0	25	0	0	0	25	100.00%
Sefrou	0	0	0	0	26	0	0	26	100.00%
Taounate	0	1	0	0	0	32	0	33	96.97%
Taza	0	0	0	0	1	0	22	23	95.65%
Total	6	28	43	26	29	34	22	188	96.28%
	External prediction ability								
	Chefchaouane	Fès	Meknès	Ouazzane	Sefrou	Taounate	Taza	Total	% correct
Chefchaouane	3	0	0	0	0	0	0	3	100.00%
Fès	0	13	0	0	0	0	0	13	100.00%
Meknès	0	0	19	2	2	0	0	23	82.61%
Ouazzane	0	0	0	11	1	0	0	12	91.67%
Sefrou	0	0	0	0	13	0	0	13	100.00%
Taounate	0	1	0	0	2	13	0	16	81.25%
Taza	0	2	0	0	0	0	9	11	81.82%
Total	3	16	19	13	18	13	9	91	89.01%

When examining the LDA classification results summarized in the confusion matrices shown in Table 3, it can be observed that the overall correct classification rate was 97.87% for the calibration and 96.28% for the cross validation method; both values are

very satisfactory. Correct classification (100%) was obtained for VOO samples from Chefchaouane, Fès, Sefrou and Taza, followed by those from Taounate (96.97%) and Meknès (93.48%). Furthermore, the LDA model provided perfect results (100%) in the validation set (external prediction ability) for samples from three regions (Chefchaouane, Fès and Sefrou), whilst the correct classification rates in the validation set obtained for Ouazzane, Meknès, Taza and Taounate were of 91.67%, 82.61%, 81.82% and 81.25%, respectively. These findings are in good agreement with previous studies where TAGs data were combined with LDA statistical analysis to determine the geographic origin of VOOs. Indeed, Stefanoudaki et al., [46] successfully used this strategy for Cretan olive oils. More recently, another interesting study carried out by Ollivier et al., [32] has reported a very satisfactory classification rate for French olive oils taking into account their botanical and geographical origin, using TAGs and fatty acids data and a stepwise LDA multivariate analysis. The same research group, a bit later, tried to combine data about sensory characteristics, fatty acid, TAG compositions and LDA for the discrimination of French olive oils from five different PDOs [33], achieving very proper results.

3.3.3 Partial Least Squares Discriminant Analysis

PLS-DA was carried out on the same data set, using seven dummy variables to code factor treatment, and the selection of the optimal number of latent variables was done using the criterion of lowest prediction error in leave-one-out cross validation. The optimal complexity of the PLS-DA model was found to be 13. Furthermore, the model classification performance (considering sensitivity, specificity and accuracy) was, preliminarily, validated internally by applying cross-validation on the training set (calibration), and then the model was applied to the external test set. Results are shown in Table 4. As can be observed within the table, the used PLS-DA model led us to quite successful results in the calibration phase and rather good predictive ability over the internal and external validation; indeed, high sensitivity and specificity levels were achieved. The model sensitivity was superior to 92% in calibration, 88% in cross-validation and 75% in prediction, whereas the specificity of the model was superior to 92.30% in calibration, 90.10% in cross-validation and 82.10% in prediction. As far as accuracy is concerned, the recognition and predictive (both cross-validation and external

validation) abilities were particularly high for samples from three regions: Chefchaouane, Fès and Sefrou. Indeed, in recognition (calibration) the accuracy was 100% for samples from these three regions; whereas in the cross-validation, values of accuracy were 99.17%, 98.77% and 94.02% for samples coming from Chefchaouane, Sefrou and Fès, respectively.

Table 4. Main parameters calculated for the PLS-DA models used for the discrimination of North Moroccan VOO samples according to the region of provenance.

Classes	LV	Calibration			Cross-validation		
		Sensitivity (%)	Specificity (%)	Accuracy (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
Chefchaouane	13	100	99.4	100	100	97.8	99.17
Fès	13	100	97.5	100	100	95.6	94.02
Meknès	13	95.7	94.4	94.95	93.5	90.1	89.51
Ouazzane	13	92	92.6	95.69	88	90.8	93.08
Sefrou	13	100	93.8	100	100	93.8	98.77
Taounate	13	93.9	92.3	96.65	93.9	90.3	94.16
Taza	13	100	98.8	97.52	100	98.2	97.52

Classes	Prediction		
	Sensitivity (%)	Specificity (%)	Accuracy (%) (%)
Chefchaouane	100	94.3	99.43
Fès	100	93.6	98.72
Meknès	87	97.1	82.61
Ouazzane	91.7	83.5	94.57
Sefrou	100	82.1	93.59
Taounate	75	96	77.46
Taza	81.8	93.8	89.66

For the other studied regions, the model was able to correctly classify (in calibration) 97.52%, 96.65%, 95.69% and 94.95% for olive oil samples produced in Taza, Taounate, Ouazzane and Meknès, respectively; whereas the predictive ability in cross-validation was 97.52% (Taza samples), 94.16% (Meknès region), 93.08% (Ouazzane) and 89.51% (Meknès). Besides, when the model was applied to the external test set samples, the prediction accuracy values were: 99.43% (Chefchaouane), 98.72% (Fès), 94.57% (Ouazzane), 93.59% (Sefrou), 89.66% (Taza), 82.61% (Meknès) and 77.46% (Taounate).

Additionally, the variable importance in projection (VIP) was evaluated, in order to identify the most significant variables for the prediction ability of the model. When the

VIP value is greater than one, the variable is normally considered as important for the model. Thus, by analyzing the obtained VIP values -indicated between brackets- it can be concluded that the most influential variables for class prediction were 5 for Chefchaouane samples (LLL (4.34), Total ECN42 (3.07), OLL (2.00), PLL (1.70) and SLL+PLO (1.70)); 3 for Fès samples (LLL (2.61), OLL (2.10) and POLn (1.90)); 3 for the samples produced in Meknès (PoOP (4.87), OOLn (2.22) and PoOO (1.85)); 4 for Ouazzane samples (OOLn (2.75), POLn (2.39), POS (1.65) and SLL+PLO (1.57)); 6 for Sefrou olive oils (LLL (2.43), PoOP (2.03), OOLn (1.69), PoOO (1.52), SLL+PLO (1.48) and POLn (1.43)); 7 for the samples coming from Taounate region (POLn (2.09), OOLn (1.70), PLL (1.69), POP (1.59), POO (1.49), SOL (1.45) and OLL (1.38)); and 6 for Taza samples (OOLn (2.91), PoOP (2.23), SLL+PLO (1.74), POLn (1.61), PoOO (1.49) and POS (1.48)).

3.3.4 Soft Independent Modeling of Class Analogies

In SIMCA, independent class models were built for each one of the 7 investigated classes. The optimum number of PCs used for each class was determined by the leave-one-out cross-validation. Three PCs were retained to describe Chefchaouane, Fès and Ouazzane classes, while four PCs were found to be optimal to properly define Meknès, Sefrou, Taounate and Taza classes. Furthermore, scores and loadings plot, of both SIMCA model and each class sub-model, were interpreted showing that all the variables included in SIMCA construction exhibited good modeling capability. The results of SIMCA analysis conducted to try to classify the studied samples are summarized in Table 5. As shown in this table, by applying SIMCA technique to the calibration set, sensitivity, specificity and accuracy were 100% for samples from Chefchaouane, Ouazzane and Sefrou. Moreover, very high values of sensitivity (99.40%), specificity (99.39%) and accuracy (99.70%) were obtained for Taza class. For the other classes, lower values of these parameters were, in general, obtained.

When the built SIMCA models were applied to the external test set, sensitivity values for the validation samples were over 75.59% for all the studied classes, with the exception of Chefchaouane model, where the achieved value was 0% as a result of the incorrect recognition of the three samples of this region used in the validation model (Table 5).

Table 5. Main parameters calculated for the SIMCA models used for the discrimination of North Moroccan VOO samples according to the region of provenance.

Clases	PCs	Calibration			Prediction		
		Sensitivity (%)	Specificity (%)	Accuracy (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
Chefchaouane	3	100	100	100	0	50	33.33
Fès	3	76.27	70	83.21	79.59	74.36	87.18
Meknès	4	78.59	73.94	84.8	75.79	75	76.63
Ouazzane	3	100	100	100	100	100	95.83
Sefrou	4	100	100	100	100	100	100
Taounate	4	92.61	92.26	94.61	91.34	94.67	75.46
Taza	4	99.4	99.39	99.7	95.1	96.25	84.49

Regarding the models specificity, good results were obtained, being for most of the studied classes higher than 74.36%; only Chefchaouane model showed a lower value (50%). Considering the accuracy of these models, in contrast to the quality of the accuracy of external prediction obtained for LDA and PLS-DA, it is possible to observe a decrease regarding this parameter for the majority of the studied classes, with the exception of Sefrou model that showed a value equal to that obtained by LDA (100%) and slightly higher than the one obtained using PLS-DA.

When comparing the external prediction ability of the three tested chemometric methods, it can be deduced that LDA was the technique that gave the best results in predicting samples from Chefchaouane, Fès, Sefrou, Meknès and Taounate; samples from Taza region were better predicted using PLS-DA, whereas the higher rate of successfully predicted samples from Ouazzane region was obtained by using SIMCA approach.

4 Conclusions

The novel contributions of this work are the following: (i) the triglycerides fraction of VOO samples coming from the main north Moroccan olive growing areas was comprehensively characterized for the first time; (ii) the number of analyzed samples is much higher than in others previously published where Moroccan samples were considered, and covers two consecutive crop seasons in order to take into account the possible inter-annual variations; (iii) we have evaluated the potential of four chemometric techniques for carrying out the data treatment.

According to the results reported in this study, it is possible to confirm that data of TAG fraction composition combined with chemometric techniques seems to be a very promising and valuable approach for discriminating VOOs of the main north Moroccan olive growing areas. LDA and PLS-DA gave the best result (in terms of accuracy for both training and test sets), but also the other classification procedure used here (SIMCA) achieved a satisfactory and correct geographical classification and demonstrated that, for most of the considered regions in the current study, useful information could be extracted from TAG data for the geographical discrimination of their VOOs.

From our point of view, our results should encourage further studies to extend the discriminant approach established here to VOOs produced in other Moroccan olive oil producing areas; moreover, this approach could stand for as a useful tool to demonstrate and check typicality of Moroccan PDO's VOOs (or for PODs from some other countries).

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Chapter 7

In-depth two-years study of phenolic profile variability among olive oils from autochthonous and Mediterranean varieties in Morocco, as revealed by a LC-MS chemometric profiling approach

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Abstract

Olive oil phenolic fraction considerably contributes to the sensory quality and nutritional value of this foodstuff. Herein, over two consecutive crop seasons (2012/2013 and 2013/2014), the phenolic fraction of 203 olive oil samples extracted from fruits of 4 autochthonous Moroccan cultivars ('Picholine Marocaine', 'Dahbia', 'Haouzia' and 'Menara'), and 9 Mediterranean varieties recently introduced in Morocco ('Arbequina', 'Arbosana', 'Cornicabra', 'Frantoio', 'Hojiblanca', 'Koroneiki', 'Manzanilla', 'Picholine de Languedoc' and 'Picual'), were explored by using liquid chromatography-mass spectrometry. A total of 32 phenolic compounds (and quinic acid), belonging to five chemical classes (secoiridoids, simple phenols, flavonoids, lignans and phenolic acids) were identified and quantified. Phenolic profiling revealed that the determined phenolic compounds showed variety-dependent levels, being, at the same time, significantly affected by the crop season. Moreover, based on the obtained phenolic composition and chemometric linear discriminant analysis, statistical models were obtained allowing a very satisfactory classification and prediction of the botanical origin of the studied oils.

Keywords: olive oil, liquid chromatography-mass spectrometry, phenolic compounds, Moroccan varieties, Mediterranean varieties, botanical origin.

INTRODUCTION

Virgin olive oil (VOO) is a quite popular and nutritionally important and valuable vegetable oil, which is widely produced in the Mediterranean Basin and areas with similar climatic conditions,[1] for human consumption, but also with cosmetic and medicinal purposes. Data from the International Olive Council (IOC) reveal that, to date, more than 11 million ha are devoted to *Olea europaea* L. cultivation all over the world, especially, in Mediterranean countries, where 98% of this area is concentrated.[2]

Morocco represents the Southwestern extreme part of the Mediterranean olive tree landscape. In this country, olive cultivation and oil production are a rooted tradition, both as a income for more than 450.000 farmers as well as a high environmental value crop, due to its role in soil protection, particularly, in mountain farms.[3] Furthermore, over the last years, Moroccan olive area has remarkably increased from 763.000 ha in

2007/2008 to 1.020.000 ha in 2013/2014.[2,4] Likewise, during the same period, Moroccan olive oil production has increased from 85.000 to 130.000 tons,[5] making this crop as one of the most profitable and strategic horticulture crop in the country. However, despite this importance, the productivity of the olive sector in most Moroccan olive growing areas is still far below the local potential, showing a strong year-to-year fluctuation.[6] One of the main reasons is that olive cultivation in Morocco has traditionally been based on planting a single cultivar so-called 'Picholine Marocaine' (occupying almost 90% of the total Moroccan olive cultivated area).[7] This autochthonous cultivar shows good adaptability to a wide range of Moroccan pedoclimatic conditions, and exhibits interesting agronomic traits, oil quality, and composition characteristics;[8] however it presents some limitations, such as an accentuated alternate bearing[6] and susceptibility to the main olive fungal diseases, particularly those caused by *Spilocaea oleagina*, *Verticillium dahliae* and *Fusarium solani*.[9] Moreover, extensive research supports the high phenotypic and genetic variability of this cultivar in different Moroccan olive growing regions (almost in the same orchard), suggesting that the name of 'Picholine Marocaine' encompass a pool of various local genotypes.[10,11] In the agricultural context, this fact can complicate the olive orchard management when nutrients, irrigation and other agricultural practices have to be applied accordingly to the cultivar demands and its agronomic characteristics. Therefore, to cope with these constraints that can hinder the development of the olive oil sector in Morocco, and meet the current needs of Moroccan olive oil industry searching for more productive cultivars with specific traits, the National Institute of Agronomic Research has extensively worked on breeding programs, mainly based on clonal selection (which allowed getting some clonal selection genotypes, such as 'Haouzia' and 'Menara'),[12] cross-breeding,[13] and comparative studies of agronomic and adaptive traits of some Mediterranean varieties recently introduced in Morocco.[14]

Similarities and differences (in terms of morphological and agronomical characteristics) among promising selected Moroccan autochthonous and recently introduced olive varieties are, to date, well established.[12,14,15] Researches characterizing oils obtained from these varieties have mostly focused on their quality parameters evaluation, although some studies can be also found regarding genetic, environmental and technological factors influencing the quality of these oils, and their fatty acids and triacylglycerols composition.[7,16-21] However, to the best of our knowledge, there are no studies evaluating

the nutritional value of these oils. Indeed, we can say that there is a knowledge gap concerning their composition and content on bioactive compounds, such as phenolic compounds, phytosterols and tocopherols; substances that are widely recognized as some of the most relevant components responsible of diverse nutritional and health benefitial properties of olive oil.[22-26]

VOO contains a number of phenolic compounds which determine important sensory characteristics, such as astringency and bitterness.[22,27] These compounds and their various biological activities, such as antioxidant, antimicrobial, anti-inflammation, anti-aging, anticancer and cardioprotective properties have been associated with the beneficial effects of this oily matrix.[28] Besides, phenolic compounds have an important role in pattern recognition studies, being described as relevant markers for the botanical and geographical origin classification of VOO.[29] All the just mentioned reasons can explain the attention paid, over the last decades, by the scientific community to these type of compounds, focusing, for instance, on the improvement of the analytical methodologies to carry out their determination,[30,31] the comprehensive characterization of their composition and content on VOOs produced from different varieties cultivated in various Mediterranean countries,[32-34] and the monitoring of changes undergone on their composition as a result of intrinsic and extrinsic factors.[35,36]

The aim of the present work was to thoroughly determine and compare, over two consecutive crop seasons (2012/2013 and 2013/2014), the phenolic profile of 'Picholine Marocaine' VOOs (as the most widespread and typical Moroccan olive oil variety) with those of oils obtained from three Moroccan clonal selected varieties ('Dahbia', 'Haouzia' and 'Menara') and nine Mediterranean varieties recently introduced in Morocco ('Arbequina', 'Arbosana', 'Cornicabra', 'Frantoio', 'Hojiblanca', 'Koroneiki', 'Manzanilla', 'Picholine de Languedoc' and 'Picual'). All the varieties were grown under identical pedoclimatic conditions in the same experimental olive orchard. Two liquid chromatography coupled to mass spectrometry (LC-MS) instruments (one of high MS resolution and another of low MS resolution) were chosen as analytical platform, achieving, for the first time, qualitative and quantitative data on the phenolic pattern of the studied oils. The possible influence of the harvest year and variety on the content of identified phenolic compounds was obviously examined. Finally, the feasibility of combining phenolic compounds data and chemometrics (Principal component analysis

(PCA) and Linear discriminant analysis (LDA)) to construct chemometric models able to trace the botanical origin of studied oils was investigated.

MATERIALS AND METHODS

Olive samples harvest, oil extraction and physico-chemical quality evaluation

Olive fruits sampling was performed over two consecutive crop seasons (2012/2013 and 2013/2014) on randomly selected trees, representing the above-mentioned 13 olive cultivars, all grown in the experimental olive grove of the Agro-pôle Olivier National School of Agriculture of Meknès, Morocco. Pest control, pruning, irrigation and fertilization practices were done following current olive orchards management practices. This location belongs to the Mediterranean bioclimate. Information about trees plantation date and spacing, as well as experimental olive grove soil characteristics and climatic conditions during the two crop seasons considered in this study, are summarized in Table 1.

From each one of the 13 cultivars investigated in this study, good quality, fresh and healthy fruits samples (30 kg each one) were randomly hand-picked from the selected trees, starting from middle of October till the end of December, collected in ventilated plastic crates and immediately sent to the laboratory for oil extraction. In the laboratory, before oil extraction, each sample was properly homogenized and 100 fruits were randomly sampled to carry out the determination of the ripening index, following the method proposed by the Agronomic Station of Jaén (Spain), based on the evaluation of the olive skin and pulp colors.[37] Thus, to avoid possible influence of the fruits maturity stage on the phenolic profiles of the studied oils, in this study, only samples picked at a ripening index within the range 3.0-3.5 were considered. This range is commonly advised for the production of high quality olive oils in Meknès region.[38] A total of 203 samples were considered, the remaining were kept for other experiments. Information about the number of samples per cultivar and crop-season are reported in Table 1.

Table 1. Data About Planting Date and Distance of the Studied Cultivars, Number of Samples per Cultivar and per Crop Season, Quality Parameters of the Obtained Oils (Mean ± Standard Deviation of Free Fatty Acids (FAAs), Peroxide Values (PV) and Ultraviolet Absorbance at 232 nm (K_{232}) and 270 nm (K_{270})), and Pedoclimatic Conditions of the Experimental Orchard During the Study Period.

	Arbequina	Arbosana	Cornicabra	Frantoio	Hojiblanca	Koroneiki	Manzanilla	Picholine Languedoc	Picual	Dahbia	Haouzia	Menara	Picholine Marocaine
Planting date	2001		2000				2001	2000	2001	2000			40 years ago
planting distances	7*5 (between rows*between trees)												10*10
2011/2012 (total = 105)	6	7	6	9	6	9	10	10	10	7	5	6	14
2012/2013 (total= 98)	10	8	5	7	7	9	7	10	8	4	6	7	10
FFAs (% oleic acid)	0.25±0.04	0.22±0.05	0.19±0.03	0.22±0.06	0.20±0.05	0.23±0.03	0.21±0.05	0.21±0.04	0.20±0.05	0.20±0.06	0.20±0.05	0.24±0.04	0.24±0.03
PV (meq O_2/kg)	4.99±0.91	5.12±0.77	5.01±0.87	5.18±0.76	5.40±0.56	5.07±0.86	5.04±0.83	5.02±0.88	5.30±0.96	4.82±0.82	5.02±0.92	5.04±0.80	5.23±0.92
K_{232}	0.94±0.11	0.90±0.15	0.92±0.10	0.95±0.13	0.95±0.14	0.94±0.13	0.95±0.12	0.95±0.12	0.94±0.13	0.95±0.12	0.93±0.15	0.98±0.10	0.95±0.12
K_{270}	0.09±0.02	0.10±0.03	0.11±0.02	0.10±0.03	0.11±0.03	0.13±0.01	0.11±0.03	0.12±0.03	0.12±0.02	0.12±0.03	0.11±0.03	0.12±0.02	0.12±0.01
Quality category	Extra-virgin												
Site climatic data													
Month		January	February	March	April	May	June	July	August	September	October	November	December
Average maximum temperatures (°C)	2012	16.3	14.9	20.9	19.6	29.9	32.9	35.4	35.9	30.7	25.48	16.67	15.25
	2013	15.51	15.52	15.59	22.7	25.19	32.3	37.53	37.97	30.83	26.8	19	16.7
Average minimum temperatures (°C)	2012	4.2	2.9	6.8	8.4	14.1	17.4	18.4	18.9	16.8	12.52	16.67	15.25
	2013	9.29	8.84	9.96	15.5	16.81	22.63	29.23	29.67	22.89	13.9	8.3	5.4
Total rainfall (mm)	2012	35.5	13	20	105	12	0	0	0	0	136.5	156	56
	2013	98.5	76.5	177	45.5	32	0	0	0	32.5	8	89	42
Soil characteristics													
	Depth (cm)	pH (H_2O)	Clay (%)	Slit (%)	Sand (%)	$CaCO_3$ (%)	Humus (%)	Al-P2O5 (mg/kg)	Al-K2O (mg/kg)	Copper (mg/kg)	Zinc (mg/kg)	Iron (mg/kg)	Manganese (mg/kg)
Horizon 1	0-30	8.5	68.3	23.8	7.9	1.7	2.59	12	493	2.91	0.36	7.98	591
Horizon 2	30-60	8.6	64.5	25.4	10.1	2.8	1.87	2	246	1	0.13	7.51	417

Afterwards, oil was extracted using an Oliomio laboratory mill (Oliomio, Italy) simulating two-phase commercial oil-extraction system. The operating mode of this instrument has been described in detail by Bajoub et al.[32] Briefly, olives were washed, deleafed, and crushed and the resulting paste was mixed at 28-30°C for 45 min, and decanted at 23-27°C without the addition of any water. Obtained oil was filtered to remove impurities, transferred into dark glass bottles without headspace and immediately frozen and stored at −18 °C for further analysis.

To evaluate the physico-chemical quality of the obtained oils, regulated criteria (free fatty acids content (given as percentage of oleic acid), peroxide value (expressed as milliequivalents of active oxygen per kilogram of olive oil (meq O_2/kg)) and K_{232} and K_{270} extinction coefficients, calculated from absorption at 232 and 270 nm, respectively) were determined, in triplicate, for each studied oil sample by using the analytical methodologies described in the European Union Standard Methods Regulations 2568/91 and the subsequent amendments.[39] Obtained results (Table 1) allow classifying all the studied oils within the "extra virgin" category.

Application of a LC-MS analytical methodology to establish the phenolic composition of the studied oils

Chemicals and reagents. All solvent used in this study were of analytical or LC-MS grade purity (depending on if they were used for the extraction or chromatographic analysis) and used without further purification. Acetonitrile and acetic acid were purchased from Lab-Scan (Dublin, Ireland) and Panreac (Barcelona, Spain), respectively. Methanol and *n*-hexane were obtained from Panreac (Barcelona, Spain). Double-deionized water with conductivity of 18.2 MΩ was purified with a Milli-Q-system (Millipore, Bedford, MA, USA). Standards of 3,4-dihydroxyphenylacetic acid (DOPAC), hydroxytyrosol, tyrosol, luteolin, apigenin, *p*-coumaric, and quinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein was purchased from Extrasynthese (Lyon, France).

Preparation of standards and quality control (QC) samples. Stock solution at 500 mg/L of each one of the above-mentioned standards, was prepared in methanol. Subsequently, to prepare working standard solutions, the appropriate volumes of the stock solution, with the exception of DOPAC (which was used as internal standard (ISt)),

were took and diluted serially in methanol to yield final concentrations of 0.5, 1, 2.5, 5, 12.5, 25, 35, 50, 100, 150 and 250 mg/L. Quality control (QC) samples at a concentration of 5 mg/L were prepared in the same way as the calibration standards, and were used to check the stability of the system over the different sequences carried out. Analytical standards solutions and QC samples were stored at −20 °C.

Liquid-liquid extraction. Phenolic compounds were extracted in triplicate from each sample as follows: an aliquot of 2.00 g of oil was accurately weighted into a 12 mL glass centrifuge tube with a screw cap. 25 µL of the ISt solution was added, methanol was evaporated using nitrogen, and the phenolic compounds were extracted after adding 1 mL of *n*-hexane by using 2 mL of methanol/water (60/40). The mixture was firstly vortexed for 1 min and then centrifuged at 3500 rpm for 6 min at room temperature. The methanol/water fraction was collected, the residue was re-extracted twice under the same conditions for recovering the totality of phenolic compounds, and the three fractions were combined. Then, the solvent was removed, using a rotary evaporator under vacuum at 30 °C and reduced pressure. Subsequently, the obtained dry extract was reconstituted in 1 mL of methanol. The final extracts were filtered through 0.22 µm membrane (nylon) syringe filters and stored at −18 °C until analyzed.

LC-MS analysis. The phenolic extracts obtained were, in a first step, analyzed using a LC coupled to a microTOF-Q IITM mass spectrometer (Bruker Daltonik, Bremen, Germany) by an electrospray ionization source. The LC system was an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany). The aim of this first step was to achieve the qualitative characterization of the phenolic fraction of representative samples of each one of the studied varieties. Afterwards, another LC-MS platform was used, coupling the same LC system to a Bruker Daltonics Esquire 2000™ Ion Trap (IT) (Bruker Daltonik, Bremen, Germany) for quantitative purposes. In both cases, the mass spectrometers were operating in negative ionization mode. LC-MS analyses were carried out by applying a previously optimized and validated analytical method.[32]

Statistical analysis. Significant differences in phenolic compounds composition ($p < 0.05$) among varieties and crop seasons were assessed with one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test. SPSS statistical package software (SPSS for Windows, Version 20, SPSS Inc., and Chicago, USA) was used for the statistical analysis

of the data. For botanical classification of the studied oils, the obtained quantitative data were subjected to PCA and LDA using XLSTAT software version 2015.04.1 (Addinsoft, France).

RESULTS AND DISCUSSION

Phenolic compounds profiles characterization. Total ion chromatograms (TICs) and Base peak chromatograms (BPC) achieved by the LC-ESI-TOF MS platform were explored and the extracted ion chromatograms (EICs) of detected phenolic compounds were depicted. Typical EICs of all the identified phenolic compounds are presented in Figure 1, together with their molecular formula, experimental and calculated mass to charge signal (*m/z*), error, mSigma value, and the proposed identity for each peak. Thus, as can be seen in this figure, apart from quinic acid **(QA)** (a polar non-phenolic compound), up to 32 phenolic compounds and derivatives were tentatively identified in all the oils from the selected cultivars. The phenolic compounds identified can be classified according to their chemical structure as follows: **secoiridoids** (22 compounds: 10-hydroxy oleuropein aglycone **(10 Hy-Ol Agl)**, dialdehydic form of decarboxymethyl oleuropein aglycone **(DOA)**, dehydro oleuropein aglycone **(Dehydro Ol Agl)**, dialdehydic form of decarboxymethyl ligstroside aglycone **(D-Lig Agl)**, desoxy elenolic acid **(Desoxy-EA)**, dialdehydic form of decarboxymethyl elenolic acid **(D-Ald-D EA)**, elenolic acid **(EA)**, hydroxy decarboxymethyl oleuropein aglycone **(Hy D-Ol Agl)**, hydroxy elenolic acid **(Hy-EA)**, ligstroside aglycone **(Lig Agl)** and 3 isomers **(Lig Agl IS1, Lig Agl IS2** and **Lig Agl IS3)**, methyl decarboxymethyl oleuropein aglycone **(Methyl D-Ol Agl)**, methyl oleuropein aglycone **(Methyl Ol Agl)**, and oleuropein aglycone **(Ol Agl)** and 6 isomers **(Ol Agl IS1, Ol Agl IS2, Ol Agl IS3, Ol Agl IS4, Ol Agl IS5** and **Ol Agl IS1)**); **simple phenols** (4 compounds: hydroxytyrosol **(Hyt)**, hydroxytyrosol acetate **(Hyt-Ac)**, oxidized hydroxytyrosol **(O-Hyt)** and tyrosol **(Ty)**); **lignans** (3 compounds: (+)-pinoresinol **(Pin)**, (+)-1-acetoxypinoresinol **(Ac-Pin)** and syringaresinol **(Syr)**), **flavonoids** (2 compounds: luteolin **(Lut)** and apigenin **(Apig)**), and **phenolic acids** (*p*-coumaric acid **(*p*-coum)**). The qualitative phenolic composition obtained herein is in good agreement with previously published reports where the phenolic fraction of monovarietal VOOs was evaluated.[31-32]

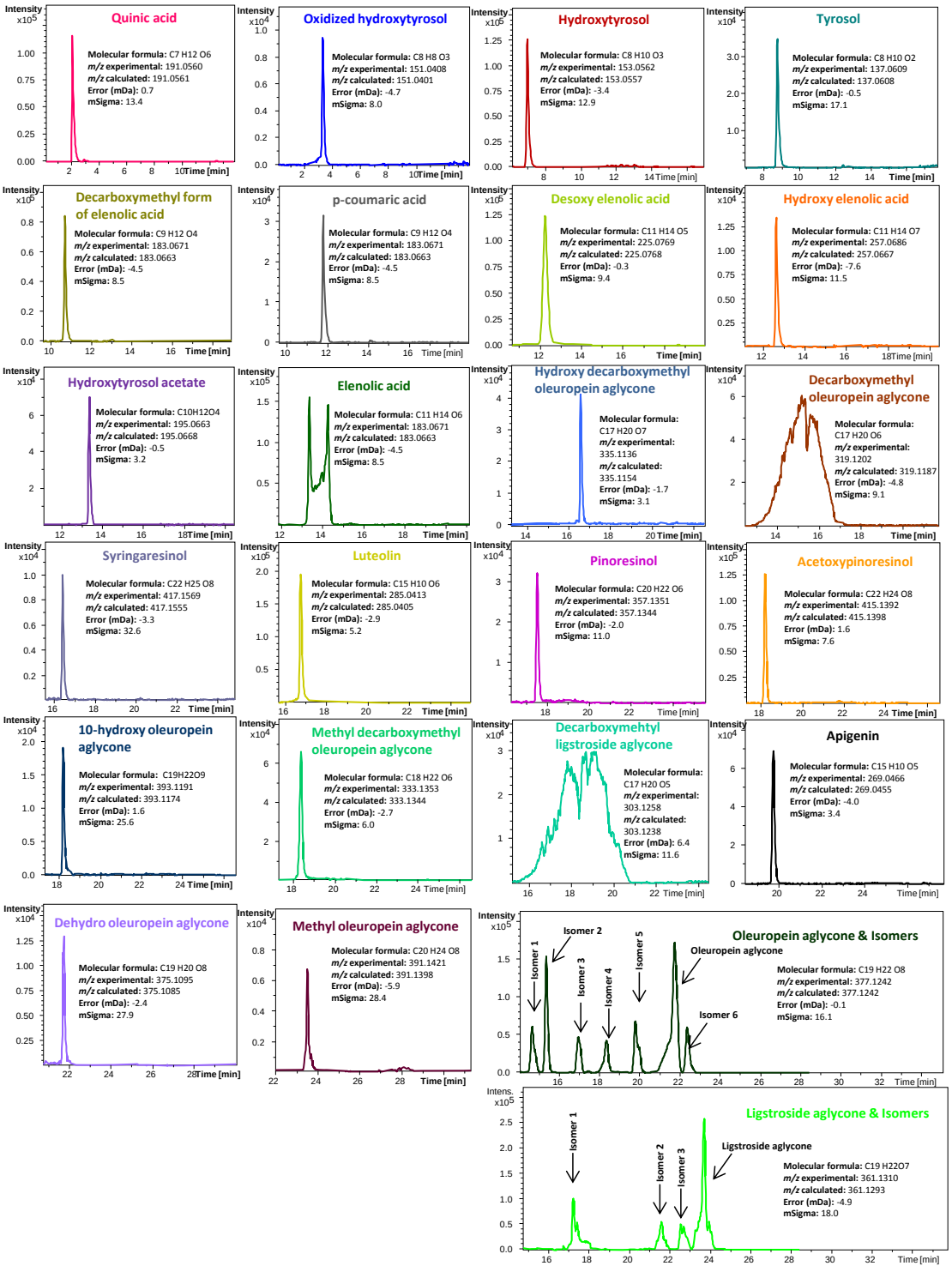

Figure 1. EICs of the main phenolic compounds identified in the studied monovarietal olive oils, together with their MS data (molecular formula, experimental and calculated accurate mass to charge (*m/z*) signal, error, and mSigma value) obtained by LC-ESI-TOF MS.

The presence of multiple isomers of Ol Agl and Lig Agl is something that we would like to clarify before continuing. Since the publication of two very complete papers written by Karkoula et al.[40,41] regarding the artificial formation of some secoiridoid derivatives

(mainly due to their reactivity with methanol (and water)), this topic is of a great interest. Our group has already discussed it in another publication,[42] where we corroborated Karkoula´s finding, after carrying out the extraction of selected olive oil samples both with the very widely used methanol/water (60:40, v/v) mixture and acetonitrile. When acetonitrile replaces the methanol-water mixture as extractant, only 3 isomers of Ol Agl (at relatively low proportions) can be observed. The same can be said for Lig Agl (observing just one isomer (instead of 3)). As a consequence, it is possible to claim that as long as methanol (and probably water and/or their mixtures) is involved in the sample preparation (or has any interaction at any point of the analytical procedure with these compounds), the "artificial isomers" will show up. We have also studied that the generation of artificial peaks (related to DOA and D-Lig Agl) in the chromatograms is not as serious as for Ol Agl and Lig Agl and could be even ignored (from a quantitative point of view).

Bearing in mind all the outcomes just mentioned and the fact that methanol-water mixtures have been extensively used (and are recommended by the International Olive Council method) and are probably present (water for sure) in most of the LC mobile phases combinations employed to analyze phenols from VOOs, we decided to keep using the extraction protocol implying the use of the methanolic mix (whose repeatability had been exhaustively checked (relative standard deviations (RSD) (in %) < 7.12%)).

To conclude the argumentation, it is necessary to add that, from our point of view, ignoring the "artificial isomers" means underestimating their initial "native amount", since they are formed from the native secoiridoids present in the VOO sample. Some other authors agree regarding this last point (Karkoulas´team, amogn others). For further information, have a look at Bajoub et al.[42] No need to mention that, apart from all the said above, the lack of commercially available pure standards (only accessible for some of the phenolic compounds found in VOO and not for secoiridoids) is a big issue and, together with the previously enumerated problems, could explain the differences found regarding the reported levels of VOO phenols.

Quantification of phenolic compounds and statistical analysis: effect of cultivar and crop season. In this work, total content of each chemical family (Figure 2) (calculated as the sum of the individual contents of the phenolic compounds belonging to each

chemical class) and individual contents of the 33 identified compounds (Table 2 a, b, c and d) were determined by LC-ESI-IT MS.

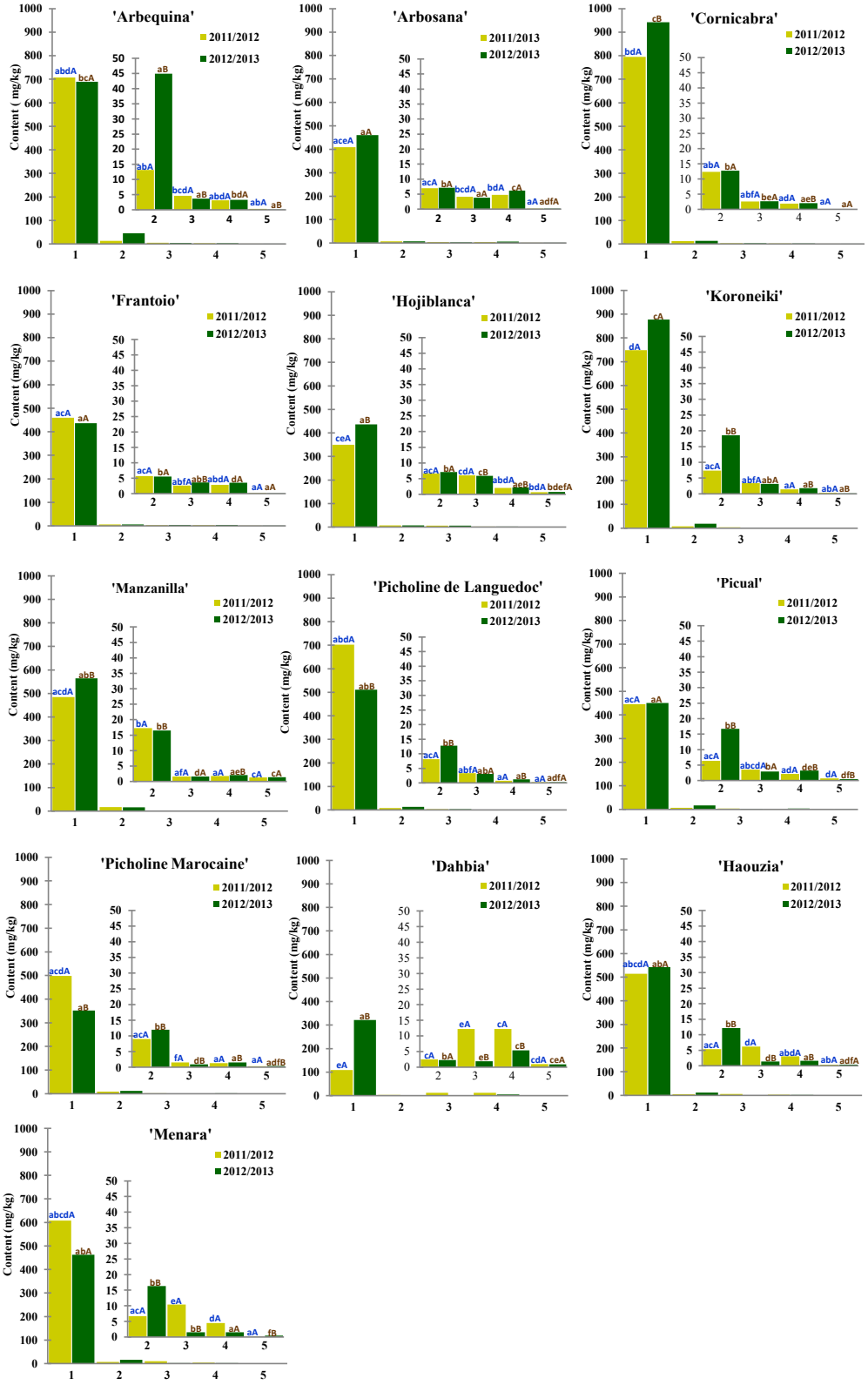

Figure 2. Total content of secoiridoids (1), simple phenols (2), flavonoids (3), lignans (4) and phenolic acids (5) of the studied monovarietal VOO samples, expressed in mg/kg.

Table 2 a. Mean ± Standard Deviation (mg/kg) of some of the Phenolic Compounds Determined in the Studied Monovarietal Olive Oils.

	QA	O-Hyt	Hyt	Ty	*p*-coum	Hyt-Ac	Syr	Lut	Apig	Pin	Ac-Pin
	2011/2012										
Arbequina	0.46abA±0.19	0.14aA±0.02	0.47aA±0.09	1.75aA±0.54	0.31abA±0.15	10.84aA±6.47	0.22aA±0.07	4.27abcfA±0.89	0.35aA±0.09	1.51aA±0.33	1.39aA±0.35
Arbosana	0.59abA±0.58	0.19aA±0.12	1.71abA±2.62	2.91acA±2.29	0.18a±0.06	2.19bA±3.93	0.69bA±0.34	3.08acA±1.23	1.08adA±0.61	2.31abA±1.11	1.78aA±0.78
Cornicabra	0.24abA±0.04	0.33abA±0.02	4.30bcA±0.33	6.39acA±0.43	0.07a±0.01	1.32bA±0.15	0.16aA±0.01	2.54acdeA±0.39	0.08aA±0.01	0.61adA±0.03	1.12aA±0.06
Frantoio	0.52aA±0.26	0.22aA±0.09	0.81aA±0.55	4.07acA±2.54	0.13a±0.09	0.59bA±0.19	0.21aA±0.04	2.45cdeA±0.79	0.20aA±0.09	0.74adA±0.19	1.89adA±0.73
Hojiblanca	0.44abA±0.37	0.19aA±0.06	0.55aA±0.29	4.53aA±1.75	0.67b±0.28	1.35bA±0.54	0.27acA±0.08	5.27abfA±1.01	0.80adA±0.18	0.80adA±0.21	0.95aA±0.29
Koroneiki	0.45abA±0.30	0.16aA±0.07	1.61aA±1.24	4.27acA±1.79	0.29ab±0.09	1.31bA±1.19	0.17aA±0.05	3.07cA±0.69	0.29aA±0.11	0.65dA±0.14	0.62aA±0.11
Manzanilla	0.43aA±0.36	0.16aA±0.05	4.05cA±3.03	11.74bA±5.98	1.28c±0.47	1.30bA±1.02	0.30acA±0.21	1.57deA±0.29	0.07aA±0.05	0.74adA±0.66	0.72aA±0.64
Picholine de Languedoc	4.89bA±7.38	0.84bA±1.04	1.31aA±0.80	5.67acA±3.08	0.24a±0.16	0.36bA±0.22	0.19aA±0.06	3.12acA±1.21	0.26aA±0.13	0.50dA±0.14	0.08acA±0.15
Picholine Marocaine	1.74abA±3.37	0.26aA±0.33	1.23aA±0.57	6.11cA±0.99	0.29a±0.09	1.43bA±1.04	0.21aA±0.06	1.47deA±0.44	0.04bA±0.07	0.46dA±0.11	0.61aA±0.15
Picual	0.28aA±0.09	0.24aA±0.16	1.47aA±1.43	4.33acA±1.01	0.75bc±0.30	0.34bA±0.22	0.22aA±0.04	3.30acA±1.04	0.28aA±0.11	1.80abA±0.72	0.13acA±0.11
Dahbia	0.08aA±0.03	0.19aA±0.03	0.35aA±0.13	1.88aA±0.43	1.00bc±0.11	0.09bA±0.10	0.49bcA±0.09	4.13abcfA±0.50	8.07cA±1.14	6.41cA±0.71	5.33bcA±1.19
Haouzia	1.18abA±2.03	0.21abA±0.19	0.51aA±0.29	3.63acA±1.90	0.29ab±0.40	0.99bA±0.80	0.13aA±0.05	3.98abcfA±1.74	2.22dA±1.30	0.37dA±0.11	2.56cdA±0.92
Menara	0.91abA±1.35	0.19aA±0.08	1.02aA±1.02	4.91acA±2.31	0.14a±0.09	0.54bA±0.52	0.17aA±0.09	5.54bfA±1.03	4.86eA±3.31	0.37dA±0.19	3.92bdA±4.66
	2012/2013										
Arbequina	0.39aA±0.14	0.30aB±0.06	6.38bcdB±2.67	11.60bB±3.28	0.11bB±0.01	26.63aB±32.66	0.36aB±0.06	3.39aB±0.21	0.32bdgfA±0.03	1.34aA±0.15	1.62bA±0.54
Arbosana	4.98aA±11.67	1.03aA±1.73	1.30aA±1.92	3.28cedA±4.00	0.16bfA±0.11	1.53bA±1.25	0.78bA±0.23	2.61cA±0.45	1.18cA±0.21	3.38bB±0.45	2.00bcA±0.54
Cornicabra	0.24aA±0.01	0.35aA±0.03	4.33abdA±0.39	6.64acedA±0.36	0.07abA±0.01	1.39bA±0.09	0.20aB±0.01	2.55acA±0.22	0.10fagB±0.01	0.66eB±0.03	1.21abA±0.14
Frantoio	0.74aA±0.15	0.45aB±0.03	0.79aA±0.36	4.08cedA±0.62	0.11bA±0.03	0.25bB±0.16	0.32aB±0.08	3.18aB±0.29	0.42dfgB±0.13	0.92aeA±0.22	2.30cA±0.67
Hojiblanca	0.36aA±0.27	0.17aA±0.03	0.51aA±0.30	5.41deA±2.14	0.76cefA±0.33	1.05bA±0.76	0.32aA±0.09	5.09bA±1.08	0.79eA±0.18	0.85aeA±0.18	1.03abA±0.25
Koroneiki	1.68aB±1.51	0.36aB±0.08	8.58bcB±2.63	7.45acdB±2.35	0.10abB±0.02	2.21bB±0.28	0.25aB±0.04	2.88aA±0.29	0.28fgA±0.04	0.94aeB±0.12	0.56adeA±0.09
Manzanilla	0.19aA±0.03	0.13aA±0.02	3.82abdA±3.29	10.76abA±5.55	1.40dA±0.57	1.77bA±1.52	0.34aA±0.24	1.52cdA±0.23	0.07aA±0.06	0.91aeA±0.92	0.86adA±0.78
Picholine de Languedoc	0.59aA±0.31	0.34aA±0.05	2.19adB±0.84	9.48abdB±1.22	0.24abfA±0.05	0.75bB±0.21	0.30aB±0.09	2.85aA±0.72	0.27bgA±0.19	0.67eB±0.16	0.32deB±0.18
Picholine Marocaine	0.59aA±0.38	0.27aA±0.07	2.21adA±1.69	8.53abdB±1.56	0.36abfB±0.06	0.95bA±0.25	0.26aB±0.04	0.94cdB±0.17	0.01aA±0.02	0.57eB±0.11	0.71adeA±0.09
Picual	0.80aB±0.58	0.55aB±0.09	4.78dB±2.74	9.19abdB±2.02	0.45acfB±0.08	2.20bB±1.97	0.31aB±0.04	2.77aA±0.27	0.19abfgB±0.06	2.80cB±0.20	0.13eA±0.04
Dahbia	0.17aB±0.01	0.11aB±0.01	0.23aA±0.03	1.73deA±0.16	0.92ceA±0.05	0.22bB±0.04	0.32aB±0.04	0.59cdB±0.25	1.82afgB±0.01	4.22dB±0.08	0.60adeB±0.02
Haouzia	0.42aA±0.21	0.32aA±0.05	3.68abdB±1.40	7.61abcdB±2.02	0.40abfA±0.17	0.54bA±0.30	0.31aB±0.06	1.34cB±0.38	0.05aB±0.03	0.50eB±0.04	0.80adeB±0.18
Menara	0.41aA±0.16	0.41aB±0.11	4.07abdB±2.72	11.41abB±2.58	0.45cfB±0.10	0.44bA±0.24	0.32aB±0.04	1.43cdB±0.27	0.05aB±0.03	0.57eB±0.11	0.64adeA±0.13

-Significant differences in the same column are indicated with different lowercase letters (comparison among the 13 cultivars investigated in this study at the same crop season, $p < 0.05$) and with different capital letters (comparison between crop seasons for the same cultivar, $p < 0.05$).

-Quinic acid, Hyt, Ty, Pin, Lut, Apig, and p-Cou were quantified in terms of their commercial pure standards. O-Hyt and Hyt-Ac were quantified in terms of Hyt. Lignans (Syr and Ac-Pin) were quantified in terms of Pin.

Table 2 b. Mean ± Standard Deviation (mg/kg) of some of the Phenolic Compounds Determined in the Studied Monovarietal Olive Oils.

		Arbequina	Arbosana	Cornicabra	Frantoio	Hojiblanca	Koroneiki	Manzanilla	Picholine de Languedoc	Picual
D-Ald-D EA	2011/2012	0.40aA±0.27	2.24aA±3.06	1.50aA±0.22	1.47aA±2.92	0.72aA±0.43	0.75aA±1.14	19.42bA±18.75	2.59aA±3.78	1.58aA±1.45
Desoxy-EA		6.83acA±3.54	5.79ac±9.59	2.42a±0.15	3.99a±5.42	9.38acd±13.76	56.30b±23.04	27.08cd±25.17	32.79d±13.70	6.67ac±11.17
Hy-EA		0.11aA±0.02	0.10a±0.04	1.51b±0.16	0.20a±0.10	0.20a±0.05	0.18a±0.09	0.16a±0.07	0.24a±0.07	0.19a±0.06
EA		27.97aA±10.54	25.09aA±11.83	58.68bcA±5.49	49.36cA±9.05	26.21aA±8.58	20.27adA±8.94	26.88aA±7.93	25.45aA±8.17	28.29aA±12.66
Hy D-Ol Agl		1.17aA±0.59	0.41abA±0.49	3.71cA±0.14	0.34bA±0.25	0.37abA±0.21	0.81abA±0.46	0.76abA±0.70	0.71abA±0.41	0.42abA±0.57
DOA		350.41aA±133.43	121.82bA±162.41	205.65acA±8.03	65.70bA±35.16	87.37bA±35.75	143.90bA±60.32	93.94bA±70.06	138.58bA±39.23	71.25bA±107.56
10 Hy-Ol Agl		0.19aA±0.06	0.65abA±1.37	3.48bA±0.24	0.45aA±0.11	0.27aA±0.10	0.34aA±0.13	0.29aA±0.19	1.82abA±4.98	0.31aA±0.15
Methyl D-Ol Agl		2.22aA±2.08	3.53abA±5.33	11.30bcdgA±1.06	14.89dgA±1.41	5.98abcA±3.95	16.59dA±4.18	9.62abgA±4.79	14.11egA±6.82	13.85degA±3.34
D-Lig Agl		203.04aA±44.28	135.98abcfA±36.20	190.46abA±5.27	80.45fA±34.30	75.32efA±25.73	106.13cfA±50.04	77.47fA±32.01	156.39abcA±50.19	55.89dA±51.71
Dehydro Ol Agl		1.67aA±1.64	1.13aA±0.73	1.52aA±0.23	5.56bceA±1.71	2.97aceA±0.32	1.29aA±0.78	1.93aA±1.01	1.85aA±0.57	4.39ceA±1.70
Methyl Ol Agl		1.80abdA±0.64	1.44aA±0.47	0.83aA±0.05	2.86bcdA±0.78	1.95abdA±0.46	3.32bcdA±0.87	1.33adA±0.70	1.25aA±0.48	1.76abdA±0.64
D-Ald-D EA	2011/2013	2.74aB±0.42	20.13aA±48.34	1.46aA±0.09	3.33aA±4.77	0.49aA±0.22	4.82aB±2.32	10.59aA±11.56	1.73aA±1.21	14.45aB±12.39
Desoxy-EA		5.65aA±0.29	1.62aA±0.62	2.81abB±0.11	2.13aA±1.57	8.95abA±13.65	10.64abB±1.77	25.82bcA±32.55	45.44bA±21.23	3.94aA±2.04
Hy-EA		1.04bB±0.24	0.60ceB±0.38	1.60dA±0.09	0.50aceB±0.07	0.14aA±0.05	0.42aceB±0.11	0.15aA±0.12	0.28aceA±0.11	0.53eB±0.19
EA		55.03aA±35.29	60.33bcA±42.65	61.30abcA±5.95	89.04bcB±20.11	38.52abdA±15.46	28.60abdB±3.03	22.42adA±8.21	43.57abdB±14.08	36.85abdA±5.22
Hy D-Ol Agl		3.30aB±0.48	1.10bA±0.83	4.23aB±0.28	0.39bA±0.32	0.14bB±0.07	2.15cB±0.68	0.68bA±0.75	0.46bA±0.19	0.86bA±0.51
DOA		221.14aB±21.08	130.97bA±96.12	221.94aA±20.22	61.84cA±51.00	101.51cA±38.84	263.20aB±88.98	110.31cA±76.53	81.55cB±27.37	79.59cA±47.61
10 Hy-Ol Agl		2.81bcA±2.97	0.19aA±0.08	4.19cB±0.25	0.48aA±0.25	0.25aA±0.10	1.33abB±0.11	0.33aA±0.18	0.62aA±0.19	1.28abB±0.88
Methyl D-Ol Agl		8.45bdeB±0.60	2.11ceA±0.35	13.59aB±0.97	10.20abdeB±2.47	7.33deA±4.30	11.50abdeB±1.02	11.58abdeA±6.61	14.06aA±3.13	10.62abdeB±1.35
D-Lig Agl		153.30bB±13.50	163.43bcA±26.55	203.75bcA±15.34	103.50aA±21.18	89.65aA±26.56	190.12bcB±37.87	108.39aA±38.15	70.38adB±31.83	78.44aA±4.17
Dehydro Ol Agl		1.39bceA±0.19	1.59ceA±0.45	1.46ceA±0.14	6.44dA±0.98	2.66abcA±0.28	0.53eB±0.12	2.19abcA±1.26	2.29abcA±0.96	2.95acA±1.58
Methyl Ol Agl		1.66bdgA±0.20	0.48ceA±0.07	0.90acdeA±0.10	0.80aceB±0.36	0.73aceB±0.35	1.76dgB±0.14	1.30abdA±0.46	0.60eB±0.20	1.61dgA±0.65

-Significant differences in the same row are indicated with different lowercase letters (comparison among the 13 cultivars investigated in this study at the same crop season, $p < 0.05$) and with different capital letters (comparison between crop seasons for the same cultivar, $p < 0.05$). Since only 9 varieties are included in Table 2b (to contain the size of the table), the results shown herewith were considered together with those from Table 2d to carry out ANOVA analysis.

-Secoiridoids were quantified in terms of Oleuropein.

Table 2 c. Mean ± Standard Deviation (mg/kg) of some of the Phenolic Compounds Determined in the Studied Monovarietal Olive Oils.

		Arbequina	Arbosana	Cornicabra	Frantoio	Hojiblanca	Koroneiki	Manzanilla	Picholine de Langaudoc	Picual
Ol Agl	2011/2012	27.66acdA±6.05	23.76adA±23.05	68.03bcefA±8.88	30.43adA±9.46	20.70adA±7.56	59.22ceA±17.16	46.32abcA±19.21	47.30abcA±8.70	39.23acA±18.15
Ol Agl IS1		3.52bA±1.66	4.84abA±9.22	17.24abdA±2.20	17.08abdA±9.57	10.78abdA±11.85	38.27cA±14.05	12.24abdA±12.68	25.04cdA±15.61	22.15abcdA±11.38
Ol Agl IS2		4.51acA±2.44	6.36acA±9.90	22.51abdeA±1.54	23.22acdeA±8.64	13.66acdA±13.27	42.02bA±15.30	15.83acdeA±12.64	29.59bdeA±15.22	26.75bcdeA±10.89
Ol Agl IS3		5.56acefA±1.97	4.06abcefA±5.33	12.29abcdgA±1.44	10.26abcgA±3.78	7.77cA±5.79	19.96dA±7.29	10.55abcgA±6.45	14.22acdgA±5.92	11.97abcgA±5.85
Ol Agl IS4		7.18abcA±2.85	4.96bcA±6.18	17.90adeA±0.91	9.04acA±3.61	8.20acA±6.48	18.15adeA±3.61	12.68adeA±8.41	15.03adeA±5.13	12.37adeA±7.21
Ol Agl IS5		10.55abcdA±2.77	7.63bdA±8.17	25.11aeA±1.42	11.66abcdA±3.79	8.90bcdA±5.69	22.39aeA±6.41	16.23abA±8.09	19.01abA±5.30	15.84abA±7.85
Ol Agl IS6		20.49abcefA±2.78	10.25bdA±7.91	19.46aeA±0.95	15.67abA±4.33	14.63abA±6.76	26.13cefA±3.46	17.66abceA±7.51	21.48aceA±6.75	18.37abceA±6.43
Lig Agl		19.83beA±5.57	28.32beA±9.06	69.51adfA±4.11	48.25abcdA±10.53	25.02ceA±9.23	73.66dfA±16.72	47.93abcdeA±19.69	64.00adfA±16.25	49.48abcdA±8.87
Lig Agl IS1		4.32bA±4.52	6.83bA±9.71	27.76abcdA±4.98	32.91acdeA±5.45	12.60abA±10.79	48.91cdeA±16.14	20.36abA±13.48	42.05cdeA±20.58	30.90acdA±11.7
Lig Agl IS2		4.49bdA±4.52	8.46bdA±7.68	19.72abcdA±2.34	26.41aceA±4.46	12.13abdA±9.71	36.49aceA±12.21	17.03abdA±11.05	34.32ceA±14.87	24.05acA±7.35
Lig Agl IS3		3.77bceA±0.97	5.46bceA±2.12	14.57adfgA±2.34	9.14abcdeA±3.11	4.88ceA±2.01	13.26adA±4.96	9.30abceA±3.91	14.24fdA±3.23	10.21abceA±1.98
Ol Agl	2011/2013	55.36aeB±6.39	21.04bdA±8.39	96.88cB±14.46	24.49adA±14.57	35.77abdeA±17.1	93.59cB±9.02	54.01aeA±33.17	46.63adeA±10.95	51.96aeA±26.81
Ol Agl IS1		9.16abcdB±1.17	1.66bdA±0.82	23.94cB±2.85	9.70abcdA±9.42	11.89abcdA±14.31	19.27acdB±1.78	14.76abcdA±20.22	14.45abcdB±2.69	11.10abcdB±7.72
Ol Agl IS2		12.95bcdeB±1.39	3.08cdeA±1.42	29.26aB±1.01	12.21deB±10.52	15.92abcdeA±14.99	27.74aB±2.73	18.88abdeA±18.98	22.97abdeA±3.09	16.65abcdeB±8.82
Ol Agl IS3		10.23aceB±1.16	3.21bceA±1.80	16.39adB±2.68	5.62ceB±4.59	9.60abceA±7.79	16.65dA±2.12	10.51acdeA±7.52	10.64acdeA±2.65	9.66abceA±4.99
Ol Agl IS4		10.85abdB±0.95	2.93bdA±1.47	20.63ceA±3.99	5.61dA±4.41	8.84abdA±6.63	20.51ceA±2.85	14.27acdeA±12.2	12.70acdeA±3.81	10.93abcdA±6.13
Ol Agl IS5		15.25abeB±2.07	6.04bA±2.24	32.83cdeB±4.91	8.68abA±6.39	11.74abA±7.25	29.00deB±4.14	19.12aeA±12.08	16.99aeA±4.18	16.16aeA±9.53
Ol Agl IS6		19.23acdA±1.41	10.97bcA±3.67	24.31adB±2.25	12.03cA±6.25	18.31abcA±7.54	26.65dA±1.80	18.98abcdA±11.01	21.19adA±1.74	16.44abcA±6.14
Lig Agl		53.97adeB±4.39	18.91beB±2.19	93.88cB±6.37	34.84abdeB±8.74	41.09abdeA±18.11	66.09dB±15.78	65.63cdA±37.07	46.29adeB±14.40	47.39adeA±6.08
Lig Agl IS1		18.67adB±2.35	3.41bA±0.44	38.32cdB±3.94	21.11adB±11.25	11.62abA±8.69	24.97acdB±4.13	22.18adA±18.25	27.03dA±12.37	16.83abdB±2.76
Lig Agl IS2		18.17adB±2.48	3.72bA±0.53	28.35cB±1.91	17.57acdB±6.29	13.17adA±8.72	17.84acdB±3.23	19.05acdA±12.52	21.19acB±7.08	13.23adB±2.54
Lig Agl IS3		9.01aB±2.23	3.29aB±0.42	20.63bcB±1.32	5.84aB±1.79	7.57aA±3.20	19.36cA±16.38	12.73abcA±7.28	9.63aB±3.03	9.53abA±2.07

-Significant differences in the same row are indicated with different lowercase letters (comparison among the 13 cultivars at the same crop season, $p < 0.05$) and with different capital letters (comparison between crop seasons for the same cultivar, $p < 0.05$). Since only 9 varieties are included in Table 2c (to contain the size of the table), the results shown herewith were treated together with those from Table 2d to carry out ANOVA analysis.

-Secoiridoids were quantified in terms of Oleuropein.

Table 2 d. Mean ± Standard Deviation (mg/kg) of some of the Phenolic Compounds Determined in the Studied Monovarietal Olive Oils.

		Picholine Marocaine	Dahbia	Haouzia	Menara
D-Ald-D EA	2011/2012	1.65aA±3.14	0.26aA±0.12	0.83aA±0.87	1.69aA±2.60
Desoxy-EA		9.60ac±4.50	0.40a±0.13	12.40acd±13.39	11.15acd±18.56
Hy-EA		0.19a±0.13	0.23a±0.05	0.15a±0.06	0.12a±0.05
EA		20.69aA±5.49	5.73dA±1.44	17.35adA±5.04	33.68acA±12.23
Hy D-Ol Agl		0.31bA±0.22	0.78abA±0.42	0.20bA±0.15	0.35abA±0.40
DOA		133.66bA±114.84	3.15dA±1.19	18.94bA±14.31	17.00bA±8.64
10 Hy-Ol Agl		0.36aA±0.07	0.37aA±0.08	0.17aA±0.09	0.17aA±0.05
Methyl D-Ol Agl		10.96cdgA±2.34	10.25abcgA±1.63	20.46eA±4.83	28.81fA±8.50
D-Lig Agl		84.50fA±47.17	0.46deA±0.04	2.78deA±1.45	2.41eA±1.07
Dehydro Ol Agl		2.30aA±1.29	15.75dA±2.32	3.59bceA±2.55	5.45eA±1.82
Methyl Ol Agl		1.61aA±1.00	2.05abcdA±0.23	3.02dA±1.80	1.93abdA±0.55
Ol Agl		45.79abcA±17.62	8.73dA±1.52	92.35efA±36.41	98.33fA±34.50
Ol Agl IS1		15.42abdA±6.11	4.59bA±3.28	26.59acdA±19.53	25.80acdA±11.15
Ol Agl IS2		23.34acdeA±7.86	8.95cA±5.45	36.03bdeA±19.49	40.59beA±15.59
Ol Agl IS3		10.65ceA±4.46	5.19cfA±2.05	20.6dgA±10.61	20.32dgA±9.00
Ol Agl IS4		14.04adeA±5.87	1.05cA±0.60	21.89deA±12.89	21.99eA±9.68
Ol Agl IS5		18.07abA±6.83	1.75dA±0.45	32.13eA±14.04	33.92eA±15.93
Ol Agl IS6		18.22abceA±4.57	2.78dA±0.81	27.69efA±9.88	31.83fA±7.31
Lig Agl		42.62abcegA±15.20	15.08eA±2.70	95.84fgA±26.78	113.01gA±48.67
Lig Agl IS1		18.55abA±5.79	9.74bA±2.04	33.34acdeA±12.23	53.91eA±29.04
Lig Agl IS2		16.10abdA±4.76	7.08dA±2.85	26.68aceA±13.61	42.72eA±13.16
Lig Agl IS3		9.38abceA±3.75	4.25eA±5.96	21.41fgA±7.65	22.54gA±7.56
D-Ald-D EA	2011/2013	1.72aA±2.07	0.33aA±0.05	7.50aA±9.24	15.58aA±17.95
Desoxy-EA		18.11abB±2.88	0.53dA±0.14	7.80abA±2.50	10.70abA±6.33
Hy-EA		0.20aA±0.12	0.10aB±0.03	0.47aceA±0.46	0.41aceB±0.08
EA		14.35dB±3.35	22.26abdB±1.63	55.48abcB±10.45	51.04abA±23.13
Hy D-Ol Agl		0.18bA±0.09	0.10bB±0.02	1.04bA±1.46	0.76bA±0.27
DOA		39.58cB±9.24	58.06cB±8.98	87.24cB±54.4	70.68cB±21.18
10 Hy-Ol Agl		0.60aB±0.08	0.14aB±0.06	1.27abA±1.12	0.87aB±0.26
Methyl D-Ol Agl		12.92aB±1.46	7.45deB±0.44	12.84abdeB±0.74	10.91abdeB±2.28
D-Lig Agl		30.68dB±9.99	93.73aB±10.60	57.94adA±55.28	61.62adB±21.87
Dehydro Ol Agl		2.87acA±0.60	6.29dB±0.63	3.46aA±1.09	3.54aB±0.67
Methyl Ol Agl		0.88aceB±0.47	2.65fB±0.35	2.23fgA±0.58	2.75fB±0.38
Ol Agl		35.20abdA±7.56	26.12abdB±0.42	63.47eA±12.68	41.56abdeB±10.47
Ol Agl IS1		19.16acdA±6.21	4.28dA±0.43	23.29acA±3.67	16.22acdA±8.80
Ol Agl IS2		28.84aA±6.80	7.31eA±1.05	31.20aA±3.31	23.85abdeB±10.83
Ol Agl IS3		11.02acdeA±2.52	5.17eA±0.52	16.97adA±3.22	12.51acdeA±4.85
Ol Agl IS4		16.02aceA±3.69	3.73dB±0.38	16.80aceA±2.93	13.36acdeA±5.23
Ol Agl IS5		15.80aeA±3.35	8.32abB±0.71	23.45eA±3.38	18.12aeB±5.06
Ol Agl IS6		20.27adA±2.82	14.75abcB±0.45	21.90abdA±1.58	16.89abcB±3.77
Lig Agl		35.94beA±3.67	33.13beB±0.96	51.14adeB±22.8	43.75abdeB±5.34
Lig Agl IS1		20.23adA±2.94	10.95abdA±1.01	26.80acdA±7.07	21.27adB±6.22
Lig Agl IS2		19.16acdA±4.03	9.28dA±1.11	20.06acA±2.04	17.53adB±4.93
Lig Agl IS3		7.93aA±2.07	6.36aA±1.08	10.39abcB±3.99	9.09abB±1.08

-Significant differences in the same row are indicated with different lowercase letters (comparison among the 13 cultivars at the same crop season, $p < 0.05$) and with different capital letters (comparison between crop seasons for the same cultivar, $p < 0.05$).

-Secoiridoids were quantified in terms of Oleuropein.

As expected and can be deduced from Figure 1 and Tables 2, in both cases (total content of each chemical family and individual phenolic compounds content), huge differences were observed among the studied oils due to the cultivar factor. These findings are in good agreement with previous works that highlighted the significant effect of the variety of origin on VOO phenolic fraction.[31,34] Furthermore, environmental factors, depicted by climatology of each season, exhibited a significant influence on the phenolic composition (regarding both total and individual contents) on almost all the investigated cultivars (Figure 1 and Tables 2). Indeed, as can be observed from Table 1, the two seasons considered in this study greatly differed concerning the climatic conditions; important differences in total precipitations and average temperatures (minimum and maximum) per month were observed, especially in the months of olive fruit growing and before olive harvesting. This could result on a great inter-annual variability of the quantitative phenolic composition of the studied oils. In this respect, the effect of crop season climatology on the phenolic composition of VOO has been already established.[32]

Over the period considered by the current study, the phenolic fractions of the studied oils were found to mainly contain secoiridoids, regardless the cultivar of origin. Numerous reports have previously claimed this fact.[29,33,34] 'Cornicabra' oils presented the highest total secoiridoids content in both crop seasons (mean content was 795.18 mg/kg in 2012/2013 and 942.64 mg/kg in 2013/2014), whereas the lowest mean concentration was calculated for 'Dahbia' samples (108.59 mg/kg and 321.06 mg/kg in crop seasons 2012/2013 and 2013/2014, respectively). Besides, in terms of total abundance, the second class of phenols was the group of simple phenols. That was observed for most of the cultivars under study, except for the Moroccan autochthonous cultivars 'Haouzia' and 'Menara', which showed higher level of flavonoids than simple phenols´ content during the first crop season (over the second crop season, this category was the second most abundant phenolic class also for the oils from these cultivars). The same was observed for 'Dahbia' variety, for which lignans were the second most abundant phenolic class in both crop seasons. Considering the simple phenols, 'Manzanilla' (17.25 mg/kg) and 'Arbequina' (44.91 mg/kg) were the cultivars with the highest mean contents over the first and second crop seasons, respectively, whereas the lowest values were again found for 'Dahbia' oils in both crop seasons (2.51 mg/kg in 2012/2013 and 2.29 mg/kg in 2013/2014, respectively). As far as the total content on flavonoids is concerned, 'Dahbia'

and 'Hojiblanca' showed the highest mean values, 12.20 mg/kg (1st season) and 5.87 mg/kg (2nd season), whilst the lowest values, in both crop seasons, were found for 'Picholine Marocaine' oil samples (1.50 mg/kg and 0.95 mg/kg in 2012/2013 and 2013/2014, respectively). With regard to the lignans, over the first crop season, the mean total content of the studied oils ranged between 12.24 mg/kg and 0.78 mg/kg, in oils from 'Dahbia' and 'Picholine de Languedoc', respectively; whereas during the second crop season mean values oscillated between 6.17 mg/kg ('Arbosana') and 1.28 mg/kg ('Picholine de Languedoc'). Finally, the total content of phenolic acids (*p*-coum) was found between 1.28 mg/kg (in 2012/2013) and 1.40 mg/kg (in 2013/2014) in 'Manzanilla' oil samples, whilst 'Cornicabra' samples showed the lowest levels (0.07 mg/kg, in both crop seasons).

When the individual phenolic compounds´ contents are considered, as expected, different concentrations were found in each of the analyzed cultivars significant, finding significant differences among them. Moreover, as occurred for the total content for each chemical phenolic class, in almost all the investigated cultivars, the effect of crop season was statistically significant ($p<0.05$). Table 2 illustrates these variations, showing the mean concentrations (± standard deviation) of the individual phenolic compounds determined by our LC-MS method. It should be highlighted that in order to simplify data visualization for readers, Table 2 has been divided into four parts (Table 2 a, b, c and d). The results of one-way ANOVA are included in each table, evaluating differences among all the investigated cultivars, as well as the influence of the crop season on their phenolic content.

Concerning the secoiridoids group, results from Tables 2 b, c and d show that the predominant compound in the analyzed oils mainly depend on the cultivar of origin and, in some cases, on the crop season. In this respect, over the studied period, DOA was by far the most abundant secoiridoid in oils from 'Arbequina', 'Cornicabra', 'Hojiblanca', 'Koroneiki', 'Manzanilla', 'Picholine Marocaine' and 'Picual' cultivars (mean concentrations ranged between 39.58 mg/kg, observed for samples from 'Picholine Marocaine' cultivar (during the second crop season) and 350.41 mg/kg, found in 'Arbequina' oils (first crop season) , whereas in oils from 'Arbosana' and 'Frantoio', D-Lig Agl showed the highest mean concentration values (between 80.45 and 135.98 mg/kg found, respectively, in 'Frantoio' and 'Arbosana' oils, both during the first crop season).

For the other investigated cultivars, the most abundant compounds were different depending on the crop season. Indeed, over the first crop season, within the secoiridoids group, D-Lig Agl was the most abundant compound in oils from 'Picholine de Languedoc' variety (156.39 mg/kg); Lig Agl showed the highest levels in oils from 'Haouzia' (95.84 mg/kg) and 'Menara' (113.01 mg/kg) cultivars; and Dehydro Ol Agl was the most abundant in oils from 'Dahbia' variety (15.75 mg/kg). In contrast, during the second crop season, DOA seemed to be the most abundant secoiridoid in oils from 'Picholine de Languedoc' (81.55 mg/kg), 'Haouzia' (87.24 mg/kg) and 'Menara' (70.68 mg/kg), while 'Dahbia' oils showed D-Lig Agl as their most prevalent individual complex phenol (93.73 mg/kg). Apart from the mentioned compounds, as detailed in Tables 2 b, c and d, the other identified secoiridoids were found in a wide range of concentrations in almost all the investigated cultivars (as depicted by the high standard deviation values obtained for each cultivar during the same crop season). Great differences between the two crop seasons were also observed.

As far as the simple phenols are concerned (Table 2 a), 'Arbequina' oils were clearly outstanding in terms of their richness on Hyt-Ac (the most abundant simple phenol in those samples, with mean concentrations ranging between 10.84 and 26.63 mg/kg, observed for 1st and 2nd crop seasons, respectively). In the other studied cultivars, Ty was the most abundant simple phenol for almost all the varieties (except for 'Koroneiki'), with amounts ranging from 1.73 mg/kg in oils from 'Dahbia' (for the second crop season) to 11.74 mg/kg in olive oils from 'Manzanilla' cultivar (for the first crop season). Hyt was found at concentration levels between 0.23 and 8.58 mg/kg, observed, respectively, in oils from 'Dahbia' and 'Koroneiki', for the second crop season. O-Hyt was found at very low levels in the studied samples (mean concentration ranged from 0.11 and 1.03 mg/kg in oils from 'Dahbia' and 'Arbosana', respectively, for the 2nd crop season).

When individual lignans are studied in depth, the evaluated cultivars could be divided in two groups, depending on their most abundant compound belonging to this chemical class. Thus, a first group including 'Arbequina', 'Arbosana', 'Koroneiki', 'Manzanilla', 'Picholine de Languedoc', 'Picual' and 'Dahbia', displayed higher levels of Pin than the other detected lignans (particularly, than Ac-Pin). Mean concentration values of this compound in oils from these varieties were between 0.50 mg/kg and 6.41 mg/kg, found in oils from 'Picholine de Languedoc' and 'Dahbia' cultivars (first crop season),

respectively (Table 2 a). The second group, which includes oils from 'Cornicabra', 'Frantoio', 'Hojiblanca', 'Haouzia', 'Menara' and 'Picholine Marocaine' was characterized by the predominance of Ac-Pin in the lignans´ group, with amounts ranging from 0.61 mg/kg in oils from 'Picholine Marocaine' to 3.92 mg/kg in 'Menara' samples (both observed for the first crop season). Finally, the Syr content of the studied oils was, in general, quite low in all the studied oils, regardless of the cultivar of origin; the obtained values were between 0.13 and 0.71 mg/kg and were found in oils from 'Haouzia' (first crop season) and 'Arbosana' (second crop season), respectively.

Moreover, the analysis of the individual flavonoids content showed that, except for 'Dahbia' oils, Lut was the most abundant compound belonging to this family in oils from the other varieties, with levels ranging between 0.94 mg/kg ('Picholine Marocaine', second crop season) and 5.54 mg/kg ('Menara', first crop season). In the case of 'Dahbia' oils, most of the samples from this variety seemed to contain more Apig than Lut. The mean Apig content in these oils was found between 1.82 mg/kg in 2012/2013 and 8.07 mg/kg in 2013/2014. *p*-Coum acid was found at relatively low levels in the studied oils, not exceeding the value of 1.4 mg/kg (in terms of mean amount). 'Manzanilla' oils showed the highest level for the second crop season, whereas the lowest amount (0.07 mg/kg) was found in 'Cornicabra' oils for both crop seasons.

In this study, the content of QA, a polar non-phenolic compound, was also determined (Table 2 a). The amount of this compound was within the range from 0.08 mg/kg found in 'Dahbia' oils to 4.98 mg/kg found in Arbosana' samples, levels measured during the first and second seasons, respectively.

The comparison of the phenolic content values obtained in this study with those from other studies (investigating the phenolic composition of some of the considered Mediterranean varieties but in their region or country of origin), is quite difficult and some considerations have to be taken into account: the analytical methodologies can differ and, sometimes, the determined compounds are even quantified in terms of another commercially available pure standard; moreover, several characteristics of the analyzed samples can be very influential, particularly, the maturation stage of the extracted olive fruits, the oil extraction system used, the pedoclimatic conditions of a particular season/location, and the filtration and conservation conditions.

If our results are compared, just for illustrative purposes, with those obtained in previously published works by García-Villalba et al.,[31] Lozano-Sánchez et al.,[34] and Bakhouche et al.,[33] where the authors used LC-MS approaches for the characterization of the phenolic profile of oils from Arbequina, Hojiblanca, Picual and Manzanilla, it can be seen that, in general, the qualitative profiles were very similar, as well as the concentration levels of some of the determined compounds.

In light of this detailed analysis of the phenolic composition of the studied oils, general traits or features of 'Picholine Marocaine' oils (the most extended variety in Morocco) could be described, trying to compare the oils coming from this cultivar with Mediterranean cultivars introduced in Morocco, and with the three other autochthonous Moroccan varieties studied herein. Based on the results reported in Figure 2 and Tables 2 a, b, c and d, it can be concluded that oils from 'Picholine Marocaine' variety (in pedoclimatic conditions of Moroccan Meknès region) showed an "intermediate phenolic composition" as compared to the one found in both Mediterranean cultivars introduced to Morocco and the three autochthonous Moroccan varieties. Just one peculiar characteristic could be revealed for 'Picholine Marocaine' oils: their low content on flavonoids. If we focus on finding differences (or peculiar characteristics) among the three autochthonous Moroccan varieties obtained through clonal selection from 'Picholine marocaine' cultivar, it can be noted that the phenolic composition of 'Menara' and 'Haouzia' oils were quite similar in general terms, being 'Dahbia' oils those showing very distinctive phenolic composition (as previously detailed in this paper) for each one of the characterized chemical classes.

Despite these findings, examination of the results obtained applying a post-hoc test (Tukey's) to the phenolic profiles of the studied oils, we found that there was not any compound that could correctly classify the studied samples according to their botanical origin, since the mean values for each variable were not significantly different for all the 13 cultivars at the same time (Figure 2 and Table 2 a, b, c, and d). The quantitative compositions of the analyzed oils also showed large differences in phenolic content within the same botanical origin and no characteristic concentration ranges could be set. That is why we decided to go for the application of chemometric data analysis to test the ability of the identified phenolic compounds for tracing the botanical origin of the oils under evaluation.

Chemometrics

Principal components analysis. An exploratory approach through non-supervised PCA, using as chemical descriptors the auto-scaled data of the phenolic content found in the analyzed olive oil samples, was performed to provide a data structure study over a reduced dimension, covering the maximum amount of the information present in the basic data, which allows to investigate any possible clustering of the samples on the basis of their botanical origin. Thus, in a first step, PCA was performed on the entire data set (including the samples from the 13 cultivars, the content of the 33 determined compounds, and the total content of the five phenolic chemical classes as dependent variables). From this analysis, 20 principal components (PCs) were identified, being 9 of them the most important (eigenvalues >1). The first four components explained 65.06% of the total variability. The obtained data structure is shown in the graphic of scores, which were based on PC1 *vs* PC2, PC2 *vs* PC3, and PC3 *vs* PC4 (Figure 3a). Examining the score plot of the objects in these sub-spaces, it was evident that no separation of the 13 cultivars was achieved, except for oils from 'Dahbia' variety (in particular, for one of the seasons), which were quite differentiated from those of other cultivars. Afterwards, to check possible dissimilarities among samples from Moroccan varieties, a second PCA was then applied just to data from the four Moroccan cultivars investigated in this study. Considering the eigenvalues >1, seven PCs contained 89.89% of total variance. Figure 3b provides an overview of the natural clustering of the studied Moroccan olive oils samples on PC1 *vs* PC2, PC2 *vs* PC3, and PC3 *vs* PC4 sub-spaces. As can be observed in this Figure, the samples from 'Dahbia' variety (from one season) were again the only ones showing a clear clustering and were distinct from those of the other three considered groups, which were strongly overlapped. This may be explained considering the fact that the phenolic composition of olive oils from 'Haouzia' and 'Menara' is, to some extent, close or similar to their parental cultivar 'Picholine Marocaine'. In other words, although differences between these cultivars can be found (Figure 2 and Table 2 a, b, c, and d), they are not distinct enough to allow the separation of samples from these varieties based only on non-supervised chemometric methods. Hence, the importance of employing supervised chemometric techniques with the purpose of obtaining classification rules for assigning categories to samples.

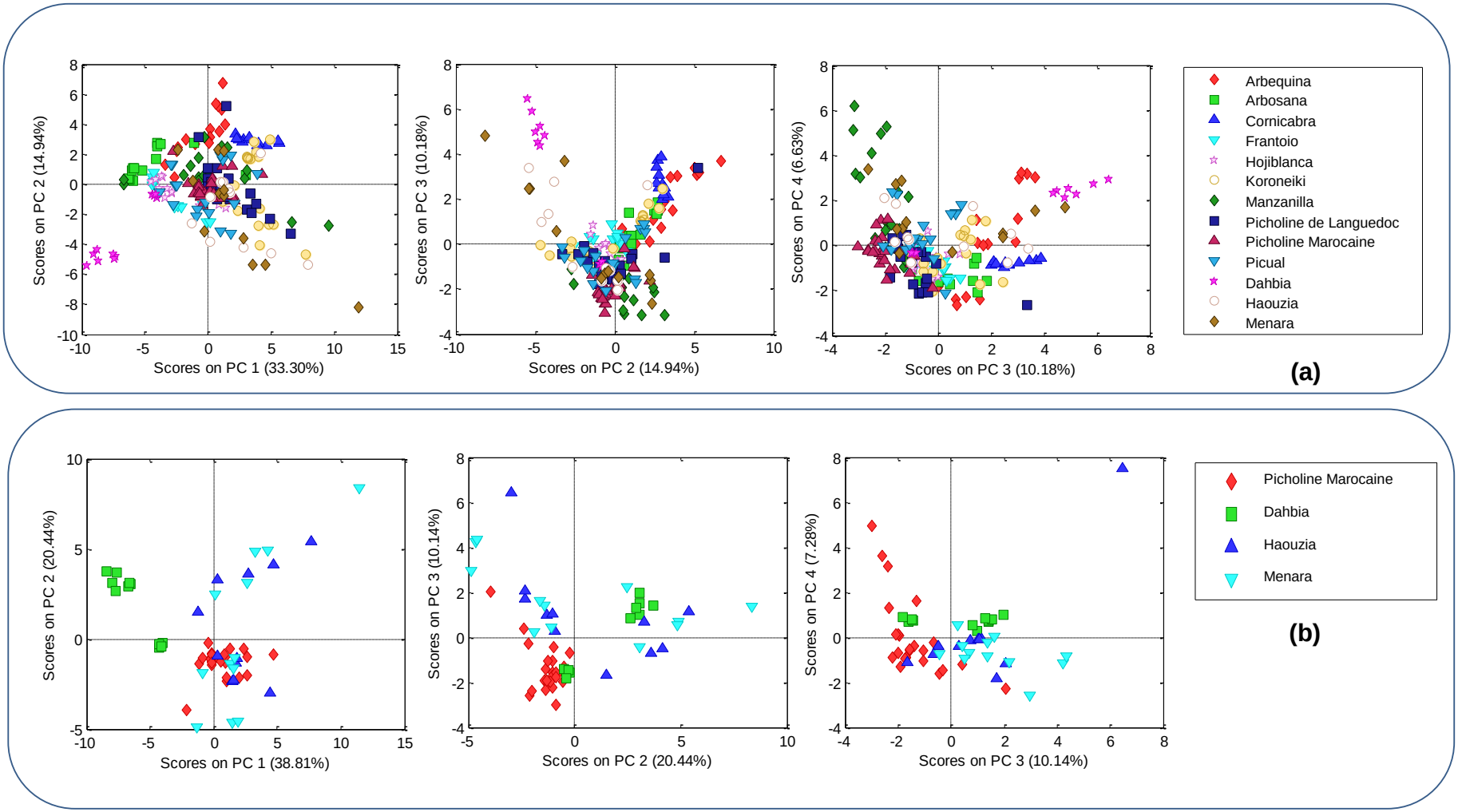

Figure 3. (a) Scores of samples from 'Picholine Marocaine' and Mediterranean cultivars on the map defined by the first four principal components. (b) Scores of samples from 'Picholine Marocaine' and the three other investigated Moroccan cultivars on the map defined by the first four principal components.

Linear discriminant analysis models. As stated above, with the aim of testing the capability of phenolic compounds variables to distinguish 'Picholine Marocaine' olive oils from the other studied oils, two LDA models were constructed: first of all, LDA was applied to a matrix containing 168 objects (which correspond to the samples from 'Picholine Marocaine' and the nine Mediterranean cultivars); secondly, LDA was carried out on a matrix composed by using samples from 'Picholine Marocaine' and the three autochthonous Moroccan varieties (59 samples). Another LDA model was also computed with the aim of discriminating among oils obtained from the nine Mediterranean cultivars and the three Moroccan varieties 'Dahbia', 'Haouzia' and 'Menara'. In the three LDA models constructed in this study, 38 predictors (the same as in PCA treatment) were used and no variable reduction was applied. The magnitude of Wilks' Lambda, which reflects the proportion of the variance in the dataset that is not accounted for the model was evaluated and significant values were obtained for the three LDA models built. Likewise, the uniformity of variability was tested (Box M index) and results were insignificant at the 95% confidence level, showing the existence of uniformity of sample variability for each botanical origin considered in the three LDA models. Moreover, the stepwise algorithm was applied to extract the best discriminant variable/s separating the studied samples according to their botanical origin. This discriminant procedure enters or removes variables by analyzing their effects on the discrimination of the groups based on the Wilks' lambda criterion. The selected variables for each model were the following: (a) Ac-Pin, Apig, Dehydro Ol Agl, DOA, EA, Hy D-Ol Agl, Hy-EA, Hyt, Hyt-Ac, Lig Agl IS1, Methyl Ol Agl, Ol Agl IS2, Ol Agl IS5, Ol Agl IS6, p-coum, Pin, total flavonoids, total lignans, total secoiridoids and total simple phenols for the first LDA model; (b) 10 Hy-Ol Agl, Apig, D-Ald-D EA, Dehydro Ol Agl, D-Lig Agl, EA, Methyl D-Ol Agl, Methyl Ol Agl, Ol Agl, Ol Agl IS4, Ol Agl IS6, p-coum, Pin, total flavonoids and Ty for the second LDA model; and (c) 10 Hy-Ol Agl, Ac-Pin, Apig, D-Ald-D EA, Dehydro Ol Agl, Desoxy-EA, D-Lig Agl, DOA, EA, H-D-Ol Agl, Hy-EA, Hyt, Hyt-Ac, Lig Agl, Lig Agl IS1, Lig Agl IS2, Lig Agl IS3, Lut, Methyl D-Ol Agl, Methyl Ol Agl, O-Hyt, Ol Agl, Ol Agl IS1, Ol Agl IS2, Ol Agl IS3, Ol Agl IS4, Ol Agl IS5, Ol Agl IS6, p-coum, Pin, QA, Syr, total flavonoids, total lignans, total secoiridoids, total simple phenols and Ty for the third LDA model. Besides, to evaluate the prediction power of the obtained models, "leave-one-out" cross-validation

was applied. Tables 3a, b and c show recognition and prediction abilities achieved by each LDA model.

Thus, as can be seen from Table 3a, the first LDA model, constructed with the phenolic compounds contents of 'Picholine Marocaine' and the other studied Mediterranean varieties, was able to correctly discriminate 'Picholine Marocaine' samples from the other monovarietal VOOs. 100% of correct classification and prediction were obtained for samples from this Moroccan variety. Similar results (100% in both classification and cross-validation) were obtained for other three monovarietal VOOs group: 'Cornicabra', 'Hojiblanca' and 'Manzanilla'. Very satisfactory results were also obtained for 'Arbequina' and 'Koroneiki' VOOs, which were 100% correctly classified, but the percentages obtained after cross-validation slightly decreased to 87.50% and 94.44%, respectively. Moreover, interesting results were obtained for 'Frantoio' samples (93.75% in both classification and cross-validation), 'Picual' (88.89% achieved in both classification and cross-validation) and 'Arbosana' (86.67 in both classification and cross-validation). The worst classification and prediction abilities were observed for 'Picholine de Languedoc' samples, where only 85.00% and 80.00% of them were correctly classified and predicted, respectively. The overall accuracy of the LDA model was, in any case, very satisfactory, with values of 95.24% in classification and 92.86% in prediction.

The LDA classification results of samples from the four Moroccan groups are shown in Table 3b. The discriminant functions achieved a classification ability of 100%, whereas their overall predictive ability was of about 86.21% (explained by the eight samples which were not correctly predicted). An analysis of these mispredicted samples showed that 4 (of 12) 'Menara' samples were predicted as 'Haouzia' oils and one as 'Picholine Marocaine' sample; two 'Haouzia' samples were predicted as 'Menara' and 'Picholine Marocaine' oils, and one 'Picholine Marocaine' sample fell within the 'Menara' group. Only 'Dahbia' samples were 100% correctly predicted, fact which again confirms the distinctive phenolic composition of the oils from this cultivar (comparatively to the other Moroccan varieties). In general, the results obtained herein (Table 3b) indicate satisfactory performances of the LDA model constructed for the classification (100%) and prediction (95.83%) of monovarietal 'Picholine Marocaine' olive oils among the other autochthonous Moroccan cultivars considered by this study.

Table 3a: Classification matrix, according to LDA, for the botanical origin discrimination between VOOs from Picholine Marocaine and Mediterranean cultivars (botanical origin discriminant Model 1).

Classification	Arbequina	Arbosana	Cornicabra	Frantoio	Hojiblanca	Koroneiki	Manzanilla	Picholine Marocaine	Picholine de Languedoc	Picual	Total	% correct
Arbequina	16	0	0	0	0	0	0	0	0	0	16	100.00%
Arbosana	1	13	0	0	0	0	0	1	0	0	15	86.67%
Cornicabra	0	0	11	0	0	0	0	0	0	0	11	100.00%
Frantoio	0	0	0	15	0	0	0	1	0	0	16	93.75%
Hojiblanca	0	0	0	0	13	0	0	0	0	0	13	100.00%
Koroneiki	0	0	0	0	0	18	0	0	0	0	18	100.00%
Manzanilla	0	0	0	0	0	0	17	0	0	0	17	100.00%
Picholine Marocaine	0	0	0	0	0	0	0	24	0	0	24	100.00%
Picholine de Languedoc	0	0	0	0	0	0	0	3	17	0	20	85.00%
Picual	0	0	0	0	0	0	0	1	1	16	18	88.89%
Total	17	13	11	15	13	18	17	30	18	16	168	95.24%
Cross-validation	**Arbequina**	**Arbosana**	**Cornicabra**	**Frantoio**	**Hojiblanca**	**Koroneiki**	**Manzanilla**	**Picholine Marocaine**	**Picholine de Languedoc**	**Picual**	**Total**	**% correct**
Arbequina	14	0	1	1	0	0	0	0	0	0	16	87.50%
Arbosana	1	13	0	0	0	0	0	1	0	0	15	86.67%
Cornicabra	0	0	11	0	0	0	0	0	0	0	11	100.00%
Frantoio	0	0	0	15	0	0	0	1	0	0	16	93.75%
Hojiblanca	0	0	0	0	13	0	0	0	0	0	13	100.00%
Koroneiki	0	0	0	0	1	17	0	0	0	0	18	94.44%
Manzanilla	0	0	0	0	0	0	17	0	0	0	17	100.00%
Picholine Marocaine	0	0	0	0	0	0	0	24	0	0	24	100.00%
Picholine de Languedoc	1	0	0	0	0	0	0	3	16	0	20	80.00%
Picual	0	0	0	0	0	0	0	1	1	16	18	88.89%
Total	16	13	12	16	14	17	17	30	17	16	168	92.86%

Table 3b: Classification matrix, according to LDA, for the botanical origin discrimination between VOOs from Picholine Marocaine and Moroccan cultivars (botanical origin discriminant Model 2).

Classification	Dahbia	Houzia	Menara	Picholine Marocaine	Total	% correct
Dahbia	11	0	0	0	11	100.00%
Haouzia	0	11	0	0	11	100.00%
Menara	0	0	12	0	12	100.00%
Picholine Marocaine	0	0	0	24	24	100.00%
Total	11	11	12	24	58	100.00%
Cross-validation	**Dahbia**	**Houzia**	**Menara**	**Picholine Marocaine**	**Total**	**% correct**
Dahbia	11	0	0	0	11	100.00%
Haouzia	0	9	1	1	11	81.82%
Menara	0	1	7	4	12	58.33%
Picholine Marocaine	0	0	1	23	24	95.83%
Total	11	10	9	28	58	86.21%

Furthermore, we tried to assess the potential of the phenolic profiles for the discrimination between Mediterranean olive oil samples and those obtained from the three Moroccan cultivars 'Dahbia', 'Haouzia' and 'Menara'. The obtained results (Table 3c) show that the LDA model had overall classification and prediction abilities of 93.26% and 84.83%, respectively. When focusing on the obtained results for each monovarietal olive oil class, it can be observed that the built LDA model gave excellent results with a success rate of 100% in both classification and cross-validation for three monovarietal groups 'Cornicabra', 'Dahbia' and 'Hojiblanca'. Classification and prediction were also reasonably successful for other cultivars, such as 'Koroneiki' (94.44% in both classification and cross-validation), 'Arbequina' (93.75% in both classification and cross-validation) and 'Frantoio' (93.75% for classification and 87.50% for cross-validation). In contrast, the worst results in classification (81.82%) and prediction (50.00%) were found for Moroccan samples from 'Haouzia' and 'Menara' varieties, respectively. Indeed, as shown by the results reported on Table 3c, the low rate obtained in classification for 'Haouzia' samples can be related to the misclassification of 2 of the 11 samples from this variety as 'Cornicabra' and 'Menara' oils, whereas only 6 from the 12 Menara samples were well predicted. The other 6 were mispredicted as 'Frantoio' (one sample), 'Picholine de Languedoc' (one sample) and 'Haouzia' (4 samples) oils. The latter also showed low rate of correct prediction (54.55%), being among the 5 misspredicted samples from this class 1 'Cornicabra', 1 'Manzanilla' and 3 classified as 'Menara'.

Table 3c: Classification matrix, according to LDA, for the botanical origin discrimination between VOOs obtained from Mediterranean and Moroccan cultivars (botanical origin discriminant Model 3).

Classification	Arbequina	Arbosana	Cornicabra	Dahbia	Frantoio	Hojiblanca	Haouzia	Koroneiki	Manzanilla	Menara	Picholine de Languedoc	Picual	Total	% correct
Arbequina	15	0	0	0	0	0	0	0	0	0	1	0	16	93.75%
Arbosana	1	13	0	0	0	0	0	0	0	0	1	0	15	86.67%
Cornicabra	0	0	11	0	0	0	0	0	0	0	0	0	11	100.00%
Dahbia	0	0	0	11	0	0	0	0	0	0	0	0	11	100.00%
Frantoio	0	0	0	0	15	0	0	0	0	0	1	0	16	93.75%
Hojiblanca	0	0	0	0	0	13	0	0	0	0	0	0	13	100.00%
Haouzia	0	0	1	0	0	0	9	0	0	1	0	0	11	81.82%
Koroneiki	0	0	0	0	0	0	0	17	0	0	0	1	18	94.44%
Manzanilla	0	2	0	0	0	0	0	0	15	0	0	0	17	88.24%
Menara	0	0	0	0	0	0	1	0	0	11	0	0	12	91.67%
Picholine de Languedoc	0	0	0	0	1	0	0	0	0	0	19	0	20	95.00%
Picual	0	0	0	0	0	0	0	0	0	0	1	17	18	94.44%
Total	16	15	12	11	16	13	10	17	15	12	23	18	178	93.26%
Cross-validation	**Arbequina**	**Arbosana**	**Cornicabra**	**Dahbia**	**Frantoio**	**Hojiblanca**	**Haouzia**	**Koroneiki**	**Manzanilla**	**Menara**	**Picholine de Languedoc**	**Picual**	**Total**	**% correct**
Arbequina	15	0	0	0	0	0	0	0	0	0	1	0	16	93.75%
Arbosana	1	11	0	0	0	0	0	0	0	1	1	1	15	73.33%
Cornicabra	0	0	11	0	0	0	0	0	0	0	0	0	11	100.00%
Dahbia	0	0	0	11	0	0	0	0	0	0	0	0	11	100.00%
Frantoio	0	0	0	0	14	0	0	0	0	1	1	0	16	87.50%
Hojiblanca	0	0	0	0	0	13	0	0	0	0	0	0	13	100.00%
Haouzia	0	0	1	0	0	0	6	0	1	3	0	0	11	54.55%
Koroneiki	0	0	0	0	0	0	0	17	0	0	0	1	18	94.44%
Manzanilla	0	2	0	0	0	0	0	0	15	0	0	0	17	88.24%
Menara	0	0	0	0	1	0	4	0	0	6	1	0	12	50.00%
Picholine de Languedoc	0	0	0	0	1	0	1	1	0	1	16	0	20	80.00%
Picual	0	0	0	0	0	0	1	0	0	0	1	16	18	88.89%
Total	16	13	12	11	16	13	12	18	16	12	21	18	178	84.83%

This proves that oil samples from these two Moroccan varieties (obtained by clonal selection from 'Picholine Marocaine' cultivar) exhibit a quite similar phenolic composition, which makes difficult their discrimination.

In conclusion, the data presented in this study are the first published on the phenolic composition of the studied cultivars in the Moroccan pedoclimatic conditions (not considering 'Picholine Marocaine' cv.). The determination of the phenolic composition of the studied monovarietal olive oils could be of great importance to select cultivars with oils showing the highest nutritional value and promoting them amongst consumers. Besides, the considerable differences among the studied cultivars in terms of their oils´ content on the identified phenolic compounds, allow to conclude that the genotype perform a major influence on the phenolic fraction of olive oil. Likewise, the effect of the crop season climatology was very significant. Finally, the application of LDA made possible the correct discrimination of 'Picholine Marocaine' olive oils, as the main one produced in Morocco, from the other studied monovarietal olive oils categories. These results can strengthen authentication controls of Moroccan VOOs certified as monovarietal from this cultivar.

ABBREVIATIONS USED

10 Hy-Ol Agl, 10-hydroxy oleuropein aglycone; Ac-Pin, (+)-1-acetoxypinoresinol; ANOVA, one-way analysis of variance; Apig, apigenin; DAD, diode-array detector; D-Ald-D EA, dialdehydic form of decarboxymethyl elenolic acid; Dehydro Ol Agl, dehydro oleuropein aglycone; Desoxy-EA, desoxy elenolic acid; D-Lig Agl, decarboxymethyl ligstroside aglycone; DOA, decarboxymethyl oleuropein aglycone; DOPAC, 4-Dihydroxyphenylacetic acid; EA, elenolic acid; EICs, extracted ion chromatograms; Hy D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone; Hy-EA, hydroxy elenolic acid; Hyt, hydroxytyrosol; Hyt-Ac, hydroxytyrosol acetate; IOC, International Olive Council; IS: isomer; ISt, internal standard; IT, Ion Trap; LC, liquid chromatography; LDA, Linear discriminant analysis; Lig Agl, ligstroside aglycone; Lut, luteolin; Methyl D-Ol Agl, methyl decarboxymethyl oleuropein aglycone; Methyl Ol Agl, methyl oleuropein aglycone; MS, mass spectrometry; O-Hyt, oxidized hydroxytyrosol; Ol Agl, oleuropein aglycone; PCA, Principal component analysis; *p*-coum, *p*-coumaric acid; Pin, (+)-pinoresinol; QA, quinic

acid; QC, quality control; Syr, syringaresinol; TICs, total ion chromatograms; Ty, tyrosol; VOO, virgin olive oil.

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Chapter

8

Assessing the varietal origin of virgin olive oil using liquid chromatography phenolic compounds fingerprints, data fusion and chemometrics

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Abstract

Herewith, HPLC with DAD and fluorescence (FLD) detection was used to acquire fingerprints of the phenolic fraction of monovarietal virgin olive oils. The chromatographic fingerprints of 140 VOO samples processed from olive fruits of seven olive varieties, collected over three consecutive crop seasons (2011-2014) were recorded at 280 nm (DAD) and λ excitation = 280 nm, λ emission = 339 nm (FLD), and statistically treated for botanical authentication purposes. First, DAD and FLD chromatographic fingerprint data sets were processed separately and, subsequently, were joined using a data fusion method. After the preliminary examination by principal component analysis (PCA), three supervised pattern recognition techniques (PLS-DA, SIMCA and k-NN) were applied to the three chromatographic fingerprinting matrices. The classification models built were very sensitive and selective, showing considerably good recognition and prediction abilities. The combination "chromatographic data set+chemometric technique" allowing the most accurate classification for each monovarietal VOO was highlighted.

Keywords: monovarietal virgin olive oils; high performance liquid chromatography; phenolic compounds fingerprints; data fusion; chemometrics; botanical origin.

1 Introduction

Virgin olive oil (VOO) is one of the most appreciated vegetable oils all over the world, although it is especially popular in the Mediterranean countries where the production of this commodity is a millenary tradition (Kapellakis, Tsagarakis, & Crowther, 2008). Likewise, VOO consumption has continuously attracted great interest, due to its beneficial effects on human health throughout its significant role in the prevention of some chronic diseases (Covas, 2007 and López-Miranda et al., 2010). These healthy properties of VOO have been mainly attributed to its chemical composition, particularly, to its richness in terms of some bioactive compounds (Bendini et al., 2007; Preedy & Watson, 2010 and Visioli & Galli, 2002). VOO chemical composition is quite complex, including the saponificable fraction (which comprises 98-99% of the total weight of the oil and is mainly formed by triacylglycerides) and the fraction formed by minor components, which, despite of being a minority group (about 2% of the total oil weight),

considerably contributes to some of the most relevant properties of this matrix. Minor components include more than 230 chemical compounds, mainly, pigments, aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile and phenolic compounds (Servili et al., 2013).

Over the last years, producers have shown a great interest in producing VOOs under differentiating systems as a market segmentation strategy, to make themselves more competitive and try to deal with the effects of the globalization process in the olive oil sector (Lamani & Khadari, 2015 and Parra-López, Hinojosa-Rodríguez, Sayadi, & Carmona-Torres, 2015). This can explain the gradual appearance of products with geographical indications (Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG)) and monovarietal certifications into the olive oil market. These products are expected to be premium high quality olive oils with peculiar and unique sensory characteristics that reflect the effects of a specific geographical origin (which includes environmental and human elements) and/or exclusive features of the processed olive variety. Thus, because of their unique characteristics, these products are commonly highly prized, and for this reason, they are occasionally subjected to various types of adulteration, such as the addition of other non-certified olive oils to enlarge the volume of the most expensive certified geographical and/or monovarietal VOOs, increasing therefore the economic profits. Hence, many researches have been performed in the last decades to develop reliable analytical methods to ensure compliance of these certified products with labeling, through authenticating their geographical and/or botanical origin (Perri, Benincasa, & Muzzalupo, 2012).

Indeed, numerous attempts have been made to find suitable analytical approaches to differentiate monovarietal VOOs according to their cultivar origin (Cichelli & Pertesana, 2004; Gurdeniz, Ozen, & Tokatli, 2008 and Sinelli et al., 2010). These analytical methodologies can be basically divided into three main conceptual approaches: conventional approaches known as targeted analysis, profiling techniques and fingerprinting methodologies. Target analysis has been applied for a long time to olive oil botanical origin authentication and includes the determination of a small set of known olive oil compounds (targets) using one particular analytical technique suitable for the compounds of interest. Extensive research has been reported about the discrimination of

the botanical origin of VOO by means of the targeted analysis of some of its compounds using, quite extensively, chromatography (Montealegre, Marina Alegre, & García-Ruiz, 2010). In contrast to this approach, profiling techniques seek to provide the qualitative and/or quantitative determination of a larger set of compounds, which are related considering their chemical nature and/or biosynthesis pathway. In practice, the profiling of olive oil secondary metabolites -such as phenolic and volatile compounds- has shown great potential for the effective differentiation of monovarietal VOOs from different cultivars (Cecchi & Alfei, 2013 and García-Villalba et al., 2011). The relevance of fingerprinting approaches is undeniable too; they offer interesting advantages over other strategies, particularly, in terms of analysis simplicity, accuracy and rapidity (a metabolic fingerprint of the sample under study is generated and, then, compared to a large sample population screening for differences among the samples under study). In that respect, spectroscopic techniques such as infrared spectroscopy in the near (NIR) and mid (MIR) regions (Sinelli et al., 2010), Fourier transform infrared (FTIR) spectroscopy (Concha-Herrera, Lerma-García, Herrero-Martínez, & Simó-Alfonso, 2009 and Gurdeniz, Tokatli, & Ozen, 2007) and nuclear magnetic resonance (NMR) spectroscopy (Mannina & Segre, 2010) are the most widely used analytical techniques for the botanical authentication of VOO employing fingerprinting analysis.

Regardless of the conceptual approach selected for the varietal origin authentication of olive oil (of the three mentioned above), the obtained data are subsequently treated using appropriate chemometric tools, including unsupervised methods (mainly Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA)), and supervised ones (such as Linear Discriminant Analysis (LDA), Partial Least Squares Discriminant Analysis (PLS-DA), Soft Independent Modeling of Class Analogies (SIMCA), K-Nearest Neighbors (k-NN), ect.) (Marini, Bucci, Magrì, & Magrì, 2010).

Previous studies focused on the development of analytical approaches for the botanical classification of VOOs have paid a special attention to phenolic compounds (Montealegre et al., 2010). Indeed, the phenolic composition of VOO is genetically determined, even if remains widely influenced by pedoclimatic conditions as well as agronomical and technological practices (Dabbou et al., 2009; Pérez et al., 2014; Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz, & Pérez, 2012 and Servili et al., 2004). Many

publications dealing with the characterization of phenolic compounds in VOOs described the use of high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS), coupling which is not affordable for many laboratories due to its cost. HPLC with diode array (DAD) and/or fluorescence (FLD) detection could be a cheaper (but useful) alternative which can cover the high-throughput analysis of the VOO phenolic fraction. Certainly, the availability of these analytical platforms in most of the olive oil laboratories and research institutes could favor their application for routine analysis evaluating the botanical authenticity of certified monovarietal VOOs.

The aim of this study was, therefore, to ascertain the potential of using phenolic compounds chromatographic fingerprints recorded by using DAD and FLD detectors (alone or combined) to discriminate among monovarietal VOOs, produced in northern Morocco, according to their cultivar of origin. The obtained three data matrices (HPLC-DAD, HPLC-FLD and HPLC-DAD-FLD (2D)) were evaluated using different chemometric techniques (PCA, PLS-DA, SIMCA and k-NN), comparing their performance (taking into account both classification and prediction results) and trying to identify which combination "chromatographic data set+chemometric technique" could lead to the most accurate botanical discrimination of each variety.

2 Materials and methods

2.1 Olive fruits sampling

Olive trees in perfect sanitary conditions and conducted under the same agronomic conditions, in the olive experimental orchard of the Agro-pôle Olivier National School of Agriculture of Meknes (Morocco), were randomly selected for olive fruits sampling. 140 fresh and healthy olive fruits samples (approximately one sample was of about 35 kg of olives), at the same maturity stage (3.0-3.5), were handpicked over three consecutive crop seasons (2011/2012 (n=47), 2012/2013 (n=56) and 2013/2014 (n=37)). The following olive varieties were considered in this study: **'Arbequina'** (2011/2012 (n=4), 2012/2013 (n=4) and 2013/2014 (n=3)), **'Arbosana'** (2011/2012 (n=5), 2012/2013 (n=6) and 2013/2014 (n=4)), **'Cornicabra'** (2011/2012 (n=5), 2012/2013 (n=6) and 2013/2014 (n=5)), **'Frantoio'** (2011/2012 (n=6), 2012/2013 (n=9) and 2013/2014 (n=6)), **'Picholine de Languedoc'** (2011/2012 (n=10), 2012/2013 (n=8) and 2013/2014 (n=5)), **'Picholine**

Marocaine' (2011/2012 (n=9), 2012/2013 (n=14) and 2013/2014 (n=8)), and **'Picual'** (2011/2012 (n=8), 2012/2013 (n=9) and 2013/2014 (n=6)). Olive samples were placed in rectangular plastic crates and immediately transported to the laboratory. Oil was extracted within 24 hours of picking.

2.2 Oil extraction

Oil extraction was carried out in a laboratory instrument (Oliomio laboratory mill (Oliomio, Italy)). First, the olives were washed and defeated, then crushed using a crusher equipped with fix hole grid and groove knives impeller at a temperature of 25-27°C. The paste produced falls into the malaxing part; malaxing was carried out for 45 min at 28-30°C. The resulting olive paste was decanted at temperature of 23-27°C without the addition of any water. All the samples were subsequently filtered, placed in amber glass bottles (250 mL) at −18°C, excluding any head space volume in order to assure their proper conservation against oxidation until the moment in which the chemical analyses were performed. Furthermore, physicochemical quality parameters (free acidity, expressed as percentage of oleic acid (%), peroxide value expressed as milliequivalents of active oxygen per kilogram of oil (meq O_2 kg^{-1}), K_{232} and K_{270} extinction coefficients (calculated from absorption at 232 and 270 nm, respectively)) were determined according to the analytical methods described in the European Union standard methods Regulations 2568/91 and the subsequent amendments (EC, 1991). Every determination was carried out in triplicate. Results allowed to classify all studied samples within the extra virgin category.

2.3 Phenolic compounds extraction and chromatographic fingerprints acquisition

Phenolic compounds were first extracted from the olive oil samples under study using a liquid-liquid extraction procedure previously described by Bajoub et al. (Bajoub et al., 2015). The solvents used to carry out the extraction (methanol and *n*-hexane) were of HPLC grade and were supplied from Panreac (Barcelona, Spain). The protocol can be briefly described as follows: 2.0 g of oil was weighed into a centrifuge tube, and 1 mL of *n*-hexane and 2.0 mL of methanol-water (60:40, v/v) were added. 3,4-Dihydroxyphenylacetic acid (DOPAC) (0.025 mL, 500 $mg.L^{-1}$), supplied by Sigma-Aldrich, was added to the oil as internal standard (with the intention of evaluating the repeatability of the extraction procedure). The mixture was stirred for 2 min in a vortex

apparatus, and afterwards, the tube was centrifuged at 3500 rpm for 6 min. The methanol layer was then separated and the extraction was repeated twice. The methanolic extracts were combined and evaporated to dryness under reduced pressure at a temperature not exceeding 30°C. The residue was redissolved in 1 mL with methanol and filtered through 0.22 μm nylon membrane filters.

After that, the phenolic compounds chromatographic analysis was performed on an Agilent 1260 LC system (Agilent Technologies, Santa Clara, CA, USA), consisting of a vacuum degassing unit, a binary pump, an autosampler injector, a column thermostat, and DAD and FLD detectors. The chromatographic separation was performed on an Agilent Zorbax C18 analytical column (4.6 x 150 mm, 1.8 μm particle size), protected by a guard cartridge of the same packing, using water with 0.5% acetic acid (Phase A) and acetonitrile (Phase B) as mobile phases at a flow rate of 0.8 mL min^{-1}. The gradient elution programme was set as follows: 0 to 10 min, 5 -30% B; 10 to 12 min, 30-33% B; 12 to 17 min, 33-38% B; 17 to 20 min, 38-50% B; 20 to 23 min, 50-95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 2.5 min. The injection volume was 10 μL. The column temperature was maintained at 25°C, and the separated compounds were monitored on-line firstly by DAD (280 nm) and, secondly, by FLD (λ excitation = 280 nm, λ emission = 339 nm). The ChemStation software package (Agilent Technologies, Santa Clara, CA, USA) was used for the chromatographic spectra acquisition and data exportation as CSV (comma-separated values) files. The chromatographic profile for each sample was composed for as many variables as retention times recorded during the data acquisition time.

2.4 Data set and chemometric treatment

HPLC-DAD and HPLC-FLD chromatographic profiles of the 140 studied olive oil samples were divided into two sub-sets. The first one included 103 chromatographic profiles of samples corresponding to the crop seasons 2011/2012 and 2012/2013, and was used as calibration set. The remaining 37 chromatographic fingerprints (corresponding to 2013/2014 olive oil samples) were used as independent test set for the external validation of the different built models. The raw data files for each chromatogram were exported as a CSV file and, then, these files were imported directly into the MATLAB® software (version 7.9, R2009b, The MathWorks Inc., Natick, MA,

USA). The Bioinformatics Toolbox™ and PLS Toolbox® (version 7.9.5, Eigenvector Research Inc., Wenatchee, WA, USA) for MATLAB software were used for all subsequent pre-processing and chemometric data treatments.

Initially, each chromatographic fingerprint was coded as a 2D vector (retention time/intensity), with a varying number of data points depending on time and data acquisition rate of each detector. In particular, the vector length of the raw chromatographic data obtained with the HPLC-DAD and HPLC-FLD detectors was 4275 and 3958 data points (variables), respectively. All the intensity data vectors, one for each oil sample, from the same chromatographic detector were merged in a single data matrix (X-block matrix). Therefore, for both training and validation data sets, two chromatographic fingerprinting matrices were obtained, having as many rows as analyzed oil samples and as many columns as the entire chromatogram data points recorded during the acquisition time with the two detection systems. Thus, for the calibration set, the obtained data matrices were: HPLC-DAD matrix [103 x 4275] and HPLC-FLD matrix [103 x 3958], whereas for the external validation data set the obtained data matrices were: HPLC-DAD matrix [37 x 4275] and HPLC-FLD matrix [37 x 3958].

Before proceeding to the data pre-processing, the training and test data matrices of each detector were merged within a single matrix, HPLC-DAD [140 x 4275] and HPLC-FLD [140 x 3958], to be processed together (for more details in this respect, have a look at section 2.4.2.).

2.4.1 Data pre-processing

Since recorded chromatograms could be affected by some sources of variability, such as variations in the operational conditions, instrumental instability and operator handling, which may lead to additional sources of variance to be modeled, the chromatographic data pre-processing is a very important step. Thus, several pre-processing techniques were applied to the raw chromatographic fingerprinting matrices. In particular, the pre-processing of chromatographic data matrices was carried out using an *ad-hoc* MATLAB function programmed by Pérez-Castaño et al. (Pérez-Castaño et al., 2015). This function makes use of some of the functions and utilities included in the MATLAB Bioinformatics Toolbox™ to improve the quality of raw chromatographic data, and also employs the *icoshift* algorithm (version 1.2.2) (Tomasi, Savorani, & Engelsen, 2011) for solving signal

alignment problems in chromatographic data. Basically, the chromatographic data pre-processing function consists of the following steps: (1) selection of the interval of interest from chromatograms; (2) de-noising and smoothing of the chromatographic data using a least-squares digital polynomial filter (*i.e.*, a Savitzky-Golay filter); (3) baseline correction using the *msbackadj* function available in the Bioinformatics Toolbox™; and (4) alignment of the chromatographic profiles with the *icoshift* algorithm. Finally, a mean centering was also applied to the chromatographic data matrix prior to the statistical analysis. The chromatographic data pre-processing on the set of 140 chromatographic fingerprints of the studied monovarietal VOOs is shown in Fig. 1, where the alignment process of the chromatographic fingerprints using the *icoshift* algorithm can be seen and the raw HPLC-DAD and HPLC-FLD chromatograms are overlapped before and after the data pre-processing.

Therefore, after the pre-processing steps, the chromatographic data matrices obtained for the training set were: HPLC-DAD matrix [103 x 3540] and HPLC-FLD matrix [103 x 3278], whereas for the validation data set the obtained data matrices were: HPLC-DAD matrix [37 x 3540] and HPLC-FLD matrix [37 x 3278].

2.4.2 Two-dimensional fingerprinting approach (data fusion into a 2D matrix)

In this work, training and validation chromatographic data sets obtained with the use of the two detectors (DAD and FLD) were combined by a data fusion method (Chen et al., 2008 and Ni, Liu, & Kokot, 2011) to form the HPLC-DAD-FLD (2D) matrix as follow: assuming that $\boldsymbol{A} = [\boldsymbol{\alpha}_1, \boldsymbol{\alpha}_2, \dots, \boldsymbol{\alpha}_m]$ is the HPLC-DAD data matrix, and $\boldsymbol{B} = [\boldsymbol{\beta}_1, \boldsymbol{\beta}_2, \dots, \boldsymbol{\beta}_m]$ is the HPLC-FLD data matrix for the olive oil samples, where m denotes the number of samples, each $\boldsymbol{\alpha}_i = [a_1, a_2, \dots, a_p]^T$ represents the ith collected HPLC-DAD chromatogram and $\boldsymbol{\beta}_j = [b_1, b_2, \dots, b_q]^T$ the jth collected HPLC-FLD chromatogram, where p and q denote the number of data points of each chromatogram. The used data fusion method simply involves the joining of a pair of chromatograms (HPLC-DAD and HPLC-FLD) of the same sample into a single chromatogram (data vector), *i.e.* $\boldsymbol{x}_m = [\boldsymbol{\alpha}_m; \boldsymbol{\beta}_m]$. Thus, the combined 2D data matrix $\boldsymbol{X} = [\boldsymbol{x}_1, \boldsymbol{x}_2, \dots, \boldsymbol{x}_m]^T$ was obtained for analysis. This matrix was normalized, and for the mth vector, $\boldsymbol{x}_m$, may be expressed as:

$$x'_{m,i} = \left(x_{m,i} - x_{m,\min}\right)/\left(x_{m,\max} - x_{m,\min}\right)$$

where $x'_{m,i}$ is the normalized intensity value of the ith element of vector, $\boldsymbol{x}_m$, and $x_{m,\min}$ and $x_{m,\max}$ are the minimum and maximum elements of $\boldsymbol{x}_m$, respectively.

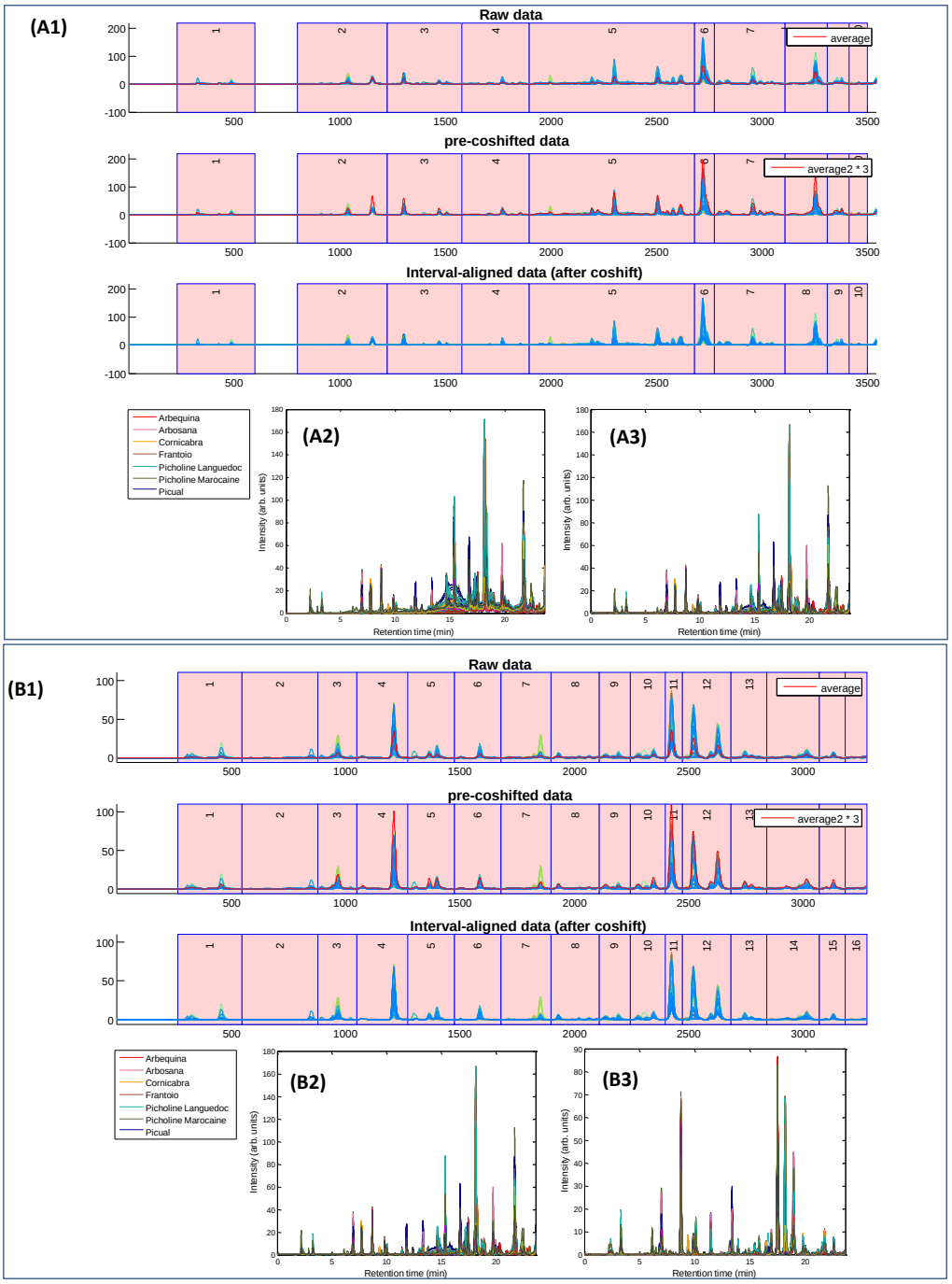

Fig.1. Chromatographic data pre-processing on a set of 140 chromatographic fingerprints of studied monovarietal VOOs. Alignment of chromatographic fingerprints using the *icoshift* algorithm on (A1) HPLC-DAD chromatograms and (B1) HPLC-FLD chromatograms. Superposition of raw chromatograms obtained from HPLC-DAD before (A2) and after (A3) data pre-processing, and superposition of raw chromatograms obtained from HPLC-FLD before (B2) and after (B3) data pre-processing.

2.4.3 Chemometrics

Each one of the six data sets (training and validation data sets for the DAD and FLD detectors measurements, and training and validation data sets resulting of combining data of both detectors) were analyzed by means of an unsupervised statistical method, for an exploratory overview, and supervised discriminant techniques in order to build statistical models able to discriminate the studied VOOs according to their botanical origin.

Unsupervised classification was carried out using PCA which is an exploratory method describing data set without *a priori* knowledge of the data structure. It provides a reduction in data set dimensionality constructing new uncorrelated variables, known as principal components (PCs) that are linear combinations of the original independent variables. These principal components, calculated from differences and similarities of all the variables for all the samples are used to explain the maximum of data set variance. The first component extracts the largest source of variance and the last one have above the random noise.

As far as supervised techniques are concerned, a discriminant technique (PLS-DA), a class modeling method (SIMCA) and a non-parametric data classification technique (k-NN) were used, since it has been widely highlighted their usefulness in literature to authenticate the varietal and/or geographical origin of different foodstuffs. PLS-DA performs a reduction in the dimension of the predictor variables using an approach similar to principal component analysis. PLS-DA is essentially based on the PLS2 algorithm (Wold et al., 1983) that searches for latent variables with a maximum covariance with the Y-variables. Of course, the main difference is related to the dependent variables, since these represent qualitative (and not quantitative) values, when dealing with classification. In PLS-DA, the Y-block describes which objects are in the classes of interest. When dealing with N classes, the class vector is unfolded and the PLS2 algorithm is applied. For each object, PLS-DA will return the prediction as a vector of size N, with values between 0 and 1: an *n-th* value closer to zero indicates that the object does not belong to the *n-th* class, while a value closer to one indicates the opposite. Since predicted vectors will not have the form (0,0,...,1,...0) but real values in the range between 0 and 1, a classification rule must be applied; the object can be assigned to the class with the maximum value in the Y vector or, alternatively, a threshold between 0 and 1 can be

determined for each class on the basis of the Bayes theorem (the class threshold is selected at the point where the number of false positives and false negatives is minimized) (Barker & Rayens, 2003).

SIMCA is a class modeling technique (Wold, 1976). A SIMCA model consists of a collection of PCA models, one for each class. Therefore, PCA is separately calculated on the objects of each class; since the number of significant components can be different for each category, cross-validation has been proposed as a way of choosing the number of retained components of each class model (*e.g.*, in this work, a 10-fold venetian blinds cross-validation method was used). In this way, SIMCA defines *N* class models, where *N* is the total number of classes. Then, a new object is projected in each subspace and compared to it in order to assess its distance from each class. Distances are calculated on the basis of normalized Q residuals and normalized Hotelling T^2 values. Q residuals and Hotelling T^2 are normalized over their 95% confidence limits. Samples can be assigned to the class with normalized Q residuals and T^2 Hotelling distance lower than a fixed threshold. The distance threshold is found for each class by increasing the threshold and maximizing class specificity and sensitivity. In this way (class modeling approach), samples could be predicted in none or multiple class spaces. Otherwise, samples can be always assigned to the closest class, *i.e.* the class with the lowest normalized Q residuals and T^2 Hotelling distance (approach used in this work).

k-NN is a supervised and non-parametric classification method (Miller & Miller, 2010). It is based on the determination of the distances between an unknown object and each of the objects of the training set. In k-NN, the K-nearest objects of the unknown sample are selected and a majority rule is applied: the unknown sample is classified in the group to which the majority of the K objects belong. The choice of K is optimized by calculating the prediction ability with different K values. Small K values between 3 and 5 are often preferred. The method has several advantages (Cruz et al., 2013): (i) its mathematical simplicity, which does not prevent it from achieving classification results as good as (or even better than) other more complex pattern recognition techniques, (ii) it is free from statistical assumptions, such as the normal distribution of the variables, and (iii) its effectiveness does not depend on the space distribution of the classes. In this work, the Euclidean distance was used as the criterion for calculating the distance between samples, and the number of neighbors (K) was selected after studying the success of the

classification with different K values applied to a training set with all the samples; this is accomplished by means of a cross validation procedure, i.e. by testing a set of K values (e.g. from 1 to 10); then, the K giving the lowest classification error in cross-validation can be selected as the optimal one. In this case, four nearest neighbors were chosen for modeling.

In the model-building step, depending on the supervised pattern recognition technique applied, classification was evaluated by calculating: the root mean square error of calibration (RMSEC), the root mean square error of cross-validation (RMSECV), the root mean square error of prediction on the test set (RMSEP), sensitivity (true positive rate, measures the proportion of positives that are correctly identified as such: SENS = 100 x [TP / (TP + FN)]), specificity (true negative rate, measures the proportion of negatives that are correctly identified as such: SPEC = 100 x [TN / (TN + FP)]) and accuracy (characterizing the correct classification rate, i.e. the percentage of samples correctly classified: ACCY= 100 x [TP+TN / (TP-TN+ FP+FN)]); where TP = true positive, the number of positive samples that are correctly identified as positive samples; FN = false negative, the number of positive samples that are misclassified as negative samples; FP = false positive, the number of negative samples that are incorrectly identified as positive samples; and TN = true negative, the number of negative samples that are correctly identified as negative samples.

3 Results and discussion

3.1 Chromatographic profiles examination

A reversed-phase HPLC procedure was used in this work for the phenolic compounds fingerprinting of the monovarietal VOOs coming from 7 varieties cultivated under the same conditions. Representative chromatograms of the phenolic extracts from each selected variety are depicted in Fig. 2. As shown in the figure, a good chromatographic separation was obtained, the complex phenolic extracts were properly resolved and a wide range of different compounds (peaks) could be observed. This proves that the used analytical chromatographic conditions, described in Section 2.3, were optimal. Indeed, the chromatographic and detections conditions (DAD (280 nm) and FLD (λ excitation = 280 nm, λ emission = 339 nm) wavelengths) were chosen on the basis of previously published results (Bajoub et al., 2015),(Godoy-Caballero, Acedo-Valenzuela, & Galeano-

Díaz, 2012), trying to maximize the number of detected compounds (and their relative intensity signals).

Carrasco-Pancorbo et al. (Carrasco-Pancorbo et al., 2005) claimed that even if routine detection of phenolic compounds in HPLC is extensively made by the measurement of UV-Vis absorption at different wavelengths (being 280 nm the most commonly used due to the chemical structures of the various chemical classes of phenolic compounds found in VOO), fluorescence detection could offer some advantages and give some complementarity (in terms of enhanced selectivity and sensitivity through optimizing λ excitation and λ emission) for the characterization of some compounds. For instance, according to Selvaggini et al. (Selvaggini et al., 2006), lignans show a good response in fluorescence. Godoy-Caballero et al. [44] identified the optimum λ excitation and λ emission for all the phenolic compounds available as commercial pure standards. Moreover, recent results from our research group (under revision to be published) point at the same direction, demonstrating the FLD can be a very suitable detector to characterize the complete phenolic fraction of VOOs (using multi-emission λ).

The HPLC-DAD and HPLC-FLD screening of the seven varieties evaluated revealed that the phenolic profiles of these varieties were, apparently, quite similar in qualitative terms, but they presented some differences regarding the peaks relative intensities (inter-variety differences). When different profiles coming from the same variety were superimposed, slight differences could be observed for all the cultivars, except for 'Picholine Marocaine'. The heterogeneity (if compared to other varieties) of the VOOs coming from this cultivar, has been already reported by our group in previous publications, where it was shown that the high phenolic composition intra-variety variability (even if it is cultivated on the same region) is due to the fact that this cultivar includes several distinct clones and local genotypes, all known under the common name 'Picholine Marocaine' (Bajoub et al., 2015). This may challenge the botanical classification of samples from this cultivar.

The following steps of this study were conducted to investigate which chemometric techniques could deal more satisfactorily with the data analysis, allowing for each variety, the best possible classification of the studied samples (with high rates of recognition and prediction).

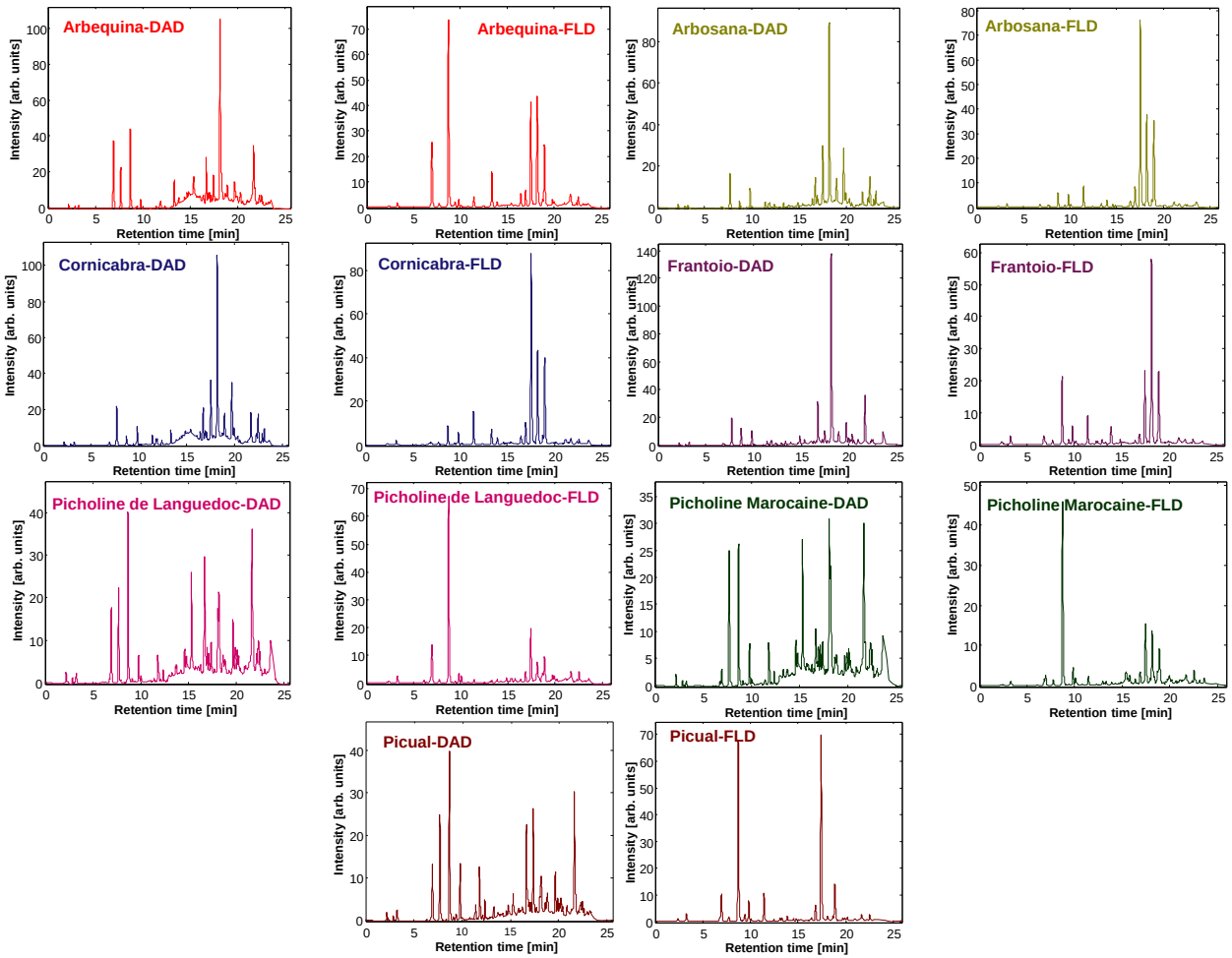

Fig. 2. Typical chromatograms of the seven monovarietal VOOs classes studied by using high performance liquid chromatography with diode array (DAD) and fluorescence (FLD).

3.2 Botanical classification

3.2.1 Unsupervised multivariate data analysis: PCA

In order to get a general overview of the data structure and distribution and detect the similarities and differences among all the studied samples, the training data sets of HPLC-DAD, HPLC-FLD and HPLC-DAD-FLD (2D) (see Section 2.4.) were subjected to PCA. On the basis of Kaiser's rule (only eigenvalues ≥ 1.0 are considered significant descriptors of data variance) (Kaiser, 1960), four principal components (PCs) were considered as significant, finding that about 88.11%, 94.58% and 87.26% of the total variance of the data was explained by these PCs when HPLC-DAD, HPLC-FLD and HPLC-DAD-FLD (2D) data sets were used, respectively. The projection of the samples onto the first four PCs shows the distribution of the samples and allows the analysis of their grouping (in Fig. 3A, B and C the variance explained by each component is indicated on each axis). In general, a good discrimination of the oils from some varieties can be observed (depending on the training data set used). Even though PCA did not lead to a total discrimination for other varieties, this analysis evaluated the overall structure of the data and identified trends and clusters within the sample set.

Thus, when PCA was applied to HPLC-DAD data set, the score plots of the first four PCs (Fig. 3A) showed that the samples of 'Cornicabra' variety were clearly discriminated from the other studied monovarietal VOOs, since there are closely clustered in the sub-space PC1 *vs.* PC2. Likewise, in the same sub-space, a general clustering trend could be observed for oils samples of 'Arbequina', 'Arbosana' and 'Frantoio'. Moreover, for the other studied varieties, PCA results showed that the oil samples from 'Picholine de Languedoc' and 'Picual' were not separated from the other studied samples, while in contrast, a clustering trend was observed for samples from the cultivar 'Picholine Marocaine' in the plans formed by PC2 *vs.* PC3 and PC3 *vs.* PC4, even if 4 samples from this cultivar were not properly separated from the other studied samples.

Subsequently, when PCA was applied to the HPLC-FLD data set (Fig. 3B), an evident separation of the 7 studied groups of monovarietal VOOs was obtained in the sub-space PC1 *vs.* PC2, even though some samples were overlapping or miss-classified.

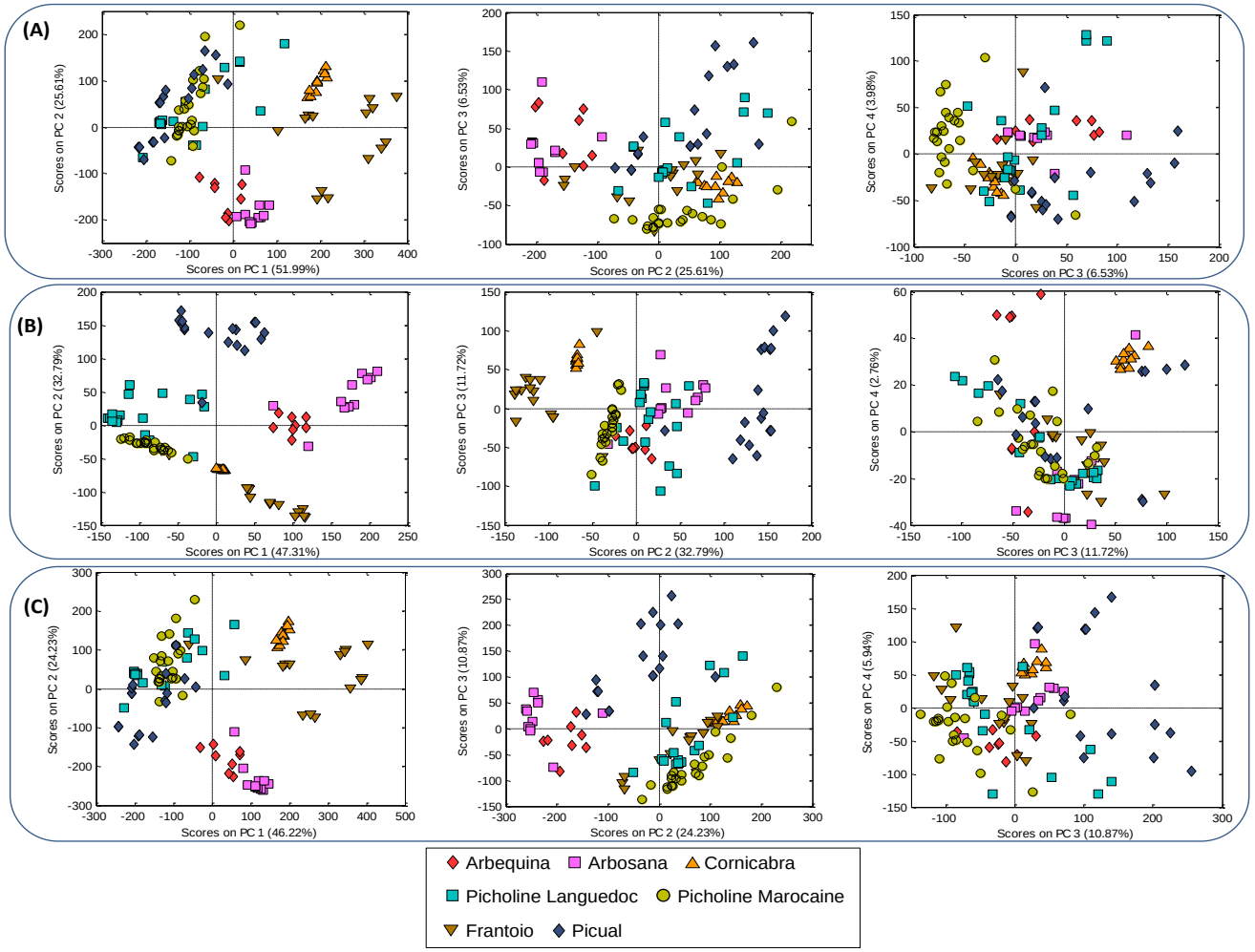

Fig. 3. Principal component analysis score plots of the studied olive oil samples on the first four principal components using the HPLC-DAD data set (3A), HPLC-FLD data set (3B), and HPLC-DAD-FLD (2D) data set (3C).

Thus, while PCA applied to HPLC-DAD data was not able to cluster, for instance, samples from 'Picual' cultivar, the latter were clearly distinguished from the other 6 monovarietal VOO groups using fluorescence data (just one sample from this variety overlapped with 'Picholine de Languedoc' samples). Similarly, 'Frantoio', 'Picholine de Languedoc', and 'Picholine Marocaine' samples were well located according to their botanical origin in the sub-space PC1 *vs.* PC2 (with the exception of one sample from 'Frantoio' and 3 samples from 'Picholine de Languedoc' which were grouped with 'Picholine Marocaine' samples, and 2 samples from 'Arbosana' which were found clustered with 'Arbequina' samples). With regard to the distribution of the studied samples on the other two sub-spaces, only 'Cornicabra', 'Frantoio' and 'Picual' samples were clearly separated from the rest on the sub-space PC2 *vs.* PC3, whereas the other samples were overlapped. In the sub-space PC3 *vs.* PC4, despite the natural grouping showed by 'Cornicabra' samples, it is possible to say that, in general terms, all samples were overlapping.

When PCA was performed on the data set created from the fusion of HPLC-DAD and HPLC-FLD data sets, score plots (Fig. 3C) showed less clearly clustered samples according to their botanical origin (if compared with the results obtained from each individual data set). Indeed, looking at samples distribution in the sub-space PC1 *vs.* PC2, it can be immediately detected that 'Cornicabra', 'Arbequina' and 'Arbosana' samples were clearly distinguished from the other samples groups (even if one sample from 'Arbosana' was very close to 'Arbequina' samples area), and also that 'Frantoio' samples showed certain clustering. The other three groups ('Picholine de Languedoc', 'Picholine Marocaine' and 'Picual') were overlapping. Considering the two other PCA sub-spaces (PC2 *vs.* PC3 and PC3 *vs.* PC4) only samples from 'Arbosana', 'Arbequina' and 'Picual' showed a slight grouping trend in PC2 *vs.* PC3 sub-space (even though two samples from 'Arbosana', three from 'Picual' and one from 'Arbequina' were not laying close to the rest of this category); all the other groups were overlapping in both PCA sub-spaces.

At this point, it is possible to say that the results of the PCA demonstrate that the data coming from fluorescence detection were more suitable (than those data from DAD detection or the combination of FLD and DAD detection) to show the natural clustering of the studied samples. The further step of this study was to evaluate the applicability and potential of three supervised multivariate data analysis techniques (PLS-DA, SIMCA and

k-NN) to correctly classify and predict the botanical origin of studied samples. The obtained results will be commented in the following sections.

3.2.2 Supervised multivariate data analysis: PLS-DA, SIMCA and k-NN analyses

3.2.2.1 Partial Least Squares Discriminant Analysis

Classification models were built using the PLS-DA algorithm. In particular, each data set (HPLC-DAD, HPLC-FLD and HPLC-DAD-FLD (2D)) was individually processed and the results were compared. In each case, the optimal complexity of the classification models (selection of the number of latent variables (LV) for building the classification models) was chosen as the one which led to less misclassification (minimum overall classification error) in cross-validation, using venetian blinds cross-validation with 10 data splits; then the optimal model was validated on the external test set. The results are summarized in Table 1, where models parameters, the correct classification rates for each one of the 7 classes and the overall one in calibration, cross-validation and prediction are reported for the three data matrices processed.

Thus, as indicated in this table, seven LVs were required to obtain the best results for PLS-DA calculation for all data matrices. Furthermore, the performance of the built PLS-DA models was examined taking into account the values of the corresponding absolute deviations of root mean square error (RMSEC, RMSECV and RMSEP). In general, it can be considered that a good PLS-DA model should have low RMSEC values, and small differences between RMSECV and RMSEP.

Thus, when the HPLC-DAD data set was used, PLS-DA models showed values of RMSEC between 0.122 and 0.259; RMSECV, from 0.139 to 0.297; and RMSEP, between 0.102 and 0.265. The absolute differences between RMSECV and RMSEP were low (0.003, 0.037, 0.012, 0.019, 0.032, 0.037, and 0.035 for 'Arbequina', 'Arbosana', 'Cornicabra', 'Frantoio', 'Picholine de Languedoc', 'Picholine Marocaine' and 'Picual' respectively), showing that the PLS-DA models for the seven studied monovarietal VOOs groups were not over fitted and exhibited considerable robustness.

Table 1. Classification parameters obtained by PLS-DA models in calibration (c), cross validation (CV) (with 10 groups split venetian blinds) and on the external test set (p) for the three chromatographic data matrices HPLC-DAD (DAD), HPLC-FLD (FLD) and HPLC-DAD-FLD (2D).

PLS-DA model [a]																					
	Arbequina			Arbosana			Cornicabra			Frantoio			Picholine Languedoc			Picholine Marocaine			Picual		
Parameters [b]	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D
RMSEC	0.135	0.171	0.138	0.122	0.133	0.131	0.185	0.175	0.196	0.196	0.206	0.198	0.258	0.263	0.247	0.259	0.26	0.249	0.185	0.147	0.143
RMSECV	0.149	0.185	0.151	0.139	0.151	0.149	0.2	0.193	0.21	0.21	0.22	0.214	0.297	0.283	0.274	0.286	0.281	0.273	0.211	0.159	0.161
RMSEP	0.146	0.222	0.154	0.102	0.125	0.117	0.188	0.157	0.198	0.191	0.192	0.195	0.265	0.258	0.259	0.249	0.261	0.23	0.246	0.221	0.23
Sensitivity_c (%)	100	87.5	100	100	100	90.9	100	100	100	93.3	93.3	93.3	88.9	88.9	88.9	95.7	91.3	95.7	94.1	94.1	94.1
Sensitivity_cv (%)	100	87.5	87.5	90.9	90.9	90.9	100	100	100	93.3	93.3	86.7	77.8	88.9	88.9	91.3	91.3	95.7	94.1	94.1	94.1
Sensitivity_p (%)	100	100	100	100	100	100	100	100	100	100	100	100	80	100	80	100	75	100	83.3	83.3	83.3
Specificity_c (%)	97.9	96.8	97.9	100	98.9	98.9	98.9	100	98.9	100	100	100	92.9	91.8	94.1	96.3	91.2	96.3	94.2	100	98.8
Specificity_cv (%)	98.9	95.8	98.9	100	98.9	98.9	97.8	100	97.8	98.9	100	98.9	88.2	90.6	88.2	96.3	92.5	96.3	94.2	100	97.7
Specificity_p (%)	94.1	94.1	97.1	100	100	100	100	100	100	100	100	100	90.6	87.5	87.5	93.1	93.1	93.1	90.3	100	100
Accuracy_c (%)	99.47	93.75	99.47	95.45	99.46	95.45	100	100	100	96.67	96.67	96.67	93.27	89.9	93.27	95.03	93.15	95.95	95.31	97.06	97.06
Accuracy_cv (%)	99.47	93.22	93.22	95.45	94.91	94.91	100	100	100	96.67	96.67	96.67	90.49	89.9	93.27	95.03	93.15	95.95	94.73	97.06	97.06
Accuracy_p (%)	100	100	100	100	100	100	100	100	100	100	100	100	88.44	96.88	88.44	93.75	85.78	98.28	88.44	91.67	91.67

Overall performance	DAD	FLD	2-D
Accuracy_c (%)	93.2	92.23	94.18
Accuracy_cv (%)	92.23	91.26	93.2
Accuracy_p (%)	91.89	91.89	94.6

[a] Seven *latent variables (LVs) were* chosen to build the PLS-DA *models* for all data sets.

[b] *Parameters: RMSEC (Root Mean Square of Calibration), RMSECV (Root Mean Square Error of Cross Validation), RMSEP (Root Mean Square Error of Prediction), Sensitivity_c (true positive rate or recall of calibration expressed as a percentage), Sensitivity_cv (true positive rate or recall of cross validation expressed as a percentage), Sensitivity_p (true positive rate or recall of prediction expressed as a percentage), Specificity_c (true negative rate of calibration expressed as a percentage) Specificity_cv (true negative rate of cross validation expressed as a percentage), Specificity_p (true negative rate of prediction expressed as a percentage), Accuracy_c (Efficiency or accuracy of the classifier for each class in calibration expressed as a percentage), Accuracy_cv (Efficiency or accuracy of the classifier for each class in cross validation expressed as a percentage), and Accuracy_p (Efficiency or accuracy of the classifier for each class in prediction expressed as a percentage).*

A similar behavior was shown by the models built with the HPLC-FLD data (Table 1), which were also characterized by low RMSEC (values within the range 0.133-0.263), RMSECV (0.151-0.283) and RMSEP (0.125-0.261), whereas the absolute differences between RMSECV and RMSEP were 0.037 , 0.026, 0.036, 0.028, 0.025, 0.020 and 0.062 for 'Arbequina', 'Arbosana', 'Cornicabra', 'Frantoio', 'Picholine de Languedoc', 'Picholine Marocaine' and 'Picual', respectively.

Even though no improvement of the accuracy of the PLS-DA models was evident, low values of calculated parameters were obtained when the calibration data of both detectors were combined, (Table 1). Thus, low RMSEC (0.131-0.249), RMSECV (0.149-0.274) and RMSEP (0.117-0.259) were obtained, whereas the absolute differences between RMSECV and RMSEP were 0.003, 0.032, 0.012, 0.019, 0.015, 0.043 and 0.069 for 'Arbequina', 'Arbosana', 'Cornicabra', 'Frantoio', 'Picholine de Languedoc', 'Picholine Marocaine' and 'Picual', respectively.

Furthermore, classification performance of PLS-DA models was assessed in terms of sensitivity, specificity and accuracy, as discussed in Section 2.4. Table 1 includes the results obtained from the PLS-DA models for the training and test sample sets. As can be deduced from this table, the sensitivity, specificity, precision and accuracy values showed excellent classification power for the three models developed by PLS-DA on HPLC-DAD and HPLC-FLD and HPLC-DAD-FLD (2D). Indeed, as far as the PLS-DA model built on the HPLC-DAD data set is concerned, the model sensitivity was always greater than 88.90% in calibration, 77.80% in cross-validation, and 80.00% in prediction, whereas the specificity of the model was superior to 92.90% in calibration, 88.20% in cross-validation, and 90.30% in prediction. Considering the accuracy, for 'Cornicabra' monovarietal VOOs, a perfect classification (100%) was obtained in recognition and prediction (both cross-validation and external validation). PLS-DA models also allowed a very good classification and prediction of samples from the other studied varieties with accuracy values higher than 93.27%, 90.94% and 88.44% in recognition, cross-validation and external validation. The lowest accuracy values obtained were those calculated for 'Picholine de Languedoc' samples, whilst 'Arbequina' VOOs showed the highest ones (not considering 'Cornicabra', with perfect scores).

When classification performance of PLS-DA model built on HPLC-FLD data set was examined (Table 1), very satisfactory results were achieved; model sensitivity was superior to 87.50%, in both calibration and cross-validation, and 75.00% in prediction, whereas the specificity of the model was higher than 91.20% in calibration, 90.60% in cross-validation, and 87.50% in prediction. If the classification accuracy obtained for each one of the seven categories is considered (Table 1), it can be said that the recognition and predictive abilities (both cross-validation and external validation) were 100% for samples from 'Cornicabra'. Accuracy values were also particularly high for 'Arbosana' (recognition accuracy 99.46%, cross-validation accuracy 94.91% and the external validation accuracy 100%), 'Frantoio' (recognition and cross-validation accuracy 96.67%, and the external validation accuracy was 100%), 'Picual' (recognition and cross-validation accuracy 97.06%, and the external validation accuracy was 91.67%), and 'Arbequina' (recognition accuracy 93.75%, cross-validation accuracy 93.22% and the external validation accuracy was 100%). For the two remaining monovarietal VOOs groups ('Picholine de Languedoc' and 'Picholine Marocaine') high accuracy were obtained as well. For 'Picholine Marocaine', the recognition and cross-validation accuracy were 93.15, and the external validation accuracy was 85.78%; in the case of 'Picholine de Languedoc', the recognition and cross-validation accuracy showed values of 89.90 and the external validation accuracy was 96.88%).

When PLS-DA was applied to the combined HPLC-DAD-FLD (2D) data set, no improvement of the classification performances was observed for, practically, most of the studied monovarietal VOOs. The best results obtained were comparable to the most positive ones obtained using one detector or the other, separately; in other words, it seemed that the data fusion did not lead to any refinement or enhancement of the results (Table 1). It is important to highlight that data fusion is normally used not for complement the results achieved by the individual detectors (as we are presenting in the current work, to feed a discussion in this direction), but to replace them. It could be particularly interesting when, for instance, HPLC-DAD works better for classifying some of the selected samples and HPLC-FLD is more advisable for the rest. In such a way, 2D data set could give us the quasi-optimal classification just working with a single matrix.

The calculated model showed good classification performances for the different studied groups. Sensitivity was superior to 88.90% in calibration, 86.70% in cross-validation and 80.00% in prediction, whereas the specificity of the model was superior to 94.10% in

calibration, 88.20% in cross-validation and 87.50% in prediction. As far as the model accuracy is concerned, it is possible to stand out, that the results were somewhat similar, in recognition and external validation, to those obtained when PLS-DA was applied to HPLC-DAD data set for 'Arbequina', 'Arbosana' and 'Picholine de Languedoc' groups, while the values of accuracy in cross-validation of the two first mentioned groups were the same as those obtained when PLS-DA was applied to HPLC-FLD data set. For the remaining studied varieties, PLS-DA applied to the HPLC-DAD-FLD (2D) data set of 'Cornicabra' and 'Frantoio' varieties led to the same results as when this chemometric technique was applied to data obtained from the two detectors separately. In the case of 'Picholine Marocaine', accuracy values were 95.95% (for the recognition and cross-validation accuracy) and 98.28% (for the external validation accuracy), whereas for 'Picual', the same values were 97.06 (recognition and cross-validation accuracy) and 91.67% (external validation accuracy), respectively.

As far as the overall performance is concerned, we can stand out that the value obtained for the overall classification accuracy was between 92.23% and 94.18% in calibration, between 91.26% and 93.20% in cross validation and it fluctuated from 91.89% to 94.60% in external validation, being the PLS-DA applied to HPLC-DAD-FLD (2D) data set the one giving rise to the best results.

3.2.2.2 Soft Independent Modelling of Class Analogies

Using the same strategy previously described, SIMCA was applied to the same data matrices subjected to PLS-DA. The models obtained were based on 5% as the significance level for critical distance and the number of PCs for the SIMCA models was chosen using venetian blinds cross-validation with 10 data splits, in such a way that PCs providing the lowest error of cross-validation were chosen for the final model construction. In general, for each studied class, the number of employed PCs explains more than 90% of class variance. Furthermore, to evaluate the predictive capability of SIMCA, the same external validation test set procedure used in PLS-DA was applied. Table 2 shows the number of PCs retained, the details of the SIMCA model for each studied class and the overall percentages obtained both in calibration and prediction.

Table 2. Classification parameters obtained by SIMCA models in calibration (c), cross validation (CV) (with 10 groups split venetian blinds) and on the external test set (p) for the three chromatographic data matrices HPLC-DAD (DAD), HPLC-FLD (FLD) and HPLC-DAD-FLD (2D).

SIMCA model [a]																					
	Arbequina			Arbosana			Cornicabra			Frantoio			Picholine Languedoc			Picholine Marocaine			Picual		
Parameters [b]	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D	DAD	FLD	2D
Number PCs	4	4	4	3	2	4	2	4	3	6	4	6	6	2	6	4	3	4	3	2	3
RMSEC	0.271	0.134	0.245	0.384	0.38	0.258	0.34	0.07	0.21	0.315	0.183	0.27	0.428	0.44	0.377	0.57	0.3	0.49	0.624	0.492	0.559
RMSECV	20.23	16.43	20.45	15.3	17.47	16.76	8.71	3.27	8.87	32.24	11.84	25.78	13.44	3.19	12.86	1.32	0.74	1.01	11.81	12.59	13.31
Sensitivity_c (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	91.3	100	100	100	100
Sensitivity_p (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	87.5	100	83.33	83.33	83.33
Specificity_c (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	97.7	100	100	100	100	100	100	100
Specificity_p (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	93.8	100	96.6	100	96.6	100	100	100
Precision_c (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	90	100	100	100	100	100	100	100
Precision_p (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	71.4	100	88.9	100	88.9	100	100	100
Accuracy_c (%)	100	100	100	100	100	100	100	100	100	100	99.43	100	98.82	87.2	99.41	96.6	96	98.8	100	97.06	100
Accuracy_p (%)	100	100	100	100	100	100	100	100	100	100	98.39	100	98.44	90.6	98.44	92	92	92	91.67	91.67	91.67

Overall performance	DAD	FLD	2-D
Accuracy_c (%)	100	98.06	100
Accuracy_p (%)	97.3	94.6	97.3

[a] *Selection of the optimal number of principal components (PCs) for each individual PCA model built on the data set from each class (it is done by means of cross-validation).*

[b] *The description of the classification parameters can be found in Table 1.*

The overall percentages obtained in both cases (classification and prediction) can be considered highly satisfactory with accuracy values in recognition and prediction higher than 98.06 and 94.60% respectively, which were slightly higher than those obtained using PLS-DA treatment. Looking at the SIMCA results, it is possible to emphasize that, among the three data matrices used in this study, HPLC-DAD and HPLC-DAD-FLD (2D) provide the best results in both recognition (100% of accuracy) and prediction (97.30% of accuracy).

After having a look at the overall performance, it makes sense to consider the model performances when the results obtained for each studied class are observed (Table 2). If so, it can be noticed that the models built in SIMCA resulted very sensitive and specific: 100% sensitivity and specificity (in both recognition and prediction) were observed for 4 studied monovarietal VOOs classes: 'Arbequina', 'Arbosana', 'Cornicabra' and 'Frantoio' regardless of the data set used. For the other three remaining classes ('Picholine de Languedoc', 'Picholine Marocaine' and 'Picual'), sensitivity and specificity values obtained were very satisfactory as SIMCA models, showing the following values: sensitivity in calibration was in all the cases 100%, with only one exception ('Picholine Marocaine' FLD); sensitivity in prediction was also 100% in most of the cases, with the same exception ('Picholine Marocaine' FLD) and the three data sets for 'Picual' (with values of 83.33%); specificity in calibration was perfect apart from 'Picholine de Languedoc' (FLD); and specificity in prediction showed values different to 100% just in three cases ('Picholine de Languedoc' (FLD), and 'Picholine Marocaine' (DAD and 2D)).

Regarding the classification and prediction accuracy, all SIMCA models exhibited similar or improved results in comparison to those obtained for the same data sets when PLS-DA technique was used (Tables 1 and 2). Indeed, perfect accuracy (100%), in recognition and external validation was obtained for three of the seven studied classes: 'Arbequina', 'Arbosana' and 'Cornicabra', regardless of the chromatographic data set used. Furthermore, similar results (100% of accuracy in recognition and prediction) were obtained when SIMCA model was built using HPLC-DAD and HPLC-DAD-FLD (2D) data sets for 'Frantoio' class, whilst for this variety, SIMCA applied to HPLC-FLD data set led to an accuracy of 99.43% in recognition and 98.39% in prediction. Finally, for the three remaining classes, best values of accuracy in both recognition and prediction were

obtained, in general, when SIMCA was applied to HPLC-DAD and HPLC-DAD-FLD (2D) data sets.

3.2.2.3 K-Nearest Neighbors

Regarding the third supervised multivariate data analysis employed in this study, we investigated the recognition and prediction accuracies of k-NN, using the three kinds of data sets examined herein (HPLC-DAD, HPLC-FLD and HPLC-DAD-FLD (2D)). Table 3 summarizes the performance of k-NN models. The optimal number of neighbors (k=4) was chosen according to the minimum percentage prediction error. When the sensitivity and specificity of k-NN model are considered (Table 3), it can be seen that quite satisfactory results were obtained, since for all the studied classes, k-NN model gave almost 100% of sensitivity and specificity in recognition and prediction independent of the chromatographic data set used, however, in cross validation sensitivity and specificity the percentages were slightly lower: sensitivity was between 66.70 (just one value was so low ('Picholine de Languedoc' DAD)) and 100% when HPLC-DAD data set was used, between 81.80 and 100% when HPLC-FLD data set was employed and between 77.80 and 100% when k-NN was applied to HPLC-DAD-FLD (2D) data set. Likewise, the model specificity in cross validation was between 94.10 and 100% when HPLC-DAD data set was used, between 95.00 and 100% when HPLC-FLD data set was employed and between 95.30 and 100% when k-NN was applied to HPLC-DAD-FLD (2D) data set.

When the global classification results included in Table 3 are studied, it can be seen that the k-NN algorithm exhibited satisfactory performance in terms of overall accuracy. . Indeed, the overall success rate of classification for all the samples ranged from 84.47% to 91.26% in calibration, and oscillated from 88.35% to 93.20% in cross-validation, being in both cases k-NN built on HPLC-FLD data the models presenting the best results, whilst those achieved by using HPLC-DAD data showed the worst performance. As far as the external validation test results are concerned, the k-NN algorithm demonstrates strong discriminatory power to differentiate among the seven botanical origins, with accuracy values found within the range comprised from 89.19% (when HPLC-FLD data were used) to 91.89% (when HPLC-DAD or HPLC-DAD-FLD (2D) data were used).

Table 3. Classification parameters obtained by k-NN models in calibration (c), cross validation (CV) (with 10 groups split venetian blinds) and on the external test set (p) for three chromatographic data matrices HPLC-DAD (DAD), HPLC-FLD (FLD) and HPLC-DAD-FLD (2D).

k-NN model [a]																					
	Arbequina			Arbosana			Cornicabra			Frantoio			Picholine Languedoc			Picholine Marocaine			Picual		
Parameters [b]	DAD	FLD	2-D	DAD	FLD	2-D	DAD	FLD	2-D	DAD	FLD	2-D	DAD	FLD	2-D	DAD	FLD	2-D	DAD	FLD	2-D
Sensitivity_c (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Sensitivity_cv (%)	87.5	87.5	87.5	90.9	81.8	81.8	100	100	100	93.3	86.7	86.7	66.7	83.3	77.8	78.3	91.3	91.3	94.1	94.1	94.1
Sensitivity_p (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Specificity_c (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Specificity_cv (%)	98.9	97.9	97.9	98.9	98.9	98.9	100	98.9	98.9	96.6	100	98.9	94.1	96.5	95.3	96.3	95	96.3	97.7	100	100
Specificity_p (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Accuracy_c (%)	93.2	99	99.5	94.9	90.9	95.5	97.8	99.5	97.8	82.2	92.2	83.3	82.8	91.1	87.1	91.9	96	95.3	96.5	97.1	97.1
Accuracy_cv (%)	93.2	99.5	93.2	94.9	90.9	94.9	100	98.9	99.5	96.1	93.3	93.3	82.2	93.9	87.1	91.9	98.1	95.3	96.5	97.1	97.1
Accuracy_p (%)	100	98.5	100	100	87.5	100	100	98.4	98.4	98.4	91.7	91.7	80	90	90	96.6	96.6	96.6	91.7	91.7	91.7

Overall performance	DAD	FLD	2-D
Accuracy_c (%)	84.5	91.3	88.4
Accuracy_cv (%)	88.4	93.2	90.3
Accuracy_p (%)	91.9	89.2	91.9

[a] *Four nearest neighbors* (k) were *chosen to build the k-NN* model *for all data sets.* [b] *The description of the classification parameters can be found in Table 1.*

If the attention is focused on the recognition and prediction abilities reached by k-NN technique for each studied class, it is possible to stand out that this technique achieved satisfactory performance in terms of accuracy. Indeed, as can be seen from Table 3, when k-NN algorithm was applied to HPLC-DAD data set, it discriminated among the studied samples according to their botanical origin with a degree of accuracy ranging from 82.20% to 97.83% for the training set; from 82.16% to 100% for the cross-validation; and between 80% and 100% for the prediction set, values which demonstrate good discriminatory power to differentiate among the seven studied classes. The best classification and prediction rates were obtained for 'Cornicabra' samples whereas the lowest rates were calculated for 'Picholine de Languedoc' samples.

When the data from HPLC-FLD were used, discrimination rates of k-NN model were higher than 90.91% in both recognition and cross-validation, and higher than 87.50% in the prediction set. Best results were showed for 'Cornicabra' and 'Arbequina' classes, whilst the lowest accuracy values were detected for 'Arbosana' category. The results for the remaining four classes can be considered as quite satisfactory. Afterwards, when k-NN algorithm was applied to the HPLC-DAD-FLD (2D) matrix, no remarkable accuracy values improvement (in recognition and prediction sets) was observed, apart from a slight enhancement for 'Arbequina' and 'Arbosana' classes; for the other classes, the results were comparable or hardly noticeable worse to those obtained when HPLC-DAD and HPLC-FLD data sets were used separately.

The last part of the current paper will try to present a comparison of the overall results achieved by the three different supervised techniques. In terms of recognition and prediction accuracy, k-NN method appears to give significantly lower correct classification rates of the oil samples on the basis of variety than the other two chemometric methods applied (Table 1, 2 and 3).

Even though excellent results were obtained by the three supervised pattern recognition techniques tested in this study, it is possible to suggest the best combination (chemometric technique+chromatographic data set) to obtain the highest classification and prediction rates for the studied oils according to their botanical origin. Within this context, SIMCA model gave perfect scores for the three data sets for the following classes: 'Arbequina', 'Arbosana' and 'Cornicabra' (both in recognition and prediction). SIMCA

model also led to perfect recognition and prediction scores for 'Frantoio' samples (DAD and 2D data sets). In the case of 'Picholine de Languedoc', we can highlight that SIMCA achieved the best accuracy rates (accuracy_cv and accuracy_p (%)) for the DAD and 2D data sets (being the values for DAD slightly better), whilst the PLS-DA model led to the best accuracy for the data coming from FLD. As far as 'Picholine Marocaine' samples are concerned, the most powerful technique seemed to be the PLS-DA model (for DAD data set it showed 95.03% in recognition and 93.75% in prediction, and for 2D data set, 95.95% in recognition and 98.28% in prediction) and the k-NN algorithm worked well for HPLC-FLD. When we pay attention to 'Picual' samples, SIMCA applied to HPLC-DAD or HPLC-DAD-FLD (2D) was the best combination (100% in recognition and 91.67% in prediction).

4 Conclusions

In view of the results attained within this study, it can be concluded that a discriminant approach based on fingerprinting of phenolic compounds jointly with suitable chemometric methods has been shown as a promising and efficient way for differentiating monovarietal VOOs according to their botanical origin. This confirms the potential of this approach to authenticate certified monovarietal VOOs. This subject is of pivotal importance for the olive oil sector, since recent requirements in the competitive current market elevate the value of monovarietal VOOs when its botanical provenance is properly protected and certified. The experimental protocols presented here and the models built could obviously have further development to fully validate the effectiveness of the method, using a higher number of samples from other olive varieties cultivated inside and/or outside Morocco.

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Chapter

9

Preliminary results of the application of a metabolic fingerprinting approach based on selected ion flow tube mass spectrometry (SIFT-MS) and chemometrics: A reliable tool for Mediterranean origin-labeled olive oils authentication

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Abstract

Selected ion flow tube mass spectrometry (SIFT-MS) in combination with chemometrics was used to authenticate the geographical origin of Mediterranean virgin olive oils produced under geographical origin labels. In particular, 130 oil samples from five different Mediterranean regions (Kalamata (Greece); Toscana (Italy); Meknès and Tyout (Morocco); and Priego de Cordoba and Baena (Spain)) were considered. The headspace volatile fingerprints were measured by SIFT-MS in full scan with H_3O^+ and NO^+ and O^{+2} as precursor ions and the results were subjected to chemometric treatments, including principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). PCA was just used for preliminary multivariate data analysis and PLS-DA was applied to build three different models (considering the three reagent ions) to classify samples according to the country of origin and regions (within the same country). The samples were satisfactorily classified bearing in mind their geographical origin, and it was even possible to distinguish those coming from the same country but from different regions.

Keywords: virgin olive oil; geographical indication labels; ion flow tube mass spectrometry; chemometrics; geographical origin authentication.

1. Introduction

The fact that virgin olive oil trade is becoming increasingly internationalized and very competitive, is causing that various olive producing regions use different instruments to protect and promote specific qualities and attributes of their olive oils, to increase their economic profitability and gain competitive advantages in the global market (Parras-Rosa, 2013). These tools are protected designation of origin (PDO), protected geographical indication (PGI) and traditional speciality guaranteed (TSG). The emergence and further proliferation of these geographical indication labeling systems as a differentiating marketing strategy on which sustainable competitive advantage is built for olive oil trading, was mainly stimulated by the recent trend in consumers purchasing habits, that are more and more placing value on products that they can associate with a certain place and/or special means of production (Barham, 2003; Lenglet, 2014; Verbeke & Roosen, 2009).

Under these labeling systems, two main types of olive oil attributes are enhanced: tangible characteristics related to olive oil distinctive quality and composition profile, and intangible features linked with the protected region cultural heritage, local know-how, ancestral traditions and legacy of olive tree (*Olea europaea* L.) cultivation and oil production. With regard to the first type of olive oil characteristics, the geographical origin is considered as one of the most important and determinant factors of olive oil quality and characteristics (Di Vita, D'Amico, La Via, & Caniglia, 2013; Galtier et al., 2007; Inarejos-García, Santacatterina, Salvador, Fregapane, & Gómez-Alonso, 2010). Indeed, the effects of factors such as cultivated variety, pedoclimatic conditions, agronomic and technological practices on olive oil quality attributes and composition profile produced have been widely demonstrated (Angerosa, Mostallino, Basti, & Vito, 2001; Dag et al., 2011; Giuffrè & Louadj, 2013; Giuffrè, 2013; Mailer, Ayton, & Graham, 2010; Morelló, Romero, & Motilva, 2006; Servili et al., 2002; Tura, Failla, Bassi, Pedò, & Serraiocco, 2008; Youssef, Youssef, Mokhtar, & Guido, 2011). These findings are the reason explaining why specific and distinctive features are expected to be exhibited by olive oils produced in some olive growing areas. In addition, when dealing with the labeled-olive oil intangible elements, the cultural identity of many Mediterranean olive oil producing regions is found at the base of the collective memory of their communities regarding olive tree cultivation, oil production and its trade as testified by archaeological evidence and literary sources (Bartolini, 2002; Bash, 2013). Overall, for all these characteristics -which constitute distinctive peculiarities of origin-labeled olive oils- this kind of products are usually commercialized at higher prices in the market. As a consequence, frauds and adulterations of these products with cheaper oils (with the obvious aim of obtaining a greater production) have become a raising problem over the last decades (López-Feria, Cárdenas, García-Mesa, & Valcárcel, 2008). As a consequence, the assessment of the traceability and authenticity of this type of products is, at the moment, a relevant concern which is attracting a lot of interest internationally.

Indeed, even if the use of production and origin labels of olive oil is strictly controlled by regulations, directives and laws that define a set of rules for its production, management, conservation and packaging (as well as internal and external control measures) (European Commission, 2008), fraudulent practices have become more sophisticated, and, for this reason, there is a need for reliable and effective methodological approaches to verify the

authenticity of origin-labeled olive oils (Galtier et al., 2007; Giacalone, Giuliano, Gulotta, Monfreda, & Presti, 2015; Korifi, Le Dréau, Molinet, Artaud, & Dupuy, 2011).

In the described context, metabolomic approaches could offer (and are already doing it) positive perspectives for the assessment of olive oil geographical origin authenticity. To date various approaches from the classical ones (based on the analysis of specific marker compounds) to the most advanced (more holistic approaches based on the use of olive oil fingerprints, considering the contribution of a wider number of compounds of this matrix, which can make adulteration detection more efficient) have been developed and successfully applied to olive oil geographical origin traceability monitoring (Beltrán, Sánchez-Astudillo, Aparicio, & García-González, 2015; Ben-Ayed, Kamoun-Grati, & Rebai, 2013; Nescatelli et al., 2014; Petrakis, Agiomyrgianaki, Christophoridou, Spyros, & Dais, 2008; Pizarro, Rodríguez-Tecedor, Pérez-Del-Notario, Esteban-Díez, & González-Sáiz, 2013; Portarena, Gavrichkova, Lauteri, & Brugnoli, 2014). In general, these approaches take advantage of the high sensitivity, selectivity and robustness of advanced analytical platforms (such as high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), spectroscopic techniques and nuclear magnetic resonance (NMR)) and the power of chemometric tools (such as principal component analysis (PCA), linear discriminant analysis(LDA), and partial least-squares discriminant analysis (PLS-DA) and soft independent Modeling of class analogy, (SIMCA), among others).

The crucial role of aroma compounds to defying sensorial attributes of olive oil is very well-known (Kesen, Kelebek, Sen, Ulas, & Selli, 2013; Morales et al., 1995). Moreover, they are among the most widely investigated components of this oily matrix for their ability to authenticate the geographical origin of this product (Ben Temime, Campeol, Cioni, Daoud, & Zarrouk, 2006; Cajka et al., 2010; Pizarro, Rodríguez-Tecedor, Pérez-del-Notario, & González-Sáiz, 2011). In most of the studies focused on olive oil aroma, theanalysis of the volatile fraction is carry out by headspace plus chromatographic techniques and selective mass spectrometry detection, especially GC-MS. The usefulness of this coupling has been, beyond a doubt, demonstrated; however, these methodologies are often time-consuming, require skilled operators and are relatively expensive.

Selected ion flow tube mass spectrometry (SIFT-MS) partially overcomes these limitations. It is a direct injection-mass spectrometric technique which allows real-time monitoring of most volatile compounds. It has been characterized by very low detection limits, typically low part-per-billion (ppb) levels (Gibson, Malek, Dekker, & Ross, 2015). Furthermore, SIFT-MS allows collecting the overall MS fingerprints of the samples which can be further processed with chemometric techniques trying to achieve a successful discrimination and classification.

Even though SIFT-MS has been previously used to investigate the volatile compounds present in olive oil headspace (Davis & McEwan, 2007; Davis, Senthilmohan, & McEwan, 2011; Davis, Senthilmohan, Wilson, & McEwan, 2005), this is the first report about the use of the same technology applied to the geographical assessment of origin-labeled olive oils. Thus, the aim of the present study was to develop a fast and reliable method for the discrimination of geographical indication labeled-olive oils by means of SIFT-MS fingerprinting measurements in combination with subsequent statistical interpretation of the obtained MS data.

2. Material and methods

2.1. Sample collection

A total of 130 samples belonging to six different Mediterranean origin-labeled regions were purchased from local stores or supplied by Agro-pôle Olivier National School of Agriculture's partners, organisms located in Meknès (Morocco). The number of extra virgin olive oil samples from each region was as follows: PDO Kalamata, Greece (n= 15); PDO Priego de Córdoba, Spain (n= 25); PDO Baena, Spain (n = 20); PDO Tyout-Chiadma, Morocco (n= 25); PGI Toscana, Italy (n = 20); and Meknès, Morocco (n= 25). A homogeneous bulk sample was prepared by mixing the same quantity of all bottles from the same region. That batch material was considered as a quality control sample and was used for the preliminary study and later on, for method optimization and validation. Sufficient quantity of each sample was stored in dark bottles at −20 °C under nitrogen atmosphere until instrumental analysis.

2.2. Samples preparation and SIFT-MS experiments

After preliminary studies, for each investigated sample, 10 g were exactly weighed in a glass bottle of 100 mL (Schott Duran bottles, Germany), which was then sealed with screw cap with two closed ports. Each one of them was equipped with a tube connection. Immediately after, glass bottles were placed in a water bath heated at 40 ± 1°C for 10 min. A temperature sensor (± 0.1 °C) was used to ensure a homogeneous temperature distribution in the hot water bath. In order to allow the equilibrium of the analytes between the liquid phase and the headspace, the sample was continuously agitated with a magnetic stirring bar. After the heating time finished, the samples were removed from the bath for headspace analysis by mean of a Voice 200Ultra SIFT-MS (Syft Technologies, Christchurch, New Zealand).

Thus, one of two tubes connection was coupled to a nitrogen gas bag and the other was connected to the inlet needle of the SIFT-MS instrument. So, to sweep volatile compounds present in the headspace of the glass bottle, the screw cap ports were opened, nitrogen gas was added by passing an adjusted air stream through the headspace of the glass bottle at 0.35 bar using a needle valve, and volatile compounds were, then, sampled via a needle into the SIFT-MS flow tube through a calibrated, heated stainless steel flow limiter at a rate of 1.69 ± 0.1 Torr L s-1 (133 ± 8 mL/min under standard conditions).

The reagent ions (H_3O^+, NO^+ and O_2^+) were generated by a microwave air discharge at ~0.5 Torr, selected by using a quadrupole mass filter and injected into a stream of carrier gas in the flow tube. Afterwards, the sample headspace was introduced into the carrier gas at a known rate, and product ions started to be formed (Figure 1). All the ion products of the chemical ionization reaction and un-reacted reagent ions were monitored by a quadrupole mass spectrometer in the full scan mode in the mass-to-charge ratio (m/z) range of 10-200 over 60 s. The full scan data (ion counts per second) averaged over the sampling time for each m/z value were used for the the statistical analyses.

The sample analysis order was randomized, at the beginning of each sequence and after every 10 analyses a blank and a test or control analysis was conducted.

Sample analyses were carried out in triplicate. For each replicate, and for each ion precursor, four full mass scans were recorded, but the first and last cycles were discarded for further calculations. The second and third scans were averaged yielding a mean mass

spectrum/replicate. Then, the three averaged mass spectrums of the three replicates of each sample were put together, to compute another average, obtaining a mean mass spectrum/sample. LabSyft software package (version 1.4.4, Syft Technologies) was used for the mass spectrum acquisition and data exportation as CSV (comma-separated values) files. In total, three data sets were obtained: H_3O^+ data matrix [130 (samples) x 187 (m/z) values]; NO^+ data set [130 x 187 (m/z) values] and O_2^+ data matrix [130 x 187 (m/z) values].

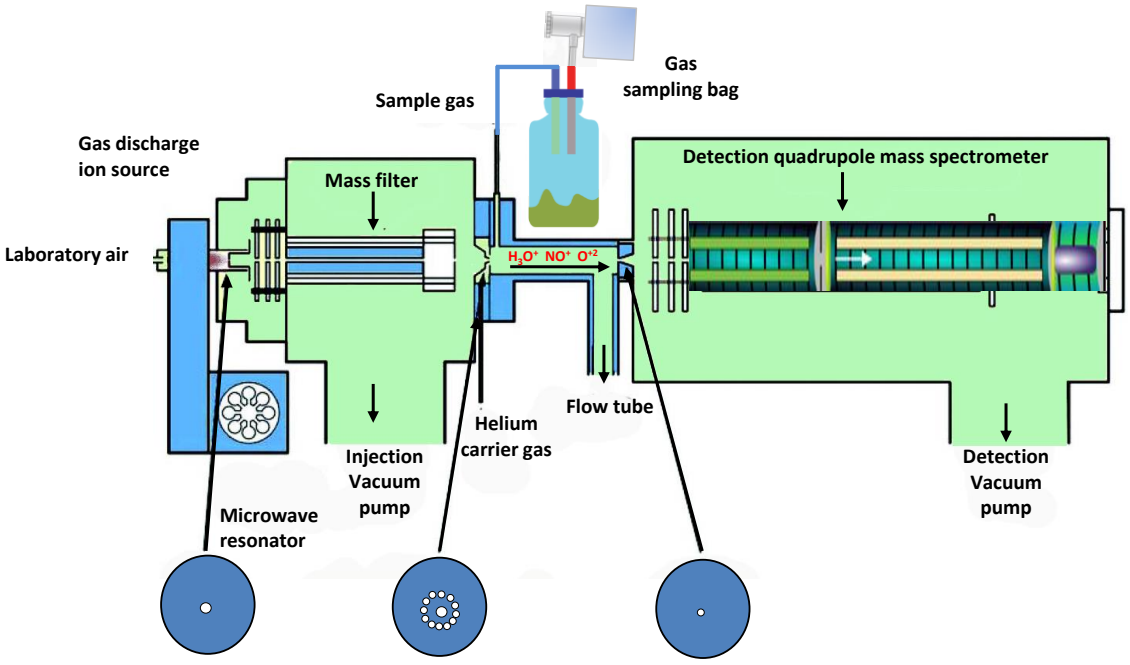

Figure 1. A schematic diagram of a selected ion flow tube, SIFT, instrument used to fingerprint the volatile compounds of the studied olive oils

2.3. Chemometrics

The usefulness of the headspace fingerprints generated using SIFT-MS to authenticate the origin of geographical labeled Mediterranean virgin olive oils was examined. For this purpose, the three built data sets were imported into the MATLAB® software (version 7.9, R2009b, The MathWorks Inc., Natick, MA, USA). The Bioinformatics Toolbox™ and PLS Toolbox® (version 7.9.5, Eigenvector Research Inc., Wenatchee, WA, USA) for MATLAB software were used for chemometric data treatments.

As a first step, data were normalized and PCA was applied as unsupervised multivariate technique just to reduce the dimensionality of the three data sets, explore samples and

variables correlations and visualize general trends within the data. In a second step, multi-class and two-class PLS-DA models were built to test for each data set obtained the ability of the proposed analytical approach to authenticate the studied oils according to their geographical origin. In multi-class models, all the countries were included within analysis, whereas the two-class models were built to discriminate between samples from the same country, but coming from different regions. Doing this was particularly interesting for samples from Morocco and Spain.

Thus, for each one of the considered data matrices, data were randomly split into a training and test set, with 70% and 30% of the samples, respectively. The training set was used to build the models and its classification efficiency was computed for each class according to the classification results for test samples.

To extract PLS-DA features, the number of latent variables (LVs) was chosen when the classification error obtained by cross-validation was minimized using venetian blinds cross-validation with 10 data splits; then, the optimal model was validated on the external test set. The performances and quality of PLS-DA models were assessed by the estimation of accurate values of the root mean squared errors for calibration (RMSEC) and prediction (RMSEP), sensitivity, specificity and accuracy. Sensitivity and specificity are defined as the number of samples correctly classified as negatives (non-defectives) or positives (defectives), respectively. Accuracy represents the correct classification rate. Obtained values were reported as percentages.

3. Results and discussion

3.1. SIFT-MS full scan headspace analysis

As stated in the introduction section, over the last years, the olive oil analytical field is showing a remarkable inclination for using fingerprinting metabolic approaches to assess or evaluate different aspects regarding olive oil quality, authenticity and traceability. This statement is particularly applicable when dealing with the authentication of geographical origin of this foodstuff (Dais & Hatzakis, 2013; Portarena et al., 2014). In this work, we tested the ability of a SIFT-MS analytical methodology in combination with chemometrics to discriminate origin labeled-olive oils coming from some of the most important olive growing Mediterranean regions. The possibility of identifying potential geographical

origin markers for oils from these regions was also explored and a deeper study of this is already ongoing in our lab.

Figure 2 shows the MS spectrum of headspace of a "control sample" using H_3O^+ (Figure 2a), NO^+ (Figure 2b) and O_2^+ (Figure 2c) as ions precursor. The possibility of using three different precursor ions is one of the advantages ofSIFT-MS, since it can provide complementary information which could be very useful for the identification of the sample compounds. For example, E-2-hexenal ($C_6H_{10}O$) reported as one of the major olive oil volatile compounds gives rise to the following reactions: with the H_3O^+, in a primary reaction a proton is transferred to the molecule producing $C_6H_{11}O^+$ with an m/z of 99.0. There is also the possibility to form clusters with the atmospheric water which is introduced alone with the sample. This secondary reaction can create two ion products $C_6H_{11}O^+{\cdot}2H_2O$ (m/z = 135) and $C_6H_{11}O^+{\cdot}H_2O$ (m/z = 117). With NO^+, however, a hydride abstraction reaction can take place leading to the formation of $C_6H_9O^+$ (m/z = 97). Finally, reactions ratios with O_2 remain very small (between 0.2 and 0.3), which indicates the low reactivity of this compounds with this precursor ion.

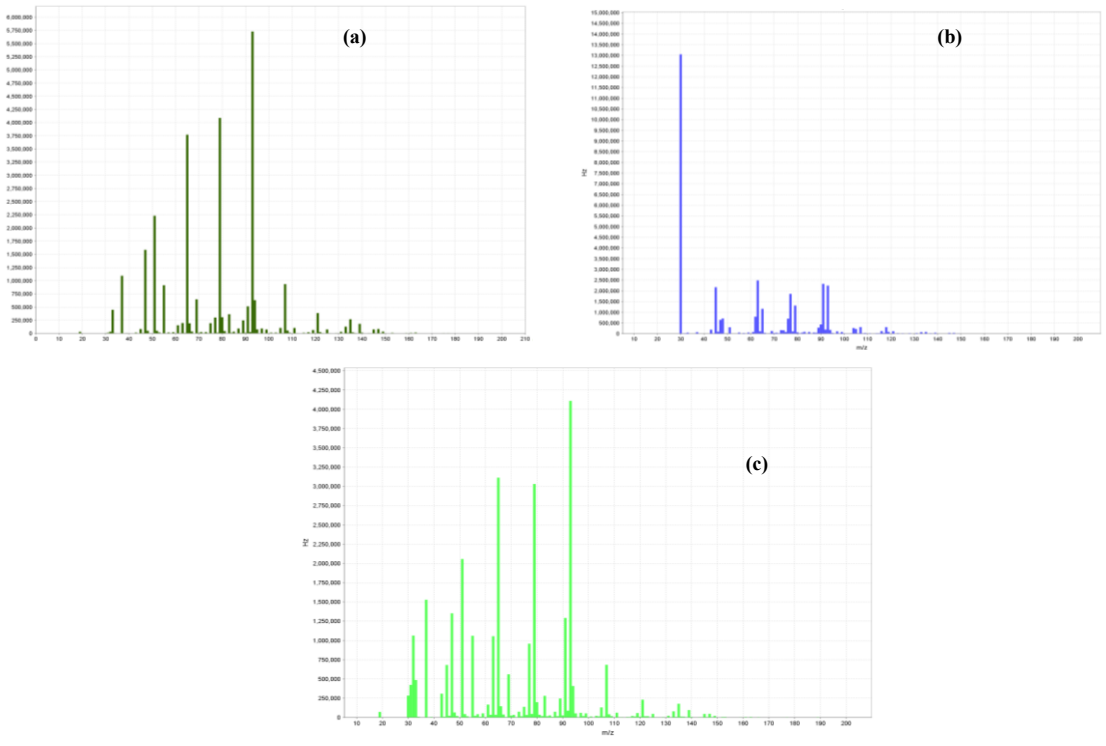

Figure 1. Example of the fingerprint mass spectra of a quality control sample-headspace volatile fraction: (a) precursor ion H_3O^+; (b) precursor ion NO^+ and (c) precursor ion O_2^+.

3.2. Geographical classification of the studied oils

The data matrices containing the whole SIFT-MS spectrum (187 *m/z* values and their intensities) for samples coming from the six studied regions were submitted to PCA without any prior class label (in an unsupervised manner) to identify outliers and visualize the underlying trends. The projection of the samples onto the first four principal components allowed the observation of the distribution of the samples and gave us the chance to have a look at their grouping. Results are given in Figure 3; there is a clear discrimination of Tyout-Chiadma (PC1 *vs* PC2) and Toscana (PC2 *vs* PC3, and PC3 *vs* PC4) from the oils coming from the remaining regions (for which a significant overlapping is shown). Two samples (from Meknès and Priego de Córdoba) were identified as outliers.

Subsequently, the attention was driven towards the application of a supervised statistical technique (PLS-DA) to classify the olive oil samples on the basis of their geographical origins. The achieved results are summarized in Tables 1 and 2. Thus, PLS-DA models were constructed after outlier's exclusion. Three models were fitted on the three different data sets; the first one was fitted on the entire 128 samples data set to classify the samples according to their country of origin (model 1). Another two models were further built; model 2 was constructed on the subset of Spanish oils (44 samples), and model 3, considering the sub-sample set composed by the Moroccan oils (49 samples). The optimum complexity of the computed models, evaluated through a 10 fold cross-validation procedure, was found to be seven latent variables (LVs) for model 1; whereas two LVs were chosen to build the PLS-DA models number 2 and 3.

Hence, when considering the multi-class PLS-DA model (Table 1), by evaluating the values of mean squared errors, it was noted that the parameters RMSEC, RMSECV and RMSEP showed low values regardless the data set used. Values lower or equal to 0.182, 0.234 and 0.217, respectively, were obtained for RMSEC, RMSECV and RMSEP when PLS-DA was applied to H_3O^+ product ions data set. Likewise, RMSEC, RMSECV and RMSEP values were lower or equal to 0.172, 0.210 and 0.240, respectively, when NO^+ data set was used. Finally, RMSEC ≤ 0.190, RMSECV ≤ 0.232 and RMSEP ≤ 0.229 were obtained when PLS-DA was applied to the O_2^+ matrix.

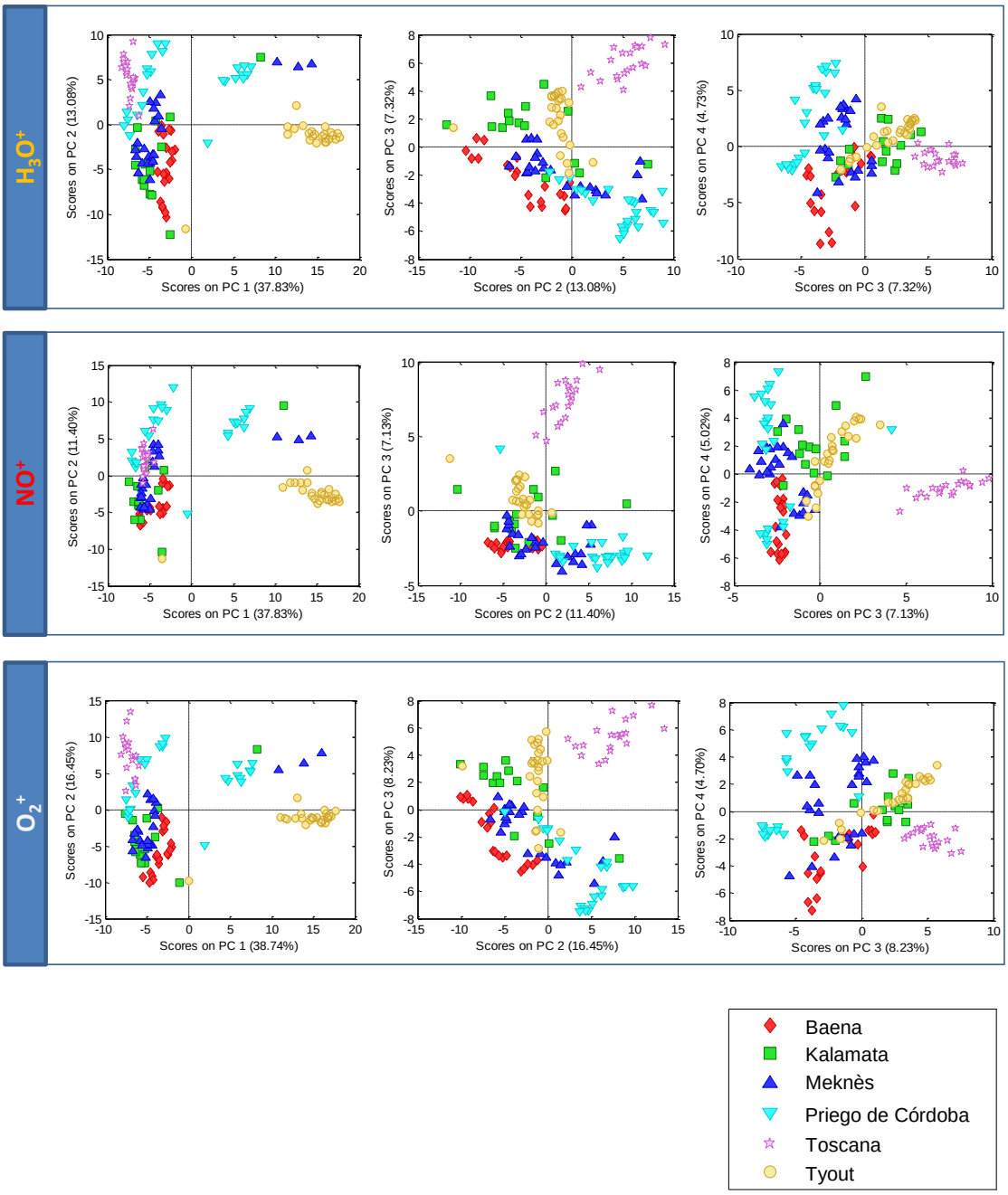

Figure 3. PCA score plots of the studied olive oil samples on the first four principal components using the H_3O^+ , NO^+ and O_2^+ data sets.

It is possible to say that regardless of the data set used the multi-class PLS-DA models provided the possibility to discriminate among the six studied groups. It ensured almost excellent specificity and sensitivity in discrimination (Table 1); the models were slightly worse for Kalamata oils. Besides, the similarity between the parameters obtained for the training and test sets indicate that overfitting did not occur, which assured the robustness and reliability of the PLS-DA models developed.

Table 1. Classification parameters obtained by PLS-DA multi-class models in calibration (c), cross validation (cv) (with 10 groups split venetian blinds) and on the external test set (p) for three SIFT-MS data sets (H_3O^+, NO^+ and O_2^+).

Parameters[b]	PLS-DA model 1[a]																	
	H_3O^+						NO^+						O_2^+					
	(1)	(2)	(3)	(4)	(5)	(6)	(1)	(2)	(3)	(4)	(5)	(6)	(1)	(2)	(3)	(4)	(5)	(6)
RMSEC	0.182	0.181	0.177	0.144	0.108	0.116	0.172	0.147	0.143	0.151	0.077	0.106	0.190	0.185	0.174	0.162	0.097	0.133
RMSECV	0.210	0.234	0.234	0.172	0.126	0.142	0.194	0.187	0.210	0.193	0.092	0.145	0.212	0.224	0.232	0.194	0.106	0.153
RMSEP	0.212	0.198	0.217	0.173	0.132	0.148	0.211	0.206	0.190	0.240	0.093	0.167	0.212	0.218	0.229	0.182	0.111	0.146
Sensitivity_c (%)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	94.12	100.00	100.00
Sensitivity_cv (%)	100.00	100.00	88.23	100.00	100.00	94.44	92.86	90.91	94.12	100.00	100.00	94.44	100.00	81.82	94.12	94.12	100.00	100.00
Sensitivity_p (%)	100.00	75.00	100.00	100.00	100.00	100.00	100.00	75.00	100.00	85.71	100.00	100.00	100.00	75.00	100.00	100.00	100.00	100.00
Specificity_c (%)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	98.63
Specificity_cv (%)	97.40	98.75	100.00	100.00	100.00	100.00	100.00	97.50	100.00	98.65	100.00	98.63	98.70	100.00	100.00	97.30	100.00	98.63
Specificity_p (%)	96.77	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	93.33	100.00	100.00	100.00	100.00	100.00	96.67
Accuracy_c (%)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	94.74
Accuracy_cv (%)	98.70	99.38	94.12	100.00	100.00	97.22	96.43	94.20	97.06	99.32	100.00	96.54	93.33	100.00	100.00	88.89	100.00	94.74
Accuracy_p (%)	98.39	87.50	100.00	100.00	100.00	100.00	100.00	87.50	100.00	92.86	100.00	96.67	100.00	100.00	100.00	100.00	100.00	87.50

[a] Seven *latent variables (LVs) were* chosen to build the PLS-DA *models* for all data sets.

[b] Parameters: RMSEC (Root Mean Square of Calibration), RMSECV (Root Mean Square Error of Cross Validation), RMSEP (Root Mean Square Error of Prediction), Sensitivity_c (true positive rate or recall of calibration expressed as a percentage), Sensitivity_cv (true positive rate or recall of cross validation expressed as a percentage), Sensitivity_p (true positive rate or recall of prediction expressed as a percentage), Specificity_c (true negative rate of calibration expressed as a percentage) Specificity_cv (true negative rate of cross validation expressed as a percentage), Specificity_p (true negative rate of prediction expressed as a percentage), Accuracy_c (Efficiency or accuracy of the classifier for each class in calibration expressed as a percentage), Accuracy_cv (Efficiency or accuracy of the classifier for each class in cross validation expressed as a percentage), and Accuracy_p (Efficiency or accuracy of the classifier for each class in prediction expressed as a percentage).

(1): Baena; (2): Kalamata, (3): Meknès; (4): Priego de Córdoba; (5): Toscana; (6): Tyout.

Furthermore, very good classification and prediction rates were obtained regardless of the ion precursor employed. Thus, in calibration PLS-DA models showed an accuracy of 100% for all the studied categories, except for Tyout-Chiadma class, for which a value of 94.74% was obtained. In term of prediction, accuracy values (in cross validation) were between 88.90% and 100%; whereas in external validation, calculated rates were between 87.5% and 100%. The applied PLS-DA models gave the best results (100%, in both calibration and prediction) for samples from Toscana.

Considering the two-class PLS-DA models (Table 2), as previously observed for the multi-class models, both models 2 and 3 showed low values of RMSEC (≤ 0.116 in model 2 and ≤ 0.127 in model 3), RMSECV (≤ 0.142 in model 2 and ≤ 0.193 in model 3), and RMSEP (≤ 0.142 in model 2 and ≤ 0.164 in model 3).

Table 2. Classification parameters obtained by PLS-DA two-class models in calibration (c), cross validation (cv) (with 10 groups split venetian blinds) and on the external test set (p) for three SIFT-MS data sets (H_3O^+, NO^+ and O_2^+).

	PLS-DA Model 2[a]			PLS-DA Model 3[a]		
Parameters	H_3O^+	NO^+	O_2^+	H_3O^+	NO^+	O_2^+
RMSEC	0.104	0.116	0.111	0.1	0.127	0.111
RMSECV	0.128	0.142	0.133	0.133	0.193	0.14
RMSEP	0.142	0.122	0.134	0.136	0.164	0.154
Sensitivity_c (%)	100	100	100	100	100	100
Sensitivity_cv (%)	100	100	100	100	94.12	100
Sensitivity_p (%)	100	100	100	100	100	100
Specificity_c (%)	100	100	100	100	100	100
Specificity_cv (%)	100	100	100	100	100	100
Specificity_p (%)	100	100	100	100	100	100
Accuracy_c (%)	100	100	100	100	100	100
Accuracy_cv (%)	100	100	100	100	97.06	100
Accuracy_p (%)	100	100	100	100	100	100

[a] two *latent variables (LVs) were* chosen to build the PLS-DA *models* for all data sets.

[b] The description of the classification parameters can be found in Table 1.

- Model 2: Discrimination between Spanish olive oils; and Model 3: Discrimination between Moroccan olive oils.

Bearing in mind the results included in Table 2, it is possible to stand out that regardless of the data sets used, model 2 showed 100% sensitivity, specify and accuracy in

calibration, cross validation and external validation. This result, from our point of view, proves the ability of the proposed methodology to correctly identify oils of the two selected Spanish geographical labeled regions (Priego de Cordoba and Baena). Likewise, model 3 -computed to discriminate between oils form the two considered Moroccan regions- showed very satisfactory results (Table 2). Sensitivity, specificity and accuracy values were 100% in all the cases, just finding one exception (when PLS-DA was applied to NO^+ data set for which values of sensitivity and accuracy in cross validation were 94.12% and 97.06%, respectively).This outcome allowed us to verify the suitability of SIFT-MS, in combination with PLS-DA chemometric treatment, to efficiently differentiate the oils from the studied Moroccan regions.

Volatile compounds analysis had been already reported to be suitable for tracing the geographical origin of olive oil. However, to the best of our knowledge, the methodology proposed herewith seems to be advantageous (in comparison, for instance, with GC-MS), as it is less time-consuming (only 21 min in total are required for sample preparation and analysis) and provides higher recognition and prediction abilities.

4. Conclusions

The developed chemometric models, based on the SIFT-MS data of the volatile fingerprints of 130 virgin olive oil samples from six of the most important Mediterranean regions, provided a strong discrimination. The use of the entire headspace volatile fingerprinting matrices, of the three precursor ions, provided a holistic approach and the possibility of deducing the best possible combination for each studied region. This study indicates that the new methodology can be considered as a non-time consuming strategy suitable for routine analysis of origin-labeled olive oils.

To the best of our knowledge, the present work is the first report highlighting the use and potential of SIFT-MS fingerprinting for geographical origin authentication.

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Chapter

10

Metabolic profiling approach to determine phenolic compounds of virgin olive oil by direct injection and liquid chromatography coupled to mass spectrometry

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Abstract

In the current work a method involving direct injection of extra-virgin olive oil (EVOO) for the LC-MS analysis of its phenolic compounds has been developed. Optimization of the most appropriate solvent for sample dilution, selection of the optimum oil/solvent ratio, and establishment of column cleaning strategy and maximum number of injections allowed (maintaining satisfactory analytical performance) were some of the most relevant steps to go through. After choosing the optimum conditions, the analytical parameters of the method were evaluated, establishing LOD (from 3.3 to 31.6 μg/l for apigenin and oleuropein, respectively) and LOQ, precision (RSD values for inter-day repeatability were found between 3.49 and 6.12% for tyrosol and ferulic acid, respectively), and trueness values (within the range 89.9-102.3% for 1.0 ppm) and checking possible matrix effect (which were no significant). Three kind of calibration were used: external standard, standard addition and calibration in a phenols-free matrix, which was subsequently applied to achieve the quantitative data from the 16 selected EVOOs (from 6 different cultivars). 21 compounds (belonging to different chemical classes) were determined without the need of using any extraction protocol. Dahbia oils were the richest in terms of *p*-coumaric acid and showed a characteristic lignans´ pattern (higher concentration of pinoresinol than acetoxy pinoresinol). Haouzia and Picholine Marocaine oils, however, had considerably high concentrations of various secoiridoid aglycones´ isomers.

Keywords: phenolic compounds; liquid chromatography-mass spectrometry; direct injection; matrix effect.

Abbreviations used (in alphabetical order)**:** acetone, **Acet**; (+)-1-acetoxypinoresinol, **Ac Pin**; apigenin, **Apig**; Base Peak Chromatogram, **BPC**; blank matrix calibration, **BlankCal**; desoxy elenolic acid, **desoxyEA**; detection limit, **LOD**; dialdehydic form of decarboxymethyl EA, **DEA**; dialdehydic form of decarboxymehtyl Lig Agl or dialdehydic form of decarboxymethyl elenoic acid linked to tyrosol, **D-Lig Agl**; dialdehydic form of decarboxymethyl Ol Agl or dialdehydic form of decarboxymethyl elenoic acid linked to hydroxytyrosol, **DOA**; 3,4-dihydroxyphenylacetic acid, **DOPAC**; direct injection, **DI**; electrospray ionization, **ESI**; elenolic acid, **EA**; external calibration, **ExtCal**; Extracted Ion Chromatograms, **EIC**; extra-virgin olive oil, **EVOO**; ferulic acid, **Fer**; hydroxytyrosol, **HYTY**; ligstroside aglycone, **Lig Agl**; liquid-liquid extraction, **LLE**; luteolin, **Lut**; number

of theoretical plates, **N**; oleuropein, **Ol**; oleuropein aglycone, **Ol Agl**; *p*-coumaric acid, ***p*-Cou**; peak width at half height, $\mathbf{w_{1/2}}$; (+)-pinoresinol, **Pin**; 1-propanol, **1-prop**; quality control, **QC**; quantification limit, **LOQ**; relative standard deviation, **RSD**; Standard addition calibration, **StdAd**; regression coefficient, $\mathbf{r^2}$; retention time, $\mathbf{t_r}$; signal to noise ratio, **S/N**; tetrahydrofuran, **THF**; tyrosol, **TY**; virgin olive oil, **VOO**.

1. Introduction

Even though people have been eating olive oil for thousands of years, it is now more popular than ever. The number of scientific studies showing that olive oil can help to prevent and treat different kind of diseases (atherosclerosis, cancer, diabetes, obesity, pulmonary diseases, cognition disorders, etc.) is constantly growing [1-4] and the benefits of a diet rich in olive oil are, indeed, nowadays absolutely undeniable. These healthy properties can be explained considering olive oil's composition regarding its high level of monounsaturated fatty acids and the fact that it also contains multiple minor components [5]. Phenolic compounds are one of the most appreciated classes of non-glyceridic constituents of this matrix [6,7], what is an easily comprehensible fact since, besides their anti-oxidant, anti-inflammatory, anti-microbial activities [3] and very promising nutraceutical uses [6], they contribute to the stability of virgin olive oil (VOO) against auto-oxidation and have an important role on its organoleptic properties [8]. These metabolites can also be considered as a very useful feature to characterize the typicality, geographical origin, genuineness and authenticity of VOOs [9-12]. Additionally, in 2011, the European Food Safety Authority stated the admissibility of specific health claim related to the levels of some VOO phenols [13], fact which is going to have obvious commercial and labelling implications.

Due to the importance of this fraction, different analytical methods have been developed to characterize its complex and heterogeneous pattern, composed by phenyl alcohols, phenolic acids, flavonoids, lignans, secoiridoids, etc [5,6,8,14]. Since the occurrence of hydrophilic phenols in VOO was firstly observed more than about 55 years ago [15], the analytical methods have considerably evolved [5,6,8]. They significantly depend on the information that the analyst would like to achieve; therefore, when the comprehensive characterization of the phenolic fraction is pursued, it implies the appropriate sample preparation and the further instrumental analysis. As far as the first stage is concerned,

two main techniques have been traditionally used for extraction: liquid-liquid extraction [16,17] and solid-phase extraction [18-21]; more recently, some other types of extraction procedures have been also applied, such as, for instance, dispersive liquid-liquid microextraction [22], matrix solid-phase dispersion [23] and ultrasound-assisted emulsification-microextraction [24].

With regard to the analysis itself, it is important to highlight that, so far, there is no internationally accepted regulation concerning the method for individual characterization of phenolic compounds [25,26]. Applications applying nonspecific colorimetric assays (using Folin-Ciocalteu reagent) can be still found, but others which draw on more advanced chromatographic or electrophoretic techniques coupled to diverse detection systems [9,10,21,22,27,28,35], electronic tongues [29], NMR [30,31], Near-infrared spectroscopy [32,33], etc. can offer to the analyst a much more complete overview about the phenolic profile of an EVOO. Among all mentioned possibilities, LC-MS is likely the coupling most widely used both with low and high MS resolution-analyzers.

Within this context, very few papers have been published proposing the direct injection of VOO instead of applying an extraction system to separate the hydrophilic phenols from the apolar matrix of olive oil. The first report in this regard was a very interesting piece of work authored by Selvaggini et al. (2006) [34] and the compounds under study (7 compounds: 2 simple phenols, 2 lignans and 3 secoiridoids) were determined by HPLC-DAD/fluorescence. Later on, three other papers showed the same strategy (i.e. DI of the oil after an appropriate dilution) in part of the experimental work that they included [35-37]. In these latter examples, CE was the analytical technique selected and it was coupled to UV-visible and fluorescence [36,37], and MS detection [35], respectively. Godoy-Caballero et al [36] determined some of the most abundant phenolic compounds (TY, HYTY and some aglycon secoiridoid derivatives (an isomer of Ol Agl, DOA and DLA)) by DI of the olive oil dissolved in 1-prop (1:1 v/v) and a nonaqueous CE method. Gómez-Caravaca et al. [35] also developed a nonaqueous CE method coupled to TOF MS (trying the DI of the investigated matrix introducing a plug of olive oil directly into the capillary) and compared their results with those achieved by CZE in aqueous buffers.

The aim of this work was to develop a LC-MS method for the determination of as many phenolic compounds as possible (belonging to different chemical classes) without the

need of carrying out an extraction protocol, but only a simple sample dilution. A complete validation of the method was done, paying particular attention to possible matrix effect. Afterwards, the method was applied to the analysis of 16 EVOO samples coming from different cultivars.

2. Materials and methods

2.1. Olive oil samples

A total of 16 monovarietal EVOO samples, from 6 different varieties were selected: 'VS 3' (2 samples), 'VS 5' (2 samples), 'Picholine Marocaine' (3 samples), 'Dahbia' (3 samples), 'Haouzia' (3 samples), and 'Menara' (3 samples). VS 3 and VS 5 are local genotypes from Picholine Marocaine variety, which were growth within the frame of a research project (RESERGEN, Olive Genetic Resources) funded by International Olive Council.

To obtain the EVOO samples, olive fruits sampling was performed over the season (2013/2014) on randomly selected trees, representing the above-mentioned 6 olive cultivars, all grown in the experimental olive grove of the Agro-pôle Olivier National School of Agriculture of Meknès, Morocco. Pest control, pruning, irrigation and fertilization practices were done following current olive orchards management practices. To avoid possible influence of the fruits ripening stage on the phenolic profiles of the studied oils, only samples picked at a ripening index within the range 3.0-3.5 were considered; range which is commonly advised for the production of high quality olive oils in Meknès region. Afterwards, oil was extracted using an Oliomio laboratory mill (Oliomio, Italy) simulating two-phase commercial oil-extraction system. The operating mode of this instrument has been described in detail by Bajoub et al [38].

To evaluate the physico-chemical quality of the obtained oils, regulated criteria (free fatty acids content (given as percentage of oleic acid), peroxide value (expressed as milliequivalents of active oxygen per kilogram of olive oil (meq O_2/kg)) and K_{232} and K_{270} extinction coefficients, calculated from absorption at 232 and 270 nm, respectively) were determined, in triplicate, for each studied oil sample by using the analytical methodologies described in the European Union Standard Methods Regulations 2568/91 and the subsequent amendments [39]. Obtained results allowed classifying all the studied oils within the "extra virgin" category.

2.2. Chemicals and reagents

All solvents were of analytical or LC-MS grade purity (depending on if they were used for the extraction or chromatographic analysis). Methanol and *n*-hexane were used for the extraction of the hydrophilic phenols from the olive oil samples and they were supplied from Panreac (Barcelona, Spain). For preparing the LC mobile phases, acetonitrile and acetic acid (purchased from Lab-Scan (Dublin, Ireland) and Panreac (Barcelona, Spain), respectively) were employed. Doubly deionised water with a conductivity of 18.2 MΩ was produced in the laboratory using a Milli-Q-system (Millipore, Bedford, MA, USA). THF, Acet, and 1-prop were used to dissolve the EVOO samples before the injection into the LC system; THF and Acet were provided by Panreac (Barcelona, Spain), and 1-prop by Sigma-Aldrich (St. Louis, MO, USA).

Commercial standards of two simple phenols (HYTY and TY), two phenolic acids (*p*-Cou and Fer) and two flavonoids (Lut and Apig) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A pure standard of a lignan (Pin) was also acquired from Arbo Nova (Turku, Finland), as well as a secoiridoid-glucoside (Ol), which was purchased from Extrasynthese (Lyon, France).

First of all, a stock solution (500 mg/L of each standard) was prepared by dissolving the appropriate amount of the compounds in methanol, and then a series of working solutions of these analytes were freshly made by diluting the mixed standard solution with methanol at appropriate ratios to yield concentrations within the range 0.1-250 mg/L. The proper amount of DOPAC, purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in methanol to prepare an internal standard (IS) solution with a concentration of 500 mg/L. All solutions were properly stored in dark flasks at −20 °C. After deciding that the EVOO dilution would be made in Acet, both the stock solutions and further serial dilutions were also prepared in this solvent in order to carry out a fair comparison of the response factor of the analytes in matrix and solvent.

2.3. Dilution of EVOO samples for direct injection into LC-MS

1 g (±0.001) of olive oil weighed in a test tube with a screw cap was mixed with 5 mL of Acet (THF or 1-prop -in the preliminary studies). In the prefatory experiments, before choosing 1 g as optimum amount of oil, different proportions olive oil/solvent were also assayed: 0.5, 1.0, 1.5, 2.0, 2.5 and 3 g were mixed with 5 mL of solvent. All the samples

(and stock solutions) were filtered through a Clarinert™ 0.22 µm nylon syringe filter from Agela Technologies (Wilmington, DE, USA) before injection into the instrument.

2.4. Phenolic compounds extraction

A LLE was also used [38**¡Error! Marcador no definido.**]. In short, 25 µL of the IS solution was added (although, in the end, correction with the IS area was not necessary) to 2 g (±0.001) of olive oil weighed in a test tube with a screw cap. 1 mL of *n*-hexane was added to the oil and the phenols were extracted by adding 2 mL of methanol/water (60:40, v/v) three consecutive times; the mixture was vortexed for 2 min and centrifuged at 3500 rpm for 6 min. The combined extracts were evaporated in a rotary evaporator (Büchi R-210) at 30ºC, and the obtained residue was reconstituted in 1 mL of methanol of LC-MS grade and filtered through 0.20 µm membrane (nylon) filter.

The extracts prepared in the described way were used to enrich or spike olive oil or sunflower oil, respectively, for the validation studies.

2.5. LC-MS analysis

2.5.1. Apparatus and software

The analyses were carried by reversed-phase LC coupled to MS. The LC system was an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (DAD), which was coupled to a Bruker Daltonic Esquire 2000™ ion trap mass spectometer (Bruker Daltonik, Bremen, Germany) by an ESI interface. Chromatographic data acquisition and examination of DAD signals was performed by using ChemStation B.04.03 software (Agilent Technologies). Bruker mass spectrometer was controlled using the software Esquire Control and the resulting files were treated with the software Data Analysis 4.0 (Bruker).

Additionally, for carrying out a proper characterization (qualitative) of the selected samples, another LC-ESI-Q TOF MS platform was used; therefore, an Acquity UPLC™ H-Class system coupled to a micrOTOF-Q IITM mass spectrometer (Bruker Daltonik) by means of an ESI source was also employed.

An exploratory analysis of the data was carried out through PCA, which was used to display a natural grouping tendency or outliers among EVOO samples. Data were analyzed by using The Unscrambler® v9.7 software (CAMO Software AS, Oslo, Norway).

2.5.2. Chromatographic and detection conditions

The phenolic compounds from the studied samples were analyzed, in triplicate, following previously reported LC-MS conditions [40]. The stock solutions, phenolic extracts and EVOO or sunflower oil diluted samples were analyzed using a Zorbax C18 analytical column (4.6 × 150 mm, 1.8 μm particle size) protected by a guard cartridge of the same packing, operating at room temperature. The mobile phases were 0.5% acetic acid in water (A) and acetonitrile (B). A flow rate of 0.8 mL/min was used and a volume of 10 μL of the extracts, standard mix or diluted oils was injected in each case. The elution gradient established was the following: 0-10 min, 5% B; 10-12 min, 30% B; 12-17 min, 38% B; 17-20 min, 50% B; 20-23 min, 95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 2.5 min.

The ion trap mass analyzer operated in negative ion mode (although several analyses in positive mode were also carried out). The capillary voltage was set at +3200 V and the MS detector was programmed to perform scans at 50-800 *m/z* range. The following parameters of ESI-MS were used: drying gas temperature, 300 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 30 psi.

A standard mixture solution with a concentration of 1 mg/L and one EVOO sample (from VS 5 *cv.*) were used as QC samples to check the stability of the system over the different sequences carried out. The QC samples were injected every ten analyses (after a blank) in each sequence.

The above mentioned MS parameters were transferred to the ESI-Q TOF MS spectrometer. The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonik). Sodium formate clusters were used for the internal calibration of the Q TOF system, pumping a solution containing 5 mM sodium hydroxide and 0.2% formic acid in water/isopropanol (1:1, v/v). The calibration solution was injected at the beginning of the run (using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA)) and all the spectra were calibrated prior carrying out the compound identification. For both mass spectrometer detectors, a flow divisor 1:4 was used, reducing the flow rate from the LC method to 0.2 mL/min. The identification of the phenolic compounds present in the analyzed samples was based on

the use of pure standards (when available), retention time data, and comparing the ESI-TOF MS and ESI-IT MS spectra (and MS/MS spectra) with previously published results [38,40].

2.5.3. Method validation

Three different kinds of calibration were used with the aim of evaluating possible matrix effects: external calibration, standard addition calibration and calibration in blank matrix.

Solutions containing pure standards of the phenolic analytes under study at 10 different concentration levels (in acetone) over the range of 0.1-50 mg/L were used in order to evaluate linearity and establish the calibration curves which could allow their quantification in the samples. After the preliminary studies, the concentration range was constrained till 10 mg/L as maximum level, covering the range in which the different metabolites under study were actually found in the selected EVOO samples. External calibration curves were established for each compound by performing a linear regression by the least-squares method. Each point of the calibration graph corresponded to the mean value of three independent injections.

Besides, standard addition calibration was also applied to, at least, one EVOO sample of each variety. Eight concentration levels were tested (0.1-10 mg/L, which is equivalent to approximately 0.61-60.9 mg/kg).

The same concentration levels as those evaluated in standard addition calibration were appraised when the calibration was done in a phenols-free oily sample (sunflower oil, which was considered as a blank sample in terms of phenolic compounds).

A matrix effect coefficient was calculated for each compound relating the slope in sunflower matrix and in solvent, and the slope in sunflower oil matrix and in olive oil, respectively, adapting the following the equation [41]:

*Matrix effect coefficient (%) = (1-(slope matrix/slope solvent))*100*

LOD and LOQ for each phenolic compound were calculated using the S/N of pure standards at the lowest concentration level injected (for every analyte) and were estimated by using both the external calibration and the calibration in blank matrix. LOD and LOQ were measured by calculating the concentration that produced a S/N equal to 3

and 10, respectively [42]. The theoretical values so achieved, were corroborated injecting the pure standards (in Acet or sunflower diluted oil) at those concentrations.

The precision of the method was evaluated as well. *Intra-day* repeatability was expressed as RSD obtained for 5 injections of the QC, carried out within the same sequence. *Inter-day* repeatability was calculated as RSD of 12 injections (belonging to 3 different sequences carried out over 3 consecutive days) of the same olive oil sample (QC).

Trueness was estimated by analyzing spiked sunflower oil at different known concentrations (0.25, 1.0 and 5.0 ppm) and calculating the effective/true concentration values.

Results and Discussions

3.1. LC-MS analyses

3.1.1. Selection of the solvent used to dissolve the oil samples and optimum oil/solvent ratio

One of the most pivotal steps in the optimization of the methodology was the selection of the most appropriate solvent to dissolve the olive oil samples before the injection into the LC-MS system. Keeping in mind the previously published reports including information about miscibility of olive oil with different organic solvents, their viscosity and polarity index [34-37,43], THF, Acet and 1-prop were selected. After the preliminary studies, we decided to prepare the samples as follows: 1 g of olive oil was dissolved adding 5 ml of the selected solvent (in the coming paragraphs the justification to this will be presented). Fig. 1 shows the peak intensity (in terms of area) of several phenolic compounds after dilution of the olive oil (VS 5-1) in Acet, THF, and 1-prop. THF and Acet gave very similar results for Pin, Lut and Ol Agl (isomer of 22.1 min); 1-prop and THF, however, produced similar peak areas for HYTY, DesoxyEA, and Ol Agl (isomer of 21.5 min). No significant differences were observed for Lig Agl (isomer of 23.6 min) regardless of the solvent used to dissolve the oil. In general, the dilution of the olive oil in Acet produced peaks with higher area values in almost all the cases. That was particularly evident for EA, DOA and Ol Agl (isomer of 21.5 min). Therefore, Acet was chosen as the most appropriate solvent. MS signal intensity was one of variables considered to make the solvent selection, but we also took into account some other factors, such as: easiness to filter (by using a syringe

filter) the dissolved sample, peak shape, and stability of the area values over consecutive injections.

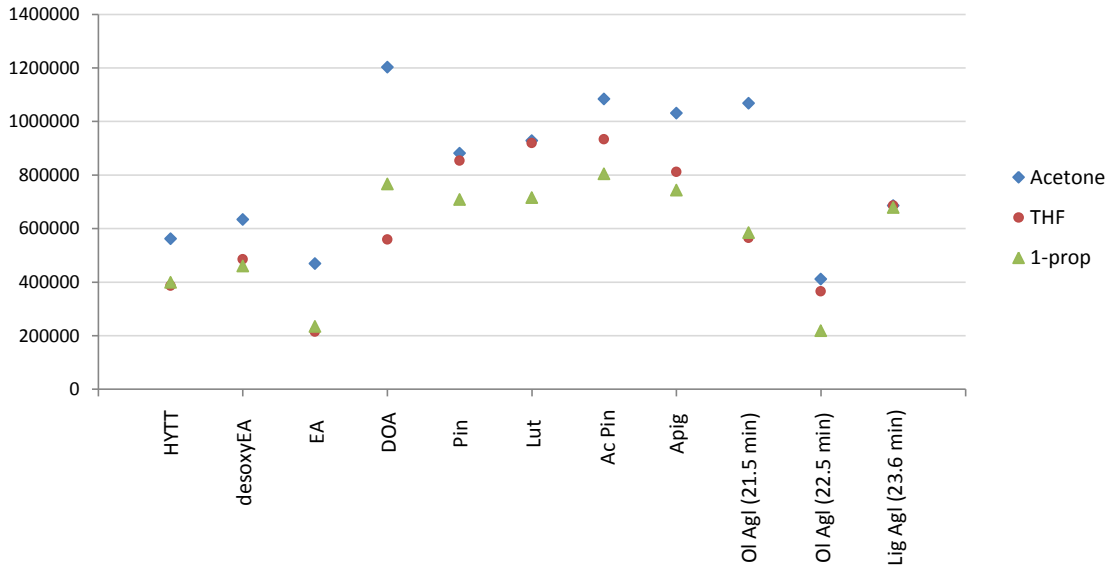
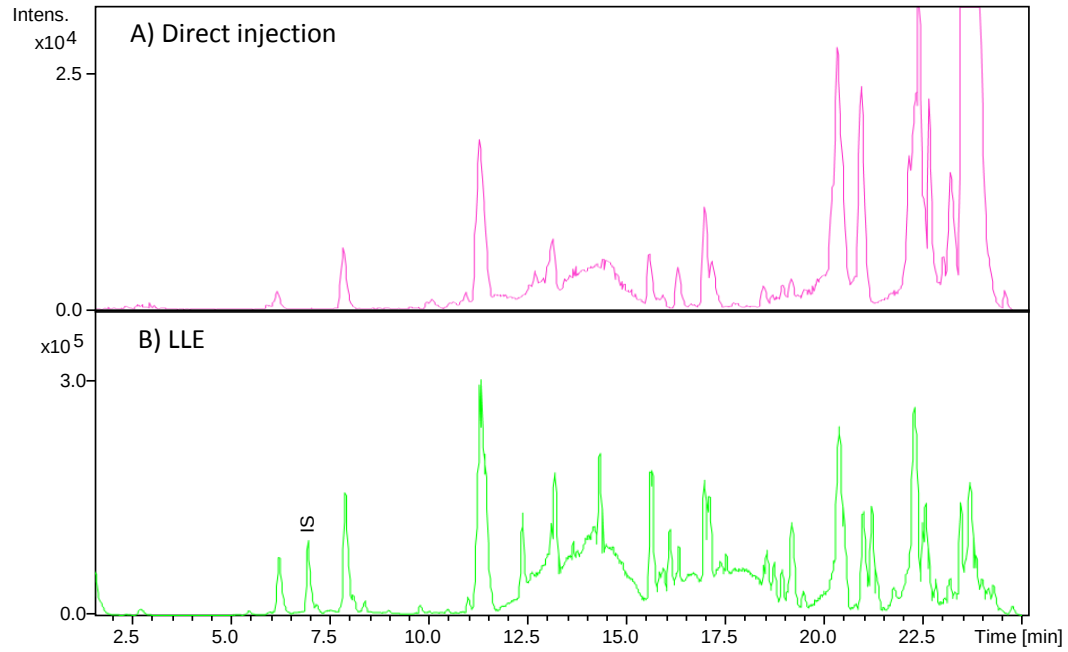


Figure 1. Peak intensity of several phenolic compounds after dilution of the olive oil (VS 5-1) in acetone, THF, 1-propanol. **Peaks nomenclature**: HYTY (hydroxytyrosol), desoxyEA (desoxy elenolic acid), EA (elenolic acid), DOA (decarboxilated oleuropein aglycone), Pin (pinoresinol), Lut (luteolin), Ac Pin (acetoxy pinoresinol), Apig (apigenin), Ol Agl (21.4 min) (oleuropein aglycone isomer with a t_r of 21.5 min), Ol Agl (22.1 min) (oleuropein aglycone isomer with a t_r of 22.5 min), Lig Agl (23.6 min) (ligstroside aglycone isomer with a t_r of 23.6 min). **Area values** represented for each compound (and solvent) are the average of five independent determinations (RSD < 6.1% in every case). Area values of HYTY, Pin, Lut, Ac Pin, Apig have been multiplied by a factor (*5, *10 or *20) in order to facilitate their representation within the same Y axis scale.

As stated before, different sample concentrations were injected in order to select the most advisable olive oil dilution. After the preliminary studies the following combinations were thoroughly evaluated: 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g, respectively, plus 5 ml of acetone. The second option was picked as optimal considering the number of compounds which could be properly detected and trying to avoid more concentrated preparations which could produce the rapid soiling of the column. Fig. 2 endeavours to illustrate the potential of our methodology and shows an example of the BPC of an EVOO VS 5 (1 g + 5 ml acetone) (Fig. 2A) and the EICs of the phenolic compounds determined by using the DI approach (Fig. 2C). Moreover, the BPC of the same oil after carrying out a LLE -as described in section 2.4- is shown as well (Fig. 2B). The profiles shown in Fig. 2A and 2B

are obviously very similar, just differing in terms of signal intensity (extracts should be about 12-fold more concentrated than the DI assay).



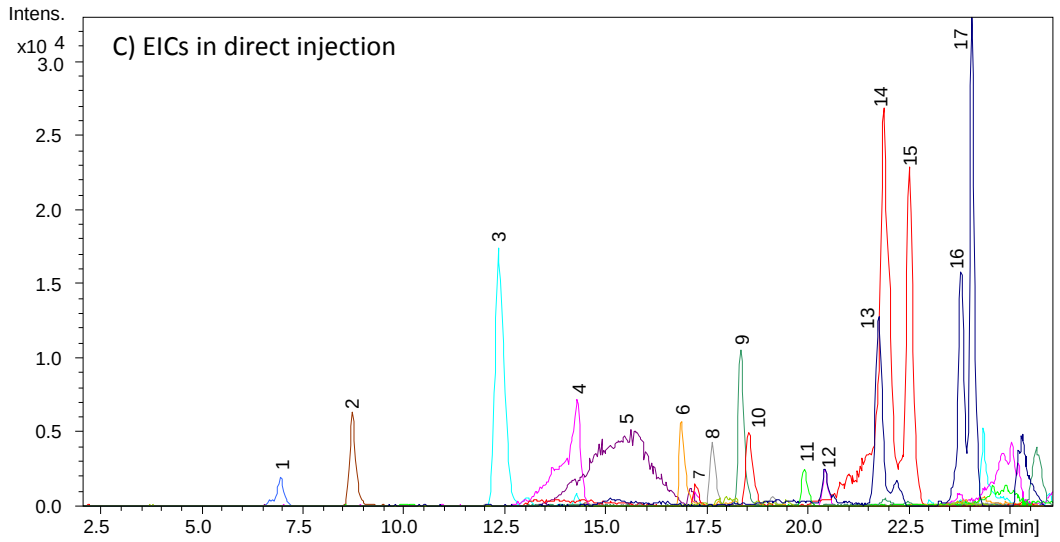


Figure 2. (A) Base Peak Chromatogram (BPC) of an EVOO sample from VS 5 variety (1 g + 5 ml acetone) using the direct injection (DI) procedure, (B), BPC of the extract of the same sample, and (C) Extracted Ion Chromatograms (EICs) of the phenolic compounds determined by the described method using the DI approach.

However, using less concentrated injections was no detriment to the potential of the new method (see Fig. 2C); indeed, compounds belonging to different chemical classes were detected: simple phenols (HYTY, TY), flavonoids (Lut and Apig), lignans (Pin and Ac

Pin) and secoiridoids or related compounds (desoxyEA, EA, DOA, and different isomers of Ol Agl and Lig Agl). In the sample chosen as exemplification in Fig. 2C very little amounts of quinic acid, DEA, *p*-Cou, Fer, syringaresinol, methyl DOA, D-Lig Agl, and methyl Ol Agl were found, that is the reason for not including the EICs of these analytes. Anyway the most relevant aim of this work was to propose an alternative methodology for carrying out a reliable quantification of the most relevant phenolic compounds present in EVOO without the need of previous extraction.

At this point, it seems necessary to make a comment about the fact of detecting multiple isomers of Ol Agl and Lig Agl (as we will explain in the coming paragraphs, these isomers showed up in a very little proportion in comparison with the results after applying LLE with methanol-water mixtures). Karkoula et al. [25,43] published two interesting manuscripts about the artificial formation of some secoiridoid derivatives (mainly due to their reactivity with methanol (and water)), and since then, this topic is awakening a lot of interest. Our group has already discussed it in another publication [44], where we have corroborated Karkoula´s findings, saying that as long as methanol (and probably water and/or their mixtures) is involved in the sample preparation (or has any interaction at any point of the analytical procedure with these compounds), the "artificial isomers" will show up. We have also studied that the generation of artificial peaks (related to DOA and D-Lig Agl) in the chromatograms is not as serious as for Ol Agl and Lig Agl and could be even ignored (from a quantitative point of view) in the samples that we have worked with. Moreover, from our point of view (in good agreement with Karkoula´s and some other research groups), ignoring the "artificial isomers" would mean underestimating their initial "native amount", since they are formed from the native secoiridoids present in the VOO sample. That is why we quantified several isomers of Ol Agl and Lig Agl. Figure 1 (supplementary material) shows that, in any case, the formation of "artificial isomers" was drastically minimized with our DI method (since extraction step is avoided), what could represent another advantage of this new approach.

3.1.2. Optimizing the column cleaning and maximum number of injections

Injection of olive oil dissolved in different solvent has been previously tried for determining, for instance, tocopherols and triacylglycerols; however, as far as phenolic compounds are concerned, there is just one report where Selvaggini et al. [34] proposed a

HPLC-fluorescence method with direct injection of the olive oil (2 g dissolved in 10 ml acetone) into the column (two C18 columns with similar dimensions (250 mm x 4.6 mm, particle size 5 µm) were used, ChromSep Inertsil ODS-3 and Spherisorb ODS-1) and compared the results with those achieved after applying LLE and HPLC-DAD/Fluorescence. Performance of both methods was satisfactory in terms of repeatability (*intra*-day and *inter*-day repeatability (variation coefficient)) after injecting 6 times the same oil and repeating the same operations 2 days in a row.

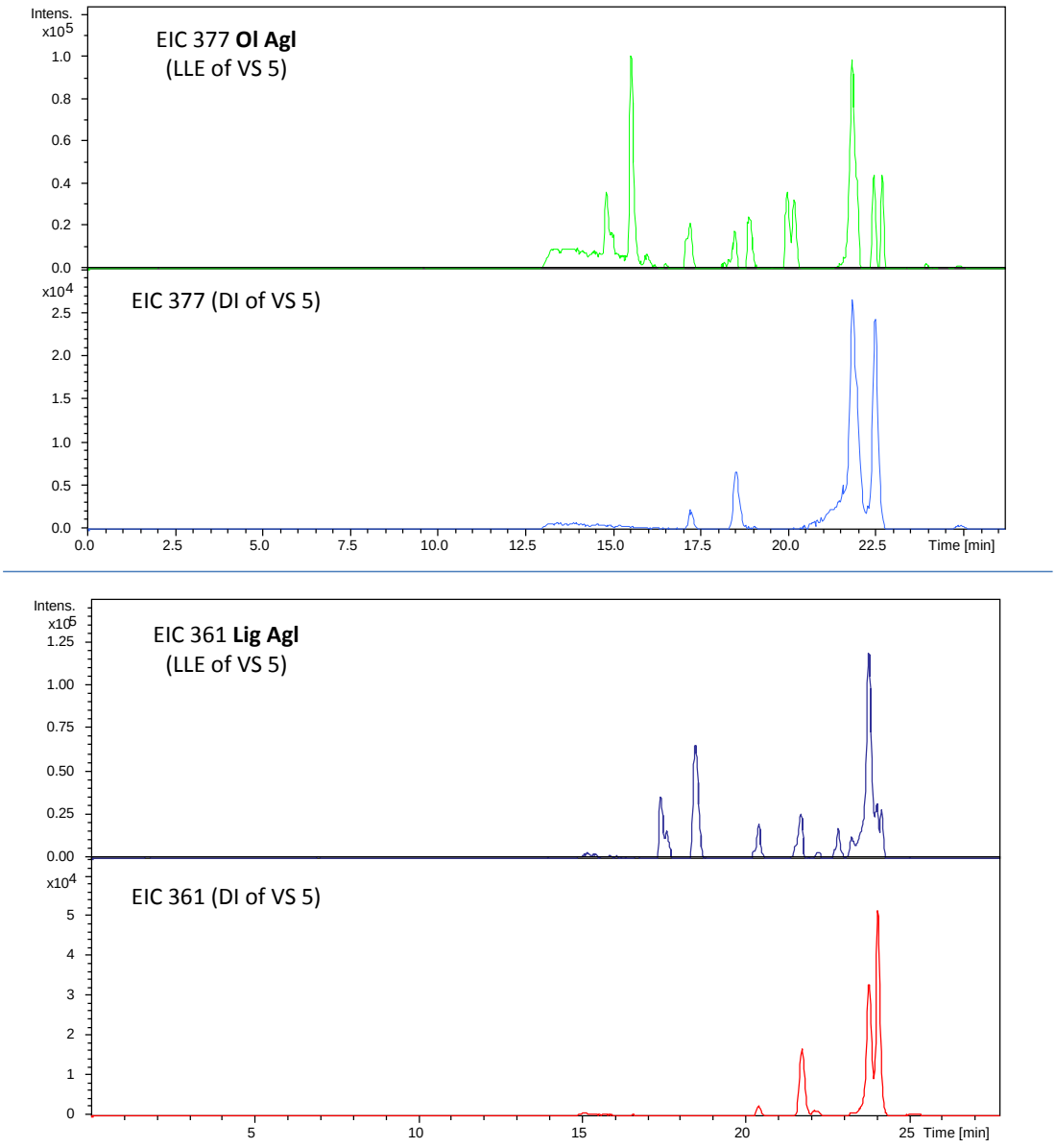


Figure 1. Supplementary material. Comparison of the EICs of Ol Agl (*m/z* 377) and Lig Agl (*m/z* 361) for an extra virgin olive oil sample (from VS 5 *cv.*) showing the "artificial isomers" which can be observed when sample extraction is carried out by using methanol-water mixtures.

The outcome of this study was very promising, but some discrepancies were observed when DI data were compared with those obtained after the extraction. The authors attributed this to the fact that the extraction procedures produce a partial and selective recovery of VOO phenols (because of the different polarities, structures and molecular weights). In other reports using similar DI approaches, CE was chosen as analytical technique.

Herewith, the evolution of the column in a sequence after 1, 5, 15, 25, 50 and 75 consecutive injections (considering the area of the selected peaks, N, S/N and retention time) was checked. To illustrate the gradual change in the column performance, Fig. 3 includes data for 5 different compounds (HYTY, desoxyEA, Pin, Apig and the main isomer of Ol Agl (21.5 min)), which were selected to have, at least, one representative metabolite from the different chemical classes determined. The values shown in the different graphics for injections number 15, 25, 50 and 75, respectively, are the mean of the different parameters calculated from those injections, but averaged together with the results from the previous and subsequent chromatographic runs. In every case, a decrease in the value of all the evaluated parameters can be observed. So the retention times tended to shorten as more injections were made, even though the column pressure did not experience any increase over the sequences. All the compounds exhibited a diminution of area value over the time; the trend was very similar for HYTY, desoxyEA, and Pin, being their areas after 15 injections about 96% (95.8-97.9%) of the initial value, and after 50 injections, about 93% (91.4-94.5%) of the starting value. After carrying out 75 analyses, HYTY, for instance, showed an area value of about 87% of the original one. The decrease was slightly more drastic for Apig and Ol Agl isomer, whose areas after 50 runs did not achieve the 86% approx. of the starting point (although values kept more stable than for the other analytes between 50 and 75 runs).

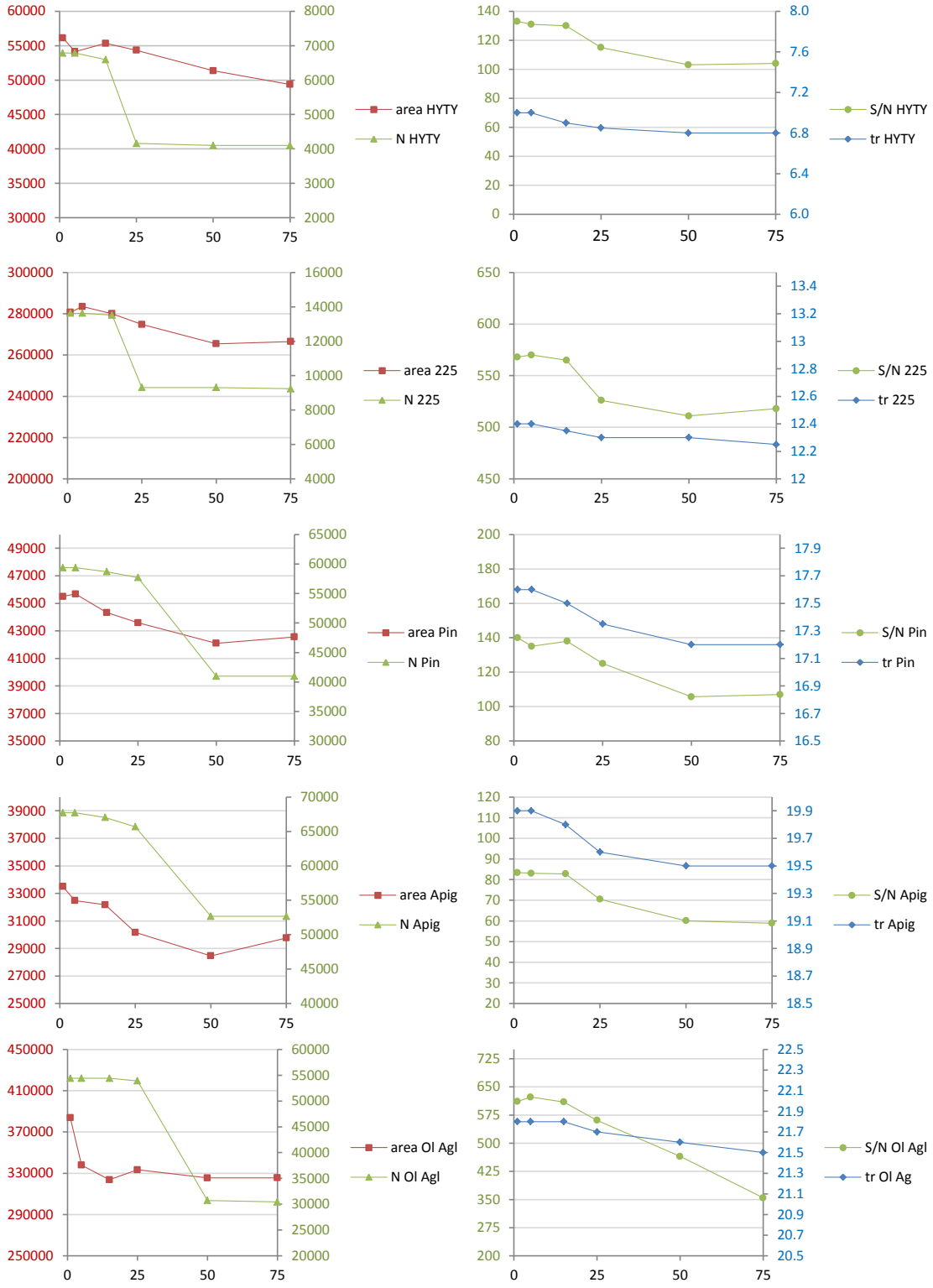


Figure 3. Evolution of the column in a sequence after 1, 5, 15, 25, 50 and 75 consecutive injections considering the area of the selected peaks (HYTY, 225 (DesoxyEA (*m/z* 225)), Pin, Apig and Ol Agl (main isomer with tr 21.5 min)), theoretical plates (N), S/N and retention time. Traces are coloured according to their scale in the Y axis.

N for the picked compounds was calculated as follows: $N = 5.54\ (t_r/w_{1/2})^2$, where both t_r and $w_{1/2}$ were expressed in minutes. The N tendency was compound-dependent; for instance, N values for Pin, Apig and Ol Agl remained very stable after 25 runs, and after 75 analyses, N were still 69.0, 77.8 and 56.0%, respectively, of the first value, showing very satisfactory values (40973.8, 52664.6 and 30450.2, respectively). N values for HYTY and desoxyEA went down after 25 analyses (showing values representing 65% of the initial ones (4159.2 and 9312.7, respectively)), but after this, they stayed very stable. As far as S/N is concerned, for HYTY, after 25 analyses, the value represented 90% of the starting one, and after 75, it was 78.2%. Very similar behavior was observed for Pin and Apig. For Ol Agl the same was noted until injection number 50; after 75 runs, S/N decreased till 354.6 (which can be estimated as 60% of the first value). The derivative of EA (desoxyEA) was the compound with a steadier S/N, after 75 runs, S/N value still accounted for 91.2% of the first registered S/N data.

The evolution of the column performance was very clear; however, the values of the tested parameters after 75 consecutive injections, from our point of view, were still reasonably adequate and satisfactory. We tried, anyway, to develop a cleaning method, which should be carried out after a certain number of analyses and would guarantee a very similar analytical performance to the one exhibited at the beginning of any sequence. After trying different sequential cleaning steps using different kind of solvents over diverse time periods, we decided to go for: acetonitrile (5 min at 0.8 ml/min), THF (5 min at 0.8 ml/min), acetonitrile (5 min at 0.8 ml/min), isopropanol (5 min at 0.4 ml/min) and acetonitrile (5 min at 0.8 ml/min). Afterwards, initial chromatographic conditions were selected and analysis of the olive oil dissolved in acetone resumed achieving comparable results to those obtained before trying any DI.

For routine analysis, we decided to include an injection of the standard mix of 1 ppm in acetone and the QC every ten analyses (after a blank). The cleaning procedure was applied every day, after about 48 injections (even knowing that some more runs could be made without cleaning), in order to assure very satisfactory analytical overall performance. This decision was, in part, made considering the logistics of the global procedure; stopping after 48 injections meant cleaning once every day (both column and ESI interface) and, to a certain extent, increase the probability of avoiding any drastic contamination problem.

3.1.3. Establishing the analytical parameters of the method

Table 1 shows the analytical parameters of the proposed method, including calibration curves and regression coefficients, LOD, LOQ, trueness, *intra/inter*-day repeatability, and matrix effect coefficients. As stated in section 2.5.3, three different kinds of calibration were used with the aim of evaluating possible matrix effects: external calibration, standard addition calibration and calibration in blank matrix.

All the resulting calibration curves showed good linearity within the indicated concentration ranges, with r^2 higher than 0.9868. LOD and LOQ (µg/L) were estimated with the data from external calibration and the calibration in sunflower matrix. The values achieved by both estimations were similar, being found between 3.3 and 31.6 µg/L for Apig and Ol, respectively (data from blank matrix calibration). The linearly was first evaluated in a wider range (till 50 ppm), but after the preliminary studies, we decided to limit the range, since fixing it at 10 ppm the range in which the different analytes were actually found in the selected EVOO samples was covered. RSD values for *intra-day* repeatability (calculated from 5 injections of the QC (a VS 5 olive oil) carried out within the same sequence) were found between 2.78 and 4.19 % for Apig and Lut, respectively. The peak areas of the evaluated compounds measured from the injections of 12 independent dilutions of the same olive oil sample (an example of an VS 5 oil) analyzed in 3 different sequences (carried out over 3 days) were used to calculate RSD values for *inter-day* repeatability, finding results within the range between 3.49 and 6.12% for TY and ferulic acid, respectively. With regard to trueness, the table shows the similarity between the effective concentration values calculated after analyzing spiked sunflower oil (at different known concentrations (0.25, 1.0 and 5.0 ppm)). A trueness value of 100% means a perfect matching between the determined concentration level (using the calibration curves built in blank matrix) and the theoretical one. For every compound, trueness was between 94.8 and 105.3% for 0.25 ppm; within the range 89.9-102.3% for 1.0 ppm; and fluctuating between 92.3 and 99.8% for 5 ppm. In order to complete the results and give an estimation about trueness regarding other phenolic compounds (not available as commercial pure standards), sunflower oil was spiked with extracts obtained after LLE at different concentration (1:5, 1:10 and 1:25, v/v diluted with acetone).

Table 1. Analytical parameters of the developed method.

Comp.	Type of calibration	Calibration curve	r^2	LOD (µg/L)	LOQ (µg/L)	Evaluated range[a]	Accuracy			Matrix Effect Coefficient (%)[e]
							Intra-day Repeatability[b]	*Inter-day* Repeatability[c]	Trueness[d]	
HYTY	ExtCal	y = 104657x + 6895.3	0.9984	7.1	23.7	10	4.12	4.65		
	BlankCal	y = 104950x - 7974.2	0.9965	12.5	41.7				98.6 (0.25 ppm) 97.7 (1.0 ppm) 94.4 (5.0 ppm)	-0.28 (SO/Ext)
	StdAd	y = 102410x + 26500	0.9938							-2.48 (SO/StdAd)
TY	ExtCal	y = 34205x + 1452.5	0.9984	13.3	44.3	10	3.21	3.49		
	BlankCal	y = 32926x + 8077.6	0.9989	19.3	64.3				99.6 (0.25 ppm) 98.7 (1.0 ppm) 98.5 (5.0 ppm)	3.74 (SO/Ext)
	StdAd	y = 32549x + 86236	0.9921							-1.16 (SO/StdAd)
*p*Cou	ExtCal	y = 72960x + 9020.2	0.9964	9.9	33	10	3.65	5.34		
	BlankCal	y = 69959x + 1927.1	0.9919	10.2	34				100.3 (0.25 ppm) 91.3 (1.0 ppm) 95.8 (5.0 ppm)	4.11 (SO/Ext)
	StdAd	y = 68811x - 2128.4	0.9977							-1.67 (SO/StdAd)
Fer	ExtCal	y = 58333x + 18496	0.9899	9.65	32.2	10	3.99	6.12		
	BlankCal	y = 60080x + 19903	0.9954	10	33.3				103.3 (0.25 ppm) 97.3 (1.0 ppm) 97.7 (5.0 ppm)	-2.99 (SO/Ext)
	StdAd	y = 59291x - 24545	0.9987							-1.33 (SO/StdAd)
Ol	ExtCal	y = 10841x + 9294.6	0.9955	25.6	85.3	10	4.11	6.01		
	BlankCal	y = 11127x + 169.34	0.9977	31.6	105.3				105.3 (0.25 ppm) 102.3 (1.0 ppm) 99.8 (5.0 ppm)	-2.64 (SO/Ext)
	StdAd	y = 11472x - 773.97	0.9913							3.01 (SO/StdAd)

Table 1 *(continued)*

	Type of calibration	Calibration curve	r^2	LOD (µg/L)	LOQ (µg/L)	Evaluated range[a]	*Intra-day* Repeatability[b]	*Inter-day* Repeatability[c]	Trueness[d]	Matrix Effect Coefficient (%)[e]
Lut	ExtCal	y = 171882x + 39155	0.9912	3.2	10.7	10	4.19	5.44		
	BlankCal	y = 171715x - 8259.1	0.9966	5.1	17				96.6 (0.25 ppm) 89.9 (1.0 ppm) 92.3 (5.0 ppm)	0.10 (SO/Ext)
	StdAd	y = 178151x + 25709	0.9976							3.61 (SO/StdAd)
Pin	ExtCal	y = 84404x + 8274	0.9987	8.2	27.3	10	3.55	5.83		
	BlankCal	y = 82023x + 13010	0.9933	8.8	29.3				93.6 (0.25 ppm) 95.5 (1.0 ppm) 99.5 (5.0 ppm)	2.82 (SO/Ext)
	StdAd	y = 79688x + 41340	0.9868							-2.93 (SO/StdAd)
Apig	ExtCal	y = 243781x + 98095	0.9909	2.9	9.7	10	2.78	4.89		
	BlankCal	y = 255554x + 54055	0.9899	3.3	11				94.8 (0.25 ppm) 93.7 (1.0 ppm) 93.3 (5.0 ppm)	-4.83 (SO/Ext)
	StdAd	y = 252160x - 27170	0.9898							-1.34 (SO/StdAd)

Abbreviations used within the table: ***Comp****: compounds´s name;* ***ExtCal****: external calibration;* ***BlankCal****: blank matrix calibration;* ***StdAd****: Standard addition calibration. (to facilitate the revision process; in subsequent versions of this paper, abbreviations will just appear in the first page of the manuscript)*

Standard addition calibration was carried out using, at least, for one EVOO sample from each variety. Results for an example of VS 5 olive oil sample are shown within this table.

[a] *Linear ranges were established from LOQ to the indicated value (mg/L).*

[b] *RSD values (%) for peak areas of the analytes under study measured from 5 injections of the quality control (olive oil VS 5) carried out within the same sequence.*

[c] *RSD values (%) for peak areas of the evaluated compounds measured from 12 injections (belonging to 3 different sequences which were carried out over 3 days) of 12 independent dilutions prepared from the same olive oil sample (VS 5-1).*

[d] *Trueness was estimated by analyzing spiked sunflower oil at different known concentrations (0.25, 1.0 and 5.0 ppm) and calculating the effective/true concentration values.*

[e] *Matrix effect coefficient (%) = (1−(slope matrix/slope solvent))×100.*

The averaged areas of three independent injections of the diluted extracts in acetone and spiked sunflower oil (at equivalent concentrations) were compared for DOA, Ol Alg isomer (21.5 min), and Lig Alg isomer with a t_r of 23.6 min. In every case, trueness was found between 87.1 and 104.3% for dilution 1:5, fluctuated from 95.1-99.7% for dilution 1:10, and varied between 96.0 to 99.5% for the mots dilute samples.

To corroborate that the response factor of each compound was equivalent in a neat solution (acetone), in sunflower oil and in EVOO matrices, different types of calibrations were carried out. Afterwards, the slopes of the obtained equations were compared using the following approach:

*Matrix effect coefficient (%) = (1-(slope matrix/slope solvent))*100*

(or the corresponding adjustment, correlating slope in sunflower/slope from standard addition)

According to Kmellár et al. [41] values from -20 to +20% mean no significant suppression or enhancement effect. Taking this criterion into account, we can claim that the matrix effect´s significance was very low in this case and, therefore, the three different calibration approaches could be equally used. The matrix effect coefficients (comparing sunflower oil calibration´s slope and the one of the external standard method) were found within the range from -4.83 and 4.11%. When the calculations were made relating the slope of the calibration curves in sunflower and in olive oils, the results were very satisfactory as well and the matrix effect coefficients fluctuated between -2.93 and 3.61%, for Pin and Lut, respectively. The standard addition calibration was carried out using, at least, one EVOO sample from each variety (corroborating the quantitative results which will be presented in the following section), however, to simplify Table 2 to the extent possible, we only show the results achieved from one VS 5 olive oil sample.

After confirming that the three tested calibration approaches were valid, the possibility of using standard addition calibration was dismissed for practical reasons, since it implies the need of carrying out a different calibration for each sample. The quantification was finally made using the calibration curves built in the phenols-free sunflower matrix.

3.2. Application of the method to analyze different EVOO samples

After evaluating the analytical parameters of the new method, we proceed to apply it for the analysis of EVOO samples coming from different varieties: 16 monovarietal extra virgin olive oil samples from 6 different varieties were selected: VS 3 (2), VS 5 (2), Picholine Marocaine (3), Dahbia (3), Haouzia (3), and Menara (3 samples). Table 2 includes the quantitative results (mg analyte/kg olive oil sample) achieved; every result is the average of three independent (sample dilution and injection) determinations (n = 3) and they are given by the mean value ± RSD (%). 21 compounds were determined: two simple phenols (HYTY and TY), two phenolic acids (*p*-coumaric and ferulic acids), two flavonoids (Lut and Apig), two lignans (Pin and Ac Pin), and 13 secoiridoids or related compounds (DEA, desoxyEA, EA, DOA, D-Lig Agl, 4 isomers of Ol Agl and other 4 isomers of Lig Agl). We just include in the table those compounds which could be properly quantified in all the evaluated samples.

When the results were observed, some evident differences were detected; we can mention, for instance, that Menara oils were the richest in terms of HYTY, DEA and EA (VS 5 oils also showed high levels of EA). VS 5 exhibited, in general, the highest levels of TY, Ac Pin, Apig and one of the isomers of Lig Agl (23.6 min). Picholine Marocaine presented high concentrations of desoxyEA, Fer, the second and fourth isomers of Ol Agl (18.5 and 22.5 min, respectively), and one of the isomers of Lig Agl (21.6). In EVOO coming from VS 3 variety, levels of DOA, Lut, D-Lig Agl and the main isomer of Lig Agl were greater than in the oils from other cultivars. Haouzia oils had considerable concentrations of two Ol Agl-isomers (16.9 y 21.5 min (which can be considered as the main isomer of this metabolite), respectively). What can be highlighted from Dahbia oils is that the levels of *p*-Cou were remarkably higher that in the other samples (oscillating between 1.39-1.47 mg/kg, meanwhile the levels in the other oils did not exceed 0.51 mg/kg in any case). This variety was also peculiar regarding its lignans´ pattern, since it was the only one presenting higher amounts of Pin (8.96-9.55 mg/kg) than Ac Pin (4.39-4.96 mg/kg).

Table 2. Quantitative results (mg analyte/kg olive oil sample) achieved by using the LC-ESI-IT MS developed method. Every result is the average of three independent (sample dilution and injection) determinations (n = 3). The results are given by the mean value ± RSD (%).

		VS 3		VS 5		Picholine Marocaine		
Compound	t_r	VS 3-1	VS 3-2	VS 5-1	VS 5-2	PM 1	PM 2	PM 3
HYTY	6.8	3.80 ± 0.19	3.47 ± 0.17	2.23 ± 0.11	10.89 ± 0.38	4.41 ± 0.15	1.69 ± 0.06	1.73 ± 0.06
TY	8.5	14.36 ± 0.72	16.77 ± 0.84	16.62 ± 0.83	21.97 ± 1.09	14.46 ± 0.72	11.90 ± 0.59	12.23 ± 0.61
DEA	10.5	0.19 ± 0.01	0.31 ± 0.02	0.46 ± 0.02	3.90 ± 0.19	1.77 ± 0.09	2.68 ± 0.13	2.75 ± 0.14
p-Cou	11.6	0.19 ± 0.01	0.20 ± 0.01	0.16 ± 0.02	0.15 ± 0.02	0.49 ± 0.02	0.48 ± 0.03	0.45 ± 0.02
DesoxyEA	12.1	6.71 ± 0.34	70.52 ± 2.53	76.57 ± 4.59	5.97 ± 0.36	42.48 ± 2.55	35.86 ± 2.15	34.24 ± 2.05
Fer	12.5	0.12 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.05 ± (<0.01)	0.31 ± 0.01	0.35 ± 0.02	0.33 ± 0.02
EA	14	44.58 ± 1.23	52.70 ± 3.63	57.51 ± 2.88	88.61 ± 3.81	20.45 ± 1.08	22.22 ± 1.33	24.31 ± 1.22
DOA	14.9	88.29 ± 5.11	37.87 ± 1.89	38.25 ± 1.91	62.41 ± 3.12	7.95 ± 0.39	7.25 ± 0.29	6.87 ± 0.27
Lut	16.5	5.86 ± 0.29	5.46 ± 0.27	2.62 ± 0.13	4.92 ± 0.24	1.55 ± 0.08	0.77 ± 0.05	0.69 ± 0.05
Ol Agl isom	16.9	0.93 ± 0.05	1.20 ± 0.06	1.83 ± 0.11	1.23 ± 0.07	1.03 ± 0.06	1.51 ± 0.09	1.47 ± 0.09
Pin	17.3	3.65 ± 0.18	4.82 ± 0.24	4.76 ± 0.22	4.26 ± 0.21	1.62 ± 0.08	1.28 ± 0.05	1.35 ± 0.06
Ac Pin	18	8.42 ± 0.42	9.95 ± 0.50	10.49 ± 0.42	9.14 ± 0.36	6.13 ± 0.27	6.56 ± 0.28	6.25 ± 0.27
Ol Agl isom	18.5	13.56 ± 0.68	4.97 ± 0.25	5.29 ± 0.26	10.07 ± 0.50	19.20 ± 1.07	8.83 ± 0.53	8.99 ± 0.55
D-Lig Agl	19	122.22 ± 6.11	106.97 ± 5.34	21.90 ± 1.09	133.06 ± 8.11	3.01 ± 0.12	4.06 ± 0.20	4.01 ± 0.20
Apig	19.5	0.96 ± 0.05	0.35 ± 0.02	0.60 ± 0.03	0.88 ± 0.04	0.31 ± 0.01	0.28 ± 0.02	0.25 ± 0.01
Lig Agl isom	20	3.47 ± 0.17	3.77 ± 0.19	4.09 ± 0.18	3.51 ± 0.15	1.36 ± 0.06	3.69 ± 0.14	3.75 ± 0.15
Lig Agl isom	21.6	82.16 ± 4.12	40.75 ± 2.04	36.60 ± 1.43	80.54 ± 3.14	85.60 ± 3.34	70.23 ± 3.02	70.33 ± 3.02
Ol Agl	21.5	36.22 ± 1.81	18.53 ± 0.93	32.11 ± 1.60	40.88 ± 2.57	31.23 ± 1.34	25.62 ± 0.84	28.32 ± 0.93
Ol Agl isom	22.5	27.03 ± 1.35	14.43 ± 0.92	17.58 ± 1.09	19.30 ± 1.19	36.33 ± 2.25	22.47 ± 1.05	21.01 ± 0.99
Lig Agl	23.6	119.51 ± 5.98	80.04 ± 3.55	72.01 ± 4.10	124.01 ± 7.07	42.79 ± 2.01	51.15 ± 3.07	49.49 ± 2.97
Lig Agl isom	24	169.07 ± 8.45	91.34 ± 5.48	93.98 ± 5.64	153.62 ± 9.22	143.12 ± 8.57	119.34 ± 7.16	108.83 ± 6.53

Table 2 *(continued)*

		Menara			Haouzia			Dahbia		
Compound	**t_r**	**Men 1**	**Men 2**	**Men 3**	**HZ 1**	**HZ 2**	**HZ 3**	**Dahbia 1**	**Dahbia 2**	**Dahbia 3**
HYTY	6.8	17.36 ± 0.78	13.48 ± 0.60	4.49 ± 0.20	4.94 ± 0.22	6.69 ± 0.30	2.23 ± 0.01	0.09 ± (<0.01)	0.09 ± (<0.01)	0.08 ± 0.003
TY	8.5	18.68 ± 0.93	15.93 ± 0.57	16.22 ± 0.44	11.70 ± 0.42	14.05 ± 0.51	10.01 ± 0.32	2.17 ± 0.08	2.14 ± 0.08	2.03 ± 0.06
DEA	10.5	20.05 ± 0.80	17.53 ± 1.14	25.65 ± 0.75	1.32 ± 0.09	11.91 ± 0.77	1.01 ± 0.06	0.10 ± (<0.01)	0.11 ± 0.01	0.11 ± 0.01
p-Cou	11.6	0.45 ± 0.02	0.51 ± 0.02	0.25 ± 0.01	0.42 ± 0.02	0.39 ± 0.02	0.41 ± 0.01	1.47 ± 0.06	1.41 ± 0.04	1.39 ± 0.05
DesoxyEA	12.1	12.75 ± 0.77	11.57 ± 0.69	13.05 ± 0.71	19.75 ± 1.18	16.12 ± 0.64	15.99 ± 0.55	2.16 ± 0.09	1.67 ± 0.06	2.00 ± 0.05
Fer	12.5	0.31 ± 0.02	0.15 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.15 ± 0.02	0.19 ± 0.01	0.15 ± (<0.01)	0.14 ± 0.01	0.13 ± 0.01
EA	14	76.85 ± 4.61	74.26 ± 2.97	62.45 ± 3.61	61.99 ± 2.48	51.74 ± 2.07	43.37 ± 2.00	26.93 ± 1.08	32.26 ± 1.29	30.66 ± 1.09
DOA	14.9	18.70 ± 0.75	20.64 ± 0.62	6.33 ± 0.25	16.86 ± 0.51	14.09 ± 0.42	13.88 ± 0.54	11.63 ± 0.35	13.97 ± 0.42	12.32 ± 0.39
Lut	16.5	1.37 ± 0.08	1.39 ± 0.08	1.40 ± 0.07	2.29 ± 0.14	1.27 ± 0.08	1.11 ± 0.09	2.28 ± 0.14	2.38 ± 0.14	2.25 ± 0.20
Ol Agl isom	16.9	0.94 ± 0.06	0.92 ± 0.06	0.31 ± 0.01	2.58 ± 0.15	1.53 ± 0.07	1.33 ± 0.04	0.75 ± 0.03	0.49 ± 0.02	0.62 ± 0.02
Pin	17.3	1.68 ± 0.07	1.63 ± 0.08	1.58 ± 0.06	1.28 ± 0.06	1.63 ± 0.07	1.75 ± 0.06	9.55 ± 0.41	9.29 ± 0.40	8.96 ± 0.35
Ac Pin	18	5.25 ± 0.23	4.72 ± 0.20	4.65 ± 0.22	6.21 ± 0.27	5.95 ± 0.28	6.08 ± 0.25	4.96 ± 0.23	4.45 ± 0.21	4.39 ± 0.31
Ol Agl isom	18.5	7.89 ± 0.48	7.02 ± 0.43	1.54 ± 0.05	14.61 ± 0.89	10.34 ± 0.63	10.43 ± 0.64	1.75 ± 0.11	2.54 ± 0.16	2.10 ± 0.29
D-Lig Agl	19	34.30 ± 1.54	49.47 ± 2.08	11.66 ± 0.51	8.94 ± 0.38	6.18 ± 0.26	9.25 ± 0.40	53.55 ± 2.25	40.61 ± 3.19	49.77 ± 3.11
Apig	19.5	0.61 ± 0.03	0.63 ± 0.02	0.75 ± 0.04	0.38 ± 0.02	0.40 ± 0.02	0.41 ± 0.02	0.36 ± 0.02	0.37 ± (<0.01)	0.31 ± 0.01
Lig Agl isom	20	1.68 ± 0.06	0.43 ± 0.02	1.01 ± 0.03	1.15 ± 0.09	1.12 ± 0.04	1.23 ± 0.05	1.04 ± 0.04	1.28 ± 0.05	1.13 ± 0.10
Lig Agl isom	21.6	65.83 ± 2.83	67.77 ± 2.91	30.21 ± 1.75	79.25 ± 3.41	68.14 ± 3.95	66.77 ± 4.02	33.07 ± 1.92	35.68 ± 2.07	34.45 ± 2.54
Ol Agl	21.5	38.11 ± 2.02	28.55 ± 1.51	12.33 ± 0.65	49.86 ± 2.64	40.85 ± 2.16	32.55 ± 1.99	17.57 ± 0.93	15.63 ± 0.82	16.76 ± 0.99
Ol Agl isom	22.5	21.02 ± 0.98	13.46 ± 0.63	5.38 ± 0.25	32.85 ± 1.54	26.12 ± 1.23	21.12 ± 1.01	11.09 ± 0.52	10.11 ± 0.47	10.65 ± 0.33
Lig Agl	23.6	97.35 ± 5.84	81.86 ± 4.50	61.28 ± 3.67	63.13 ± 3.47	57.75 ± 3.12	58.00 ± 2.88	39.12 ± 2.11	36.36 ± 1.96	39.54 ± 1.89
Lig Agl isom	24	142.99 ± 7.29	133.90 ± 6.83	63.23 ± 4.01	128.97 ± .658	123.01 ± 7.50	131.32 ± 8.25	66.96 ± 4.08	72.74 ± 4.44	68.74 ± 3.55

Abbreviations used within the table (alphabetical order): **Ac Pin**, (+)-1-acetoxypinoresinol; **Apig**, apigenin; **DEA**, dialdehydic form of decarboxymethyl elenolic acid; **D-Lig Agl**, dialdehydic form of decarboxymethyl ligstroside aglycone; **DOA**, dialdehydic form of decarboxymethyl oleuropein aglycone; **EA**, elenolic acid; **HYTY**, hydroxytyrosol; **isom,** Isomer; **Lig Agl**, ligstroside aglycon; **Lut**, luteolin; **Ol Agl**, oleuropein aglycon; **Pin**, (+)-pinoresinol; **TY**, tyrosol. (to facilitate the revision process; in subsequent versions of this paper, abbreviations will just appear in the first page of the manuscript)

-HYTY, TY, Pin, Lut, Apig, and *p*-Cou were quantified in terms of their commercial pure standards. Ac Pin was quantified in terms of Pin. As far as secoiridoids are concerned, Ol Agl-derivatives were quantified in terms of HYTY, and Lig Agl-derivatives were quantified in terms of TY. -Ol Agl (21.5 min) and Lig Agl (23.6 min) are underlined since they can be considered as the most extensively determined isomers (or main peaks with m/z 377 and 361, respectively).

Principal Component Analysis (PCA) was applied to evaluate the whole structure of the data set and highlight general trends in the phenolic profiles of the samples under evaluation. Fig. 4 shows the score and loading plots of PC1 vs. PC2 for the matrix composed by 21 variables (the number of phenolic compounds that were quantified in the EVOO samples) and 48 samples (16 EVOO analyzed in triplicate).

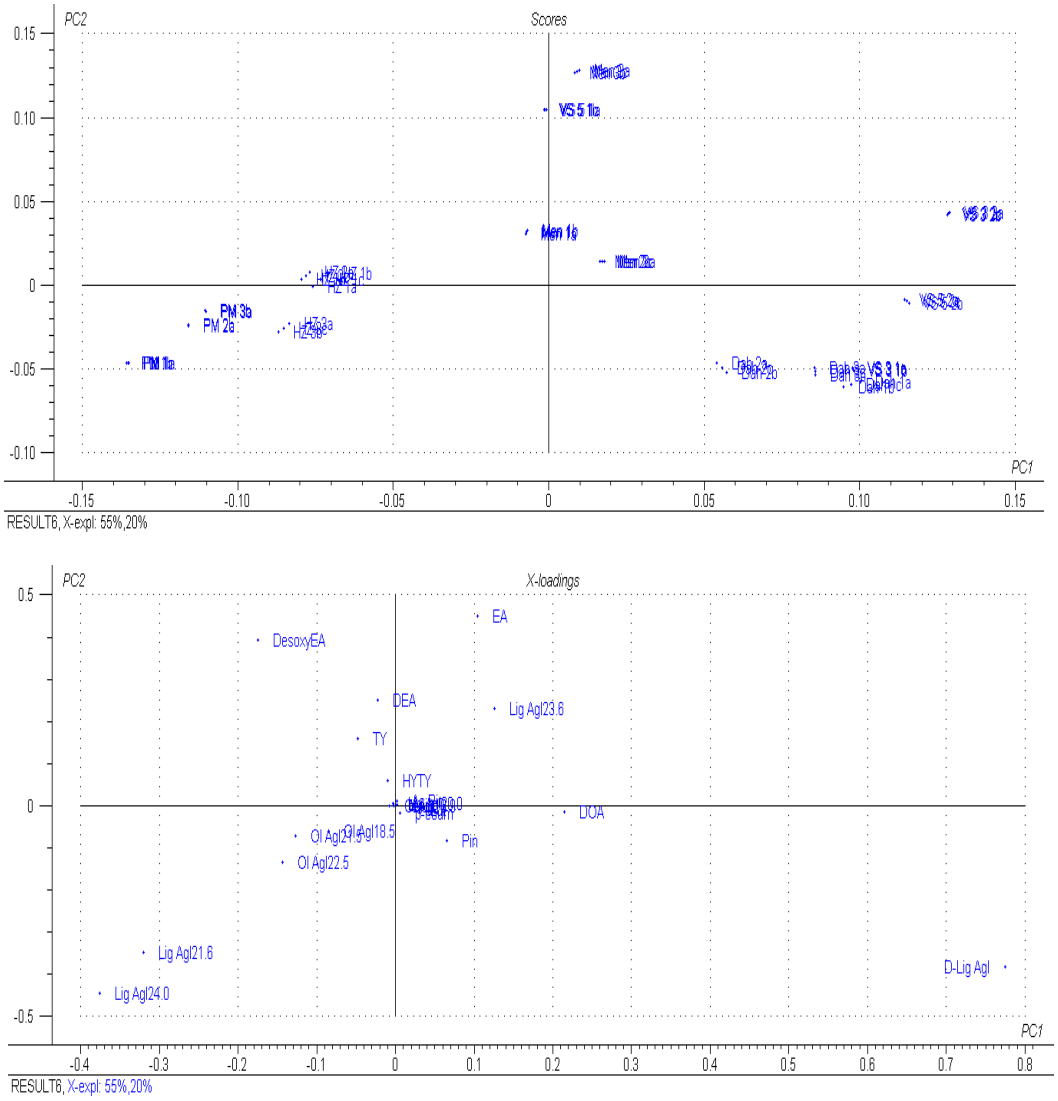


Figure 4. Score and loading plots of PCA modelling of LC-MS data considering the individual concentration of each quantified phenolic compound. **Identification legends of varieties**: **Dah**: Dahbia; **Hz**: Haouzia; **Men**: Menara; **PM**: Picholine Marocaine. **VS 3** and **VS 5** are identified with the complete name of the variety. **Identification legends of compounds**: The abbreviations regarding the identity of the different compounds have been explained in other parts of the manuscript. In the case of secoiridoid isomers, the different analytes´ names include the retention time.

The first two PCs explained 75.40 % of total variance in raw data, whereas PC3 and PC4 accounted for 16.55 % and 3.39 %, respectively. All the possible combinations of PCs

were studied; however, the figure only shows PC2 vs. PC1, since they were those which provided the best separation. Picholine Marocaine samples were properly separated from the rest, although were laying quite close within the graph to Haouzia oils, fact which can be partially explained considering their concentration in terms of Lig Agl (isomers at 21.6 and 24.0 min) and Ol Agl isomers (at 18.5, 21.5 and 22.5 min). The two samples from VS 5 cultivar were substantially separated from each other, and the same was observed for VS 3. Indeed, the replicates of one of the VS 5 sample (VS 5-1) were very close to Menara oils (since they showed similar levels of EA, simple phenols and Lig Agl isomer at 23.6 min); the replicates of VS 5-2 were in between VS 3 samples and relatively close to Dahbia oils. The latter can be related to its high level of D-Lig Agl.

Further experiments are needed to get a more comprehensive insight into the complete phenolic patter of these varieties and their main differences; that was logically not the aim of the current study. Our purpose was developing an accurate and reliable (but simple) LC-MS methodology which could be subsequently applied to analyze a higher number of samples. To the best of our knowledge, this is the first method which allows the determination of such a high number of compounds within a single run by using the direct injection approach.

4. Conclusions

Food is not considered as mere source of energy any longer and consumers are very aware about its importance for health; that explains the need of comprehensive characterization of the composition of what we eat. Even though EVOO phenols have been studied for decades, extensive research is continuously carried out pursuing the simplest but very powerful and reliable analytical methodology. Herewith, the analysis of the phenolic fraction of EVOO (dissolved in acetone) without the need of carrying out any previous extraction step has been done for the first time using LC coupled to ESI-MS. The possibility of implementing direct injection of olive oil into the LC (after a simple dilution) could be one of the greatest advantages of this method, since it could prevent partial and selective recovery of some phenolic compounds after the extraction or their possible partial oxidation during the sample preparation. The reliability of the quantification was demonstrated, as possible matrix effects were thoroughly evaluated and

the method was fully validated; afterwards, it was applied for the analysis of 16 samples coming from 6 different varieties and the most remarkable differences were underlined.

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SECTION

3

"A proposal for a methodological approach to the implementation of virgin olive oil geographical indication labels: An application to Meknès Moroccan region"

Chapter 11

Contribution to the establishment of a protected designation of origin for Meknès virgin olive oil: a 4-years study of its typicality

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Abstract

Geographical indications (GIs) implementation is one of the most prominent differentiation strategies used nowadays in olive oil market. However, the proliferation of these labels causes debate and controversy, especially about their usefulness and effectiveness, as well as the suitability of some protected areas to acquire them. The current chapter firstly introduces the global situation of using GI labels in olive oil market; after exploring the concept of GI, the chapter gives a four-stage methodological approach to examine the potential of a Moroccan olive growing area -"Meknès region"- to acquire a GI label and ensure its effectiveness. Thus, based on this approach, Meknès region territorial dimensions were defined, the typicality of its olive oil was characterized, a general scheme for the GI recognition was proposed, and the adopted strategy to enhance the meaning of this label on domestic, national and international markets was highlighted. The main findings of this study profoundly justify the suitability of Meknès region to protect its olive oil with a GI label. The applied methodological approach could be an appropriate tool to support the implementation of GI labels inside and outside Morocco.

Keywords: olive oil, geographical indication, differentiation strategy, Meknès region.

1. Introduction

In the Mediterranean Basin area, olive oil production is both a science and a blend of Mediterranean populations' creativity and innovation, but above all it is a lifestyle and a unique and ancestral tradition passed down through history from one generation to another. This historical interaction between Mediterranean populations, environment and olive tree cultivation has created a specific cultural identity, which is crucial to understand the emergence and spread of olive tree cultivation and oil production all over the world [1]. It is also very important to mention the fundamental socio-economic role that olive oil production and trade have played in different parts of the Mediterranean area throughout history.

In many Mediterranean regions, nowadays, an integral form of revitalization, enhancement and appreciation of the historical legacy of olive oil production is the establishment of GIs. In these regions, GIs are often perceived as valuable tools that allow

not only the safeguard of their cultural identity, but also to gain market benefits and profitability and competitiveness in a growing olive oil globalized market [2].

GIs are names of places or regions used to brand goods, in this case olive oil, with a distinct geographical connotation, being the main feature of geographically-differentiated olive oils the fact that their specific quality attributes are considered to be inherently linked to (or determined by) their geographical origin's characteristics. In this way, olive oil companies, besides using private trademarks, have an excellent opportunity to promote the uniqueness of their products through the use of these labels. Thereby, olive oil producers generally demonstrate the quality of this product by regulatory standardized parameters (mainly content of free fatty acids, peroxide value, ultraviolet specific extinction coefficients and sensory characteristics); in contrast, GIs broaden the olive oil quality concept to added-value attributes such as provenance, local know-how, cultural traditions and distinctive quality, which taken as a whole, helps to differentiate the origin-labeled olive oil among similar products. By adopting these labels, added-value, premium price and a competitive positioning on either traditional or emerging olive oil markets are, obviously, expected. It is also assumed that they can provide rural areas (where they are established) with additional social and economic benefits [3,4]. Given their importance, an unprecedented recognition of these labels was recorded over the last years in many parts of the Mediterranean area. According to the information published by different entities [5-9], it is possible to state that till 2015 about 123 olive growing regions have registered their olive oil production under GI schemes; all of them are located in Mediterranean countries, with Italy (43 GIs), Greece (29 GIs), and Spain (28 GIs) at the highest positions of the list.

However, because of the rapid proliferation of GIs in the olive oil sector, their usefulness and effectiveness are currently lively debated topics. Within this context, some very successful stories of olive oil GIs can be told (for example: the Spanish Baena olive oil [10], French GIs olive oils [11] and Italian Toscana olive oil [12], that have achieved a considerably higher price (premium price) than other no GI labeled olive oils). Nevertheless, these prosperous cases represent a limited number of examples if compared with the high number of existing GI olive oils. In this respect, some authors state a lack of reputation/notoriety of several of these olive oils produced under GI schemes, which

would have negative consequences on the overall value of the olive oil GI system [13,14]. Therefore, the main questions that arise are: WHEN implementing an olive oil GI is appropriate? and HOW to benefit from this? As properly pointed out by Aubard (2012), GIs should be adopted as response to an identified (product chain) need and must be designed pragmatically and realistically, so as to be useful to the business. Furthermore, the author brings to mind the false idea that many producers have about GIs, thinking that once a GI is recognized, the markets will just be more accessible for it. This is not true; any procedure to increase the value of products, such as GI, necessary requires a smart and properly planned management and considerable marketing efforts [15].

Morocco has traditionally been a land of olive tree cultivation and olive oil production, and it currently stands out as one of the most important olive oil producing countries, ranking sixth worldwide [16]. In this country, the olive oil sector is increasingly being viewed as an economic and social development engine of various regions. Under that perspective, olive oil sector modernization, yield increasing, and olive oil quality enhancement, have been -and remain- a priority in Moroccan agricultural policy [17]. In this respect, promoting the olive oil quality linked to the geographical origin through the establishment of GIs is the cornerstone of the current Moroccan olive oil quality policy. So, an optimistic atmosphere has been created considering the potential of using GI strategies to strengthen the quality and competitiveness of the produced olive oil in various Moroccan regions. Protected designations of origin (PDO), protected geographical indications (PGI) and traditional specialities guaranteed (TSG) are the instruments created for this purpose [18]. Indeed, till 2016, 2 PDOs and 3PGIs have been registered, while some others are in the scrutiny process.

Meknès region is one of these Moroccan olive growing areas that aim to provide their olive oil production with a GI label and, particularly, a PDO. Geographical names have been used for centuries to distinguish olive fruits and olive oil produced in this region, since its name "Meknassa Zeytona" (meaning: Meknès, land of olive tree) was closely linked to olive tree cultivation and oil production. Currently, there is a real willingness to formalize this linkage by means of acquiring a PDO label.

Therefore, with the purpose to contribute to the implementation of this GI label for Meknès olive oil, our research group carried out a multidisciplinary and pluri-annual

study, bearing in mind other Mediterranean experiences in this field, and obviously considering all the assets and constraints abovementioned that have led to the current scenario in which some olive oil GIs have experienced exceptional success while others have failed. In the mentioned study, we basically tackled the following broad questions: is setting up a PDO in Meknès region appropriate? and what should it be done to assure that this label will be long-lastingly effective and not only one more without any interest and benefit? In others words, how can Meknès region position itself to benefit from an olive oil PDO?

Thus, in this chapter, we are going to present and discuss the methodological approach adopted and the main results obtained from that experience.

2. Setting up a Meknès olive oil PDO: methodological approach and main findings

The developed approach in this study was based on a comprehensive literature review of what drives to successful processes of implementation and valorization of different GIs. Every success factor identified in literature were listed and adapted during our research, with additional insights gained over the study period.

Thus, based on a literature review [19-23], it was found that no universal model exists for a successful GI labels implementation and valorization; indeed, there is a wide diversity of practices for implementing registered GIs all over the world. In addition, literature clearly points out that the process of determining the suitability of a product in a given region to pursue a GI label should be scientifically grounded, or built on a robust methodology taking into account the current knowledge in the field, as well as able to stimulate the participation of all the product supply chain actors in order to integrate different points of view and interests. Indeed, the collective dimension of the concept of GI necessarily requires the efforts and skills of the different producers and/or processors to build a common vision concerning the quality of the product and the specific characteristics of its production process.

Moreover, from the very beginning, our multidisciplinary team was convinced about the fact that the implication of the future Meknès olive oil PDO stakeholders was strongly

encouraged and could represent the key to success. We refer to the members of the association "Union pour le Développement de l'Olivier de Meknès (UDOM)", including farmers, olive oil producers and processors.

The scientific approach was designed around the basic definition of a PDO. The Lisbon Agreement for the Protection of Appellations of Origin and their International Registration (Lisbon Agreement) adopted in 1958, as a special Agreement under Article 19 of the Paris Convention for the Protection of Industrial Property [24], defines the PDO, also called "geographical designation of origin" or "appellations of origin", as: "*the geographical denomination of a country, region, or locality, which serves to designate a product originating therein, the quality or characteristics of which are due exclusively or essentially to the geographical environment, including natural and human factors*". Two features are noteworthy in this definition:

- The first is related to the fact that a PDO consists on a denomination that identifies a geographical entity, including natural and human factors. In the GI field, this is commonly designed by the term "Terroir".
- The second is the existence of a unique bond between the quality, characteristics and reputation of the PDO product and the "Terroir" where is produced. The term "Typicality" is the one employed to highlight this concept.

Therefore, only those products that fulfil the condition of exhibiting typicality linked to a delimited terroir can be considered as suitable to be certified under a PDO scheme.

A methodological approach for the implementation of a PDO label should certainly be built on the delimitation of the terroir, the definition of its factors, and on the characterization of the typicality of the product. That is exactly what was made within this study.

2.1. A first step towards Meknès virgin olive oil PDO: "terroir" dimensions construction

Terroir is a derivative of the French word for soil or land "terre", with no exact English counterpart or equivalent nomeclature, that can be conceptualized in several different

disciplines [25,26]. In the subject of the GI products, the concept of terroir is practically the base of any GI system, and therefore, many researchers have worked on the definition of this concept and the determination of its components [27]. Thus, various definitions are nowadays available, but herewith we quote the one most widely used [19]. Terroir: "*is a delimited geographical space, where a human community has constructed over the course of history a collective intellectual or tacit production know-how, based on a system of interactions between a physical and biological milieu, and a set of human factors, in which the socio-technical trajectories put into play reveal an originality, confer a typicality and engender a reputation for a product that originates in that terroir*" [28,29]. Such definition illustrates the different sides of the concept of terroir that goes far beyond the physical environment in which the production is made, as it also includes the social and cultural features emerging from the human intervention. What comes out from this definition is that an efficient way of approaching terroir should basically imply the determination of its three dimensions:

- ***Geographical dimension:*** the natural environment (mainly climate, topography, geology and soil);
- ***Social dimension:*** the local know-how, traditions and cultural aspects in relation to the production, trade and use of the product; and
- ***Technical dimension:*** the agronomical and technological techniques used in the elaboration of the product.

Overall, it can be said that it is the combination and the strong interaction among these three dimensions, which is reflected on the special quality and particular characteristics of a GI product.

2.1.1. Meknès terroir geographical dimension

Meknès region is located in the North-Center of Morocco (33 ° 53 '36 N, 5 ° 32' 50 W), covering an area of approximately 400,700 ha, including a total olive growing area of 43,000 ha. It is a region with dramatic topographic contrasts and its landscape has a complex geomorphology (Figure 1). Climatically, Meknès terroir is situated in a Mediterranean sub-humid to semi-arid climatic zone, with cool winter and warm dry summer. Rainfall is mostly uniformly distributed over the year. On average, this terroir annually receives 400-600 mm of rainfall, which is favourable for various crops, including

olive tree. Meknès soils are constituted by fluvisols, regosols, lithosols, rendzinas, yermosols, xerosols, vertisols, kastanozems, chernozems, phaezems, luvisols, and acrisols, varying in thickness, depending on the depth of the substrate and the old and recent manifestations of anthropogenic erosion and runoff.

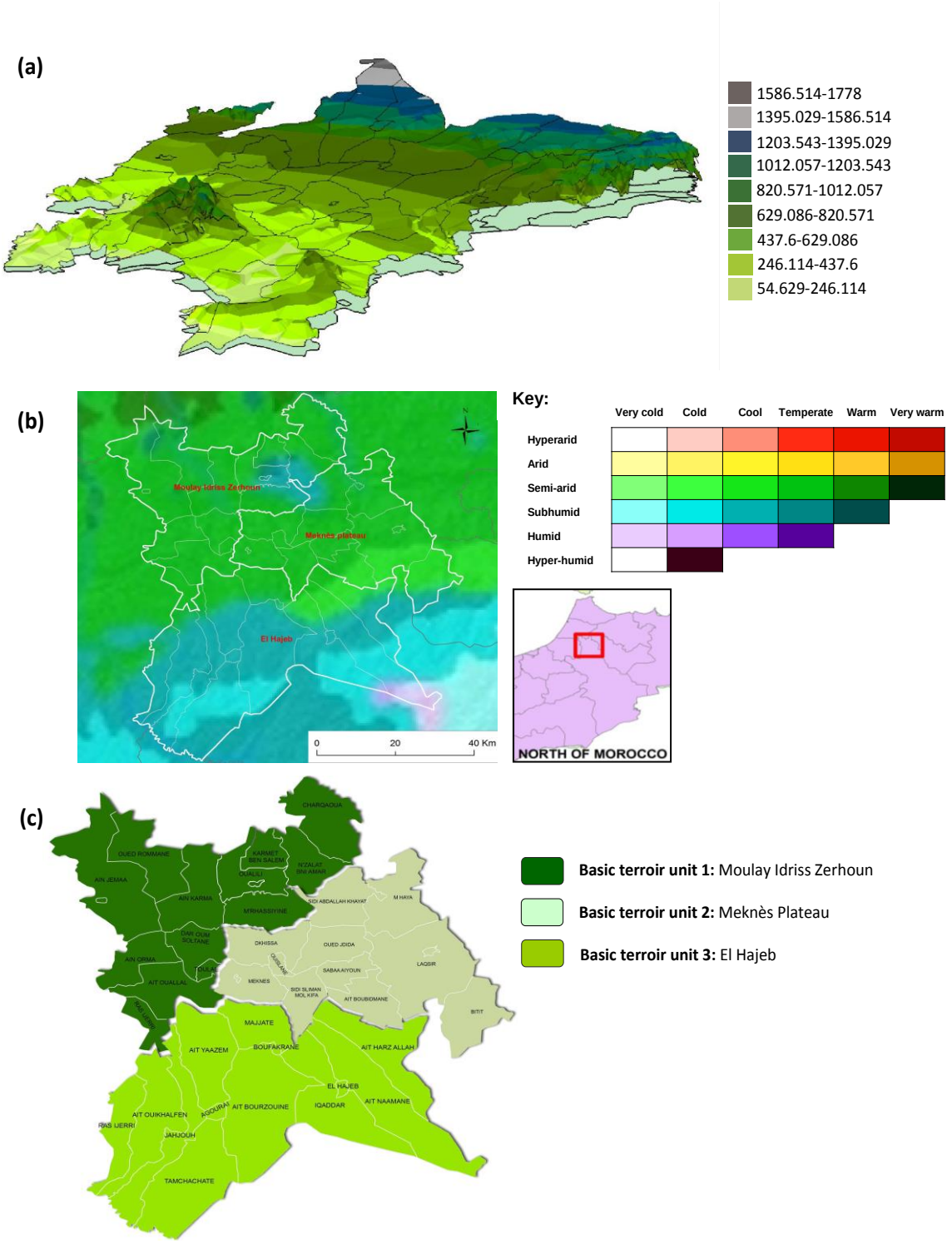

Figure 1. Meknès terroir geographical dimension construction, (a) altitude map ; (b) climate map ; (c) Meknès terroir delimitation showing the localization of the three basic terroir units identified.

The first step in the characterization of the geographical dimension of Meknès terroir has revealed a relatively complex variability of natural environmental factors in this area, which can affect the homogeneity of qualitative and compositional profile of the olive oils obtained in this terroir. Therefore, the identification of basic terroir units (homogenous geographical areas, from the environmental point of view) inside this region was the following step. The model proposed by Morlat [30] was used for that. Practically, each unit is defined by three associated components: a geological component, a pedoclimatic element and a landscape component. Therefore, the workflow of this methodology involved three main tasks:

- Delimitation, characterization and cartography of Meknès terroir.
- Characterization of the landscape and pedoclimatic conditions in this terroir.
- A multifactorial analysis which integrates all the results obtained from the first two activities and allows the determination of the basic terroir units.

Thus, by applying this methodology, it was possible to identify three basic terroir units in Meknès region: Moulay Idriss Zerhoun, Meknès Plateau and El Hajeb (Figure 1).

2.1.2. Social dimension of Meknès terroir

Once the geographical dimension of Meknès terroir was defined, the study went on with an exploratory and qualitative approach, examining the main material and intangible cultural heritage of olive tree cultivation and oil production in this region. To this end, a careful investigation of ancient documents, archive maps and books that report the history of olive tree and oil production in this territory was made. Gastronomy habits and ancient practices and uses of this product throughout the history of Meknès were also documented. Finally, historical structures and archeological evidences which testify the long-standing olive growing and oil production practices in this zone were explored and inventoried.

It was clearly demonstrated that Meknès region constitutes the cradle of olive tree cultivation and oil production in Morocco, since the oldest evidence of these practices in this country (dating for the roman era, about 2,000 years ago) are found in this area. Significant examples corroborating it are the millenary olive trees found in the Moulay Idriss Zerhoun area and the archaeological site of Volubilis, where the ruins of a Roman

settlement stand and numerous ancient olive mills showing mill stones, for crushing the olives, olive press systems and the tanks for collecting and separating the oil can be visited (Figure 2).

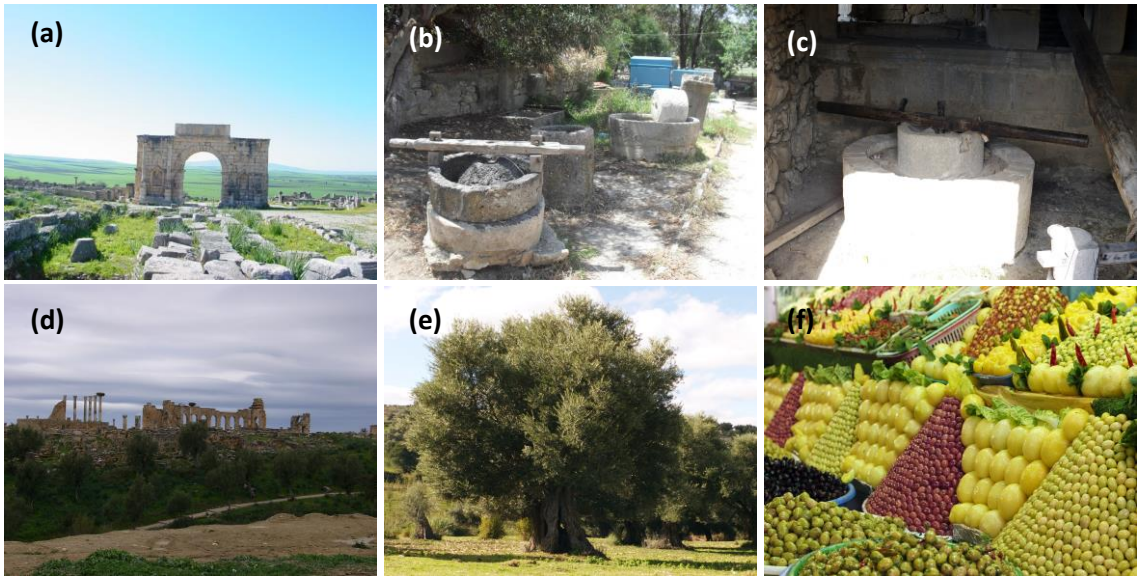

Figure 2. Illustrations of the historical legacy of olive tree and olive oil in Meknès region, (a), (b) and (c) ancient Roman olive mills in the archaeological site Volubilis; (d) monumental olive trees in Volubilis; (e) millenary olive trees found in the Moulay Idriss Zerhoun area; and (f) olive market in the monumental Place el-Hedim in Meknès.

2.1.3. Technical dimension of Meknès terroir

At this point, we analyzed the olive-growing and olive oil processing sector characteristics of Meknès terroir. Thus, the work was firstly based on the study of data coming from the national and local institutions in charge of agriculture development, and, then, several surveys were performed among farmers and olive oil processors.

At this level, a first category of surveys focusing on olive-growing farms were conducted to characterize the practiced agronomical techniques for the management of olive orchards (mainly planted olive varieties, plantation density, soil management, irrigation, fertilization, pruning, disease management and harvesting), and also the productivity of the olive orchard. The collected data were analyzed in depth and allowed to distinguish four main different olive growing cultivation systems: traditional rainfed, intensive rainfed, intensive irrigated and super-intensive system (Figure 3), with 93.4% of Meknès olive orchard planted using 'Picholine Marocaine' cultivar.

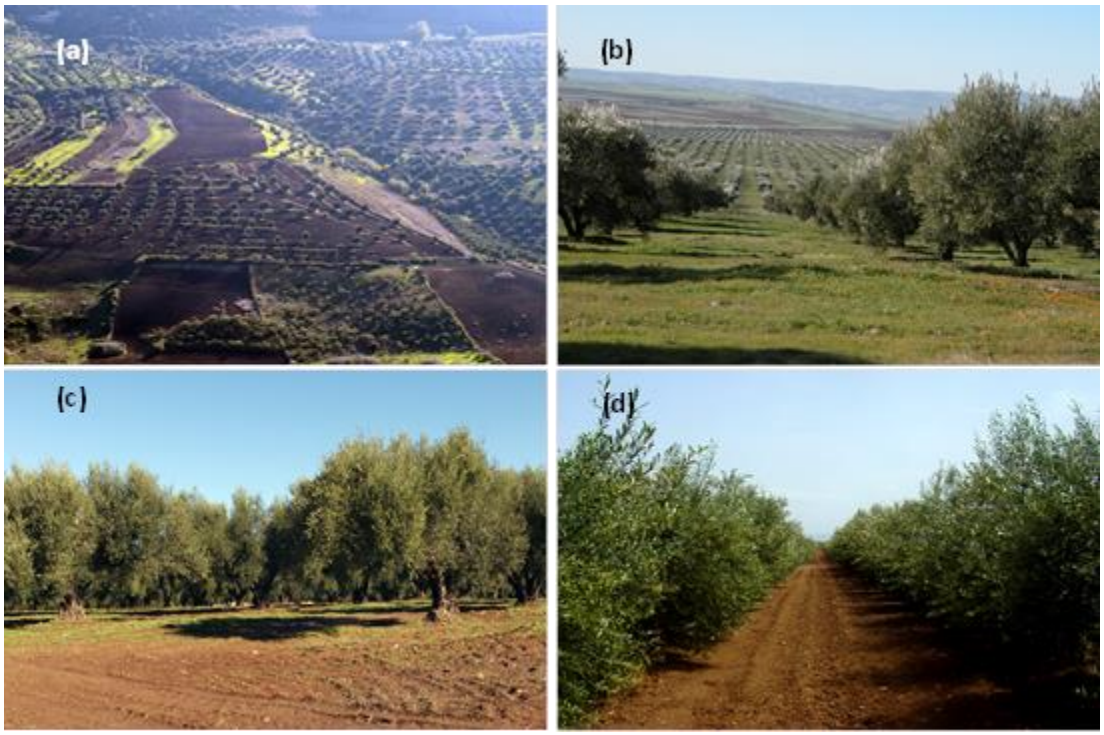

Figure 3. Olive growing cultivation systems in Meknès, (a) traditional rainfed; (b) intensive rainfed; (c) intensive irrigated; and (d) super-intensive system.

A second type of surveys for Meknès olive oil producers was carried out focusing on olive oil mills. The collected data about provenance of olive fruits, olive oil processing systems, olive oil filtration, and conservation and bottling allowed us to obtain very valuable information regarding the used olive oil making processes in Meknès terroir. The collected data were compiled in a geo-database and integrated with digital maps of Meknès region (Figure 4). As can be seen in this figure, a total of 245 olive oil mills were listed; 102 of them were traditional processing mills, so-called massars, 91 semi-moderns oil mills, and 52 moderns oil mills (28 two-phase and 24 three-phase). It should be noted however, that even though the number of traditional and semi-traditional mills in Meknès region is considerably higher than the number of modern mills, the vast majority of olive oil produced is made by modern mills, showing a mean processing capacity of 3,533.6 tons/day. Furthermore, all the olive oils commercialized in package are coming from these mills.

Once this step was brought to completion, the first phase of the study was accomplished, and, therefore, the territorial dimensions of Meknès region had been characterized. The results obtained from this first stage of the study logically conditioned the selected methodological approach to carry out the second stage of the project, in particular,

regarding the selection and collection of olive oil samples for the determination of the typicality of Meknès olive oil.

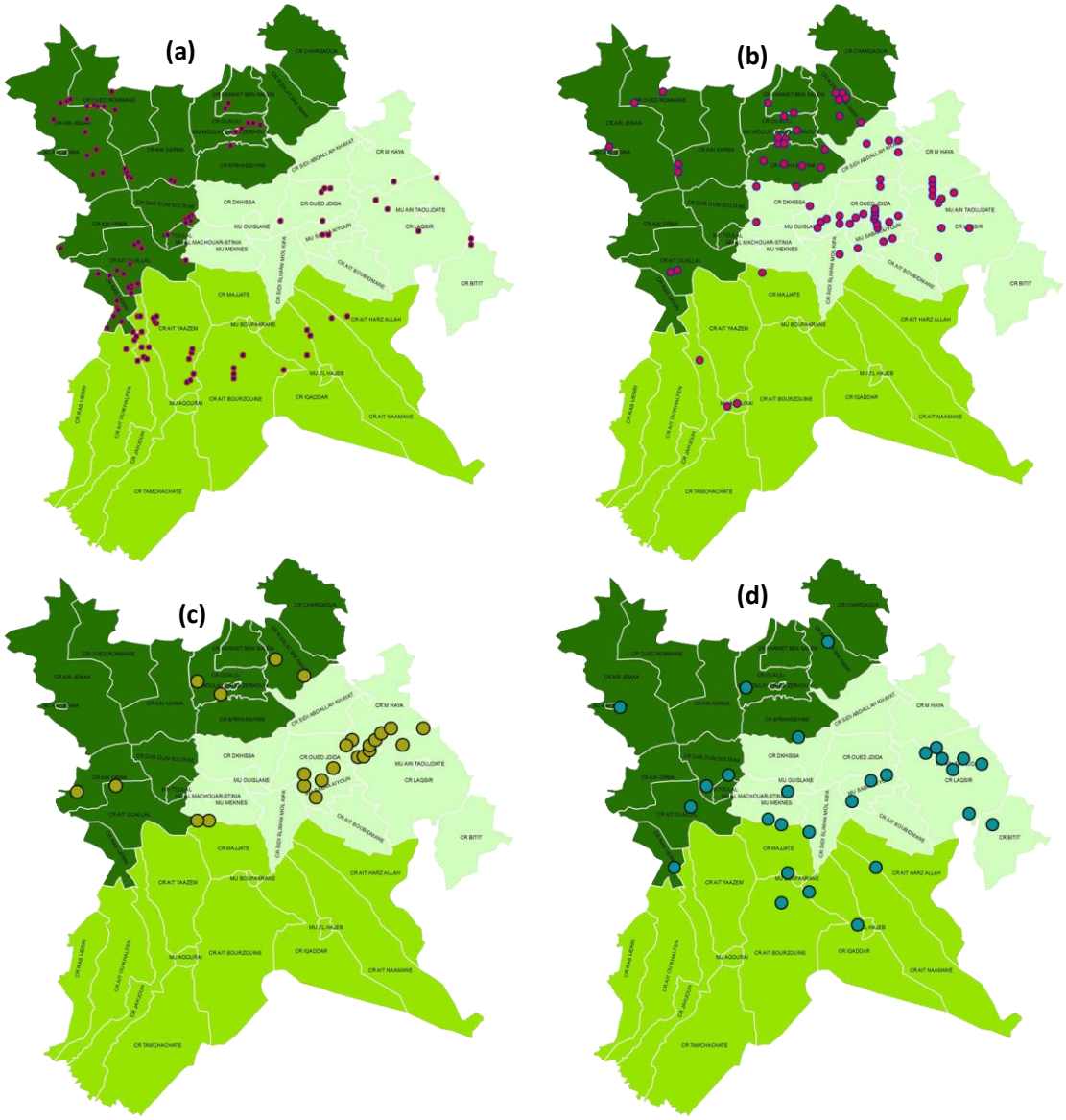

Figure 4. Geographical localization maps of Meknès olive oil mills, (a) traditional; (b) semi-modern; (c) three-phase; (d) two-phase.

2.2. Characterization of typicality of Meknès virgin olive oil

2.2.1. The typicality concept: definition, dimensions and attributes

According to the definition of the concept of typicality of an agricultural product this term means: *"the property of belonging to a particular type defined and recognized as such by a specific human group, the different members of which have acquired areas of knowledge or know-how relative to their role in the production process. Know-how, then, as regards the setting up of the*

process, and the making, testing and tasting of the product. Typicality must not be mistaken for conformity with a norm; it allows for internal variations within the type. Among the diverse manifestations of typicality, which is linked to the land (notion of terroir) is a particular construction that aims to materialize the effect of the land on a given product" [28]. Inspired by this definition, various authors describe as mandatory the determination of a set of **properties of belonging** (level of representativeness of an item in a category) and **distinction** (properties that make possible to differentiate, identify, and recognize the product among others similar) for the ascertainment of the typically of a product [31,32]. These properties are based on the know-how distributed among numerous participants, including producers of raw materials used, processors, regulators and connoisseur consumers.

2.2.2. Typicality of Meknès virgin olive oil: findings of the main studies

Based on the previous definition, the evaluation of the typicality of Meknès olive oil was performed by determining:

- ***Its properties of belonging:*** by assessing the qualitative and compositional profile of olive oils produced within Meknès region. Considering the high diversity of the environmental factors in the Meknès terroir, it was expected that olive oils produced in this area would show some differences among them, but sharing certain common characteristics. Indeed, the determination of these common features could allow defining the "usual type" of olive oils produced in this region. Moreover, as the construction stage of terroir dimensions highlighted the existence of three basic terroir units inside this region, the matter about the homogeneity of the qualitative and compositional profile of oils produced in the whole Meknès region was decisive. Indeed, if this "usual profile" would differ among the three basic terroir units identified, there will be no way to consider the whole Meknès region as an eligible area to be certified by a PDO scheme. In that case, the study should be then redirected towards considering each one of the three basic terroir units identified as possible terroir.
- ***Its properties of distinction:*** which involves the characterization and identification of qualitative and compositional properties of Meknès olive oils that distinguish them among others oils produced outside this terroir. In other words, it is a

question of identifying those elements responsible of the uniqueness and singularity of the oils produced in Meknès region.

Thus, for the determination of Meknès olive oil properties of belonging, two pluri-annual studies were carried out.

The first work aimed the characterization of the physicochemical and sensorial quality and compositional profile of 298 olive oil samples from 'Picholine marocaine' cultivar, obtained from 12 industrial olive oil mills, located in the three identified basic terroir units. Both variations induced by crop season and those expected between the three basic terroir units were assessed over four consecutive crop seasons (from 2010 to 2013). The results obtained reveal that, besides an interannual variation, olive oils produced in Meknès region are characterized by high physicochemical and sensory quality, as well as a homogeneous composition regardless of the production sub-area. More details can be found in [33].

In the second study [34], particular attention was paid to the characterization of phenolic compounds from oils produced in Meknès region. These compounds are of unquestionable importance since they have a noticeable influence on some olive oil sensory characteristics and biological properties [35,36]. These reasons made us going for their chracterization in Meknès olive oils. The study was conducted over three consecutive crop seasons (2011, 2012 and 2013) on 142 'Picholine Marocaine' olive oil samples obtained by extracting olive fruits collected from orchards located on the three Meknès basic terroir units. A liquid chromatography-mass spectrometry platform was used to this purpose. Results showed that the variation of the content of phenolic compounds was mainly related to the crop season, which proves, once again, the homogenous character of the profile of olive oils produced in the entire Meknès terroir.

With regard to the properties of distinction of Meknès olive oils, various phenolic compounds profiling studies combining compositional data and chemometric treatments were performed in an attempt to discriminate olive oils produced in Meknès terroir from others produced outside this region. Within this context, the potential of merging quality and chemical profiles data and multivariate statistical analysis was tested on 279 olive oil samples (among which 69 were from Meknès region and the others were collected from 6 North Moroccan regions). The obtained chemometric models were able to correctly

discriminate Meknès olive oils from the rest, with rate of 100% and 91.30% in recognition and prediction abilities, respectively [37].

In another work carried out on the same samples set, the triacylglycerols fraction was determined and chemometric data analysis was applied to build geographical discriminant models for the differentiation of the studied samples. When considering Meknès terroir samples, rates of 93.48% in both classification and cross-validation and 82.61%, in external validation, were obtained [38].

In addition, a phenolic compounds profiling approach was applied to discriminate Meknès olive oils (36 samples) from those produced in 6 North Moroccan regions (120 samples) [39]. The developed methodology (using liquid chromatography coupled to mass spectrometry and a discriminant data analysis treatment) allowed differentiating 100% of Meknès samples in recognition and 91.67% of them in prediction. Besides, a similar approach was used to discriminate Meknès olive oil from those coming from two existing olive oil GIs in Morocco (PGI Ouazzane and PDO Tyout-Chiadma). In this case, 136 commercial extra virgin olive oil samples were collected and analyzed. The results were very satisfactory, sincethe 57 samples belonging to Meknès terroir were 100% correctly classified and 94.70% accurately predicted.

Moreover, the potential of volatile compounds (determined by gas chromatography coupled to mass spectrometry) combined to chemometric data analysis was also tested to distinguish Meknès olive oils from other olive oil samples produced in diverse Moroccan zones. Among the 92 samples analyzed, very good rates of classification (100%) and prediction (90.48%) were obtained for the 21 studied samples from Meknès region [40].

In general, the good discriminant rates achieved within all the above-mentioned studies, as well as the identified geographical markers demonstrate, from our point of view, the uniqueness and specificity of the olive oil produced in Meknès region.

We can conclude this section stating that, in light of the results obtained by the mentioned studies, in which about 967 samples were analyzed, the typicality of Meknès olive oil was properly characterized, defining, at the same time, an average qualitative and compositional profile of these oils and their distinctive characteristics when compared to

other Moroccan olive oils. The information obtained within these studies was (and is being) of great practical use in redacting the specifications report.

3. General scheme for the recognition of Meknès virgin olive oil PDO

Once the potential of Meknès olive oil to be certified under a PDO scheme was verified, the next step was the elaboration of a general scheme to be applied for registration in front of the relevant authorities. Figure 5 briefly illustrates the main activities undertaken within this stage of the study. Thus, working sessions have been arranged with the producers of the future PDO and a participatory approach, articulated around four actions, was adopted:

- ***Preparation of the production specification:*** in the broadest terms, a manual containing all the relevant information about Meknès olive oil, as well as a clear and concise description of the practices to be complied with (and those which are not permitted) along the production chain of this product was prepared. This manual was obviously made in strict conformity with Moroccan legislation [18]. In particular, this manual includes the name of the product "Meknès olive oil"; definition of the geographical area of Meknès region (the limits of a production and processing area are defined); description of the product through indication of the raw materials and the main organoleptic and physico-chemical characteristics of Meknès olive oil; explanation of the method by which this product is obtained; information justifying the link with the geographical area; information about the inspection structure; and specific labeling rules.
- ***Elaboration of the internal monitoring system:*** corresponding to a plan which details in-situ controls and documents that should be adopted (and filled in) by Meknès olive oil producers to monitor and check the activities, techniques and processes employed during the elaboration of this product. This monitoring plan is of crucial importance as it enables the assurance of the traceability and compliance with specifications.
- ***Elaboration of the external monitoring system:*** an external monitoring plan which details the main points to be checked and the relevant evaluation methods to be used for ensuring compliance with specifications was prepared. This has to be logically made with the assistance and support of an accredited certification body.

- ***Definition of specific rules*** concerning packaging and labeling of the future Meknès olive oil PDO.

All the elaborated documents during these steps of the study were given to the Meknès olive oil PDO stakeholders to start the procedure to apply for the official registration (in front of the relevant authorities). The further procedure for the registration is set out in Food and Agriculture Organization of the United Nations [41].

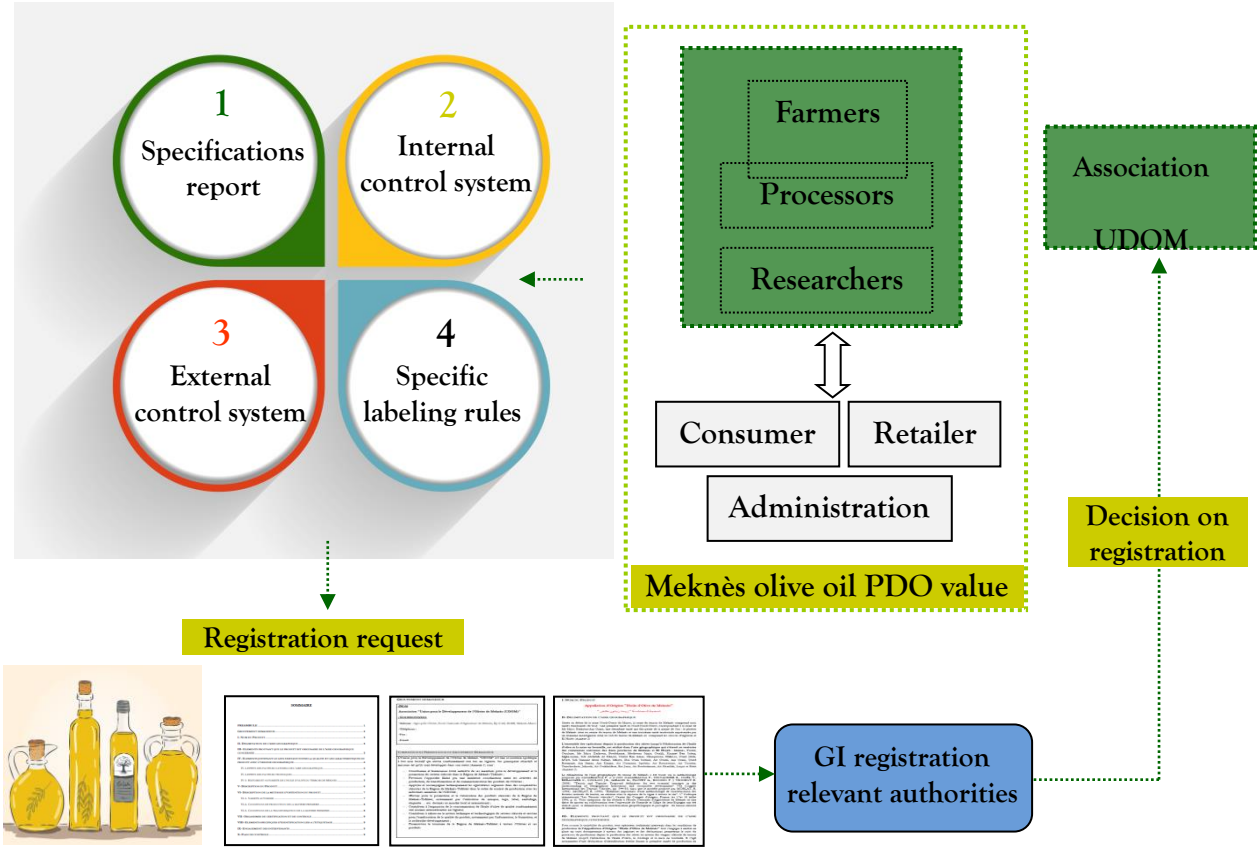

Figure 5. Proposed scheme for Meknès virgin olive oil PDO recognition.

However, it is important to point out that, the official recognition of Meknès olive oil PDO cannot be considered the end of the process, but rather, it is the beginning of a huge amount of work to maintain, monitor, and promote this PDO. Indeed, the acquisition of this label is not the goal itself; the final objective is the creation of added-value products and benefits for Meknès farmers and olive oil producers, enhancing therefore the access to national and international markets. In other words, the overall outcome is to achieve a real contribution to the sustainable development of this region.

So, as clearly emphasized within this chapter, the success of the PDO label, in Meknès or anywhere else, widely depends on proper implementation, management and further development of marketing and promotional strategies to intensify its effectiveness. To that end, specific emphasis has to be made on the promotion of Meknès olive oil PDO within the communication charter and action plans jointly elaborated by the association UDOM and other public and private organisms, to ensure the development of Meknès olive oil sector.

4. Enhancement of the effectiveness of PDO Meknès: promoting a terroir through olive tree and olive oil

The regional charter for promoting Meknès terroir through the olive tree and its products reflects a common ambition (shared by all the participants) to make olive growing and oil production activities the basis of the economic regional development. It summarizes a collective vision derived from the alliance between different institutions that take part in the olive oil sector advancement in this region with the intention of stimulating cultural, touristic and commercial activities in a way which is advantageous for all regional partners. Mechanisms to accomplish these objectives could be principally structured around the following actions:

- Developing common activities to improve cost-effectiveness of management, marketing and promotion of Meknès olive oil;
- Implementing a cooperative communication strategy and promoting information about Meknès olive oil in regional, national and international markets;
- Organization of promotional events around the culinary uses of olive oil with special emphasis on Meknès olive oil characteristics. The participation of recognized and influential chefs could be a way to enhance both the consumption of Meknès olive oil and its image or perception as a high quality product;
- Promoting the health benefits of olive oil standing out the Meknès olive oil composition and its richness on bioactive compounds. This activity involves the participation of nationally and internationally reputed doctors and experts to encourage or motivate the consumption of Meknès olive oil;

- Enhancing the recognition of Meknès PDO olive oil on domestic and regional markets by means of developing elements for visual identity of this product (product image) and informing about its uniqueness and specificity (promotional video materials and publicity spots that could be presented on national and international TV stations, flyers, Meknès olive oil route map, website, profiles on social networking services,ect.);
- Building credibility and reputation of Meknès olive oil and promotion of it on the market via the participation in important national and international fairs, exhibitions, sensory competitions or other events to promote a more appealing image of Meknès olive oil;
- Organization of Meknès olive oil festival (trade show) as an opportunity for discovery, learning, and even commemorating all legacy related-things from Meknès olive oil and olive tree cultivation;
- Organization of a national sensory quality competition with the participation of well-known international experts, which could stand for an valuable opportunity to promote the quality and sensory characteristics of Meknès olive oil;
- Membership in other related domestic and foreign olive oil organizations;
- Supporting the development of olive oil tourism in Meknès region.

Overall, this strategy is already getting positive effects on Meknès olive oil, in terms of recognition on the international market (oils from this region are, nowadays, listed among the best ones worldwide in specialized manuals and guides) and premium prices achieved by regional producers in national and international markets.

5. Conclusions

The results presented in this chapter offer two main contributions regarding olive oil GIs. The current situation of the use of these labels all over the world was discussed, paying particular attention on their usefulness for achieving consumers' recognition, quality signalling, control and differentiation, and competitive benefits that producers may obtain from the GI protection. The role that these GIs can play in sustainable development of the geographical area where they are implemented was also debated. Moreover, the main factors that affect the success or failure of these geographical labeling schemes and the support needed to make them effective were underlined. After doing

this general analysis, we assert that what makes GIs both feasible and operationally effective is the methodological approach followed for their setting up and the capacity of stakeholders to collectively manage, promote and transform territorial resources into quality attributes recognized by consumers.

Afterwards, we used the case of Meknès olive oil to contextualize the matter and suggest a methodological approach to support the implementation of a PDO label of an olive oil. Within this approach, the suitability of this region to acquire a PDO label was verified and the cooperative management, marketing and promotion strategy adopted to ameliorate the recognition of this label in local, national and international markets was outlined.

We really believe that the proposed methodology can be of great importance and assistance to better guide olive oil GI labels implementation both inside and outside Morocco.

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Chapter

12

Contribution to the establishment of a protected designation of origin for Meknès virgin olive oil: a 4-years study of its typicality

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Abstract

Protected designations of origin (PDO) constitute, nowadays, one of the most important differentiation strategies used for the promotion and commercialization of virgin olive oil (VOO). Obtaining these distinctive labels entails a deep study of the inherent features and characteristics of a VOO linked to a given geographical production area. The aim of this study was to perform the physicochemical and sensorial quality characterization of Meknès VOO, as well as the determination of its compositional profile. Thus, a total of 298 olive oils samples from 'Picholine Marocaine' cultivar, obtained from 12 industrial olive oil mills, located in three sub-areas of Meknès region, were analyzed over four consecutive seasons, paying particular attention to the determination of physicochemical quality indices, sensorial evaluation, total phenol content, pigment content, tocopherol and fatty acid composition. Results show that, besides an interannual variation, VOOs from Meknès region are characterized by high physicochemical and sensory quality, as well as a homogeneous composition regardless of the production sub-area.

Keywords: protected designations of origin; virgin olive oil; physicochemical and sensorial quality, olive oil composition.

1 Introduction

The olive tree (*Olea europea L.*) has deeply marked the rural landscape of both shores of the Mediterranean Basin since ancient times, and influenced from different points of view (economically, socially and culturally) the customs and traditions of Mediterranean populations, creating therefore, real genuine cultures in some olive growing areas (Blondel, 2006; Caramia, Gori, Valli, & Cerretani, 2012). Thus, in these regions, the historical legacy and the symbolic meaning of olive tree, together with the intimate link of the cultures to olive farming and VOO production, make the establishment of geographical indications such as PDOs a very interesting approach to promote and protect agricultural products and foodstuffs with high quality standards and distinctive characteristics. Within a PDO, the quality and other essential and exclusive characteristics are guaranteed due to a particular geographical environment.

In 1975, this distinctive sign of quality was used by first time in the field of VOO production for a PDO in Catalonia (Spain). Afterwards, very few Mediterranean regions were recognized as PDOs till 2001 (Angles, 2007), when olive oil PDO recognition experienced a remarkable increase in terms of protected surface, as well as VOO production (Avilés, Navarro, Barea, & Vázquez, 2007). According to the International Olive Council (IOC) there are 91 PDO of VOO around the world: 35 in Italy, 22 in Spain, 16 in Greece, 7 in France, 6 in Portugal, 3 in Turkey, 1 in Morocco and 1 in Slovenia (IOC, 2010b). The noticeable proliferation of PDOs observed over the last years has, nevertheless, created a growing concern about the need of demonstrating those elements proving the uniqueness and high quality of the products with this label. Indeed, according to Sanz (2009), the recognition of olive oil PDOs is, nowadays, a phenomenon in expansion phase but with a notable reputation deficit in many Mediterranean regions. That means that the importance of studying olive oil typicality at the moment of the establishment of a PDO quality sign is undeniable; doing it, the new olive oil PDOs will be well-founded, appreciated and will clearly represent an added-value.

The concept of typicality is strongly linked to the quality distinctive signs and, especially, to geographical indications. It represents the product characteristics in all, meaning that this product is representative of its growing area, reflecting the effects of the exclusive environmental factors, agronomic and technological practices that characterize that region (Salette, 1997). A most recent definition of this concept considers that typicality is a set of properties of similarity and distinction that determine the belonging of a PDO product to one determined category or "Type", making possible to differentiate, identify, and recognize this product as original and unique (Casabianca et al., 2005).

Keeping all this in mind, the concept of typicality applied to olive oil can represent a comprehensive description of a given VOO as a whole entity, including physicochemical characteristics, sensory quality and compositional parameters that reflect the effects of the olive growing area factors (genetic, environmental and human elements).

Thus, in our opinion, a general procedure to establish an olive oil PDO must rely mainly upon two points: the first one is that the profile of produced olive oils inside the evaluated area should be well defined throughout a deep and comprehensive characterization of their quality and composition. Moreover, it is also necessary assure the

homogeneity among the profiles obtained for the olive oils produced in the entire evaluated region. The second one is to provide elements to certify the singularity of these oils versus others produced outside the labelled area. As far as this point is concerned, over the past decades, there has been a remarkable proliferation of research works focused on the development of suitable methods (chromatographic, spectroscopic/spectrometric and DNA-based techniques) to classify and discriminate among olive oils from different geographical origins (Beltrán, Sánchez-Astudillo, Aparicio, & García-González, 2015; Lerma-García et al., 2009; Longobardi et al., 2012; Pizarro, Rodríguez-Tecedor, Pérez-del-Notario, & González-Sáiz, 2011; Sinelli, Cerretani, Egidio, Bendini, & Casiraghi, 2010). Most of these studies have been conducted in order to establish the geographical origin of the selected olive oils by the assessment of different chemical components as geographical markers, in combination with chemometric tools. Recent interesting reviews summarize some of these researches (Ben-Ayed, Kamoun-Grati, & Rebai, 2013; Mannina & Segre, 2010; Perri, Benincasa, & Muzzalupo, 2012; Rabiei & Enferadi, 2005).

Meknès is a north-central region of Morocco, with historical heritage and deep-rooted traditions regarding VOO production. As a matter of fact, this region presents the oldest vestiges of olive oil production in Morocco. Indeed, in the archaeological site of Volubilis situated in Meknès region, remains of Roman olive presses and olive mills dating from the 3rd century are still present (Panetier & Limane, 2002). In this region, about 43,000 ha are currently devoted to the olive crop, producing approximately 21,000 tons of olive oil per year. *Picholine marocaine* remains the main olive variety cultivated in this region (covering 96.1% of the olive growing area), being the olive trees, generally, growing under rain-fed conditions (DPA, 2014). Furthermore, in Meknès region, farming and olive oil processing techniques have globally undergone considerable changes over the last years, stimulating the improvement of the quality of Meknès VOOs, which are achieving considerable success and receiving national and international recognition.

In spite of the long tradition of VOO production in Meknès region, deep and comprehensive studies to establish the physicochemical and sensory standard properties of the produced oils have not been carried out so far; indeed, there is practically no substantial information on Meknès olive oil overall composition and quality. Therefore, the aim of this study was to perform a physicochemical and sensorial quality

characterization of Meknès olive oil, as well as to determine its compositional profile. To achieve this purpose, we have being collaborating since 2010, with the Association "*Union pour le Développement de l'Olivier de Meknès (UDOM)*", which is the promoter group of *"Meknès Virgin Olive Oil PDO"* and the determination of Meknès olive oil typicality. Moreover, interregional variations of the composition and quality of Meknès olive oils were evaluated within the aim of ensuring certain homogeneity in the profile of Meknès VOO.

Therefore, considering the approach detailed above, the work described here constitutes a contribution to the establishment of a PDO for Meknes VOO through the definition of a qualitative and compositional profile of the olive oils produced in this region, as well as a deep evaluation of the homogeneity of this profile. However, it remains necessary to demonstrate the singularity of Meknès olive oils providing those elements which allow differentiating it from other olive oils produced outside Meknès region. In this regard, some data are already available (Bajoub, Carrasco-Pancorbo, Ajal, Ouazzani, & Fernández-Gutiérrez, 2014), demonstrating the possibility of using phenolic compounds as geographical markers to discriminate among olive oils from Meknès and other north Moroccan regions.

2 Materials and methods

2.1 Olive and olive oil sampling

In a previous work carried out by our group (no published data) to characterize Meknès olive growing area factors, different elements were taken into account (genetic factors (olive tree cultivars), pedoclimatic conditions, agronomic and technological practices). It was possible to differentiate three olive growing sub-areas in this region (Figure 1): Moulay Idriss Zerhoun (sub-area 1), Meknès Plateau (sub-area 2) and El Hajeb (sub-area 3). Moreover, the mentioned study indicated that more than 96% of olive oil produced in Meknès region comes from the standard cultivar "*Picholine marocaine*". Therefore, only olive oil samples of this variety were considered in the current work. Bearing in mind the possible effects of the variations in the climatic conditions on the characteristics of Meknès olive oil, monthly information regarding rainfall, and maximum and minimum temperatures were obtained over the four years of the study (Figure 2).

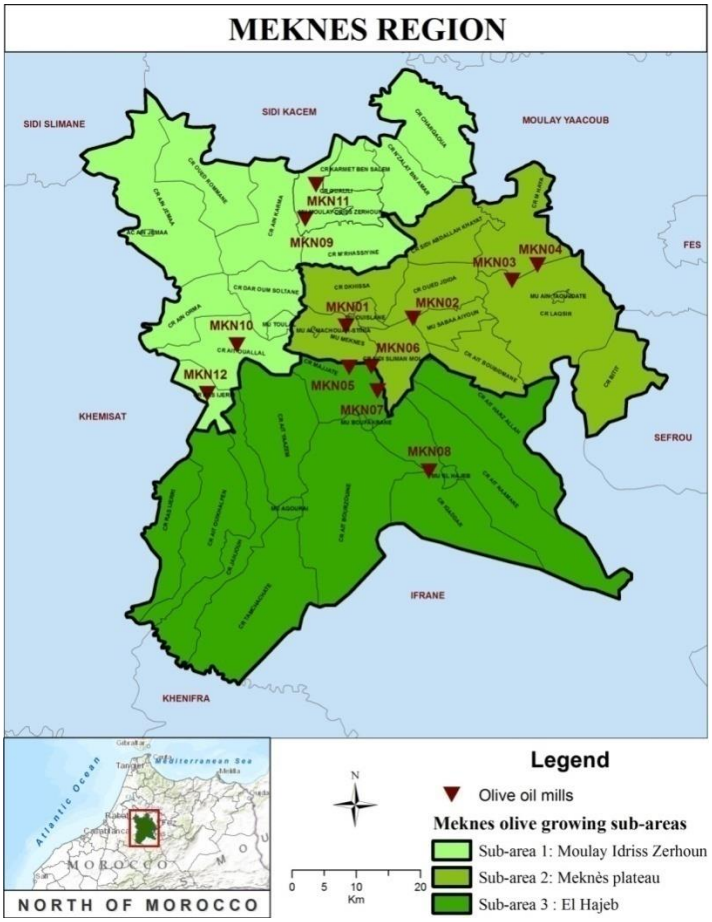

Figure 1: Map of Meknès region, showing its three sub-areas delimitation and sampling olive oil mills localization.

Industrial olive oil mills (belonging to the Association UDOM) were selected for each olive growing sub-area. The selection was made based on several criteria: geographical localization and production capacity of the mills and our intention of considering all possible sources of variability in the final products (different olive oil extraction systems, for instance). So, the selected olive oil mills should cover different geographic positions of Meknès region. Moreover, we chose those olive oil mills with a high capacity and combining mills using two and three phases continuous olive oil extraction systems. 12 industrial olive oil mills were selected (Figure 1).

Over the olive crop seasons from 2010 to 2013, a total of 298 "*Picholine marocaine*" olive oil samples were collected from the selected industrial olive oil mills. The sampling period in each season was from middle of October until the end of December, which represents

the normal period of olive oil production in Meknès region. 250 mL of olive oil were collected after vertical centrifugation at each olive oil mill.

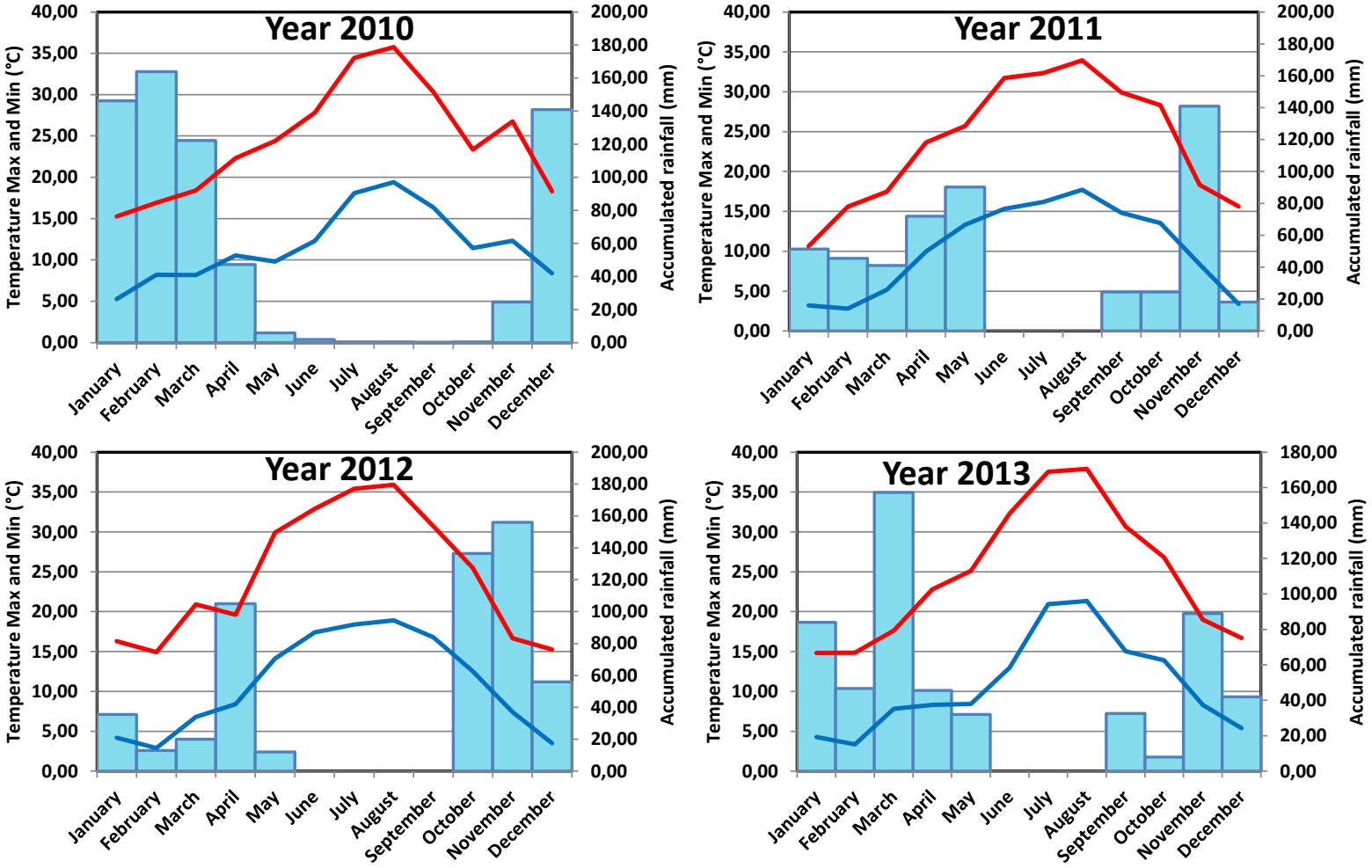

Figure 2: Monthly patterns of minimum and maximum air temperature (line) and rainfall (bars) over the four years of the study.

At the same time, a sample of approximately 1 kg of the processed olives was collected from the conveyor belt to determine the ripening index. Samples were immediately transported to the laboratory, where the olive oils were filtered and stored in dark glass bottles at 4ºC avoiding any head space volume to assure a better preservation of the materials from oxidation until the moment in which the chemical analyses were performed.

2.2 Ripening index

The olive ripening index was determined according to the method developed by the Agronomic Station of Jaén (Uceda & Frías, 1975) based on the evaluation of the olive skin and pulp colours. Ripening index values can be found between 0 (100% intense green skin) and 7 (100% purple flesh and black skin).

2.3 Physicochemical quality indices

Free fatty acids, expressed as the percentage of oleic acid, peroxide value expressed as milli-equivalents of active oxygen per kilogram of oil (meq O_2/kg), and K_{232} and K_{270} extinction coefficients calculated from absorption at 232 and 270 nm, respectively, were determined according to the analytical methods described in the European Union standard methods Regulations 2568/91 and the subsequent amendments (EC, 1991). All parameters were determined in triplicate for each sample.

2.4 Compositional parameters

All compositional parameters were determined in triplicate for each sample.

2.4.1 Pigment content

Carotenoids and chlorophylls were determined as described by Minguez-Mosquera et al. (1991). 3 g of olive oil were accurately weighed and dissolved in cyclohexane up to a final volume of 10 mL; the carotenoid and chlorophyll content were determined by measuring the absorbance at 470 and 670 nm, respectively. The absorbance measurements were performed in a Varian Cary 50 UV-Vis Spectrophotometer. The results were expressed as mg kg^{-1} and calculated using the following equations:

[Chlorophylls] mg kg^{-1}= $(A_{670}\times10^6)/ (E_1\times100\times d)$.

[Carotenoids] mg kg^{-1}= $(A_{470}\times10^6)/ (E_2\times100\times d)$.

Where A is the absorbance, d is the spectrophotometer cell thickness (1 cm) and E_1 and E_2 are, respectively, the values of the specific extinction coefficients: $E_1 = 613$ for pheophytin (as major component in the olive oil chlorophyll fraction), and $E_2 = 2000$ for lutein (as major component in the olive oil carotenoid fraction).

2.4.2 Total phenol content

Total phenol content was determined as described by Vázquez, Janer, and Janer (1973). The phenolic compounds were isolated from a solution of olive oil in hexane (1 g of olive oil in 5 mL of hexane) by a triple extraction with 2 mL of a water/methanol mixture (60:40, v/v). 1 to 5 mL of the combined extract was diluted in 35 mL of distilled water, and then 2.5 mL of the Folin-Ciocalteu reagent was added. After homogenization, 5 mL of Na_2CO_3 solution (6%) and distilled water was added to have a total volume of 50 mL. The mixture was kept in dark for 10 min, and afterwards, the absorbance was evaluated at 725 nm using a Varian Cary 50 UV-Vis Spectrophotometer. Calibration curve was prepared using caffeic acid as pure standard. The results were expressed as mg caffeic acid/kg of olive oil. Depending on the volume used of the combined extract, dilution coefficients were: 500 for 1 mL, 250 for 2 mL, 167 for 3 mL, 125 for 4 mL and 100 for 5 mL.

2.4.3 Tochopherol content

The tocopherol content in the selected olive oil samples was determined according to the IUPAC 2432 method (IUPAC, 1992). 1.5 g oil was dissolved in 10 mL of the mobile phase (0.5% isopropanol in *n*-hexane). The chromatographic separation was performed using a Perkin-Elmer liquid chromatography equipped with an isocratic pump LC200 and a UV-Vis detector. Tocopherol compounds were separated by using a normal phase column Lichrosphere Si60, Merck, (4.6 x 250 mm, 5µm particle size), operating at 25°C and a flow rate of 1.0 mL/min. Injection volume was 20 µL and the total run time was 20 min. Three calibration curves were made with standard solutions of each compound (α-, β- and γ-tocopherols; $r^2 = 0.999$, 0.986 and 0.999 respectively) and used for quantification. Results are given in mg of α-, β-, and γ-tocopherols per kg of olive oil.

2.4.4 Fatty acids composition

Determination of the fatty acids composition was performed via trans-esterification into fatty acid methyl esters following the analytical methods described in the European Regulation (EC) no. 2568/91 and later amendments (EC, 1991). The chromatographic separation was performed using a Perkin-Elmer gas chromatography system with a split/splitless injector and a FID detector. The GC system was equipped with a BPX 70 capillary column of 50 m of length, 0.22 mm i.d. and 0.25 μm of film thickness (SGE, Australia). The carrier gas was helium, with a flow rate of 1 mL/min. Injector and detector temperatures were set at 240 and 250 °C, respectively, and the oven temperature was gradually increased from 180 to 210 °C in 20 min. The injection volume was 1 μL. Fatty acids composition was expressed as relative percent of total area.

2.5 Sensory analysis

Aiming the determination of a general sensory quality standard for Meknès olive oils over each olive crop season, a commercial extra VOO bottle of 500 mL, coming from each olive oil mill (of those selected in the current study) was collected. Sensorial evaluation was performed by a fully trained analytical taste panel composed by 11 judges recognized by the IOC. The evaluation of the samples was carried out under the conditions described in the IOC official method COI/T.20/Doc. nº 15 and the subsequent amendments (IOC, 2010a). Each taster rated the intensity of the sensory attributes using a continuous unstructured line scale of 10 cm, ranging from low to high intensity. The data obtained were used to define the sensory profile of each sample using median of all the panelists´ scores. Moreover, the presence of secondary sensory positive attributes contributing to "pleasant flavors" of extra VOO was determined using an open generic profile; then those for which the presence was confirmed by all panel test judges in all tested samples, an intensity level was indicated.

2.6 Statistical analysis

Statistical analysis was performed by means of SPSS statistical package software (SPSS for Windows, Version 20.0, SPSS Inc., and Chicago, USA). Results were reported as tables of mean values and standard deviations. One-way analysis of variance (ANOVA) was used to evaluate the influence of sub-area and crop season on all analyzed parameters and the significance of differences at 5% ($p<0.05$) level between mean values was determined

using Tukey's test. Additionally, physicochemical quality indices and compositional parameters were used to perform a principal component analysis (PCA) to better visualize the data structure and evaluate samples possible clustering.

3 Results and Discussion

3.1 Ripening Index

Olive fruit ripening is a complex process. Over the ripening a series of physiological changes and metabolic processes take place (Conde, Delrot, & Geros, 2008; Dağdelen, Tümen, Ozcan, & Dündar, 2013). All of them are reflected on olive oil quality, oxidative stability and composition properties (Baccouri et al., 2008; Rotondi et al., 2004; Salvador, Aranda, & Fregapane, 2001). Thus, to avoid the effect of the ripening index on possible variations of the studied parameters, olive oil samples were distributed, considering the ripeness stage of the olives used for its manufacture, in three ranges: [1-2];]2-3.5] and]3.5-7], corresponding, respectively, to green, spotted and ripe stages. Afterwards, for each studied parameter, statistical analysis was performed on data coming from samples of the same ripening stage, evaluating the effect of the sub-area and the crop season as variation factors. As can be seen from Table 1, from a total of 298 olive oil samples collected over the 4 years of the study, 21.48% were classified into the green stage, 46.31% into the spotted stage and the rest, 32.21%, were classified into the ripe stage.

3.2 Quality indices

Table 1 includes the mean and standard deviation values of the quality criteria of the 298 studied olive oils samples according to the European Commission Regulations (EC, 1991). In order to facilitate the discussion of the achieved results and structure properly the current section, we will have a look at free fatty acids, and peroxide value and UV spectrophotometric indices in two independent sub-sections.

3.2.1 Free fatty acids

As shown by Table 1, mean value of free fatty acids content, expressed as percentage of oleic acid, ranged from 0.16 up to 0.55%. Only 5 of the studied samples showed values higher than 0.8% (which is the upper threshold limit established for the Extra VOO category) and there were no samples exceeding the limit of 2% for the Virgin category.

Table 1. Mean values and standard deviations (SD) for the regulated physicochemical quality parameters (Free fatty acids (FFAs), Peroxide value (PV) and extinction coefficients K_{270} and K_{232}) in Meknès olive oil samples.

			Sub-area 1			Sub-area 2			Sub-area 3			Category	
			Green	Spotted	Ripe	Green	Spotted	Ripe	Green	Spotted	Ripe		
Crop season 2010	Samples number		6	18	8	5	19	8	5	20	7	Virgin-extra	Virgin
	FFAs (% oleic acid)	Mean ± S.D.	0.26^{a}a±0.07	0.26^{a}a±0.06	0.43^{a}a±0.05	0.24^{a}a±0.05	0.26^{a}a±0.07	0.38^{a}a±0.04	0.29^{a}a±0.02	0.27^{a}a±0.11	0.40^{a}ab±0.05	96	0
	PV (meq O_2/kg)		5.16^{a}a±1.00	4.07^{a}a±0.98	3.55^{a}a±1.66	4.55^{a}a±0.93	4.38^{a}a±0.85	3.59^{a}a±1.39	4.97^{a}a±0.61	4.56^{a}a±0.68	4.93^{a}ab±2.52		
	K_{232}		1.30^{a}a±0.10	1.44^{a}a±0.18	1.09^{a}a±0.04	1.34^{a}a±0.18	1.43^{a}a±0.21	1.16^{b}a±0.09	1.44^{a}ab±0.19	1.41^{a}a±0.21	1.36^{a}a±0.21		
	K_{270}		0.14^{a}a±0.02	0.15^{a}a±0.02	0.14^{a}a±0.01	0.12^{a}a±0.01	0.14^{a}a±0.02	0.13^{a}a±0.01	0.14^{a}ab±0.02	0.14^{a}a±0.02	0.14^{a}a±0.01		
Crop season 2011	Samples number		3	4	5	3	7	4	7	7	11		
	FFAs (% oleic acid)	Mean ± S.D.	0.33^{a}a±0.04	0.48^{a}b±0.33	0.33^{a}a±0.09	0.27^{ab}a±0.10	0.27^{b}a±0.04	0.29^{a}a±0.05	0.23^{b}b±0.09	0.50^{a}b±0.27	0.55^{b}a±0.36	47	4
	PV (meq O_2/kg)		7.78^{a}b±0.61	7.56^{a}bc±0.24	6.12^{a}ab±0.28	8.78^{a}b±0.63	7.54^{a}b±0.85	6.88^{a}b±0.48	7.97^{a}b±0.68	7.25^{a}b±1.11	4.43^{a}b±0.89		
	K_{232}		1.74^{a}a±0.07	1.83^{a}b±0.16	1.37^{a}b±0.31	1.75^{a}a±0.19	1.70^{a}a±0.30	1.35^{a}a±0.29	1.81^{a}b±0.20	1.84^{a}b±0.19	1.32^{a}a±0.25		
	K_{270}		0.13^{a}a±0.02	0.13^{a}a±0.04	0.13^{ab}a±0.02	0.14^{a}a±0.05	0.15^{a}a± 0.03	0.12^{b}a±0.01	0.17^{a}a±0.03	0.14^{a}a±0.03	0.15^{a}a±0.03		
Crop season 2012	Samples number		3	3	5	4	8	4	10	14	23		
	FFAs (% oleic acid)	Mean ± S.D.	0.16^{a}a±0.03	0.20^{a}a±0.07	0.47^{a}a±0.10	0.21^{a}a±0.03	0.23^{a}a±0.07	0.47^{a}a±0.8	0.18^{a}b±0.04	0.22^{a}a±0.07	0.41^{a}b±0.05	74	0
	PV (meq O_2/kg)		5.33^{a}ab±0.76	4.67^{a}ab±2.02	3.60^{a}a ± 0.89	7.25^{b}ab±0.87	5.63^{a}a±1.96	7.63^{b}b±2.50	4.50^{a}a±0.58	4.21^{a}a±2.22	3.52^{b}a±1.31		
	K_{232}		1.49^{a}a±0.38	1.58^{a}ab±0.20	1.24^{a}a±0.25	1.38^{a}a±0.36	1.48^{a}a±0.23	1.28^{a}a±0.31	1.67^{a}ab±0.26	1.42^{a}a±0.25	1.27^{a}a±0.30		
	K_{270}		0.13^{a}a±0.03	0.14^{a}a±0.03	0.14^{a}a±0.01	0.12^{a} a±0.01	0.14^{a}a±0.03	0.16^{a}a±0.04	0.13^{b}b±0.01	0.15^{a}a±0.03	0.15^{a}a±0.02		
Crop season 2013	Samples number		6	15	8	4	5	3	8	18	10		
	FFAs (% oleic acid)	Mean ± S.D.	0.20^{a}a±0.07	0.29^{a}ab±0.14	0.41^{a}a±0.13	0.28^{a}a±0.03	0.30^{a}a±0.17	0.46^{a}a±0.24	0.17^{a}b±0.07	0.33^{a}a±0.15	0.48^{a}a±0.10	76	1
	PV (meq O_2/kg)		8.33^{a}bc±1.29	7.97^{a}c±2.76	7.56^{a}b±2.80	9.38^{a}b±2.39	6.80^{b}a±1.15	5.00^{a}ab±2.65	9.16^{a}b±2.92	6.11^{ab}b±2.10	4.25^{a}ab±2.84		
	K_{232}		1.51^{a}a±0.25	1.65^{a}ab±0.32	1.31^{a}a±0.41	1.36^{a}a±0.08	1.61^{a}a±0.41	1.18^{a}a±0.60	1.38^{a}a±0.25	1.47^{a}a±0.30	1.17^{a}a±0.34		
	K_{270}		0.13^{a}a±0.02	0.15^{a}a±0.02	0.14^{a}a±0.03	0.16^{ab}a±0.05	0.16^{a}a±0.03	0.14^{a} a±0.04	0.17^{b}a±0.03	0.15^{a}a±0.03	0.15^{a}a±0.04		
	Total		84			74			140			293 (98.32%)	5 (1.68%)
Total samples/ripening stage			Green n=64			Spotted n=138			Ripe n=96				
			21.48 %			46.31 %			32.21 %				

-Significant differences in the same column are indicated with different lowercase letters (comparison among crop seasons at the same maturation stage, $p < 0.05$).

-Significant differences in the same row are indicated with different superscript letters (comparison between sub-areas at the same maturation stage, $p < 0.05$).

The same table shows that, in general, over the four years of the study, there were no statistically significant differences in free fatty acids values between the three studied sub-areas, except for the second crop season when significant differences between the sub-areas 1 and 3 were observed at the green and ripe stage, between the sub-areas 2 and the other two sub-areas at the spotted stage, and between the sub-areas 3 and the other sub-areas for samples belonging to the ripe stage. Moreover, interannual variations of the free fatty acids mean values were detected, especially, between the second and the third crop seasons.

For the four seasons tested, it can be observed as well an increase in free fatty acids content over ripening. These results are in good agreement with the findings of previous studies (Anastasopoulos, Kalogeropoulos, Kaliora, Kountouri, & Andrikopoulos, 2011; Gutiérrez, Jímenez, Ruíz, & Albi, 1999), where the authors ascribed this increase to the free fatty acids content to a progressive activation of the lipolytic activity in olive fruits over the ripening process. It can be also related to the fact that ripe olive fruits are more sensitive to pathogenic infections and mechanical damage, which results in olive oils with higher acidity values.

3.2.2 Peroxide value and UV spectrophotometric indices

Peroxide value and spectrophotometric UV absorption of olive oil at 232 nm (K_{232}) and 270 nm (K_{270}) measure the oxidative deterioration of olive oil samples. As shown in Table 1, the peroxide and K_{232} and K_{270} values of all analyzed samples were lower than 20 meq O_2/kg, 2.50 and 0.22, respectively, which are the legal limits established for Extra VOO category (EC, 1991). The average of peroxide value ranged from 3.52 up to 9.38 meq O_2/kg, whereas K_{232} and K_{270} ranged, respectively, from 1.09 up to 1.84, and from 0.12 up to 0.17. The low values of these quality parameters confirmed the good overall quality of the analyzed olive oils. Moreover, as can be seen in Table 1, the oils obtained from olive fruits at advanced stage of maturity (ripe stage) showed lower peroxide and K_{232} values in all the crop seasons considered in the current study, even if in most of the cases, an increase in K_{232} value at the intermediate stage (spotted stage) was observed. As far as peroxide value is concerned, the same behavior was reported in other olive oils cultivars (Dag et al., 2011; Gutiérrez et al., 1999). According to these authors the observed increase for this parameter could be related to a decrease in the activity of the enzyme lipoxygenase

over the ripening process. However, we cannot claim that a clear trend was observed for the K_{270} values when the three ripening stages were compared.

Furthermore, the ANOVA analysis showed that, in general, there is no effect of the growing sub-area on peroxide value, K_{232} and K_{270} extinction coefficients; although significant differences were observed in few cases.

All things considered and bearing in mind the legal olive oil physicochemical quality parameters, it was possible to classify 98.32 % of studied olive oil samples as Extra VOO, whereas the rest (1.68%) were within the Virgin category.

3.3 Compositional parameters

3.3.1 Pigment content

The evaluation of chlorophylls and carotenoids pigments profile in olive oil is of great importance, given their antioxidant properties, as well as their crucial role as colorful substances responsible of greenness (chlorophylls) and yellowness (carotenoids) of olive oil (Giuliani, Cerretani, & Cichelli, 2011). The color given to the olive oil by these substances has an enormous commercial importance because, although it is not included among the legal quality parameters to be checked, there is a strong relationship between such attribute and the acceptability of olive oil by the consumers (Moyano, Heredia, & Meléndez-Martínez, 2010).

Quantitative results of pigment content in Meknès olive oils samples are given in Table 2. Chlorophylls concentration was found within the range of 14.01 to 21.44 mg kg^{-1}. A quite clear decrease in chlorophylls content was detected throughout the maturation stages, in particular, between the green and ripe stages. Likewise, the olive oils samples from the first ripening stage showed as well higher concentrations of carotenoid pigment. Their concentration levels were decreasing from the green stage to the ripe one, finding the lowest values for the ripe samples; the amounts were found ranging from 7.79 to 12.80 mg kg^{-1}. However, this decrease was more easily observed for the chlorophylls than for the carotenoid fraction. These results are in good agreement with those finding by other authors (Benito et al., 2012).

Table 2. Pigments content (expressed in mg kg^{-1}) of Meknès olive oil samples (mean ± standard deviation).

				Sub-area 1			Sub-area 2			Sub-area 3		
				Green	Spotted	Ripe	Green	Spotted	Ripe	Green	Spotted	Ripe
Crop seasons	2010	Pigments (mg kg^{-1})	Carotenes	$12.49^{a}a\pm0.32$	$8.73^{a}a\pm1.59$	$8.48^{a}a\pm0.45$	$12.32^{a}a\pm0.61$	$8.70^{a}ab\pm1.82$	$8.32^{a}a\pm0.49$	$12.66^{a}a\pm0.29$	$8.59^{a}a\pm1.86$	$8.30^{a}a\pm0.54$
			Chlorophylls	$18.54^{a}a\pm1.63$	$17.89^{a}a\pm1.01$	$14.46^{a}a\pm0.21$	$18.03^{a}a\pm0.55$	$17.53^{a}a\pm1.27$	$14.27^{a}a\pm0.23$	$17.81^{a}a\pm0.24$	$17.68^{a}a\pm1.01$	$14.40^{a}a\pm0.23$
	2011	Pigments (mg kg^{-1})	Carotenes	$11.96^{a}a\pm4.09$	$10.16^{a}a\pm0.43$	$9.49^{a}a\pm3.39$	$12.80^{a}ab\pm2.31$	$8.73^{a}a\pm1.67$	$8.74^{a}a\pm1.26$	$10.50^{a}b\pm1.68$	$9.53^{a}ab\pm2.33$	$9.50^{a}a\pm2.31$
			Chlorophylls	$18.21^{a}ab\pm0.85$	$17.55^{a}a\pm0.54$	$14.71^{a}a\pm2.74$	$19.25^{a}a\pm1.88$	$17.60^{a}ab\pm3.06$	$15.74^{a}b\pm1.26$	$18.08^{a}ab\pm3.78$	$17.77^{a}ab\pm2.26$	$14.81^{a}a\pm4.14$
	2012	Pigments (mg kg^{-1})	Carotenes	$11.88^{a}a\pm0.80$	$7.79^{a}a\pm0.20$	$8.48^{a}a\pm0.55$	$10.19^{b}b\pm0.57$	$9.52^{ab}ab\pm2.09$	$9.40^{a}a\pm3.02$	$12.17^{a}a\pm0.42$	$10.52^{b}b\pm0.80$	$8.82^{a}a\pm1.85$
			Chlorophylls	$18.67^{a}ab\pm2.62$	$18.70^{a}a\pm0.80$	$14.40^{a}a\pm0.12$	$19.75^{a}a\pm1.83$	$18.89^{a}ab\pm1.39$	$14.01^{a}a\pm1.71$	$20.39^{a}b\pm0.26$	$19.07^{a}b\pm1.09$	$14.76^{a}a\pm1.54$
	2013	Pigments (mg kg^{-1})	Carotenes	$12.76^{a}a\pm0.58$	$10.60^{a}a\pm0.96$	$8.58^{a}a\pm1.94$	$12.18^{a}a\pm0.18$	$10.98^{a}b\pm1.00$	$7.89^{a}a\pm0.05$	$12.06^{a}a\pm0.71$	$10.45^{a}b\pm0.78$	$9.25^{a}a\pm2.36$
			Chlorophylls	$21.44^{a}b\pm1.17$	$17.13^{a}a\pm1.30$	$15.29^{a}a\pm2.02$	$19.88^{a}a\pm0.76$	$17.84^{a}b\pm0.77$	$14.65^{a}ab\pm0.26$	$20.47^{a}b\pm1.57$	$17.04^{a}b\pm1.08$	$14.88^{a}a\pm 2.05$

-Significant differences in the same column are indicated with different lowercase letters (comparison among crop seasons at the same maturation stage, $p < 0.05$).

-Significant differences in the same row are indicated with different superscript letters (comparison between sub-areas at the same maturation stage, $p < 0.05$).

Furthermore, as can be seen in Table 2, there were no statistically significant differences among the three sub-areas of Meknès region in terms of the chlorophylls and carotenoid pigment concentrations over the four years of the study. The observed subtle differences were mainly due to crop season factor. Indeed, significant differences were observed between the first and the fourth seasons of the study. This variation could be due to the differences of climatic conditions between both crop seasons, as shown in Figure 2.

3.3.2 Total phenol content

The determination of total phenol content in VOO is of great nutritional and commercial importance, since these compounds have become the subject of intensive research because of their biological activities, their influence on the organoleptic properties of VOO and their contribution to its oxidative stability (Bendini et al., 2007). As shown in Table 3, the overall range observed for Meknès olive oil samples for the mean of total phenol content was from 216.83 up to 668.67 mg kg^{-1}. Certain differences can be observed when comparing the content in samples coming from the three olive fruit maturation stages. Indeed, there was an increasing tendency in total phenol content between the green and spotted stage (where the highest levels were observed); however, from spotted to ripe samples, a marked decreasing trend was detected. The quantitative differences detected between the analyzed samples can be mainly assigned to the crop season.

Particularly, the highest mean amounts of total phenols in Meknès olive oils were observed during the first crop season. Interestingly, for that crop year, the olive oils samples from the three sub-areas of Meknès region showed a high amount of total phenols at the green stage in comparison to those observed at the same maturation stage of the others crop seasons. This result could be related to climatic conditions differences between the four years of the study. Indeed, as shown in Figure 2, a drought period was registered during the summer-autumn months of 2010 and could be one of the main factors responsible for the high content of total phenol in olive oil samples belonging to the green stage of that crop year. As reported by different authors, periods of water deficit influence significantly the activity of phenylalanine ammonia-lyase (PML), which is a key enzyme in the biosynthetic pathway of phenolic compounds and is directly involved in the accumulation of polyphenols in the olive fruit and, consequently, in VOO.

Table 3. Total phenols and tocopherols contents (mg kg^{-1}) of Meknès olive oil samples (mean ± standard deviation).

			Sub-area 1			Sub-area 2			Sub-area 3		
			Green	Spotted	Ripe	Green	Spotted	Ripe	Green	Spotted	Ripe
Crop season 2010	Tocoperols (mg kg^{-1})	α-tocoferol	266.50^{a}a±0.84	255.42^{a}a±16.73	212.00^{a}ab±5.15	266.60^{a}a±0.89	256.96^{a}a±16.35	214.50^{a}a±7.53	266.20^{a}a±1.48	248.83^{a}a±23.72	208.34^{a}a±1.66
		β-tocoferol	3.33^{a}a±0.52	3.15^{a}a±1.32	3.16^{a}a±0.75	3.40^{a}a±0.55	3.31^{a}a±1.11	2.35^{a}a±1.40	3.40^{a}a±0.55	3.24^{a}a±0.77	3.06^{a}a±1.22
		γ-tocoferol	18.00^{a}a±0.00	18.45^{a}a±0.61	17.92^{a}a±0.36	18^{a}a±0.00	18.45^{a}a±0.58	18.12^{a}a±0.66	18.00^{a}a±0.00	18.38^{a}a±0.72	18.17^{a}a±0.25
		Total	287.83^{a}a±0.41	277.02^{a}a±16.54	233.08^{a}ab±5.01	288.00^{a}a±0.71	278.73^{a}a±16.11	234.97^{a}ab±7.88	287.60^{a}a±1.52	270.46^{a}a±23.92	229.56^{a}a±2.24
	Total phenol (mg kg^{-1})		542.24^{a}a±72.13	668.67^{a}a±23.83	444.94^{a}a±10.41	538.34^{a}a±78.95	662.40^{a}a±9.86	440.63^{a}a±28.08	521.95^{a}a±80.20	655.80^{a}a±12.19	437.57^{a}a±8.46
Crop season 2011	Tocoperols (mg kg^{-1})	α-tocoferol	228.07^{a}ab±51.99	241.58^{a}ab ± 57.00	272.52^{a}a±38.51	266.70^{a}a±52.44	218.86^{a}a±36.60	204.10^{a}a±25.19	228.53^{a}a±58.81	236.56^{a}a±51.38	218.48^{a}a±54.96
		β-tocoferol	1.00^{a}b±1.73	0.50^{a}bc±0.58	0.40^{a}b ± 0.55	0.33^{a}b±0.58	0.29^{a}b±0.49	0.25^{a}b±0.50	0.62^{a}b±0.96	0.51^{a}b±0.50	0.66^{a}b±0.83
		γ-tocoferol	17.33^{a}ac±1.73	16.75^{a}b±0.50	16.80^{a}ab±0.84	17.33^{a}a±1.15	16.57^{a}a±1.62	16.75^{a}ab±2.22	17.43^{a}ab±1.40	17.14^{a}ab±1.46	17.36^{a}ab±1.63
		Total	246.40^{a}ab±53.48	258.83^{a}ab±57.01	289.72^{a}a±38.84	248.37^{a}a±52.02	235.71^{a}a±57.01	221.10^{a}a±23.88	246.53^{a}a±60.46	254.21^{a}a±51.08	236.51^{a}ab±55.26
	Total phenol (mg kg^{-1})		334.14^{a}b±59.16	664.13^{a}b±198.40	427.38^{a}a±54.38	356.81^{a}b±66.45	614.78^{a}a±66.47	405.88^{a}a±135.58	311.85^{a}b±52.82	649.52^{a}b ± 48.99	356.43^{a}a±101.06
Crop season 2012	Tocoperols (mg kg^{-1})	α-tocoferol	211.33^{a}ab±67.12	245.67^{a}ab±28.29	181.80^{a}b±32.51	223.50^{ab}a±19.35	250.63^{b}a±78.09	285.00^{a}b ± 46.87	264.00^{b}a±6.18	249.57^{a}a±64.47	228.57^{c}a±23.79
		β-tocoferol	1.00^{a}b±1.00	1.00^{a}b±0.00	0.40^{a}b±0.55	0.50^{a}b ± 0.58	0.38^{b}b±0.52	1.00^{b}ab±0.00	1.00^{a}b±0.00	0.79^{ab}b±0.43	0.96^{b}b±0.21
		γ-tocoferol	15.33^{a} bc±0.58	16.00^{a}b±0.00	15.20^{a}b±0.45	17.25^{b}a ± 0.96	16.13^{a}a±0.47	15.50^{a}b±1.00	16.00^{a}b±0.67	16.29^{a}b±0.47	16.74^{b}b±0.96
		Total	227.67^{a}ab±66.46	262.67^{a}ab±28.29	197.40^{a}b±32.97	241.25^{ab}a±19.16	267.13^{a}a±78.00	301.50^{b}b±46.39	281.00^{b}ab±6.39	266.64^{a}a±64.51	246.26^{c}b±24.64
	Total phenol (mg kg^{-1})		320.42^{a}b±126.98	559.80^{a}b±44.11	353.21^{a}a±140.66	321.04^{a}b±137.20	588.13^{a}a±52.61	428.24^{a}a ± 95.92	329.65^{a}c±97.60	595.13^{a}ab±82.17	595.13^{a}b±82.17
Crop season 2013	Tocoperols (mg kg^{-1})	α-tocoferol	199.33^{a}b±37.25	214.33^{a}b ± 28.08	206.25^{a}ab±32.59	250.75^{a}a±45.18	235.00^{a}a±44.07	228.00^{a}a±22.52	227.13^{a}a±33.71	257.72^{b}a±58.90	225.40^{a}a±32.29
		β-tocoferol	0.50^{a}b±0.84	0.47^{a}c±0.52	0.50^{a}b±0.76	0.25^{a}b±0.50	1.20^{a}b±1.10	1.33^{a}ab±1.53	0.25^{a}b±0.46	0.61^{a}b±0.85	0.60^{a}b±0.70
		γ-tocoferol	16.17^{a}c±0.98	16.00^{a}b±0.38	15.75^{a}b±0.89	16.50^{a}a ± 1.00	17.40^{ab}a±1.67	16.67^{ab}ab±1.15	16.88^{a}ab±1.36	18.28^{b}a±2.63	17.20^{b}ab±1.14
		Total	216.00^{a}b±38.12	230.80^{a}b±28.26	222.50^{a}ab±32.59	267.50^{a}a±45.93	253.60^{ab}a±45.38	246.00^{a}a±20.00	244.25^{a}b±33.39	255.50^{b}a±51.37	243.20^{a}ab±32.11
	Total phenol (mg kg^{-1})		291.12^{a}b±93.99	550.19^{a}b±57.02	404.29^{a}a±138.20	308.69^{a}b±51.90	537.88^{a}a±60.69	476.94^{a}a±102.69	339.94^{a}bc±68.78	638.20^{b}b±145.64	216.83^{b}a±37.63

-Significant differences in the same column are indicated with different lowercase letters (comparison among crop seasons at the same maturation stage, $p < 0.05$).

-Significant differences in the same row are indicated with different superscript letters (comparison between sub-areas at the same maturation stage, $p < 0.05$).

Therefore, a water deficit induced the activity of PML enzyme in olive fruits and, consequently, caused an increase in total phenol content in the obtained VOO (Morelló, Romero, Ramo, & Motilva, 2005).

3.3.3 Tocopherol composition

The contents of Meknès olive oil samples in terms of α-tocopherol, β-tocopherol and γ-tocopherol are shown in Table 3. The α-tocopherol is by far the most abundant tocopherol isomer found in the samples under study with a mean value ranging from 181.80 to 285.00 mg kg^{-1}. If these results are compared considering the sub-area of origin, no significant differences were observed in general, even though statistical significant differences were detected between the studied sub-areas in the 3rd crop season. However, α-tocopherol content was significantly different between crop years. As far as the other tocopherols are concerned, concentrations ranged from 0.25 to 3.40 mg kg^{-1} and from 15.20 to 18.45 mg kg^{-1} were found, respectively, for β-tocopherol and γ-tocopherol. The quantitative differences in the content of these tocopherols isomers found over the evaluated period (2010-2013) are mainly related to the crop season. Furthermore, regarding the total tocopherols content, a concentration range from 197.40 to 301.50 mg kg^{-1} was found. These results allow classifying VOOs of Meknès within the category of "low to intermediate content in tocopherol", according to the classification established by Uceda (2009).

3.3.4 Fatty acids profile

The detailed fatty acids composition (%) of Meknès olive oils samples is summarized in Table 4. A total of nine fatty acids were identified. Monounsaturated fatty acids (MUFA), including palmitoleic acid (C16:1), oleic acid (C18:1), and eicosanoid acid (C20:1) were within the range of 74.01 to 79.09%; Palmitic (C16:0) and stearic (C18:0) acids were the major saturated fatty acids (SAFA) found in Meknès olive oils, whist the margaric (C17:0) and behinic (C22:0) acids were found in very low amounts. SAFAwere detected within the range of 11.62 to 14.51%. The polyunsaturated fatty acids (PUFA), including linoleic (C18:2) and linolenic (C18:3) reached an average value ranging from 8.51 to 13.41%.

Table 4. Fatty acids composition (expressed as percentage of oleic acid) of Meknès olive oil samples (mean ± standard deviation).

			C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3
2010	Sub-area 1	G	10.59^{a}a ± 0.35	0.75^{a} a ± 0.05	0.03^{a}a ±0.01	2.27^{a}a±0.18	75.85^{a}a±1.41	8.90^{ab}a±1.07	0.88^{a}a±0.05
		S	9.81^{ab}a±0.95	0.75^{a}a±0.16	0.04^{a}a±0.01	2.28^{a}a±0.22	76.05^{a}a±1.96	9.27^{a}a±0.91	0.84^{a}a±0.05
		R	9.01^{a}a±0.69	0.72^{a}a±0.1	0.04^{a}a±0.01	2.45^{ab}a±0.18	76.52^{ab}a±0.75	9.63^{a}a±0.31	0.83^{a}ab±0.03
	Sub-area 2	G	9.94^{a}a±0.24	0.67^{a}a± 0.02	0.03^{a}a±0.01	2.04^{a}b±0.12	78.05^{a}a±0.89	7.61^{b}b±0.73	0.91^{a}a±0.02
		S	9.27^{b}b±0.77	0.69^{a}b±0.08	0.04^{a}a±0.00	2.18^{a}±0.25	77.91^{b}a±1.24	8.21^{a}b±0.76	0.89^{b}a±0.07
		R	9.08^{a}a±0.33	0.67^{a}a±0.02	0.04^{a}a±0.00	2.18^{b}b±0.14	77.89^{b}a±0.68	8.44^{b}a±0.41	0.83^{a}a±0.03
	Sub-area 3	G	10.04^{a}a±1.1	0.69^{a}a± 0.08	0.04^{a}ab±0.01	2.33^{a}a±0.22	75.43^{a}ab±2.53	9.81^{a}ab±1.41	0.89^{a}ab±0.03
		S	10.30^{a}a±1.3	0.79^{a}a±0.19	0.04^{a}a±0.01	2.35^{a}ab±0.26	74.90^{a}ab±2.66	9.97^{a}a±1.62	0.87^{ab}ab±0.05
		R	9.79^{a}±1.55	0.80^{b}ab±0.21	0.04^{a}ab±0.01	2.48^{a}a±0.31	75.52^{a}ab±2.5	9.67^{a}a±1.19	0.83^{a}ab±0.02
2011	Sub-area 1	G	8.96^{a}b ±0.05	0.65^{a}a ±0.01	0.04^{a} a ±0.00	2.26^{a}a±0.22	73.83^{a}a±0.96	12.53^{a}a±1.18	0.89^{a}a±0.04
		S	9.80^{a}a±0.57	0.75^{a}a±0.07	0.04^{a}a±0.00	2.18^{a}a±0.13	74.97^{a}a±1.83	10.47^{a}a±1.96	0.93^{a}a±0.06
		R	9.15^{a}a±0.3	0.66^{a}a±0.02	0.04^{a}a±0.00	2.24^{a}a±0.13	74.43^{a}a±1.55	11.74^{a}b±1.63	0.90^{a}b±0.04
	Sub-area 2	G	9.38^{a}a±0.91	0.70^{a}a±0.08	0.04^{a}a±0.00	2.36^{a}a±0.06	76.82^{a}a±1.6	9.00^{b}a±1.61	0.83^{a}ab±0.07
		S	11.11^{a}b±2.93	1.01^{a}a±0.64	0.05^{a}a±0.02	2.13^{a}b±0.33	74.47^{a}a±5.71	9.48^{a}b±2.77	0.80^{a}a±0.16
		R	10.14^{a}ab±1.98	0.86^{a}a±0.31	0.04^{a}a±0	2.25^{a}a±0.17	75.88^{a}ab±1.45	9.15^{a}a±3.11	0.85^{a}a±0.11
	Sub-area 3	G	10.41^{a}a±2.45	0.85^{a}a±0.36	0.06^{a}b±0.03	2.41^{a}a±0.54	73.95^{a}b±1.86	10.47^{a}b±1.53	0.92^{a}b±0.15
		S	11.47^{a}a±3.12	1.08^{a}a±0.56	0.06^{a}b±0.03	2.32^{a}ab±0.83	72.48^{a}b±3.69	10.82^{a}a±1.9	0.84^{a}ab±0.18
		R	10.93^{a}a±2.47	0.96^{a}b±0.47	0.06^{a}b±0.04	2.27^{a}a±0.39	75.17^{a}a±4.02	8.86^{a}a±2.69	0.80^{a}a±0.13
2012	Sub-area 1	G	10.49^{a}abc±1.07	0.66^{a}a±0.13	0.04^{ab}a± 0.01	2.23^{a}a±0.27	76.41^{ab}a±1.01	8.85^{ab}a±1.60	0.73^{a}a±0.1
		S	10.52^{a}a±0.38	0.71^{a}a±0.08	0.04^{a}a±0.01	2.13^{a}a±0.22	76.07^{a}a±1.62	9.16^{a}a±1.32	0.84^{a}ab±0.1
		R	9.65^{a}a±1.43	0.68^{a}a±0.12	0.04^{ab}a±0.01	2.47^{a}a±0.69	75.40^{a}a±1.53	10.38^{a}±ab1.05	0.80^{a}ab±0.1
	Sub-area 2	G	10.55^{a}a±0.67	0.77^{a}a±0.07	0.05^{b}a±0.01	2.12^{a}a±0.42	74.70^{b}a±1.75	9.70^{b}a±0.89	0.84^{ab}ab±0.02
		S	10.53^{a}ab±1.29	0.74^{a}a±0.15	0.04^{a}a±0.01	2.21^{a}b±0.19	77.36^{a}a±1.05	7.73^{a}ab±2	0.78^{a}a±0.1
		R	11.85^{b}b±1.51	0.88^{b}a±0.26	0.05^{b}a±0.03	1.86^{b}b±0.21	74.92^{a}b±1.83	8.91^{a}a±1.34	0.83^{a}a±0.15
	Sub-area 3	G	10.71^{a}a±0.69	0.83^{a}a±0.1	0.03^{a}a±0.00	2.12^{a}a±0.19	77.17^{a}a±0.62	8.05^{a}a±0.71	0.87^{b}ab±0.06
		S	10.50^{a}a±0.64	0.76^{a}a±0.05	0.04^{a}a±0.01	1.97^{a}b±0.16	76.43^{a}a±0.55	8.61^{a}b±1.07	0.90^{a}b±0.08
		R	10.29^{a}a±0.46	0.73^{a}ab±0.05	0.02^{a}a±0.02	1.86^{b}b±0.08	75.27^{a}a±1.75	9.14^{a}a±1.76	0.94^{b}b±0.04
2013	Sub-area 1	G	9.83^{a}ac ±0.4	0.68^{a}a ±0.04	0.04^{a} a ± 0.01	2.48^{a}a±0.4	75.42^{a}a±3.22	10.27^{a}a±2.66	0.76^{a}a±0.14
		S	10.23^{a}a±0.69	0.71^{a}a±0.06	0.04^{a}a±0.01	3.97^{a}a±7.18	76.51^{a}a±2.74	9.06^{a}a±2.36	0.75^{a}b±0.08
		R	10.08^{a}a±0.9	0.69^{a}a±0.08	0.04^{a}a±0.01	2.29^{a}a±0.41	76.3^{a}a±1.61	9.27^{a}a±1.7	0.79^{a}a±0.07
	Sub-area 2	G	10.44^{a}a±0.46	0.76^{a}a±0.05	0.03^{a}a±0.01	2.07^{a}a±0.18	77.05^{a}a±2.4	8.35^{a}a±2.49	0.82^{a}b±0.05
		S	10.72^{a}ab±0.37	0.75^{a}a±0.09	0.04^{a}a±0.00	2.03^{a}b±0.35	76.52^{a}a±1.13	8.59^{a}ab±1.08	0.83^{a}a±0.11
		R	10.12^{a}ab±0.5	0.71^{a}a±0.02	0.04^{a}a±0	2.15^{a}ab±0.06	77.73^{ab}a±0.22	7.9^{a}a±0.39	0.75^{a}a±0.09
	Sub-area 3	G	10.59^{a}a±1.88	0.84^{a}a±0.39	0.04^{a}ab±0.03	2.12^{a}a±0.38	76.70^{a}a±2.85	8.07^{a}a±1.2	0.75^{a}a±0.13
		S	10.27^{a}a±1.26	0.79^{a}a±0.29	0.04^{a}a±0.03	2.45^{a}a±0.66	76.94^{a}a±2.39	8.08^{a}b±0.92	0.80^{a}a±0.1
		R	9.72^{a}a±0.34	0.68^{a}a±0.05	0.04^{a}a±0.01	2.20^{a}a±0.29	78.09^{b}b±0.8	7.86^{a}a±0.72	0.79^{a}a±0.06

Table 4. (continued)

			C20:1	C22:0	SAFA	MUFA	PUFA	MUFA/PUFA	O/L
2010	Sub-area 1	G	0.31^{a}a±0.02	0.07^{a}a±0.02	13.28^{a}a±0.47	76.97^{a}a±1.4	9.77^{ab}a±1.12	7.97^{ab}a±1.02	8.64^{ab}a±1.16
		S	0.33^{a}a±0.02	0.07^{a}ab±0.01	12.53^{ab}a±0.87	77.21^{a}a±1.87	10.11^{a}a±0.89	7.70^{a}a±0.78	8.29^{a}a±0.93
		R	0.36^{a}a±0.02	0.07^{a}a±0.01	11.89^{a}a±0.58	77.67^{ab}a±0.68	10.45^{a}ab±0.3	7.44^{ab}ab±0.25	7.96^{a}ab±0.31
	Sub-area 2	G	0.32^{a}a±0.01	0.06^{a}±0.01	12.38^{a}a±0.18	79.10^{a}a±0.88	8.53^{b}a±0.74	9.34^{b}a±0.91	10.33^{b}a±1.1
		S	0.34^{a}a±0.03	0.07^{a}a±0.02	11.88^{b}b±0.6	79.01^{b}a±1.2	9.1^{b}a±0.76	8.74^{b}a±0.78	9.57^{b}a±0.92
		R	0.35^{a}a±0.03	0.07^{a}a±0.02	11.69^{a}a±0.27	78.98^{b}a±0.67	9.35^{b}a±0.42	8.47^{b}a±0.46	9.25^{b}a±0.53
	Sub-area 3	G	0.32^{a}abc±0.01	0.06^{a}ad±0.01	12.79^{a}a±1.16	76.51^{a}ab±2.48	10.70^{a}ab±1.38	7.27^{a}ab±1.17	7.85^{a}a±1.38
		S	0.33^{a}a±0.02	0.07^{a}a±0.01	13.09^{a}a±1.23	76.09^{a}ab±2.56	10.84^{a}a±1.61	7.23^{a}ab±1.46	7.78^{a}ab±1.73
		R	0.34^{a}a±0.02	0.07^{a}a±0.01	12.72^{a}ab±1.41	76.74^{a}ab±2.38	10.54^{a}a±1.18	7.39^{a}a±1.09	7.94^{a}a±1.29
2011	Sub-area 1	G	0.37^{a}b±0.02	0.07^{a}a±0.00	11.62^{a}b±0.28	74.92^{a}a±0.94	13.41^{a}b±1.22	5.62^{a}b±0.61	5.94^{a}b±0.67
		S	0.35^{a}a±0.02	0.08^{a}b±0.01	12.41^{a}a±0.59	76.14^{a}a±1.84	11.39^{a}a±1.97	6.86^{a}a±1.39	7.39^{a}a±1.6
		R	0.37^{a}a±0.02	0.07^{a}a±0	11.80^{a}a±0.34	75.51^{a}b±1.52	12.64^{a}b±1.66	6.09^{a}b±1.05	6.47^{a}b±1.18
	Sub-area 2	G	0.34^{a}b±0	0.09^{a}a±0.03	12.20^{a}a±0.93	77.93^{a}a±1.57	9.83^{a}a±1.67	8.08^{a}ab±1.38	8.72^{b}ab±1.56
		S	0.33^{a}a±0.03	0.10^{a}a±0.03	13.72^{a}b±2.79	75.90^{a}b±5.05	10.29^{a}b±2.75	7.89^{a}a±2.29	8.49^{a}a±2.62
		R	0.33^{a}a±0.05	0.08^{a}a±0.01	12.81^{a}ab±2.01	77.13^{a}ab±1.6	10.00^{a}a±3.21	8.62^{a}a±3.81	9.40^{a}a±4.42
	Sub-area 3	G	0.34^{a}b±0.03	0.10^{a}b±0.03	13.31^{a}a±2.13	75.24^{a}b±1.67	11.39^{a}b±1.67	6.75^{a}b±1.14	7.21^{a}a±1.17
		S	0.33^{a}a±0.03	0.09^{a}a±0.02	14.27^{a}a±2.62	74.01^{a}b±3.29	11.66^{a}a±1.98	6.55^{a}b±1.47	6.94^{a}b±1.65
		R	0.33^{a}a±0.03	0.11^{a}b±0.03	13.71^{a}b±2.24	76.56^{a}ab±3.65	9.67^{a}a±2.73	8.81^{a}a±3.7	9.61^{a}a±4.46
2012	Sub-area 1	G	0.25^{a}c±0.02	0.07^{a}ab±0.03	13.10^{a}ab±1.07	77.4^{ab}a±1.06	9.57^{ab}a±1.56	8.23^{ab}a±1.3	8.82^{ab}a±1.53
		S	0.24^{a}b±0.01	0.05^{a}ac±0.02	12.97^{a}a±0.33	77.09^{a}a±1.55	10.00^{a} a±1.41	7.82^{a}a±1.2	8.43^{a}a±1.32
		R	0.25^{a}b±0.03	0.05^{a}ab±0.01	12.46^{a}a±0.91	76.39^{a}ab±1.43	11.18^{a}ab±1.13	6.9^{a}b±0.83	7.34^{a}ab±0.88
	Sub-area 2	G	0.27^{ab}c±0.01	0.06^{a}ab±0.01	12.99^{a}a±0.76	75.81^{b}a±1.71	10.54^{b}a±0.87	7.24^{b}b±0.73	7.76^{b}b±0.85
		S	0.24^{a}b±0.03	0.09^{a}a±0.03	13.10^{a}b±1.23	78.42^{a}ab±1.16	8.51^{a}ab±2.1	9.93^{a}a±3.4	10.9^{a}a±4.01
		R	0.26^{a}b±0.01	0.07^{a}a±0.02	14.07^{a}b±1.48	76.18^{b}b±1.59	9.74^{a}a±1.39	7.96^{a}a±1.27	8.57^{a}a±1.5
	Sub-area 3	G	0.29^{b}c±0.02	0.07^{a}b±0.01	13.13^{a}a±0.54	78.36^{a}a±0.6	8.92^{a}a±0.73	8.84^{a}a±0.75	9.66^{a}a±0.89
		S	0.28^{a}b±0.02	0.02^{a}b±0.04	12.78^{a}a±0.69	77.54^{a}ac±0.57	9.51^{a}b±1.12	8.31^{a}ac±1.44	9.06^{a}ac±1.64
		R	0.26^{a}b±0.08	0.01^{b}c±0.02	12.40^{b}a±0.51	76.33^{a}b±1.75	10.08^{a}a±1.78	7.89^{a}a±1.95	8.67^{a}a±2.40
2013	Sub-area 1	G	0.23^{a}c±0.01	0.02^{a}b±0.03	12.6^{a}ab±0.66	76.38^{a}a±3.19	11.04^{a}ab±2.67	7.26^{a}a±1.73	7.75^{a}a±1.97
		S	0.21^{a}b±0.03	0.04^{a}c±0.02	14.51^{a}a±7.23	77.48^{a}a±2.74	9.81^{a}a±2.38	8.29^{a}a±1.82	8.92^{a}a±2.06
		R	0.22^{a}b±0.03	0.04^{a}b±0.02	12.68^{a}a±0.71	77.27^{a}ab±1.63	10.05^{a}a±1.7	7.89^{a}a±1.38	8.49^{a}a±1.58
	Sub-area 2	G	0.19^{b}d±0.01	0.02^{a}b±0.03	12.78^{a}a±0.4	78.05^{a}a±2.37	9.17^{a}a±2.53	8.97^{a}a±2.23	9.80^{a}a±2.58
		S	0.21^{a}b±0.02	0.04^{a}b±0.02	13.05^{a}b±0.26	77.54^{a}ab±1.16	9.42^{a}ab±1.05	8.32^{a}a±1.00	9.03^{a}a±1.18
		R	0.22^{ab}b±0.02	0.04^{a}b±0.00	12.60^{a}ab±0.45	78.73^{ab}a±0.22	8.65^{a}a±0.32	9.11^{ab}a±0.33	9.85^{ab}a±0.47
	Sub-area 3	G	0.24^{a}d±0.02	0.04^{a}d±0.03	13.07^{a}a±1.76	77.86^{a}a±2.45	8.82^{a}a±1.11	8.98^{a}a±1.33	9.73^{a}a±1.68
		S	0.23^{a}c±0.03	0.04^{a}b±0.02	13.08^{a}a±1.6	78.04^{a}c±2.13	8.88^{a}b±0.93	8.90^{a}c±1.08	9.66^{a}c±1.28
		R	0.25^{b}b±0.02	0.04^{a}d±0.03	12.25^{a}a±0.3	79.09^{b}a±0.84	8.65^{a}a±0.71	9.20^{b}a±0.81	10.01^{b}a±0.97

-Significant differences in the same column are indicated with different lowercase letters (comparison among crop seasons at the same maturation stage, $p < 0.05$).

-Significant differences in the same row are indicated with different superscript letters (comparison between sub-areas at the same maturation stage, $p < 0.05$).

- Meaning of letters used to contain the size of the table: G: green; S: spotted; R: ripe.

Furthermore, as can be seen in Table 4, Meknès olive oil samples are characterized by high MUFA/PUFA and oleic/linoleic ratios. Other particular characteristic of the oils under evaluation is the predominance of monounsaturated oleic acid compared to polyunsaturated linoleic and linolenic acids; this fact can confer to Meknès olive oils a high nutritional value and a high oxidative stability. If these results are compared with those found for olive oils of *Picholine marocaine* variety cultivated in southern (El Antari et al., 2003) and central (Houlali et al., 2014) Moroccan zones, it is possible to highlight that olive oils from Meknès region present much higher values of MUFA and MUFA/PUFA ratios. Moreover, in Meknès olive oil samples, the overall ranges observed in this study were: 8.96-11.85% for palmitic acid, 0.65-1.08% for palmitoleic acid, 0.02-0.06% for margaric acids, 1.86-3.97% for stearic acid, 72.48-78.09% for oleic acid, 7.61-12.53% for linoleic acid, 0.73-0.94% for linolenic acid, 0.19-0.37% for eicosanoid acid and 0.01-0.11% for behinic acid. All these levels fulfill the requirements of the IOC regulation for a VOO (IOC, 2011). As observed for the parameters evaluated in previous sections, the fatty acid profile (Table 4) showed some significant differences mainly due to the crop season.

Furthermore, PCA was applied to a matrix composed by 23 analytical parameters (corresponding to the determined physiochemical quality indices and compositional parameters) for the 298 samples to evaluate the overall data structure and try to identify possible samples clustering based on the sub-area of origin. After applying PCA, six principal components were extracted, using varimax method. Percentages of variance explained by each PC were 19.51%, 18.37%, 11.50%, 8.25%, 7.36% and 5.90%, respectively. In Figure 1 (Supplementary material), a scatter plot of PC1 vs. PC2 (describing 37.88% of the sample variability) is shown. As can be seen, an overlapping of the samples coming from the different areas is observed and no grouping of VOOs according to the geographical origin can be achieved.

3.4 Sensory evaluation

Taking into account their sensory quality, all the 48 analyzed samples were classified as Extra VOOs, according to IOC regulations (IOC, 2010a), since the median of the defects was equal to 0 and the median of the fruity above 0. Figure 3 depicts the

standard/average sensory profile of the studied samples, having two categories: intense fruitiness and medium fruitiness.

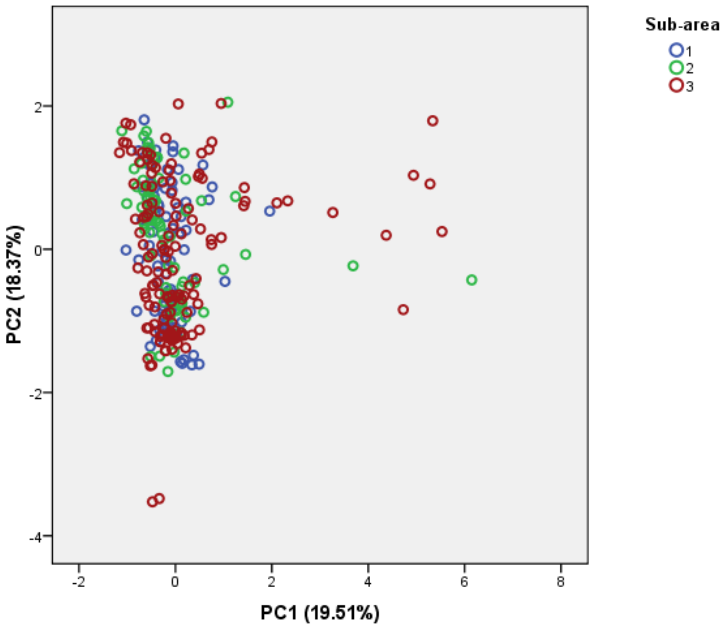

Figure 1-supplementary material: Score plot for the first two principal components of all the 298 olive oil samples coming from the three studied Meknès sub-areas.

Indeed, on basis of the fruity attribute and according to the IOC regulation, 28 samples were classified into the fruity intense category because they showed fruitiness superior to 6, whilst 20 samples were classified into the medium fruitiness category, since their fruity attribute was between 3 and 6. Furthermore, the positive attributes, fruity, bitterness, pungency, tomato, apple and almond were perceived in all analyzed samples by all the tasters. Slight variations of these attributes were observed over the four years of the study and were mainly due to the crop season.

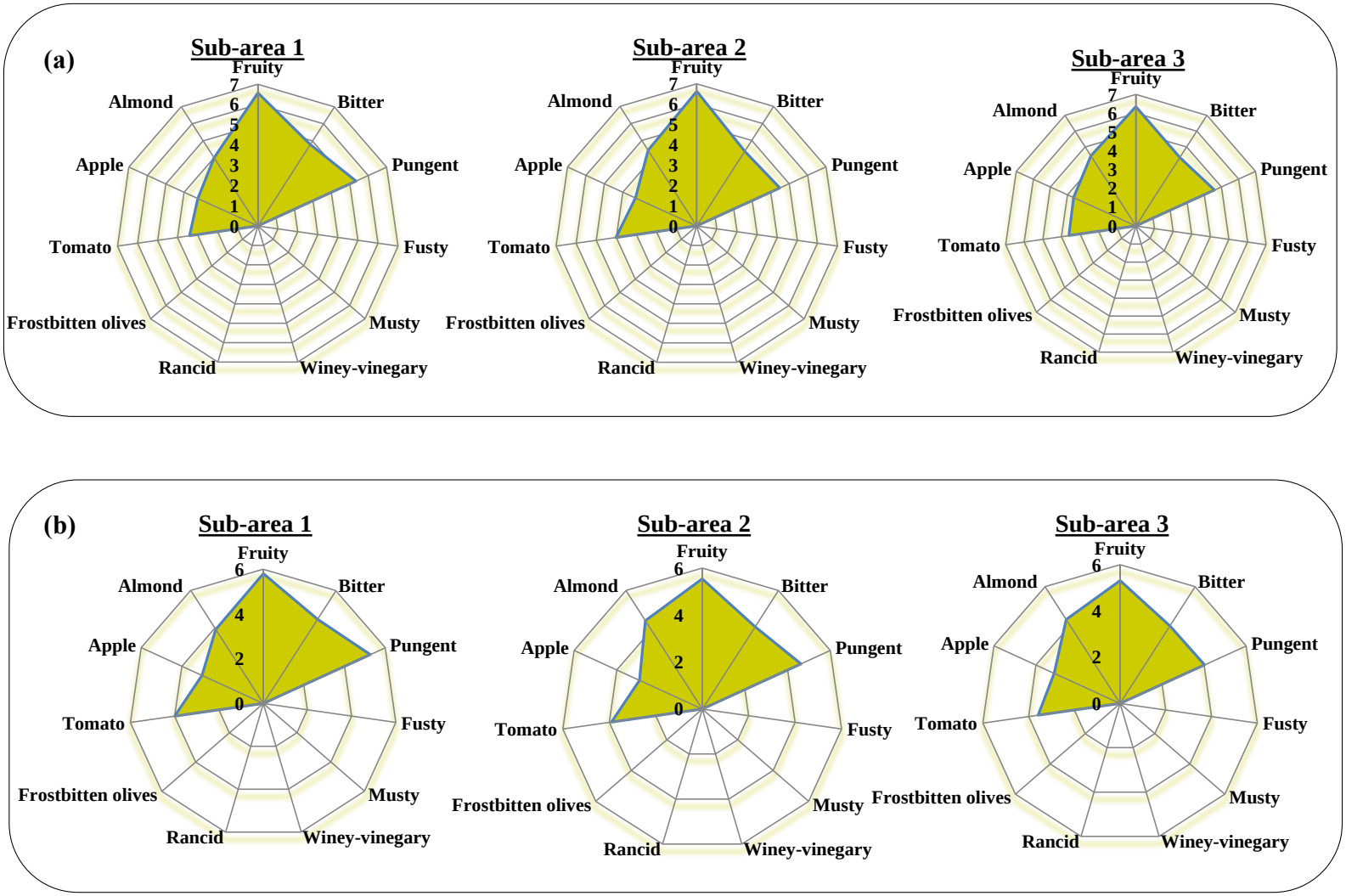

Figure 3: Sensory averaged profiles of Meknès monovarietal olive oils, (a) intense fruitiness profile, and (b) medium fruitiness profile

4 Conclusions

There is a great interest in foods that belong to PDOs because the quality and other essential and exclusive characteristics are guaranteed due to a particular geographical environment. However, to demonstrate the uniqueness and high-standards of quality of the products under this label is a very challenging task.

This work is the first pluri-annual characterization of the physicochemical and sensorial quality and composition profiles of Meknès olive oils. With the results obtained in this study, it is possible to conclude that Meknès region seems to produce olive oil with high physicochemical and sensory standards of quality. In terms of chemical composition, depending on the maturation stage of processed olive fruits, the obtained oils show a medium to very high content in total phenol, low to medium content in tocopherol and a pigment content that decrease over ripening. The acidic composition of Meknès olive oils shows a high content of monounsaturated fatty acids. The quantitative differences observed in all the analyzed parameters could be attributed to crop season conditions rather than sub-area of production.

The current study could contribute to the establishment of a PDO for Meknès VOOs, since includes a comprehensive characterization of the VOOs produced in this area and an appropriate evaluation of their homogeneity.

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Chapter

13

A comprehensive 3-years study of the phenolic profile of Moroccan monovarietal virgin olive oils from Meknès region

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Abstract

The phenolic fraction of monovarietal virgin olive oils (VOOs) from the main Moroccan cultivar 'Picholine Marocaine' (142 samples coming from three different sub-areas of Meknès region) was studied over three consecutive crop seasons (2011, 2012 and 2013) using a powerful LC-MS methodology. First, LC-ESI-TOF MS was used to get a comprehensive characterization of the phenolic fraction; afterwards, LC-ESI-IT MS was utilized for further identification (MS/MS experiments) and quantitation purposes. A total of 28 phenolic compounds (and quinic acid) were determined, revealing the complex profile of Meknès VOO, composed, in order of abundance, by secoiridoids, phenolic alcohols, lignans, flavonoids and phenolic acids. Tukey´s test was applied to ascertain possible significant intra-regional and/or inter-annual variations of the phenolic content of the Meknès VOOs under study. Results showed that the content on phenolic compounds was mainly related to the crop season.

Keywords: phenolic compounds; virgin olive oil; liquid chromatography-mass spectrometry; 'Picholine Marocaine'; inter-annual and intra-regional study.

INTRODUCTION

The way in which virgin olive oil (VOO) is obtained, exclusively by mechanical extraction from the fruit of *Olea europeae L.* (*Oleaceae* family) [1], provides this matrix with a very distinctive composition, as well as a unique aroma and taste properties which make it especially appreciated by consumers. Moreover, apart from been one of the oldest known vegetable edible oils in the Mediterranean countries, it has been considered as "the great therapeutic" [2], since Hippocrates called it so, due to its relevant beneficial effects on human health.

The health-promoting effects attributed to VOO are, nowadays, increasingly supported by scientific evidences; indeed, VOO consumption has been associated with a lower incidence of certain diseases, such as different types of cancer [3], coronary heart diseases [4], obesity, metabolic syndrome and diabetes [5]. These evidences have prompted the recognition by the European Union in 2012 that a daily intake of 20 g of VOO containing, at least, 5 mg of hydroxytyrosol and its derivatives, contributes to improve

human health and well-being [6]. Furthermore, this regulation has authorized health claims on VOOs labels.

It has been shown that the predominance of monounsaturated fatty acids on VOO composition is closely related to its medicinal properties; however, it has been also demonstrated that the presence of some other bioactive compounds, such as phenolic compounds, tocopherols, pigments and phytosterols [7], is of great importance in that regard. Among these components, phenolic compounds have acquired a well-deserved reputation, since they contribute to lengthen the oxidative stability of VOO [8,9], are partially responsible of some sensory attributes (they impart bitterness, pungency and astringency to VOO [10,11]), and are secondary metabolites with remarkable biological activities, including anticancer [3,12], anti-bacterial [13], anti-inflammatory and anti-oxidant effects [14,15]. As a consequence, phenolic compounds are, nowadays, among the most studied constituents of VOO. This group of analytes is very heterogeneous, with a wide range of phenolic compounds belonging to different chemical classes, mainly to: phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavonoids [10].

Over the past decade, the structural and quantitative analysis of the individual phenolic compounds present in VOO has attracted a lot of attention, and numerous methodologies have been developed and used for applications such as the characterization of VOOs from different olive varieties, evaluation of the influence of agro-technological parameters on the final composition of the oils, assessment of the bioavailability of some relevant phenols, or the determination of the geographical origin of samples obtained from cultivars grown in different countries, among others. Even though Morocco is currently the VOO world's 6th largest producer, it can be stated that the information about the phenolic compounds levels and properties of Moroccan VOOs from different regions [16] is very scarce. Meknès region, located in the north-center of Morocco, is considered as one of the most relevant and productive areas of the whole country. Its favorable edaphoclimatic conditions and the fact that new farming practices and olive oil processing techniques have been embraced over the last years, contribute to assure the production of VOO with high quality. The annual production of VOO has experienced a substantial increase, being over the last years, between 20,000 and 32,000 tons per year [17]. However, in spite of the importance of VOO production in the above

mentioned Meknès region, there is practically no detailed pluri-annual qualitative and quantitative characterization of the phenolic fraction of VOOs produced in this region.

Therefore, the aim of this work was to solve the lack of information in this regard, carrying out the comprehensive characterization and evolution of the phenolic fraction of Meknès VOOs over three consecutive seasons. A powerful LC-MS (using different analyzers) was used and a total of 142 samples (coming from 3 diverse areas) were analyzed. The possible effect of inter-annual and intra-regional variations on the content of the identified phenolic compounds was also evaluated. To our best of knowledge, this work represents the first pluri-annual study reporting data on the phenolic fraction of VOOs from a Moroccan region.

MATERIALS AND METHODS

Olives sampling

Monovarietal virgin olive oils from the most relevant Moroccan cultivar Picholine marocaine were considered. A total of 142 olive fruit samples were collected over three consecutive seasons (harvesting period 2011-2013) from three sub-areas of Meknès region (Figure 1). Sampling was carried out from middle of October until the end of December. Olive samples (one sample is approximately 35 kg of olives) were handpicked in olive groves located in the three selected sub-areas belonging to Meknès region. Only good quality, fresh and healthy fruits were selected. Ripening stage -for being a parameter with a great influence on olive oil characteristics- was evaluated by determining the ripening index following the method developed at the Agronomic Station of Jaén, based on the evaluation of the olive skin and pulp colors [18]. After harvesting, the olive fruit samples were immediately transported to the laboratory in ventilated rectangular plastic crates and the oil was extracted within 24 h.

Oil extraction

Oil extraction was carried out in a laboratory instrument, SPREMOLIVA C30 Oliomio (Oliomio, Italy) with a work capacity of 30 kg/h, and equipped with a knife crusher, an horizontal malaxator and a two phase decanter. Fruits were processed as follows: olives were washed to eliminate any foreign material and poured into the receiving hopper, where a screw feeds the crusher that is equipped with fix hole grid and groove knives impeller at a temperature of 25-27 °C. The paste produced falls into the malaxing part;

malaxing was carried out for 45 min at 28-30 °C. The resulting olive paste was decanted at temperature of 23-27 °C without the addition of any water. All the samples were subsequently filtered, placed in amber glass bottles and stored in the dark at 4 °C.

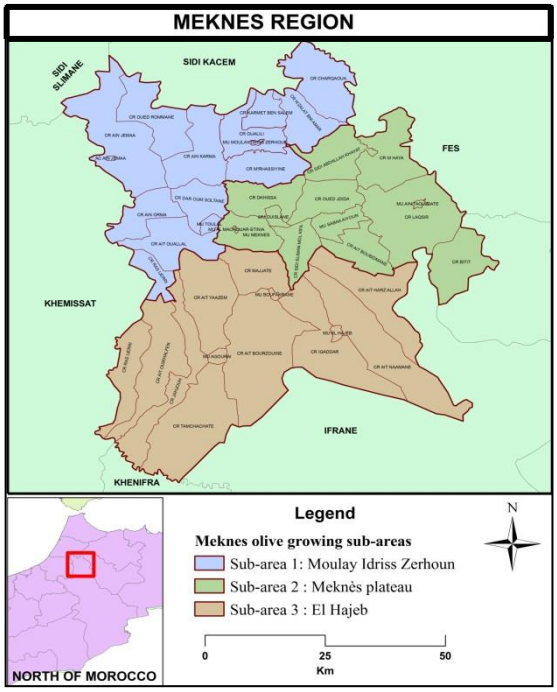

Figure 1. Localization map of Meknès region showing the three sub-areas considered within the current study.

Physicochemical quality indices

Determination of free fatty acids content (given as percentage of oleic acid), peroxide value (expressed as milliequivalents of active oxygen per kilogram of olive oil (meq O_2/kg)) and K_{232} and K_{270} extinction coefficients calculated from absorption at 232 and 270 nm, respectively, were carried out by using the analytical methodologies described in the European Union Standard Methods Regulations 2568/91 and the subsequent amendments [19]. All the parameters were determined in triplicate for each sample.

Phenolic compounds analysis

Chemicals and reagents

All the reagents were of analytical grade and used as received. Methanol and *n*-hexane of HPLC grade supplied from Panreac (Barcelona, Spain) were used for the extraction of the phenolic compounds from the olive oil samples. Acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Panreac (Barcelona, Spain) were used for preparing the mobile phases. Water was deionized using a Milli-Q-system (Millipore, Bedford, MA,

USA). Several phenolic standards were used for identification and quantification purposes, namely hydroxytyrosol, tyrosol, luteolin, apigenin, *p*-coumaric, quinic acid, ferulic and caffeic acids were supplied by Sigma-Aldrich (St. Louis, MO, USA) and (+)-pinoresinol was purchased from Arbo Nova (Turku, Finland). Oleuropein was acquired from Extrasynthese (Lyon, France). 3,4-dihydroxyphenylacetic acid (DOPAC) was used as internal standard and supplied by Sigma-Aldrich (St. Louis, MO, USA).

Stock standard solutions of individual compounds at a concentration of 500 mg/L were prepared by exact weighing of the appropriate amount of the pure compound and dissolving it in methanol. These solutions were stored at -20 ºC in the dark. Multi-analyte working standard solutions at different concentration levels within the range 0.5-250 ppm were prepared by appropriate dilutions of the stock solutions and stored in screw-capped glass tubes at -20 ºC in the dark.

Phenolic compounds extraction

The extraction of the phenolic compounds was carried out as previously described by Bajoub et al. [16], with little modifications. Briefly, 2 g of olive oil and 0.025 mL of internal standard solution (at a concentration of 500 mg/L) were extracted (repeating the same process three times in a row), adding every time 1 mL of *n*-hexane and 2 mL of methanol/water (60/40); the extract was centrifuged at 3500 rpm for 6 min. The combined extracts were evaporated to dryness using a rotary evaporator under reduced pressure and a temperature of about 30 °C. The residue was reconstituted up to 1 mL with methanol and filtered through a 0.20 µm membranes (Nylon) filters.

Chromatographic separation

In a first step and for initial characterization purposes of the phenolic fraction of the studied samples, the analyses were carried out on a Waters LC Acquity system (LC, Acquity H-Class) (Milford, MA, USA) equipped with a Waters Acquity quaternary solvent manager (QSM), an Acquity sample manager (SM), a column heater, a degassing system, a diode-array detector and an ESI-Time of Flight MS detection system. Afterwards, an Agilent 1260-LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, degasser, quaternary pump, thermostated column compartment, a diode-array detector, and an ESI-Ion Trap MS detection system was used for further identification and quantification purposes. In both cases, the analysis of phenolic

compounds was performed by following a previously reported methodology [16]. Separation was achieved on a Zorbax C18 analytical column (4.6 x 150 mm, 1.8 µm particle size), protected by a guard cartridge of the same packing. The injection volume was 10 µL in every case. The binary mobile phase consisted of water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B) and was delivered at a flow rate of 0.8 mL/min. Gradient elution was performed using the following solvent gradient: 0 to 10 min, 5% B; 10 to 12 min, 30% B; 12 to 17 min, 38% B; 17 to 20 min, 50% B; 20 to 23 min, 95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 2.5 min. As stated before, the separated compounds were monitored in sequence first with DAD (240, 280 and 330 nm), and then with a mass spectrometry detector (ESI-TOF MS or ESI-IT MS).

ESI-TOF MS and ESI-IT MS conditions

To avoid the introduction of humidity into the system and achieve stable electrospray ionization and reproducible results, the flow delivered into the MS detectors from LC was reduced to 0.2 mL/min using a flow divisor 1:4. MS detection was firstly performed in a Synapt G2 time-of-flight (TOF) mass spectrometer (Milford, MA, USA) equipped with an electrospray interface. Spectra were recorded in negative mode, operating within the range of 50-1200 Da, and at a scan speed of 240 ms. The source temperature was set at 100 ºC with a cone gas flow of 800 L/h, a desolvation gas temperature of 500 ºC, and a desolvation gas flow of 100 L/h. The capillary voltage was set at 2.2 kV. Internal calibration was performed using sodium formate cluster by using a solution containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 v/v. The calibration solution was injected at the beginning of the run and all the spectra were calibrated prior carrying out the compound identification. The accurate mass data of the molecular ions were processed through the software MassLynx 4.1 (Waters), which provided a list of possible elemental formulas, giving a Fit Conf (%) parameter that shows the prediction confidence.

After characterizing the phenolic fraction of the samples under study by TOF MS, a Bruker Daltonics Esquire 2000TM ion trap (Bruker Daltonik, Bremen, Germany) was used. The phenolic extracts were injected using the chromatographic conditions described above and both Full Scan (for quantitation purposes) an auto MS/MS (for

further characterization purposes, in both negative and positive polarity). As far as MS/MS conditions are concerned, two methods were used: in the first one, the selected fragmentation energy (using nitrogen (N_2) as collision gas) was automatically applied to all the ions that overtook the threshold intensity of 15,000; in the second method, however, the analytical window was divided into two segments and different threshold intensities were fixed for each one (the first one up to 12.5 min with an intensity threshold of 10,000 and, the second one, from 12.5 min till the end of the run, with a threshold intensity of 27,000). *Full Scan* mass spectra (50-800 *m/z*) were recorded in negative mode using a capillary voltage of 3.2 kV. The nebulizer gas was set at 30 psi, dry gas at 9 L/min, and drying gas (N_2) temperature at 300 °C. MS data were processed through Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany).

Phenolic compounds identification and quantification

Peak identification was done bearing in mind retention time, spectral data and ESI-TOF MS and ESI-IT MS/MS information obtained from olive oil samples and pure standards. We also used the previously reported information [12]. The linearity of the detector response was verified with standard solutions at 11 different concentration levels over the range from the quantification limit to 250 ppm (0.5; 1; 5; 12.5; 25; 35; 50; 100; 150; 200 and 250 mg/L). Each point of the calibration graph corresponded to the mean value from three independent replicate injections. Calibration curves were obtained for each standard by plotting the standard concentration as a function of the peak area obtained from LC-ESI-IT MS analyses. The following equations were obtained for each calibration curves: apigenin ($y = 298095x + 53202$; $r^2 = 0.999$); caffeic acid ($y = 121649x + 45541$; $r^2 = 0.995$); ferulic acid ($y = 69884x + 26610$; $r^2 = 0.992$); hydroxytyrosol ($y = 117686x-12928$; $r^2 = 0.998$); luteolin ($y = 259366x + 17767$; $r^2 = 0.999$); oleuropein ($y = 20535.20x+5444.60$; $r^2 = 0.995$); *p*-coumaric acid ($y = 82728x+23247$; $r^2 = 0.997$); pinoresinol ($y = 79815+44395$; $r^2 = 0.997$); quinic acid ($y = 77315x-6950.41$; $r^2 = 0.997$) and tyrosol ($y = 33975x+9524.70$; $r^2 = 0.995$). The compounds, for which pure standards were not available, were quantified on the basis of other analytes with similar chemical structures. In particular, secoiridoids were quantified in terms of oleuropein, lignans in terms of pinoresinol and oxidized hydroxytyrosol was quantified by comparison with hydroxytyrosol.

Statistical analysis

As the number of samples selected in the current study was considerably high (142 samples coming from different areas over 3 seasons), the quantitative data have been reported as mean ± standard deviation (SD) values of triplicate experiments, and were analyzed using SPSS 20.0. One-way analysis of variance (ANOVA) and Tukey´s test (with a significance level of 0.05) were used to determine the significance of the difference among the studied parameters regarding the sub-area and the crop season.

RESULTS AND DISCUSSION

Ripening and quality indices

Table 1 summarizes the mean ± standard deviation, and maximum and minimum values, of ripening index and the regulated quality criteria (free fatty acids, peroxide value, K_{232} and K_{270}) for all the olive oil samples extracted from fruits of Picholine marocaine cultivar population grown in the areas considered within the current study. As can be seen, the ripening index values of the olives fruits used for the extraction of the olive oils were between a minimum value of 1.14 (crop season 2013) and a maximum value of 6.78 (crop season 2012). This wide range allowed evaluating the phenolic composition of the olive oil samples at three main maturation stages: green, spotted and ripe. Furthermore, the ripening index was practically unaffected by the olive cultivation sub-area or the crop season over the studied period. Therefore, the effect of the maturity stage on the differences found for the other studied parameters (in particular, for the phenolic composition) can be dismissed.

As far as the quality indices are concerned, and taking into account the limits set by the Commission of the European Community for the olive oil classification [19], the 97.18% of the studied samples were falling within the "Extra-virgin" category, and the rest (2.82%) were found within the "Virgin" category. This result is not surprising since the raw material was carefully selected, picked and processed. Thus, as shown in Table 1, mean values for free fatty acids content (expressed as percentage of oleic acid) ranged from 0.18 up to 0.46%. Only 4 olive oil samples showed a value higher than 0.8%, which is the upper threshold limit established for the Extra VOO category; none exceeded the limit of 2% for the "Virgin" category [19].

Table 1. Average Values and Intervals of Ripening Index and Quality Parameters (Free Fatty Acids (FAAs), Peroxide Values (PV) and Ultraviolet Absorbance at 232 nm (K_{232}) and 270 nm (K_{270})) Determined for Meknès Monovarietal Olive Oils Samples Over the Three Crop Seasons Considered (2011, 2012 and 2013).

			Sub-area 1			Sub-area 2			Sub-area 3			Category	
			Mean ± S.D	Max	Min	Mean ± S.D	Max	Min	Mean ± S.D	Max	Min	Extra- Virgin	Virgin
2011 (n = 43)	Ripening Index		3.16Aa±1.25	4.85	1.67	2.99Aa±0.95	4.63	1.60	2.96Aa±1.19	5.60	1.44	41	2
	Quality indices	FAAs (% oleic acid)	0.31Aa±0.07	0.41	0.21	0.31Aa±0.13	0.62	0.20	0.46Ba±0.20	0.86	0.22		
		PV (meq O_2/kg)	8.18Aa±0.46	8.89	7.35	8.69Aa±0.69	9.50	7.46	8.11Aa±0.84	9.75	6.80		
		K_{232}	1.28Aa±0.17	1.59	1.09	1.24Aa±0.16	1.54	1.02	1.28Aa±0.18	1.65	1.03		
		K_{272}	0.13Aa±0.02	0.16	0.08	0.14Aa±0.03	0.16	0.08	0.13Aa±0.02	0.16	0.08		
2012 (n = 34)	Ripening Index		3.52Aa±1.67	6.29	1.47	3.27Aa±1.43	6.78	1.40	3.43Aa± 0.75	4.26	2.40	34	0
	Quality indices	FAAs (% oleic acid)	0.20Ab±0.08	0.38	0.12	0.22Aa±0.06	0.35	0.11	0.18Ab± 0.04	0.23	0.11		
		PV (meq O_2/kg)	5.55Ab±1.13	8.00	4.50	7.53Ba±2.19	5.00	12.00	6.71ABa±3.40	12.50	3.50		
		K_{232}	1.40Ab±0.23	1.74	1.03	1.49Ab±0.18	1.74	1.20	1.37Aab±0.23	1.73	1.08		
		K_{272}	0.14Aa±0.04	0.21	0.09	0.15Aa±0.04	0.18	0.09	0.14Aa±0.02	0.16	0.11		
2013 (n=65)	Ripening Index		3.12Aa±0.93	5.45	1.14	2.56Aa±0.93	4.12	1.56	3.42Aa± 1.37	6.72	1.75	63	2
	Quality indices	FAAs (% oleic acid)	0.24Ab±0.11	0.51	0.11	0.26Aa±0.12	0.42	0.12	0.26Ac± 0.15	0.89	0.12		
		PV (meq O_2/kg)	9.17Aa±2.59	15.00	5.00	7.94Aa±2.48	12.50	5.00	7.73Aa± 2.60	15.00	5.00		
		K_{232}	1.60Ab±0.36	1.78	1.12	1.61Ab±0.36	1.85	1.12	1.52Ab± 0.28	1.95	1.12		
		K_{272}	0.15Aa±0.03	0.17	0.10	0.15Aa±0.03	0.18	0.09	0.14Aa± 0.04	0.18	0.09		
									Total			**138 (97.18%)**	**4 (2.82%)**

[a] *Significant differences in the same row are indicated with different superscript letters (comparison between sub-areas at the same crop season, $p < 0.05$). Significant differences in the same column are indicated with different lowercase letters (comparison among crop seasons, $p < 0.05$).*

[b] *In the current Table and Table 3 the mean values are those calculated for all the samples coming from the same area, therefore, SD gives to the reader only an idea about the variability of the olive oils in terms of composition, and obviously not about the repeatability of the analytical methods used.*

The peroxide value was lower than the upper limit of 20 meq O_2/kg established for "Extra-virgin" category for every sample, ranging from 5.55 to 9.17 meq O_2/kg. Likewise, all the values of spectrophotometric UV absorption at 232 nm (K_{232}) and 270 nm (K_{270}) were below the limits of 0.22 and 2.25, respectively, established for the "Extra-virgin" category. Their mean values ranged from 1.24 up to 1.61 and 0.13 up to 0.15, for K_{232} and K_{270}, respectively. Results of ANOVA test revealed that no statistically significant differences were observed for the quality indices according to production sub-area. These results are in good agreement with the findings of other authors [20,21], who claimed that these analytical parameters are mainly affected by factors causing damage to the fruits, such as olive fly attacks or not appropriate harvesting practices, transport and storage of olives.

Phenolic compounds profiling

Qualitative characterization

Figure 2 shows a typical LC-ESI-TOF MS profile of a phenolic extract of Picholine marocaine, where the Extract Ion Chromatograms (EICs) of the determined compounds are shown. The identification of these compounds was performed considering the information provided by the TOF MS detector, the information collected from MS/MS experiments in negative and positive modes and the use of commercial pure standards (when available). The components were efficiently separated, and it was possible to identify 29 peaks, corresponding to a polar no phenolic compound (quinic acid), and 28 phenolic substances (belonging to five different chemical categories) (see Table 2). The phenolic profile of Meknès olive oil samples was dominated by the presence of a high number of secoiridoids, mainly oleuropein aglycone (Ol Agl), ligstroside aglycone (Lig Agl), and 6 and 3 isomers of each one, respectively, decarboxymethyl oleuropein aglycone (DOA), dehydro oleuropein aglycone (Dehydro Ol Agl), methyl oleuropein aglycone (Methyl Ol Agl), methyl decarboxymethyl oleuropein aglycone (Methyl D-Ol Agl), decarboxymethyl ligstroside aglycone (D-Lig Agl), dialdehydic form of decarboxymethyl elenolic acid (D-Ald-D EA), desoxy elenolic acid (Desoxy-EA) and elenolic acid (EA). Besides, it was also possible to determine various simple phenols (hydroxytyrosol (Hyt), oxidized hydroxytyrosol (O-Hyt) and tyrosol (Ty)), lignans ((+)-pinoresinol (Pin), (+)-1-

acetoxypinoresinol (Ac-Pin) and syringaresinol (Syr)), flavonoids (luteolin (Lut) and apigenin (Apig)), and a phenolic acid (*p*-coumaric acid).

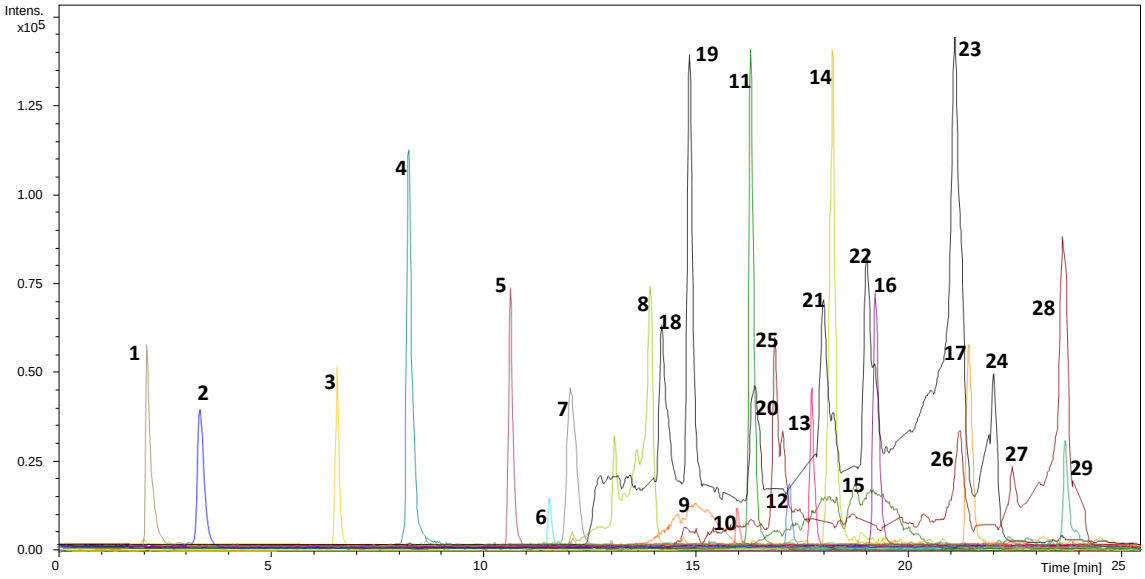

Figure 2. Extracted ion chromatograms (EICs) of the main phenolic compounds identified in Meknès VOOs. Peak identification: 1, quinic acid, 2, O-Hyt; 3, Hyt; 4, Ty; 5, D-Ald-D EA; 6, *p*-cou; 7, Deoxy-EA; 8, EA; 9, DOA; 10, Syr; 11, Lut ; 12, Pin; 13, Ac-Pin; 14, Methy D-Ol Agl; 15, D-Lig Agl;16, Apig; 17, Dehydro Ol Agl; 18, Ol Agl isomer 1; 19, Ol Agl isomer 2; 20, Ol Agl isomer 3; 21, Ol Agl isomer 4; 22, Ol Agl isomer 5; 23, Ol Agl; 24, Ol Agl isomer 6; 25, Lig Agl isomer 1; 26, Lig Agl isomer 2; 27, Lig Agl isomer 3; 28, Lig Agl; 29, Methyl Ol Agl.

Variation of the phenolic compounds content over the studied period

It is well established that phenolic content in olive oil widely depends on intrinsic (genetic) and extrinsic (geographical, seasonal, agronomic, environmental, maturation stage, processing and storage conditions) factors [22-25]. In the current study, the effect of the harvest season and production sub-area on the composition of monovarietal olive oil from Meknès region (in terms of phenolic compounds) was evaluated (Table 3). Moreover, the total content for each chemical class (expressed in mg/kg) was calculated as the sum of the content of the individual phenols belonging to that chemical category (Table 3). It is possible to highlight the existence of an important intra-sub-zone variability, as attested by the wide range of obtained values. This variability can be the result of the genetic diversity of the cultivar Picholine marocaine. Indeed, several studies carried out in order to get the comprehensive genetic characterization of this cultivar, have reported that it includes several distinct clones and local genotypes all known under the common name 'Picholine Marocaine' [26,27].

Table 2. Phenolic compounds in Extracts from Meknes VOOs identified by LC–TOF MS and LC-IT/MS/MS analysis

Compounds	Retention time (min)	Molecular formula	m/z experimental	m/z calculated	Error (mDa)	Fit Conf (%)	Fragments of MS/MS Spectra
Quinic acid	2.06	C7 H12 O6	1.910.568	1.910.556	1.2	100	191; 127.0;93.1
Oxidized hydroxytyrosol	3.17	C8 H8O3	1.510.384	1.510.395	-1.1	100	151.1;122.9;169.9
Hydroxytyrosol	6.74	C8 H10 O3	1.530.557	1.530.552	0.5	100	153.0;123
Tyrosol	8.49	C8 H10 O2	1.370.591	1.370.603	-1.2	100	137.0;119.0;105.9
Decarboxymethylated form of elenolic acid	10.47	C9 H12 O4	1.830.654	1.830.657	-0.3	100	183.0;139.0;95.1
p-coumaric acid	11.6	C9 H8O3	1.630.378	1.630.395	-1.7	100	163.1;119
Desoxy elenolic acid	12.13	C11 H14 O5	2.250.764	2.250.763	0.1	100	225.0;123.0;101.0
Elenolic acid	14.03	C11 H14 O6	2.410.709	2.410.712	-0.3	88.11	241.0;138.9;127,0;95.1;110.1;164.9
Decarboxymethyl oleuropein aglycone	15.05	C17 H20 O6	3.191.158	3.191.182	-2.4	99.69	319.1;194.9;165.0;300.9
Syringaresinol	16.13	C22 H25 O8	4.171.233	4.171.251	-1.8	95.99	
Luteolin	16.38	C22 H6 O	2.850.409	2.850.438	-2.9	92.74	285.0;150.9;164.9
Pinoresinol	17.2	C20 H22 O6	3.571.279	3.571.261	1.8	87.76	357.0;151.0;136.0;311.0;342.0
Acetoxypinoresinol	17.85	C22 H24 O8	4.151.386	4.151.393	-0.7	98.14	415.1;325.0;295.0;371.0;235.0
Methyl decarboxy oleuropein aglycone	18.04	C11 H26 O11	3.331.356	3.331.397	-4.1	95.99	
Decarboxymehtyl Ligstroside aglycone	18.64	C17 H20 O5	3.031.132	3.031.126	0.6	98.86	303.0;284.9;165.0;179.0
Apigenin	19.27	C15 H10 O5	2.690.472	2.690.451	2.1	99.51	269.0; 150.9;183,5;224.8;201,0
Dehydro-oleuropein aglycone	21.43	C19 H20 O8	3.751.072	3.751.068	0.4	95.99	375.0;307.0;275.2;345,0;139.2
Oleuropein aglycone	21.5	C19 H22 O8	3.771.235	3.771.236	-0.1	100	377.0;307.0;275.1;345.0;149.1
Isomer 1	14.5	C19 H22 O8	3.771.256	3.771.236	2	99.23	
Isomer 2	15.2	C19 H22 O8	3.771.254	3.771.236	1.8	99.88	
Isomer 3	16.8	C19 H22 O8	3.771.263	3.771.236	2.7	99.96	
Isomer 4	18	C19 H22 O8	3.771.225	3.771.236	-1.1	99.35	
Isomer 5	19.5	C19 H22 O8	3.771.237	3.771.236	0.1	100	
Isomer 6	22.1	C19 H22 O8	3.771.248	3.771.236	1.2	99.76	
Methyl oleuropein aglycone	23.4	C20 H24 O8	3.911.403	3.911.393	1	92.86	391.1;321.0;275.1;209.1;344.9
Ligstroside aglycone	23.6	C19 H22O7	3.611.263	3.611.287	-2.4	99.96	361.1;291.0;259.1;223.1
Isomer 1	16.9	C19 H22O7	3.611.288	3.611.287	0.1	100	
Isomer 2	21.3	C19 H22 O7	3.611.294	3.611.287	0.7	100	
Isomer 3	22.5	C19 H22 O7	3.611.282	3.611.287	-0.5	100	

Even if the qualitative phenolic composition of all the analyzed olive oil samples from Meknès over the studied period was quite similar, the abundance of the analytes under study (both in terms of individual phenol concentration levels and global content by chemical categories) widely differed as illustrated by Figure 3 and Table 3. In general, **secoiridoids** were the most abundant group of phenolic compounds in every sample regardless of the sub-area of origin and the harvest season, fact which is in good agreement with the findings of other author for various monovarietal olive oils [12,28,29]. This type of phenolic compounds is of great importance since, for instance, contributes to the organoleptic characteristics of VOO, by conferring bitterness and pungency [10]. The highest mean concentration value of secoiridoids (1106.96 mg/kg) was observed for olive oil samples from the sub-area 3 during the crop season 2011 whereas the lowest mean concentration was found for the samples coming from sub-area 2 in the crop season 2013, with 540.12 mg/kg. With regard to individual secoiridoids, the most abundant one over the evaluated period was the DOA, with mean concentrations of 165.86, 168.34 and 101.39 mg/kg (for sub-area 3), respectively, during the first, second and third crop seasons. Other secoiridoids found at relatively high concentration levels were: Ol Agl, with amounts ranged from 47.40 mg/kg in oils from the sub-area 2 for the third crop season to 155.45 mg/kg in olive oils from the same sub-area for the crop season 2011; Lig Agl, with mean values ranging from 70.42 to 111.23 mg/kg, respectively, in olive oils coming from the sub-area 1 (at the second crop season) and the sub-area 2 (at the third crop season); and EA, whose lowest concentration was found in oils from the sub-area 3 at the third crop season (36.83 mg/kg) and the highest concentration value was registered in oils from the sub-area 2 during the first crop season (109.46 mg/kg). Isomers, both of Ol Agl and Lig Agl, were also determined at relatively high concentration levels. Indeed, in the case of Ol Agl, over the crop season 2011, the isomers 5 and 2 showed the highest mean concentration level (60.79 mg/kg and 55.95 mg/kg) in olive oils from the sub-area 3, whereas the isomer 6 showed the highest mean concentration value (57.17 mg/kg) for the samples coming from sub-area 2. As far as Lig Agl isomers are concerned, the isomer 1 remains the prevailing one in all the analyzed samples over the three crop seasons with a mean concentrations ranging from 33.20 mg/kg in oils from the sub-area 1 (for the first crop season) to 40.25 mg/kg in olive oils from the same sub-area over the 2013 crop season.

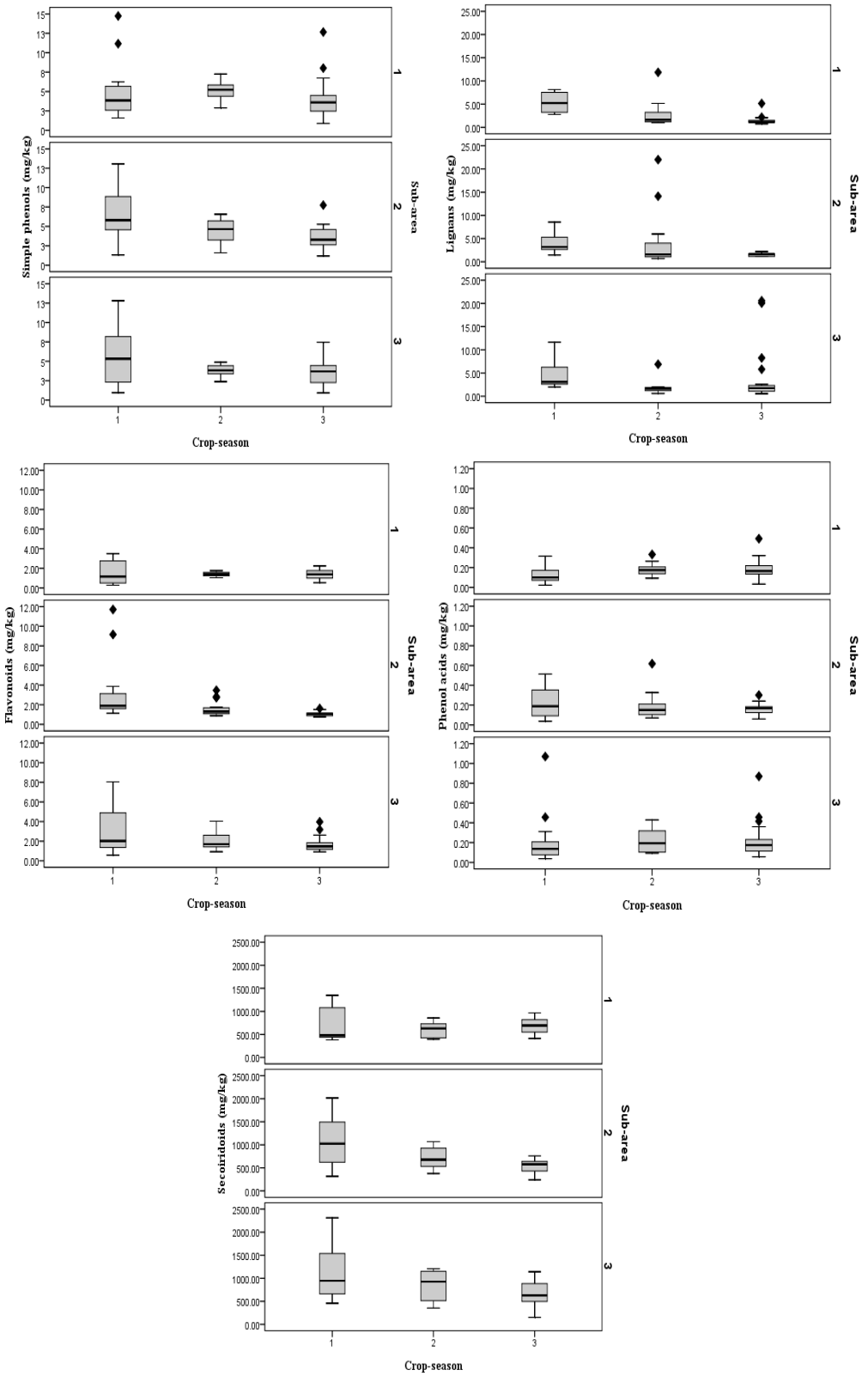

Figure 3. Box and whisker plots of total content of simple phenols, lignans, flavonoids, phenolic acids, and secoiridoids determined on Meknès VOOs. For box-plots, the line within the box indicates the median; the box boundary marks the 25th and 75th percentiles; percentiles and whiskers above and below the box indicate the 90th and 10th percentiles. Outlier data are shown as◆.

Besides the above mentioned compounds, other secoiridoids were found at relatively lower amounts: Dehydro Ol Agl that showed mean concentration values ranged between 3.06 mg/kg (found in oils from the sub-area 2 during the second crop season) and 15.02 mg/kg (found in oils from the sub-area 1 for the crop season 2011); D-Ald-D EA, with mean concentration levels fluctuating from 0.39 mg/kg in oils from the sub-area 3 during the second crop season to 37.60 mg/kg in olive oils from the sub-area 1 over the crop season of 2011; Desoxy-EA, which was found at mean concentration levels between 8.23 mg/kg (in oils from the sub-area 1 in 2011) and 23.70 mg/kg (in olive oils from the sub-area 3 in 2012); D-Lig Agl, at concentrations ranging between 36.96 mg/kg (for oils from the sub-area 1 in 2012) and 157.90 mg/kg (in 2013, for oils from the sub-area 3); Methyl D-Ol Agl, in amounts comprised between the following values 15.80-27.27 mg/kg, for oils from the sub-area 2 in 2012 and samples coming for sub-area 1 over the first crop season, respectively; and Methyl Ol Agl, whose lowest concentration was determined in oils from the sub-area 2 at the second crop season (1.28 mg/kg) and the highest one was registered in oils from the sub-area in 2011 (4.15 mg/kg).

Simple phenols constitute the second group of phenols in terms of abundance in Meknès olive oil samples, with a total average amount which varies between 6.90 mg/kg (sub-area 3, crop season 2013) and 13.07 mg/kg (sub-area 2, crop season 2011). Within this group, Ty was the predominant substance, with levels found between 4.84 mg/kg, in oils from sub-area 3 over the third crop season, and 7.80 mg/kg, in oils from sub-area 1 during the second crop season. In terms of relative abundance, Ty was followed by Hyt that showed mean concentration values oscillating between 1.07 mg/kg (sub-area 3 at crop season 2013) and 5.62 mg/kg (sub-area 2 at crop season 2011); O-Hyt that was also found in relatively lower amounts, varying its mean values between 0.16 mg/kg (sub-area 1 and 3, crop season 2013) and 1.02 mg/kg (sub-area 3, crop season 2011). If these results are compared with those achieved in previous studies about the phenolic composition of monovarietal olive oils from different Mediterranean countries [12,30], some particular characteristics can be highlighted for Meknès olive oils. The first feature is that Ty concentration levels in the evaluated oils are, in general, higher than Hyt concentration values; the second characteristic is that Hyt levels are lower than the previously published concentration values for other Mediterranean monovarietal olive oils.

Table 3. Mean ± standard deviation (mg/kg) of phenolic compounds evaluated on Meknès monovarietal VOOs

Phenolic group	Phenolic compounds	Crop season 2011			Crop season 2012			Crop season 2013		
		Sub-area 1	Sub-area 2	Sub-area 3	Sub-area 1	Sub-area 2	Sub-area 3	Sub-area 1	Sub-area 2	Sub-area 3
Simple phenols	O-Hyt	$0.22^{a}a\pm0.11$	$0.65^{b}a\pm0.50$	$1.02^{b}a\pm1.56$	$0.40^{a}b\pm0.21$	$0.25^{b}b\pm0.06$	$0.26^{ab}ab\pm0.11$	$0.16^{a}a\pm0.07$	$0.23^{a}b\pm0.32$	$0.16^{a}b\pm0.19$
	Hyt	$4.16^{a}a\pm4.90$	$5.62^{a}a\pm4.12$	$4.33^{a}a\pm3.75$	$2.01^{a}b\pm0.85$	$1.96^{a}b\pm0.99$	$1.46^{a}b\pm0.63$	$2.02^{a}b\pm2.72$	$1.31^{a}b\pm1.53$	$1.07^{a}b\pm0.94$
	Ty	$5.83^{a}a\pm3.11$	$6.79^{a}a\pm3.38$	$4.84^{a}a\pm2.62$	$7.80^{a}a\pm2.13$	$6.57^{a}a\pm2.47$	$5.92^{a}a\pm1.88$	$5.93^{a}a\pm2.34$	$5.98^{a}a\pm2.41$	$5.68^{a}a\pm2.51$
	Total	$10.22^{a}a\pm7.97$	$13.07^{a}a\pm6.98$	$11.19^{a}a\pm7.02$	$10.19^{a}a\pm2.63$	$8.77^{a}ab\pm2.98$	$7.64^{a}ab\pm1.78$	$8.11^{a}a\pm4.83$	$7.51^{a}b\pm3.95$	$6.90^{a}b\pm3.15$
Lignans	Syr	$2.42^{a}a\pm1.78$	$1.18^{b}a\pm1.00$	$0.90^{b}a\pm0.46$	$0.23^{a}b\pm0.08$	$0.20^{a}b\pm0.06$	$0.20^{a}b\pm0.07$	$0.17^{a}b\pm0.04$	$0.21^{a}b\pm0.07$	$0.69^{a}ab\pm2.34$
	Pin	$0.30^{a}a\pm0.23$	$0.75^{ab}a\pm0.97$	$1.15^{b}a\pm0.68$	$0.51^{a}b\pm0.19$	$0.61^{a}a\pm0.49$	$0.44^{a}b\pm0.16$	$0.35^{a}a\pm0.08$	$0.45^{a}a\pm0.1$	$0.47^{a}b\pm0.38$
	Ac-pin	$2.62^{a}a\pm1.16$	$2.16^{a}a\pm1.75$	$2.36^{a}a\pm2.79$	$2.25^{a}ab\pm3.04$	$3.28^{a}a\pm5.85$	$1.55^{a}a\pm1.96$	$0.91^{a}b\pm0.81$	$0.92^{a}b\pm0.35$	$1.91^{a}a\pm3.53$
	Total	$5.34^{a}a\pm2.16$	$4.08^{a}a\pm2.23$	$4.41^{a}a\pm2.96$	$2.99^{a}b\pm3.25$	$4.09^{a}ab\pm5.83$	$2.19^{a}a\pm2.12$	$1.43^{a}b\pm0.87$	$1.58^{a}b\pm0.42$	$3.08^{a}a\pm4.77$
Flavonoids	Lut	$1.22^{a}a\pm1.11$	$2.63^{a}a\pm2.19$	$2.50^{a}a\pm1.6$	$1.29^{a}a\pm0.23$	$1.39^{a}b\pm0.53$	$1.77^{a}ab\pm0.81$	$1.29^{a}a\pm0.45$	$1.02^{a}b\pm0.25$	$1.42^{a}b\pm0.53$
	Apig	$0.32^{a}a\pm0.12$	$0.59^{a}a\pm1.00$	$0.65^{a}a\pm1.01$	$0.11^{a}b\pm0.04$	$0.18^{a}a\pm0.24$	$0.33^{a}ab\pm0.30$	$0.09^{a}b\pm0.07$	$0.08^{a}b\pm0.06$	$0.21^{a}b\pm0.43$
	Total	$1.55^{a}a\pm1.17$	$3.23^{a}a\pm3.18$	$3.15^{a}a\pm2.47$	$1.40^{a}a\pm0.24$	$1.57^{a}ab\pm0.76$	$2.10^{a}ab\pm1.1$	$1.38^{ab}a\pm0.5$	$1.10^{a}b\pm0.29$	$1.63^{b}b\pm0.67$
Phenol acids	p-Cou	$0.13^{a}a\pm0.09$	$0.22^{a}a\pm0.15$	$0.22^{a}a\pm0.27$	$0.18^{a}a\pm0.07$	$0.19^{a}a\pm0.14$	$0.22^{a}a\pm0.15$	$0.18^{a}a\pm0.09$	$0.17^{a}a\pm0.07$	$0.21^{a}a\pm0.15$
	Total	$0.13^{a}a\pm0.09$	$0.23^{a}a\pm0.15$	$0.22^{a}a\pm0.27$	$0.18^{a}b\pm0.07$	$0.19^{a}a\pm0.14$	$0.22^{a}a\pm0.15$	$0.18^{a}b\pm0.09$	$0.17^{a}a\pm0.07$	$0.21^{a}a\pm0.15$

Table 3. (continued)

Phenolic group	Phenolic compounds	Crop season 2011			Crop season 2012			Crop season 2013		
		Sub-area 1	Sub-area 2	Sub-area 3	Sub-area 1	Sub-area 2	Sub-area 3	Sub-area 1	Sub-area 2	Sub-area 3
Secoiridoids	D-Ald-D EA	37.60^{a}a±44.96	30.90^{a}a±36.95	29.30^{a}a±49.79	2.76^{a}b±3.06	0.70^{b}b±0.70	0.39^{b}b±0.34	0.91^{a}b±1.42	6.57^{b}c±11.6	2.45^{ab}b±4.82
	D-EA	8.23^{a}a±7.92	15.57^{a}ab±13.54	13.98^{a}a±23.6	12.89^{a}b±10.91	16.48^{a}a±8.98	23.70^{a}a±17.12	12.84^{a}b±8.69	8.48^{a}b±5.38	16.05^{a}a±14.36
	EA	83.22^{a}a±43.82	109.46^{a}a±64.90	92.66^{a}a±38.96	94.61^{a}a±50.69	51.74^{b}b±17.17	50.20^{b}b±10.94	45.35^{ab}b±21.99	56.3^{b}b±17.82	36.83^{a}b±14
	DOA	32.84^{a}a±43.35	99.52^{ab}a±96.64	165.86^{b}a±161.13	51.33^{a}ab±33.75	116.7^{ab}a±76.38	168.34^{b}a±124.71	80.44^{ab}b±59.23	49.51^{b}b±32.26	101.39^{a}b±70.18
	Methy D-Ol Agl	27.27^{a}a±7.19	23.72^{a}a±12.44	18.85^{a}a±13.39	17.68^{a}b±9.21	15.80^{a}a±8.34	16.67^{a}a±7.73	20.94^{a}b±5.5	20.15^{a}a±6.47	17.36^{a}a±7.63
	D-Lig Agl	39.25^{a}ab±22.6	96.71^{ab}a±80.47	157.9^{b}a±137.72	36.96^{a}a±20.56	67.20^{ab}b±37.57	117.47^{b}ab±89.51	60.21^{a}b±30.39	61.71^{a}b±19.18	72.82^{a}b±35.59
	Dehydro Ol Agl	15.02^{a}a±6.94	8.61^{a}a±6.96	8.73^{a}a±10.23	5.23^{a}b±2.38	3.06^{a}b±2.53	3.24^{a}b±3.04	3.91^{a}b±2.47	5.50^{a}a±1.15	3.99^{a}b±2.84
	Ol Agl	109.01^{a}a±61.78	155.45^{a}a±73.37	152.29^{a}a±72.17	60.24^{a}b±16.68	77.83^{a}b±29.26	81.57^{a}b±31.64	75.13^{a}b±21.99	47.4^{b}c±14.25	70.60^{a}b±28.00
	Ol Agl-Isomer 1	19.52^{a}a±26.59	31.99^{a}a±29.70	35.23^{a}a±39.44	27.35^{a}ab±20.78	31.71^{a}a±17.62	26.95^{a}a±14.30	39.77^{a}b±20.19	18.20^{b}a±11.32	37.10^{a}a±26.19
	Ol Agl-Isomer 2	37.96^{a}a±43.32	54.68^{a}a±44.53	55.94^{a}a±53.73	38.70^{a}a±31.7	44.43^{a}a±21.95	39.69^{a}a±22.24	53.22^{a}a±23.11	28.51^{b}b±14.2	49.06^{a}a±30.98
	Ol Agl-Isomer 3	17.49^{a}ab±14.86	27.34^{a}a±17.49	25.47^{a}a±20.08	14.57^{a}a±7.04	18.80^{a}ab±7.79	19.06^{a}a±8.31	21.99^{a}b±8.96	11.72^{b}b±5.94	19.74^{a}a±9.84
	Ol Agl-Isomer 4	22.80^{a}a±20.32	39.66^{a}a±25.74	38.98^{a}a±28.55	20.84^{a}a±9.82	28.42^{a}a±12.56	30.22^{a}a±15.24	28.67^{a}a±10.79	17.27^{b}b±8.27	27.99^{a}a±16.17
	Ol Agl-Isomer 5	34.80^{a}a±21.91	60.54^{a}a±34.12	60.79^{a}a±36.83	26.90^{a}a±9.62	35.67^{a}b±13.91	39.63^{a}ab±19.13	32.49^{a}a±9.89	18.79^{b}c±5.59	31.50^{a}b±13.72
	Ol Agl-Isomer 6	44.23^{a}a±20.43	57.17^{a}a±25.77	53.42^{a}a±26.44	28.70^{a}b±8.18	31.98^{a}b±7.64	31.61^{a}b±5.32	32.49^{a}b±8.18	21.07^{b}c±7.59	33.16^{a}b±12.1
	Methyl Ol Agl	2.05^{a}a±1.19	4.15^{a}a±4.71	2.16^{a}a±1.58	1.61^{a}a±0.64	1.28^{a}b±0.58	1.67^{a}a±0.64	2.66^{ab}a±2.22	3.67^{b}a±1.86	2.03^{a}a±1.33
	Lig Agl	93.01^{a}a±34.7	111.23^{a}a±64.32	108.27^{a}a±64.48	70.42^{a}a±29.71	84.65^{a}a±26.25	103.43^{a}a±51.85	83.75^{a}a±24.57	77.39^{a}a±32.8	85.67^{a}a±31.5
	Lig Agl-Isomer 1	33.20^{a}a±17.97	39.44^{a}a±33.23	39.25^{a}a±35.86	33.71^{a}a±18.02	34.61^{a}a±16.45	33.53^{a}a±19.63	40.25^{a}a±12.69	38.88^{a}a±16.57	35.33^{a}a±17.41
	Lig Agl-Isomer 2	29.58^{a}a±18.08	29.29^{a}a±22.36	25.87^{a}a±23.07	29.71^{a}a±12.53	27.42^{a}a±12.13	24.47^{a}a±11.67	32.19^{a}a±10.56	32.80^{a}a±15.07	28.50^{a}a±14.8
	Lig Agl-Isomer 3	17.49^{a}a±5.90	22.55^{a}a±13.85	22.02^{a}a±13.22	20.31^{a}a±19.45	17.68^{a}a±6.10	19.64^{a}a±11.13	17.43^{a}a±5.37	16.21^{a}a±7.01	16.57^{a}a±7.37
	Total	704.59^{a}a±361.56	1018.01^{a}a±524.23	1106.96^{a}a±553.85	594.50^{a}a±175.16	706.15^{a}ab±218.98	831.46^{a}ab±363.83	684.64^{a}a±177.52	540.12^{a}b±158.91	688.14^{a}b±253.54
	Quinic acid	1.04^{a}a±1.05	12.87^{b}a±16.60	9.34^{b}a±15.2	2.80^{a}a±3.89	0.51^{b}b±0.53	1.32^{ab}a±1.18	1.84^{a}a±2.35	5.48^{a}a±12.38	2.34^{a}b±3.83

-Significant differences in the same row are indicated with different lowercase letters (comparison among crop seasons, $p < 0.05$) and with different superscript letters (comparison between sub-areas at the same crop season, $p < 0.05$).

Both factors could be potentially considered as markers or discriminative characteristics of Meknès oils when compared with olive oils coming from other different Mediterranean locations.

Lignans were another relevant group of phenolic analytes determined in the selected samples; in terms of abundance, they were the third group, with average contents ranging from 1.43 to 5.34 mg/kg. The highest value was observed for oils from sub-area 1 at the first crop season, whilst the lowest mean concentration level was found for oils from the same sub-area over 2013-crop season. When the concentration levels of individual compounds are taken into account within this group, we observe that Ac-pin levels were found between 0.91 mg kg^{1} (sub-area 1, crop season 2013) and 3.28 mg/kg (sub-area 2, crop season 2012); Syr concentration values were falling within the range spanned between 0.17 mg/kg (sub-area 1 and crop season 2013) and 2.42 mg/kg (sub-area 1 and crop season 2011); and concentration values for Pin fluctuated within the range 0.30-1.15 mg/kg, defined by the mean value obtained for the samples from sub-areas 1 and 3, respectively, over the season 2011.

With regard to **flavonoids** family, in terms of total content, average values were found within the range from 1.10 to 3.23 mg/kg, levels observed, respectively, in oils from the sub-area 3 during the crop season 2013 and the sub-area 2 in 2011. Lut and Apig are the most relevant compounds within this group; Lut, which was the most abundant flavonoid occurring in the analyzed olive oil samples, was found at mean concentration levels ranging from 1.02 mg/kg (sub-area 2, crop season 2013) to 2.63 mg/kg (sub-area 2, crop season 2011), and Apig was found at lower concentration levels than those of Lut, with amounts oscillating between 0.08 and 0.65 mg/kg, observed for samples from sub-area 2 over the third crop season and olive oils from sub-area 3 in 2011.

In the group composed by **phenolic acid**, p-Cou was the most abundant analyte. Its values (mean concentration) were found from 0.13 (in olive oils from the sub-area 1 during the first crop season) to 0.22 mg/kg (which was a common concentration value for samples from sub-areas 2 and 3 in 2011 and those from sub-area 3 in 2012).

The results obtained in this study are in good agreement with those achieved in a previous work in which the phenolic fraction of VOOs from seven north Moroccan olive growing areas were compared [16]. Also, some distinctive features of the phenolic fraction

of Picholine marocaine VOOs -when compared to other Mediterranean VOOs- revealed by the mentioned work could be confirmed by the present study.

Furthermore, the study of intra-regional and interannual variability of both the total and individual concentrations of the determined phenolic compounds in Meknès VOOs was carried out in order to evaluate the possible influence of the sub-area and the crop season factors on the phenolic fraction of the samples under study. Thus, an analysis of variance (ANOVA) was performed on the obtained quantitative data; the results are reported in Table 3.

Among the studied compounds, it should be pointed out that Ty and Lig Ag and one of its isomers (isomer 3) were the analytes unaffected by both sub-area and year of production factors. Furthermore, considering the influence of the sub-area of origin, the examination of the results obtained applying a Post-hoc test (Tukey's) showed that the total amount of the considered chemical classes seems to be unaffected by the sub-area factor over the three crop seasons. However, as far as individual phenolic compounds content is concerned, we observed that in 2011, analytes such as O-Hyt, Syr, Pin, DOA and D-Lig Agl were influenced by the sub-area of origin. In 2012, the effect of sub-area factor was observed for O-Hyt, D-Ald-D EA, EA, DOA and D-Lig Agl. The third crop season, 2013, was the period with the highest number of compounds affected by the sub-area factor, finding 10 compounds significantly affected ($p < 0.05$) by it, including O-Hyt, D-Ald-D EA, DOA, Methyl Ol Agl and the six Ol Agl Isomers. Despite these differences, there was no compound that could differentiate samples according to the sub-area of origin, since the mean values for each variable were not significantly different ($p < 0.05$) at the same time for the three studied sub-areas (Table 3).

When the effect of crop season was evaluated, the ANOVA test revealed that this factor shows most pronounced influence both in the global amount of phenols (determined by families) and the content of individual phenolic analytes. Thus, olive oil samples coming from the sub-area 1 showed statistically significant differences ($p < 0.05$) for the following compounds: O-Hyt, Hyt, D-Ald-D EA, Desoxy-EA, EA, DOA, Syr, Pin, Ac-pin, Methyl D-Ol Agl, D-Lig Agl, Apig, Dehydro Ol Agl, Ol Agl, Ol Agl Isomers 1, 3 and 6, and Methyl Ol Agl. When the same was made to evaluate the effect of harvest season factor on the samples coming from the sub-area 2, significant differences were found for O-Hyt, Hyt,

D-Ald-EA, Desoxy-EA, EA, Syr, DOA, Lut, Ac-pin, Dehydro Ol Agl, Apig, D-Lig Agl, Ol Agl, Ol Agl Isomers 2, 3, 4, 5 and 6, and Methyl Ol Agl; in olive oils from sub-area 3, crop season significantly affected O-Hyt, Hyt, D-Ald-EA, p-Cou, EA, DOA, Syr, Lut, Pin, Methyl D-Ol Agl, Dehydro Ol Agl, Apig, D-Lig Agl, Ol Agl and Ol Agl Isomer 5.

In conclusion, the work conducted here, demonstrate that Picholine marocaine VOOs produced in Meknès region, could be considered as potential sources of phenolic bioactive compounds, since a total of 28 phenolic compounds (and quinic acid), belonging to five chemical classes, were identified and quantified in all the analyzed samples. Differences were observed for the phenolic compounds under study as a function of the sub-area of origin and harvest season, been the latter the most relevant variation factor. As a matter of fact, Picholine marocaine is the dominant olive cultivar in Morocco olive growing regions. We believe that our approach might be reasonably adopted for future studies of comprehensive characterization of the selected variety (or any other), as well as for comparative studies of the composition of olive oils from a particular cultivar obtained in different growing areas.

ABBREVIATIONS USED

Ac-Pin, (+)-1-acetoxypinoresinol; ANOVA, one-way analysis of variance; Apig, apigenin; DAD, diode array detector; D-Ald-D EA, dialdehydic form of decarboxymethyl elenolic acid; Dehydro Ol Agl, Dehydro oleuropein aglycone; Deoxy-EA, desoxy elenolic acid; D-Lig Agl, decarboxymethyl ligstroside aglycone; DOA, decarboxymethyl oleuropein aglycone; DOPAC, 3,4-dihydroxyphenylacetic acid; EA, elenolic acid; EICs, extract ion chromatographs; ESI, electrospray ionization; FAAs, free fatty acids; HMR, High Mass Resolution; Hyt, hydroxytyrosol; IT, Ion Trap; MS, Lig Agl, ligstroside aglycone; LRM, Low-resolution mass; Lut, luteolin; Methy D-Ol Agl, methyl decarboxy oleuropein aglycone; Methyl Ol Agl, methyl oleuropein aglycone; MS, Mass Spectrometry; MS/MS, tandem mass spectrometry; O-Hyt, oxidized hydroxytyrosol; Ol Agl, oleuropein aglycone; *p*-cou, *p*-coumaric. Pin, (+)-pinoresinol; PV, peroxides value; Syr, syringaresinol; TIC, Total Ion Current chromatogram; TOF, Time-of-Flight; Ty, tyrosol; VOOs, virgin olive oils.

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Chapter

14

Evaluating the potential of phenolic profiles as discriminant features among extra virgin olive oils from Moroccan controlled designations of origin

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Abstract

Herewith, the potential of an approach based on the combination of the determination of phenolic compounds and the use of chemometric treatments has been evaluated to establish, for the first time, promising models to authenticate the provenance of Moroccan monovarietal olive oils produced under different geographical origin indication systems. To achieve this purpose, 136 commercial extra virgin olive oil samples from three diverse production areas (Meknès territory; the Protected Geographical Indication Ouazzane; and the Protected Designation of Origin Tyout-Chiadma) were collected over two consecutive crop seasons (2012/2013 and 2013/2014). Their phenolic fraction composition was investigated by using high performance liquid chromatography coupled to mass spectrometry (HPLC-ESI IT MS). The results showed that geographical provenance and harvest season had a marked influence on the content of identified phenolic compounds. Principal components analysis (PCA) and linear discriminant analysis (LDA) were applied to test the potential of the determined compounds as geographical discriminant features, achieving a noticeable discrimination among the three evaluated regions. The contribution of each analyte to the statistic model has been evaluated in depth.

Keywords: phenolic compounds; chemometrics; geographical origin authentication; Moroccan monovarietal olive oils; geographical origin indication systems; liquid chromatography-mass spectrometry.

1 Introduction

Linking foodstuffs and agricultural products to its geographical provenance is an ancient universal practice which allow differentiating them from other conventional products by means of certain characteristics, qualities and/or reputations, attributed to their historic legacy and their distinctive features linked to environmental and human factors such as soil, climate, production practices, local know-how and traditions (Couillerot, 2000). Geographical Indication Protection systems, in particular, Protected Designations of Origin (PDOs) and Protected Geographical Indications (PGIs) are, nowadays, increasingly considered, in many countries, as great tools giving producers the opportunity to move forward from commodity markets into more lucrative niche markets through differentiation. As such, geographic origin becomes a strategic tool granting high added

value to local production systems, since the use of geographical indications allows producers to obtain market recognition and, often, a premium price (Barham, 2003; Verbeke & Roosen, 2009).

Unfortunately, in spite of the importance of these differentiation systems, the general procedure to assess whether the claims of origin are valid remains, till now, mainly limited to the traceability of the production by regional institutions, which act as regulatory councils ensuring rigorous controls of the entire management conditions and giving certification to those products which comply with the quality standards demanded and show typical characteristics of the geographic label. However, because of the economic importance of these labeled products, new and increasingly sophisticated strategies can be used in their adulteration, so it is, sometimes, very difficult to be certain about the geographical authenticity of these products based only on the regulatory council's controls. Therefore, there is a continuous demand for effective, robust and reliable analytical strategies to verify the declared geographical origin, which can constitute an priceless tool for helping regulatory councils to trace foods back to their provenance (Luykx & van Ruth, 2008).

Virgin olive oil (VOO) represents an important example of food for which the establishment of geographic indications is widespread. Indeed, over the past two decades, olive oil sector has experienced a significant motion towards market differentiation, and consequently, origin-labeled VOOs have proliferated in many producing countries. Indeed, in 2010, there were about 105 origin-labeled extra VOOs all over the world (both PDOs and PGIs); almost all of them (101) are produced in European Union countries, mainly in Italy, Greece and Spain (International Olive Council (IOC), 2010). Additionally, according to Chever et al. (2012), the production value of marketed European origin-labeled extra VOOs was, on average, 215 M€ per year from 2006 to 2008, and 203 M€ in 2010. Therefore, it is easy to understand that, the characterization of the quality and composition of origin-labeled VOOs, as well as the development of quick and powerful analytical approaches to authenticate their geographical origin are, nowadays, emerging topics within the olive oil sector (Antonini et al., 2015; García-González, Tena, & Aparicio, 2012; López-Feria, Cárdenas, García-Mesa, & Valcárcel, 2008; Pardo, Cuesta, Alvarruiz, Granell, & Álvarez-Ortí, 2011). Certainly, several studies have focused on the quality and compositional profiling of VOO, underlining the

relationship between these parameters and geographical origin characteristics. They clearly demonstrated that differences in geographical provenance factors (including intrinsic factors, such as those related to the characteristic of the cultivated olive tree variety, and extrinsic factors such as soil, climate, cultivation and manufacturing methods used for olive oil extraction) and storage conditions actually cause differences in the physico-chemical quality, sensorial attributes, and the chemical composition of the produced olive oils (Arslan, Karabekir, & Schreiner, 2013; Ben-Hassine et al., 2013; Issaoui et al., 2009; Noorali, Barzegar, & Sahari, 2014). Furthermore, great effort has been made to investigate the potential of using some chemical components contents combined with the adequate chemometric treatments to trace the geographic origin of this foodstuff (Giacalone, Giuliano, Gulotta, Monfreda, & Presti, 2015; Gurdeniz, Ozen, & Tokatli, 2008; Marini, Magrì, Bucci, Balestrieri, & Marini, 2006; Ollivier, Artaud, Pinatel, Durbec, & Guérère, 2006; Youssef et al., 2011). Among the various olive oil compounds used with this scope, phenolic compounds have shown a remarkable potential as geographical markers of oils produced in various Mediterranean regions (Lerma-García et al., 2009; Nescatelli et al., 2014; Ouni et al., 2011; Romero, Saavedra, Tapia, Sepúlveda, & Aparicio, 2015; Servili et al., 2004). The relevance of these compounds is also irrefutable considering, among other reasons, their biological effects (Alesci et al., 2014; Clodoveo et al., 2015).

Morocco is among the leading producing countries of olive oil, and its oils show a wide range of peculiar characteristics which mainly depend on the pedoclimatic conditions and the production process of the predominant olive variety 'Picholine Marocaine' cultivated in this country. Moroccan oils are gaining noteworthy international reputation, but the process for adopting geographic indications systems in this country is still in its earliest stages. Indeed, till October 2013, there was only one recognized PDO for the extra VOO (Tyout-Chiadma, located in the South of Morocco) and one official PGI (Ouazzane, located in the North of this country). Another PDO for the extra VOO produced in Meknès territory is in the process of characterization of its typicality and it is expected that will enlarge the list in the near future (Bajoub et al., 2014).

The present study can be contextualized within a research line devoted to the establishment of efficient analytical approaches combining the comprehensive characterization of the composition of Moroccan monovarietal VOOs and chemometric

treatments in order to develop trustworthy and effective geographical origin tracing models. Hence, the main objectives of this work can be formulate as follows: 1) carrying out the characterization of the phenolic fraction of commercial extra VOO samples (coming from Meknès territory, PGI Ouazzane and PDO Tyout-Chiadma over two consecutive crop seasons 2012/2013 and 2013/2014) using a LC-MS; 2) investigating the geographical provenance and crop season effects on the phenolic content of the studied samples; and 3) evaluating the potential of combining phenolic data and chemometric treatments (PCA and LDA) for the development of models which will allow the geographical classification of oils under evaluation. To the best of our knowledge, the current work is the first one studying the phenolic fraction from Moroccan origin-labeled VOOs and no geographical discriminant approach for such kind samples has been previously performed and reported in literature.

2 Materials and methods

2.1 Olive oil samples

A total of 136 commercial monovarietal 'Picholine Marocaine' extra virgin olive oil samples, from three different Moroccan geographical locations: Meknès region (57 samples); PGI Ouazzane (42 samples) and PDO Tyout-Chiadma (37 samples), have been collected over two consecutive crop seasons (2012/2013 and 2013/2014). Each sample was a commercial bottle of approximately 250-500 mL, purchased from supermarkets or directly from their own producers ("Coopérative Tyout de Production et de Commercialisation de l'Huile d'Olive" in the case of PDO Tyout-Chiadma samples; and "Groupement d'Intérêt Economique Femmes du Rif (GIE)" for PGI Ouazzane samples), whereas Meknès samples were kindly supplied by "Agro-pôle Olivier Ecole Nationale d'Agriculture de Meknès". About 20 g of each sample were weighed and stored in dark glass bottles (22 mL) at -18 °C, excluding any hade space volume in order to ensure the proper conservation of the olive oils. These aliquots were used when the subsequent analyses had to be carried out. The geo-locations of the three studied zones are given in Fig. 1.

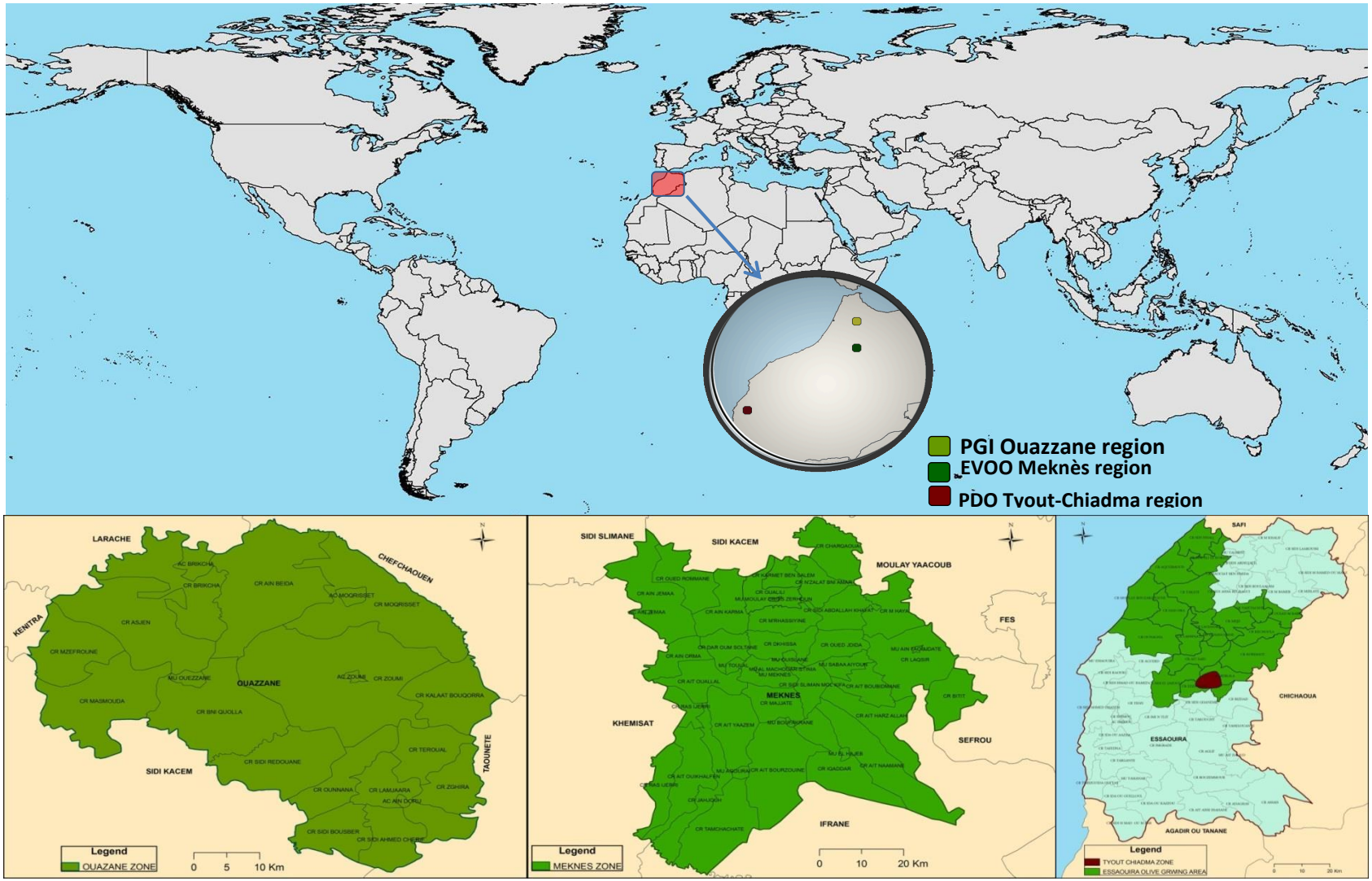

Figure 1: Map showing the geographic locations of the three Moroccan olive oil producing areas considered in the study.

The extra VOOs produced in Tyout-Chiadma were the first receiving the designation PDO in Morocco in 2010, and it has a very limited production (approximately 20 tons of 'Picholine Marocaine' extra VOO per year) because of a cultivated surface of only 100 ha. The olive production is conducted under irrigated system, and oil extraction is made by means of a peculiar production process (a pressing method where olive fruits crushing is carried out in grinder with two cylindrical granite millstones, afterwards the obtained past is mixed by a slow and continuous stirring and, finally, the oil is extracted using a pressing system). This production area is characterized by a sandy limestone soil and a semi-arid climate with a moderate oceanic influence (the rainfall in the area does not exceed 300 mm, the annual mean temperature varies between 20 °C and 21 °C and the temperature fluctuation between the coldest month (January) and the warmest month (August) can reach 17 °C (Chamchati & Bahir, 2011). As far as the PGI Ouazzane is concerned, the production of its oils is made using 'Picholine Marocaine' olive fruits coming from the entire province of Ouazzane (the olive growing area is approximately 40,885 ha), extracted by two or three phases extraction systems. The most remarkable pedoclimatic characteristics of Ouazzane region are: minimum mean temperatures: 17.4 °C, annual mean maximum temperatures: 25.6 °C; annual mean rainfall: 676 mm; soil classes: fluvisols, regosols, lithosols, vertisols, rendzinas, yermosols, xerosols, cambisols, luvisols, acrisols, gleysols and planosols. Finally, regarding Meknès EVOOs, their production comes from 'Picholine Marocaine' olive fruits grown in Meknès region (about 43,000 ha leading to 21,000 tons of olive oil), by various olive oil mills belonging to the association "Union pour le Développement de l'Olivier de Meknès (UDOM)" using two and three phases continuous olive oil extraction systems. The main pedoclimatic characteristics of Meknès region are: annual mean minimum temperatures: 11 °C, annual mean maximum temperatures: 23 °C; annual mean rainfall: 400 to 600 mm; soil classes: fluvisols, regosols, lithosols, rendzinas, yermosols, xerosols, vertisols, kastanozems, chernozems, phaezems, luvisols and acrisols.

2.2 Phenolic compounds analysis

The phenolic compounds from the studied samples were analyzed, in triplicate, following a previously reported procedure (Bajoub et al., 2015). The following sections (Sections

from 2.2.1 to 2.2.3) will describe the chemical and reagents, the extraction protocol, and the methodology used for their analysis and quantitative characterization.

2.2.1 Chemicals and reagents

All the solvents were of analytical or high pressure liquid chromatography-mass spectrometry (LC-MS) grade purity (depending on if they were used for the extraction or chromatographic analysis) and used without further purification. Methanol and *n*-hexane used for the extraction of the phenolic compounds from the olive oil samples were supplied from Panreac (Barcelona, Spain). Acetonitrile and acetic acid used for preparing mobile phases were purchased from Lab-Scan (Dublin, Ireland) and Panreac (Barcelona, Spain), respectively. Doubly deionised water with a conductivity of 18.2 MΩ was produced in the laboratory using a Milli-Q-system (Millipore, Bedford, MA, USA). Commercial standards of hydroxytyrosol, tyrosol, luteolin, apigenin, *p*-coumaric, and quinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein was purchased from Extrasynthese (Lyon, France).

Firstly, a stock solution (at a concentration of 500 mg/L for each standard) was prepared by dissolving the appropriate amount of the compounds in methanol, and then a series of working solutions of these analytes were freshly prepared by diluting the mixed standard solution with methanol at appropriate ratios to yield concentrations within the range 0.5-250 mg/L. The proper amount of 3,4-dihydroxyphenylacetic acid (DOPAC), purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in methanol to prepare an internal standard (IS) solution with a concentration of 500 mg/L. All solutions were stored in dark flasks at −20 °C and the stability controls assured their appropriate conservation for at least 6 months.

2.2.2 Phenolic compounds extraction

The methodology used to prepare the phenolic extracts in this study was a liquid-liquid extraction. Briefly, 25 µL of the IS solution was added to 2 g (±0.001) of olive oil weighed in a test tube with a screw cap. An aliquot of 1 mL of *n*-hexane was added to the oil and the phenolic compounds were extracted three consecutive times adding every time 2 mL of methanol/water (60:40, v/v); the mixture was then vortexed for 2 min and centrifuged at 3500 rpm for 6 min. The combined extracts were evaporated under reduced pressure

at 30 °C (rotary evaporator, Büchi R-210), and the obtained residue was reconstituted in 1 mL of methanol of LC-MS grade and filtered through 0.20 µm membrane (nylon) filter.

Bearing in mind the interesting results obtained by Karkoula´s research team (Karkoula, Skantzari, Melliou, & Magiatis, 2012; Karkoula, Skantzari, Melliou, & Magiatis, 2014) claiming the reactivity of some secoiridoids with methanol (and water) producing derivatives from the native secoiridoids (isomers (or alteration of their original proportions), hemiacetals or acetals), the same extraction protocol as described above was applied replacing the methanol/water extractant by acetonitrile. The residue obtained after the evaporation of the final combined extracts was, obviously, redissolved in acetonitrile and filtered before the injection into the LC-MS.

2.2.3 Separation, identification and quantification of the analytes under study

The phenolic extracts were analyzed using an Agilent 1260-LC system (Agilent Technologies, Waldbronn, Germany) with a vacuum degasser, auto-sampler and a diode-array detector (DAD) coupled to a HP Chemstation data-processing software. A Zorbax C18 analytical column (4.6 × 150 mm, 1.8 µm particle size) protected by a guard cartridge of the same packing, operating at room temperature was used. The mobile phases were 0.5% acetic acid in water (A) and acetonitrile (B). A flow rate of 0.8 mL/min was used and an injection volume of 10 µL of the methanolic extracts of olive oil or standards mix was injected in each case. The elution gradient established was the following: 0-10 min, 5% B; 10-12 min, 30% B; 12-17 min, 38% B; 17-20 min, 50% B; 20-23 min, 95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 2.5 min. Double on-line detection was carried out using a DAD (with 240 nm and 280 nm as optimum wavelengths) and a mass spectrometer connected to the LC system.

Mass spectrometry detection was performed in a Bruker Daltonik Esquire 2000™ Ion Trap (Bruker Daltonik, Bremen, Germany) equipped with Electrospray Ionization (ESI) and an ion trap mass analyzer operating in negative ion mode and controlled by the software Data Analysis 4.0 (Bruker Daltonik). The capillary voltage was set at +3200 V and the MS detector was programmed to perform scans at 50-800 m/z range. The following parameters of ESI-MS were used: drying gas temperature, 300 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 30 psi.

A standard mixture solution with a concentration of 5 mg/L was used as quality control (QC) sample to check the stability of the system over the different sequences carried out. The quality control sample was injected every five analyses (after a blank) in each sequence.

Additionally, for carrying out a proper characterization of the high resolution MS signals detected for each extract, several representative samples belonging to each geographic area were analyzed using the same chromatographic instrument coupled to a micrOTOF-Q II™ mass spectrometer (Bruker Daltonik, Bremen, Germany) by means of an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The above mentioned MS parameters were transferred to the ESI-QTOF spectrometer. The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonik), which provides a list of possible elemental formulas by using the SmartFormula™ Editor tool. Internal calibration was performed using sodium formate cluster by using a solution containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 v/v. The calibration solution was injected at the beginning of the run (using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA)) and all the spectra were calibrated prior carrying out the compound identification. For both mass spectrometer detectors, a flow divisor 1:4 was used, reducing the flow rate from the LC method to 0.2 mL/min, which is low enough to avoid the introduction of humidity in the system.

The identification of the phenolic compounds present in the analyzed samples was based on the use of pure standards (when available), retention time data, and comparing the ESI-TOF MS and MS/MS spectra with previously published results (Bajoub et al., 2015; Dierkes et al., 2012; García-Villalba et al., 2010; Gilbert-López et al., 2014; Sánchez de Medina, Priego-Capote, & de Castro, 2015, among others). For the quantitative analysis, a calibration curve of every compound was constructed using eleven different concentrations (0.5, 1, 2.5, 5, 12.5, 25, 35, 50, 100, 250 and 500 mL/L) of the standard mixture solution and plotting the peak areas versus the concentrations, obtaining the following results: apigenin ($y = 253332x + 51956$; $R^2 = 0.997$); hydroxytyrosol ($y = 68325x + 52766$; $R^2 = 0.996$); luteolin ($y = 197530x + 15246$; $R^2 = 0.999$); oleuropein ($y = 5845.9x + 26977$; $R^2 = 0.9967$); *p*-coumaric ($y = 49140x + 5243.7$; $R^2 = 0.999$);

pinoresinol (y = 60211x + 18014; R^2 = 0.998); quinic acid (y = 92639x - 3640.9; R^2 = 0.999) and tyrosol (y = 29745x + 8476.7; R^2 = 0.999). When a pure standard was not available, the quantification was made using the calibration curve of a similar compound (approach commonly used by numerous authors): oleuropein was used for all secoiridoids and related compounds; hydroxytyrosol was used for oxidized hydroxytyrosol; and lignans were quantified in terms of pinoresinol. The results were expressed in mg per kg of olive oil, as mean ± standard deviation for three independent analyses. When necessary, a dilution of the extracts was injected to make sure about carrying out the quantification within the linear dynamic range.

2.3 Statistical analysis

The quantitative data obtained for the studied variables (phenolic compounds) were subjected to univariate and multivariate statistical treatments. First, a homogeneity of variances test was applied (Levene test), indicative of the type of analysis to use (parametric or non-parametric). On the basis of the obtained results, for all the variables studied, a parametric analysis of variance (one-way analysis of variance (ANOVA)) was applied. When significant differences were found ($p < 0.05$), means were compared using the Tukey test. This analysis was carried out to evaluate both the effect of the geographical provenance and the crop season on the concentration of the identified phenolic compounds. The statistical treatment was carried out using SPSS statistical package software (SPSS for Windows, Version 20, SPSS Inc., and Chicago, USA).

Moreover, multivariate data analyses were applied to the obtained data set, using a 136 × 29 matrix (136 samples, 30 variables (phenolic compounds)), without any pre-treatment, in order to ascertain to which extend a geographical discrimination could be achieved using the phenolic fraction composition. Principal component analysis (PCA) -an unsupervised technique- was first used in order to reduce the dimensionality of the data matrix by calculating a number of components (Principal components (PCs)), which reduce the number of variables retaining the maximum amount of variability present in the data. It is a way to provide a better visualization of the data structure in a reduced dimension. Afterwards, linear discriminant analysis (LDA) -a supervised method- was applied to construct classification models. Discriminant functions were obtained as a linear combination of variables that maximize the ratio of between-class variance and

minimize the ratio of within-class variance. The statistical significance of each discriminant function was evaluated on the basis of the Wilks' λ factor after the function was removed. The variables are determined with a stepwise analysis. The prediction capacity of the discriminant models was studied by "leave-one-out" cross-validation. The Statistical software XLSTAT version 2015.04.1 (Addinsoft, France) was used for multivariate data analysis.

3 Results and discussion

3.1 Phenolic compounds profiling

LC-MS was used to determine the phenolic profile of the monovarietal 'Picholine Marocaine' extra VOOs produced in the three different Moroccan regions over two consecutive crop seasons (2012/2013 and 2013/2014). Typical base peak chromatograms (BPCs) of the methanolic extracts of representative samples from the three studied regions are shown in Fig. 2, where peak numbers have been assigned in accordance with the elution order in the chromatogram. As can be observed from this figure, a similar phenolic composition -from the qualitative point of view- was found in the samples from the three regions studied in this work; in all cases, the BPC consisted of 30 peaks corresponding to 29 phenolic compounds and a polar non-phenolic compound (quinic acid (peak **1**)). The identification was achieved on the basis of criteria described in Section 2.2.3 of Material and methods and our results were in good agreement with previous report focused on the analysis of olive oils from different countries and cultivars (Dierkes et al., 2012; García-Villalba et al., 2010; Gilbert-López et al., 2014; Kesen, Kelebek, & Selli, 2014; Montedoro et al., 1993; Ouni et al., 2011; Sánchez de Medina, Priego-Capote, & de Castro, 2015; Taamalli, Arráez-Román, Zarrouk, Segura-Carretero, & Fernández-Gutiérrez, 2012).

Table 1 summarizes retention time, molecular formula, experimental and calculated mass to charge signal (*m/z*), error, mSigma value, and the proposed identity for each peak. An important number of the identified compounds belong to the group of secoiridoids: oleuropein aglycone (Ol Agl) (peak **26**) (widely designated as 3,4 DHPEA-EA), ligstroside aglycone (Lig Agl) (peak **30**) (extensively named as *p*-HPEA-EA), and 6 (peaks **10, 12, 15, 20, 23** and **27**) and 3 (peaks **16, 24** and **28**) isomers of each one, respectively; decarboxymethyl oleuropein aglycone (DOA) (peak **11**) (also known as dialdehydic form

of decarboxymethyl elenolic acid linked to (3,4-Dihydroxyphenyl)ethanol (3,4 DHPEA-EDA)), dehydro oleuropein aglycone (Dehydro Ol Agl) (peak **25**), methyl oleuropein aglycone (Methyl Ol Agl) (peak **29**), methyl decarboxymethyl oleuropein aglycone (Methyl D-Ol Agl) (peak **19**), decarboxymethyl ligstroside aglycone (D-Lig Agl) (peak **21**) (known as dialdehydic form of decarboxymethyl elenolic acid linked to (*p*-Hydroxyphenyl)ethanol (*p*-HPEA-EDA) to), dialdehydic form of decarboxymethyl elenolic acid (D-Ald-D EA) (peak **5**), desoxy elenolic acid (Desoxy-EA) (peak **7**), hydroxy elenolic acids (Hy-EA) (peak **8**) and elenolic acid (EA) (peak **9**).

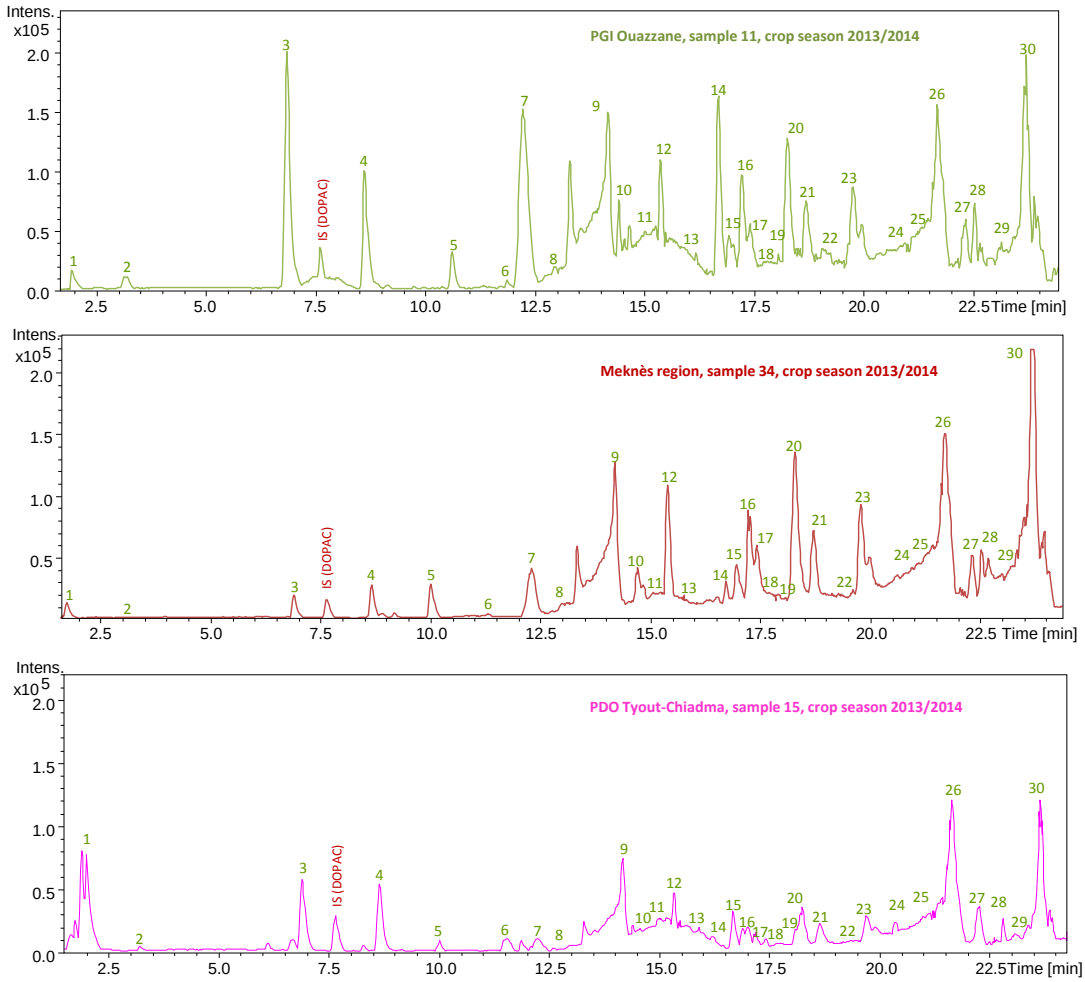

Figure 2: Base peak chromatograms (BPCs) typical for Meknès, PGI Ouazzane and PDO Tyout-Chiadma extra VOOs samples. The peak numbers are the same as those used in Table 1.

Apart from secoiridoids, three simple phenols, hydroxytyrosol (Hyt) (peak **3**), oxidized hydroxytyrosol (O-Hyt) (peak **2**) and tyrosol (Ty) (peak **4**)), were also found in all the studied samples. The other identified peaks were lignans ((+)-pinoresinol (Pin) (peak **17**),

(+)-1-acetoxypinoresinol (Ac-Pin) (peak **18**) and syringaresinol (Syr) (peak **13**)), flavonoids (luteolin (Lut) (peak **14**) and apigenin (Apig) (peak **22**)), and a phenolic acid (*p*-coumaric acid (peak **6**)).

The presence of multiple isomers of aldehydic Ol Agl and Lig Agl is, nowadays, attracting a lot of attention and deserves special mention, in particular, after the inspiring reports published by Karkoula et al. (Karkoula, Skantzari, Melliou, & Magiatis, 2012; Karkoula, Skantzari, Melliou, & Magiatis, 2014) about the artificial formation of some secoiridoid derivatives, mainly due to their reactivity with methanol (and water) or even because of their interaction with the silica-based stationary phase used in chromatography. The quoted authors´ assertion is, from our point of view, absolutely right, and in the coming paragraphs we will try to demonstrate this statement.

According to Karkoula, acetonitrile does not react with the aldehydic Ol and Lig aglycons or with the aldehydic form of DOA or D-Lig Agl; therefore, some samples were extracted both with the very widely used methanol/water (60:40, v/v) mixture and acetonitrile. When acetonitrile replaces the methanol-water mixture as extractant, Ol Agl (peak 26) and 3 isomers (instead of 6), at relatively low proportions, were detected. Likewise, Lig Agl (peak 30) plus one of its isomers (instead of 3) were observed. Consequently, we can confirm that the formation of what could be so-called "artificial isomers" happens as long as methanol (and probably water and/or their mixtures) is involved in the sample preparation, dilution of the extract or has any interaction at any point of the analytical procedure with these compounds.

Apart from this, the same research team also observed something similar concerning DOA and D-Lig Agl, corroborating the formation of acetals or hemiacetals for these compounds. Our experience with the samples used in this study suggests that the generation of artificial peaks (related to DOA and D-Lig Agl) in the chromatograms is not as drastic as for Ol Agl and Lig Agl and could be even ignored from a quantitative point of view. Further experiments are ongoing in our lab to keep delving into these topics and clarify additional details.

Table 1. Main phenolic compounds (or related analytes) identified in the studied extra VOO samples by using LC-ESI-TOF MS.

Peak	Compounds	Retention time (min)	Molecular formula	*m/z* experimental	*m/z* calculated	Error (mDa)	mSigma
	Simple phenols						
2	Oxidized hydroxytyrosol	3.39	C8 H8O3	1.510.408	1.510.401	-4.7	8.0
3	Hydroxytyrosol	6.95	C8 H10 O3	1.530.562	1.530.557	-3.4	12.9
4	Tyrosol	8.69	C8 H10 O2	1.370.609	1.370.608	-0.5	17.1
	Secoiridoids						
5	Decarboxymethylated form of elenolic acid	10.72	C9 H12 O4	1.830.671	1.830.663	-4.5	8.5
7	Desoxy elenolic acid	12.27	C11 H14 O5	2.250.769	2.250.768	-0.3	9.4
8	Hydroxy elenolic acid	12.66	C11 H14 O7	2.570.686	2.570.667	-7.6	11.5
9	Elenolic acid	14.20	C11 H14 O6	2.410.733	2.410.718	-6.4	4.8
11	Decarboxymethyl oleuropein aglycone	15.23	C17 H20 O6	3.191.202	3.191.187	-4.8	9.1
19	Methyl decarboxy oleuropein aglycone	18.30	C18 H22 O6	3.331.353	3.331.344	-2.7	6.0
21	Decarboxymehtyl ligstroside aglycone	18.84	C17 H20 O5	3.031.258	3.031.238	6.4	11.6
25	Dehydro oleuropein aglycone	21.63	C19 H20 O8	3.751.095	3.751.085	-2.4	27.9
26	Oleuropein aglycone	21.71	C19 H22 O8	3.771.242	3.771.242	-0.1	16.1
10	Oleuropein aglycone (Isomer1)	14.70	C19 H22 O8	3.771.257	3.771.242	-3.9	19.4
12	Oleuropein aglycone (Isomer2)	15.39	C19 H22 O8	3.771.259	3.771.242	-4.5	19.4
15	Oleuropein aglycone (Isomer3)	16.94	C19 H22 O8	3.771.260	3.771.242	-4.7	11.9
20	Oleuropein aglycone (Isomer4)	18.37	C19 H22 O8	3.771.263	3.771.242	-5.5	12.7
23	Oleuropein aglycone (Isomer5)	19.74	C19 H22 O8	3.771.260	3.771.242	-4.7	16.9
27	Oleuropein aglycone (Isomer6)	22.25	C19 H22 O8	3.771.253	3.771.242	-3.1	14.7
29	Methyl oleuropein aglycone	23.73	C20 H24 O8	3.911.421	3.911.398	-5.9	28.4
30	Ligstroside aglycone	23.84	C19 H22O7	3.611.305	3.611.293	-3.5	12.5
16	Ligstroside aglycone (Isomer1)	17.22	C19 H22O7	3.611.310	3.611.293	-4.9	18.0
24	Ligstroside aglycone (Isomer2)	21.49	C19 H22O7	3.611.311	3.611.293	-5.0	23.8
28	Ligstroside aglycone (Isomer3)	22.75	C19 H22O7	3.611.308	3.611.293	-4.1	16.8
	Lignans						
13	Syringaresinol	16.38	C22 H25 O8	4.171.569	4.171.555	-3.3	32.6
17	Pinoresinol	17.44	C20 H22 O6	3.571.351	3.571.344	-2.0	11.0
18	Acetoxypinoresinol	18.07	C22 H24 O8	4.151.392	4.151.398	1.6	7.6
	Flavonoids						
14	Luteolin	16.70	C15 H10 O6	2.850.413	2.850.405	-2.9	5.2
22	Apigenin	19.51	C15 H10 O5	2.690.466	2.690.455	-4.0	3.4
	Phenols acids						
6	*p*-coumaric acid	11.77	C9 H8O3	1.630.395	1.630.401	3.6	40.1
1	***Other polar compounds: Quinic acid***	2.08	C7 H12 O6	1.910.560	1.910.561	0.7	13.4

Within this context and bearing in mind that methanol-water mixtures have been very extensively used (being even recommended by the International Olive Council method) and the fact that water and/or methanol are probably present (water for sure) in most of the LC mobile phases combinations employed to analyze phenols from VOOs, we decided to keep using the extraction protocol implying the use of the methanolic mix. Before continuing, we corroborated something already known for us: the reproducibility of all the reactions that occur because of the presence of this solvent. If 10 independent extracts of the same sample were prepared and analyzed by our method, comparing the areas of the 30 peaks observed within the profiles (and expressing the repeatability in terms of relative standard deviations (RSD) (in %)), the outcome was very satisfactory (RSD < 7.12%, in all the cases).

All these ideas bring along with the following: (1) several "artificial peaks" (isomeric forms, acetals or hemiacetals) are always formed when methanol is present in any step of the analytical procedure to determine phenols from VOO; (2) these peaks will be obviously emerge in the LC profiles; and (3) they are formed from the native forms of the secoiridoids present in the VOO sample, so ignoring them is, to a certain extent, underestimating their initial "native amount". Points (1) and (2) have been confirmed by other authors (Angerosa, d'Alessandro, Corana, & Mellerio, 1996; Caruso, Colombo, Patelli, Giavarini, & Galli, 2000; Dierkes et al., 2012; Fu, Arráez-Román, Menéndez, Segura-Carretero, & Fernández-Gutiérrez, 2009; Gilbert-López et al., 2014; Obied, Bedgood, Prenzler, & Robards, 2007; Pérez-Trujillo, Gómez-Caravaca, Segura-Carretero, Fernández-Cutiérrez, & Parella, 2010). Moreover, in some other papers, even if the authors do not mention any isomeric form for some secoiridoids (in particular for Ol Agl and Lig Agl), when they show the extracted ion chromatograms of the m/z signal of these compounds in GC-MS or LC-MS, multiple peaks can be observed. Point 3 was partially addressed by Karkoula´s team, claiming that, in some applications, the produced "artificial peaks" are often not measured, just considering part of the unreacted native secoiridoids and leading, therefore, to questionable conclusions. To the best of our knowledge, Ol Agl (peak 26 at 21.7 min) and Lig Agl (peak 30 at 23.8 min) are the only ones that are quantified in numerous publications. Some authors, however, give a quantitative estimation considering of all the isomers together (Dierkes et al., 2012; Gilbert-López et al., 2014). This approach seems correct to us, but, from our point of

view, taking into account the considerable amounts of secoiridoids that we found in the oils included in this study, the total area, for instance, of Ol Agl, could very easily be found beyond the linear range of our method. That is the reason explaining the fact that we prefer to give an individual quantitative estimation for each isomer, since we consider this option as more accurate from an analytical point of view (taking into account the linear dynamic range of our method). It is interesting to mention that other authors carry out a hydrolysis after the extraction of polyphenols from olive oil to evaluate total hydroxytyrosol and tyrosol, avoiding all the mentioned difficulties (Purcaro, Codony, Pizzale, Mariani, & Conte, 2014). It is not possible to conclude this argumentation without mentioning that the lack of commercially available pure standards (only accessible for some of the phenolic compounds found in VOO and not for secoiridoids) is a big issue and, together with the previously enumerated problems, can explain the differences found regarding the reported levels of VOO phenols.

3.2 Quantitative analysis

Table 2 includes the quantitative results expressed as means and standard deviations (mean ± SD) for the identified phenolic compounds. It also includes an estimation of the total content for each chemical category, expressed as the sum of the individual phenols belonging to that chemical class.

Secoiridoids resulted to be the most abundant chemical family found in the phenolic fraction of the studied samples, regardless of their geographic origin. For the two crops seasons considered in this study, the samples with the highest mean content in terms of total secoiridoids were those from PGI Ouazzane region (744.68 mg/kg in 2012/2013 and 1224.81 in 2013/2014), followed by Meknès olive oil samples (594.51 mg/kg in 2012/2013 and 844.54 in 2013/2014), and PDO Tyout-Chiadma samples (507.67 mg/kg and 562.91 in the first and second seasons, respectively). The difference is particularly remarkable in the season 2013/2014, when PGI Ouazzane samples total content for secoiridoids was nearly double of the found amount in the PDO Tyout-Chiadma oils.

Table 2. Mean ± standard deviation (mg/kg) of the phenolic compounds determined in the evaluated extra VOOs.

Phenolic compounds	Crop season 2013 (n=51)			Crop season 2014 (n=85)		
	PGI Ouazzane (n=17)	PDO Tyout-Chiadma (n=12)	Meknès Extra VOO (n=22)	PGI Ouazzane (n=25)	PDO Tyout-Chiadma (n=25)	Meknès Extra VOO (n=35)
Simple phenols						
O-Hyt	$1.07^{a}a\pm0.08$	$0.58^{b}a\pm0.09$	$0.27^{c}a\pm0.22$	$0.80^{a}b\pm0.11$	$0.31^{b}b\pm0.07$	$0.51^{c}b\pm0.34$
Hyt	$19.61^{a}a\pm0.82$	$11.78^{b}a\pm2.55$	$3.83^{c}a\pm3.98$	$11.75^{a}b\pm0.76$	$4.12^{b}b\pm1.83$	$3.50^{b}a\pm3.12$
Ty	$18.33^{a}a\pm1.36$	$15.12^{b}a\pm5.12$	$8.54^{c}a\pm2.78$	$10.29^{a}b\pm5.58$	$8.82^{a}b\pm2.97$	$5.51^{b}b\pm3.80$
Total	$39.01^{a}a\pm1.77$	$27.48^{b}a\pm6.33$	$12.63^{c}a\pm6.29$	$22.84^{a}b\pm5.44$	$13.25^{b}b\pm4.34$	$9.52^{c}b\pm7.11$
Lignans						
Syr	$0.14^{a}a\pm0.02$	$0.36^{b}a\pm0.04$	$0.16^{a}a\pm0.08$	$0.10^{a}b\pm0.01$	$0.26^{b}b\pm0.03$	$0.08^{a}b\pm0.05$
Pin	$0.76^{a}a\pm0.05$	$0.64^{b}a\pm0.04$	$0.38^{c}a\pm0.12$	$0.14^{a}b\pm0.02$	$0.71^{b}b\pm0.05$	$0.64^{b}a\pm0.62$
Ac-pin	$0.67^{a}a\pm0.05$	$0.93^{a}a\pm0.08$	$1.27^{a}a\pm2.12$	$0.10^{a}b\pm0.02$	$2.16^{b}a\pm2.61$	$1.00^{a}a\pm0.91$
Total	$1.92^{a}a\pm0.14$	$1.81^{a}\pm2.27$	$1.98^{a}a\pm0.07$	$0.34^{a}b\pm0.03$	$3.13^{b}b\pm2.62$	$1.72^{c}b\pm1.56$
Flavonoids						
Lut	$1.78^{a}a\pm0.06$	$1.20^{b}a\pm0.05$	$1.26^{b}a\pm0.51$	$3.51^{a}b\pm0.27$	$0.93^{b}b\pm0.10$	$1.47^{c}a\pm0.72$
Apig	$0.20^{a}a\pm0.02$	$0.13^{b}a\pm0.01$	$0.18^{ab}a\pm0.10$	$0.43^{a}b\pm0.02$	$0.09^{b}b\pm0.01$	$0.19^{c}a\pm0.10$
Total	$1.98^{a}a\pm0.07$	$1.33^{b}a\pm0.05$	$1.44^{b}a\pm0.54$	$3.94^{a}b\pm0.28$	$1.01^{b}b\pm0.11$	$1.65^{c}b\pm0.79$
Phenolic acids						
p-Coum	$0.21^{a}a\pm0.03$	$0.08^{b}a\pm0.02$	$0.22^{a}a\pm0.16$	$0.29^{a}b\pm0.08$	$0.12^{b}b\pm0.02$	$0.08^{c}b\pm0.04$

Table 2. (continued)

Phenolic compounds	Crop season 2013 (n=51)			Crop season 2014 (n=85)		
	PGI Ouazzane (n=17)	PDO Tyout-Chiadma (n=12)	Meknès Extra VOO (n=22)	PGI Ouazzane (n=25)	PDO Tyout-Chiadma (n=25)	Meknès Extra VOO (n=35)
Secoiridoids						
D-Ald-EA	19.12^{a}a±5.00	40.52^{b}a±7.84	2.40^{c}a±7.05	8.31^{a}b±0.70	14.77^{b}b±5.16	2.19^{c}a±2.68
Desoxy-EA	2.43^{a}a±0.46	1.16^{a}a±1.84	11.54^{b}a±3.21	4.18^{a}b±2.16	5.77^{b}b±1.34	13.92^{c}a±9.13
Hy-EA	4.09^{a}a±0.37	2.95^{b}a±0.80	0.16^{c}a±0.06	2.41^{a}b±7.64	0.65^{b}b±0.51	0.34^{c}b±0.33
EA	282.91^{a}a±10.77	51.51^{b}a±4.75	83.90^{c}a±34.22	153.05^{a}b±12.91	71.40^{b}b±5.89	120.71^{c}b±38.05
DOA	37.98^{a}a±2.82	53.69^{b}a±3.99	72.30^{b}a±48.17	127.32^{a}b±10.41	64.04^{b}b±6.50	103.92^{c}a±78.14
Methyl D-Ol Ag	18.49^{a}a±1.45	5.52^{b}a±0.67	22.21^{c}a±4.69	22.24^{a}b±1.06	8.13^{b}b±1.23	19.70^{a}a±9.14
D-Lig Agl	26.10^{a}a±1.78	31.37^{a}a±2.32	32.49^{a}a±18.15	140.29^{a}b±8.39	35.36^{b}a±10.50	68.62^{c}b±46.20
Dehydro Ol Agl	4.40^{a}a±0.41	1.40^{b}a±0.18	3.84^{a}a±2.26	0.40^{a}b±0.29	0.19^{a}b±0.29	1.24^{c}b±0.70
Ol Agl	99.28^{a}a±12.38	85.80^{b}a±7.72	91.32^{c}a±18.34	149.68^{a}b±14.1	106.64^{b}b±7.70	119.14^{b}b±40.46
Ol Agl-isomer1	6.68^{a}a±1.64	2.03^{b}a±0.26	14.33^{c}a±11.25	27.70^{a}b±3.24	8.10^{b}b±2.07	30.53^{a}b±17.42
Ol Agl-isomer2	12.15^{a}a±1.79	4.65^{b}a±0.91	24.59^{c}a±17.82	38.13^{a}b±3.72	15.14^{b}b±2.53	41.44^{a}b±20.82
Ol Agl-isomer3	5.68^{a}a±1.72	5.73^{a}a±0.97	11.83^{b}a±5.46	25.66^{a}b±3.89	14.13^{b}b±2.50	23.23^{a}b±10.10
Ol Agl-isomer4	16.13^{a}a±2.11	10.41^{b}a±1.07	14.15^{a}±5.98	35.79^{a}b±3.93	18.47^{b}b±3.49	30.82^{a}b±13.48
Ol Agl-isomer5	32.12^{a}a±3.80	23.88^{b}a±2.52	21.39^{b}a±6.17	54.13^{a}b±5.49	32.80^{b}b±8.08	45.64^{c}b±17.96
Ol Agl-isomer6	12.97^{a}a±0.76	8.14^{b}a±0.40	14.34^{a}a±5.72	42.13^{a}b±2.17	20.42^{b}b±3.06	31.01^{c}b±8.57
Methyl Ol Agl	4.43^{a}a±0.23	0.82^{b}a±0.08	1.71^{c}a±1.40	5.55^{a}b±0.34	0.98^{b}b±0.11	0.91^{c}b±0.57
Lig Agl	84.42^{a}a±6.25	64.71^{b}a±4.34	75.68^{c}a±7.61	132.49^{a}b±8.57	67.99^{b}a±6.42	102.99^{c}b±52.42
Lig Agl-isomer1	24.15^{a}a±2.77	5.48^{b}a±0.29	21.10^{a}a±10.97	55.42^{a}b±8.39	11.16^{b}b±1.54	39.56^{c}b±22.04
Lig Agl-isomer2	14.52^{a}a±1.36	3.36^{b}a±0.26	22.66^{c}a±13.62	30.67^{a}b±3.89	6.75^{b}b±1.67	24.90^{c}a±13.32
Lig Agl-isomer3	19.48^{a}a±2.33	8.15^{b}a±1.72	10.55^{c}a±1.48	26.61^{a}b±4.48	12.80^{b}b±2.10	21.00^{c}b±12.96
Total	744.68^{a}a±27.07	507.67^{b}a±15.18	594.51^{c}a±72.59	1224.81^{a}b±68.20	562.91^{b}b±37.30	844.54^{c}b±190.54
Other compounds						
Quinic acid	1.74^{a}a±0.23	1.68^{a}a±0.70	1.43^{a}a±1.62	0.73^{a}b±0.55	3.45^{b}b±0.50	0.97^{a}a±0.83

-In the current table, the mean values are those calculated for all the samples coming from the same area for each season, and therefore, SD gives to the reader only an idea about the variability of the olive oils in terms of phenolic composition, and obviously not about the repeatability of the analytical methods used.

-Significant differences in the same row are indicated with different lowercase letters (comparison among crop seasons, $p < 0.05$) and with different superscript letters (comparison among the three studied regions at the same crop season, $p < 0.05$).

These results can be explained considering the differences regarding the climatic conditions and, especially, the olive oil extraction systems used. Indeed, several interesting studies focused on evaluating the effect of the extraction systems on the phenolic content if the olive oil, showed that continuous centrifugation systems markedly increase the total phenols content (Fregapane, Salvador, Aranda, & Go, 2003). In the particular case of secoiridoids, crushing and kneading (malaxation) appear to highly modulate their content in the obtained oils, mainly by affecting the activity of enzymes related to the biosynthesis and transformation of phenolic compounds, such as β-glucosidase and esterase (Frankel, Bakhouche, Lozano-Sánchez, Segura-Carretero, & Fernández-Gutiérrez, 2013; Fregapane & Salvador, 2013), being the hammer crusher (used in the current study for the production of the oils coming from PGI Ouazzane and Meknès) pointed out as the crushing system which leads to higher content of secoiridoids, if compared with the traditional stone mills (used, in this case, for extracting oils of the PDO Tyout-Chiadma).

When the quantitative values of the individual secoiridoids are studied, it can be observed that EA was the dominant compound for Ouazzane region (for first and second seasons, with 282.91 and 153.05 mg/kg, respectively) and Meknès (crop season 2014, with 120.71 mg/kg), being Ol Agl the most abundant complex phenol for the oils coming from Tyout-Chiadma region (both seasons (85.80 for the 1st season and 106.64 mg/kg for the second one)) and Meknès area in 2012/2013 (91.32 mg/kg). The extra VOOs coming from PGI Ouazzane showed the highest mean values for a great number of secoiridoids, being the richest in terms of 10 compounds over the first season. In that season, Meknès oils exhibited the highest mean concentration levels for 9 secoiridoids, and PDO Tyout-Chiadma for one analyte. PGI Ouazzane produced again the richest extra VOOs over the season 2013/2014, showing the highest levels of 15 compounds (Hy-EA, EA, DOA, Methyl D-Ol Ag, D-Lig Agl, Ol Agl, Ol Agl-isomer 3, 4, 5 and 6, Methyl Ol Agl, Lig Agl and Lig Agl-isomer 1, 2 and 3). Meknès oils showed, for the second season, the highest concentrations of Desoxy-EA, dehydro Ol Agl and Ol Agl isomers 1 and 2; and the PDO Tyout-Chiadma only showed the highest mean concentrations for one of the complex phenols determined, D-Ald-EA (with 14.77 mg/kg); indeed, the oils coming from this PDO were, in general, those with lowest levels of secoiridoids.

Simple phenols were the second group in terms of abundance. The simple phenols total contents of olive oil samples from PGI Ouazzane (39.01 mg/kg in 2012/2013 and 22.84 mg/kg in 2013/2014) were comparatively higher than those from Tyout-Chiadma region (27.48 mg/kg in 2012/2013 and 13.25 mg/kg in 2013/2014) and Meknès area (12.63 mg/kg in 2012/2013 and 9.52 mg/kg in 2013/2014). These results confirm the findings achieved by our group in previous works, where low levels of simple phenols were found in VOOs produced in Meknès region (Bajoub et al., 2015). Extra VOOs from PGI Ouazzane were the richest in terms of O-Hyt (1.07), Hyt (19.61) and Ty (18.33 mg/kg) in season 2012/2013 and also in the second year of the study (O-Hyt (0.80), Hyt (11.75), Ty (10.29 mg/kg)). The samples coming from Ouazzane region exhibited higher concentrations of Hyt than Ty, finding an opposite trend (higher levels of Ty than Hyt) for the oils from Tyout-Chiadma region and Meknès.

When lignans are considered, it is possible to highlight that Meknès extra VOOs were the samples with the highest mean value in the season 2012/2013 (1.98 mg/kg) and those coming from Tyout-Chiadma region, the richest in 2013/2014 (3.13 mg/kg). A remarkable decrease of lignans levels was observed for PGI Ouazzane from the first season (1.92 mg/kg) to the second one (0.34 mg/kg). Olive oils from PGI Ouazzane were distinctive from those of Meknès and Tyout-Chiadma, since over the period of our study, Pin was the most abundant lignan in these samples (with a mean value concentration of 0.76 and 0.14 mg/kg in the first and second crop seasons, respectively). In contrast, for the oils coming from Meknès and Tyout-Chiadma, Ac-pin concentrations were significantly higher than the levels of the two other detected lignans. All the analyzed oils showed low content of Syr, being the mean concentration values within the range from 0.08 mg/kg, detected in Meknès oils during the second crop season, and 0.36 mg/kg, found for PDO Tyout-Chiadma oils.

As far as flavonoids are concerned, the levels were fluctuating between 1.01 and 3.94 mg/kg for the three different regions over the two consecutive seasons, being PGI Ouazzane the provenance with the oils exhibiting the highest levels (1.98 in 2012/2013 and 3.94 mg/kg in 2013/2014). Lut was by far the most abundant flavonoid, with mean concentration values ranging between 0.93 mg/kg (found on Tyout-Chiadma oils) and 3.51 mg/kg (showed by samples of PGI Ouazzane), both values obtained in season 2013/2014. Apig showed mean concentration values within the range of 0.09 mg/kg

(Tyout-Chiadma oils) and 0.43 mg/kg (PGI Ouazzane oils), both values obtained over the second crop season.

The phenolic acid *p*-Coum was found in all the analyzed oils; it levels ranged from 0.08 mg/kg (PDO Tyout-Chiadma, first crop season and Meknès, second season) to 0.29 mg/kg (PGI Ouazzane, 2013/2014).

As stated in Section 3.1, beyond the phenolic compounds characterized in this study, one polar non-phenolic compound, quinic acid, was also determined. Its highest content was found in PDO Tyout-Chiadma oils (3.45 mg/kg), while the lowest levels were determined for Meknès olive oils (0.97 mg/kg), in both cases, over the second crop season.

ANOVA analysis was performed to test the effect of the geographic area of origin and crop season on the content on the phenolic compounds of the studied samples. Differences in all the identified phenolic compounds were observed considering the geographical origin, except for two compounds, Ac-pin and D-Lig Agl, for which the geographic provenance of the samples had no statistically significant effect during the first crop season (Table 2). Additionally, the influence of the crop season resulted to be very prominent for all the compounds in the case of PGI Ouazzane olive oils, whereas in the two other studied regions, some compounds did not show significant differences in this respect (Table 2). Thus, ANOVA results suggest that variations in the concentration of D-Lig Agl and Lig Agl in oils from Tyout-Chiadma PDO are not associated to crop season factor. The phenolic compounds contents from Meknès extra VOOs seem to be less affected by the crop season than those of oils from PGI Ouazzane and PDO Tyout-Chiadma; indeed, no statistically significant differences were observed for Hyt, Pin, Ac-pin, Lut, Apig, D-Ald-EA, Desoxy-EA, Methyl D-Ol Agl and Lig Agl-isomer 2 in the oils coming from Meknès region when the crop season factor was considered (Table 2). All the other phenolic compounds identified in these oils were affected by the crop season. These results ratify previous results obtained by our group concerning the effect of the crop season on the phenolic compounds variation in olive oils produced in Meknès region (Bajoub et al., 2015). Moreover, the current results revealing the significant influence of both crop season and geographic area on the phenolic compounds of the studied oils, are, from our point of view, in good agreement with those obtained by other authors who investigated and proved their influence on VOOs produced from different

cultivars in other Mediterranean regions (Kesen et al., 2014; Ouni et al., 2011; Romero et al., 2015; Taamalli et al., 2012).

3.3 Geographical provenance discrimination

One that the quantitative characterization of the samples under study was achieved we faced the multivariate analysis trying to classify the samples according to their geographical origin. In a first step, the data set was initially subjected to PCA, to get a general overview of the data distribution and detect the natural grouping of studied samples. Afterwards, the supervised classification technique linear discriminant analysis (LDA) was applied. No specific pre-treatment of the data (e.g. mean centering, auto-scaling or dimensionality reduction) was carried out.

When PCA was performed, a new set of latent factors or principal components (PCs) was generated. The first principal component (PC1) had the highest eigenvalue of 12.84, and accounted for 40.11% of the variability in the data set. The second, third, fourth, fifth, and sixth PCs (PC2, PC3, PC4, PC5, and PC6) had eigenvalues of 5.60, 3.87, 2.43, 1.24 and 1.10, and explained 17.48%, 12.10%, 7.60%, 3.88% and 3.41% of the variance in the data, respectively. The remaining generated PCs (PC7 to PC32) yielded progressively smaller eigenvalues (<1.00%) and explained less than 16% of the total variance in the data set. Afterwards, taking into account the Kaiser Rule (only eigenvalues of >1.00 are considered significant descriptors of data variance) (Kaiser, 1960), only the first sixth PCs were considered. Plotting the scores of the samples in the sub-spaces PC1 vs. PC2 (Fig.3a) (accounting for 57.59% of the total variance of the data) no clear grouping of samples was observable according to the geographical origin. What can be observed in Fig. 3a is the influence of the crop season, in particular in samples from PGI Ouazzane, where the effect of this factor was more remarkable. Indeed, there are two groups clearly distinguished within the samples belonging to this PGI. The samples which are more spread out on the plane defined by PC1 and PC2 are the extra VOOs from Meknès, fact that can be explained considering that in this area different olive oil mills (two and three phases extraction systems) were used to produce the samples (what obviously could bring a bit more of heterogeneity), whereas in the case of the other two areas, just one mill was producing all the samples coming for each geographic location. The projection of the samples along the directions identified by PC2 and PC3 (Fig.3b) and PC3 and PC4 (Fig.

3c) reveled a certain visual clustering of samples from PGI Ouazzane (Fig. 3b) and PDO Tyout-Chiadma (Fig. 3c).

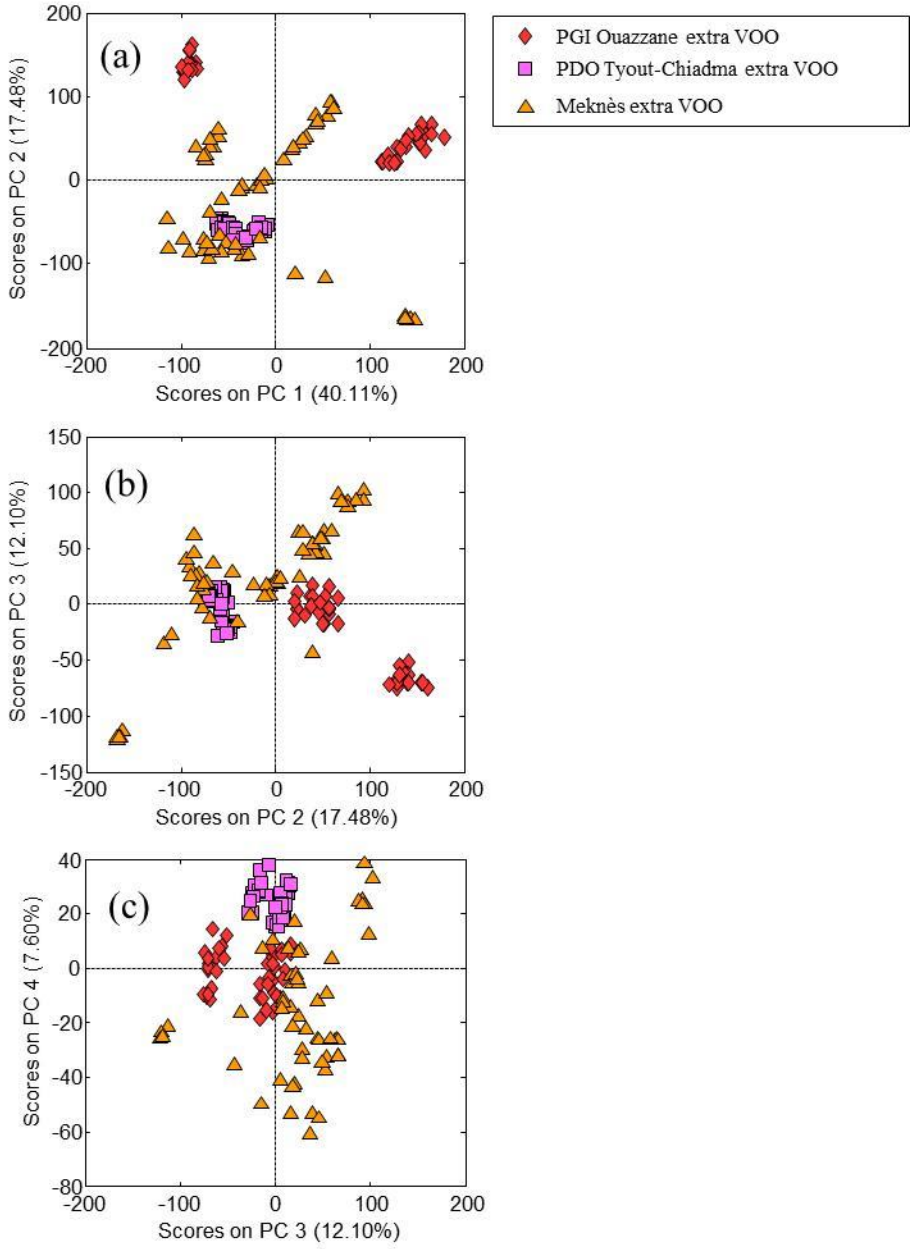

Figure 3: Principal component analysis score plots of the studied olive oil samples on the first two principal components (3a); the sub-plane defined by the second and the third principal components (3b); and the sub-plane defined by the third and the fourth principal components (3c).

Subsequently, a LDA model was constructed for classifying the olive oil samples according to their geographic origins. The forward stepwise variable selection method was used to select the most relevant phenolic compounds for the discrimination of geographical origins, and 10 variables were excluded from the LDA modeling: 5 secoiridoids (Ol Agl, Lig Agl, Ol Agl-isomer 4, Lig Agl-isomer 2 and Lig Agl-isomer 3), 2

lignans (Pin and Ac-Pin), 1 simple phenol (Tyr), 1 phenolic acid (*p*-Coum) and quinic acid. Table 3 lists the standardized coefficients, the eigenvalues and cumulative proportions of total variance and canonical correlation of the two canonical discriminant functions obtained when LDA was performed. As shown in the table,

the first discriminant function explains 86.32% of the variance in the dataset, whereas, the second function explains 13.68%. The magnitude of Wilk's lambda, which reflects the proportion of the variance in the dataset that is not accounted by the model, encompassing both functions of the model, was 0.005, reflecting the good discriminating ability of the LDA model.

Table 3. Variable standardized canonical discriminant function coefficients along with discriminant functions classification parameters.

		Discriminant functions	
Variables		Function 1	Function 2
O-Hyt	Standardized Canonical Discriminant Function Coefficients	0.796	-0.576
Hyt		1.190	-0.306
D-Ald-D EA		-1.056	0.322
Desoxy-EA		0.451	1.255
Hy-EA		0.316	0.537
EA		2.323	0.719
DOA		0.784	0.817
Syr		-1.248	0.506
Lut		0.789	0.400
Methyl D-Ol Agl		1.000	-0.296
D-Lig Agl		-0.561	-1.162
Apig		1.641	-0.712
Dehydro Ol Agl		0.616	-1.032
Ol Agl-isomer 1		-1.397	0.228
Ol Agl-isomer 2		1.110	-0.047
Ol Agl-isomer 3		-0.713	-0.842
Ol Agl-isomer 5		-0.533	-0.264
Ol Agl-isomer 6		-2.041	1.538
Methyl Ol Agl		-0.091	0.957
Lig Agl-isomer 1		0.771	-0.373
Eigenvalue		35.142	5.568
Variance (%)		86.32	13.68
Cumulative variance (%)		86.32	100.00
Canonical correlation		0.986	0.921

A closer examination of the coefficients of the discriminant functions (the higher is the absolute value of a standardized coefficient, the more significant is the related selected variable in the canonical variable) indicates that the compounds EA, Apig, Ol Agl-isomer 1 and Ol Agl-isomer 6 are those with a stronger contribution to the first discriminant function, whilst the second canonical discriminant function was mainly related to Desoxy-EA, D-Lig Agl, Dehydro Ol Agl and Methyl Ol Agl. Apart from them, we could mention O-Hyt, Hyt, D-Ald-D EA, Syr, Lut, Methyl D-Ol Agl, Ol Agl-isomer 2, Ol Agl-isomer 5, and Lig Agl-isomer 1 are other relatively important contributors to the first discriminant function, whereas Hy-EA, DOA, and Ol Agl-isomer 3 are features of certain relevance for the second canonical discriminant function. The location of the olive oil samples within the plane defined by the two obtained canonical functions is shown in Fig. 4.

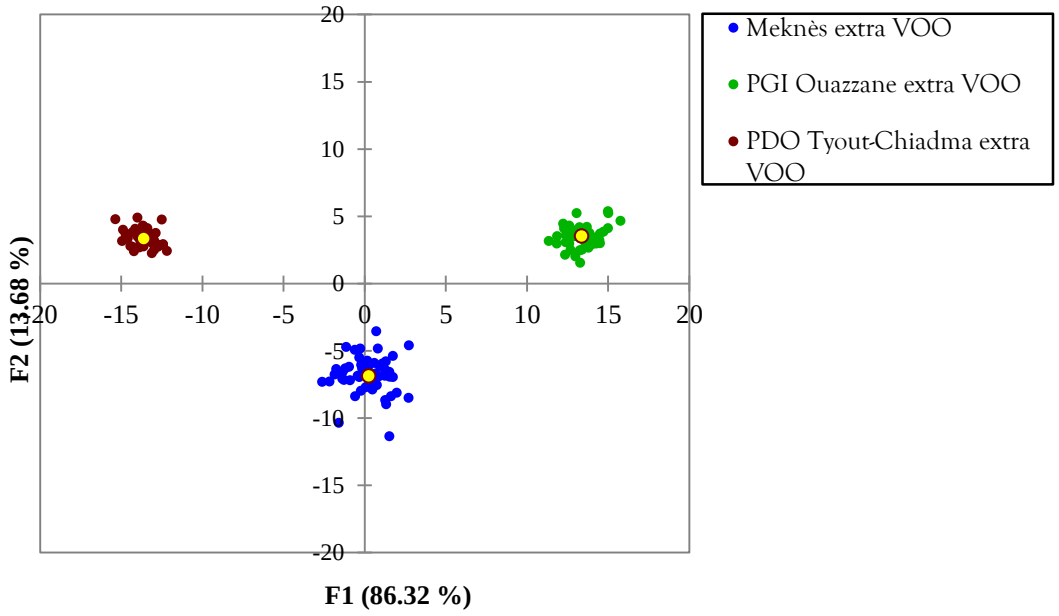

Figure 4: Discriminant analysis similarity map obtained for the geographical classification of the studied samples determined by discriminant factors 1 and 2.

The scatter plot shows a quite good separation among the samples as a function of the geographic origin. It can be said that the first discriminant function allowed an excellent separation among the samples from PGI Ouazzane and PDO Tyout-Chiadma (PGI samples are laying at positive values of F1, and PDO Tyout-Chiadma oils appear at negative values of F1 axis), being the second discriminant function which mainly differentiates Meknès extra VOOs from the rest.

Additionally, the results reported in Table 4 showed the suitability of this discriminant approach for achieving an accurate classification of the studied samples according to their geographical origin (all the studied samples were correctly classified (100%)).

Table 4. Classification matrix, according to LDA, for the geographical origin of the studied extra VOO samples.

		Zone	Predicted Group Membership			Total
			PGI Ouazzane	PDO Tyout Chiadma	Meknès	
Original	Count	PGI Ouazzane	42	0	0	42
		PDO Tyout Chiadma	0	37	0	37
		Meknès	0	0	57	57
	%	PGI Ouazzane	100.0	0	0	100.0
		PDO Tyout Chiadma	0	100.0	0	100.0
		Meknès	0	0	100.0	100.0
Cross-validated	Count	PGI Ouazzane	42	0	0	42
		PDO Tyout Chiadma	0	37	0	37
		Meknès	1	2	54	57
	%	PGI Ouazzane	100.0	0	0	100.0
		PDO Tyout Chiadma	0	100.0	0	100.0
		Meknès	1.8	3.5	94.7	100.0

Furthermore, the predictive abilities of the model were validated using the leave-one-out cross-validation. This technique was used to check the percentage of correctly classified oils, since it could be overestimated, considering that the oil samples being classified were also used to build the classification functions. The obtained results after applying the cross-validation (Table 4) evidenced a good overall predictive ability in terms of low misclassification of sample origin. The prediction ability obtained was higher than 94%; the misclassified samples were three samples from Meknès territory that were incorrectly classified as Ouazzane (1 of them) and Tyout-Chiadma olive oils (2 samples coming from Meknès) , whereas 100% of the samples belonging to PGI Ouazzane and PDO Tyout-Chiadma were correctly classified.

These results lead us to suggest that combining phenolic compounds data and chemometrics is a very advisable and reliable approach to trace the origin of VOOs from different provenance geographical indications systems in Morocco; some other authors have observed the same for other cultivars originating from different Mediterranean countries (Gurdeniz et al., 2008; Lerma-García et al., 2009; Ouni et al., 2011; Taamalli et al., 2012).

4 Conclusions

This is the first reported study about the composition of the phenolic compounds in commercial extra VOOs from Meknès territory, PGI Ouazzane and PDO Tyout-Chiadma. The content of these compounds was significantly influenced by both crop season and geographic region. As a general statement, it can be said that PGI Ouazzane samples showed the highest contents of a large number of the identified compounds, whilst olive oils from Tyout-Chidama showed lowest levels. The usefulness of our contribution could be described considering two aspects: 1) we have highlighted the richness of the extra VOOs produced in the three studied zones in terms of bioactive compounds (in this case, phenolic compounds) what can be particularly relevant for the producers to promote the commercialization of these oils as healthy products ; 2) this procedure may be an important tool for the detection of fraud and control of the geographic authentication of oils coming from these regions and produced under geographical origin indication systems. Moreover, the proposed approach could be easily adapted to other olive growing areas certified under geographical origin indication systems.

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CONCLUSIONS AND PERSPECTIVES/ CONCLUSIONES Y PERSPECTIVAS

CONCLUSIONS AND PERSPECTIVES

This thesis investigated multiple aspects regarding metabolomics approaches development and their application in the field of geographical and botanical authentication of virgin olive oil. We have demonstrated that appropriate analytical determinations of some components of this matrix and the subsequent application of chemometric tools are promising techniques for reliable and efficient geographical and/or botanical origin traceability. In the following paragraphs, we have formulated the partial conclusions achieved in each section:

In section 1: A comprehensive and deep analysis of the actual status of the international olive oil regulation frameworks has been presented in chapter 1, highlighting the ongoing debate about the risks caused by the current proliferation of regulation standards systems all over the world for olive oil trade. Furthermore, the analytical methods used, nowadays, to official assessment of the quality and authenticity of olive oil have been revisited, emphasizing their drawbacks and demonstrating the absence of official procedures for the geographical and botanical origin authentication (despite the increasingly importance of the commercialization of olive oil under geographical and botanical certification scheme). Finally, the main advantages shown by recent analytical approaches to overcome the found issues have been described.

Afterwards, attention was paid to the definition of quality and purity criteria indices for North Moroccan olive oils and the development of chemometric models for their geographical origin authentication (Chapter 2). This topic is of paramount importance to Moroccan olive oil sector if we bear in mind the relevance of the studied zones which account for over 49% of Moroccan overall olive oil production. The main findings of this are, among others, definition of the variation ranges of quality and composition profiles of the studied oils, geo-location of olive-growing areas whose oils showed high level of linolenic acid and the compilation of a two-years database of Moroccan olive oil quality and composition. Moreover, the built chemometrics models when applied to North Moroccan olive oils seemed to be very efficient for geographical origin traceability purpose.

In section 2: different metabolomic approaches have been optimized and efficiently applied to trace the geographical and/or botanical origin of olive oil. The olive oil compounds investigated within this section were triacylglycerols and flavor compounds (phenolic and volatile compounds). Thus, when chemometrics were applied, for instance, to triacylglycerols data (Chapter 6), the developed multivariate statistical models were able to successfully classify and predict the origin of the studied oils. In addition, even if some differences existed between the different chemometric methods tested in this study, all of them presented a great potential with regard to the study aim.

As fa as flavor compounds are concerned, the investigation of the phenolic and volatile fraction of a wide sample collection coming from different regions, countries and/or cultivars gave us the opportunity to compile a very interesting database on their occurrence and variability, depending on the geographical and genetic origin.

Phenolic compounds were analyzed by using different approaches (liquid-liquid extraction and LC-MS analysis, solid-phase extraction and LC-MS analysis, solid-phase extraction and GC-MS analysis, and direct injection and LC-MS analysis) and the obtained data were treated by different multivariate statistical methods (PCA, k-NN, PLS-DA, SIMCA and s-LDA). Both profiling and fingerprinting metabolomic approaches were developed and successfully applied for the geographical or botanical origin classification of olive oil.

Volatile compounds were analyzed using two analytical platforms: HS-SPME-GC FID/MS and SIFT-MS (Chapters 4 and 9). The first one allowed the comprehensive characterization of the quali-quantitative volatile profiles of North Moroccan olive oils. Further, through the application of s-LDA, it was possible to correctly classify and predict the geographical origin of the analyzed oils. When SIFT-MS technique was used to reveal fingerprints of volatile headspace of olive oil and a chemometric (PLS-DA) treatment was applied to the achieved data, it was possible to satisfactorily discriminate between Mediterranean origin labeled olive oils. The developed method offers some interesting advantageous when compared to classical methods.

In section 3: the potential of analytical methods to resolve some problems related to the olive oil geographical indication establishment was fully verified. It is important to look for methodologies able to characterize the quality and compositional profiles and also to prove the distinctiveness of an origin-labeled (or a candidate to be certified) olive oil.

Meknès region case was taken as an example, to illustrate our argumentation. Thus, the proposed methodology, through a pluriannual study of the composition and the physico-chemical and sensory quality of Meknès olive oil, was able to verify the homogenous character of the qualitative and compositional profile of the oil coming from this region. The phenolic profile was also established and a database summarizing the main characteristics of olive oil produced within the studied region was compiled. Furthermore, distinctiveness of Meknès olive oil (compared with Moroccan olive oils produced in other origin-labeled regions) was tested and efficiently proved. This was possible taking advantage of a metabolomic approach optimized within this thesis, combining phenolic compounds profiling and chemometric treatment.

Certainly, the outcomes of this thesis are of interest for olive oil Moroccan sector (and also from a more international point of view), since most of the topics considered in this dissertation have been treated and discussed for the first time. However, there is still a lot of work to be done. Some open questions are: the evaluation of the applicability of the developed metabolomic approaches to the characterization and/or geographical and botanical origin authentication of oils produced in other important Moroccan olive growing areas; the deep characterization of nutritional value of Moroccan olive oils; an assessment of the causes of linolenic acids high level in Moroccan olive oils; the establishment of analytical approaches to control possible Moroccan olive oil adulteration; and the execution of comparative studies between Moroccan olive oils and those produced in other countries.

CONCLUSIONES Y PERSPECTIVAS FUTURAS

En la presente tesis se han estudiado muchos aspectos concernientes al desarrollo y aplicación de aproximaciones ómicas en el campo de la autentificación del origen geográfico y botánico del aceite de oliva. Se ha demostrado que la determinación de los compuestos apropiados (de entre todos los presentes en la matriz objeto de estudio), y el posterior tratamiento de datos mediante quimiometría, pueden representar una combinación analítica fiable y eficiente para asegurar la trazabilidad geográfica/botánica. En los siguientes párrafos, se formulan las conclusiones que se han ido alcanzando en cada una de las secciones, así:

En la sección 1, se presenta un análisis exhaustivo y profundo del estado actual de los marcos reguladores en el ámbito del aceite de oliva (Capítulo 1), poniendo de manifiesto el debate actual en relación a los riesgos que afloran en este campo como consecuencia de la proliferación y coexistencia de diferentes sistemas reguladores de aplicación en diversas partes mundo. En dicho capítulo, se ha dado también una visión general de todos los métodos oficiales (ventajas e inconvenientes) y se ha resaltado la carencia de métodos para la autentificación geográfica y botánica. De igual manera, se han presentado los avances metodológicos más relevantes que podrían resolver algunos de los problemas de los métodos usados hasta el momento.

Tras ello, la atención se centró en definir los criterios de pureza y calidad para aceites del norte de Marruecos y en el desarrollo de modelos quimiométricos para la autentificación de su origen geográfico (Capítulo 2). Este tema posee gran importancia para el sector oleícola en Marruecos, sobre todo si tenemos en cuenta la relevancia de las zonas consideradas en el estudio, que representan el 49% de la producción global del país. Los principales hallazgos fueron, entre otros, la definición de los rangos típicos de calidad y perfil composicional de los aceites estudiados, la geo-localización de las áreas de producción en las que el aceite presenta niveles de ácido linolénico por encima de los establecidos por la norma, y la elaboración de una base de datos bi-anual con datos de calidad y composición del aceite marroquí. Los modelos estadísticos que se construyeron,

cubrían nuestro objetivo, pudiendo asegurar la trazabilidad en términos de origen geográfico.

En la sección 2, diferentes aproximaciones metabolómicas fueron desarrolladas y aplicadas para evaluar el origen (geográfico o botánico) de aceites de oliva virgen. Los compuestos del aceite que se determinaron fueron los triglicéridos y los analitos relacionados con su flavor (compuestos fenólicos y fracción volátil). Cuando se aplicaron herramientas estadísticas a los datos de triglicéridos (Capítulo 6), por ejemplo, se pudo determinar correctamente el origen de los aceites. Además, si bien se observaron algunas diferencias entre los diferentes modelos, todos y cada uno de ellos, mostraron un gran potencial en relación con el objetivo planteado.

En lo que respecta a las otras dos familias de compuestos mencionadas, la fracción volátil y el perfil polifenólico de un buen número de aceites (provenientes de diferentes regiones, países y variedades) fueron estudiados, dándonos la oportunidad de recabar una cantidad de información muy interesante para conformar una base de datos acerca de sus niveles habituales y variabilidad en función del origen genético y geográfico del aceite.

Los compuestos fenólicos, en esta tesis, han sido estudiados empleando diferentes herramientas (extracción líquido-líquido y LC-MS, extracción en fase sólida y LC-MS, extracción en fase sólida y GC-MS, e inyección directa de la matriz de aceite (con una simple dilución) y LC-MS); los datos obtenidos en cada caso han sido tratados mediante métodos estadísticos multivariantes (PCA, k-NN, PLS-DA, SIMCA y s-LDA). Aproximaciones metabólicas *profiling* y *fingerprinting* se han aplicado con éxito para garantizar la trazabilidad de los aceites seleccionados.

En relación a los compuestos volátiles, indicar que se han estudiado utilizando dos plataformas: HS-SPME-GC FID/MS y SIFT-MS (Capítulos 4 y 9). En el primer caso, se logró la caracterización exhaustiva del perfil de volátiles (cualitativo y cuantitativo) de aceites del norte de Marruecos. A través de la aplicación de s-LDA, nos fue posible clasificar correctamente (y predecir el origen geográfico de) los aceites seleccionados. Cuando SIFT-MS fue elegida como plataforma analítica y sus datos tratados mediante PLS-DA, también se logró la discriminación de aceites provenientes de sistemas de indicación geográfica certificada. Las características analíticas y ventajas de esta plataforma (si se compara con otras más ampliamente utilizadas) han sido discutidas.

En la sección 3: se ha verificado el potencial de diferentes métodos analíticos para resolver problemas relacionados con la indicación geográfica certificada de distintos aceites. Es muy importante desarrollar metodologías capaces de caracterizar la calidad y el perfil composicional de un aceite dato, así como aptas para demostrar la tipicidad o carácter distintivo de aceites pertenecientes a sistemas de indicación geográfica certificada (o candidatos a serlo). La región de Meknès en Marruecos, se tomó como ejemplo para ilustrar los aspectos más importantes de la disertación. Así, la metodología que se propuso, a través de un estudio a lo largo de dos campañas consecutivas en el que se evaluaba la calidad físico-química y sensorial del aceite de Meknès, fue capaz de verificar la homogeneidad del perfil cualitativo y composicional del aceite producido en esta región.

El perfil fenólico de los aceites se evaluó y, tras ello, se construyó una base de datos para plasmar las principales características del aceite producido en la mencionada región. Además, las características distintivas del aceite de Meknès (en comparación con otros aceites producidos al amparo de sistemas de indicación geográfica certificada en Marruecos) fueron puestas de manifiesto. Esto fue posible gracias al empleo de aproximaciones metabolómicas que se aplicaron durante esta tesis doctoral, combinando el estudio del perfil fenólico de los aceites con tratamientos estadísticos avanzados.

Todos los logros de esta tesis son, desde nuestro punto de vista, muy interesantes para el sector oleícola de Marruecos (y también a nivel internacional), ya muchos de los temas aquí tratados no habían sido estudiados con anterioridad. Sin embargo, es más que evidente que queda mucho trabajo por hacer. Algunas cuestiones permanecen abiertas: evaluación de la aplicabilidad de algunas de las herramientas aquí desarrolladas para la caracterización y autentificación del origen geográfico/botánico de aceites de otras áreas productoras de Marruecos; caracterización profunda del perfil nutricional de los aceites marroquíes; evaluación de las causas que expliquen altos niveles de ácido linolénico en aceites marroquíes; establecimiento de aproximaciones analíticas para el control de adulteraciones y fraudes en el país; y la realización de estudios que permitan llevar a cabo una comparación profunda entre aceites marroquíes y otros provenientes de distintos países.