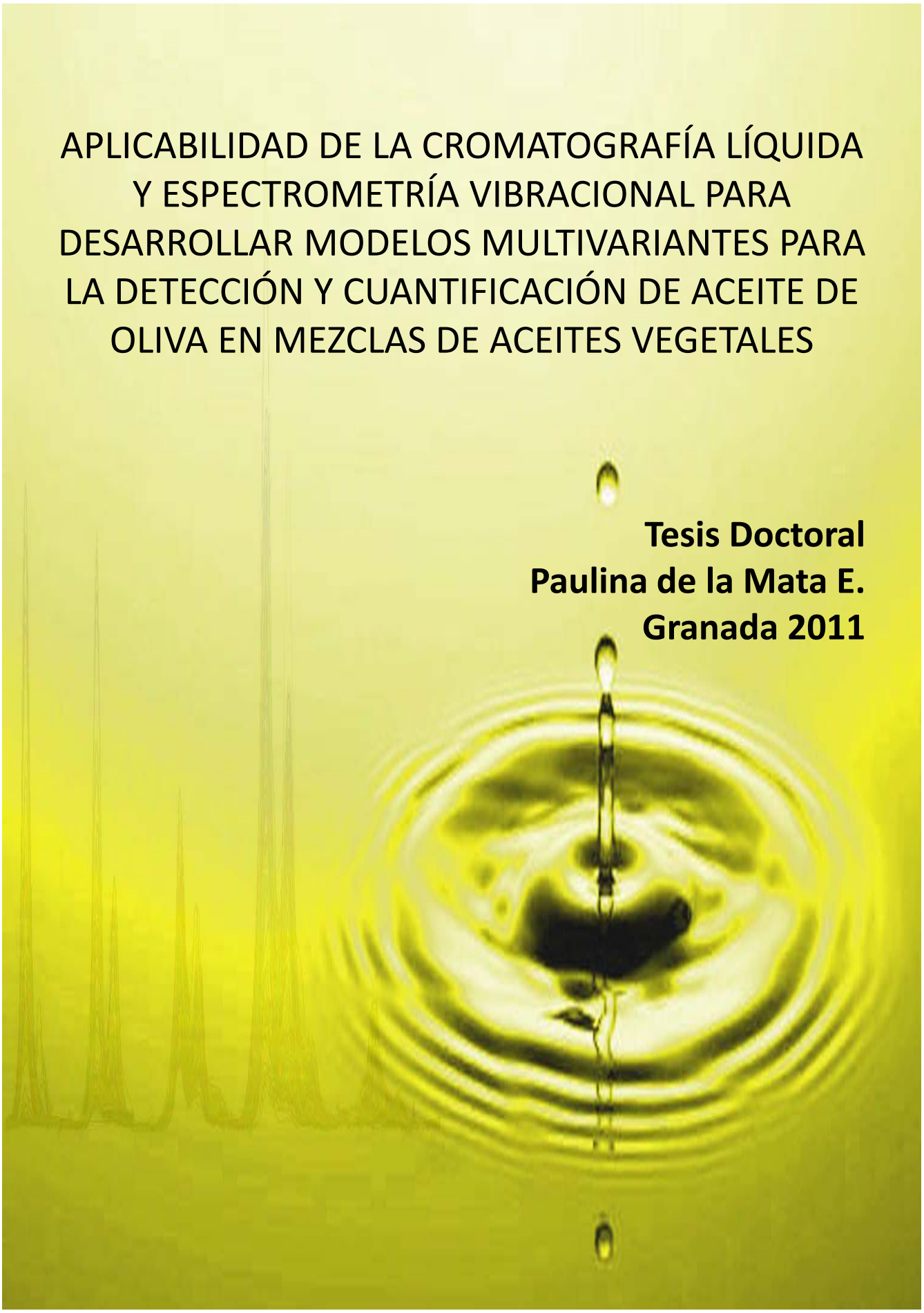
APLICABILIDAD DE LA CROMATOGRAFÍA LÍQUIDA Y ESPECTROMETRÍA VIBRACIONAL PARA DESARROLLAR MODELOS MULTIVARIANTES PARA LA DETECCIÓN Y CUANTIFICACIÓN DE ACEITE DE OLIVA EN MEZCLAS DE ACEITES VEGETALES



Tesis Doctoral
Paulina de la Mata E.
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Aplicabilidad de la cromatografía líquida y espectrometría vibracional para desarrollar modelos multivariantes para la detección y cuantificación de aceite de oliva en mezclas de aceites vegetales

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Tesis doctoral

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Índice

PRESENTACIÓN

El aceite de oliva se consume directamente (sólo o mezclado con otros aceites) o como ingrediente en numerosos alimentos (conservas, salsas y aliños, galletas y productos de panadería, embutidos, patatas fritas, margarinas y mantequillas, sopas (líquidas o concentrados sólidos en pastilla), etc. El Reglamento CE 1019/2002 sobre las normas de comercialización del aceite de oliva, regula el contenido del mismo en productos alimenticios. La verificación de los requisitos especificados en este Reglamento requiere la existencia de métodos analíticos que permitan cuantificar la proporción de aceite de oliva en alimentos. En la actualidad, existe un creciente interés por parte de las empresas oleícolas de comercializar mezclas de aceites con objeto de ofrecer productos con un composición lipídica nutricionalmente óptima.

Sobre la base de este problema analítico, los estudios experimentales que conforman esta Memoria de tesis doctoral son consecuencia del planteamiento de una hipótesis de trabajo que radica en que es posible diseñar y desarrollar de metodologías analíticas, genéricas y fácilmente transferibles, de uso rutinario en laboratorios de control de la calidad del aceite de oliva para verificar los requisitos especificados en el ya mencionado Reglamento CE 1019/2002; dichos métodos se basarán en medir atributos intrínsecos del aceite de oliva relacionados con el contenido de compuestos característicos como son los triglicéridos, que son estables y por tanto permanecen bastante inalterados en el aceite. Las técnicas a aplicar en los estudios experimentales serán fundamentalmente la cromatografía de líquidos acopladas a un detector de partículas cargadas en aerosol (CAD) y la espectrometría vibracional en el infrarrojo (FTIR).

Por tanto, los objetivos que en su día se programaron fueron:

1. Se estudiarán los perfiles de contenidos de los triglicéridos y los datos encontrados se utilizarán para buscar índices y/o funciones de discriminación entre aceite de oliva y otros aceites vegetales, basado en aquellos que sean específicos del aceite de oliva.

2. Los resultados obtenidos, a partir de dichos índices y/o funciones que antes se comentan, y que estará altamente correlacionados con el contenido en aceite de oliva, podrán ser aplicados como variables predictoras en modelos univariantes de regresión.

3. Se evaluarán la aplicación de técnicas quimiométricas de análisis de datos multivariantes (reconocimiento de pautas) para desarrollar modelos de discriminación y clasificación.

4. Sobre la base de los modelos de clasificación encontrados, se desarrollarán modelos multivariantes para cuantificar aceite de oliva en mezclas de aceites vegetales y alimentos.

Todos los estudios experimentales se llevarán a cabo bajo un sistema de gestión en la calidad en la investigación y de los análisis realizados a fin de facilitar la transferencia de dichas metodología a sectores sociales relacionados con el control alimentario del aceite de oliva, lo que comporta una puesta en valor de los resultados obtenidos.

INTRODUCCIÓN

Tortilla Española

Ingredientes para 4 personas:

- 1/2 Kg. de patatas
- 1 cebolla
- 4 Huevos
- Aceite de Oliva Virgen Extra
- Sal

Preparación

Pelar las patatas y cortarlas en dos mitades a lo largo y luego en finas lonchas. Calentar el Aceite de Oliva Virgen Extra en una sartén y cortar las cebollas finamente y añadir al Aceite de Oliva Virgen Extra caliente y rehogar. Seguidamente añadir las patatas y dejar unos 25 minutos aproximadamente, comprobar que las patatas están blandas pinchándolas con un tenedor, escurrir las patatas quitando el Aceite de Oliva Virgen Extra sobrante y sazonarlas. Batir los huevos en un bol, y añadir las patatas. Remover para que se mezclen bien. Colocar la sartén de nuevo al fuego y añadir un poco de Aceite de Oliva Virgen Extra; cuando coja calor, volcar la mezcla, y remover con una espumadera de madera, rebajar la intensidad de la llama y dejar que la tortilla se vaya haciendo durante 5 - 6 minutos. Para evitar que se nos peque al fondo, moveremos la sartén de vez en cuando. Por último cubrir la sartén con una tapa del mismo diámetro y de un golpe volcar la tortilla sobre la tapa, dándole la vuelta. Volver a echar la tortilla en la sartén y dejar unos minutos para que termine de cuajar por el otro lado.

Aceite de oliva

Aceite de oliva, el recuerdo de estas tres palabras nos hacen sentir una multitud de sensaciones: aromas del mediterráneo, tomates aliñados o una tostada recién hecha. Por muchos años se ha considerado como un ingrediente típico de la cocina mediterránea, hoy, el aceite de oliva pasa las fronteras de su origen.

¿Qué es el aceite de oliva?

Las aceitunas son los frutos del olivo y dan lugar al aceite de oliva. Los olivos pertenecen a la familia de las Oleáceas, la cual tienen una diversidad muy grande. La familia contiene más de una veintena de géneros, en total cerca de trescientas especies. Carl von Linné, en el siglo XVIII, agrupó una treintena de ellos en un género único denominado *Olea* (a partir del latín *oleum*, término que proviene del griego *elaia*, aceite). Dentro de todas las especies que existen sólo la especie *Olea europea L* es la que se utiliza en alimentación.

El uso del aceite viene de muchos años atrás donde se utilizaba no sólo como alimento sino que era un producto básico para la medicina tradicional, higiene o belleza. También se utilizó como combustible o lubricante para herramientas. El aceite de oliva se considera el 4º alimento por su importancia a nivel mundial y su excelente calidad. El mayor productor de aceite de oliva es Europa, la producción de la campaña 2009/2010 fue de 2.148 (1000 toneladas). Dentro de Europa, España es el mayor productor con 1.200 (1000 toneladas), seguido de Italia y Francia [1]. El comercio del aceite de oliva representa un 15% del comercio mundial debido a que su valor por unidad del producto es mayor que otros aceites comestibles. Este porcentaje puede variar dependiendo del país. Por ejemplo, en España representa alrededor del 6% [2].

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[1] International Olive Oil Council, Olive Oils, Table 1: Production http://www.internationaloliveoil.org/downloads/production2_ang.PDF, nov 2009

[2] Aparicio R., Harwood J., Manual del Aceite de Oliva, AMV, Madrid 2003

El aceite de oliva y la salud

Varios estudios médicos recomiendan el consumo regular de aceite de oliva para tener una vida mejor.

Las aceitunas están esencialmente compuestas por agua, la cual va disminuyendo y dando a lugar al aceite, al fin de la maduración. En las olivas se pueden encontrar azúcares, proteínas, clorofila, sales minerales (sodio, azufre, fósforo, entre otros) y vitaminas (A, B1, C y E).

El aceite de oliva como los aceites de maíz, girasol, canola, etc., es un producto graso de origen vegetal y tiene un valor energético igual que los otros aceites vegetales: 900 Kcal/100 g, o sea, 90 Kcal por una cucharada sopera.

Sin embargo, el aceite de oliva se distingue de otros aceites vegetales de varias maneras. Primero, el aceite de oliva se obtiene de la pulpa de la aceituna por métodos a presión, a diferencia de los aceites de semillas donde el aceite procede de la extracción del grano con disolventes. Esta diferencia hace que el aceite de oliva mantenga vitaminas y compuestos polifenólicos que son de beneficiosas para la salud por su actividad antioxidante.

Otros compuestos saludables que están presentes en el aceite de oliva son los ácidos grasos, los cuales pueden ser de dos clases: saturadas e insaturadas (poliinsaturadas y monoinstauradas). Las grasas insaturadas son propias de los alimentos de origen vegetal, mientras que las saturadas de las grasas animales. El aceite de oliva contiene en una proporción alta de un ácido graso monoinstaurado, 65-80%, acido oleico. Gracias a este ácido graso el aceite de oliva es una de las grasas alimentarias de mejor digestión.

Por otra parte la vitamina E, presente en el aceite de oliva, evita la oxidación de las lipoproteínas o transportadores en sangre del colesterol, con lo que se puede decir que el aceite de oliva colabora en la prevención de enfermedades cardiovasculares.

Variedades de aceite de oliva

En la antigüedad se conocía al olivo como árbol inmortal gracias a la facilidad que tiene de reproducirse.

Existen una numerosa cantidad de variedades de aceitunas que se cultivan en un gran número de regiones. Todas ellas se adaptan al tipo de suelo y al clima de su región. En función de la variedad de la aceituna, el aceite producido tiene diferentes características sensoriales y fisicoquímicos.

Cada región oleícola es reconocida por una variedad de aceituna. En España existen varias comarcas olivareras, con diferentes variedades de aceituna. En la Tabla 1 se observan algunos ejemplos de las diferentes variedades de aceituna que podemos encontrar en España, Italia y Francia [3].

Proceso de elaboración

Para obtener el aceite de oliva es necesario romper las células vegetales mediante trituración hasta obtener una pasta homogénea que se somete a un prensado mediante dispositivos mecánicos que aplican presión a la pasta para después exprimirla. Este sistema de extracción del aceite de oliva es muy importante dentro de las características cualitativas del aceite. Un aceite de alta calidad se obtiene con aceitunas maduras y enteras. El proceso debe de comenzar inmediatamente al recibirse en las aceitunas, ya que los procesos fermentativos comienzan muy rápido y deterioran la calidad del aceite. Estos mecanismos pueden provocar mal olor, sabor defectuoso y elevar su acidez.

[3] Uceda Ojeda M., Aceites de Oliva Vírgenes Extra. Calidad y Diversidad, PROEDI, Zaragoza 2000

Tabla 1. Ejemplos de variedades de aceituna de España, Francia e Italia

Variedad	País	Región
Picual	España	Jaén, Córdoba, Granada
Cornicabra	España	Castilla-La Mancha
Hojiblanca	España	Córdoba, Málaga, Granada, Sevilla
Lechín de Sevilla	España	Sevilla, Córdoba, Málaga, Cádiz
Verdial de Badajoz	España	Badajoz, Cáceres
Empeltre	España	Tarragona, Navarra, Castellón
Arbequina	España	Lérida, Zaragoza, Tarragona, Huesca
Picudo	España	Córdoba, Granada, Málaga, Jaén
Verdial de Huevar	España	Sevilla
Morrut	España	Tarragona, Castellón
Sevillenca	España	Tarragona, Castellón
Verdial de Vélez-Malaga	España	Málaga
Blanqueta	España	Alicante, Valencia, Albacete, Murcia
Tanche	Francia	Nyons
Cailletier	Francia	Niza
Picholine	Francia	La Germaine, Languedoc
Frantoio	Italia	
Leccino	Italia	

El proceso de elaboración del aceite se puede dividir en los siguientes pasos [4]:

1. Recolección
2. Molienda
3. Batido
4. Separación sólido-Líquido
5. Almacenamiento

[4] Alba J., Izquierdo J.R., Gutiérrez F., Aceite de Oliva Virgen, Análisis Sensorial, Agrícola Española S.A., Madrid 2003

1. Recolección

Las aceitunas que se utilizan para obtener un aceite de calidad deben de estar recién recolectadas, exclusivamente de vuelo, sanas y con un punto óptimo de maduración. Deben de ser transportadas en condiciones que sufran el mínimo daño posible. La limpieza de la aceituna debe hacerse en las mejores condiciones para no afectar las características del aceite, como el sabor a hoja verde (si no se retira los restos de tallos) o el aumento de acidez y olor a humedad (por exceso de lavado con agua).

2. Molienda

En el proceso de molienda se debe tener en cuenta el tipo de trituradores a utilizar para no transferir trazas metálicas o catalizar su oxidación.

3. Batido

El batido se realiza con el objetivo de reunir el mayor número de gotas de aceite dispersas en la masa molida, que forman una película sobrenadante. Los tiempos de batido no deben superan los 90 minutos y la temperatura los 30°C para no tener repercusiones en las características organolépticas del aceite.

4. Separación sólido-líquido

El proceso de separación se puede realizar: i) por extracción parcial, utilizando cilindros giratorios de malla metálica; ii) por extracción utilizando prensas hidráulicas; o iii) por centrifugación con decantadores centrífugos de dos o tres salidas. Por cualquier medio por el que se realice se debe tener cuidado con las pequeñas cantidades residuos de sólido que puede deteriorar la calidad del aceite.

5. Separación líquido-líquido

Está separación se realiza porque los líquidos, el aceite, siguen teniendo un determinado grado de impurificación de otros componentes. La separación se puede realizar por decantación o por centrifugación. Para eliminar fase acuosa y sólidos suspendidos, se añade en la separadora una cantidad de agua caliente que no debe sobrepasar el 30-40% del aceite y de 35-40°C de temperatura para evitar influencia en la

calidad fisicoquímica y organoléptica del aceite como puede ser olores de humedad, avinagrado, sucio, borras o la oxidación del aceite.

6. Almacenamiento

El aceite producido debe clasificarse según sus características y composición para almacenarse en los depósitos para su maduración en bodega. Los depósitos deben mantener una temperatura en su interior de 18-20°C, ser de materiales inertes, con cierres herméticos, de fácil limpieza, con fondos que faciliten la decantación de impurezas y con válvulas que faciliten la carga y descarga. Con estas condiciones se protege la calidad del aceite de oliva.

Categorías de aceites

Aceite de oliva es definido, por la norma del Consejo Oleícola Internacional (COI) [5], como el aceite procedente del fruto del olivo, con exclusión de los aceites obtenidos por disolventes o por procedimientos de esterificación y de todas mezclas con aceites de otra naturaleza. Los aceites de oliva tienen la siguiente clasificación:

Aceites de oliva vírgenes son obtenidos únicamente del fruto del olivo por medio de procedimientos mecánicos en condiciones térmicas que no alteren el aceite y que las aceitunas no hayan sido tratadas más que en el lavado, decantación centrifugación y filtrado. Los aceites de oliva vírgenes se dividen dependiendo de su calidad y pureza en (mayor calidad a menor calidad):

a) Aceite de oliva virgen extra.

b) Aceite de oliva virgen

c) Aceite de oliva corriente

[5] COI/T.15/NC No 3/Rev. 4, Trade standard applying to olive oils and olive-pomace oils (2009). International Olive Council, Madrid

d) Aceite de oliva lampante (no apto para consumo)

Aceite de oliva refinado se obtiene a partir del aceite de oliva virgen mediante técnicas de refinado.

Aceite de oliva mezcla de aceites de oliva vírgenes y refinados para el consumo.

Aceite de orujo de oliva. Los orujos son los subproductos que se obtiene de las aceitunas una vez que se les ha extraído el aceite, suele estar formado por restos de huesos, piel, pulpa, agua, etc. Pero, todavía, puede quedar cierta cantidad de aceite dentro de él, normalmente menos del 4%. Este aceite es obtenido por medio de tratamientos con disolventes u otros procedimientos físicos con exclusión de los aceites obtenidos por procedimientos de reesterificación y de toda mezcla con aceite de otra naturaleza. El aceite de orujo de oliva se clasifica en:

a) Aceite de orujo de oliva crudo

b) Aceite de orujo refinado

c) Aceite de orujo de oliva. Mezcla de aceite de oliva crudo y aceite de orujo refinado.

Calidad del aceite de oliva

Dependiendo de la categoría a la que pertenezca el aceite de oliva, deben de cumplir con algunos criterios de calidad y de pureza. Estos criterios pueden ser químico-físicos u organolépticos. Para estos criterios existen límites que están establecidos en las normas oficiales como la del Consejo Oleícola Internacional [5] o la del Departamento de Agricultura de Estados Unidos [6].

[6] AMS-FV-08-0073-0006 (2010). United States Standards for Grades of Olive Oil and Olive-Pomace Oil. US Department of Agriculture, Washington

Los criterios de calidad son:

a) características organolépticas (atributos positivos y negativos)
b) contenido de ácidos grasos libres (%)
c) índice de peróxidos
d) absorbancia UV

Los criterios de pureza:

a) contenido de ácidos grasos trans
b) contenido de esteroles totales
c) contenido de triglicéridos ECN42
d) acidez
e) ceras
f) alcoholes terpénicos
g) esteradienos (estigmatadieno)
h) alcoholes alifáticos

Composición Química del Aceite

Los aceites son insolubles en agua pero solubles en la mayoría de los disolventes orgánicos. Su densidad es menor que la del agua y su consistencia a temperatura ambiente es líquida. Los principales componentes de los aceites son los triglicéridos. Otros componentes minoritarios son mono y diglicéridos, ácidos grasos libres, fosfolípidos, esteroles, vitaminas solubles en grasas, tocoferoles, pigmentos, ceras y alcoholes grasos, entre otros [7].

Los triglicéridos están compuestos por tres ácidos grasos unidos a una molécula de glicerol por medio de un enlace éster. Las propiedades físicas y químicas de los

[7] Technical Committee of the Institute of Shortening and Edible Oils, Inc, Food Fats and Oils, 9ed, Institute of Shortening and Edible Oils, Inc., NY 2006

aceites están influenciadas por el tipo y la posición de los ácidos grasos en la molécula de glicerol (o glicerina), por ejemplo en su punto de fusión. Los ácidos grasos, que componen los triglicéridos son cadenas de carbonos saturadas e insaturadas con un grupo hidroxilo (Figura 1).

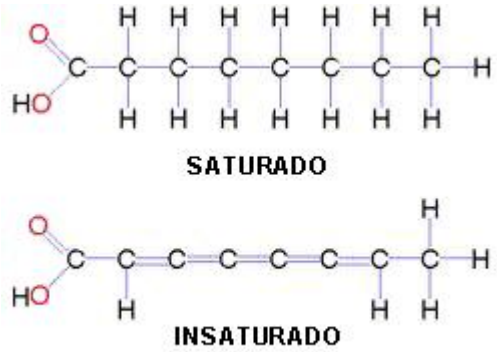

Fig1. Ácidos Grasos

Los ácidos grasos saturados son los que contienen enlaces simples en la cadena de carbono, mientras que los insaturados presentan dobles enlaces en la cadena. Dentro de los insaturados podemos encontrar a los monoinsaturados, con un solo doble enlace, y los poliinsaturados con dos o más. No todos los ácidos grasos se encuentran en los aceites, algunos de los más comunes se presentan en la Tabla 2 y Figura 2.

Como se menciono anteriormente los aceites están compuestos principalmente de moléculas de triglicéridos que pueden contener ácidos grasos ambos saturados e insaturados. Los triglicéridos se pueden dividir en dos tipos, simples y mixtos. Los simples son los que contienen los tres ácidos grasos iguales, mientras que los mixtos presentan un ácido graso diferente. Los triglicéridos simples pueden ser trisaturados o triinsaturados, los mixtos monosaturados o disaturados [7]. En la Figura 3 se muestra una molécula de triglicérido mixto disaturado.

Tabla2. Ejemplos de ácidos grasos

Nombre sistemático	Nombre común	No. de carbonos	No. de dobles enlaces.
Hexadecanoico	Palmítico	16	0
9-Hexadecenoico	Palmitoleico	16	1
Octadecanoico	Esteárico	18	0
9-Octadecenoico	Oleico	18	1
9,12-Octadecadienoico	Linoléico	18	2
9,12,15-Octadecatrienoico	Linolénico	18	3

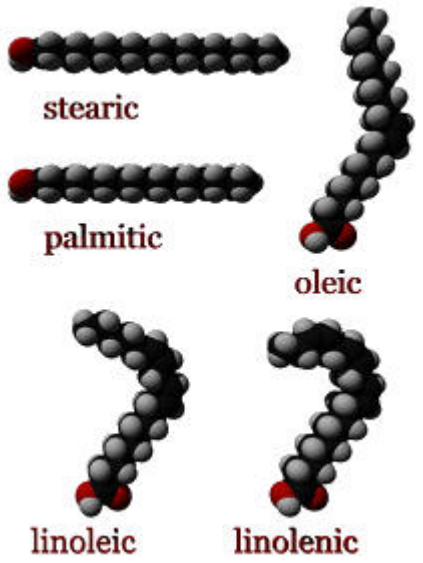

Fig2. Ejemplos de ácidos grasos [pustakalaya.org]

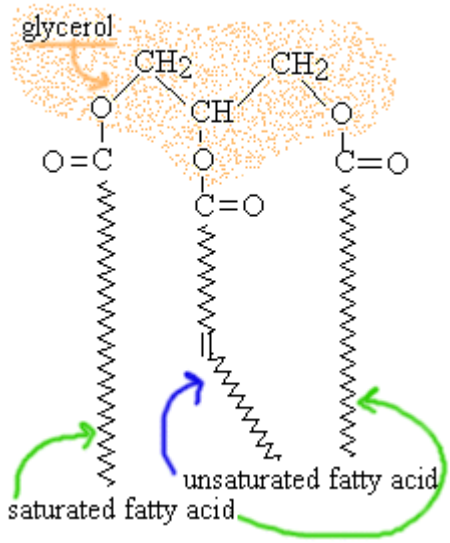

Fig3. Triglicérido mixto disaturado

Por lo tanto, los ácidos grasos pueden dar lugar a diferentes triglicéridos que puede ser diferenciados en estereoisómeros, es decir, dependiendo de la posición de los tres ácidos grasos en la molécula de triglicérido [8]. El número de triglicéridos presentes en un aceite, teniendo en cuenta los diferentes estereoisómeros, puede ser muy elevado. En la Tabla 3 se indican el número de isómeros de triglicéridos posibles en función del número de ácidos grasos presentes. Los aceites de vegetales contienen entre 5 y 10 ácidos grasos, mientras que las grasas animales del orden de 10 a 40 ácidos grasos [9].

[8] IUPAC-IUB Commission on Biochemical Nomenclature (1967) The nomenclature of lipids, J. Lipid Res. 8: 523-528.

[9] Buchgraber M, Ulberth F, Emons H, Anklam E (2004) Triacylglycerol profiling by using chromatographic techniques, Eur. J. Lipid Sci. Technol. 106: 621-648.

Tabla 3. Número de isómeros de triglicéridos [9]

Ácidos Grasos	Número de triglicéridos		
x	Isómeros totales x3	N° Isómeros ópticos (x3 + x2)/2	N° Isómeros (x3 + 3x2 + 2x)/6
2	8	6	4
3	27	18	10
4	64	48	20
5	125	75	35
10	1000	550	220
20	8000	4200	1540
40	64000	32800	11480

Caracterización de los triglicéridos en aceite de oliva

Para la determinación de los triglicéridos se han propuesto varias técnicas instrumentales en donde podemos destacar la utilización de cromatografía líquida de alta presión (HPLC) con diferentes detectores: absorción UV a longitud de onda definida, o de fila de diodos ("diode array detector", DAD), índice de refracción ("refractive index detector, RID) y el detector de dispersión de luz por vapor ("evaporative light scattering detector", ELSD). Sin embargo, el método oficial utilizado comúnmente para la determinación de triglicéridos en aceites vegetales en los laboratorios de rutina, está establecido por el Consejo Oleícola Internacional (COI) en el documento: COI/T.20/Doc. No 20/Rev. 2 [10], así como por la Unión Internacional de Química Pura y

[10] COI/T.20/Doc. No 20/Rev. 2 (2008), Method of analysis. Difference between actual and theoretical content of triacylglycerols with ECN 42. International Olive Council, Madrid

Aplicada (IUPAC) [11]. La metodología utilizada en dichos documentos es realizada utilizando HPLC y un detector de refractómetría diferencial, usando como parámetro el número equivalente de carbono (ECN equivalent carbon number), el cual está definido:

$$ECN = CN - 2n$$

Donde CN es el número de carbonos acilos (en las cadenas grasas) y n la suma de los enlaces dobles presentes en los ácidos grasos de los triglicéridos. Otro documento que utiliza la HPLC es el de American Oil Chemists´Society (AOCS) en el método oficial Ce 5b-89 [12], este documento permite el uso de tres diferentes detectores i) refráctometría diferencial, ii) absorción ultravioleta y iii) espectrometría de masas.

El aceite de oliva se caracteriza por cuatro picos mayoritarios con ECN 44, 46, 48 y 50. El ECN42 está presente en cantidades traza y el ECN 40 no está presente en este tipo de aceite. Los aceites de girasol, soja y lino se caracterizan por concentraciones de ECN 42 muy elevadas.

Los triglicéridos se han utilizado para caracterizar aceites vegetales. Se han analizado para cuantificar y clasificar aceites de oliva. Sin embargo, aunque sea el compuesto mayoritario en los aceites, presenta la dificultad, de encontrar un gran número de isómeros en un aceite. En este momento no existe ningún método analítico, que nosotros tengamos conocimiento, que identifique todos los isómeros.

Quimiometría

El término de quimiometría fue introducido en 1972 por el sueco Svante Wold y el estadunidense Bruce R. Kowalski. La definición de quimiometría es: “Disciplina química que usa métodos matemáticos y estadísticos para a) designar o seleccionar

[11] Wolff J.P., Mordret F.X., Dieffenbacher A. (1991) Determination of triglycerides in vegetable oils in terms of their partition numbers by high performance liquid chromatography, Pure & Appl. Chem. 63 No.8: 1773-1182

[12] AOCS Official Method Ce 5b-89 (1997). American Oil Chem.Soc., S. Boulder

medidas, procedimientos y experimentos óptimos; y b) proveer la máxima información química analizando datos químicos". Los métodos quimiométricos son aplicados generalmente para desarrollar evaluaciones de los datos analíticos. Las herramientas más utilizadas, entre otras, son [13,14]:

- Reconocimiento de pautas
- Procesamiento de señales químicas
- Diseños de experimentos
- Procesamiento de imágenes químicas
- Métodos de inteligencia artificial
- Resolución matemática de mezclas complejas

El reconocimiento de pautas o *Pattern Recognition* es una de las áreas de la quimiometría que tiene como objetivo, como su propio nombre indica, el determinar pautas (o patrones) de comportamiento de las muestras utilizando los datos analíticos obtenidos, extrayendo la mayor cantidad posible de información de dichos datos. La información obtenida puede ser de tipo clasificatoria o de agrupamiento. Se puede dividir en dos clases: i) análisis exploratorio de datos; y ii) reconocimiento de pautas supervisadas y no supervisadas.

Análisis exploratorio de datos

El análisis exploratorio se utiliza para resaltar la información contenida en una matriz de datos multidimensional. Se emplea para tener un enfoque de los datos, es decir, que tipo de modelo sigue los datos. Puede contestar preguntas como: "¿qué se busca?", "¿cómo se busca?", y "¿cómo se interpreta?" la información que contiene la matriz de datos.

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[13] Otto M. Chemometrics 2ed Wiley, Alemania 2007

[14] Talavera Bustamante I., Rodríguez Hierrezuelo J.L., Reconocimientos de Patrones, CENATAV, Cuba 2008

El análisis exploratorio tiene como objetivo: i) extraer las variables o información importante; ii) detectar valores anómalos (outliers); e iii) identificar relaciones entre muestras. El análisis de componentes principales (PCA) (ver capítulo 3) y el análisis factorial son dos técnicas que se utilizan en el análisis exploratorio de datos. Se recomienda utilizarlo antes que otra técnica quimiométrica.

Reconocimiento de pautas no supervisadas

Los métodos de reconocimiento de pautas no supervisadas forman grupos de muestras sin tener información *a priori.* Por lo tanto, tiene como objetivo encontrar los grupos existentes en los datos a partir de agrupamiento de muestras similares. Entre los métodos empleados se encuentran análisis de agrupamientos (o "cluster"). [14,15,16].

Reconocimiento de pautas supervisadas

Existen numerosos métodos de reconocimiento de pautas supervisadas, la mayoría son con el objetivo de clasificar muestras. En este tipo de métodos la pertenencia de las muestras a un grupo o clase es conocida, estas muestras se denominan el conjunto de entrenamiento, con el cual se construye el modelo de clasificación. Una vez construido el modelo, se utiliza para predecir las clases de otras muestras, éste es denominado conjunto de validación. Usualmente se utilizan muestras adicionales a las del conjunto de entrenamiento, sin embargo se puede realizar una validación cruzada, método de dejar uno fuera [15,16].

Los pasos que sigue un modelo de reconocimiento de pautas supervisadas se pueden resumir en: i) construcción del modelo; ii) validación del modelo; iii) mejoramiento de datos (si es necesario); y iv) aplicación del modelo. Los métodos más comunes del reconocimiento de pautas supervisadas son el análisis discriminante, k-vecino

[15] Miller J.N., Miller J.C., Estadística y Quimiometría para Química Analítica, 4ed Prentice Hall, Madrid 2002

[16] Mongay Fernandez C., Quimiometría, Universidad de Valencia, Valencia 2005

más próximo, regresión múltiple, regresión sobre mínimos cuadrados, entre otros [17].

Las técnicas que se utilizaron en realización de esta tesis, análisis de componentes principales, análisis discriminante de mínimos cuadrados parciales, resolución de curvas multivariante, procesamiento de señales y mínimos cuadrados parciales se explicarán detalladamente en los capítulos posteriores.

Quimiometría y el aceite de oliva

Generalmente, los estudios de aceite de oliva en combinación con modelos quimiométricos se han realizado utilizando los espectros de infrarrojo (IR), infrarrojo cercano con transformada de Fourier (FT-NIR) o utilizando directamente los cromatogramas obtenidos tanto de la cromatografía de líquidos (HPLC) como de la cromatografía de gases (GC).

El uso y aplicaciones de diferentes herramientas quimiométricas en aplicaciones que conciernen al aceite de oliva ha sido recopilado, hasta el año 2003, en el capítulo 10 del Manual del Aceite de Oliva de Aparicio y Harwood [17]. En este texto de referencia se puede encontrar, entre otros temas, procedimientos estadísticos multivariantes.

Una revisión más reciente la podemos encontrar en el artículo de Arvanitoyannis and Vlachos [18], y, más específicamente, en relación con los métodos de clasificación, en el capítulo de F. Marini [19],

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[17] Vandeginste B.M.G, Massart D.L., Buydens L.M.C, De Jong S., Lewi P.J. , Smeyers-Verbeke J., Handbook of Chemometrics and Qualimetrics Part B, Elsevier, Netherlands 2008

[18] Arvanitoyannis, I.S., Vlachos, A. (2007) Implementation of physicochemical and sensory analysis in conjunction with multivariate analysis towards assessing olive oil authentication/adulteration. Crit Rev. Food Sci, 47:441–498.

[19] Marini F., Bucci R., Magrí A.L., Magrí A.D. (2010) An overview of chemometric methods for the authentication of the geographical and varietal origin of olive oils in Victor R. Preedy and Ronald Ross Watson Olives and Olive Oil in Health and Disease Prevention, Elsevier Inc.

En otras referencias bibliográficas encontradas se puede comprobar la utilización de técnicas quimiométricas para depurar y extraer información oportuna de los datos experimentales, y que sin estas técnicas, las conclusiones obtenidas no serían concluyentes o incluso posibles. De estas referencias recogidas podríamos destacar, por su importancia e interés para esta tesis las que seguidamente se comentarán.

Galtier *et al* realizaron el estudio de ácidos grasos y triglicéridos utilizando NIR, el tratamiento de los datos se llevo a cabo con el método de análisis discriminante por mínimos cuadrados parciales (PLS-DA) [20]. En un estudio, Marini *et al.* utilizaron el modelado quimiométrico en la autentificación de aceites Italianos Protegidos con la Denominación de Origen (PDO). Se utilizaron 22 parámetros químicos y químico-físicos para establecer las ecuaciones. Las herramientas quimimétricas utilizadas fueron Soft Independent Modeling of Class Analogy (SIMCA) y Unequal Class Modeling (UNEQ) [21]. Por otra parte Priego Capote *et al.* realizaron un estudio de detección, identificación y cuantificación de adulteración en aceite de oliva por medio del análisis de los ácidos grasos en GC-MS. Utilizaron análisis de componentes principales y posteriormente utilizó SIMCA y Análisis se Agrupamientos (Cluster Analysis) de K-vecinos más cercanos para la clasificación cualitativa. Para el análisis cuantitativo se utilizó regresión de mínimos cuadrados parciales (PLS) [22].

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[20] Galtier O., Dupuy N., Le Dréau Y., Ollivier D., Pinatel C., Kister J., Artaud J. (2007) Geographic origins and compositions of virgin olive oils determinated by chemometric analysis of NIR spectra. Anal. Chim. Acta 595:136-144.

[21] Marini F., Magri A.L., Bucci R., Balestrieri F., Marini D. (2006) Class-modeling techinques in the authentication of Italian oils from Sicily with a Protected Denomination of Origin (PDO), Chemometr. Intell. Lab. 80: 140-149

[22] Priego Capote F., Ruiz Jiménez F., Luque de Castro M.D. (2007) Sequential (step-by-step) detection, identification and quantification of extra virgin olive oil adulteration by chemometric treatment of chromatographic profiles. Anal Bioanal Chem 388:1859-1865

A través de la composición de triglicéridos y ácidos grasos se ha establecido una diferenciación entre distintas denominaciones de origen de aceites de oliva virgen francés [23,24]. El contenido de ácido grasos se estableció utilizando cromatografía de gases y un detector FID, mientras que para determinar la composición de los triglicéridos se utilizó un HPLC con un detector de refractómetría. La clasificación y discriminación de las distintas denominaciones de origen se consiguió utilizando un análisis discriminante lineal (LDA).

Las variedades y el origen de 37 aceites de oliva del sureste italiano fue establecido utilizando 44 variables analíticas (11 FA, 14 esteroles y 19 TAG) que fueron determinadas siguiendo los métodos oficiales y NMR [25]. El tratamiento quimiométrico de los dos grupos de datos experimentales utilizando análisis de componentes PCA, análisis de cluster y LDA permitió obtener conclusiones similares.

El perfil de esteroles en aceites de oliva virgen de distintas variedades [26] y en aceites de oliva virgen de distintas zonas de Sicilia [27] ha permitido autentificar y clasificar los distintos aceites de oliva estudiados. En el primer caso, el contenido de estero-

[23] Ollivier D., Artaud J., Pinatel C. Durbec J.P., Guérère M. (2003) Triacylglycerol and fatty acid compositions of French virgin olive oils. Characterization by chemometrics. J Agric Food Chem 51: 5723-5731

[24] Ollivier D., Artaud J., Pinatel C. Durbec J.P., Guérère M. (2006) Differentiation of French virgin olive oil RDOs by sensory characteristics, fatty acid and triacylglycerol compositions and chemometrics. Food Chem. 97: 382-393

[25] Galtier O., Le Dréau Y., Ollivier D., Kister J., Artaud J., Dupuy N. (2008) Lipd Compositions and French Registered Designations of Origins of Virgin Olive Oils Predicted by Chemometric Analysis of Mid-Infrared Spectra, Appl. Spectr. 62: 583-590

[26] Rui Alves M., Cunha S.C., Amaral J.S., Pereira J.A., Oliveira M.B. (2005) Classification of PDO olive oils on the basis of their sterol composition by multivariate analysis, Anal. Chim. Acta 549: 166-178

[27] Nagy K., Bongiorno D., Avellone G., Agozzino P., Ceraulo L., Vékey K. (2005) High performance liquid chromatography–mass spectrometry based chemometric characterization of olive oils, J. Chromatogr. A 1078: 90–97

les fue establecido mediante GC/FID y se utilizaron varias técnicas quimiométricas para el tratamiento de los datos, MANOVA, PCA y análisis canónico. En el segundo caso, el perfil de esteroles se estableció utilizando HPLC y MS y las técnicas quimiométricas utilizadas fueron PCA y análisis discriminante (DFA y LDA).

El cromatograma de los triglicéridos obtenido utilizando HPLC y un detector ELSD permitió establecer el perfil de triglicéridos de tres variedades de aceites de oliva de varias zonas de Portugal [28] y de diferentes aceites de semillas vegetales: girasol, maíz, cacahuete, avellana, nuez, sésamo y aceites de oliva virgen [29]. El tratamiento del contenido de triglicéridos mediante ANOVA y PCA ha permitido en el primer estudio establecer una clasificación entre las variedades de aceite, mientras que en el segundo estudio, permitió la identificación y discriminación de los distintos aceites vegetales.

La determinación de los triglicéridos y de los ácidos grasos en la composición de aceites de oliva de distintas variedades (Cornicabra, Arbequina, Hojiblanca y Picual) ha permitido clasificarlos según la variedad [30]. GC/FID y HPLC con un refractómetro como detector fueron las técnicas analíticas empleadas para las determinaciones. Para el tratamiento de los datos experimentales se recurrió al ANOVA de una vía, y a técnicas multivariantes como el análisis de componentes principales (PCA) y el análisis discriminante lineal (LDA).

[28] Cunha S.C., Casal S., Oliveira MB.P.P. (2005) Triacylglycerol profile by HPLC/ELSD as discriminant parameter pf varietal olive oil from Portugal, Ital. J. Food Sci. 17: 447-454

[29] Cunha S.C., Oliveira M.B.P.P. (2006) Discrimination of vegetable oils by triacylglycerols evaluation of profile using HPLC/ELSD, Food Chem. 95: 518–524

[30] Aranda F., Gómez-Alonso S., Rivera del Álamo R. M., Salvador M. D., Fregapane G. (2004) Triglyceride, total and 2-position fatty acid composition of Cornicabra virgin olive oil: comparison with other Spanish cultivars, Food Chem. 86: 485–492

Por último, Montealgre *et al* presentan un reciente review utilizando diferentes analitos junto con varias técnicas quimiométricas para el estudio del origen botánico del aceite de oliva [31].

En los capítulos siguientes se ejemplifican más trabajos realizados con aceite de oliva en combinación con modelos quimiométricos.

[31] Montealegre C., Marina Alegre M.L., Garcí-Ruiz C. (2010), Traceability markers to the botanical origin in olive oils, J. Agric. Chem. 58: 28-38

CAPÍTULO 1

CERTIFICACIÓN DE MATERIALES DE REFERENCIA DE ACEITE DE OLIVA

Migas

Ingredientes:

- 2 barras de pan del día anterior
- 1 cabeza de ajos
- 1/2 l. de **Aceite de Oliva Virgen Extra**
- 1/4 Kg. de tocino
- Chorizo
- Pimientos rojos secos
- Sal
- 1 vaso y medio de agua

Preparación

En una sartén se fríen en **Aceite de Oliva Virgen Extra** los pimientos y se reservan. A continuación, se fríen en **Aceite de Oliva Virgen Extra** el tocino y el chorizo y se apartan. Se parte el pan en trozos pequeños, se pelan los ajos y se fríen en **Aceite de Oliva Virgen Extra**. Seguidamente, se añade el agua y el pan, se remueve todo durante 30 minutos; se fríe el tocino y el chorizo aparte y una vez frito se mezcla con las migas. Se suele comer en la misma sartén en la que se han hecho las migas. Se suelen acompañar de aceitunas, melón y sardinas.

1.1 Presentación

El inicio de esta tesis coincidió con una propuesta del Servicio de Calidad Agroalimentaria de la Consejería de Agricultura y Pesca de la Junta de Andalucía para el desarrollo y certificación de materiales de referencia de aceite de oliva. La finalidad última de estos materiales de referencia era su utilización en laboratorios de análisis de control de la calidad del aceite de oliva.

En este capítulo se recogen los resultados de dicho estudio que, bajo la denominación de "*Campaña InterOLEO-MRC 2006 para la elaboración, certificación y distribución de cuatro materiales de referencia certificados de aceite de oliva*", fue desarrollado durante los años 2006 a 2009. Este estudio de certificación fue promovido por la Junta de Andalucía y realizado bajo la coordinación de la Unidad de Metrología Química y Cualimetría (CMQ) de la Universidad de Granada.

Los materiales de referencia fueron diseñados de manera que cubrieran el máximo rango de valores para todos los parámetros físico-químicos que se describían el Reglamento (CE) 1989/2003 [1], es decir, acidez, índice de peróxidos, absorción de luz UV, ácidos grasos, isómeros trans del ácido oleico (C18), ácidos grasos saturados en posición 2 de los triglicéridos, ECN42, esteroles, uvaol + eritrodiol, estigmastadienos, ceras y alcoholes grasos alifáticos.

En el apartado 1.2 se presenta el artículo **Elaboration of four olive oil certified reference materials: InterOleo-CRM 2006 certification study**, que fue publicado en 2008 en la revista Food Analytical Methods (1:259-269), donde se recogen los resultados y la discusión del citado estudio de caracterización de los cuatros materiales de referencia de aceites de oliva.

A pesar de existir un cierto número de artículos donde se plantean estudios de estabilidad de aceites de oliva en relación a las condiciones y al proceso de almace-

[1] Commission Regulation (EC) No 1989/2003 amending Regulation (EEC) No 2568/91, On the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis, Off. J. Eur. Union, Brussels, Belgium, L295/57-77, (2003).

namiento, no existen protocolos donde se desarrollen las condiciones de un estudio de estabilidad en procesos de caracterización y certificación de aceites de oliva. Por ello, creemos de especial interés el estudio desarrollado para el establecimiento de la estabilidad de los materiales certificados. El estudio de estabilidad, tal y como se recoge en el apartado 1.3, se llevó a cabo simultáneamente a la utilización de los materiales certificados por los laboratorios. Para su desarrollo, se consideraron dos grupos de parámetros. El grupo que considerábamos crítico, en el que se incluyeron los parámetros: acidez, peróxidos, valores de absorción a 232 y 270nm (K232 y K270) y ceras, fue controlado mensualmente. El segundo grupo, donde se incluyeron el resto de los parámetros fisicoquímicos (ácidos grasos, isómeros trans-C18, ácidos grasos saturados en posición 2 de los triglicéridos, ECN42, esteroles, uvaol + eritrodiol, estigmastadienos y alcoholes alifáticos) pero que considerábamos de menor influencia en el estudio de estabilidad, fue controlado trimestral.

Los resultados del estudio de estabilidad se muestran en el apartado 1.3., donde se recoge el artículo que con el título: **Stability for olive oil control materials**, ha sido publicado en la revista Food Chemistry, 125(2010) 1418-1422.

Todos los análisis fueron realizados en el laboratorio agroalimentario de Granada, sede en Atarfe, que depende de la Consejería de Agricultura y Pesca de la Junta de Andalucía.

1.2 Artículo publicado en Food Analytical Methods (2008) 1: 259-269

ELABORATION OF FOUR OLIVE OIL CERTIFIED REFERENCE MATERIALS: INTEROLEO-CRM 2006 CERTIFICATION STUDY

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ABSTRACT

The study for the elaboration and certification of four olive oil reference materials covering all the physical-chemical characteristics, defined in Commission Regulation (EC) 1989/2003, is reported herein. The different steps of the process: preparation of reference materials, homogeneity and stability studies and characterization study have been carried out for 14 laboratories from five European countries. The certificate of analysis for these CRMs provide assigned values for concentrations of more than 50 parameters or characteristics of olive oils in compliance with the European legislation about the labeling of food products. The certified values and its corresponding uncertainties were obtained applying robust statistics to the raw data provided by the laboratories taking part in the interlaboratory study. The certificate is valid for a maximum period of 18 months from the elaboration date. These four CRMs are intended for use as a primary material for quality control and for validation of analytical methods for measurements in authentication and adulteration problems of olive oils.

Keywords*:* Certified reference material, Olive oil analysis, Food quality, Chemical metrology

Introduction

A reference material is a material whose property values are sufficiently homogeneous and well established to be used for the calibration, the assessment of measurement method, or for assigning values to materials (ISO 30 1992). There are two categories of RMs recognized by ISO, certified reference materials (CRMs) and the reference materials (RMs) (EA-04/14 2003; Walker and Lumley 1999). The difference between a CRM and an RM is the certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realization of the unit in which the property values are expressed, and for which each certificate values is accompanied by an uncertainty at a stated level of confidence (ISO 30 1992). To consider a material like a CRM it has to accomplish some properties: homogeneity, stability, traceability, uncertainty and similitude with the real samples. The purpose of the CRMs is to improve the comparability of measurement results and they can be used for calibration or for quality control (Walker and Lumley 1999; BCR/01/97 1997).

The certification of a CRM is a procedure that establishes the value(s) of one or more properties of a material or substance by a process ensuring traceability to an accurate realization of the units in which the property values are expressed, and that leads to the issuance of a certificate (ISO 30 1992). The CRMs are produced by organizations, private or publics, technically competent in accordance with the general and statistical principles in ISO Guides 31, 33, 34 and 35 (ISO 31 2000; ISO 33 2000; ISO 34 2000; ISO 35 2006). The CRMs are used as measurement standards; they allow highly reliable measurements in a low cost and traceable. They should be used according to the state of the good practice of the different analytical methods employed (EA-04/14 2003). For the RMs certification, a characterization interlaboratory study needs to be carried out, which establishes the best estimate of the true value of an analyte in a reference material (Walker and Lumley 1999: Eurachem and LGC 2000; Wernimont and Spendley 1993).

It is important to consider the number of analytes that can be determined accurately given the facilities, the requirements and the capabilities of the laboratories. Another factor to be included is the cost of the determination. The number of analytes must be determined by the organization group (BCR/01/97 1997; IAEA-TECDOC-1350 2003). Generally the number of analytes to certify it is reduced, however in the case of some certified reference materials are multiparametrics, for example in foods or medicine (Polkowska-Motrenko and Rossbach 2007; Brown Thomas et al. 1995).

Olive oil is defined (COI/T.15/NC no.3/Rev 1. 2003) as the oil obtained solely from the fruit of the olive tree (Olea Europaea L.). The definitions for the different types of olive oil are (Commission Regulation (EC) No. 1513/2001 2001):

1. Virgin olive oils: Oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil, which have not undergone any treatment other than washing, decantation, centrifugation or filtration, to the exclusion of oils obtained using solvents or using adjuvants having a chemical or biochemical action, or by re-esterification process and any mixture with oils of other kinds. Virgin olive oils are exclusively classified and described as follows:

(a) Extra virgin olive oil: Virgin olive oil having a maximum free acidity, in terms of oleic acid, of 0.8 g per 100 g, the other characteristics of which comply with those laid down for this category.

(b) Virgin olive oil: Virgin olive oil having a maximum free acidity, in terms of oleic acid, of 2 g per 100 g, the other characteristics of which comply with those laid down for this category.

(c) Lampante olive oil: Virgin olive oil having a free acidity, in terms of oleic acid, of more than 2 g per 100 g, and/or the other characteristics of which comply with those laid down for this category.

2. Refined olive oil: Olive oil obtained by refining virgin olive oil, having a free acidity content expressed as oleic acid, of not more than 0.3 g per 100 g, and the other characteristics of which comply with those laid down for this category.

3. Olive oil - composed of refined olive oils and virgin olive oils: Olive oil obtained by blending refined olive oil and virgin olive oil other than lampante oil, having a free acidity content expressed as oleic acid, of not more than 1 g per 100 g, and the other characteristics of which comply with those laid down for this category.

4. Crude olive-pomace oil: Oil obtained from olive pomace by treatment with solvents or by physical means or oil corresponding to lampante olive oil, except for certain specified characteristics, excluding oil obtained by means of re-esterification and mixtures with other types of oils, and the other characteristics of which comply with those laid down for this category.

5. Refined olive-pomace oil: Oil obtained by refining crude olive-pomace oil, having a free acidity content expressed as oleic acid, of not more than 0.3 g per 100 g, and the other characteristics of which comply with those laid down for this category.

6. Olive-pomace oil: Oil obtained by blending refined olive-pomace oil and virgin olive oil other than lampante oil, having a free acidity content expressed as oleic acid, of not more than 1 g per 100 g, and the other characteristics of which comply with those laid down for this category.

Adulteration of olive oil usually implies the dilution of olive oil with other inferior quality olive oil or a cheaper vegetable seed oils.

Authentication of the olive oil is important since the consumption per head in the European Union (EU) has increased considerably, and the EU is by far the biggest olive oil producer. The Mediterranean members produce approximately 75% of the world's olive oil. In order to asses both the quality and purity

of the olive oil, detect the fraud and defend the consumers, a very strict control system, based on analytical methodologies to establish the value of certain physical and chemical characteristics of olive oil, has been set up by the International Olive Oil Council (IOOC) in collaboration with olive oil associations to monitor the features of product sold (Luchetti 2002). Such methodology was adopted as own by the EU in the Commission Regulation (EEC) No 2568/91 (1991) and it has been successively updated in later revisions of the initial Regulation until the most recent Commission Regulation (EC) No 702/2007 (2007).

Compliance with the European legislation on the labeling of food products requires standardization and validation of authentication methods. The laboratories that perform the (official) analytical control of olive oils have to prove they are proficient in applying the testing methods described in Commission Regulation (EEC) No 2568/91 (1991) or the corresponding later modifications that are applicable, for instance, by an ISO 17025 accreditation. For this, the laboratory shall have validation and quality control procedures for assuring the validity of results. The monitoring should preferably include regular use of CRMs. However, there are not suitable olive oil CRMs covering some or all the physical and chemical characteristics defined in the above quoted EU Regulation. According to our knowledge, there is not any information about RM, certifying none specific parameters about olive oil. As background knowledge of vegetables oils CRMs, it can be emphasized the one of palm oil (Hishamuddin 2005) representing parameters such fatty acid profile, slip melting point, iodine value and triacylglycerol profile.

This paper describes the full process for the elaboration and certification of four olive oil reference materials, elaborated so that they cover the range of values for all the physical-chemical characteristics defined in Commission Regulation (EC) 1989/2003 (2003). The latest Commission Regulation (EC) 702/2007 (2007) was not considered to go into effect after the study development because the study started on April 2006 and the four materials were ready on July 2006. Certificates are valid for 18 months from this date. The certified reference ma-

terial has been obtained through the "*InterOleo-CRM 2006 Campaign*" certification study. This is the first time that such certified reference materials are elaborated and they can be used by laboratories. In addition, it can be emphasized that the laboratories which participated in this study are recognized by the IOOC due to their maximum excellence in the quality of the olive oil analyses.

Materials and Methods

Analytical techniques used for certification. Different analytical techniques were used for the certification study, which are described in Regulation (EC) No 2568/91 (1991) and its later modifications. Acidity and peroxide value were analyzed by volumetric methods; GC has been applied for fatty acids, trans isomers of fatty acids, saturated fatty acids in 2-position, theoretical ECN42, steradienes, waxes and aliphatic alcohols; TLC+GC was employed to determine sterols and terpenic alcohols; total ECN42 was analyzed by HPLC; and UV spectrometry was used to quantify K232, K270 and ΔK.

Preparation of reference materials. A CRM can be prepared synthetically or artificially with material from a natural or commercial source (BCR/01/97 1997). The material batches have been made from commercial and homogenized oils. They were prepared from human-intake olive oils obtained directly from oil industries in Andalusia (Spain). For oil blends, oils of palm and sunflower were used; those oils were obtained from the appropriate food industry.

The composition of the different materials has been carried out looking for that includes all the physical-chemical characteristics described in the Regulation (EC) 1989/2003 (2003) (Table 1). For that purpose the addition of a significant proportion of sunflower oil to a virgin olive oil provides a blend in which it has been significantly changed the polyunsaturated fats and sterols profile; the addition of palm oil increases the saturated fat content.

The homogenization of the material was carried out with an industrial blender and it was mixed for a 1 h in stainless steel deposits. The blend rested the whole night. Before bottling, the blend was mixed for 15 min.

The temperature of the deposits was controlled and registered at the beginning of the homogenization and in the bottling process. The temperature did not overcome at any point of the process the 20 ºC. Seven hundred and fifty unitary samples of each material were prepared. The bottled method assured the stability of the samples. They were bottled in topaz-colored glass 250 ml bottles and sealed with an oil-inert plastic stopper. Each bottle bore a label in which was indicated, in an unequivocal way, the characteristics of the material: the title of the campaign, the name of the material and an alphanumeric code made up by two letters specific for each material (see below), and a number taken from the range 010 to 750 (BCR/01/97 1997) (the first 9 samples were discarded as security criteria).

The materials obtained have been identified by a code of two letters: VG Material: lampante olive oil 100%; RF Material: blend of refined olive oil (approx. 80%) and palm oil (approx. 20%); PM Material: refined pomace-olive oil 100%; and BL Material: blend of extra virgin olive oil (approx. 60%) and sunflower oil (approx. 40%).

The appropriate conditions of transportation had been assured with low temperatures, equal or less than 8 ºC and avoiding direct light from sun. The samples were stored in darkness at controlled temperature 0-8 ºC.

Homogeneity study. The homogeneity for a CRM is a condition of being of uniform composition with respect to one or more specified properties (ISO 30 1992). The non-homogeneity can be presented in the material due to the decanting oil.

The method selected for the study must have a high precision and it is recommended to use a different method than in the characterization study, unless the method used in the characterization measurements are sufficiently precise. The method should be applied under best repeatability conditions and must be performed by a single laboratory (BCR/01/97 1997).

Table 1. Physical-chemical characteristics to be determined in the Certification Study.

Physical and chemical characteristics defined in Commission Regulation (EC) 1989/2003	
Acidity	**Sterols:**
Peroxide value	• cholesterol
Fatty acids:	• brassicasterol
• myristic (C14:0)	• 2,4-methylencholesterol
• palmitic (C16:0)	• campesterol
• palmitoleic (C16:1, n-7)	• campestanol
• heptadeconic (C17:0)	• stigmasterol
• heptadecenic (C17:1)	• Δ7-campesterol
• stearic (C18:0)	• Δ5,23-stigmastadienol
• oleic (C18:1, n-9)	• clerosterol
• linoleic (C18:2, n-6)	• β-sitosterol
• linolenic (C18:3, n-3)	• sitostanol
• arachidic (C20:0)	• Δ5-avenasterol
• elcosenoic (C20:1, n-11)	• Δ5,24-stigmastadienol
• behenic (C22:0)	• Δ7-stigmastenol
• lignoceric (C24:0)	• Δ7-avenasterol
Aliphatic alcohols:	• apparent β-sitosterol
• docosanol (C22)	• total sterols
• tetracosanol (C24)	**Terpenic alcohols:**
• hexacosanol (C26)	• erythrodiol + uvaol
• octacosanol (C28)	**Steradienes:**
• total alcohols	• 3,5-stigmastadienes
ECN42 triglycerides:	**Saturated fatty acids in 2-position**
• total ECN42 (HPLC)	***Trans* isomers of fatty acids :**
• theoretical ECN42 (GC)	• *t*-oleic (*t*-C18:1)
• difference ECN42	• *t*-linoleic (*t*-C18:2)
Waxes:	• *t*-linolenic (t-C18:3)
• C40	• *t*-linoleic + *t*-linolenic
• C42	**UV spectrometry:**
• C44	• K232
• C46	• K270
• total waxes	• DK

The characterization methods were sufficiently established and had a high level of confidence for being employed in the homogeneity study

The physical-chemical characteristics chosen are: acidity, K270 and content in major fatty acid. These characteristics to assess the homogeneity were considered depending the total time invested in the analyses and the types of the blends, they are able to detect the difference in the proportions of the mixtures of oils or the effects due to sedimentation and/or decantation. In addition, the analytical methods are precise and simple. The contents in majority fatty acid are those whose chromatographic peak area is equal or bigger than 3-5% of the oleic acid peak area. The acids selected were four: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2).

A total of 25 samples of each material were analyzed. These samples were selected so that they could cover the whole lot of each material; a systematic sampling was used using the equation $N_H = 26+29(n-1)$, where N_H is the numeric code of the unitary sample selected for the study of homogeneity and n indicates a natural number series (beginning with the 1).

For each material, the analyses of the different samples were carried out under repeatability conditions or, in their defect, in the smallest time and, whenever it was possible, using the same equipment and the same operator. The analytical methods applied are described in the Regulation (EC) 2568/91 (1991) and later modifications. The Official Food Quality Control Laboratory of Granada, Atarfe venue, has carried out the analysis.

There were not replicates of the determinations except for possible incidences that could invalidate one or more results of a sample. However, the standard deviation of repeatability ($s_{repeatib}$) assumed by the laboratories is request to the laboratory for statistical treatment. The statistical criterion for the homogeneity was:

$$s_{homog} \leq 2 \times s_{repeatib}$$

Stability study. The stability of a reference material is the ability, when it is stored under specified conditions, to maintain a stated property value within specified limits for a specified period of time (ISO 30 1992). There are two types of stability in the CRMs: the short -term stability and the long -term stability of the material (ISO 35 2006).

The short-term instability of the samples could be due to transportation conditions, and the long-term stability is associated with the conservation and storage of the material. The stability of the samples of each lot was verified under the established storage conditions, stored in the darkness and in a controlled temperature between 0 and 8 ºC.

According to the ISO Guide 35 (2006), a classical stability study was applied; it was programmed for 18 months from the elaboration date; the period coincides with the validity term of the certificate; however, the stability studies have been extended for a period of 2 years. The study is carried out under reproducibility conditions.

The study is made with 25 unitary samples of each material. The selection of the samples of each material was developed with the intention of covering the entire batch of each material, a systematic sampling was used, using the equation $N_S = 27+58(n-1)$, where N_S is the numeric code of the unitary sample selected for the study of stability and n indicates a natural number series (beginning with the 1).

The method chosen for the study must detect small differences over long periods of time, which is a long-term reproducibility (BCR/01/97/1997; Eurochem and LGC 2000). In this study the characterization methods were sufficiently reproducible to use them for the homogeneity study.

The characteristics considered for the stability study are divided in 2 groups based on the sensitivity of the physico-chemical changes in function of the time: critical and non critical groups. The critical characteristics are those which value is quite sensible to changes in short periods of time (acidity, perox-

ide value, K values and waxes). This group was determined every month. The non critical group (fatty acids, *trans* isomers of fatty acid C18, saturated fatty acids in 2-position of the triglycerides, ECN42, sterols, erythrodiol+uvaol, stigmastadienes and aliphatic alcohols) was determined quarterly due to the characteristics of this group are less sensitive in short periods of time. The stability of the material was analyzed by the Official Food Quality Control Laboratory of Granada, Atarfe venue.

Shewhart control charts are used for statistical treatment. The charts were established with the next rule:

$$\text{initial value} \pm 2(\text{or } 3) \times s_{reprod}$$

The acceptance intervals are defined from reproducibility standard deviation values, which were provided by the laboratory.

Characterization study. The characterization of a CRM is the determination of one or more physical, chemical, biological, or technological property values that are relevant to its intended end use (ISO 30 1992). For the determination of the values the guide BCR (BCR/01/97 1997)) indicates that at least 6 laboratories have to participate; but in case that the certification is attempted for the first time, the guide recommended to include more laboratories.

For the characterization study, 14 laboratories from five European Countries, all of them recognized, by their quality in the analysis of olive oil, by the IOOC has taken part in the interlaboratory characterization study (Table 2).

To each participant laboratory a unitary sample of each material was sent. The samples were selected so they cover the whole range of unitary samples of each one of the materials, using the equation $N_C = 30+49(n-1)$, where N_C is the numeric code of the unitary sample selected for the certification study and n indicates the natural (beginning with the 1) number series.

Analytical methods for the characterization of reference materials may be classified in (IAEA-TECDOC-1350 2003): (1) Definitive method; (2) Independent reference methods; (3) Independent reference and validated method by selected expert analysts; (4) Volunteer analysts, various methods; (5) Method-specific. The characterization scheme applied in this study was the method-specific, which is the characterization by a specific, validated method by selected experienced analysts, and carry out, in an interlaboratory study. The reason to apply this type of scheme was because of the use of official methods, described in the Regulation (EC) 2568/91 (1991), without modifications and the study was made with recognized laboratories evaluated by de IOOC in such methods.

Of each sample, all the physical-chemical characteristics included in the Regulation (EC) 2568/91 (1991) were determined. Results were expressed with a unique numeric data by each parameter. Although, it is recommended to perform replicates of the analysis (BCR/01/97 1997), in this study the laboratories did not carry out them due to the large number of physical-chemical characteristics to analyze; however the laboratories were able to repeat one or several analyses when they had founded doubts that the result could be erroneous. In the cases in which the procedure of the laboratory required that way, the result was the average of the determinations.

The mentioned Regulation was used as a guideline for the number of decimals and units. The participant laboratories emitted a testing report of the results and the uncertainty values, when it was possible, to the organizers.

In order to avoid the outlier test detection, robust statistics were used to estimate representative values. The assigned value of each parameter was estimated as the median of the raw data from the different laboratories, and the standard deviation was calculated from the MADe (Eurachem and LGC 2000; Peña 2001). Due to the laboratories do not have implemented the uncertainty as estimation in their quality system; standard deviation was used as an estimation of the variability of the characterization measurements.

Table 2. Laboratories recognized by the IOOC which have participated in the characterization study

Country	Laboratory
Spain	Laboratorio Agroalimentario de Atarfe, Dirección General de Industrias y Promoción Agroalimentaria, Conserjería de Agricultura y Pesca, Atarfe (Granada).
	Laboratorio Agroalimentario de Córdoba, Dirección General de Industrias y Promoción Agroalimentaria, Conserjería de Agricultura y Pesca, Córdoba.
	Laboratorio Arbitral Agroalimentario, Subdirección de Control de la Calidad Alimentaría, Ministerio de Agricultura, Pesca y Alimentación, Madrid.
	Laboratorio Central de Aduanas, Ministerio de Economía y Hacienda, Madrid.
	Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Sevilla.
	Laboratorio del Centro de Asistencia Técnica e Inspección de Comercio Exterior (SOIVRE), Dirección Regional de Comercio en Andalucía, Sevilla.
France	Laboratoire Interrégional de Marseille, Direction générale de la Concurrente, de la Consommation et de la Répression des Fraudes, Marseille.
Greece	General Chemical State Laboratory, Ministry of Finance and Economy, Athens.
Italy	Laboratorio del Servizio Revisione Analisi Istituto Sperimentale per la Elaiotecnica, Citta Santangelo (Pescara).
	Stazione Sperimentale per le Industrie degli Oli e dei Grassi, Milano.
	Laboratorio Chimico Direzione Regionale per il Lazio e L'Umbria Agenzia delle Dogane, Roma.
	Laboratorio Chimico-Microbiologico Assocazione Granaria di Milano, Rozzano (Milano).
Portugal	Laboratório Central de Qualidade Alimentar, Direção-Geral de Fiscalização e Controlo de Qualidade Alimentar, Ministério de Agricultura, do Desenvolvimento Rural e das Pescas, Lisboa.
	Laboratório de Estudos Técnicos, Instituto Superior de Agronomia, Lisboa.

The median absolute deviation (MAD) is a robust statistic what measures the scattering of a data set. It is given by the median of all absolute difference of each result with regard to the median of the raw data:

$$MAD = \text{median} (| x_i - \text{median} (x_i)|)$$

MAD can be related to the standard deviation of a normal distribution using the MADe. The MADe can be calculated from the MAD by applying:

$$MADe(x_i) = 1.483 \times MAD(x_i)$$

Certified values and their uncertainties. A certified value for a CRM is defined (ISO 30 1992) as the value that appears in the certificate accompanying the material and the uncertainty is defined (VIM Final Draft 2006) as a parameter characterizing the dispersion of the quantity values being attributed to a measurand. In the practice the uncertainty is the interval of values in which we can find the true value with a high probability when all the error sources are considered.

In this study the certified values were expressed as:

$$X_{refer} \pm 2 \times u_{refer} \quad \text{or} \quad X_{refer} \pm U_{refer}$$

where X_{refer} = median (x_i); u_{refer} is the corresponding standard uncertainty and U_{refer} is the expanded uncertainty calculated a coverage factor k=2. The standard uncertainty value is calculated as a combination (quadratic addition) of the two variability components:

1. The precision of the analytical procedure (repeatability) used for each parameter, obtained from the results of other studies reported by recognized institutions (IOOC, IUPAC, Instituto de la Grasa, Spain); the same methods, implicated in the characterization study, were used in these studies. This was carried out with the purpose of unifying criterions, in view of the fact that most of the characterizing laboratories did not provide the precision values;

2. The precision of the material characterization study (reproducibility).

$$u_{refer} = \sqrt{\frac{1}{p}\left(s^2_{repetib} + s^2_{caract}\right)}$$

where $s_{repeatib}$ is the repeatability standard deviation; s_{caract} is the MADe (values obtained in the interlaboratory study), and p is the number of laboratories.

The statistical criterion used to certify a value was: $2 \cdot u_{refer} \leq 0.25 \cdot X_{refer}$. The uncertified values are included in the certificate without the corresponding uncertainty (ISO 30 1992).

Results and Discussion

The result obtained in the elaboration process of the CRMs has demonstrated that the study planning has been correct. The homogeneity studies proved that the VG, PM and RF material unitary samples are homogeneous, however the BL material unitary samples appear to be no-homogeneous with respect to three physical-chemical characteristics. The BL material had showed a non-significant homogeneity test for acidity, palmitic and stearic acid, however for K270, and linoleic and oleic acid had a significant test. As an example, Figure 1 shows clearly the results of the K270 homogeneity study for the RF and BL materials: (A) all the bars, corresponding to RF material, have the same length indicating that the 25 analytical results are similar to 0.69, hence the material is homogeneous with respect to K270; (B) however, the bars of the BL material show two very different blocks by far, centered on 0.81 and 0.92, therefore the material is no homogeneous with respect to K270.

In view of the fact that it does not exist a property that measures the chemical homogeneity as a whole, the homogeneity study was carried out with some of the same parameters of the characterization study because it not feasi-

ble to use all parameters. The characterization study will verify, *a posteriori,* the results of the homogeneity study on the selected parameters.

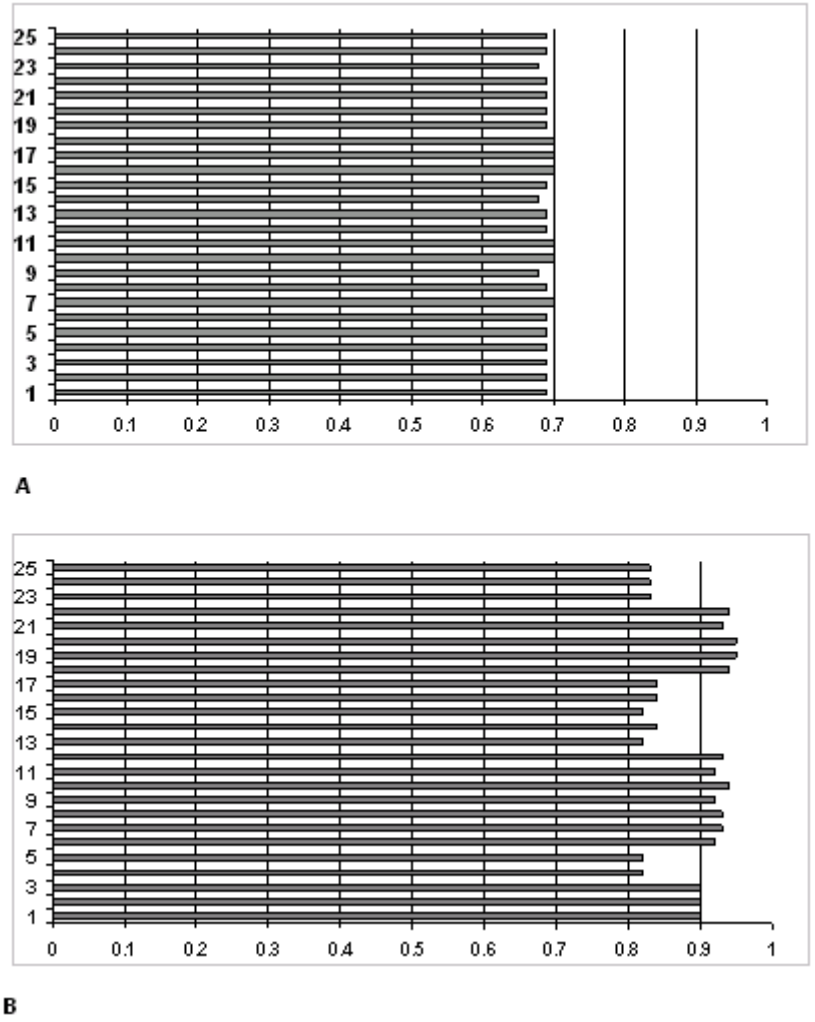

Figure 1. Bar chart results of the K270 homogeneity study from two materials. A: RF material; B: BL material.

From the stability study developed on a representative sample set of each material could be verified that the four materials are stable for 18 months,

what agrees with the initially schedule (Figure 2); however, to be able to prolong the time of life of the certificate, the stability are extended for a period of 2 years.

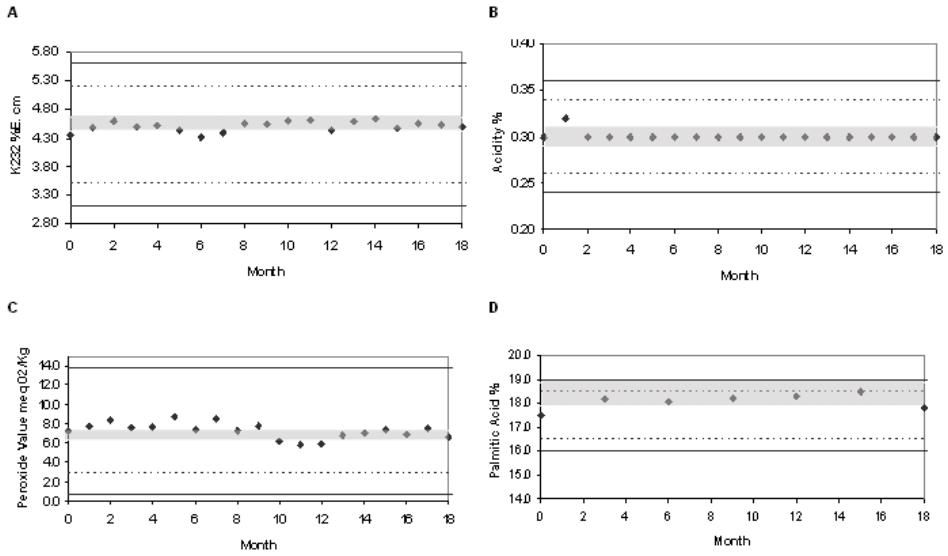

Figure 2. Control chart results of the stability study from four representative materials with four different characteristics. A: K232 PM Material; B: Acidity Level BL Material; C: Peroxide Value VG Material; D: Palmitic Acid RF Material (Dashed lines: ±2s interval; Solid lines: ±3s interval; Grey stripe: certified uncertainty).

As the stability study was being carried out simultaneously to the use of the CRMs, a warning system was designed: in case 2 consecutives physical-chemical characteristics values were found "off stability" in the study, laboratories using that material will be warned so they would not make use of the faulty value as certified reference value.

In order to obtain the reference concentration values, fourteen laboratories from five European Countries, all of them recognized by the International Olive Oil Council (IOOC) (www.internationaloliveoil.org/downloads/labo2.pdf), have taken part in the characterization interlaboratory study. The laboratories taking part are listed in Table 2.

Because not all the laboratories provided the results of all parameters and, in some cases, the laboratories results were found discordant, the certified values and its corresponding uncertainties were obtained applying robust statistics to the raw data provided by the laboratories. Although ISO Guide 35 recommend applying classical parametric statistics for data treatment in the characterization study, when there are suspicion of several outliers, a methodology alternative, based in robust statistics, could be applied to the data (BCR/01/97/1997). The certified values of the CRMs are showed in Table 3. The certificate includes the values of the certified analytical parameters and their uncertainties. All olive oil physical-chemical characteristics, defined in Commission Regulation (EC) 1989/2003, are certified except in the following situations:

1) The BL material showed non-homogeneity in six characteristics, the values appear as a indicating interval.

2) The characteristic data which were not trustworthy due to: (i) a scarce number of results; (ii) a low parameter value; (iii) a high data scattering; or (iv) a final uncertainty that was over 25%. An indicative value is available and no uncertainty is stated. They appear in italics and between parentheses.

Uncertainty of the certified values is assessed as an expanded uncertainty.

In addition, the characterization study results confirm that the selection of the parameters for the homogeneity study was appropriated. As it can be observed in Figure 3, the behavior of the parameters used in the characterization study follows a similar pattern that in the homogeneity study (see Figure 1). Figure 3 shows clearly the results of the K270 characterization study for the RF and BL materials: (A) the results corresponding to RF material, have the same length (≈ 0.7), except for three results.

Table 3. Certified values and their uncertainty for the four reference materials

Characteristics	VG		PM		RF		BL		Unities
	CV	***± U***	***CV***	***± U***	***CV***	***± U***	***CV***	***± U***	
Acidity	3.4	0.02	0.1	0.02	0.1	0.02	0.3	0.01	% m/m
Peroxide value	6.8	0.5	7.2	0.7	*-1.2*		14	0.6	mEq O_2 / kg
UV Spectrom:									
K232	2.21	0.06	4.54	0.12	1.94	0.09	3.4	0.15	$E_{1cm}^{1\%}$
K270	0.38	0.01	1.34	0.012	0.69	0.02	0.81- 0.92		$E_{1cm}^{1\%}$
ΔK	0.02	0	*-0.09*		*-0.07*		0.106	0.01	$E_{1cm}^{1\%}$
Fatty acids:									
myristic (C14:0)	*-0.01*		*-0.02*		0.22	0.01	*-0.03*		% m/m
palmitic (C16:0)	10.9	0.2	10.9	0.2	18.4	0.5	8.8	0.1	% m/m
palmitoleic (C16:1. n-7)	0.8	0.03	0.9	0.01	0.71	0.03	0.6	0.03	% m/m
heptadecanoic (C17:0)	0.08	0.02	0.1	0.01	0.11	0.01	0.09	0.01	% m/m
heptadecenoic (C17:1)	0.1	0.01	0.1	0.01	0.16	0.03	0.1	0.01	% m/m
stearic (C18:0)	4	0.09	3	0.06	3.2	0.1	3.3	0.07	% m/m
oleic (C18:1. n-9)	77.7	0.3	74.3	0.3	66	0.6	62.70-65.9		% m/m
linoleic (C18:2. n-3)	5	0.08	8.7	0.1	9.5	0.2	19.7-22.8		% m/m
linolenic (C18:3. n-6)	0.62	0.02	0.7	0.02	0.6	0.08	0.44	0.03	% m/m
arachidic (C20:0)	0.4	0.01	0.45	0.03	0.4	0.02	0.31	0.02	% m/m
eicosenoic (C20:1. n-11)	0.22	0.02	0.31	0.01	0.26	0.04	0.22	0.02	% m/m
behemic (C22:0)	0.1	0.01	0.2	0.01	0.11	0.02	0.3	0.01	% m/m
lignoceric (C24:0)	0.05	0.01	0.1	0.01	*-0.06*		0.12	0.02	% m/m
Fatty acid *trans* isomers:									
t-oleic (t-C18:1)	*-0.04*		0.23	0.05	*-0.05*		*-0.02*		% m/m
t-linoleic (t-C18:2)	*-0.02*		*-0.05*		*-0.09*		0.25	0.04	% m/m

Table 3. (continued)

	VG		PM		RF		BL		Unities
Characteristics	***CV***	***± U***	***CV***	***± U***	***CV***	***± U***	***CV***	***± U***	
t-linolenic (t-C18:3)	*0*		*-0.03*		*-0.06*		*-0.01*		% m/m
t-C18:2 + t-C18:3	*-0.02*		*-0.08*		*-0.15*		0.26	0.04	
Saturated acid in 2-position	*-0.65*		*-0.86*		*-2.85*		*-0.47*		% m/m
ECN42 triglycerides:									
Total ECN42 (HPLC)	0.33	0.03	0.71	0.05	0.49	0.02	6.9 – 8.0		% mol
Theoretical ECN42 (GC)	0.21	0.01	0.43	0.03	0.4	0.03	1.2 – 1.8		% mol
Difference ECN42	*-0.11*		0.29	0.06	*-0.1*		5.6 – 6.3		% mol
Steradienes:									
3.5-stigmatadienes	1.1	0.09	103.5	11	5.9	0.4	6	0.5	mg/kg
Sterols:									
cholesterol	0.13	0.03	0.12	0.03	*-0.3*		*-0.12*		% m/m
brassicasterol	*0*		*-0.08*		*-0.03*		*0*		% m/m
2.4-methylcholesterol	*-0.2*		*-0.1*		*-0.1*		*-0.15*		% m/m
campesterol	3.4	0.05	3.1	0.06	4.6	0.2	6.4	0.1	% m/m
campestanol	*-0.1*		*-0.15*		*-0.13*		*-0.1*		% m/m
stigmasterol	0.71	0.03	1.1	0.03	2.3	0.1	4.5	0.1	% m/m
Δ7-campesterol	*-0.09*		*-0.09*		*-0.1*		*-1.6*		% m/m
Δ5.23-stigmastadienol	*-0.03*		*-0.8*		*-0.35*		*-0.22*		% m/m
clerosterol	*-1*		*-1.2*		*-1*		*-0.9*		% m/m
β-sitosterol	*84.9*		*-87.27*		*-79.6*		*-68.26*		% m/m
sitostanol	*-0.6*		*-1.8*		*-1*		*-0.5*		% m/m
Δ5-avenasterol	*-7.5*		*-1.52*		*-7.58*		*-4.3*		% m/m
Δ5.24-stigmastadienol	*-0.5*		*-1.74*		*-1.4*		*-1.2*		% m/m

Table 3. (continued)

Characte-ristics	VG		PM		RF		BL		Unities
	CV	***± U***	***CV***	***± U***	***CV***	***± U***	***CV***	***± U***	
Δ7-stigmastenol	0.5	0.04	0.47	0.07	0.47	0.2	8.5	0.2	% m/m
Δ7-avenasterol	0.4	0.06	*-0.12*		*-0.8*		2.9	0.3	% m/m
apparent β-sitosterol	94.4	0.2	95	0.3	91	0.6	76	0.4	% m/m
total sterols	1420	40	2680	80	1290	70	1940	70	mg/kg
Terpenic alcohols:									
erythrodiol + uvaol	1.9	0.2	18	0.6	2.4	0.5	0.8	0.1	% m/m
Waxes :									
C40	*-52.1*		*-656*		*-68*		*-50*		mg/kg
C42	*-90.4*		*-1146.3*		*-68*		*-43.5*		mg/kg
C44	*-101*		*-810*		*-66.9*		-18		mg/kg
C46	*-31*		*-194*		*-33*		*-16*		mg/kg
Total waxes	270	17	2800	150	230	41	118	30	mg/kg
Aliphatic alcohols:									
docosanol (C22)	*-38.7*		-399		*-40.71*		-20.5		mg/kg
tetracosanol (C24)	*-66*		-751		*-45.26*		-28.6		mg/kg
hexacosanol (C26)	*-144*		-637		*-63.34*		-39		mg/kg
octacosanol (C28)	*-39.5*		-158		*-31.04*		-15.5		mg/kg
Total aliphatic alcohols	290	16	1990	57	184	24	101	10	mg/kg

These results do not affect the study conclusions due to the use of robust statistic; therefore the material is homogeneous with respect to K270; (B) the BL material results show two different blocks, like in the homogeneity study, centered on 0.9 and 0.8, thus the material is no homogeneous with respect to K270.

The characterization study found that the parameters, K270, oleic and linoleic acid are non-homogenous in the BL material. And, like it was expected, the ECN42 also appeared as non-homogenous, due to the non-homogeneity of the linoleic and oleic acids, which are the majority fatty acids. In the other materials (VG, PM and RF) the characterization study confirms the homogeneity in all the parameters.

The four olive oil CRMs prepared are the only reference materials available that have certified values for the most of the physical-chemical characteristics included in the Regulation (EC) 1989/2003 for the olive oil. These values are been certified for the first time. These materials could be used for method validation or in quality control programmes carried out by the testing laboratories operating under Regulation (EC) 2568/91. These CRMs can also be used for assigning values to in-house reference materials and to ensure a proper interlaboratory data interpretation. The use of these CRMs will help to improve analytical measurement capability of the different physical-chemical characteristics used to characterize olive oils. They suppose a new significant tool for the quality assurance of the official olive oil quality control laboratories.

Acknowledgments

The authors would like to thank to the Spanish Ministry of Education and Science (Dirección General de Investigación, Project No CTQ2006-15066-C02-02) and the Andalusia Regional Government (Consejería de Agricultura y Pesca, Junta de Andalucía) for financial assistance. This work has been also partially supported for the European Regional Development Funds (ERDF). One of the authors, A.P.M.E., is also grateful to the Mexican National Council of Sciences and Technology (Consejo Nacional de Ciencia y Tecnología), for the personal postgraduate grant.

This work could be done thanks to the disinterested collaboration of IOC for facilitated the laboratories to the study.

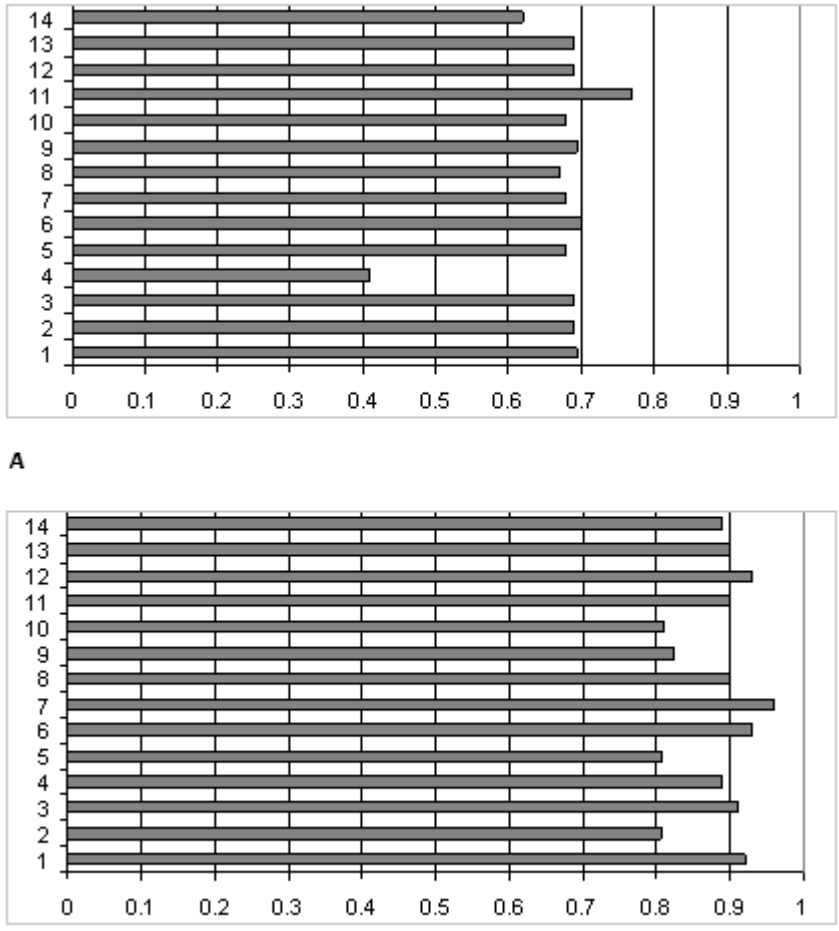

Figure 3. Bar charts results of the K270 characterization study from two materials. A: RF material; B: BL material.

References

BCR/01/97, Guidelines for the production and certification of BCR reference material, European Commission, Standards, Measurements and Testing Programme: Luxemburg, (1997).

COI/T.15/NC no. 3/Rev. 1, Trade standard applying to olive oils and olive-pomace oils, 1st ed., International Olive Oil Council, Madrid, Spain, (2003).

Commission Regulation (EC) No 1989/2003 amending Regulation (EEC) No 2568/91, On the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis, Off. J. Eur. Union, Brussels, Belgium, L295/57-77, (2003).

Commission Regulation (EC) No 702/2007 amending Regulation (EEC) No 2568/91, On the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis, Off. J. Eur. Union, Brussels, Belgium, L161/11-27 (2007).

Commission Regulation (EC) No. 1513/2001, On descriptions and definitions of olive amending Regulations No 136/66/EEC and (EC) No 1638/98 as regards the extension of the period of validity of the aid scheme and the quality strategy for olive oil

Commission Regulation (EEC) No 2568/91, On the characteristics of olive oil and olive-residue oil on the relevant methods of analysis, Off. J. Eur. Commun., Brussels, Belgium, L248/1-83 (1991).

D. Peña, Fundamentos de Estadística. 1st ed; Alianza Editorial, S.A.: Madrid, Spain, p65, (2001).

E. Hishamuddin, MPOB Information Series, 291, 288-289 (2005).

EA-04/14, The Selection and Use of Reference Materials, 1st ed, European Co-operation for Accreditation (2003). see also: http://www.european-accreditation.org/n1/doc/ea-4-14.pdf

Eurachem and LGC, Selection, use and interpretation of proficiency testing (PT) schemes by laboratories, 1st ed.; Teddington, United Kingdom, (2000).

F. Luchetti, Eur. J. Lipid Sci. Technol., 104, 559-563, (2002).

G. Wernimont, Use of Statistics to Develop and Evaluate Analytical Methods. 1st ed.; Spendley W, Ed.; AOAC: Arlington, Virginia, pp 87-141 (1993).

H. Polkowska-Motrenko, M. Rossbach, Accred Qual Assur, 12, 343-350 (2007).

IAEA-TECDOC-1350, Development and use of reference materials and quality control materials and quality control materials, 1st ed.; International Atomic Energy Agency: Vienna, Austria (2003).

ISO 30, Terms and definitions used in connection with reference materials, 2nd ed.; ISO: Geneve Switzerland (1992).

ISO 31, Uses of certified reference materials, 2nd ed.; ISO: Geneva, Switzerland, (2000).

ISO 33, Reference materials. Contents of certificates and labels, 2nd ed.; ISO: Geneva, Switzerland (2000).

ISO 34, General requirements for the competence of reference material producers, 2nd ed; ISO: Geneva, Switzerland, (2000).

ISO 35, Reference materials. General and statistical principles for certification, 3rd ed.; ISO: Geneva, Switzerland, (2006).

J, Brown Thomas, M. Kline, S. Schiller, P. Ellerbe, L. Sniegoski, D. Duewer, K. Sharpless, Fresenius J Anal. Chem., 356,1-9 (1995).

R. Walker, I. Lumley, J. Trends in Analytical Chemistry 18, 9-10 (1999).

VIM Final Draft., International Vocabulary of Metrology–Basic and General Concepts and Associated Terms (VIM), 3rd ed. (2006)

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STABILITY FOR OLIVE OIL CONTROL MATERIALS

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ABSTRACT

For the reliability of analytical Quality Control Materials (QCMs), stability studies are essential. In spite of the importance of the stability studies, there is no article relating to QCMs of olive oil. This paper describes an intralaboratory stability study aimed for assuring olive oil QCMs concerning to the physical-chemical characteristics defined in Commission Regulation (EC) No. 1989/2003. The study concluded that, in storage conditions of darkness and low controlled temperature (0-8ºC), critical physical-chemical parameters of the olive oil QCMs, such as acidity level, peroxide value, K-values and total waxes, are stable at least during 24 months (2 years) with, in general, light variability and up trends. Theoretically, according to this study a homogeneous sample olive oil could be used as a QCM during two years if this material were stored in the dark and low temperature. Then, olive oil quality control testing laboratories have an alternative to dispose stable in-house QCMs for the control of its measurement.

Keywords: olive oil storage stability, analytical quality control material, metrology, acidity, peroxide value, K-values, waxes

1. INTRODUCTION

Olive oil is only obtained from the fruits of the olive tree, to the exclusion of oils using solvents or re-sterification process and of any mixture with oils of other kind (COI/T.15/NC no. 3/Rev. 2, 2006). Olive oil, unlike vegetable oils, is a fresh squeezed juice from olives; maintains its properties including natural antioxidant components that will keep the olive oil longer than the other vegetables oils without a refined processing.

The importance of olive oil is due to the increasingly consumption around the world, because of its nutritional and sensory properties. European Union (EU) is the leading producer and inside the EU, the Mediterranean members are the biggest producers. In order to asses to establish the quality and purity of olive oils, certain physical and chemical characteristics has been set up by the International Olive Oil Council (IOOC) in collaboration with olive oil associations to monitor the features of the products sold (European Commission Directorate-General for Agriculture, 2003; Luchetti, 2002). EU uses the Commission Regulation (EEC) which adopted the methodology used by the IOOC in the No. 2568/91 (Commission Regulation (EEC) No. 2568/91, 1991,COI/T.15/NC no. 3/Rev. 2 , 2006). The characteristics have to be analyzed by testing laboratories recognized by the IOOC (Resolution No. RES-2/78-IV/98, 1998), which should have validation and quality control procedures for assuring the validity of their results by including the use of suitable standards, formally named Reference Materials (RM) (ISO Guide 30, 1992; Emons, Linsinger & Gawlik, 2004; ILAC-G9, 2005). RM definition has been updated in the VIM latest edition (BIPM, IEC, IFCC,ILAC, ISO, IUPAC, IUPAP & OIML, 2008). In due course, the concept and uses of RM have been clarified in different papers (Emons, Fajgelj, van der Veen, & Watters, 2006; Emons, 2006). Within RMs family, QCMs are significant materials. These materials have their homogeneity and stability well established but they are not sufficiently characterized to be used for calibration or provide traceability (Emons, Linsinger & Gawlik, 2004; Emons, 2006). However, they are very useful in quality control (Emons, Linsinger & Gawlik, 2004). Homogeneity and stability studies are essential in the process of preparation and characteriza-

tion of RMs (Linsinger, Pauwels, Van der Veen, Schimmel, & Lamberty, 2001). This kind of studies are assessed and controlled during the time-scale of the material preparation, for maintaining a stated property value during their life-time (ILAC-G12, 2005; ISO Guide 35, 2006).

In spite of the importance of the stability studies, there are not many articles that contain information about stability studies in samples of olive oil and none relating to RMs of this oil. Reports on RMs are found that studied another types of oils such as palm oil and a blend of soy-corn oils (Pocklington, Pearse, Lognay, Wagstaffe, Boenke, & Schurer, 1993; Dabrio., Marini, Ricci., Ulberth., & Emons, 2007; Ahmad Tarmizi, Wai Lin, & Kuntom, 2008). Different papers have been published in relation of olive oil stability. The effect of the storage conditions on acidity, FA profile and peroxide index as criteria of the quality of the extra-virgin and virgin olive oil were studied in different types of containers during 6 months at 20-22ºC (Méndez, & Falqué, 2007). Also, Secoiridoid and Tocopherol contents and antioxidant activity have been studied during 8 months of storage in dark at 40ºC and 25ºC (Roca, Gandul-Rojas, Gallardo-Guerrero, & Mínguez-Mosquera, 2003). Pigment composition was analyzed every 30 days during one year at 15ºC to predict a model, using discriminant criterion, for olive variety classification (Lavelli, Fregapane, & Salvador, 2006).

Another research work studied the olive oil oxidation, during 24 months at 18-28ºC, analyzing some parameters like, chlorophyll, carotenoid, total polar, squalene, α-tocopherol content (Psomiadou, & Tsimidou, 2002). There are also research works which studied the stability of olive oils, in relation of lighting room and ambient temperature, using some physical-chemical parameters like acidity, peroxide value, K232, K270, tocopherols, fatty acids, and sterols (Gutiérrez, & Fernández, 2002; De Leonardis, & Macciola, 1998).

This paper describes an intralaboratory stability study aimed for assuring the stability of olive oil QCMs concerning to the physical-chemical characteristics defined in Commission Regulation (EC) No. 1989/2003, (2003). The final objective of this study is to check the stability of four different homogeneous olive oil

materials in order to establish general conditions of stability to obtain a cheap and simple in-house QCM for the olive oil control laboratories as an alternative of commercial RMs.

2. MATERIALS AND METHODS

2.1 Material preparation. The stability study was performed using 30 unitary samples resulting from the InterOLEO-CRM 2006 certification study (Cuadros-Rodríguez, Bosque-Sendra, De la Mata-Espinosa, González-Casado, & Rodríguez-García, F, 2008). They were: BL: mixture of virgin extra olive oil (60%) and sunflower oil (40%); PM: refined pomace olive oil (100%); RF: mixture of refined olive oil (80%) and palm oil (20%); and VG: lampante olive oil (100%).The homogenization of every material was carried out with an industrial blender and it was mixed for an 1 hr in stainless steel deposits. The blend rested the whole night. Before bottling, the blend was mixed for 15 min.

The four materials were analyzed by the Official Agricultural and Food Quality Control Laboratory of Granada, Atarfe venue (LAA), which is a testing laboratory recognized by the IOOC and accredited under the ISO/IEC 17025 standard. All the analysis were performed according to the described methods in the Regulation (CEE) 2568/91 (1991).

2.2 Homogeneity Study. The homogeneity study was carried out on 25 samples of each material. They were selected to cover the whole lot of each material according to the establishing procedure in the InterOLEO-CRM 2006 certification study (Cuadros-Rodríguez, Bosque-Sendra, De la Mata-Espinosa, González-Casado, & Rodríguez-García, F, 2008). The analyses of the different samples were carried out under repeatability conditions. The analytical methods applied are described in the Regulation (EC) 2568/91 and later modifications.

2.3 Stability Study. The stability of the samples was analyzed under the best storage conditions as found in literature (Gutiérrez, & Fernández, 2002; De Leonardis, & Macciola, 1998): darkness and controlled low temperature (0-8ºC). A classical stability study was applied (ISO Guide 35, 2006). The samples were stored in the established conditions and they were analyzed monthly during the

duration of the study. The stability study was carried out under reproducibility conditions, these conditions were established due to accuracy limitations of the applied methods. The study was programmed for 28 months from the QCMs elaboration date, from July 2006 to December 2008.

2.4 Statistics. The statistical treatment was carried out by the Unit of Chemical, Metrology and Qualimetrics (CQM) of the Department of Analytical Chemistry, University of Granada.

The statistical criterion for the homogeneity study was: $s_{homog} \leq 2 \times s_{repeatib}$, where the standard deviation of repeatability ($s_{repeatib}$) is that assumed by LAA.

Control limits were established to monitor the stability for each parameter studied and for each material according to ISO Guide 33 (ISO Guide 33, 2000):

$$x \pm 2 \cdot s_D$$

where x is the initial assigned value obtained, this is, the certified value obtained from the InterOLEO-MRC 2006 certification study, and s_D is the standard deviation associated with the measurement process motivated by the methods used in the analysis:

$$s_D = \sqrt{s_{inter} + \frac{s_{intra}}{r}}$$

where s_{inter} is the standard deviation from the between-laboratories fluctuation, obtained from the InterOLEO-MRC 2006 certification study, s_{intra} is within-laboratory standard deviation or the short-term fluctuation and r the number of replicates analyses made of each reference material.

The criteria for acceptance were divided by two conditions: (i) the stability values have to be inside the control limits, and (ii) if two continued stability values were outside the control limits, the parameter was considered out of control.

This criterion was established due to the accuracy of some of the applied methods in the studio are not sufficient high as it is recommended, but these are the only methods recommended by official regulations.

5. RESULTS AND DISCUSSION

The production of the materials and the homogeneity study were carried out in the framework of the InterOLEO-CRM 2006 certification study (Cuadros-Rodríguez, Bosque-Sendra, De la Mata-Espinosa, González-Casado, & Rodríguez-García, F, 2008). These materials were chosen as representatives of the materials analyzed in olive oil control laboratory and due to its natural heterogeneity. Then, if the essays will confirm the homogeneity and stability of the materials this fact could be assimilated to more homogeneous olive oil materials. The physical-chemical characteristics chosen for the homogeneity study were: acidity, K270 and content in major fatty acid. They are able to detect the difference in the proportions of the mixtures of oils or the effects due to sedimentation and/or decantation. The contents in majority fatty acid are those whose chromatographic peak area is equal or bigger than 3-5% of the oleic acid (C18:1) peak area: palmitic acid (C16:0), stearic acid (C18:0), and linoleic acid (C18:2). The homogeneity studies proved that the unitary samples of the different materials are homogeneous (Cuadros-Rodríguez, Bosque-Sendra, De la Mata-Espinosa, González-Casado, & Rodríguez-García, F, 2008).

In vegetables oils the stability can be higher due to a partial hydrogenation employed in the processing, additives used in the oils, like antioxidants, and biotechnological engineering techniques have been also developed to increase oxidative stability and shelf-life in oils (Institute of Shortening and Edible Oils, 9 ed, 2006). While, the olive oil stability is only due to its properties since cannot use any additive or suffer any process to enlarge their life-time. The election of the physical-chemical parameters considered for the study was based on the IOOC classification criteria, purity and quality (COI/T.15/NC no. 3/Rev. 2, 2006) and the previous results obtained in the InterOLEO-CRM 2006 certification study. Parameters such as: acidity, peroxide value, K-values and total wax content were considered critical characteristics due to they are reasonably sensible to changes in short periods of time and they were determined every month. On the other hand, previous results of the parameters such as: fatty acids, fatty acid trans isomers of C18, saturated fatty acids in 2-position of the triglycerides,

ECN42, sterols, erythrodiol + uvaol, stigmastadienes and aliphatic alcohols showed a non-significant alteration or trend and then, they were considered non-critical group parameters and they were not analyzed. In Table 1 are collected the statistical data used for the elaboration of control charts.

Figure 1 shows representatives behaviour and trend of the different parameters in BL material.

Acidity levels. Figure 1 A shows that the acidity level in BL material is stable, like as in PM material but in RF material there is a minimum increment inside the limits. In VG material this parameter shows an uptrend starting in the 24th month but this increase is always lower than the control upper limit.

Peroxide value. In Figure 1 B is presented a light down trend under control of this parameter in BL material. In PM material peroxide value shows a data increase which is emphasized in the 25th month but it is inside the limits. RF material presents a similar behaviour than PM material but with more variability. In contrast, in VG material, the peroxide value has a down trend with short variability (Fig 2).

K270 and K232 parameters. In BL material, K232 an indicator of formation of hydroperoxide and conjugated dienes, like as K270, increases its value starting from month 25th but the change is inside the limits (Figure 1 C). The RF and PM material present a similar behavior among the parameters: K270 and K232, these parameters are stable and they do not show tendency in their data. Therefore, there was no evidence of formation of hydroperoxide or secondary oxidation products. The parameter K232, in VG material, does not present any variability or trend in the data. On the other hand, the K270 trend starts in the 24th month and it is not so pronounced like acidity level. Therefore, these changes in K270 parameter show formation of ethylenic diketones.

Table 1. Statistical data for the elaboration of control charts

	PARAMETER	x	s_{inter}	s_{intra}	n	s_D	x + ($2s_D$)	x - ($2s_D$)
BL	Acidity level (%)	0.30	0.04	0.01	7	0.04	0.38	0.22
	Peroxide value (meqO2/kg)	14.0	1.16	1.00	7	1.2	16.4	11.6
	K232	3.40	0.23	0.15	7	0.23	3.87	2.92
	Total waxes	118.0	19.20	8.55	7	19.5	156.9	79.1
PM	Acidity level (%)	0.10	0.08	0.03	7	0.08	0.28	-0.08
	Peroxide value (meqO2/kg)	7.20	2.00	0.42	7	2.0	11.92	2.48
	K232	4.54	0.75	0.09	7	0.751	6.16	2.92
	Total waxes	2800.0	151.6	286.26	7	186.2	3322.56	2277.44
VG	Acidity level (%)	3.40	0.131	0.15	7	0.14	3.69	3.11
	Peroxide value (meqO2/kg)	6.80	1.426	0.52	7	1.43	9.68	3.92
	K232	2.21	0.09	0.04	7	0.09	2.41	2.01
	Total waxes	270.0	5.9	13.8	7	7.9	285.8	254.15
RF	Acidity level (%)	0.10	0.09	0.03	7	0.09	0.3	-0.1
	Peroxide value (meqO2/kg)	0.00	0.86	0.31	7	0.87	1.74	-1.74
	K232	1.94	0.08	0.01	7	0.08	2.21	1.67
	Total waxes	230.0	5.9	12.2	7	7.5	286.1	173.9

Where x is the certified value and s_D is the standard deviation associated with the measurement process.

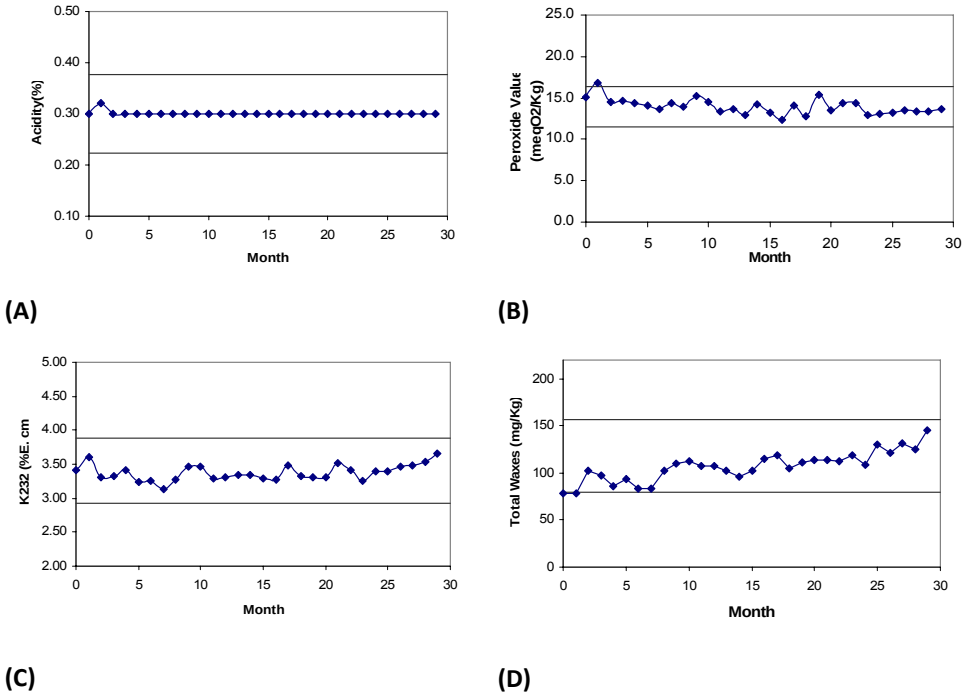

Figure 1. Control chart for stability monitoring of critical parameters from BL material: (A) Acidity, (B) Peroxide value, (C) K232, (D) Total waxes.

CONCLUSIONS

It is concluded that, in the storage conditions of darkness and low controlled temperature (0-8ºC), the critical physical-chemical parameters of the olive oil QCMs are stable at least during 24 months (2 years) with, in general, light variability and up trends. Consequently, this study has confirmed that these olive oil materials have the required stability to be used as QCMs for the physical-chemical characteristics established in the European Regulation (CE) 1989/2003. According to this study, theoretically, a homogeneous sample olive oil could be used as a QCM if this material were stored in the dark and low temperature. Therefore olive oil quality control testing laboratories have a new alternative to dispose stable in-house QCMs for their measurements. For this pur-

pose, testing laboratories could use a proper quantity of a batch of olive oil with appropriate and confirmed homogeneity, previously analyzed in a recognized laboratory (preferably accredited by ISO 17025), to prepare unitary samples and maintaining them in the coolness (temperature < 8ºC) and in the dark. Then, taking all the necessary precautions, these olive oil samples could be used as in-house QCMs for a shelf-life of two years.

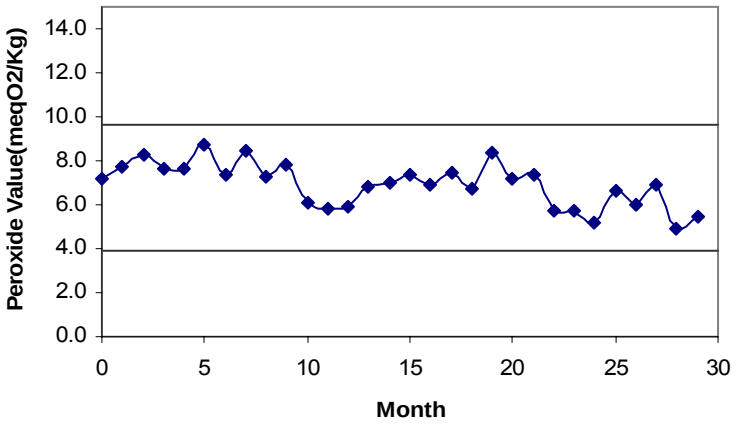

Figure 2. Peroxide value stability in VG material.

ABBREVIATIONS

QCM Quality Control Materials

RM Reference Materials

SD Standard deviation

S_{inter} Standard deviation interlaboratories

S_{intra} Standard deviation intralaboratories

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REFERENCES

Ahmad Tarmizi, A.H., Wai Lin, S., & Kuntom, A. (2008). Development of Palm-Based Reference Materials for the Quantification of Fatty Acids Composition, J. Oleo Sci., 57(5), 275-285.

BIPM, IEC, IFCC,ILAC, ISO, IUPAC, IUPAP & OIML (2008). International vocabulary of basic and general terms in metrology. 2nd edn., ISO, Geneva

Christie, W. W. (2003). Lipid Analysis, 3ed. Bridgwater: The oily Press.

COI/T.15/NC no. 3/Rev. 2 (2006). Trade standard applying to olive oils and olive-pomace oils, 1st ed. Madrid: International Olive Oil Council.

Commission Regulation (EC) No. 1989/2003 amending Regulation (EEC) No. 2568/91 (2003). On the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis. Off. J. Eur. Union, Brussels, Belgium, L295/57-77.

Commission Regulation (EEC) No. 2568/91 (1991). On the characteristics of olive oil and olive-residue oil on the relevant methods of analysis. Off. J. Eur. Commun. L248/1–83, and further revisions.

Cuadros-Rodríguez, L., Bosque-Sendra, J. M., De la Mata-Espinosa, A. P, González-Casado, A., & Rodríguez-García, F. P. (2008). Elaboration of Four

Olive Oil Certified Reference Materials: InterOLEO-CRM 2006 Certification Study. Food Anal. Methods, 1, 259-269.

Dabrio, M., Marini, M., Ricci, M., Ulberth, F., & Emons H. (2007). Certification of the fatty acid profile of soya-maize oil blend BCR-162R. EUR 22877 EN. Luxemburg: European Commission Joint Research Centre. Institute for Reference Materials and Measurements

De Leonardis, A., & Macciola V. (1998). Evaluation of the Shelf-Life of Virgin Olive Oils. Riv. Ital. Sostanze Grasse, 75, 391-397.

Emons, H., Linsinger T.P.J. & Gawlik, B. M. (2004). Reference materials: terminology and use. Can't one see the forest for the trees?. Trends Anal Chem 23, 442-449

Emons, H., Fajgelj, A., van der Veen, A.M.H. & Watters, R. (2006). New definitions on reference materials. Accred. Qual. Assur 10, 576-578

Emons, H. (2006). The 'RM family'–Identification of all of its members. Accred. Qual. Assur.10, 690-691

European Commission Directorate-General for Agriculture. (2003). The olive oil sector in the European Union.

Gutiérrez, F., & Fernández, J. L. (2002). Determinant Parameters and Components in the Storage of Virgin Olive Oil. Prediction of Storage Time beyond Which the Oil Is No Longer of "Extra" Quality. J. AOCS, 50, 571-577.

ILAC-G9. (2005). Guidelines for the selection and use of reference materials. http://www.ilac.org/guidanceseries.html. Accessed 25 January 2010

ILAC-G12. (2000). Guidelines for the Requirements for the Competence of Reference Materials Producers. http://www.ilac.org/guidanceseries.html. Accessed 25 January 2010

Institute of Shortening and Edible Oils, 9 ed. (2006). Food Fats and Oils, Washington, USA.

ISO Guide 30. (1992). Terms and definitions used in connection with reference materials, 2nd ed. Geneva: International Standardization Organization.

ISO Guide 33 (2000). Uses of certified reference materials. 2nd ed. Geneva: International Standardization Organization.

ISO Guide 35 (2006). Reference materials. General and statistical principles for certification, 3rd edn. Geneva: International Standardization Organization

Lavelli, V., Fregapane, G., & Salvador, M. D. (2006). Effect of Storage on Secoiridoid and Tocopherol Contents and Antioxidant Activity of Monovarietal Extra Virgin Olive Oils. J. Agric. Food Chem., 54, 3002-3007.

Linsinger, T. P. J., Pauwels, J., Van der Veen, A. M. H., Schimmel, H., & Lamberty, A. (2001). Homogeneity and stability of reference materials. Accred. Qual Assur, 6, 20-25.

Luchetti, F. (2002). Importance and future of olive oil in the world market- an introduction to olive oil. Eur. J. Lipid Sci. Technol., 104, 559-563.

Méndez, A.I., & Falqué, E. (2007). Effect of storage time and container type on the quality of extra-virgin olive oil. Food Control, 18, 521-529.

Pocklington, W.D, Pearse, J., Lognay, G., Wagstaffe, P.J., Boenke, A., & Schurer B. (1993). The Certification of the fatty acid profile and the cholesterol mass fraction of two edible oil and fat materials. Soya-Maize oil blend CRM162 & Beed-Pork Fat blend CRM163. EUR15278 EN. Luxembourg: Commission of the European Communities.

Psomiadou, E., & Tsimidou, M. (2002). Stability of Virgin Olive Oil. 1. Autoxidation Studies. J. Agric. Food Chem., 50, 716-721.

Resolution No. RES-2/78-IV/98. (1998). IOOC certificate for the recognition of olive oil testing laboratories, 1st ed. Madrid: International Olive Oil Council.

Roca, M., Gandul-Rojas, B., Gallardo-Guerrero, L., & Mínguez-Mosquera, M.I. (2003). Pigment Parameters Determining Spanish Virgin Olive Oil Authenticity: Stability During Storage. J. AOCS, 80, 1237-1240.

1.4 Discusión

El estudio InterOLEO-MCR 2006, financiado por la Junta de Andalucía, a través de la Empresa Pública "Desarrollo Agrario y Pesquero", ha permitido la elaboración y certificación de cuatro materiales de referencia (MRC) de aceite de oliva para su utilización en operaciones de aseguramiento y control de la calidad en los laboratorios, tanto oficiales como privados, de control de la calidad del aceite de oliva; este estudio ha supuesto un hito internacional ya que estos materiales de referencia constituyen el primer ejercicio de certificación que se ha llevado a cabo en el sector del aceite de oliva.

Se puede concluir, que en las condiciones de almacenamiento (oscuridad y temperatura baja de 0-8ºC, los parámetros críticos de estabilidad del aceite de oliva son estables, al menos por 24 meses. De acuerdo con el estudio realizado, muestras homogéneas de aceite de oliva se pueden utilizar como materiales de control de calidad, si se almacenan y mantienen en las condiciones antes mencionadas.

EL presente capitulo se considera como una iniciación al mundo del aceite de oliva. Ya que el trabajo realizado en los estudios de certificación y estabilidad de materiales de referencia, ha servido de preparación para los siguientes estudios con muestras de aceites y los alimentos que lo contienen. Así, estos estudios me han permitido conocer: i) el aceite de oliva, su importancia y sus aspectos industriales; ii) los parámetros físicos-químicos y las técnicas analíticas que se usan para el control de su calidad y caracterización; iii) sus propiedades sensoriales y las técnicas para su caracterización

sensorial; y iv) como se pueden preparar muestras patrón de aceite y sus condiciones de almacenamiento. Todo ello ha ayudado a encontrar la solución de los problemas planteados en el desarrollo de los siguientes capítulos de esta tesis doctoral sobre el aceite de oliva, los aceites vegetales y sus mezclas.

CAPÍTULO 2

DETERMINACIÓN DEL PERFIL DE TRIGLICERIDOS EN ACEITES VEGETALES UTILIZANDO HPLC-CAD

Patatas a lo pobre

Ingredientes:

- 2 patatas
- 1 cebolla
- 1 pimiento
- Aceite de oliva virgen extra
- sal

Preparación

Se pone un poco de **Aceite de oliva** en el fondo de una sartén, y se añaden la cebolla y los pimientos cortados en aros y también las patatas, cortadas en láminas, como cuando se va a hacer tortilla patatas.

Se sazonan con un poco de sal y se deja a fuego bajo y se tapan para controlar mejor la cocción y evitar que se queme. Su propia agua y el vapor que suelta la verdura hace que se cocinen sin necesidad de mucho aceite.

De vez en cuando se mueven con un poco de cuidado, para evitar que queden convertidas en un puré, y cuando esté todo bien pochado, se sube el fuego, para dorarlas un poco y así terminar de hacerlas

Se pueden tomar solas, o si se quiere un plato redondo, sólo hay que freír un huevo y estrellarlo encima

2.1 Presentación

Uno de los objetivos del proyecto QuOLEO, en el que se enmarca la presente tesis doctoral, es el poner a punto un método analítico para la caracterización de triglicéridos (TAGs) en aceites vegetales y alimentos que contengan aceite de oliva, tales como productos de panadería, frituras y embutidos, utilizando para ello el sistema de cromatografía líquida de alta presión (HPLC por sus siglas en inglés) en fase inversa y elución en gradiente, con un detector de partículas cargadas en aerosol (CAD por sus siglas en inglés) (Fig 2.1).

Fig 2.1 Equipo HPLC-CAD utilizado para la caracterización de triglicéridos

Los objetivos para el que se desarrolló el método analítico HPLC-CAD son: i) identificación de los triglicéridos presentes en aceite de oliva y aceites vegetales, utilizando para ello: patrones, comparación de los cromatogramas obtenidos con el método HPLC-CAD con cromatogramas obtenidos mediante el método oficial (recogido en los apartados 2.3 y 2.4) y los tiempos de retención relativos (apartado 2.3); ii) validación inicial del método, efecto matriz, linealidad y la señal del blanco (aparta-

do 2.4); iii) validación de la precisión y veracidad del método (apartado 2.4); y iv) estudiar las posibles aplicaciones del método, tales como: estudio de la composición de triglicéridos, composición de ácidos grasos, y composición nutricional de ácidos grasos, a partir del contenido en triglicéridos, de diferentes categorías de aceite de oliva (apartado 2.4) y por último, la caracterización de aceite de oliva en productos alimenticios (embutidos, patatas fritas y productos de panadería) (apartado 2.5).

2.2 Aspectos generales

Para la determinación de los triglicéridos se han propuesto varias técnicas en donde podemos destacar la utilización de HPLC con diferentes detectores: absorción en el ultravioleta (UV), diodos en fila (DAD), índice de refracción ("refractive index detector, RID) y el detector de dispersión de luz por vapor ("evaporative light scattering detector", ELSD). Sin embargo, el método “oficial” utilizado comúnmente en los laboratorios de rutina para la determinación del contenido triglicéridos en aceites de oliva, está establecido por el Consejo Oleícola Internacional (COI), cuya versión más reciente está descrita en el documento: COI/T.20/Doc. No 20/Rev. 2 [1]. Dicho método es equivalente al recomendado por la Unión Internacional de Química Pura y Aplicada (IUPAC) [2]. Así mismo, la American Oil Chemists´Society (AOCS) lo ha aceptado como método oficial uno de características similares, Ce 5b-89 [3]. En este último caso, se permite el uso de tres diferentes detectores i) RID, ii) UV y iii) MS.

[1] COI/T.20/Doc. No 20/Rev. 2, (2008) Method of analysis. Difference between actual and theoretical content of triacylglycerols with ECN 42, International Olive Council, Madrid

[2] Wolff J.P., Mordret F.X., Dieffenbacher A. (1991) Determination of Triglycerides in Vegetable oils in terms of their partition numbers by high performance liquid chromatography, Pure & Appl. Chem. 63 No.8: 1773-1182

[3] AOCS Official Method Ce 5b-89 (1997). American Oil Chemists' Society, S. Boulder

Adicionalmente, también la Unión Europea ha adoptado una adaptación de este método como oficial para evaluar las diferentes categorías de aceite de oliva [4].

Las limitaciones que presentan los detectores mencionados anteriormente, RID, ELSD y UV, así como las ventajas del detector CAD y su funcionamiento se recogen en el apartado 2.4. Un diagrama del funcionamiento del detector CAD se explica detalladamente en la Figura 2.2

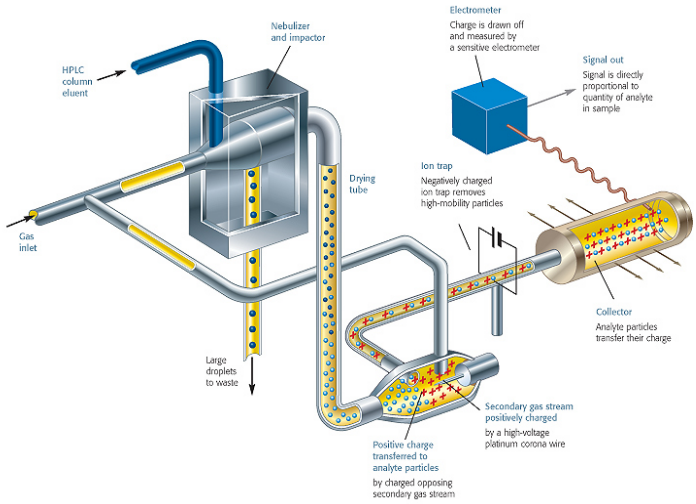


Fig2.2 Funcionamiento del detector Corona CAD. Imagen obtenida de la página web del fabricante [5]

Adicionalmente, en la Tabla 2.1 se establece una comparativa de las características de diferentes detectores habitualmente utilizados en HPLC, en donde se ponen de manifiesto las ventajas del CAD [5].

[4] Commission Regulation (EEC) No. 2568/91 (1991) On the characteristics of olive oil and olive-residue oil on the relevant methods of analysis. Off. J. Eur. Commun., Brussels, Belgium, L248/1–83

[5] http://coronacad.com/CAD_Overview.htm, A new era in HPLC detection, junio 2010, Corona CAD

Tabla 2.1 Comparación de las características de diferentes detectores utilizados con HPLC [5]

	CAD	UV	ELSD	RID
Sensibilidad	***	***	**	*
Rango Dinámico	***	***	*	**
Consistencia en Respuesta	***	*	**	*
Aplicabilidad	***	**	***	**
Reproducibilidad	***	***	*	**
Compatibilidad Cromatográfica	***	**	***	*
Fácil de usar	***	***	**	***

El método HPLC-CAD fue optimizado en sus distintos parámetros, dando como resultado cromatogramas con una resolución satisfactoria. La metodología, equipos y materiales se muestran en el apartado 2.4.

2.3 Identificación de picos cromatográficos

En esta sección se amplía la información recogida en el apartado 2.4., sobre las diferentes metodologías utilizadas para la identificación de los picos cromatográficos de los triglicéridos.

Dicha identificación se realizó utilizando dos aceites vegetales, soja y oliva. Estos aceites se escogieron debido a que su perfil de triglicéridos esta descrito en documentos oficiales de referencia. Esta identificación se realizó utilizando tres métodos: i) preparando una disolución simulando los aceites de oliva y de soja a partir de patrones de triglicéridos; ii) realizando una comparación de los cromatogramas de los aceites de soja y oliva obtenidos, con cromatogramas de referencia publicados en documentos oficiales de la Unión Internacional de Química Pura y Aplicada (IUPAC) y del Consejo Oleícola Internacional (COI), cabe señalar que, ambos cromatogramas de referencia fueron obtenidos utilizando la metodología HPLC-RID

para su análisis [6,7]; y iii) tiempos de retención relativos (t_{RR}) con respecto a un patrón interno.

En el método (i), se prepararon dos disoluciones de triglicéridos simulando su contenido en los aceites de oliva y soja, para ello se usaron patrones de triglicéridos en hexano. Los triglicéridos utilizados fueron los que se encuentran en más abundancia en dichos aceites. Para la identificación de cada uno de los triglicéridos, se aumentó su concentración en un 25% respecto a la inicial. Es decir, en el cromatograma obtenido, el pico cromatográfico que aumentaba era el correspondiente al del triglicérido cuya concentración se había aumentado en un 25%. Como ejemplo, en la Tabla 2.2 se muestran los patrones de triglicéridos usados para la preparación de la simulación del aceite de soja y en la Figura 2.3 se muestra el cromatograma obtenido de esta disolución de triglicéridos de aceite de soja simulado con los picos cromatográficos identificados.

Para el método (ii), el cromatograma de aceite de soja obtenido con el método HPLC-CAD se comparó con el cromatograma correspondiente de la IUPAC [6] que se establece como referencia para la identificación de triglicéridos. La metodología aplicada por el método HPLC-CAD fue la misma que se recoge en el apartado 2.4, utilizando 250 ppm de muestra de aceite y 4 µl de volumen de inyección. Mientras que el método utilizado por la IUPAC realiza la identificación de triglicéridos con una concentración de muestra del 5 % (50.000 ppm) y el volumen de inyección es de 10 µl. Los resultados obtenidos se muestran en la Figura 2.4. En el cromatograma de

[6] COI/ T.20/ Doc. nº 20/ Rev.1 (2001), Determinación de la diferencia entre el contenido real y el contenido teórico en triglicéridos con ECN42, 1ª ed. Consejo Oleícola Internacional, Madrid.

[7] Wolff J.P., Mordret F.X., Dieffenbacher A., Determination of triglycerides in vegetable oils in terms of their partition numbers by high performance liquid chromatography, Pure&Appl. Chem. Vol. 63, No. 8, pp. 1173-1182, 1991.

Tabla 2.2 Concentraciones de triglicéridos utilizadas en la preparación del aceite de soja simulado

TAG		Concentración utilizada en la mezcla (ppm)	Concentración real presente en el aceite de soja (%)
Acrónimo	Nombre		
OLL	1,2-linoleín-3-oleína	50	18.1
LLP	1,2-linoleín-3-palmitina	50	12.6
OOL	1,2-oleín-3-linoleína	50	17.0
LLS	1,2-linoleín-3-estearina	50	2.5
POL	1-palmitín-2-oleín-3-linoleína	50	9.7
PPL	1,2-palmitín-3-linoleína	50	1.8
OOO	trioleína	50	2.8
OOP	1,2-oleín-3-palmitina	50	2.3
PPO	1,2-palmitín-3-oleína	50	0.7
PPP	tripalmitina	50	0.2
OOS	1,2-oleín-3-estearina	50	1.5
PSO	1-palmitín-2-estearin-3-oleína	50	0.6
PPS	1,2-palmitín-3-estearina	50	0.4
SSO	1,3-estearin-2-linoleína	50	0.8
LLL	Trilinoleína	50	20.5

la IUPAC (Fig. 2.4A) se identifican 13 triglicéridos (por orden de elución):1-linoleín-2,3-linolenina (LLnLn), 1,2 linoleín-3-linolenina (LLLn), LLL, 1-oleín-2-linoleín-3-linolenina (OLLn), 1-palmitín-2-linoleín-3-linolenina (PLLn), OLL, PLL, OOL, POL, 1-estearín-2,3-linoleína (StLL), PPL, OOO y 1-estearín-2-oleín-3-linoleína (StOL). En el cromatograma obtenido con el método HPLC-CAD (Fig. 2.4B) se identificaron 8 triglicéridos, LLL, OLL, LLP, OOL, LLS, POL, PPL y OOO. Comparando los dos cromatogramas, se observa una gran similitud en los dos perfiles cromatográficos del aceite de soja, tanto en el orden como en la posición relativa en los cromatogramas; únicamente hay una diferencia en la asignación del pico del triglicérido LLS. En el cromatograma de la IUPAC (Fig. 2.4A) el LLS se asigna al pico cromatográfico solapado con el triglicérido POL y en el cromatograma obtenido con el método HPLC-CAD está solapado con el triglicérido OOL.

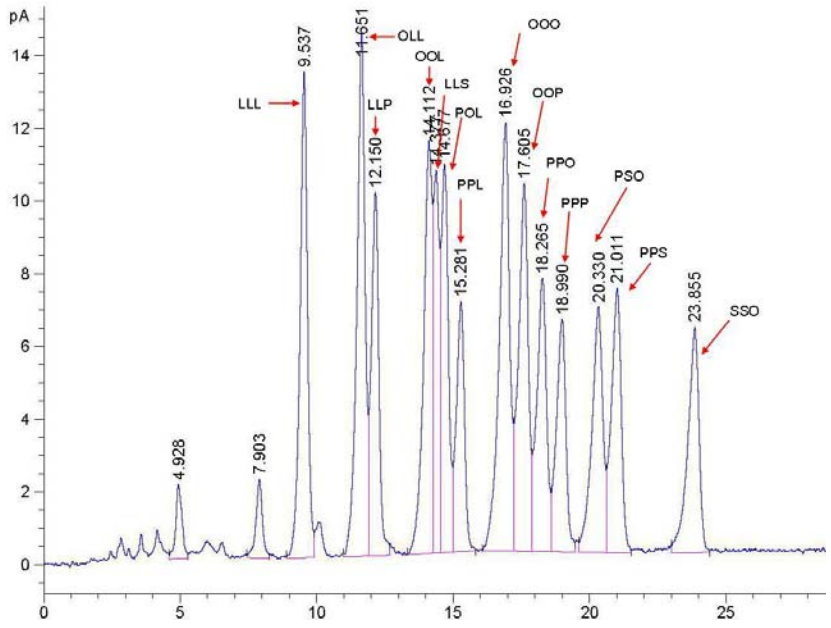
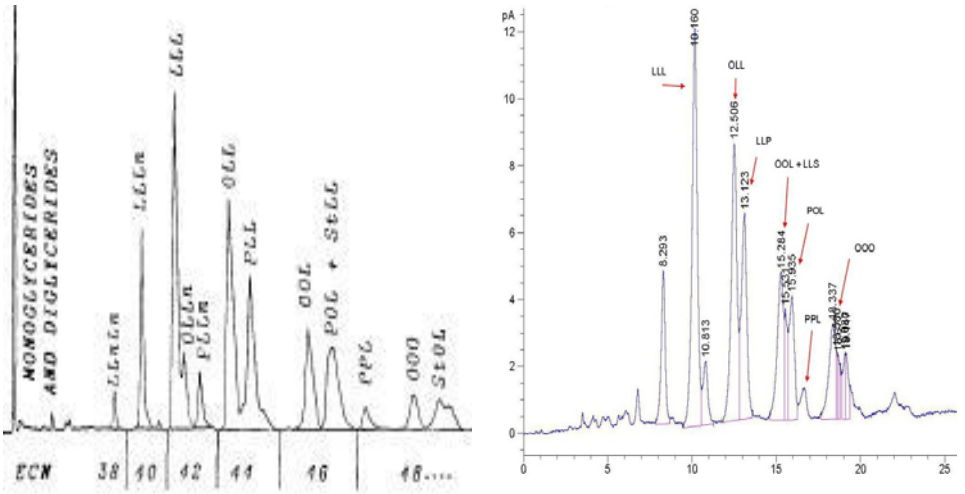


Fig 2.3 Cromatograma de la disolución de simulación de triglicéridos de aceite de soja utilizando patrones de triglicéridos

Del mismo modo, el cromatograma obtenido de la muestra del aceite de oliva se comparó con el que presenta el COI en el documento para la determinación de triglicéridos [6]. El COI utiliza las mismas condiciones de concentración de muestra y volumen de inyección que la IUPAC. En la figura 2.5 se recogen ambos cromatogramas. En el cromatograma del COI (Fig. 2.5A) se identifican 16 triglicéridos (por orden de elución): LLL, 1-oleín-2-linolenín-3-linoleína (OLnL), 1-palmitín-2-linolenín-3-linoleína (PLnL), LOL, 1-oleín-2-linolenín-3-oleína (OLnO), PLL, 1-palmitín-2-linolenín-3-oleína (PLnO), LOL, PLO, OOO, 1-estearín-2-linoleín-3-oleína (SLO), POO, POL, 1-estearín-1,2-oleína (SOO), 1,3-estearín-2-linoleína (SLS) y POS. En el cromatograma del aceite de oliva obtenido con el CAD (Figura 2.5A) se identificaron 13 triglicéridos, LLL, LLO, LLP, OOL, LLS, POL, OOO, OOP, PPS, PPO, OOS, SLS y PSO. Los dos cromato-

gramas presentan el mismo perfil cromatográfico de triglicéridos, tanto en lo que se refiere al orden en la elución como respecto a su posición relativa.



A B

Figura 2.4 Cromatogramas de aceite de soja. (A): Cromatograma de referencia publicado por la IUPAC utilizando HPLC-RID; (B): Cromatograma obtenido con el método HPLC-CAD

Utilizando el método HPLC-CAD se puede concluir que, aún con las diferencias metodológicas, se obtienen similares cromatogramas con picos cromatográficos bien resueltos, utilizando menor concentración de muestra y en un tiempo sensiblemente menor (30 min en lugar de los 120 min).

Por último, en el tercer método, los picos cromatográficos atribuidos a los triglicéridos fueron identificados comparando los tiempos de retención relativos ($t_{R,r}$) con respecto a un patrón interno. El patrón interno utilizado fue el triglicérido triestearina (SSS), por no estar presente en los aceites vegetales. Los $t_{R,r}$ fueron calculados utilizando la ecuación 2.1.

$$t_{R,r} = \frac{t_{R,\text{triglicérido}}}{t_{R,\text{patrón}}} \qquad \text{Ec. 2.1}$$

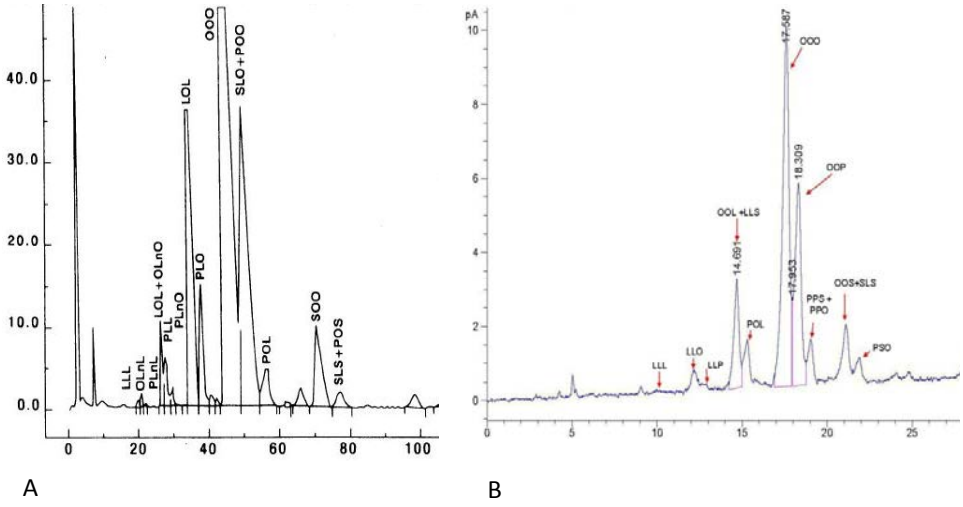


Figura 2.5 Cromatograma de aceite de oliva. (A): Cromatograma de referencia publicado por el COI, obtenido mediante HPLC-RID; (B): Cromatograma obtenido utilizando el método HPLC-CAD. Es de hacer notar que el cromatograma del documento COI hay un error tipográfico, ya que el pico identificado como POL en el conjunto de ECN 46, debe de referirse efectivamente a PPS o PPO ya que el POL tiene un ECN de 44.

Los resultados obtenidos de los $t_{R,r}$ para los dos aceites se muestran en la Tabla 2.3. Los $t_{R,r}$ no mostraron diferencias significativas entre los dos aceites, soja y oliva, en los seis triglicéridos presentes en ambos aceites, LLO, LLP, OOL, LLS, POL, OOO como se muestra en la Tabla 2.3 (marcados en negritas y cursiva), por lo que se utilizó este método para la identificación de los triglicéridos en otros aceites vegetales, utilizados en esta tesis doctoral, tales como: maíz, semillas, uva, colza, girasol, canola, cacahuete, lino, sésamo, avellana y mezclas de aceites comerciales.

Tabla 2.3 Tiempos de retención relativos al patrón SSS para los aceites de Soja y Oliva

	Aceite de Soja		**Aceite de Oliva**	
TAG	t_R	$t_{R,r}$	t_R	$t_{R,r}$
LLL	9.538	0.349		
LLO	11.623	***0.426***	12.2	***0.427***
LLP	12.138	***0.444***	12.759	***0.447***
OOL	14.084	***0.516***	14.691	***0.515***
LLS	14.084	***0.516***	14.691	***0.515***
POL	14.653	***0.537***	15.278	***0.535***
PPL	15.278	0.559		
OOO	16.901	***0.619***	17.587	***0.616***
OOP			18.309	0.641
PPS			19.027	0.667
PPO			19.027	0.667
OOS			21.098	0.739
SLS			21.098	0.739
PSO			21.881	0.767

t_R patrón (SSS) Aceite de Soja: 27.31

t_R patrón (SSS) Aceite de Oliva: 28.54

En el apartado siguiente (2.4) se presenta una publicación, en la que se recogen los aspectos fundamentales del trabajo realizado por medio del método HPLC-CAD para la caracterización de los perfiles de triglicéridos en aceites de oliva. La publicación bajo el nombre de **Quantification of triacylglycerols in olive oils using HPLC-CAD**, ha sido enviada a la revista Food Analytical Methods.

2.4 Artículo envíado a Food Analytical Methods (en revisión)

Quantification of Triacylglycerols in Olive Oils using HPLC-CAD

(Short running head: TAGs quantification in Olive Oils)

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Abstract

This paper describes an RP-HPLC method for the quantification of triacylgycerol (TAG) compositional profile in olive oils. The chromatographic separation was achieved using a C18 column and the detection was accomplished using a charged aerosol detector (CAD). TAG peaks were identified using retention times and by addition of TAGs standards.

The linearity, matrix effect, and accuracy (precision and trueness) were validated; detection and quantification limits of the method were estimated. The developed analytical method was applied to the determination of the TAGs composition and the estimation of the concentration of each individual TAG, using an internal quantification standard (tristearin). From the TAG compositional profile, a fatty acid (FA) compositional profile could be calculated. The proposed method appears to be rapid and simple to determine TAGs in olive oil.

Keywords: HPLC, Charged aerosol detection, TAGs, olive oils

Introduction

Vegetable oils are mainly composed by triacylglycerols (TAGs), diacylglycerols (DAGs), free fatty acids (FAs), phospholipids, phytosterols, tocopherols and other minor components (Boskou, 1998, Buchgraber et al.2004). However, the main compounds found in oils are the TAGs (Adrikopoulos, 2002). Each TAG is chemically characterized by its total number of carbons (CN), the degree of unsaturation of each FA and the position and configuration of double bonds of each FA.

Several instrumental techniques have been applied for analyzed TAGs, one of the most used is high performance liquid chromatography (HPLC) (Ruiz-Gutiérrez and Barron 1995, Buchgraber et al.2004). The parameter that is used for the separation and identification of TAGs in HPLC is the "equivalent carbon number" (ECN), defined as:

$$ECN = CN - 2n \quad (1.1)$$

where, CN, the acyl number of carbons; and n, the number of the double bonds per TAG present in the three FAs (Buchgraber et al.2004). Different detectors have been used to analyze TAGs with this technique, the most common are: ultraviolet-visible spectroscopy (UV-Vis) (Ruiz-Gutiérrez and Barron 1995), refractive index (RID) (Kiritsakis et al. 2002, Moreda et al. 2003, Osorio et al. 2006, Lee et al. 2001) and evaporative light scattering (ELSD) (Cunha and Oliveira 2006, Neff et al. 1999, Heron et al. 2010, Amaral et al. 2004). There are several limitations for analyzing complex molecules, like TAGs, with these detectors a consequence of some disadvantages, like low sensitivity or moderate dynamic range, low specificity or absence of standards, among others (Vehovec and Obreza 2010).

The European Commission has adopted, like official method for the determination of TAGs, the difference between actual and theoretical content of ECN 42 in olive oils, the method uses a RID (EC No. 2474/97 1997). This method has been also proposed by the United States Department of Agriculture

(AMS-FV-08-0073-0006, 2010). However, RID presents an inconvenient, it is not able to use gradient, so it is strongly dependent on the mobile phase composition which makes difficult its applicability. Close to the limitations found in the detectors, TAGs present some complications in its characterization and quantification due to its structure. Quantification of TAGs, in conventional detectors, is based on the use of different response factors (RFs) for each TAG, these RFs are due to the different length from its carbon chains, and to the number and position of the double bonds that present the TAGs. In addition of these limitations, ELSD gives nonlinear calibration functions, so the values of corresponding RFs vary with the TAG concentration. All these disadvantages can lead to frequent errors in individual TAGs quantification.

In previous years, mass spectrometry has been used for detection and quantification of TAGs in vegetal oils [Holapek et al. 2005) and olive oils (Mottram et al. 1997, Jakab 2003, Kiritsakis et al. 2002). Compared with conventional detectors, mass spectrometry provides a better identification and quantification of TAGs, since it can identify more of them. However, in routine methods, the relative high cost of this detector prevents its use and also, specialists are required for working with it.

Charged Aerosol Detector (CAD), developed by ESA Biosciences in 2004 (Górecki et al. 2006, Moreu 2006), could be a possible solution. A review about its principles and applications has been published in 2010 (Vehovec and Obreza 2010). To our knowledge there are only two papers about olive oils using CAD (Lísa et al. 2007, de la Mata-Espinosa et al 2011). CAD, as ELSD, could be affected by mobile phase composition but this limitation could be solved by the use of inverse gradient compensation (Moreu 2006, Lísa et al. 2007). However, ELSD presents disadvantages in front CAD, like moderate sensitivity, moderate dynamic range and wide variation in response between analytes (Armstrong 2009).

CAD also could provide a solution to some of the limitations that presents RID, which is used, as mention before, in the official method for determination of TAGs in olive oils: i) a uniform answer to non-volatile analytes independently of their nature; ii) consistency in the RFs and reproducibility and iii) the most important, it accepts the use of gradient for better TAGs separation by its ECN. In the present study, an RP-HPLC-CAD method is proposed for the quantification of TAGs in olive oil and in food products containing olive oil. This method can be used as an alternative to routine methods or to the official method in the determination of the content of TAGs in olive oil.

Material and Methods

Instrumentation

The assays utilized an HPLC 1100 Series system from Agilent Technologies (Santa Clara, CA USA) equipped with thermostatic column compartment, Eppendorf TC-50. Detection was carried out with a Corona CAD (ESA Biosciences Inc., Chemlsford, MA USA). Experimental data were analyzed using Chemstation for LC systems (Rev. B.02.01-SR1 [260]). Standards and samples were filtered using a 0.22 µm polytetrafluoroethylene (PTFE) membrane, purchased from CAMEO (General Electric, Belgium).

Food fat extractions were performed on an Accelerated Solvent Extractor ASE 100 (Dionex, Sunnyvale, CA, USA), using 34 ml steel extraction cells. A calibrated three-figure analytical balance (Mettler Toledo PB303) was used for weight measurements. A Büchi RE-124 rotatory evaporator equipped with a vacuum pump V-700 (Büchi) was used to remove the remaining solvents after extraction. A household grinder (Taurus) was used for previous sample homogenization

Chemicals, reagents and standards

Acetonitrile HPLC grade was purchased from PANREAC (Barcelona, Spain). Hexane and isopropanol HPLC grade were obtained from PROLABO (Barcelona, Spain). The nitrogen (99%) was from Air Liquide (Madrid, Spain). The pure standards trilinolein (LLL), tripalmitin (PPP), triolein (OOO), tristearin (SSS), 1,2-linolein-3-palmitin (LLP), 1,2-olein-3-palmitin (OOP), 1,2-olein-3-stearin (OOS), 1,2-palmitin-3-linolein (OOL) were purchased from Sigma (Barcelona, Spain). And 1,2-palmitin-3-stearin (PPS), 1,2-palmitin-3-olein (PPO), 1,2-palmitin-3-linolein (PPL), 1,2-stearin-3-olein (SSO), 1,2-linolein-3-stearin (LLS), 1,2-linolein-3-olein (LLO), 1-palmitin-2-stearin-3-olein (PSO), 1-palmitin-2-olein-3-linolein (POL) were acquired from Larodan Fine Chemicals AB (Malmö, Sweden). An extra virgin olive oil, previously analyzed by an official Food Quality Control Laboratory, sited in Atarfe (Granada, Spain), was used to validate FA compositional profile. For food fat extraction hexane and 2-propanol, analytical grade, were supplied by PANREAC. Diatomaceous earth as inert solid was supplied by Dionex.

Oil samples and food products

Five olive oil samples were used in the study, purchased on retails stores in Spain. The samples were maintained in dark at −2ºC until analyses. The oil samples analyzed were classified, according to their label, as three extra virgin olive oils, one virgin olive oil and one olive oil (blend of virgin and refined olive oil).

The fat of nine bread snacks, two cured Spanish sausages (salchichón and chorizo) and two potato chips were analyzed. All food products were purchased in retail stores from Spain.

Chromatographic conditions

Chromatographic analysis was carried out using a LiChrosphere C-18 (5µm; 250 x 4mm) purchased from Agilent Technologies (Waldbronn, Germany). The column temperature was kept at 30 ºC. In order to obtain a maximum reproducibility and repeatability, the injection volume used was 4 µl. A binary mobile phase composed of acetonitrile and hexane/isopropanol (1:1), were used for gradient analysis (60:40 to 45:55 in 40 min with a post time of 5 min), the flow rate was 1.0 ml/min. The CAD nitrogen gas pressure was adjusted to 35 psi. For CAD monitoring none filter and a 100 pA output range was used.

Standards and samples preparation

For working reasons, stock solutions of standards were prepared by dissolving 100 mg of each TAG standard into 10 g of hexane. Stock solutions were diluted by hexane to achieve the required working concentrations.

Stock olive oil and fat extracted of food products sample solutions were prepared in the same way than stock standards solutions. Working sample solutions were diluted by hexane to obtain a final concentration of 250 µg/g.

Both standard and sample working solutions were filtrated prior to injection through a 0.22 µm PTFE membrane.

For accuracy test and matrix effect assessment, standards were added into working samples at a 25 µg/g concentration. Only one extra virgin olive oil was used for the validation test, the others olive oils were used for the application study.

The fat extraction was performed using a pressurized liquid extraction method (Ruíz-Samblás et al. 2010).

Calibration and quantification with internal standard

There are some analytical situations in which an internal standard (IS), employed as a calibrant, is added to the sample and analyzed as a whole together with the analyte. From the same analytical preparation, a response factor is obtained from the IS which is applied on the analyte signal for quantification using the equations 1.2 and1.3. From a metrological point of view, this is a true internal calibration (IC), a formally different methodology to the signal-ratio calibration (Cuadros-Rodríguez et al. 2007).

SSS was selected for using as an internal quantification standard, because it is not present in olive oils. For the quantification of individuals TAGs, the following equations were applied

$$RF_{SSS} = \frac{y_{SSS}}{x_{SSS}} \tag{1.2}$$

$$x_{TAG} = \frac{y_{TAGs}}{RF_{SSS}} = x_{SSS} \cdot \frac{y_{TAG}}{y_{SSS}} \tag{1.3}$$

where, RF is the response factor; y is the height or area of the peak; and x is the concentration.

For quantification a SSS concentration of 25 µg/g was added to working solutions.The detection (LOD) and quantitation (LOQ) limits were calculated using the signal/noise ratio (S/N). LOD was estimated as three times the S/N of the chromatogram and the LOQ, ten times this ratio.

TAGs and FA profiles

The TAGs composition in percentage of the different olive oil samples were determined using an internal quantification standard (SSS) and applying equations 1.2 and 1.3.

Results and discussion

TAGs identification

The identification of TAGs present in olive oil was obtained using TAGs standards, and also, applying two methodologies: (i) adding TAGs standard into test sample solution; and (ii) making the comparison of chromatographic retention times obtained from standard solution with sample solution.

Once all the peaks were identified by standards addition, the chromatograms were compared with a reference chromatogram published in official documents by the International Olive Council (IOC). As it is reported in the IOC document, olive oil chromatogram was obtained by applying an RP-HPLC-RID method (COI/T.20/Doc. No. 20/Rev.2 2008), olive oil sample was analyzed using a concentration of 5 % (50 mg/mL) and an injection volume of 10 μl. On the other hand, the proposed method, using the HPLC-CAD methodology, was carried out with a sample concentration of 250 μg/g and an injection volume of 4 μl.

Thirteen major TAGs (LLL, LLO, LLP, OOL, LLS, POL, OOO, OOP, PPS, PPO, OOS, SLS, and PSO) were identified. TAGs that do not exceed 1 % of total TAGs concentration in olive oil (Adrikopoulos 2002) were those not recognized. Although there are six TAGs (OOL+LLS, PPS+PPO, OOS+SLS) overlapped, the number of TAGs that were identified, are similar to those identified using others methodologies, such as MALDI-TOF/MS (Chapagain and Weisman 2009), HPLC-IR (Kiritsakis et al. 2002) and GC (Rezanka and Rezanková 1999).

The chromatogram obtained by RP-HPLC-CAD (Figure 1) presents the same TAGs profile and the same ECN array than the chromatogram reported by IOC (COI/T.20/Doc. No. 20/Rev.2 2008). The IOC methodology presents the same three sets of TAGs overlapped as Figure 1. This fact is due to the two methods use the same chromatographic column. Therefore, it is not possible to make an actual individual quantification of them. However, with the proposed method the chromatogram is obtained in only 40 minutes as opposed to the 80 minutes use in the official method. The RP-HPLC-CAD methodology presents

lower analysis time and better sensitivity than the IOC methodology. The better sensitivity is shown in the use of lower concentrations. This aspect is important when the aim is to analyze fat extracts of foods which contain small quantities of oil. Therefore, the proposed method can be used as an alternative to routine methods in the determination of the content of TAGs in olive oil. Even though the price of the CAD could be somewhat higher than RID, around 30%, but the CAD has advantages of lower cost of operation, because it needs less analysis time by using a gradient. The solvent used could be 40-50% less than the solvent consume by a method which used RID.

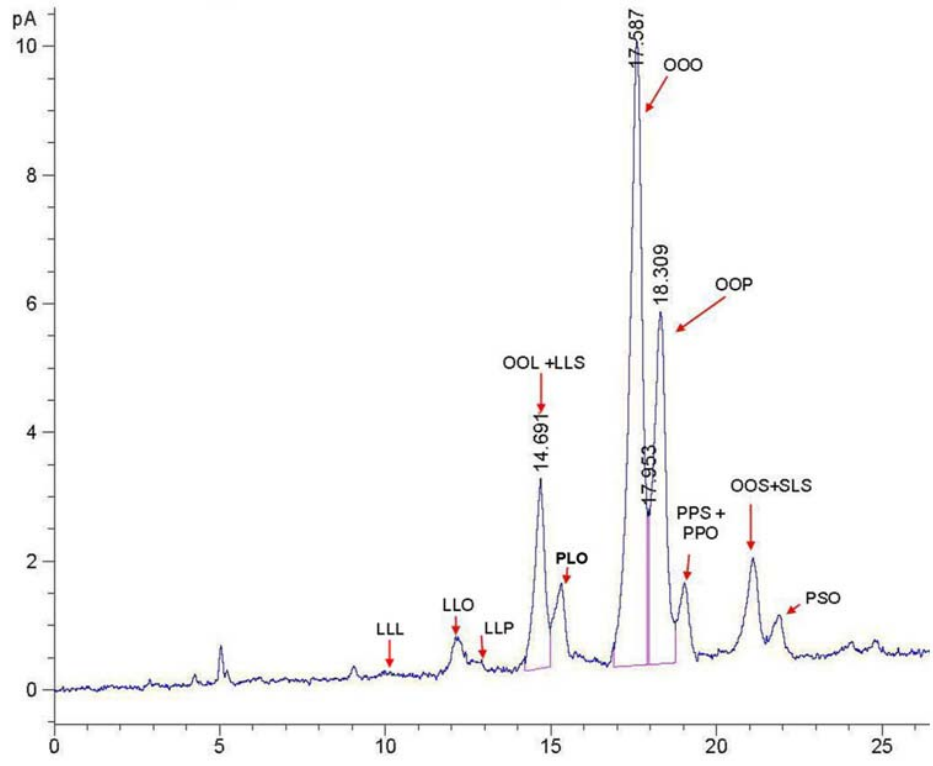

Figure 1. Olive oil chromatogram using HPLC-CAD method. Thirteen major TAGs of olive oil.

Initial validation (or pre-validation)

An internal calibration was performed from only one calibrant (one-standard internal calibration) when three conditions are fulfilled: (i) the blank signal (intercept of the calibration function) must be null in the interval of analyte amount ranged from the standard value to zero; (ii) the calibration function must be linear in the interval previously referred to; and (iii) the presence of sample matrix does not affect the RF value of the TAGs. These basic assumptions were tested for a proper application of the proposed quantification methodology.

Intercept and linearity

For this, calibration curves were established for four single TAG standards. RP-HPLC methodology operates on the principle of chain length and number of unsaturation of fatty acids, considering this principle, the selection of the TAGs was based on TAGs commodity groups and also in their percentage found in olive oils. Commodity groups are formed by their equivalent carbon number (ECN). The TAGs selected, in order to their ECN covering all the ECN range existing in vegetable oils, were LLL (ECN 42), OOL (ECN 46), OOP (ECN 48), and SSS (ECN 54). The concentration range used was 25-75 µg/g, this range was selected according to the maximum amount of TAGs found in olive oil (20%) (Andrikopoulos 2002). Fifteen calibration points (five replicates of 3 concentrations levels) were obtained for each TAG.

All the calibration curves were performed from two types of CAD response for each corresponding analyte concentration, peak area and peak height.

Non-significant intercept was tested, which means, that the zero must be included in the range $a \pm t\ sa$ (or $a \leq t\ sa$), where a is the intercept, sa is the intercept standard deviation, and t is the corresponding two-side student-t.

Linearity of the method was evaluated for the four TAGs standards. In order to establish if the calibration curves are equivalent, a statistic linearity test was used. Linearity test used % relative standard deviation (RSD), which must be lower than 10% (RSDb ≤ 0.10).

The results of calibration curves for peak area were linear in the studied range; however, the height response shows better results in tests, linearity and intercepts (Table 1). Then, only the height response was used in the other tests and applications.

Table 1. Calibration curve features for initial validation (a intercept; b slope; s_a standard deviation of intercept; s_b standard deviation of slope; $\%RSD_b$ relative standard deviation of slope; R^2 determination coefficient)

		SSS	LLL	OOL	OOP
Areas	a	7.4532	24.5351	24.77	5.02
	s_a	2.44917	12.07	1.47	22.35
	b	3.25	2.34	2.04	3.07
	s_b	0.045	0.22	0.03	0.41
	% RSD_b	1.38	9.39	1.47	13.35
	R^2	0.999	0.99	0.999	0.982
Heights	a	0.31	0.95	0.88	0.47
	s_a	0.16	0.46	0.08	0.69
	b	0.12	0.12	0.08	0.098
	s_b	0.003	0.008	0.001	0.009
	% RSD_b	2.5	6.66	1.25	9.18
	R^2	0.999	0.993	0.999	0.985

Due that the intercept was considered non-significant (p-values > 20% in all cases), response factors (RFs) were assigned from slope of each curve. The average, SDRF and RSDRF also were calculated for the four TAGs.

To check the linearity of calibration of this data, a test was determined by comparing RSDRF to a limit specified, RSDRF ≤ 0.10 (United States Environmental Protection Agency 2007). . Then, if the RSDRF fulfils with the criterion, linearity is assumed and permit to use an average RF or the response factor of some of the four TAGs evaluated, for example the corresponding to SSS. Since the RSDRF is 0.09 (Table 2) the criterion was accomplished

The response factors of the TAGs selected, containing between 16-18 atoms, show a variation lower than 10%, then, no mobile phase compensation were used in order to propose a simpler method. In addition, these calibration curves do not required plotting on logarithmic coordinates to obtain linearity as in a precedent paper (Górecki et al. 2006).

Matrix effect

A one-standard addition calibration was used in order to evaluate the absence of matrix effect. As it has been previously stated, for quantification purposes, this type of calibration can be used due to the fact that standard calibration functions, of the selected TAGs, resulted linear and also the intercept was non-significant (Cuadros-Rodríguez et al. 2007).

The absence of matrix effect was demonstrated by comparing the RF average of the TAGs, obtained in the linearity test (external RF), with the RF average of the added TAG standards into oil sample, obtained by the one-standard addition calibration (matrix RF). RFs were calculated using the following equation:

$$\text{matrix RF} = \frac{y_{TAG(oil+TAG)} - y_{TAG(oil)}}{x_{TAG}} \quad (1.4)$$

where yTAG(oil+TAG) and yTAG(oil) are the TAG heights in the added and non-added sample; and xTAG is the added standard TAG amount. The TAGs added in the oil sample were the same than in the linear test (LLL, OOL, OOP, SSS), 25 µg/g of each TAG was added to a 250 µg/g extra virgin olive oil sample, each TAG was analyzed separately.

The similarity of both RFs showed the absence of significant matrix effect, the average external RFs from TAGs in solvent is 0.11 and average matrix RFs of TAGs in presence of oil is 0.12 (Table 2).

Table 2. Both external and matrix RFs values for initial validation

TAGs	External RF	Matrix RF
SSS	0.125	0.132
LLL	0.124	0.123
OOL	0.1	0.126
OOP	0.102	0.115
Average	0.11	0.124
SD_{RF}	0.01	0.007
% RSD_{RF}	9.1	5.7

The LOD and LOQ obtained from de S/N using the matrix RF were 0.054 µg/g and 0.18 µg/g, respectively.

Accuracy (Precision and Trueness) validation

Accuracy indicates an existence of agreement between the assay result and an accepted value as reference. Accuracy must be expressed in terms of precision and trueness (ISO 5725-1 1998). The accuracy of the method was

evaluated in repeatability conditions, using twenty olive oil injections of working samples, divided in two lots of ten injections each, one for precision and the other for trueness. The quantification was carried out using the RF of an internal standard (SSS) for each chromatogram.

Trueness essay was prepared using three solutions of 250 µg/g of extra virgin olive oil containing three standards with concentrations of 25 µg/g : i) OOL and OOP, selected because of their abundance in olive oil, their ECN (46 and 48 respectively) and for their good peak resolution in the chromatogram (Figure 1); and ii) SSS as internal standard. For the trueness of the method the first solution was injected four times and the other two solutions, three times each. The trueness was evaluated for a concentration of 25 µg/g of both standards and expressed as recovery. OOL and OOP presented a recovery of 105.44% and 90.67% respectively.

Precision essay was prepared using three extra virgin olive oils with concentration of 250 µg/g and an internal standard (SSS) with a concentration of 25 µg/g. As trueness essay, the first solution was injected four times and the other two solutions, three times each. The precision of the method was expressed as %RSD. The results show that in all cases RSD was lower than 5% except for LLP and PSO that was lower than 8%.

Therefore, it can be concluded that quantification of TAGs in olive oil is possible using an internal RF; in this case, an RF from the internal standard SSS was used.

Application study: TAGs profile and FA profile

TAGs profile

A study of fat profile of three olive oil samples (extra virgin A and B, and virgin) and the fat extracted of food products (bread snacks, potato chips and

cold meat) was obtained from the quantification of the TAGs using an internal quantification standard (SSS) and applying equations 1.2 and 1.3. All the results are inside the ranges found in literature (Andrikopoulos 2002). Table 3 shows the TAGs composition in percentage of the different olive oil and food products samples.

Table 3. Triacylglycerols (TAGs) molecular composition (wt%) of various olive oils analyzed using the HPLC-CAD system and calibrations described in this study. PF potato chips; BS bread snacks; CM cold meat; EV extra virgin olive oil; V virgin olive oil. ***Values significantly different*** (see text)

	PF1	PF2	BS1	BS2	BS3	BS4	BS5	BS6
LLL	1.32	***3.01***	2.83	***7.24***	1.51	2.37	***14.72***	2.01
LLO	3.37	***6.51***	4.27	***6.57***	2.95	3.39	***16.73***	3.64
LLP	1.44	2.35	3.65	***5.72***	1.37	3.04	***7.15***	2.76
OOL+LLS	11.88	12.75	9.88	10.76	11.16	10.21	11.78	11.53
POL	6.02	5.86	6.71	6.02	4.85	5.19	5.67	5.35
OOO	40.58	38.66	38.38	35.47	43.68	42.57	***28.83***	41.17
OOP	23.58	20.77	23.75	19.37	21.54	22.52	***9.83***	22.43
PPS+PPO	3.92	3	3.66	2.62	4.4	3.33	***0.88***	3.75
OOS+SLS	7.04	6.43	5.69	5.11	7.6	6.43	***3.99***	6.44
PSO	0.87	0.67	1.2	1.13	0.94	0.95	0.44	0.92
	BS7	BS8	BS9	CM1	CM2	EV(A)	EV(B)	V
LLL	1.42	1.58	2.8	0.22	1.01	~0	~0	~0
LLO	3.25	3.62	4.22	0.88	2.36	1.05	0.95	1.48
LLP	2.03	1.49	3.19	0.44	1.41	1.26	1.95	1.48
OOL+LLS	10.7	11.54	11.97	9.63	8.67	11.2	9.86	12.4
POL	5.42	3.36	5.53	7.23	7.57	4.43	3.85	5.52
OOO	42.07	44.77	39.91	39.76	35.32	46.7	48.2	42.2
OOP	23.19	22.18	21.82	25.33	25.53	22.8	23.6	23.5
PPS+PPO	3.74	2.93	3.77	4.46	4.83	2.93	2.89	4.59
OOS+SLS	6.53	6.86	5.84	7.01	6.97	8.15	8.18	6.34
PSO	1.62	1.65	0.94	***5.05***	***6.34***	1.62	1.49	2.45

Some TAGs values increased significantly (italics and bold in Table 3), maybe, due to the presence of other fat. In the case of samples PF2 and BS2, the difference in LLL, LLO and LLP could be cause by the addition of vegetable oil, owing to the fact that these TAGs correspond to vegetable oils. Cold meats

samples present high values in the TAG PSO, possible due to the meat's own fat.

Instead the sample BS5 shows clearly that does not contain olive oil. This product stated on its label that contains only olive oil as fat but the results did not verify this fact.

FA profile

From the TAG compositional profile, an estimate of compositional profiles of the majority FA could be obtained. These profiles can be calculated by summarizing all the FA percentage found in each TAG. In olive oils, FA exist as free FA and also as bonded FA like mono, di and triglycerides, phosphatides, waxes and esters of sterols (Boskou 1998). Therefore, this methodology is only valid for virgin (extra) olive oils because, the only significant source of FA is bonded compounds, TAGs.

Table 4 shows the percentage of the FA for three olive oils. The validity of this methodology has been able to check using one sample of extra virgin olive oil, which was analyzed by the official Food Quality Control Laboratory Atarfe, Granada. This analysis was developed using the official method of IOC, GC-FID of fatty acid methyl ester (FAMES) of olive oil (COI/T.20/Doc. No. 24 2001). A comparison was made with the results of FAs obtained using the methodology proposed. Results are given in Table 4, showing that the differences between the two values are around 2 percentage points.

A nutritional fat profile can be obtained using majority FA compositional profiles. The nutritional profile is given by unsaturated FA, polyunsaturated fatty acid (PUFA), monounsaturated fatty acid (MUFA), and by saturated FA (Table 5).

Table 4. Mayor Fatty Acid (FA) composition (wt%) of various olive oils analyzed using the HPLC-CAD system and calibrations described in this study, calculated based on fatty acids in TAG molecular species. Number of samples, n = 3
L linoleic; O oleic; P palmitic; S stearic acids.
[a] Results obtained from FAMEs using GC-FID.

	L	O	P	S
Extra Virgin (A)	6.71	78.47	11.56	3.26
Extra Virgin (B)	5.82	79.39	11.58	3.22
Virgin	7.98	75.57	13.56	2.95
Atarfe Lab. Values[a]	5.58	77.72	10.8	3.46
Experimental values[b]	8.13	76.87	11.68	3.31

Table 5. FAs nutritional composition (%) of different olive oil samples

FA		Extra Virgin (A)	Extra Virgin (B)	Virgin	Atarfe Lab. values	Experimental Values
	PUFA	6.71	5.82	7.98	6.24	8.13
Unsaturated	MUFA	78.47	79.39	75.57	78.91	76.87
Saturated		14.82	14.81	16.51	14.84	14.99

Conclusions

An HPLC method is proposed for the characterization and quantification of TAGs in olive oil using a CAD detector. The proposed HPLC-CAD method presents two advantages: lower analysis time and better sensitivity, in comparison with the official method adopted by European Commission for the identification of TAGs in olive oil. No mobile phase compensation was necessary because response factors of the TAGs selected show a variation lower than 10 %.

Calibrations curves for three TGs present in olive oil were established using SSS as internal standard and response factors (RFs) were assigned from slope of each curve. Due to the absence of matrix effect and that the linearity

can be assumed, the quantification of the TAGs can be estimated using an average response factor or the response factor corresponding to SSS.

The developed method was applied for the quantification of TAGs in three different olive oils and thirteen food products. Good precision and excellent recoveries were achieved. The TAGs compositional profiles obtained of the olive oils are in concordance with those found in literature. In addition, for nutritional information purposes, fatty acid compositional profile can be calculated from obtained TAGs profiles using a simple and fast chromatographic analysis.

List of Symbols and Acronyms

CAD charged aerosol detector

CN carbon number

DAG diacylglycerol

ECN equivalent carbon number

ELSD evaporative light scattering detector

FA fatty acid

HPLC high permance liquid chromatography

IC internal calibration

IOC International Olive Council

IS internal standard

LLL trilinolein

LLO 1,2-linolein-3-olein

LLP 1,2-linolein-3-palmitin

LLS 1,2-linolein-3-stearin

LOD limit of detection

LOQ limit of quantitation

MUFA monounsaturated fatty acid

OOL 1,2-palmitin-3-linolein

OOO triolein

OOP 1,2-olein-3-palmitin

OOS 1,2-olein-3-stearin

POL 1-palmitin-2-olein-3-linolein

PPL 1,2-palmitin-3-linolein

PPO 1,2-palmitin-3-olein

PPP tripalmitin

PPS 1,2-palmitin-3-stearin

PSO 1-palmitin-2-stearin-3-olein

PTFE polytetrafluoroethylene

PUFA polyunsaturated fatty acid

RF response factor

RF response factor

RID refractive index detector

RP reverse phase

RSD relative standard deviation

S/N signal/noise ratio

SD standard deviation

SLS 1,3-stearin-2-linolein

SSO 1,2-stearin-3-olein

SSS tristearin

TAG triacylglycerol

UV-Vis ultraviolet-visible spectroscopy

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References

Amaral JS, Cunha SC, Rui Alves M, Pereira JA, Seabra RM, Oliveira PP (2004) J Agr Food Chem 52:7964-7969

AMS-FV-08-0073-0006 (2010) United States Standards for Grades of Olive Oil and Olive-Pomace Oil. U. S. Department of Agriculture, Washington.

Andrikopoulos NK (2002) Food Rev Int 18:71-102

Amstrong D. (2009), Lipidomics Volume 1: Methods and Protocols, Human Press, USA,

B.P. Chapagain, Z. Weisman, J. Agric. Food Chem., 57 (2009) 1135-1142.

Boskou D (1998) Química y Tecnología del Aceite de Oliva, 1ª ed., AMV Ediciones, Madrid, España.

Buchgraber M, Ulberth F, Emons H, Anklam E (2004) Eur J Lipid Sci Tech 106:621-648

COI/ T.20/ Doc. nº 20/ Rev.2 (2008) Determination of the difference between actual and theoretical content of triacyglycerols with ECN 42, International Olive Council, Madrid

COI/ T.20/ Doc. nº 24/ (2001)Preparación de los ésteres metílicos de los ácidos grasos del aceite de oliva y el aceite de orujo de oliva, International Olive Council, Madrid

Commission Regulation (EC) No 2472/97 of 11 December 1997 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on their methods of analysis, Annex XVIII. Off. J. Eur. Commun. L341: 25-39

Cuadros-Rodríguez L, Bagur-González MG, Sánchez-Viñas M, González-Casado A, Gómez-Sáez A (2007) J Chromatogr A 1158:33-46

De la Mata-Espinosa P, Bosque-Sendra JM, Bro R, Cuadros-Rodríguez L (2011) Anal Bioanal Chem DOI XXX

Cunha SC, Oliveira MBPP (2006) Food Chem 95:518-524

Górecki T, Lynen F, Szucs R, Sandra P (2006) Anal Chem 78:3186-3192

Heron S, Maloumbi MG, Silou T, Verette E, Dreux M, Tchapla A (2010) Food Anal. Methods 3:67-74

Holcapek M, Lisa M, Jandera P, Kabatova N (2005) J Sep Sci 28:1315-1333

ISO 5725-1 (1998) Accuracy (truenes and precision) of measurement methods and results – Part 1: General principles and definitions. ISO, Geneva

Jakab A, Jablonkai I, Forgács E (2003) Rapid Comm Mass Spec 17: 2295-2302

Kiritsakis A, Kanavouras A, Kiritsakis K (2002) Eur J Lipid Sci Technol 104: 628-638

Lee DS, Lee ES, Kim HJ, Kim SO, Kim K (2001) Anal Chim Acta 429: 321-330

Lísa M, Lynen F, Holčapek M, Sandra P (2007) J Chromatogr A, 1176:135-142

Moreda W, Pérez-Camino MC, Cert A (2003) Grasas Aceites 54:175-179

Moreu RA (2006) Lipids AOCS 41:727-734

Mottram HR, Woodbury SE, Evershed RP (1997) Rapid Comm Mass Spec 11:1240-1252

Neff WE, List GR, Byrdwell WC (1999) J Liq Chromatogr RT 22:1649-166

Osorio Bueno E, Sánchez Casas JJ, Martínez Cano M, Montaño García AM (2003) Grasas Aceites 54:1-6.

Rezanka T, Rezanková H (1999) Anal Chim Acta 398:253-261

Ruiz-Gutiérrez V, Barron LJR (1995) J Chromatogr B 671:133-168

Ruiz-Samblás C, Cuadros-Rodríguez L, González- Casado A (2010) Talanta 83: 25-30

United States Environmental Protection Agency (March 2007) Solutions to Analytical Chemistry Problems with Clean Water Act Methods, EPA, Washington USA

Vehovec T, Obreza A (2010) J Chromatogr A 1217:1549-1556

2.5 Resultados no publicados:

Aplicación del método HPLC-CAD para la caracterización de aceite de oliva en alimentos (embutidos, patatas fritas y productos de panadería)

La caracterización de grasa extraída de alimentos se realizó con objeto de identificar la presencia de aceite de oliva en alimentos tales como, embutidos, patatas fritas y productos de panadería. Como paso previo, se realizó una separación de la materia grasa de dichos productos por medio de un proceso de extracción con líquidos presurizados (Pressurized Liquid Extraction, PLE) y posteriormente se realizó la caracterización por medio de perfiles de TGs utilizando el método HPLC-CAD, ya descrito.

La técnica PLE está basado en la utilización de disolventes orgánicos a alta presión (500-3000 psi) y temperatura (hasta 200ºC), pero manteniendo condiciones subcríticas. La base del funcionamiento de este tipo de extracción es la siguiente: el incremento de temperatura reduce la viscosidad del disolvente y su tensión superficial, además de favorecer la penetración del mismo en la matriz de la muestra, acelerando la cinética de extracción. El empleo de presión elevada mantiene el disolvente en estado líquido, favoreciendo la interacción del disolvente con la matriz de la muestra y permitiendo una extracción rápida y segura. La extracción PLE presenta numerosas ventajas respecto a métodos similares de extracción sólido-líquido, en cuanto a tiempo, cantidad disolvente necesario, automatización, eficacia etc. [8].

Las extracciones se llevaron a cabo en un extractor con líquidos presurizados ASE 100 (Dionex), usando celdas de acero inoxidable de 34 mL. Este equipo había sido

[8] Carabias-Martínez R., Rodríguez-Gonzalo E., Revilla-Ruiz P., Hernández-Méndez J. (2005) Pressurized liquid extraction in the analysis of food and biological samples, J. Chrom. A 1089: 1-17

validado previamente para la extracción de grasa de muestras sólidas, utilizando un procedimiento interno, para asegurar la calidad de los resultados. [9].

El procedimiento operatorio fue el siguiente: para las medidas de peso se utilizó una balanza analítica calibrada. Para la eliminación del disolvente tras la extracción se utilizó un rotavapor RE-124 (Büchi) equipado con una bomba de vacío V-700 (Büchi). Para realizar la homogeneización previa de la muestra se utilizó un molinillo eléctrico (Taurus). Los disolventes utilizados para la extracción (hexano y propanol) fueron suministrados por PANREAC (calidad analítica). Como diluyente sólido inerte se utilizó tierra de diatomeas suministrada por Dionex y sulfato sódico anhidro suministrado por PANREAC. El agua desionizada fue obtenida de un sistema de purificación (Milli-Q; Millipore), y el nitrógeno 99,9 % de pureza (Air Liquide).

Para el análisis de las muestras se pesaron entre 3-4 gramos de muestra sobre una porción de papel de filtro, las muestras son molidas con el objetivo de reducir el tamaño de partícula e incrementar la superficie de contacto con el disolvente extractor. Adicionalmente, se pesan 6 gramos de diluyente sólido inerte, tierra de diatomeas, que se mezclan con la muestra previamente homogeneizada. La mezcla de ambos fueron colocados en la celda de extracción de 34 mL del ASE. Las condiciones óptimas que se utilizaron para la extracción fueron: temperatura de 175 °C, periodo de calentamiento de 5 min, tiempo estático de extracción de 5 min con un ciclo estático por muestra, volumen de disolvente 40 % y tiempo de purga de 100 s. La presión de trabajo fue de 1.500 psi (100 bar). Se eliminó el disolvente por evaporación utilizando un rotavapor. El matraz fue pesado para determinar la masa de grasa extraída.

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[9] Guerrero García C., De la Mata Espinosa P., Ruiz Samblás C., Cuadros Rodríguez L., Validación de un método de extracción con líquidos presurizados y cuantificación del contenido de grasa total en patatas fritas envasadas y en embutidos, Libro de comunicaciones V Congreso Virtual Iberoamericano sobre Gestión de Calidad en Laboratorios, internet 2009, NIPO 770-09-264-6

Los extractos fueron estabilizados por adición de BHA (hidroxibutilanisol) y diluidos con hexano hasta una concentración del 10%. La disolución resultante fue almacenada en un congelador, en la oscuridad, a una temperatura controlada de −20 ºC.

Las muestras utilizadas fueron cuatro embutidos (salchichón y chorizo), dos con aceite de oliva como materia grasa principal y dos con otro tipo de grasas. 3 tipos de marcas comerciales de patatas fritas en aceite de oliva y 13 productos de panadería (regañas, piquitos, rosquillas, entre otros).

Los cromatogramas obtenidos de todas las muestras se compararon con un cromatograma de aceite de oliva, lo que permitió identificar sus picos cromatográficos. Como ejemplo de los resultados obtenidos para los embutidos, en la Figura 2.6A se muestra el cromatograma del salchichón con aceite de oliva. Su comparación con el cromatograma correspondiente de un salchichón sin aceite de oliva (FIg. 2.6B), mostró picos cromatográficos diferentes entre estos dos cromatogramas. Sin embargo, al ser comparado con un cromatograma de aceite de oliva (Fig. 2.6C) presentó el mismo perfil de triglicéridos. Estos resultados fueron similares para las otras muestras de embutidos.

En la Figura 2.7 se observa un ejemplo de los resultados obtenidos para las muestras de patatas fritas y panadería. Estas muestras se compararon con un perfil de triglicéridos de aceite de oliva. La Figura 2.7A muestra el cromatograma para una muestra de patatas fritas con aceite de oliva y la Figura 2.7B la de panadería. Se puede observar que muestran el mismo perfil cromatográfico que un aceite de oliva (Figura 2.7C).

Los resultados de esta aplicación fueron presentados en una comunicación en el XIV simposium cinetífico-técnico EXPOLIVA 2009 [10].

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[10] De la Mata Espinosa P., Guerrero García C., Ruiz Samblás C., González Casado A., Cuadros Rodríguez L., Extracción (con líquidos presurizados) y caracterización de la fracción grasa de embutidos que contienen aceite de

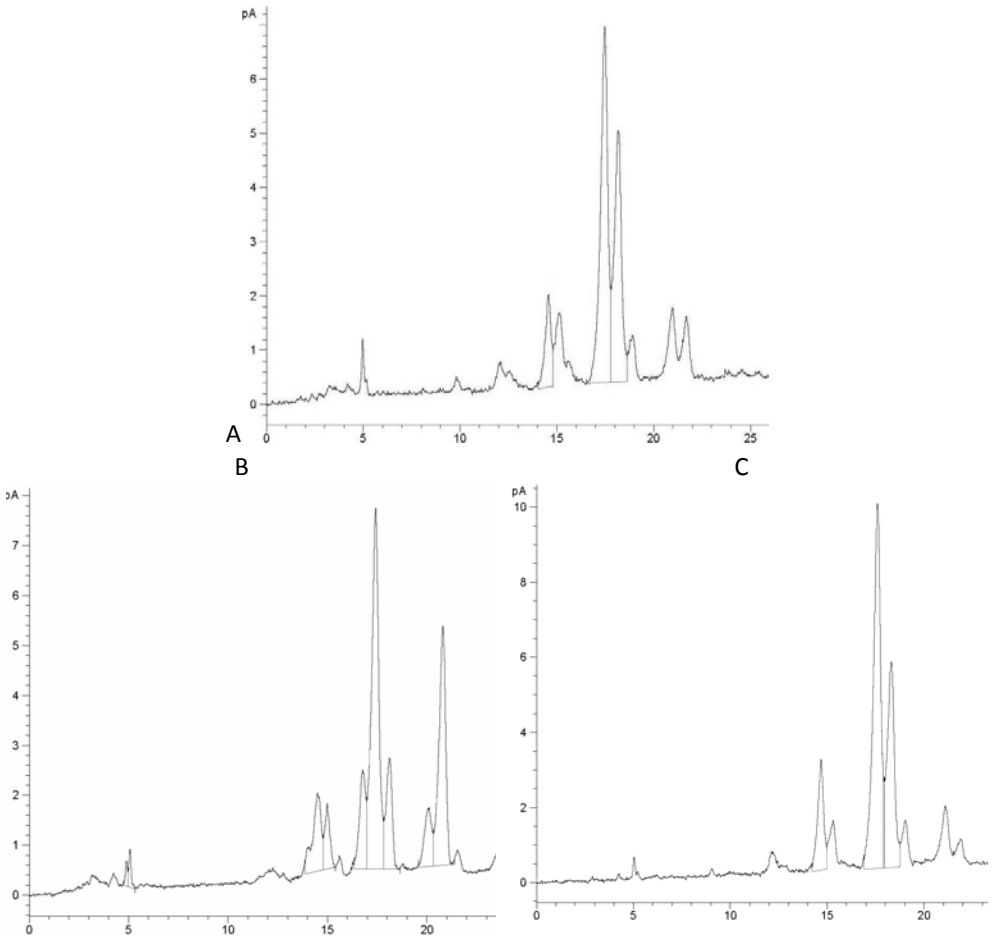

Fig 2.6 (A): Cromatograma de salchichón con aceite de oliva; (B): Cromatograma de salchichón sin aceite de oliva; (C) Cromatograma de aceite de oliva

2.6 Discusión

El método propuesto para obtener los perfiles de triglicéridos utilizando con el detector CAD proporciona mejor sensibilidad, así como una disminución considerable en los tiempos totales de análisis. El cromatograma de aceite de oliva presentado por el COI necesita por lo menos 80 minutos para obtener una separación de los triglicéridos,

oliva, Actas del XIV Simposium Científico-Técnico del aceite de oliva. Expoliva 2009 IND-17 1-6, Jaén (2009)

mientras que el método propuesto, consigue una separación similar y perfiles iguales en tan solo 25 minutos. Asimismo, la concentración de la muestra de aceite utilizada en los métodos del COI y la IUPAC es del 5%, mientras que en el método que utiliza el CAD como detector es de 250 ppm.

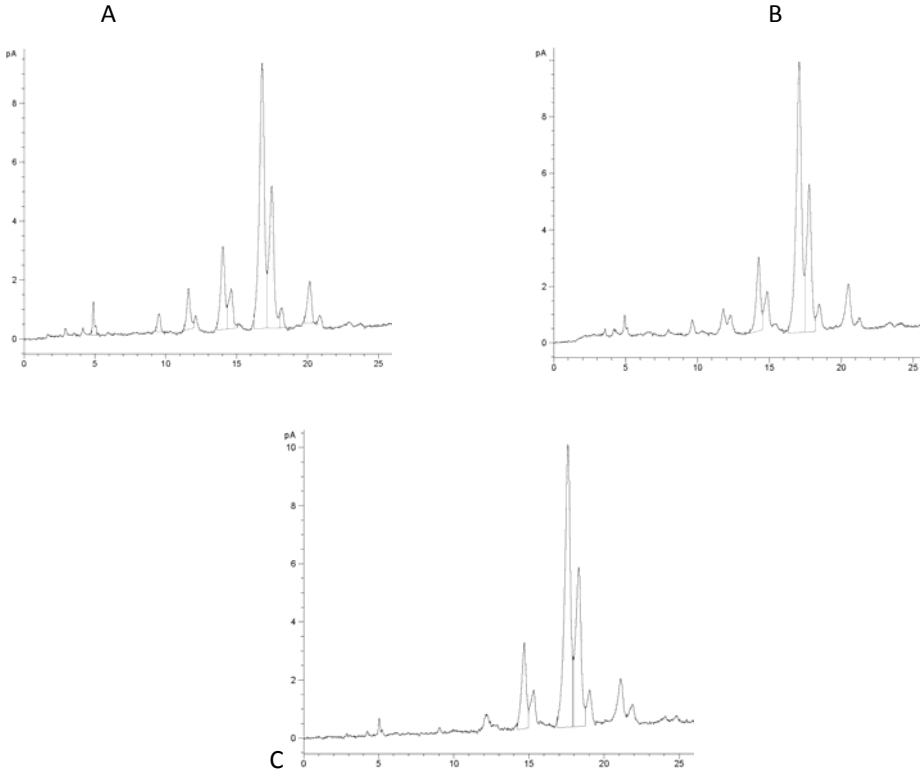

Figura 2.7 Cromatogramas de aceite de oliva. (A): Cromatograma de patatas fritas con aceite de oliva; (B): Cromatograma de un producto de panadería con aceite de oliva; (C) Cromatograma de aceite de oliva

Los factores de respuesta de los triglicéridos obtenidos utilizando el CAD son prácticamente iguales a la unidad e iguales entre sí, e independientes de la concentración, por lo que el perfil composicional se puede obtener directamente de los valores medidos de altura de pico. El único inconveniente que presenta el CAD, similar al de

otros detectores convencionales en la identificación de triglicéridos, es que no permite diferenciar isómeros con el mismo valor de ECN, y que siempre coeluyen juntos.

Se obtuvieron resultados satisfactorios en los estudios para la caracterización de los diferentes alimentos, embutidos, patatas fritas y productos de panadería, a partir del perfil de triglicéridos. Utilizando dicho perfil, se pudo identificar la presencia de aceite de oliva en el extracto graso de alimentos que lo contienen como ingrediente.

A partir de los buenos resultados obtenidos con el método HPLC-CAD, se planteó realizar nuevas aplicaciones: la clasificación y discriminación entre aceites de oliva y aceites vegetales, así como la cuantificación del contenido de aceite de oliva en mezclas con diferentes aceites vegetales. En ambos casos, se pretende tener en cuenta la variabilidad que aportan las diferentes categorías y variedades del aceite de oliva. Los siguientes capítulos de la tesis se enfocan a estos objetivos utilizando técnicas quimiométricas como herramientas de apoyo.

CAPÍTULO 3

DISCRIMINACIÓN Y CLASIFICACIÓN DE ACEITES VEGETALES Y ACEITE DE OLIVA UTILIZANDO HPLC-CAD Y TÉCNICAS QUIMIOMÉTRICAS

Bacalao confitado

Ingredientes para 4 personas:

- 1 Kilo de lomos de bacalao con su piel
- 2 cabezas de ajos
- Aceite de oliva

Preparación

Se pone a desalar el bacalao la noche anterior y se le procura cambiar el agua dos o tres veces durante el proceso.

Se pone abundante aceite y se fríen los ajos enteros, pelados y con una pequeña raja solamente.

A continuación se reservan los ajos y se añaden los lomos de bacalao con la piel hacia abajo. Antes hemos dejado que se temple el aceite (hasta que casi podamos meter el dedo sin abrasarse).

El Aceite de oliva ha de cubrir totalmente el bacalao, que se hará a fuego muy lento (para los que tengan instrumentos sofisticados, a unos 80º) durante unos veinte minutos.

Seguidamente se le quita el Aceite de oliva dejando muy poco en el cazo en el que hemos confitado el bacalao y se va rotando la sartén muy despacio para que el pescado vaya soltando la gelatina. Se añade poco a poco Aceite de oliva como si de una mayonesa se tratara y cuando vemos que ya está la gelatina, se puede servir.

El plato es delicado de hacer por el toque final de la gelatina (que no es imprescindible) y porque si nos pasamos guisando el pescado el bacalao nos quedará seco y no muy atractivo, pero una vez cogido el punto, es muy fácil y muy rico.

3.1 Presentación

Los resultados obtenidos por el método HPLC-CAD, descrito en el capitulo anterior, abrieron la posibilidad de nuevas opciones de aplicación de esta técnica. En este tercer capítulo se presentan los estudios realizados para lograr la identificación y diferenciación entre aceites de oliva y diferentes tipos de aceites vegetales utilizando los perfiles cromatográficos de los triglicéridos junto con distintas técnicas quimiométricas.

Para realizar este estudio, se establecieron los perfiles cromatográficos de triglicéridos de 126 muestras de diferentes categorías y variedades de aceites de oliva y de distintos aceites vegetales comestibles, entre ellos se incluyeron aceites de maíz, girasol, cacahuete, soja, colza, canola, sésamo, uva, además de varias mezclas comerciales de los citados aceites. Los cromatogramas obtenidos, previamente pretratados, fueron analizados con varias herramientas quimiométricas, tales como: análisis de componentes principales (PCA), análisis discriminante por mínimos cuadrados parciales (PLS-DA) y resolución de curvas multivariantes (MCR).

3.1.1. Fundamentos teóricos

3.1.1.1 Análisis de Componentes Principales

El análisis de componentes principales es una herramienta quimiométrica que permite buscar posibles modelos en los puntos experimentales a través de unas nuevas coordenadas espaciales con mejor visión del problema. Estos nuevos ejes de coordenadas, denominados “componentes principales” (PC) son combinaciones lineales de las variables manifiestas y describe las principales tendencias de

los datos [1]. Es decir, trata de definir, a partir de un conjunto inicial de variables correlacionadas otro conjunto de variables no correlacionadas, obtenidos por combinación lineal de las variables originales, de forma que, reproduciendo la estructura de varianza/covarianza de las variables originales, las primeras componentes sean aquellas que explican la mayor variabilidad del problema. Así, aunque se obtienen tantas nuevas variables como variables originales existen, es posible reducir el número de ellas sin pérdida de información [2,3].

Si **X** es una matriz de datos con *m* filas y *n* columnas, las variables son las columnas y las muestras las filas, PCA descompone X como la suma de *r* **ti y pi**, donde *r* es el rango de la matriz **X**:

$$X = tipT1 + t2pT2 + \cdots + tkpTk + \cdots + trpTr \qquad 3.1$$

r debe de ser menor o igual a la menor dimensión de **X**. Los pares **ti y pi** están ordenados por la cantidad de varianza explicada. Los vectores **ti** son conocidos como scores o puntuaciones, contienen información de cómo las muestras se relacionan entre ellas. Los vectores **pi** son conocidos como pesos (loadings) y contienen información de cómo las variables manifiestas se relacionan entre ellas y con las componentes principales. Usualmente si los modelos de PCA describen bien la tendencia de los datos con un número k de componentes, la matriz residual E con-

[1] Wise BM, Gallagher NB, Bro R, Shaver JM, Windig W, Koch RS, PLS_Toolbox 4.0 Manual for use with MATLAB™, Eigenvector Research, Inc. Wenatchee, 2006

[2] Mongay Fernaández Carlos, Quimiometría, Universidad de Valencia, Valencia, 2005

[3] Miller J.N., Miller J C., Estadística y Quimiometría para Química Analítica, 4ed, Prentice Hall, España 2002

tendrá una pequeña cantidad de la varianza restante no explicada por los componentes.

$$X = t1pT1 + t2pT2 + \cdots + tkpTK + E \qquad 3.2$$

En el modelo PCA se puede realizar una descomposición de vectores propios (eigenvectors) de la covarianza. Para una matriz **X** con *m* filas y *n* columnas, la covarianza de la matriz se define como:

$$cov(X) = \frac{X^T X}{m-1} \qquad 3.3$$

Suponiendo que las columnas de **X** han sido centradas substrayendo del valor original la media de cada columna y si las columnas han sido autoescaladas, dividiendo cada columna por su desviación estándar, es decir, ajustadas la media a cero y la varianza a uno, entonces la ecuación 3.1 proporciona la matriz de correlación de **X**. En la descomposición del PCA, los vectores pi son los vectores propios de la matriz de covarianza para cada pi:

$$cov(X)pi = \lambda ipi \qquad 3.4$$

Donde ***λi*** es el autovalor (eigenvalue) asociado al vector propio ***pi***. Para la forma ortogonal **ti** (***t****i*T***t****j* = 0 para *i* ≠*j*), cuando pi son ortonormales (***p****i*T***p****j* = 0 para *i* ≠*j*, ***p****i*T***p****j* = 1 para *i* =*j*)**:**

$$\boldsymbol{Xpi = ti} \qquad \mathbf{3.5}$$

ti son proyecciones de ***X*** en ***pi***. Los pares ***tipi*** son ordenados en orden descendente de acuerdo a su autovalor ***λi***, el cual mide la cantidad de varianza descrita por los pares ***tipi***. Es decir, el primer par es el que explica más varianza y así sucesivamente [1], estableciendo los componentes en orden a su significación, es decir, el primer componente principal es el de mayor autovalor del grupo de datos. Con esta información se puede decidir con cuantos componentes principales se va a trabajar, ya que los componentes con bajos autovalores, que explican poca varianza, se dejan

fuera. Con los componentes que se elijan se forma un nuevo vector, el cual se transpone y se multiplica por el vector original traspuesto [4]. En la Figura 3.1 se puede observar que los datos siguen un patrón. La componente uno es la que muestra la mayor variabilidad de los datos, mientras que el segundo nos da menos información.

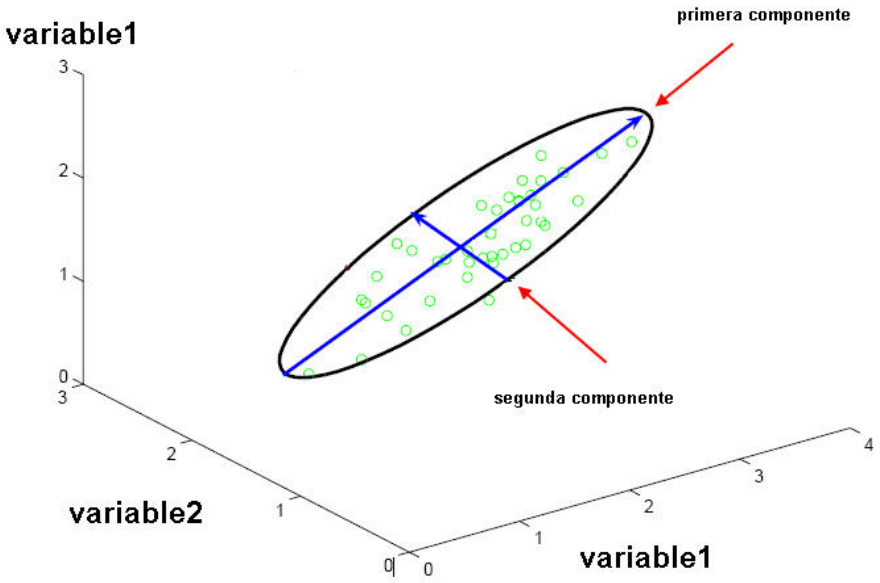

Fig3.1. Representación gráfica de un Análisis de Componentes Principales [1]

[4] Smith L.I (2002) A tutorial on Principal Components Analysis, University of Otago, Nueva Zelanda

3.1.1.2 Análisis Discriminante por Mínimos Cuadrados Parciales (PLS-DA)

El análisis discriminante por mínimos cuadrados parciales (PLS-DA) fue usado con el objetivo de obtener la mejor separación entre grupos y poder entender qué variables permiten dichas separación. Los resultados obtenidos son una categoría o clase, es decir predice la clase de cada muestra. El método PLS-DA encuentra la varianza entre las variables predictoras (datos-X) y la correlaciona con la varianza en las variables de respuesta (datos-Y)

Este método se aplica en dos pasos, en el primero, se aplica un modelo de regresión PLS (ver el Capítulo 4) donde la variable respuesta es una variable categórica, en realidad, un conjunto de variables ficticias que establecen las categorías de los objetos. En el segundo, con el análisis discriminante se realiza la clasificación de los objetos a partir del resultado de la regresión obtenida con el modelo de PLS. Esta clasificación se realiza con una matriz de clases (Y), es decir, un conjunto de variables ficticias que indica si las muestras pertenecen a una clase o a otra.

El análisis discriminante (DA) [3] es un método de reconocimiento de pautas supervisado, por tanto las clases a las que pueden pertenecer los objetos son conocidas. Existen dos tipos de variables, la variable dependiente que es categórica e indica las distintas categorías o clases en las que pueden clasificarse los objetos y las variables independientes que son continuas y determinan a qué clase o categoría, al menos deben existir dos, pueden pertenecer los objetos. Mediante DA se pretende establecer las relaciones lineales entre las variables continuas que mejor discriminen en las categorías existentes los objetos. También se busca construir una regla de decisión (variable discriminante, una por cada dos categorías existentes) que permita asignar un objeto nuevo, no clasificado previamente, a una de las categorías prefijadas con un cierto grado de confianza. Para el caso de dos categorías, la variable discriminante lineal sería:

$$Y = a_1 + X_1 + a_2 + X_2 + \cdots + a_n X_n \qquad 3.6$$

Con las dos categorías que se asignan a una serie de objetos y n variables medidas sobre ellos (X_1, . . . , X_n), se trata de obtener para cada objeto una variable discriminante , Y, que indican el grupo al que pertenece de modo que sea función lineal de X_1, . . . , X_n. Esta combinación lineal de las n variables debe maximizar la varianza entre los grupos y minimizar la varianza dentro de los grupos.

Mediante el uso de PLS se pretende reducir dimensiones para hacer más sencilla y efectiva la clasificación. La diferencia entre el uso del PLS o PCA junto al DA es que PCA provee una reducción de dimensión de la variabilidad total, mientras que, en una aplicación discriminante, PLS provee una reducción de dimensión de la variabilidad entre grupos. Este uso del PLS fue propuesto por Barker and Rayens en 2003 [5].

3.1.1.3 Resolución de curvas multivariantes (MCR)

El método de resolución de curvas multivariantes se utilizó para extraer información sobre la composición química a partir del perfil cromatográfico de triglicéridos obtenido de las muestras de aceite. Como resultado se pudieron establecer las respuestas cromatográficas puras, es decir los componentes químicos que participan de forma significativa en la clasificación de las muestras en estudio.

El método MCR [6,7] utiliza una matriz **D**, cuyas dimensiones son las respuestas y tiempos de retención de un cromatograma. La matriz **D** se obtiene:

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[5] Barker M., Rayens W. (2003) Partial least squares for discrimination, J. Chemometrics 17: 166-173

[6] Tauler R., Kowalski B. Fleming S. (1993) Multivariate curve resolution applied to spectral data from multiple runs of an Industrial process, Anal. Chem. 65: 2040-2046

[7] Tauler R., Barceló D. (1993) Multivariate curve resolution applied to liquid chromatographic-diode array detection, Trends Anal. Chem. 12: 319-327

$$D = CB \qquad 3.6$$

donde C y B son las matrices de los perfiles de concentración y de respuesta, respectivamente. Las dimensiones de las matrices son C (NC x NQ) y B (NQ x NV), donde NC es el número de cromatogramas, NC es el número de componentes químicos presentes en la muestra y NW es el número de variables cromatográficas. La matriz D tiene el mismo número de filas que cromatogramas adquiridos.

El método MCR pretende obtener las matrices C y B que forman parte de la matriz D. El primer paso es determinar el número de componentes principales o la fuente de variación. Aplicando PCA a la matriz **D**, podemos realizar una descomposición de la matriz:

$$D = UV^T + E \qquad 3.7$$

donde U y V son las puntuaciones (scores) y los pesos (loadings) correspondientes al número de componentes principales elegido y E es el error residual.

Otra forma es utilizar un análisis de factores evolutivos (EFA [8], el cual estima cuantas especies o analitos se encuentran en una región. Se basa en el uso de diferentes análisis de PCA. El proceso comienza utilizando un cromatograma y añadiendo uno nuevo para cada nuevo PCA y así sucesivamente. Se realiza en dos direcciones de principio a fin (forward EFA) y viceversa (backward EFA).

El segundo paso es realizar una estimación de los perfiles de los cromatogramas con los componentes principales antes escogidos. Estas estimaciones iniciales ayudan a obtener una solución real. Se pueden utilizar perfiles de cromatogramas conocidos o se puede utilizar EFA como estimación inicial. Sin embargo, si no se tiene

[8] Maedere M.(1987) Evolving factor analysis for the resolution of overlapping chromatographic peaks, Anal. Chem. 59: 527-530

información previa para poder estimar C y B, se pueden encontrar un número infinito de soluciones. Pueden existir dos ambigüedades en el método MCR, rotacional e intensidad. La ambigüedad de intensidad viene dada por la posible existencia del escalado por un factor *m* del cromatograma, este factor es propio de cada componente. Este tipo de ambigüedad es necesario resolverla cuando se hace un análisis cuantitativo. Por su parte, la ambigüedad de rotación se presenta cuando existen dos o más componentes independientes linealmente superpuestos.

En el último paso, los perfiles de concentración encontrados por EFA son usados como estimados iniciales en la optimización restringida por mínimos cuadrados alternados (ALS) [1]. En cada iteración de optimización se obtiene una nueva estimación de la matriz del cromatograma B y de los perfiles de concentración C usando las ecuaciones siguientes:

$$B = C^{+}\widehat{D} \qquad 3.8$$

$$C = \widehat{D}\,A^{+} \qquad 3.9$$

donde las matrices C^{+} y B^{+} son los pseudoinversos de las matrices C y B respectivamente. El uso de la matriz $\widehat{D}$ en lugar de la matriz D mejora los resultados conseguidos, ya que en ellos se ha realizado un filtrado del ruido.

Asimismo, para limitar el número de posibles soluciones de estas ecuaciones se aplican restricciones, como no-negatividad y forzar la concentración de un analito a cero.

3.1.1.4 Preprocesamiento de los datos

El preprocesamiento es una preparación de los datos antes de su análisis. Su objetivo es linealizar las respuestas y eliminar fuentes extrañas de variabilidad. La linealización es importante porque las respuestas lineales son más fáciles de modelar. Con respecto a las fuentes de variabilidad, un número excesivo puede incrementar la difi-

cultad en la estimación del modelo y además puede entorpecer el poder aislar la varianza de interés [1].

Los procesamiento que se utilizaron fueron: i) centrado en la media, ii) normalización, iii) corrección de la línea base mediante mínimos cuadrados ponderados y iv) correción del desplazamiento de los picos cromatográficos utilizando el algoritmo "interval Correlation Optimised shifting" (icoshift).

Centrado en la media

Algunos métodos son influenciados por las magnitudes de los datos. Para remover esta influencia los datos deben de ser centrados. Para lo cual, se calcula la media de cada columna y se resta a cada uno de los datos de esa columna [9].

$$X_{CM} = X - \bar{X} \qquad 3.10$$

Normalización

En algunas circunstancias, algunas variables medidas pueden estar sujetas a ser escaladas, por incremento o decremento de su valor verdadero por un factor multiplicativo. Esto puede originar problemas en su interpretación conjunta debido a los distintos valores de las variables, ya que puede no tenerse en cuenta su contribución a la varianza del modelo. La normalización permite que todas las variables tengan un impacto similar en los modelos. La normalización fue realizada dividiendo cada variable por la suma de los valores absolutos de todas las variables de cada muestra. El resultado de la normalización es un vector de área igual a 1 [9,10].

[9] Vandeginste B.G.M., Massart D.L., Buydens L.M.C, De Jong S., Lewi P.J., Smeyers-Verbeke J., Hanbook of Chemometrics and Qualimetrics: Part B, Data Handling in Science and Technology 20B, Elsevier, Netherlands 1998

[10] Ramis Ramos G., García Álvarez-Coque, Quimiometría, Sintesis, España 2001

Corrección de la línea base mediante mínimos cuadrados ponderados

La corrección de línea base se realizó con el propósito de corregir la deriva de los cromatogramas debido al uso de gradiente en el método HPLC-CAD. El algoritmo de mínimos cuadrados ponderados (WLS) permite establecer los puntos que son más parecidos a los correspondientes de la línea base. Este procedimiento realizado iterativamente, permite determinar qué variables están por encima de la línea base o por debajo de está. El efecto final es la eliminación del fondo evitando crear picos negativos. Típicamente la línea base está aproximada a un polinomio de bajo orden, el cual se usa para sustraer de la línea base [1].

Alineación de cromatogramas

El último preprocesamiento empleado para la corrección de los cromatogramas fue el icoshift. Se utilizó para eliminar el posible desplazamiento de los cromatogramas debido al propio uso de la metodología de HPLC-CAD, permitiendo alinear dichos cromatogramas [11].

En el apartado siguiente (3.2) se presenta el artículo titulado **Discriminating olive and non-olive oils using HPLC-CAD and chemometrics** en el que se recogen los aspectos fundamentales de una eficiente determinación de clases entre aceite de oliva y diferentes tipos de aceites vegetales, usando las técnicas quimiométricas anteriormente indicadas. Para este estudio se utilizaron los perfiles de triglicéridos, obtenidos con la metodología HPLC-CAD, de 126 muestras de aceite.

[11] Savorani F, Tomasi G, Engelsen SB (2010) icoshift: A versatile tool for the rapid alignment of 1D NMR spectra, J Magn Reson 202: 190-202

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Discriminating olive and non-olive oils using HPLC-CAD and chemometrics

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Abstract

This work presents a method for an efficient differentiation of olive oil and several types of vegetable oils using chemometric tools. Triacylglycerides (TAGs) profiles of 126 samples of different categories and varieties of olive oils, and types of edible oils, including corn, sunflower, peanut, soybean, rapeseed, canola, seed, sesame, grape seed and some mixed oils, have been analyzed. High-performance liquid chromatography coupled to a Charged Aerosol Detector (CAD) was used to characterize TAGs. The complete chromatograms were evaluated by PCA, PLS-DA and MCR in combination with suitable preprocessing. The chromatographic data show two clusters; one for olive oil samples and another for the non-olive oils. Commercial oil blends are located between the groups, depending on the concentration of olive oil in the sample. As a result, a good classification among olive oils and non-olive oils and a chemical justification of such classification was achieved.

Keywords: Olive oil authentication, pattern recognition, Principal Component Analysis, Partial Least Square-Discriminant Analysis, Multivariate Curve Resolution, liquid chromatography

Introduction

Olive oil is obtained from the fruit of the olive tree (Olea Europaea L.) [1]. The principal producer of olive oil is Europe. In the campaign 2009/2010 the production was 21484 of thousands of tons in Europe. Spain is the major producer with 1200 of thousands of tons, followed by Italy and France [2]. Olive oil is produced from different botanical varieties of olive trees. Worldwide there are more than one thousand varieties. However, only less than a hundred are used to produce edible olive oils. Usually, commercial olive oil is a blend of a number of varieties. Nevertheless, it is possible to find olive oil produced from a single variety, and these are named "monovarietal" olive oil.

Depending on the final quality product that is traded, the International Olive Council recognizes four main commercial olive oil categories (or grades) [1]. The "virgin" olive oil is the juice of freshly harvested olive fruits; the "extra-virgin" olive oil is virgin olive oil of the highest quality based on its physical-chemical and sensory characteristics. The "refined olive oil" is an olive oil that has been chemically purified; the commercial "olive oil" is a blend of virgin olive oil and refined olive oil. Finally, the "pomace" olive oil is the oil that is obtained by solvent extraction from the solid remains of olives after pressing for juice. These olive oil categories, with some minor modifications in the nomenclature, have been recognized by EU [4] and USA [5] regulations in order to be used for human consumption.

Vegetable oils as olive oils are complex mixtures which contain a wide range of compounds, such as diglycerides, free fatty acids, phospholipids and other minor components. The main compound present in vegetable oils is the triacylglycerols. There is an official method of the International Olive Council for analyzing triacylglycerols in olive oil. This method is based on the use of the reverse phase-liquid chromatography with a refractive index detector, using as parameter the Equivalent Carbon Number, ECN which is defined as CN-2n, where CN is the acyl number of carbons and n, the number of double bonds present in the fatty acids of the TAGs [6]. Olive oil is characterized by four major

ECN peaks: 44, 46, 48 and 50. The ECN40 is not present in these types of oils, and ECN42 (mainly associate to trilinolein LLL) is present in trace amounts, unlike some vegetable oils that are characterized by high concentrations of LLL.

The Ce 5b-89 official method of the American Oil Chemists' Society (AOCS) is similar to the International Olive Council method and uses also HPLC [7]. However, three detector options are given: (a) differential refractometer, (b) UV detector, or (c) mass detector.

There is a need to discriminate between olive oils and edible oils, due to the requirement of verifying if food stuffs (as potato chips, bakery foods) indeed have olive oil as an ingredient.

Some studies use chemometrics as a tool for classifying or discriminating olive oil from edible oils. There are two principal ways for applying chemometric models to analytical results: i) directly to the raw signal profiles (e.g. chromatograms, spectra) or ii) to the information obtained from raw signal profiles (e.g. integrated signals, analyte contents after the quantification). For example in IR spectrometry, it is common to use the spectrum directly, but in chromatography, it is more common to use derived parameters from the chromatogram such as peak heights or areas. The use of the raw signal profiles has the advantage that it is not necessary to know the chemical compound that causes each analytical signal of these profiles (blind assay). This can be useful in chromatographic procedures when it is not possible to obtain a good chromatographic resolution of all peaks.

For classification, discrimination or quantification of olive oil, TAGs analysis [8,9] and different chemometric techniques, have been applied to ECNs, ratios or abundance compositions [10,11,12,13,14].

This work presents a method for an efficient discrimination between olive oil and different types of other edible vegetable oils using PCA and PLS-DA. This study, unlike most of previous methods, used the whole TAG chroma-

togram profile obtained by HPLC coupling to Charged Aerosol Detector (CAD). TAGs profiles of 126 samples of different categories and varieties of olive oils and different types of edible oils, including commercial blends, were used for the study. The chemical explanation for the obtained discrimination is confirmed by performing a discriminant analysis using the most influent TAGs.

Experimental

Instrumentation

Oil samples were analyzed with an HPLC 1100 Series system from Agilent Technologies (Santa Clara, CA USA) equipped with thermostatic column compartment, Eppendorf TC-50. Detection was carried out with a Corona CAD (ESA Biosciences Inc., Chemlsford, MA USA). And data were analyzed using MATLAB® 7.8.0 (R2009a The Maths Inc., Natick, MA, USA) and PLS toolbox 5.5 (Eigenvector Research Inc., West Eaglerock Drive, Wenatchee, WA).

Chemicals, reagents and standards

Acetonitrile HPLC grade was purchased from PANREAC (Barcelona, Spain). Hexane and isopropanol HPLC grade was obtained from PROLABO (Barcelona, Spain). The nitrogen (99%) was from AirLiquid (Madrid, Spain).

Oil samples

The oils samples were purchased in retail from Spain, France, Mexico, Italy and USA. 126 oils samples were used for the study; 47 were edible vegetable oils samples (Table 1), 68 were olive oils of different categories: extra-virgin, virgin, refined (for simplicity, under this designation we consider the market blend of virgin and refined olive oil) and pomace.

Table 1 Vegetable oil samples

Sample Code	Type of Oil	Composition	Commercial Container	Place Purchase	Quantity
CAN	canola	100% canola	plastic	USA	4
COR	corn	100% corn	plastic	Spain	5
FLA	flaxseed	100% flaxseed	glass	Spain	3
GRA	grape seed	100% grape seed	glass	Spain	4
HAZ	hazelnut	100% hazelnut	plastic	Spain	1
PEA	peanut	100% peanut	plastic	France	2
RAP	rapeseed	100% rapeseed	plastic	France	4
SAF	safflower	100% safflower	plastic	Mexico	1
SEE	seed	100% vegetable seeds	plastic	Spain	4
SES	sesame	100% sesame	glass	Spain	3
SOY	soybean	100% soybean	glass	Spain	5
SUN	sunflower	100% sunflower	plastic	Spain	9
SUNH	high oleic sunflower	100% sunflower	plastic	Spain	2

It is necessary to note that the "monovarietal" olive oils are only marketed under the categories of extra-virgin or virgin olive oil. Therefore, in extra-virgin or virgin olive oils categories, there are (i) the "monovarietal" ones, named by the variety designation and (ii) those which contain a blend of varieties, named generically as virgin-extra or virgin (Table 2).

Chromatographic analysis

Chromatographic analysis was carried out using a Licrosphere C-18 (250 × 4mm 5µm) purchased from Agilent Technologies (Waldbronn, Germany). The column temperature was kept at 30ºC. The injection volume was 4 µL. A binary mobile phase composed of acetonitrile and hexane-isopropanol (1:1), were used for gradient analysis (60:40 to 42.5:57.5 in 40 min with a post time of 5 min), the flow rate was 1.0 mL/min.

Table 2 Olive oil samples

Sample Code	Type of Oil	Category	Variety	Composition	Commercial Container	Place Purchase	Quantity
O1	olive	extra-virgin olive		100% olive	plastic	Spain	12
O2	olive	virgin olive		100% olive	plastic	Spain	3
O3	olive	refined olive		100% olive	plastic	Spain	4
O4	olive	pomace olive		100% olive	plastic	Spain	3
O5	olive	extra-virgin olive	arbequina	100% olive	plastic	Spain	8
O6	olive	extra-virgin olive	blanqueta	100% olive	glass	Spain	1
O7	olive	extra-virgin olive	cornicabra	100% olive	plastic	Spain	6
O8	olive	extra-virgin olive	empeltre	100% olive	plastic	Spain	1
O9	olive	extra-virgin olive	frantoio	100% olive	plastic	Italy	6
O10	olive	extra-virgin olive	hojiblanca	100% olive	plastic	Spain	8
O11	olive	extra-virgin olive	lechin	100% olive	plastic	Spain	1
O12	olive	extra-virgin olive	manzanilla	100% olive	plastic	Spain	3
O13	olive	extra-virgin olive	picual	100% olive	plastic	Spain	9
O14	olive	extra-virgin olive	picudo	100% olive	plastic	Spain	1
O15	olive	extra-virgin olive	royal	100% olive	plastic	Spain	1
O16	olive	extra-virgin olive	verdial	100% olive	plastic	Spain	1

Eleven commercial blend oils were also used for the study. Seven of these contain olive oil and four were blends of vegetable oils (Table 3).

Table 3. Blend commercial oil samples

Sample Code	Type of Oil	Composi-tion	Commercial Container	Place Purchase	Quantity
MIX	blend with olive				
		rap55%, olive20%, sun20%, saf5%	plastic	France	1
		olive, caroten solution	glass	France	1
		rap50%, ses25%, olive25%, nut15%, canna-bis5%	glass	France	1
		olive51%, rap44%, gra5%	glass	France	1
		olive,sun, rap,gra	glass	France	1
		olive50%, vegeta-ble47%, blue fish3%	glass	Spain	1
		sunh, olive, fish	glass	France	1
VEG	vegetable blend				
		sun,rap,gra	plastic	France	1
		soy70%, sun20%, see10%	plastic	France	1
		sun,soy	plastic	Spain	1
		ses,soy	glass	Spain	1

CAD nitrogen gas pressure was adjusted to 35 psi. None filter for signal was used and a 100 pA output range was used for CAD monitoring.

The acquiring time of the detector was 0.003 min. Hence, each chromatogram was a vector of 143.000 points which correspond to the intensities (height) of the detected signal at each time point.

Samples preparation

Stock solutions were prepared by dissolving 100 mg of each oil sample into 1.0 g of hexane. Working sample solutions were diluted by hexane to obtain a final concentration of 250 µg/g. The samples were filtrated prior to injection through a 0.22 µm PTFE membrane. The samples, after opening, were bottled in amber glass flask and maintained in dark at −2 ºC until analysis.

Statistical analysis

Data analysis was performed using multivariate statistical methods with Matlab (version 7.8.0347 R2009a) and PLS_Toolbox (version 5.5). For the grouping and classification models, PCA and PLS-DA were used respectively and MCR for extracting the chemical information of chromatograms. LDA was performed using Statgraphics (Plus 5.0).

Initial baseline correction was performed on the chromatographic data by a weighted least squares correction method using a second-order polynomial basis; peak shifting was corrected with interval Correlation Optimized shifting (*i*coshift) [15]. The chromatograms were normalized and mean centered for PCA and PLS-DA models.

The procedure followed in this study is summarized as:

1.Collect the chromatograms.

2.Use PCA to characterize variance sources.

3.Preprocessing of the chromatograms by removing baseline and correcting for elution time shifts.

4.Re-use PCA to check that the signal preprocessing indeed gets rid of the uninteresting variance (chromatographic artifacts) while keeping the interesting information (differences between oils).

5.Attempt classification using fit-for-purpose classification tools such as PLS-DA.

6.Interpret loadings to identify the chromatographic regions of most utility.

7.Identify the chemical component(s) in the interesting chromatographic regions.

8. Use LDA, with the chemical profile obtained in point 7, in order to check the classiffication strategy.

Results and discussion

Due to the complexity and similarity of TAG profiles, both in olive oils and vegetable oils, a chromatogram, including all TAGs existing in these oils, is difficult to obtain with good resolution using this HPLC-CAD method. This fact prevents establishing a one-to-one relationship between each TAG and its corresponding chromatographic peak. Applying chemometric techniques on the entire chromatographic profile could be a possible remedy to this problem.

A preliminary PCA model was applied to 40 oil samples to provide an overview of the capability of distinguishing non-olive oils and olive oils, based on TAGs profiles by HPLC-CAD (figure 1). Nine components were chosen, explaining 90% of the variance. The score plot for the first two components shows two clusters separated by principal component one (PC1). The two components explained 49% of the variance. The olive oil samples mainly have positive

scores in component one and non-olive samples mainly negative scores. However, the clusters are overlapping because some non-olive samples are positioned close to the olive sample cluster. To improve the discrimination model, preprocessing was applied to the raw data.

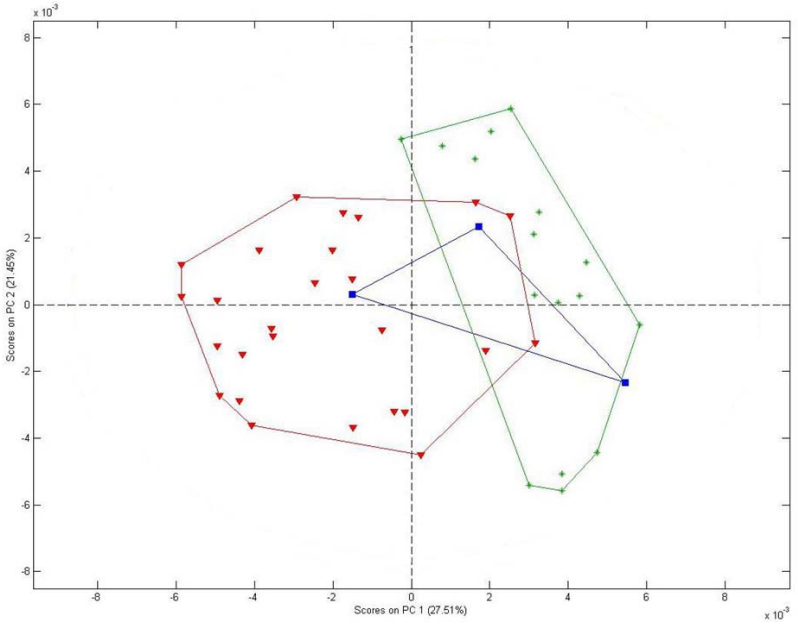

Fig 1 Raw data PCA score plot for first two principal components. Olive oils (*), non-olive oils (▲) and oil blends (■)

Data preprocessing

The chromatograms have significant 'non-chemical' variation such as baseline and retention time shifts (Figure 2A). Such variation will affect the multivariate models and obscure the relevant chemical information. The aim of pre-

processing is to reduce peak misalignment and drift in the baseline and thereby remove sources of irrelevant variation of the analysis [19].

The first eight minutes and the last ten minutes of the chromatograms were eliminated due to lack of information. Figure 2B shows the chromatograms after applying pretreatment. The baseline correction was performed using a weight least squared baseline correction and the peaks misaligned with icoshift. Icoshift is an algorithm that independently aligns each signal to a target by maximizing the cross-correlation between user-defined intervals [18]. Six intervals were used to align the chromatograms. The intervals were defined based on the TAGs peaks and the maximum peak in each interval was used as target for the alignment. Fig. 3 shows the results of the alignment using icoshift. Icoshift used two steps: i) the correlations-shifted of the full-chromatograms and ii) the interval correlation-shifted using a maximum peak as target in each interval. Raw chromatogram is presented in (a), (b) shows the full-chromatograms correlations-shifted chromatogram and finally in (c) the interval data after customized interval-correlation-shifted chromatogram.

PCA Results after pre-processing

After applying pre-processing to raw data, a PCA model was fitted. 90% of variance was explained with only three principal components as compared to nine components for the raw data. The two first principal components explain 86% of the variance (figure 4). The score plot shows two well differentiated groups, non-olive and olive oil and a third group of oil blends. The oil blends are located in the non-olive oils cluster but more or less displaced towards the olive oil cluster, depending on the percentage of olive oil presented in the blend. The sample of the blend oil that is outside of both groups appears to be an outlier. It is different from the rest of the samples due to the type of blend that contains olive oil and “high oleic” sunflower.

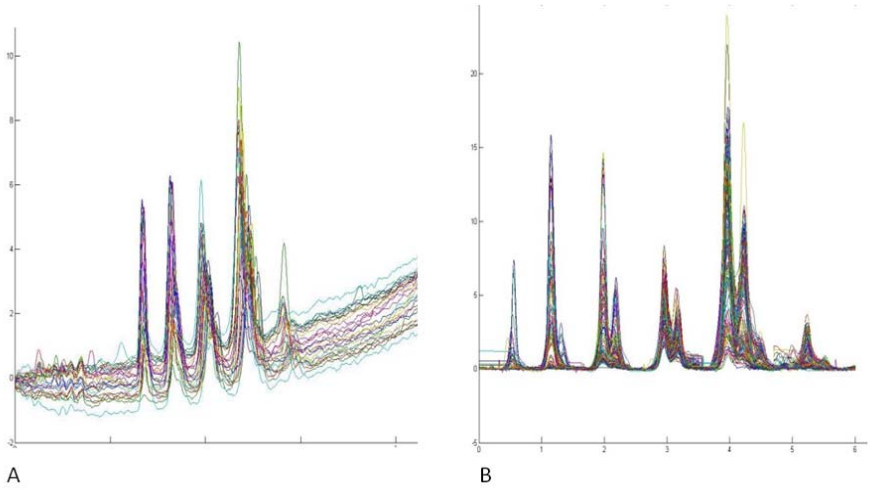

Fig 2 Oil samples HPLC-CAD chromatograms; A. raw data; B. after pre-processing

Figure 4 also shows that the groups are mainly defined by the first component; the second and the third components separate the different types of oils within the two clusters. In figure 4A four groups can be seen in the non-olive cluster: i) with negatives scores (corn, soy, flaxseed oils); ii) with values around zero (sesame and seed oils); iii) with low positives values (grape seed and sunflower); and iv) with high positive scores (rapeseed, canola, and high oleic sunflower). However, within the olive oil cluster, it is not clear if there is meaningful separation in types due to the variability from the olive oil categories. These results are confirmed by the loadings shown in figure 5. The first loading corresponds to the main peaks of TAGs in olive oil chromatogram, positive values, and to the peaks for non-olive oils samples, negative values. The second and the third loadings reflect differences between types of vegetable oils. The second loading groups according to the TAG triolein and the third loading by trilinolein and 1,2-olein-3-linolein.

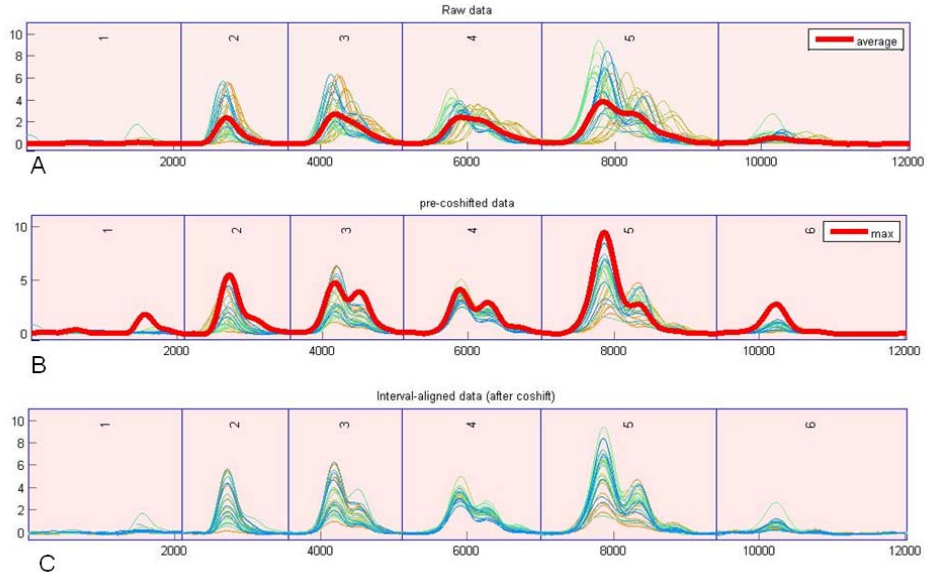

Fig 3 Shift correction using icoshift. A Raw chromatogram; B shows the full-chromatograms correlations-shifted chromatogram; C the interval data after customized interval-correlation-shifted chromatogram

PLS-DA Results

In accordance with the above results, a classification model was proposed. PLS-DA was chosen to classify the non-olive and olive oil samples. This method finds variance in the set of predictor variables (X-data) that correlates with variance in the response variables (Y-data). The Y-data set is a dummy matrix reflecting the classes; in this case non-olive and olive oils, of observations in the calibration set.

The model was performed using as calibration set 101 samples (non-olive, olive oils). Two latent variables were used for developing the model, because the third and beyond PLS components did not contribute significantly to the explained variance. With these two latent variables 83% of the variance was

explained in X. All the non-olive oils were classified correctly except for the hazelnut oil.

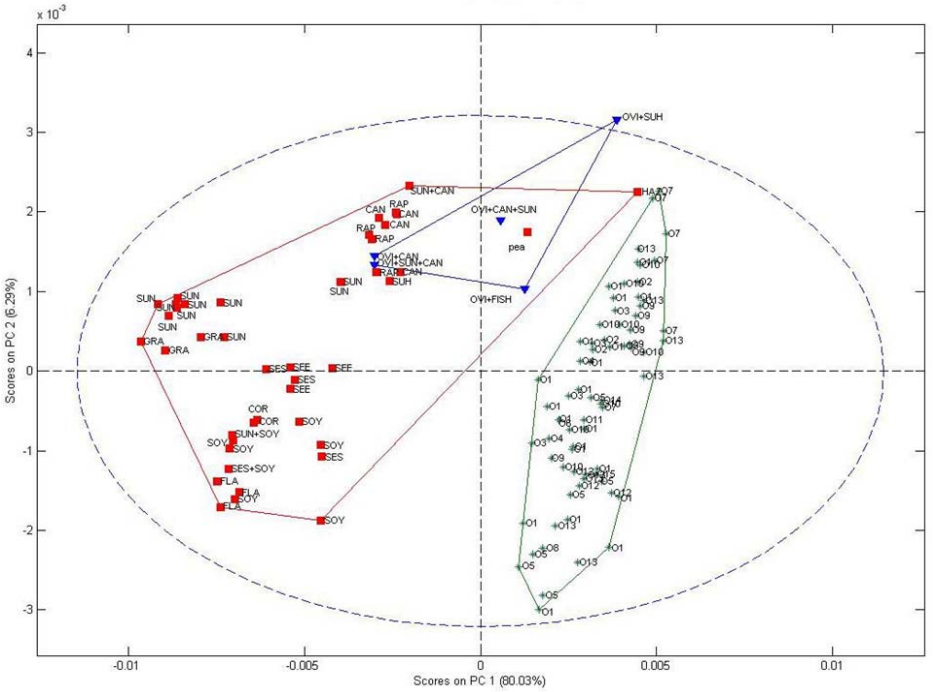

Fig 4 PCA scores plot of data after pre-processing. A. First component **vs** second component B. First component vs third component **.**Olive oils (*), non-olive oils (■) and oil blends (▼).

All the olive oil calibration samples were predicted as olive oils. The oils blends, olive with vegetable oils, were classified between both oils. Figure 6 shows the classification for olive oil samples.

A validation set was performed using 25 samples (non-olive and olive oils). The non-olive and olive oils were classified 100% as non-olive oils and olive oils, respectively.

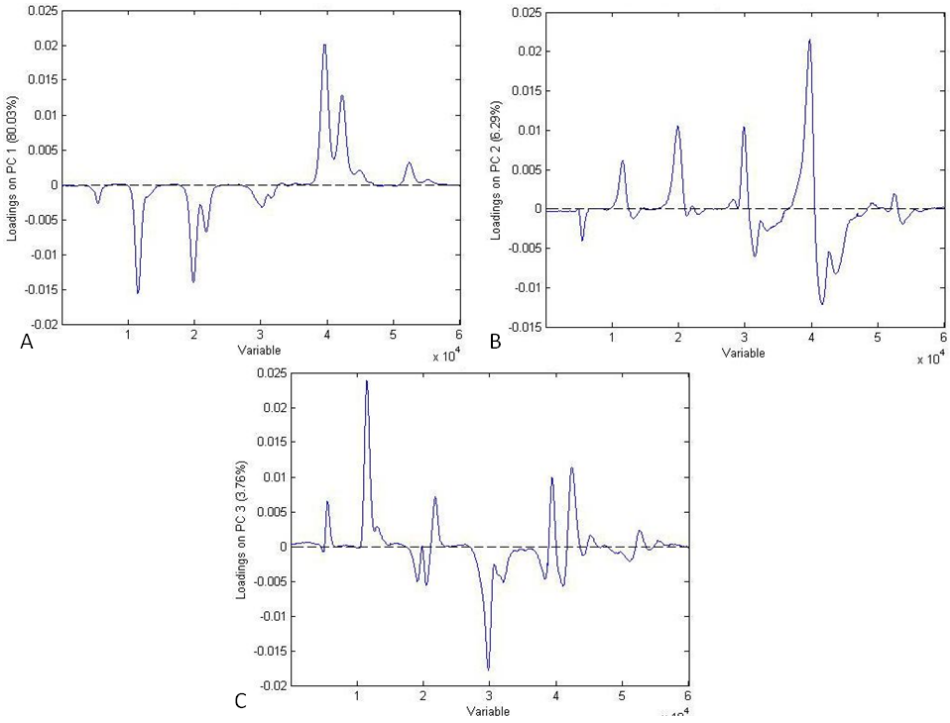

Fig 5 PCA loadings of oils samples. A. Loading on PCA1; B. Loading on PCA2; C. Loading on PCA3

MCR Results

Multivariate Curve Resolution (MCR) can be used to estimate pure chromatographic profiles and the corresponding relative concentrations [16,18]. Hence, MCR can be a useful tool for providing a chemical explanation of the contribution of the chromatographic peaks to the classification of oil samples.

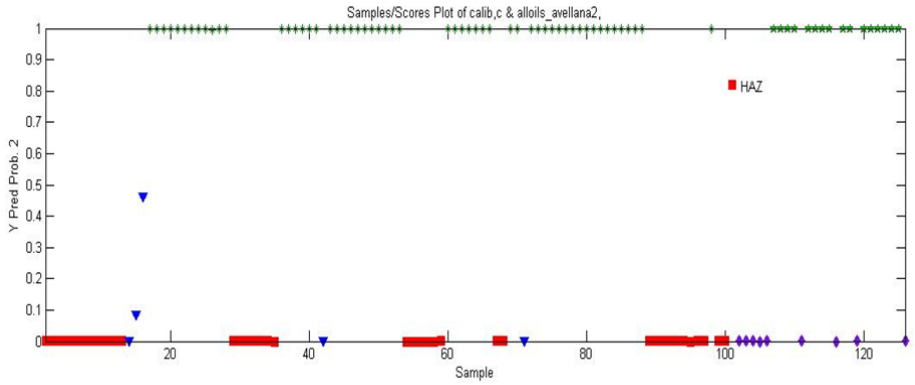

Fig. 6 PLS-DA score plot for non-olive (A) olive (B) oils. Calibration set: olive oils (*), non-olive oils (■) and oil blends (▼). Validation set: olive oils (★), non-olive oils (◆**)**, **HAZ** hazelnut

MCR was applied to the 126 samples, including non-olive, olive and blend oils. Due to that there are five groups of peaks in the sample chromatograms, five components were chosen in the MCR model. This was also confirmed by looking into MCR models with more and less components (results not shown). Figure 7 shows the score plots of the combination of the five components (figure 7A). The best separation into the known groups was provided with component one and three as shown in score plot 7A-2. The corresponding loadings are shown in figure 7B. In figure 7B the first component represents contributions of the principal TAGs of olive oil (triolein, 1,2-olein-3-palmitin, and 1,2-olein-3-linolein) and the second component represents a contribution of trilinolein,1,2-linolein-3-olein and 1,2-linolein-3-palmitin, which are the most abundant TAGs in non-olive oils. Hence, the samples that lie on the first component (figure 7A-2) are olive oils; the samples that lie between the two components are mixtures of both groups of TAGs.

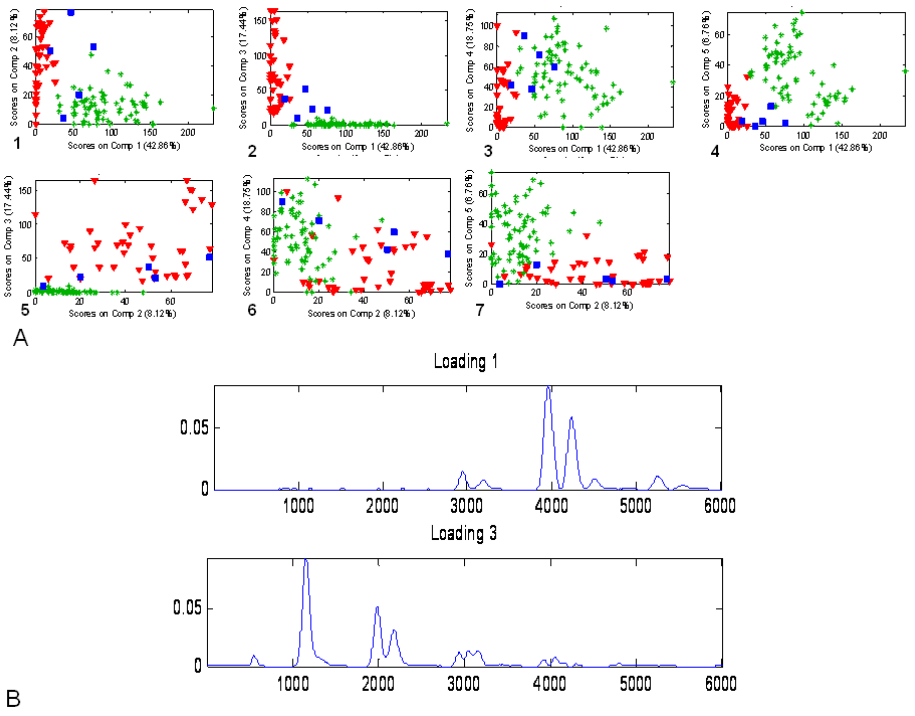

Fig. 7 MCR plot. A. Score plots for the different combinations of components. B. Loadings plots

Classification using distinct TAGs

From the MCR study it can be concluded that there are six TAGs (triolein; 1,2-olein-3-palmitin; 1,2-olein-3-linolein; trilinolein; 1,2-linolein-3-olein; and 1,2-linolein-3-palmitin) that contain information on olive oils versus vegetable oils. To verify this, a linear discriminant analysis (LDA) was performed using the peak heights of these six TAGs as input variables of the three possible categories, olive oils, vegetable oils and blends of oils.

Figure 8 shows the canonical variates from LDA. Each canonical variates represents a direction (discriminate function) with maximum separation

among categories. In this figure it can be seen that there is a good separation between the categories, olive and vegetable oils, whereas blend oils are included in vegetable oils category. In addition, if only two categories, olive and non-olive oils, were considered, it was proved that the blends of oils were grouped in the vegetable oils cluster.

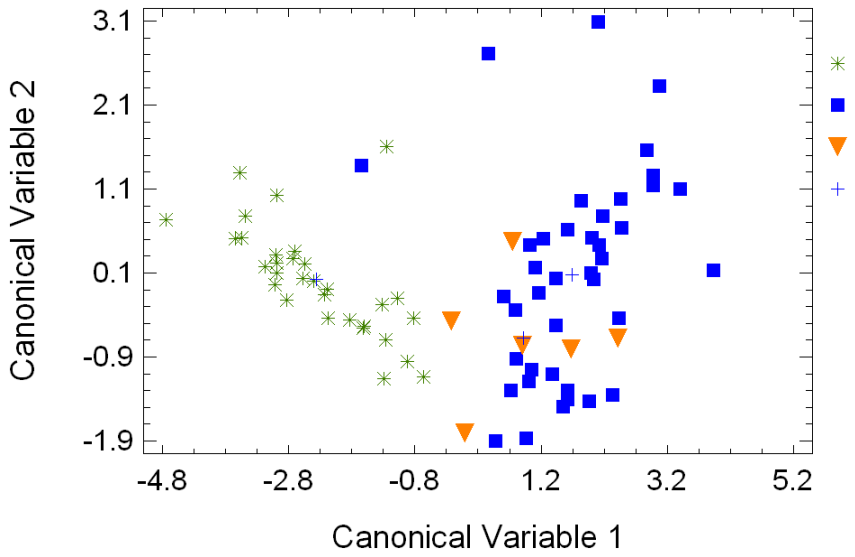

Fig 8 LDA score plots on canonical variables of oil samples: olive oils (*), non-olive oils (■) and oil blends (▼).

However, these LDA results, in comparison with those obtained by PLS-DA, are not the best for grouping. For example, hazelnut oil is closer to the olive oil group, and inside the vegetable oil cluster. Maybe, this grouping was obtained because the study was performed using only six TAGs, as opposed to the other techniques that use the complete chromatographic profile.

Therefore, it can be argued that TAGs profile obtained by HPLC-CAD can be used to discriminate between olive and non-olive oils, both using the

whole TAG chromatogram profile and a selection of six TAGs, which present a special discriminate effect.

Conclusion

From the study of TAGs by HPLC-CAD and chemometric tools, grouping of two clusters (olive and non-olive oils) was obtained from properly preprocessed data using PCA. Using PLS-DA, the classification of olive oils were 100% and the chemical behavior was further detailed by MCR, explaining the classification and grouping chemically.

Two groups of TAGs are responsible for the classification of the olive and non-olive oils. These results were achieved using whole chromatogram profiles obtained by HPLC coupled to CAD, and also taking into account different categories and varieties of olive oil. In further studies, this can be expanded to performing quantification of olive oil in blends with edible oils, using whole HPLC-CAD chromatograms, regardless all categories and varieties of olive oils, due to the need of identifying and quantifying the olive oil used in foodstuffs, when it is specified the percentage of olive oil in the labeling.

Acknowledgments

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References

[1] COI/T.15/NC No 3/Rev. 4, Trade standard applying to olive oils and olive-pomace oils (2009). International Olive Council, Madrid

[2]International Olive Oil Council, Olive Oils, Table 1: Production http://www.internationaloliveoil.org/downloads/production2_ang.PDF, nov 2009AOCS Official Method Ce 5b-89 (1997). American Oil Chemists' Society, S. Boulder

[3] Aparicio R., Harwood J., Manual del Aceite de Oliva, AMV, Madrid 2003

[4] Commission Regulation (EEC) No 1989/2003 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-pomace oil and on relevant methods of analysis, Off J Eur Commun L295/57

[5] AMS-FV-08-0073-0006 (2010). United States Standards for Grades of Olive Oil and Olive-Pomace Oil. US Department of Agriculture, Washington

[6] COI/T.20/Doc. No 20/Rev. 2, Method of analysis. Difference between actual and theoretical content of triacylglycerols with ECN 42 (2008). International Olive Council, Madrid

[7] AOCS Official Method Ce 5b-89 (1997). American Oil Chemists' Society, S. Boulder

[8] Cristopoulou E, Lzaraki M, Komaitis M, Kaselimis K (2004) Food Chem 84: 463-474

[9] Piravi-Vanak Z, Ghavami M, Ezzatpanah H, Arab J, Safafar H, Ghasemi JB (2009) J Am Oil Chem Soc, 86:827-833

[10]Aparicio R, Aparicio-Ruíz R (2000) J Chromatogr A 881: 93-104

[11] Dourtoglou VG, Dourtoglou T, Antonopoulos A, Stefanou E, Lalas S, Poulos C (2003) J AOCS Int 80: 203-208

[12] Park JR, Lee DS (2003) Bull Korean Chem Soc 24: 527-530

[13]Ollivier D, Artaud J, Pinatel C, Durbec JP, Guérère M (2003) J Agric Food Chem 51: 5723-5731

[14] Ollivier D, Artaud J, Pinatel C, Durbec JP, Guérère M (2006) Food Chemistry 97: 382–393

[15]Savorani F, Tomasi G, Engelsen SB (2010) J Magn Reson 202: 190-202

[16]Wise BM, Gallagher NB, Bro R, Shaver JM, Windig W, Koch RS (2006) PLS_Toolbox 4.0 Manual for use with MATLABTM, Eigenvector Research, Inc. Wenatchee

[17]Jaumot J, Gargallo R, de Juan A, Tauler, R (2005) Chemom Intell Lab Syst 76:101-110

[18]Amigo JM, Skov T, Bro R (2010) ChroMATHography: Solving Chromatographic Issues with Mathematical Models and Intuitive Graphics. Chem Rev. Doi:10.1021/cr900394n

3.3 Discusión

En este capítulo se ha presentado una estrategia para agrupar y clasificar aceite de oliva y aceites vegetales. Se utilizó el perfil cromatográfico de triglicéridos obtenido mediante el método HPLC-CAD y distintas herramientas quimiométricas para alcanzar este objetivo. Un aspecto importante a destacar de la metodología utilizada, fue que se recurrió al uso de los cromatogramas completos y no al uso de área de pico o altura de pico como se han utilizado generalmente en otros trabajos existentes en bibliografía. Esto da como resultado un mejor agrupamiento y clasificación de las muestras, ya que no se desecha ningún tipo información y al mismo tiempo, el ruido (línea base, desplazamiento de picos) se ha eliminado. Se ha podido comprobar que con el uso de esta metodología se obtuvieron dos grupos bien definidos, aceite de oliva y aceites vegetales.

A partir de estos resultados se planteó la posibilidad de utilizar el perfil cromatográfico de triglicéridos obtenido mediante HPLC-CAD y técnicas de regresión quimiométricas para conseguir la cuantificación de mezclas de aceite de oliva y aceites vegetales.

CAPÍTULO 4

CUANTIFICACIÓN DE ACEITE DE OLIVA EN MEZCLAS DE ACEITES VEGETALES

Gambas al píl-píl

Ingredientes para 4 personas:

- 1/2 kilo de gambas frescas y peladas
- 3 ó 4 dientes de ajo.
- Perejil picado.
- Guindilla.
- 1 cucharadita de pimentón picante o dulce, según los gustos.
- Aceite de oliva
- sal.

Preparación

Picar los dientes de ajo a trocitos. Poner una cazuela de barro a calentar con **Aceite de oliva** suficiente. Echar los ajos y la guindilla (el nivel de pique lo marcará la calidad y cantidad de la guindilla de cayena). Una vez fritos incorporar las gambas y dejar hacer 3 minutos hasta que las gambas estén cocinadas. Antes de retirar del fuego, incorporar el pimentón picante o dulce según os guste, moverlo para mezclarlo. Retirar la cazuela de barro del fuego y espolvorear con el perejil picado.

Servir muy caliente en la misma cazuela. Lo mejor de esto es la salsa para mojar pan.

4.1 Presentación

Los resultados obtenidos en capitulo anterior, abrieron nuevas posibilidades de análisis de las muestras de aceite de oliva y vegetales. El presente capitulo presenta la utilización de los triglicéridos para la cuantificación del aceite de oliva en mezclas de aceites vegetales. En estas mezclas se incluyeron numerosas combinaciones entre aceites vegetales y aceites de oliva a diferentes concentraciones. Los análisis se realizaron con el método descrito en el capítulo 2, HPLC-CAD en combinación con diferentes herramientas quimiométricas: mínimos cuadrados parciales (PLS) y mínimos cuadrados parciales por intervalos (iPLS)

En este estudio, se usaron 39 muestras, 29 de ellas se utilizaron para la calibración del modelo y 10 para la validación del mismo. Los cromatogramas de triglicéridos obtenidos fueron preprocesados, de la misma forma que los que se utilizaron en la clasificación (capitulo anterior).

4.1.1. Fundamentos teóricos

4.1.1.1 Regresión por mínimos cuadrados parciales.

La regresión por mínimos cuadrados parciales (PLS) es un método para la construcción de modelos de predicción. Fue desarrollado por Herman Wold como técnica econonmétrica, pero sus mayores usuarios son los quimiométras [1]. A diferencia de los métodos de regresión lineal múltiple (MLR) y regresión por componentes principales (PCR), el método PLS intenta encontrar factores en donde se obtenga la máxima covarianza, correlación y varianza, entre *X* y *Y*. MLR encuentra un factor que mejor correlacione *X* (variable predictora) con *Y*

[1] Randall D.T., An introduction to partial least squares regression, SAS Institute Inc. Cary, NC, DOI. 10.1.1.136.8520

(variable predicha), mientras que PCR trata de encontrar factores que capturen la mayor cantidad de varianza de *X*, es decir explicar *X* más que *Y*.

El objetivo del PLS es construir un modelo de la forma:

$$Y = XB + E \qquad 4.1$$

donde ***Y*** es una matriz de ***n*** x ***m*** (variables respuesta), ***X*** es una matriz de ***n*** x ***p*** (variables predictoras), ***B*** es una matriz de coeficientes ***p*** x ***m*** y ***E*** es el error del modelo con dimensiones ***Y***. La regresión PLS produce puntuaciones de factores como combinación lineales de las variables predictoras originales. En un conjunto de datos con variables respuesta ***Y*** y variables predictoras ***X*** la regresión por PLS se obtiene una matriz de puntuaciones de factores:

$$T = XW \qquad 4.2$$

donde ***W*** es el peso de la matriz, que es la covarianza que existe entre las variables predictoras y de respuesta. A partir de la ecuación 4.2 se puede obtener el modelo de regresión:

$$Y = TQ + E \qquad 4.3$$

donde ***Q*** es la matriz de las cargas de las puntuaciones y ***E*** es el error del modelo. Igualando la ecuación 4.1 con la 4.3 se obtiene la ecuación 4.4, la cual puede usarse como modelo de regresión predictivo.

$$B = WQ \qquad 4.4$$

4.1.1.2 Regresión por mínimos cuadrados parciales por intervalos

El método PLS por intervalos (iPLS) selecciona un subconjunto de variables para obtener una mejor predicción en comparación a la utilización de todas las variables. Para obtener el subconjunto de variables, iPLS realiza una búsqueda de la mejor o mejores combinaciones de variables. Esta búsqueda se puede realizar en un modo “hacia delante” o “hacia atrás”. En el modo “hacia

delante" los intervalos son incluidos sucesivamente en el análisis y en el modo "hacia atrás" los intervalos son removidos sucesivamente. El intervalo utilizado en iPLS puede ser una sola variable o una "ventana" de variables adyacentes [2]. Las ventanas son de igual anchura. La selección del número de intervalos se realiza comparando la raíz cuadrada del error cuadrático medio de la "validación cruzada" (RMSECV) entre los modelos de cada intervalo [3].

En el caso del modo "hacia delante", el intervalo elegido es el cual contenga el menor RMSECV. Si solo se requiere un solo intervalo el algoritmo se detiene en este punto, pero si se quiere más intervalos se realiza otro ciclo, es decir, se utiliza el primer intervalo seleccionado adicionando los intervalos restantes, uno por uno, hasta encontrar el RMSECV más bajo de las combinaciones de intervalos. Este ciclo se repite hasta n intervalos.

En el modo "hacia atrás" todos los intervalos son incluidos en el modelo inicial y el algoritmo selecciona un intervalo para removerlo. El intervalo escogido es el peor intervalo, es decir, el intervalo, que al quitarlo, produce un menor RMSECV del modelo. Si se quieren quitar mas intervalos se realiza otro ciclo para quitar otro intervalo.

En el apartado 4.2 se presenta la publicación, en la que se recogen los aspectos fundamentales del trabajo realizado por medio del método HPLC-CAD y técnicas quimiométricas, PLS e iPLS para la cuantificación de aceite de oliva en mezclas con aceites vegetales utilizando los perfiles de triglicéridos. La publicación fue enviada bajo el nombre de ***Olive oil quantification of edible vegetable oil blends using triacylglycerols profiles and chemometric tools***, a la revista Talanta.

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[2] Nørgaard L., iToolbox Manual, www.models.kvl.dk, April 2005

[3] Leardi R., Nørgaard L.(2004) Sequential application of backward interval partial least squares and genteic algoritms for the selection of relevant spectral regions, J. Chemometrics 18: 486-497

4.2 Artículo enviado a Talanta (en revision)

Olive oil quantification of edible vegetable oil blends using triacylglycerols chromatographic fingerprints and chemometric tools

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ABSTRACT

The present work studies the effectiveness of the use of triacylglycerols (TAGs) for the quantification of olive oil in blends with vegetable oils. The determinations were obtained using high-performance liquid chromatography (HPLC) coupled to a Charged Aerosol Detector (CAD), in combination with Partial Least Squares (PLS) regression and using interval PLS (iPLS) for variable selection.

Results revealed that PLS models can predict olive oil concentrations with low errors. Variable selection through iPLS did not improve predictions significantly, but revealed the chemical information important in the chromatogram to quantify olive oil in vegetable oil blends.

Keywords: *TAG, HPLC, PLS, iPLS, olive oil, quantification*

1. Introduction

Olive oil (OO) is defined as the oil obtained solely from the fruit of the olive tree (Olea Europaea L.). There are different botanical varieties of olive trees, so olive oils can be obtained as monovarietal oils (arbequina, picual, hojiblanca, etc.) or as a blend of two or more of these varieties. Moreover, the International Olive Council (IOC) recognizes four main categories of olive oil according to the final quality product: extra-virgin, virgin, refined and pomace [1]. Refined olive oil is a tasteless product and, in the market, it is always blended with a low proportion of virgin olive oil. OO consumption per person in the European Union (EU) has increased considerably in recent decades and EU is the biggest olive oil producer in the world [2]. Moreover OO is recognized nowadays as oil with beneficial effects for health (skin, cardiovascular system, cancer, etc) [3]. Due to these facts, companies have been taking advantage of selling OO blends at the same price as pure OO, obtaining important economic benefits. The adulterants used in blends are the ones with similar physical and chemical properties and usually they are cheaper and easy to obtain. In the case of OO this usually implies the dilution with other inferior quality olive oils or cheaper vegetable oils [4].

It is necessary to be able to verify authenticity of blends with edible oils. When the prepared blend deviates from the mixture proportions given on the product label, it is considered that the oil is adulterated [5]. In the EU, requirements has being established in Regulation (EC) No. 1019/2002, concerning commercialization and labeling of products which contain olive oil. For example, foodstuffs containing olive oil have to specify the percentage of olive oil in the labeling. In addition, if blends of olive and other edible vegetable oils are marketed, the presence of olive oil higher than 50% has to be indicated on the label, but if the percentage is lower than 50% the name of olive oil cannot be used in the label [6].

There is a demand for the development of rapid methods to detect adulteration of OO present in edible oil blends. For this, authentication and/or quanti-

fication methods have been developed. Different chemical and physical techniques have been studied to quantify olive oil in blends with other vegetable oils in combination with chemometric tools, for example, Headspace and Mass Spectrometry [7], 1H and 31P Nuclear Magnetic Resonance [8,9], and Fourier Transform-IR, Near IR, Mid-IR and FT-Raman [10, 11, 12, 13, 14]. Chromatographic techniques, such as Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) are the most frequently used for this purpose [15,16,17,18,19,20]. Nevertheless, the official method of the International Olive Council (IOC) is based on the use of the reverse phase-liquid chromatography with a refractive index detector (HPLC-RID), to establish the difference between actual and theoretical content of TAGs with Equivalent Carbon Number 42 (ECN42) [21].

There are a limited number of published studies that take into account all the variability (different categories and varieties of olive oil) that can be found in products containing mixtures of olive oil with edible vegetable oils, since the mixtures present in the products do not always specify the category/variety of olive oil.

Analytical signals can typically be analyzed in two different ways: a) using raw analytical signals, which come directly from the analytical instrument (i.e. an elution profile) as input to a multivariate model; or b) Use derived information such as peak areas (~concentrations). Such concentrations may be used in a univariate manner one at a time or in a multivariate chemometric modeling using all available information (Figure1).

In chromatography, chemometric tools are usually applied to the information derived from the signal profile such as peak heights, areas or parameters as ECN for TAGs analysis. Peak areas or similar measures are used partly for historical reasons and partly because several problems typically prevent straightforward use of raw chromatograms. These problems are mainly caused by baseline drift and peak shifting.

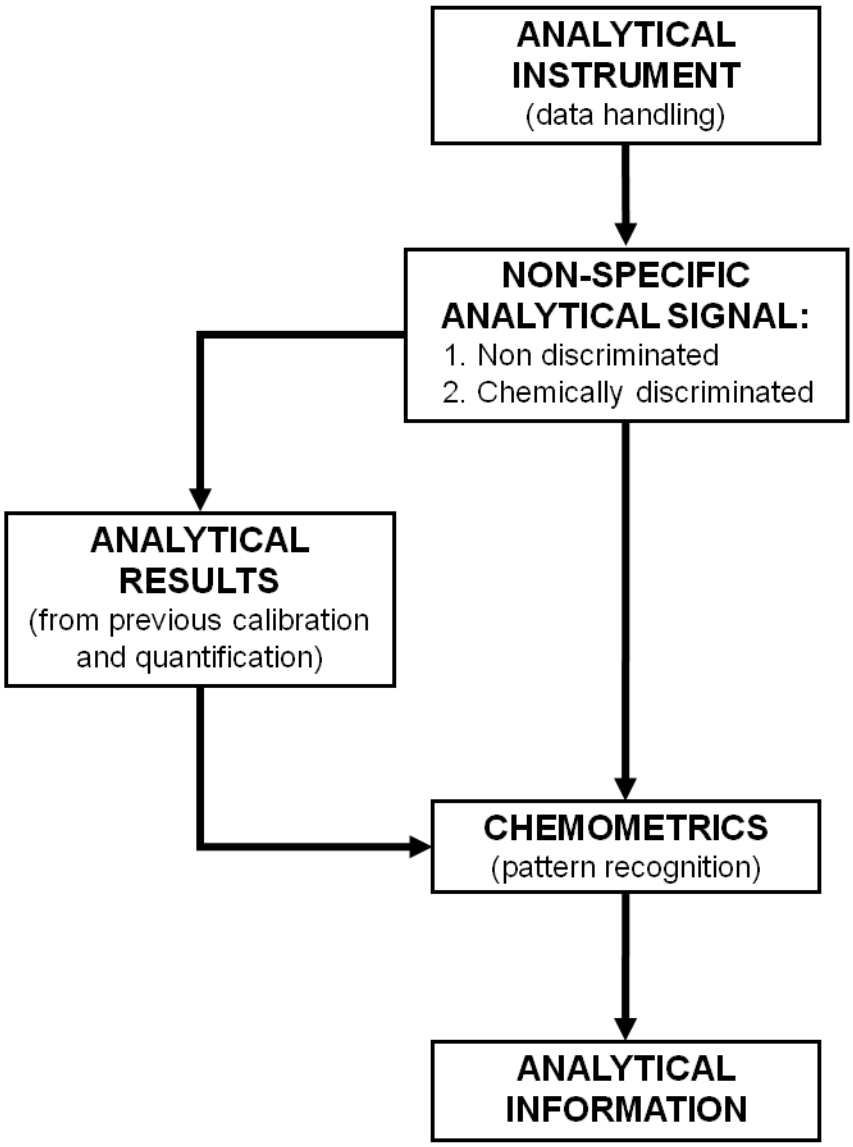

Fig. 1. Application of chemometric techniques on analytical signals

The present work studies the effectiveness of the use of TAGs for the quantification of olive oil in vegetable oil blends, including almost all possible

combinations of vegetable oils and considering the different categories and varieties of olive oils at several percentages. The analyses were obtained using a HPLC-Charged Aerosol Detector (CAD) method, applying chemometrics tools to full TAG chromatogram profiles. To our knowledge, there is only one study about olive and vegetable oils using CAD [22]. This detector has some advantages, among others, it presents sensitive, wide dynamic range and little variation in response between analytes [23], a broader discussion of the advantages of this detector can be found in the paper Quantification of Triacylglycerols in Olive Oils using HPLC-CAD[24]

2. Experimental

2.1. Instrumentation

The samples were analyzed by a chromatographic system HP Agilent HPLC 1100 Series system composed of quaternary pump, degasser, automated sampler and 1100 ChemStation software (Santa Clara, CA USA). A thermostatic column compartment from Eppendorf TC-50 was used. Data analysis was performed using multivariate statistical methods by MATLAB® 7.8.0 R2009a (The Maths Inc., Natick, MA, USA) and PLS_Toolbox 5.5 (Eigenvector Research Inc., West Eaglerock Drive, Wenatchee, WA).

2.2 Chemicals, reagents

Acetonitrile HPLC grade were purchased from PANREAC (Barcelona, Spain). Hexane and isopropanol HPLC grade were obtained from PROLABO (Barcelona, Spain). The Nitrogen (99%) was acquired from AirLiquid (Madrid, Spain).

2.3 Oil samples

Ten olive oils samples, including four categories: extra virgin, virgin, olive oil (blend of virgin and refined) and pomace, and two varieties: arbequina

and picual. Ten vegetable oils, representative of the most used edible oils, were used in the study. These vegetal oils were 2 sunflower, 1 high-oleic sunflower, 1 rapeseed, 1 soybean, 1 canola, 1 corn, 1 grape seed and 2 commercial vegetable seed, they are seeds mixtures and in the label it is not specified the type of seed.) oils,. All the oils were purchased in retail stores and were maintained in dark at −2ºC until analysis.

2.4 Chromatographic conditions

Chromatographic analysis was carried out using a LiChrospher 100 RP-18 (250 x 4mm 5µm) purchased from Agilent Technologies (Waldbronn, Germany). The column temperature was kept at 30 ºC. The injection volume was 4 µL. A binary mobile phase composed of acetonitrile and hexane-isopropanol (1:1), was used for gradient analysis (60:40 to 42.5:57.5 in 40 min with a post time of 5 min); the flow rate was 1.0 mL/min. CAD conditions were: nitrogen gas pressure was adjusted to 35 psi, none filter was used for detector signal and a 100 pA output range was used for CAD monitoring.

2.5 Samples preparation

The working samples were prepared mixing one olive oil with one vegetable oil in different percentages. Four batches were prepared: i) one olive oil and one sunflower oil; ii) one olive oil and nine different vegetable oils; iii) one sunflower oil with nine different olive oils iv) a validation batch. Composition of the samples of the different batches is presented in Table 1. The selection of the sunflower oil for preparing the batch 1 and 3 was due to the importance of this oil as ingredient in foodstuffs, like in chips, bakery products or preserves foods. The concentrations were chosen to cover all the concentration ranges of the blends.

Stock solutions were prepared by dissolving 100 mg of each oil sample into 1.0 g of hexane. Working sample solutions were diluted by hexane to obtain a final concentration of 250 µg/g. The samples were filtrated prior to injection through a 0.22 µm PTFE membrane.

TABLE 1. Samples composition

BATCH 1		BATCH 2		BATCH 3		BATCH 4	
Sun-flower (%)	Olive (VE) (%)	Vege-table (%)	Olive (VE) (%)	Sun-flower (%)	Olive (%)	Vegeta-ble (%)	Oliv e (%)
100	0						
90	10	Soy 90	10	90	Pom 10	Soy 90	V10
80	20	Canola 80	20	80	V 20	Rape-seed 90	OO 10
70	30	Seed 70	30	70	OO 30	Sun-flower 70	V 30
60	40	HO Sun-flower 60	40	60	VE (arbequi-na) 40	Corn 70	OO 30
50	50	Corn 50	50	50	OO 50	Soy 50	V 50
40	60	Rape-seed 40	60	40	VE (pi-cual) 60	Rape-seed 50	OO 50
30	70	Grape 30	70	30	VE 70	Sun-flower 30	V 70
20	80	Sun-flower 20	80	20	V 80	Corn 30	OO 70
10	90	Seed 10	90	10	OO 90	Soy 10	V 90
0	100					Rape-seed 10	OO 90

HO: High Oleic; VE: virgin extra; V: virgin; OO: Olive Oil (mixture refined olive oil + virgin); Pom: pomace Sun: Sunflower; Veg: vegetable. Batch1-3 calibration sets and Batch 4 validation set

2.6 Statistical analysis

Pre-processing was used to make the data suitable for statistical analysis. Peak shifting was corrected with interval Correlation Optimized shifting, *i*co-shift [25], and initial baseline correction was performed by a weighted least

squares approach using a second-order polynomial basis. The chromatograms were subsequently mean centred. The prediction capability of the regression models was assessed by cross-validation by removing one sample at a time.

3. Results and discussion

The quantification of olive oil in samples mixed with different vegetable oils was carried out using PLS and iPLS models, *abscissa* was the measured TAG values (%) and *ordinate* contained the percentage of olive oil in the samples. Therefore, the results, predicted values, were obtained in percentage units of olive oil.

In this study, 29 samples, batch one to three, were used for the calibration model. Firstly, each of the three calibration batches was analyzed separately; and then, the three batches were combined to obtain a more comprehensive model. Finally the batch four, with ten samples, was used as external validation.

3.1 Data preprocessing

To avoid bias from chromatographic sources of variation that are unrelated to the chemistry of the samples, preprocessing of the data was used. The aim of this preprocessing is to reduce peak misalignment and drift in the baseline [26].

The first eight minutes and the ten last minutes of the chromatograms were eliminated due to lack of information. Figure 2 shows the chromatograms after baseline correction and alignment. Initial baseline correction was performed on the chromatographic data by a weighted least square correction based on a second-order polynomial basis [26,27]. The misaligned peaks were corrected with *i*coshift [25]. Six intervals were used for aligning the chromatograms. These intervals were chosen based on the TAGs peaks, and the maximum peak in each interval was used as target (Figure 2).

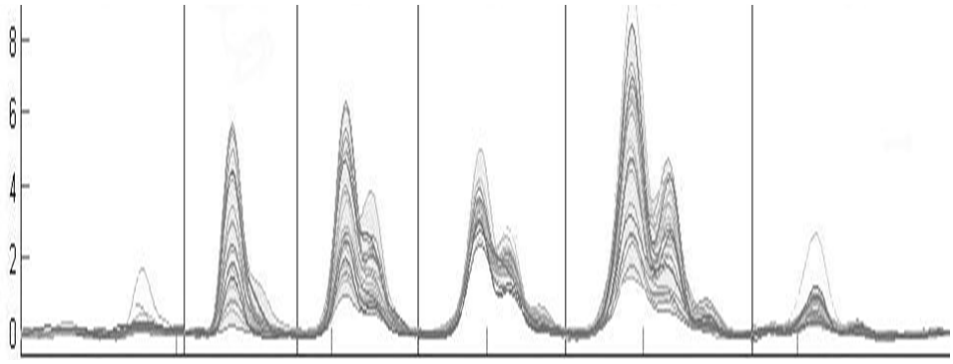

Fig. 2. HPLC-CAD chromatograms of oil samples after pretreatment of chromatographic raw data

3.2 PLS Results

Regression models were created using PLS applied to a matrix composed of 29 samples by 11969 variables from the three calibration batches. The performance of model was evaluated by R^2. In addition, calibration set was also evaluated with root-mean-squared error of calibration (RMSEC) and validation set with root-mean-squared error of cross-validation RMSECV, using a leave-one-out validation. External validation of the model was quantified by the root-mean-squared error of prediction (RMSEP) [26,27].

Figure 3 shows the plots of predicted versus actual concentrations of olive oil. The model of the batch of one olive oil and one sunflower oil (Figure 3A) presents a good model due to its R^2 (0.997) and a RMSECV of 1.76, error percentage prediction of the model, but the model is simple in the sense that it only models the variability of a blend of two oils. However, a more complete model is obtained when a batch of different vegetable oils and one olive oil is used, Figure 3B shows an R^2 of 0.959 and a RMSECV of 6.79, the model predicts concentrations of olive oil in different blend samples of different vegetable oils (soybean, canola, seeds, sunflower, corn, rapeseed, grape), hence the model include more variability. Figure 3C presents the results of different olive oils with one sunflower; the plot indicates an R^2 of 0.980 with a RMSECV of 3.74. This model shows better fit than the Figure 3B, even though it uses nine

different olive oils. To obtain the results for the aim of the study, the quantification of olive oil in different mixed oil samples, it was necessary to develop a model that contains all the variability, that is, four different categories and two varieties of olive oil and different types of vegetable oils. Figure 3D shows the results for this model (batch one to three) using four latent variables, although the model presents an R^2 of 0.910, and a RMSECV 8.64, it is a good model considering that the model uses ten different vegetable oils and ten different olive oils.

3.3 iPLS Results

In order to investigate if the results could be improved, variable selection was applied. Using iPLS to find, if exists, a peak or peaks that give a lower RMSECV and better R2 than the full-chromatogram. The iPLS approach splits the data into a number of intervals and calculates local PLS model for each interval [28]. The models were constructed using up to four latent variables. The matrix used in the model was 29 samples by 601variables. The results did not improve dramatically; RMSECV decreased from 8.64 to 7.79 (Figure 4B). Figure 4A shows the two segments that are considered in the model, which are the segments that presented the lowest RMSECV. The segments are two of the four groups of peaks characteristic of olive oil. Olive oil is characterized by four major ECN peaks: 44, 46, 48 and 50. The ECN40 is not present in this types of oils, and ECN42 (trilinolein LLL) is present in trace amounts, unlike some vegetable oils that are characterized by high concentrations of LLL.

In the first segment are the TAGs with ECN44, which are present in olive oil and in vegetable oils, the second segment is the peak of triolein, the TAG most abundant presents in olive oil, therefore the variable selection confirm that both peaks are important to quantify olive oil [29].

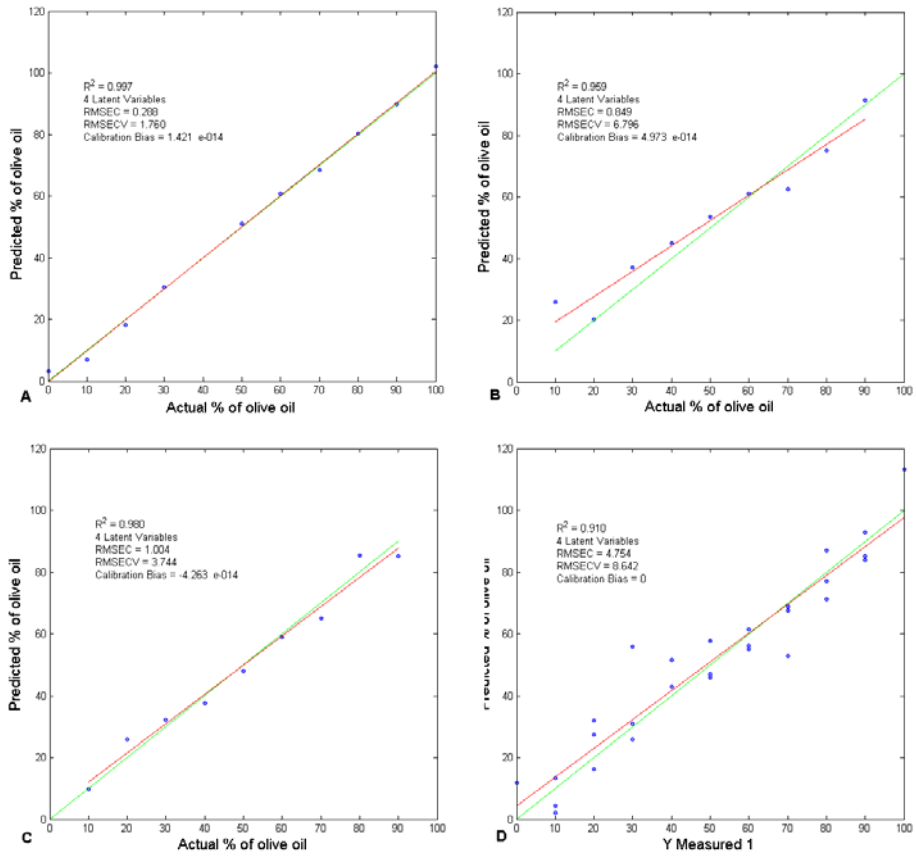

Fig. 3. Predicted % of olive oil in different batches. See Table 1 for details of oil sample composition. **A.** Batch1; **B.** Batch2; **C.** Batch3 **D.** Batch1-3

The original PLS model (Figure 3D) was validated with ten samples (Table 1), including blends of four different types of vegetable oils and two varieties of olive oils at five different percentages. The selection of the vegetable oils was due to the common blends that are presented in food stuffs, like potato chips or bakery products [24]. In Figure 5 are shown the results obtained for the

training set and the validation set. The RMSEP obtained for the validation set was 10% (Figure 5).

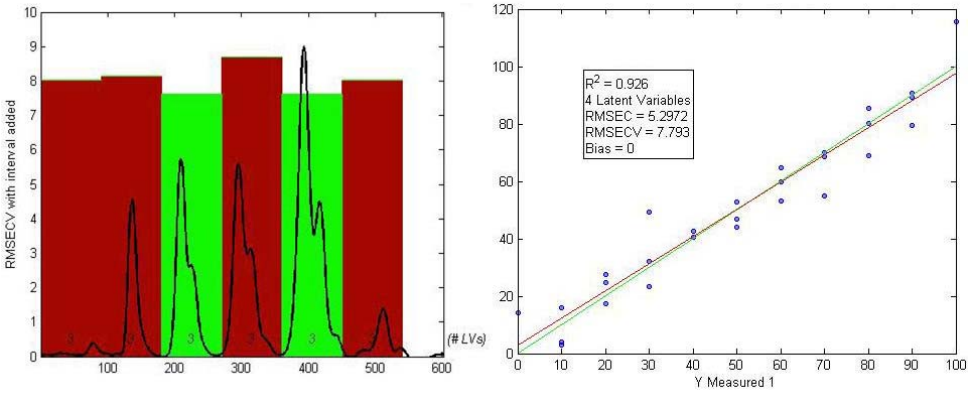

Fig. 4. iPLS using all the oil samples; A. Selected interval for the four batches, B. Predicted values using the selected intervals

4.Conclusions

Olive oil was able to quantify in edible oils blends using HPLC-CAD, applying chemometric tools (PLS and iPLS) to the full chromatogram. Different categories of olive oils (extra-virgin, virgin, refined and pomace) and various vegetable oils (sunflower, rapeseed, corn, soybean, canola, seed and grape seed) were used at different concentrations. As a result, using the whole variability of the samples, the PLS obtained a low RMSECV of 8.6%. Also iPLS was applied; using two peak segments obtaining also, low RMSECV of 7.8% a slight improvement in RMSECV and also providing a chemically meaningful selection of peaks. The external validation was applied to the PLS model obtaining a 10% of RMSEP.

The proposed method provides a way to quantify olive oils using the full TAG chromatogram, chromatographic fingerprint, instead of using the information derived from the signal profiles.

The results obtained show the ability to establish and quantify the presence of various vegetable oils blended with different varieties and types of olive oils with errors not exceeding 10%.

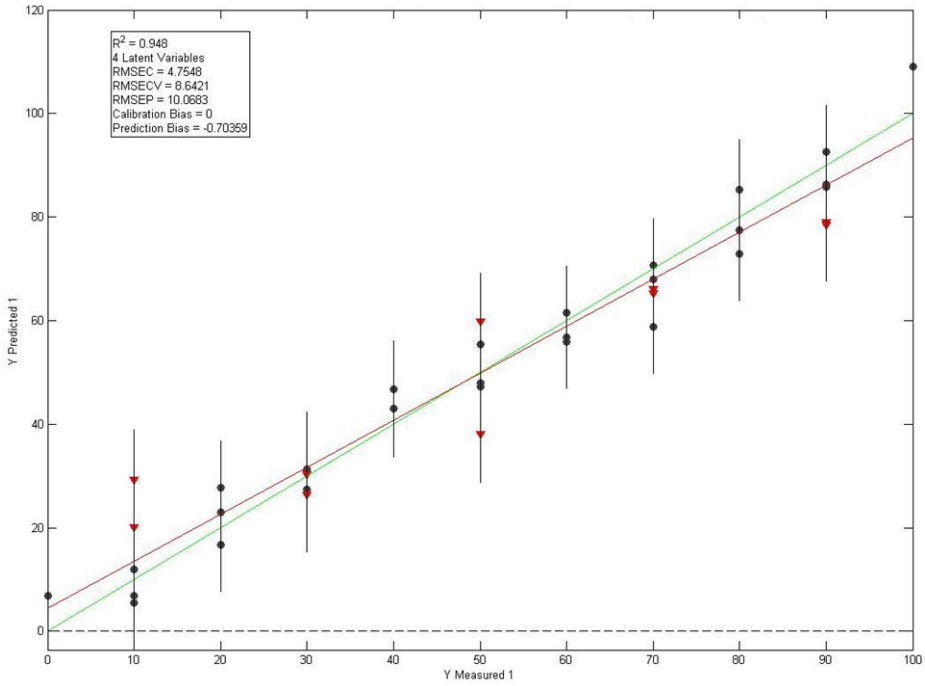

Fig. 5. External validation using PLS model with 10 vegetable oils and 10 olive oils (• training set; ▼validation set)

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References

1 COI/T.15/NC no. 3/Rev. 4, Trade standard applying to olive oils and olive-pomace oils, International Olive Oil Council, Madrid, Spain, 2009.

2 F. Luchetti, Eur. J. Lipid Sci. Technol.104 (2002) 559-563

3 Consejo Económico y Social de la Provincia de Jaén Universidad de Jaén, El aceite de Oliva Virgen Alimento Saludable, Diputación provincial de Jaén, España, 2009

4 D. Filmore, Today´s Chemist at work (TCAW), February (2004), 29-31

5 F. Ulberth, M. Buchgraber, Eur. J. Lipi Sci. Technol. 102 (2000) 687-694

6 Commision Regulation (EC) No. 1019/2002, on marketing standards for olive oil, Official Journal of the European Communities, L 155/27, 2002

7 F. Peña, S. Cárdenas, M. Gallego, M. Valcárcel, J. Chromatogr. A 1074 (2005) 215-221

8 C. Fauhl, F. Reniero, C. Guillou, Magn. Reson. in Chem. 38 (2000) 436-443

9 G. Vigli, A. Philippidis, A. Syros, P. Dais, J. Agric. Food Chem. 51 (2003) 5715-5722

10 M.A. Allam, S.F. Hamed, J. Appl. Sci. Res. 3 (2007) 102-108

11 N. Vlachos, Y. Skopelitis, M. Psaroudaki, V. Konstantinidou, A. Chatzilazarou, E. Tegou, Anal. Chim. Acta 573-574 (2006) 459-465

12 A. A. Christy S. Kasemsuran, Y. Du, Y. Ozaki, Jpn. Soc. Anal. Chem. 20 (2004) 935-940

13 G. Gurdeniz, B. Ozen, Food Chem. 116 (2009) 519-525

14 V. Baeten, R. Aparicio, Biotechnol. Agron. Soc. Environ. 4 (2000) 196-203

15 E. Cristopoulou, M. Lzaraki, M. Komaitis, K. Kaselimis, Food Chem. 84 (2004) 463-474

16 J. R. Park, D.S. Lee, Bull. Korean Chem. Soc. 24 (2003) 527-530

17 J. Parcerisa, I. Casals, J. Boatella, R. Codony, M. Rafecas, J. Chromatogr. A 881 (2000) 149-158

18 R. Aparicio, R. Aparicio-Ruíz, Authentication of vegetable oils by chromatographic techniques, J. Chromatogr. A 881 (2000) 93-104

19 F. Priego Capote, J. Ruiz Jiménez, M.D. Luque de Castro, Anal. Bioanal. Chem. 388 (2007) 1859-1865

20 V.G. Dourtoglou, T. Dourtoglou, A. Antonopoulos, E. Stefanou, S. Lalas, C. Poulos, J. Am.Oil Chem. Soc. 80 (2003) 203-208

21 COI/T.20/Doc. No 20/Rev. 2, Method of analysis. Difference between actual and theoretical content of triacylglycerols with ECN 42, International Olive Council, Madrid, 2008

22 M. Lisa, F. Lynen, M. Holčapek, P. Sandra, J. Chromatogr. A 1176 (2007) 135-142

23 D. Amstrong, Lipidomics Volume 1: Methods and Protocols, Human Press, USA, 2009

24 P. de la Mata-Espinosa, J.M. Bosque-Sendra, L.Cuadros-Rodríguez, Food Anal. Method. (in press)

25 Savorani F., Tomasi G., Engelsen S.B., J. Magn. Reson. 202 (2010) 190-202

26 Wise B.M., Gallagher N.B., Bro R., Shaver J.M., Windig W., Koch R.S., PLS_Toolbox 4.0 Manual for use with MATLABTM, Eigenvector Research, Inc., Wenatchee, WA, USA, 2006

27 D.L Massart, B.G.M. Vaneginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke J.,Handbook of Chemometrics and Qualimetrics Part A, Data Handling in Science and Technology 20A, Elsevier, Amsterdam, 2007

28 L. Nørgaard, iToolbox Manual, Quality & Technology, Department of Food Science, Faculty of Life Science, University of Copenhagen, 2005

29 N.K. Andrikopoulos, Food Rev. Int. 18 (2002) 71-102

4.3 Discusión

El método propuesto, HPLC-CAD en combinación con técnicas quimiométricas, ha proporcionado buenos resultados en la predicción de concentraciones de aceite de oliva en mezclas de diferentes tipos de aceite de oliva y aceites vegetales.

Los modelos de PLS pusieron de manifiesto la posibilidad de predecir las concentraciones de aceite de oliva con RMSECV bajos. Sin embargo, para mejorar el modelo, aumentando el poder de predicción y facilitar su interpretación, se realizó una selección de variables a través de modelos iPLS. A pesar de la utilización de dos intervalos, este último modelo no mejoró significativamente la predicción, pero puso de manifiesto los intervalos o zonas del cromatograma que son importantes para la cuantificación de aceite de oliva en mezclas con aceites vegetales.

A partir de estos resultados se planteó la posibilidad de utilizar otra técnica instrumental para mejorar los resultados obtenidos. La técnica elegida fue la espectroscopia de infrarrojo, ya que como se comprueba en la bibliografía existente, es una técnica bastante utilizada en estudios sobre el aceite de oliva.

CAPÍTULO 5

ESTUDIOS DE ACEITES DE OLIVA, ACEITES VEGETALES Y MEZCLAS DE ELLOS UTILIZANDO FTIR Y QUIMIOMETRÍA

Receta de habas con jamón

Ingredientes:

- 3 kg. de habas tiernas
- 2 cebolletas
- 8 lonchas de jamón serrano
- 1 trozo de tocino ibérico
- Aceite de oliva virgen extra
- sal.

Preparación

Desgranamos las habas, picamos la cebolleta.

En una sartén con un chorrito de Aceite de oliva virgen extra, mareamos el trozo de tocino, añadimos la cebolla, pochamos, agregamos las habas, tapamos y cocinamos a fuego lento 10-15 minutos, añadimos el jamón, le damos un meneillo a la sartén y apartamos del fuego.

2.1 Presentación

En este capítulo se desarrolla un método de espectrométrico en el infrarrojo medio con transformada de Fourier (FTIR) para la clasificación y discriminación de aceites de oliva y aceites vegetales. Adicionalmente se realizó un estudio para la clasificación de mezclas de aceites oliva con aceites vegetales. Estos objetivos se establecieron cumpliendo con la necesidad de verificar los requerimientos especificados en el Reglamento de la Unión Europea (EC) No. 1019/2002, en el cual se regula, entre otros temas, el contenido de aceite de oliva en productos alimenticios [1]. En este Reglamento se indica que el porcentaje del aceite de oliva utilizado en el producto debe de ser declarado en el etiquetado y cuando este contenido es destacado mediante imágenes o gráficos es obligatorio que este porcentaje sea mayor del 50%.

Los análisis de las muestras se llevaron a cabo en un equipo de FTIR espectrometro Varian 660, equipado con un detector de banda estrecha merucrio cadmio telurico (Fig 5.1), utilizando un accesorio de reflectancia total atenuada (Attenuated Total Reflectance, ATR) MIRacle (Pike technologies) con un elemento de reflexión interna de diamante de tres reflexiones muy adecuada para el tipo de muestras medidas, aceites vegetales. Para este tipo de muestras de gran viscosidad, la celda de medida del equipo ATR presenta ventajas frente a las celdas de flujo al ser sencillas de medir y más fácil de limpiar.

La técnica de ATR, desarrollada por Harrick N.J. y Fahrenfort J. [2,3], se basa en la reflectancia interna que es transmitida por un material con un alto índice de refrac-

[1] Commision Regulation (EC) No. 1019/2002, on marketing standards for olive oil, Official Journal of the European Communities, L 155/27, 2002

[2] Harrick N J (1960) Surface chemistry spectral analysis of totally internally reflected radiation, J Phys Chem 64:1110-1114

[3] Fahrenfort J (1961) Attenuated total reflectance, Spectrochim Acta 17: 698-709

ción (IRE) al recibir un haz infrarrojo. Un cristal construido con un material IRE permite una reflexión interna total creando una onda evanescente sobre la superficie del mismo. Esta onda penetra en la muestra, distribuida en una fina capa sobre la superficie del cristal, produciendo un espectro de infrarrojo de la misma. La profundidad de la pe-

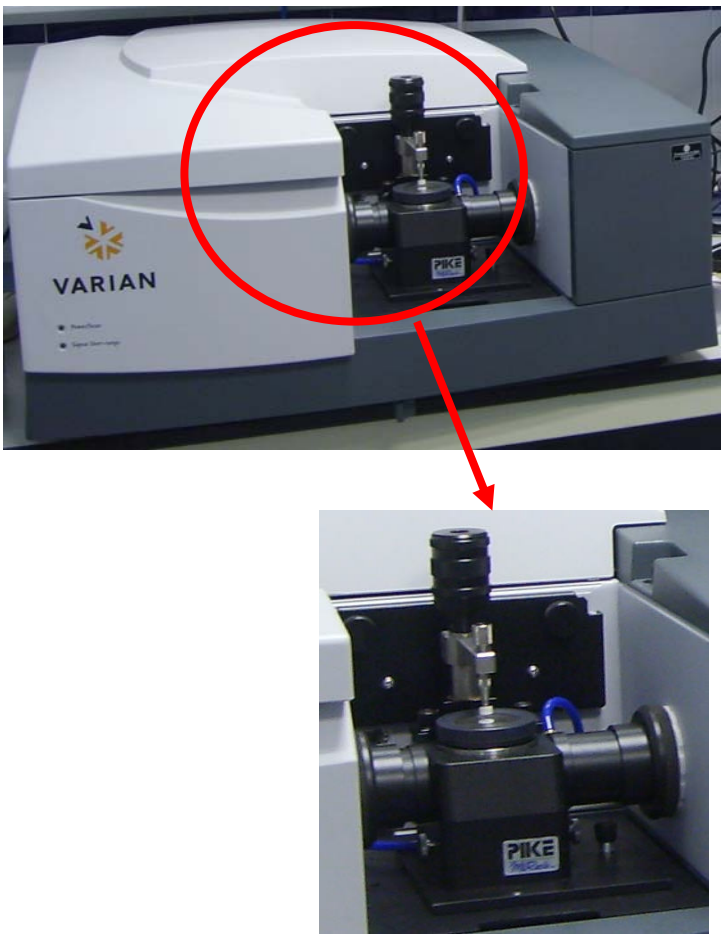

Fig1. Equipo FTIR con ATR utilizado en las medidas

netración de la onda depende del ángulo de incidencia del haz infrarrojo, del tipo de material del cristal y del tipo de muestra. Generalmente la profundidad de penetración oscila entre 0.5-2 µm [4] (Fig. 5.2).

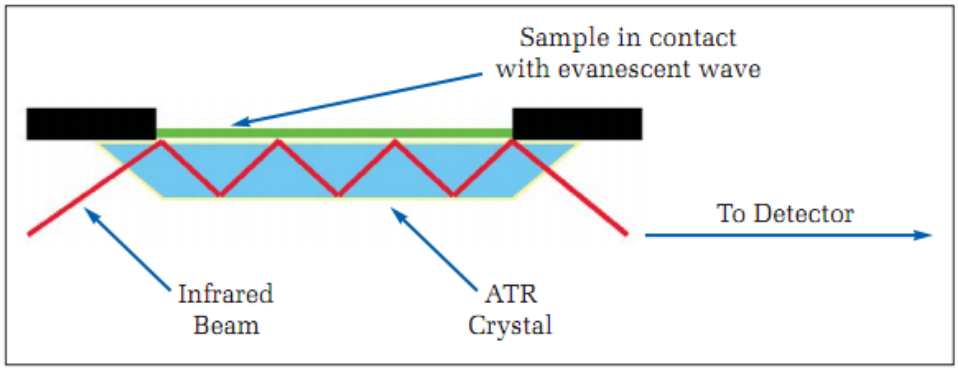

Fig 5.2. Funcionamiento del ATR [5]

Los aceites vegetales presentan picos bien definidos en las regiones 3050-1700 cm^{-1} y picos solapados en la región de la huella espectral entre 1500 y 700 cm^{-1}. Las bandas más representativas se presentan en la tabla 5.1. Diferentes aceites vegetales presentan diferencias en la posición de los picos, así como en la absorbancia de las bandas, esto es debido a la diferente composición química de los aceites en triglicéridos, ácidos grasos, etc (Fig. 5.3) [6,7].

[4] Wilson R H (1990) Fourier transform mid-infrared spectroscopy for food anlaysis, Trends Anal Chem 9:127:131

[5] PerkinElmer Life and Analytical Science, Technical note, FT-IR spectroscopy, http://las.perkinelmer.com/content/TechnicalInfo/TCH_FTIRATR.pdf, Diciembre 2010

[6] Tay A, Singh R K, Krishnan S S, Gore J P (2002) Authentication of olive oil adulterated with vegetable oils using Fourier transform infrared spectroscopy, Lebensm-Wiss. u. Technol. 35:99-103

Tabla 5.1 Bandas de FTIR más representativas de aceites comestibles [8,9].

Número de onda (cm^{-1})	Banda
Ligeramente arriba de 3000	Estiramiento de ═C—H (cis)
2927-2854	Estiramiento vibracional simétrico y asimétrico de —C—H (CH_2)
1745	Estiramiento vibracional de —C═O de los triglicéridos
1654	Estiramiento vibracional —C═C— (cis) de las olefinas
1400-1200	Flexión vibracional de grupos alifáticos —C—H (CH_2, CH_3)
1125-1095	Estiramiento vibracional de grupos ester C—O
723	Balanceo o rocking de —$(CH_2)_n$, —HC═CH—(cis)

Los aspectos y resultados principales del trabajo realizado con el método FTIR-ATR para la clasificación y cuantificación de aceite de oliva en mezclas de aceites vegetales, se recogen en el siguiente apartado (5.2). La publicación bajo el nombre de **Olive oil assessment in edible oil blends by means of mid-infrared spectroscopy and chemometrics**, enviada a la revista Analytica Chimica Acta.

[7] Yang H, Irudayaraj J, Paradkar M M (2005) Discrimination analysis of edible oils and fat by FTIR, NT-NIR and FT-Raman, Food Chem 93:25-32

[8] Ayora-Cañada M.J, Domínguez-Vidal Ana, Lendl B. (2010), Monitoring Oxidation of Lipids in Edible Oils and Complex Food Systems by Vibrational Spectroscopy, Hanbook of Vibrational Spectroscopy. DOI: 10.1002/0470027320.s8946

[9] Guillén M.D., Cabo N(1997) Infrared spectroscopy in the study of edible oils and fats, J Sci Food Agric 75:1-11

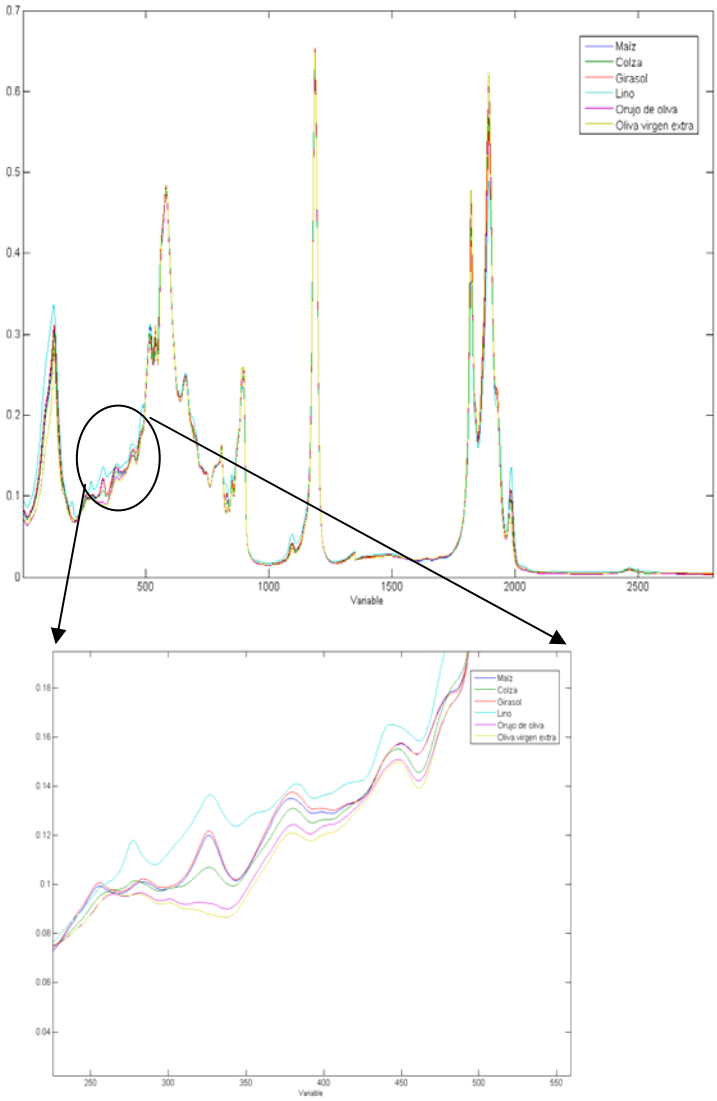

Fig5.3 Espectro de FTIR-ATR de diferentes aceites vegetales

5.2 Artículo enviado al Analytica Chimica Acta

Olive oil assessment in edible oil blends by means of mid-infrared spectroscopy and chemometrics

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ABSTRACT

According to European trade standards only if an oil blend contains more than 50% of olive oil it can be marketed with images or graphics referring to the presence of olive oil. In this work a fast method for verifying the correct labeling of oil blends based in FTIR spectroscopy and chemometrics was developed. First, the capability of the technique to distinguish olive oil samples of different varieties, categories and origins from other types of vegetable oils was tested using a wide collection of pure samples. Then, using a method based on partial least squares discriminant analysis (PLS-DA) it was possible to differentiate between blends with olive oil content higher than 50% and below 50% on the basis of their first derivative ATR-FTIR spectra.

Keywords: Olive oil blends, pattern recognition, Principal Component Analysis, Partial Least Square-Discriminant Analysis, ATR-FTIR

Introduction

The market for olive oil is continuously increasing in the last years due to the organoleptic properties of olive oil and because it is considered important for a healthy diet. Apart from the direct intake of olive oil, many foodstuffs, such as, sauces, bakery foods, can food, chips, cold meats, soups, etc. contain olive oil as ingredient. The European Union legislation attempts to protect the consumer against misleading commercial practices, like highlighting the reputation of olive oil without clearly specifying the real composition of the product. Thus, Commission Regulation (EC) No. 1019/2002 [1], concerning commercialization and labeling of products which contain olive oil, establishes that the percentage of olive oil must be always clearly indicated on the labeling if its presence is emphasized by means of words, images or graphics. In addition, in the case of blends of olive oil with other edible vegetable oils, the presence of olive oil can only be highlighted if it accounts for more than 50% and the exact percentage of olive oil in the blend has to be also indicated on the label.

To the authors' knowledge, nowadays, there are not official methods to verify the requirements indicated in this Regulation. Therefore, there is a need to develop analytical methods to detect and quantify olive oil in edible oil blends. Detection and quantification of olive oils are not simple tasks because olive oil is a complex substance. Some of the difficulties are based on the production system and others are related to final quality. There is a large quantity of varieties of olive trees and olive oil could be produced from a single variety of olive, named monovarietal, or it could be marketed as a blend of olive varieties. The final quality of the olive oil is related to different categories (grades) recognized by the International Olive Council [2]: "extra-virgin" (EVOO), "virgin" (VOO), "olive oil" (OO), "refined" (ROO) and "pomace" (POO), which is a blend of virgin and refined olive oil. These olive oil categories, with some minor modifications in the nomenclature, have also been recognized by regulation in EU [3] and USA [4].

Table 1. Vegetable oil samples

Sample Code	Type of Oil	Composition	Quantity
CAN	canola	100% canola	4
COR	corn	100% corn	5
FLA	flaxseed	100% flaxseed	3
GRA	grape seed	100% grape seed	4
PEA	peanut	100% peanut	2
RAP	rapeseed	100% rapeseed	4
SAF	safflower	100% safflower	1
SES	sesame	100% sesame	2
SOY	soybean	100% soybean	5
SUN	sunflower	100% sunflower	9
SUNO	high oleic sunflower	100% sunflower	2

Up to date most of the effort has been dedicated to the detection of adulterants in olive oil. Different analytical techniques have been used to quantify the amount of different cheaper vegetable oils employed as adulterants in olive oils. Chromatographic methods, such as HPLC and GC, are the most common used [5,6,7]. The principal problematic of these techniques is the intensive use of organic solvents and the production of chemical waste. Therefore, spectroscopic techniques, as Fourier transform infrared (FTIR), present an alternative solution when it is used in combination with chemometric tools. These spectroscopic methods have the advantage that time consuming sample preparation steps are kept to a minimum and even completely overcome. There are diverse approaches to determine authenticity of olive oil using FTIR and chemometric techniques. Principal component analysis (PCA) has been used with different vegetable oils and EVOO, obtaining acceptable results [8]. Blends of edible oils with OO, particularly with EVOO, have been studied using partial least squares-dicriminant analysis, PLS-DA [9]. Other studies had been applied using FTIR and PLS to different blends with vegetable oils [10,11,12,13]. However, these previous researches had considered a limited number of categories

and varieties of olive oils in the studies. These different variables should be taken into account if the aim is to discriminate and quantify between olive oils and edible oil blends.

The aim of this study is filling out the need of an analytical method to verify the percentage of olive oil presents in edible oil blends using a fast analytical technique. For this objective, first we have conducted a feasibility study with pure oils to test the capability of the technique to distinguish a wide collection of different olive oil samples from several types of edible vegetable oils. Then, we have developed and tested methods to classify and quantify blends of olive oils with vegetable oils.

Experimental

Instrumentation and FTIR measurements

Infrared spectra were recorded in the range from 3800 to 600 cm^{-1} with a Varian 660 Fourier transform infrared spectrometer equipped a narrow-band mercury cadmium telluride (MCT) detector. A MIRacle attenuated total reflexion (ATR) accessory (Pike technologies) with a three-reflection diamond internal reflection element (IRE) was used. For measurement, a drop of oil was deposited on the surface of the diamond IRE. All spectra were recorded at 2 cm^{-1} resolution and were average of 128 scans. The oil was removed with a dry tissue and surface of the IRE was cleaned first with methanol and then with distilled water. Finally, it was dried with a clean tissue. Spectra of the clean and dry diamond IRE against air were recorded before each sample measurement and used as background.

Oil samples

Oils samples were purchased in retail from Spain, France, Mexico, Italy and USA. 111 oils samples were used; 41 were edible vegetable oils samples

(Table 1) and 70 were olive oils of different categories: extra-virgin (blends of varieties and monovarietal), virgin, refined (for simplicity, under this designation we consider the market blend of virgin and refined olive oil) and pomace (Table 2).

76 blend samples were prepared by mixing one olive oil with one vegetable oil in different percentages obtaining five different levels of concentration. The blends that are presented in Table 3 cover all the possible combinations of vegetable oils, considering the different categories and varieties of olive oils.

The samples, after opening, were bottled in amber glass flask and maintained in dark at −2 ºC until analysis.

Statistical analysis

Data were analyzed using MATLAB® 7.8.0 (R2009a The Maths Inc., Natick, MA, USA) and PLS toolbox 5.5 (Eigenvector Research Inc., West Eagleрock Drive, Wenatchee, WA). For the grouping and classification models, cluster, PCA, and PLS-DA were used respectively. PLS were used for olive oil quantification.

Results and discussion

Infrared spectra of oil samples

Figure 1 shows typical FTIR spectra of oil samples. The major bands in mid-IR can be attributed to the following vibrations: from high to low wavenumber, the band at 3008 cm^{-1} is due to the CH stretching of *cis* double bonds, whereas bands centered at 2924 and 2854 cm^{-1} are due to the antisymmetric and symmetric stretching vibrations, respectively, of aliphatic C-H in CH_2 and

Table 2. Olive oil samples

Sample Code	Type of Oil	Category	Variety	Composition	Quantity
EVOO	olive	extra-virgin olive		100% olive	14
VOO	olive	virgin olive		100% olive	3
REF	olive	refined olive		100% olive	4
POM	olive	pomace olive		100% olive	3
ARB	olive	extra-virgin olive	arbequina	100% olive	8
BLA	olive	extra-virgin olive	blanqueta	100% olive	1
CORNI	olive	extra-virgin olive	cornicabra	100% olive	6
EMP	olive	extra-virgin olive	empeltre	100% olive	1
FRA	olive	extra-virgin olive	frantoio	100% olive	6
HOJ	olive	extra-virgin olive	hojiblanca	100% olive	8
LEC	olive	extra-virgin olive	lechin	100% olive	1
MAN	olive	extra-virgin olive	manzanilla	100% olive	3
PIC	olive	extra-virgin olive	picual	100% olive	9
PID	olive	extra-virgin olive	picudo	100% olive	1
ROY	olive	extra-virgin olive	royal	100% olive	1
VER	olive	extra-virgin olive	verdial	100% olive	1

terminal CH_3 groups; the strong single peak of the C=O stretching vibration of carbonyl groups of the triglycerides is observed at about 1745 cm^{-1}; the extremely weak band near 1654 cm^{-1} corresponds to the stretching vibration of the C=C group of *cis*-olefins; bands in the 1400-1200 cm^{-1} region are mainly attributed to bending vibrations of CH_2 and CH_3 aliphatic groups like symmetric HCH

Table 3 Oil blend samples

Sample	Composition	Sample	Composition
mz-034	POM 10% + SUN 90%	mz-072	POM 30% + RAP 70%
mz-035	POM 50% + SUN 50%	mz-073	POM 70% + RAP 30%
mz-036	POM 90% + SUN 10%	mz-074	EVOO 50%+ RAP 50%
mz-037	EVOO 30%+ SUN 70%	mz-075	VOO 10%+ RAP 90%
mz-038	VOO 70%+ SUN 30%	mz-076	VOO 90%+ RAP 10%
mz-039	ARB 10% + SUN 90%	mz-077	ARB 30%+ RAP 70%
mz-040	ARB 50%+ SUN 50%	mz-078	ARB 70%+ RAP 30%
mz-041	ARB 90%+ SUN 10%	mz-079	HOJ 10% + RAP 90%
mz-042	HOJ 30%+ SUN 70%	mz-080	HOJ 50%+ RAP 50%
mz-043	HOJ 70%+ SUN 30%	mz-081	HOJ 90%+ RAP 10%
mz-044	PIC 10% + SUN 90%	mz-082	PIC 30%+ RAP 70%
mz-045	PIC 50%+ SUN 50%	mz-083	PIC 70%+ RAP 30%
mz-046	PIC 90%+ SUN 10%	mz-084	POM 10% + SES 90%
mz-047	POM 30% + COR 70%	mz-085	POM 50% + SES 50%
mz-048	POM 70% + COR 30%	mz-086	POM 90% + SES 10%
mz-049	EVOO 10%+ COR 90%	mz-087	EVOO 30%+ SES 70%
mz-050	VOO 50%+ COR 50%	mz-088	VOO 70%+ SES 30%
mz-051	EVOO 90%+ COR 10%	mz-089	ARB 10% + SES 90%
mz-052	ARB 30%+ COR 70%	mz-090	ARB 50%+ SES 50%
mz-053	ARB 70%+ COR 30%	mz-091	ARB 90%+ SES 10%
mz-054	HOJ 10% + COR 90%	mz-092	HOJ 30%+ SES 70%
mz-055	HOJ 50%+ COR 50%	mz-093	HOJ 70%+ SES 30%
mz-056	HOJ 90%+ COR 10%	mz-094	PIC 10% + SES 90%
mz-057	PIC 30%+ COR 70%	mz-095	PIC 50%+ SES 50%
mz-058	PIC 70%+ COR 30%	mz-096	PIC 90%+ SES 10%
mz-059	POM 10% + SEED 90%	mz-097	POM 30% + SOY 70%
mz-060	POM 50% + SEED 50%	mz-098	POM 70% + SOY 30%
mz-061	POM 90% + SEED 10%	mz-099	EVOO 10%+ SOY 90%
mz-062	EVOO 70%+ SEED 30%	mz-100	VOO 50%+ SOY 50%
mz-063	VOO 30%+ SEED 70%	mz-101	EVOO 90%+ SOY 10%
mz-064	ARB 10% + SEED 90%	mz-102	ARB 30%+ SOY 70%
mz-065	ARB 50%+ SEED 50%	mz-103	ARB 70%+ SOY 30%
mz-066	ARB 90%+ SEED 10%	mz-104	HOJ 10% + SOY 90%
mz-067	HOJ 30%+ SEED 70%	mz-105	HOJ 50%+ SOY 50%
mz-068	HOJ 70%+ SEED 30%	mz-106	HOJ 90%+ SOY 10%
mz-069	PIC 10% + SEED 90%	mz-107	PIC 30%+ SOY 70%
mz-070	PIC 50%+ SEED 50%	mz-108	PIC 70%+ SOY 30%
mz-071	PIC 90%+ SEED 10%	mz-109	EVOO 70%+ SUN 30%

bending at 1377 cm^{-1} and CH_2 scissoring at 1462 cm^{-1}; bands in the 1125-1095 cm^{-1} region correspond to the stretching vibration of C-O ester groups and CH_2 wag; finally, below 1000 cm^{-1}, the band near 723 cm^{-1} is due to the overlapping of the $(CH2)_n$ rocking vibration and the out-of-plane vibration (CH wag) of *cis*-di-substituted olefins. Different oils show slight differences both in the position and absorbance of the bands because of their different triglyceride composition.

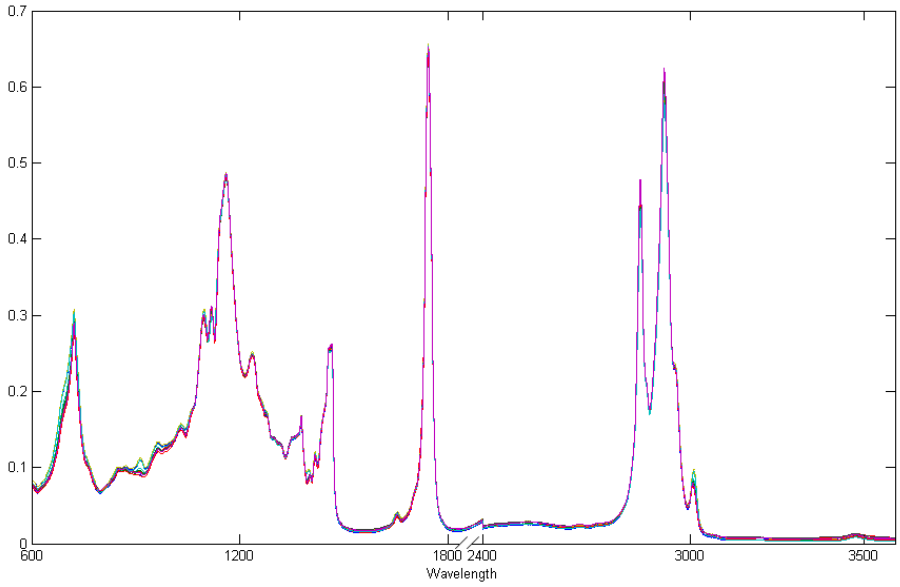

Fig 1. FTIR spectra of different vegetable oils

Data preprocessing

Data were transformed in order to be suitable for chemometric analysis. The region used for data analysis was from 3588 to 650 cm^{-1} (Fig.1) where the characteristic bands of vegetable oils appear. The spectral region from 1870 to

2350 cm^{-1} was eliminated because of the absence of useful bands and the strong absorbance of the diamond IRE.

Two matrices (samples by wavenumbers) were prepared, one for pure oils samples (111x2584) and another one for blend oil samples (74x2584) (Figure 1). Different pre-processing tools were applied to the raw data, obtaining the best results with a baseline correction that was performed on the 1st derivative spectra with order 2. Spectra were also normalized and mean centered for PCA, PLS-DA and PLS models.

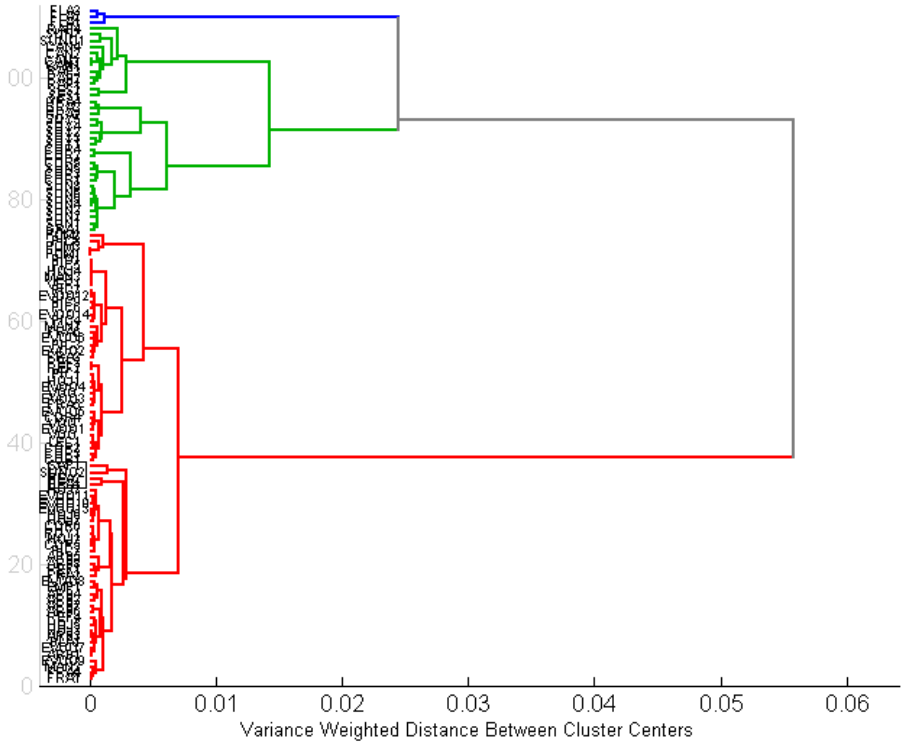

Fig. 2 Dendrogram of pure oils. Green: non-olive oil; red: olive oils and peanut, safflower and high oleic sunflower oil and blue flaxseed oils

Studies of pure oils

Hierrchical Cluster

Hierarchical Cluster analysis (HCA) was performed in order to observe similarities or dissimilarities between the oil samples. HCA was applied to data using the Ward method [14]. The results were presented in a dendogram structure, showing the different groups. Figure 2 shows the plot obtained from the

111 pure oil samples with three main clusters with a similarity of about to 0.75. The first cluster (from bottom to top), in red color, comprises all olive oil samples. It also includes four non-olive samples (peanut, safflower and high oleic sunflower oil). This result suggests that these oils have a physical-chemical profile more similar to olive oil than the others. In the second cluster, green color, most of non-olive oil samples are grouped and the third, blue color is a small cluster formed by a group of flaxseed oils. Detection of such natural groupings suggests that discrimination between olive oil and the rest of the vegetables oils may be possible.

PCA

The PCA analysis was applied to the matrix that contained the FTIR spectra of the pure oils (111 samples). The first three principal components explained 98.68% of variance and the results are shown in Figure 3 and 4 (score and loading plots, respectively). The score plots show certain separation between olive oil samples (green color) and non-olive oil samples (red color). In fact, in the space defined by the two first components (Figure 3A) that accounted for the 97.63% of the variance olive oil samples form a very tight cluster. The first principal component is the one which differentiates better the two groups; olive oil samples have negative scores while most of non-olive oil samples have

positive scores. This component is possibly related to the total unsaturation as can be seen in the loading plot. The loading of PC1 has the strongest contribution in the region form 2800-3030 cm^{-1}, and concretely in 3003 and 3017 cm^{-1}, the characteristic region of CH stretching in *cis* olefins. The samples that are outside the 95% confidence ellipse are flaxseed (linseed) oil (Figure 3A) with very high positive scores in both PC1 and especially in PC2. Flaxseed oil is a drying oil with a very high content in poly-unsaturated fatty acids, particularly linolenic acid, which explains the difference with the rest of the oils. Looking at the loading of the PC2 the most intense contribution is the band at 2956 cm^{-1}. This band can also be found in the first derivative FTIR spectra of triglycerides containing linolenic acid. PC3 is a minor component that enhances differences among all the samples. Samples of rapeseed and canola oils show high positive scores for PC3, whereas sesame and some samples of olive oil show the largest negative scores.

PLS-DA

According to the results obtained by PC analysis, a classification model was proposed to distinguish two categories within the vegetable oil samples, olive oil and non-olive. To do this, PLS-DA was performed using a set of predictor variables (X-data) which are correlated with response variables (Y-data). Response variables are a dummy matrix consisting of zeros and ones. The dummy matrix has as many columns as classes in the predictor variables. In this model, each sample has a value of one for the class that it belongs and zeroes for the others. One sample is assigned to a certain class when tha predicted value is higher than 0.5. The performance of classification was evaluated using root-mean-squared error of calibration (RMSEC), and root-mean-squared error of prediction (RMSEP) for the external validation of the model [15]. These parameters were determined by the following equations:

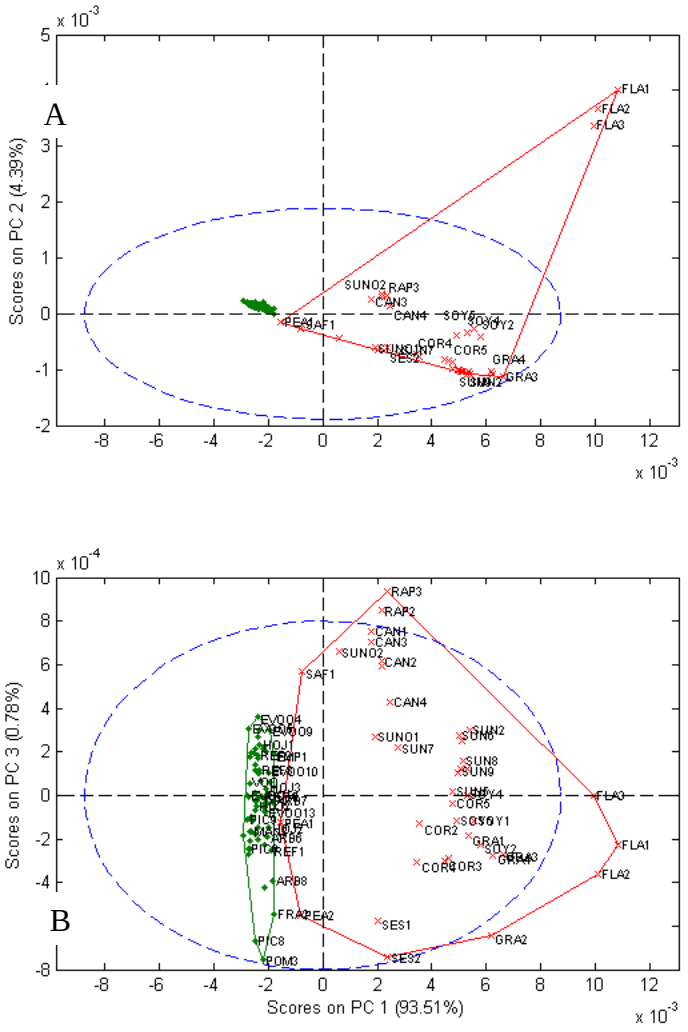

Figure 3. PCA scores of pure oils. Red: non-olive oil; green: olive oils. 95% confidence ellipse in blue

$$\text{RMSEC} = \sqrt{\frac{\sum_{I=1}^{n}(\hat{y}_i - y_i)^2}{n - k - 1}} \qquad (1)$$

$$RMSEP = \sqrt{\frac{\sum_{I=1}^{n} (\hat{y}_i - y_i)^2}{n}} \qquad (2)$$

where: $\hat{y}_i$ is the values of the predicted variable when all samples are included in the model; n is the number of calibration samples; and k is the number of components or latent variables.

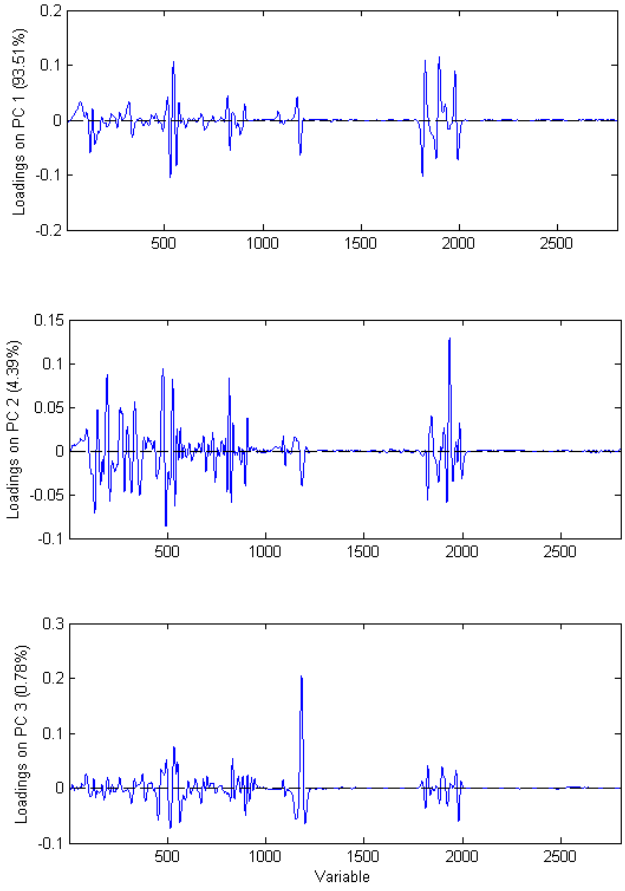

Figure 4. First three PCA loadings of pure oils

A calibration set of 94 samples was used to build the model and 17 samples were used for external validation. Both sets included olive and non-olive oils and were randomly constructed. Two latent variables were used for the PLS-DA model because the third and beyond latent variables did not contribute significantly. In these conditions, 97.2% of the variance was explained in X and 83.7% in Y. As can be seen in Figure 5, all the samples were correctly classified in both the calibration and the validation sets with RMSEC and RMSEP values of 0.190 and 0.168, respectively. The good performance of the method demonstrates the capability of the technique for the discrimination between olive oil and other vegetable oils. These findings suggest the possibility of using FTIR spectroscopy to analyse samples that contain olive oil in blends with different vegetable oils.

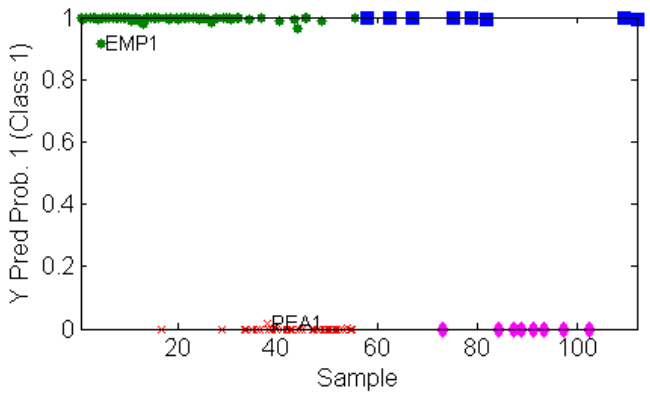

Figure 5. PLSDA of pure oils. Red: non-olive oil calibration set; green: olive oils calibration set. Blue: non-olive oil validation set; pink: olive oils validation set.

Studies of blend oil samples

This study attempts to develop a method to verify the requirements indicated in the Regulation (EC) No. 1019/2002. Table 3 summarizes the 76 samples employed for this study.

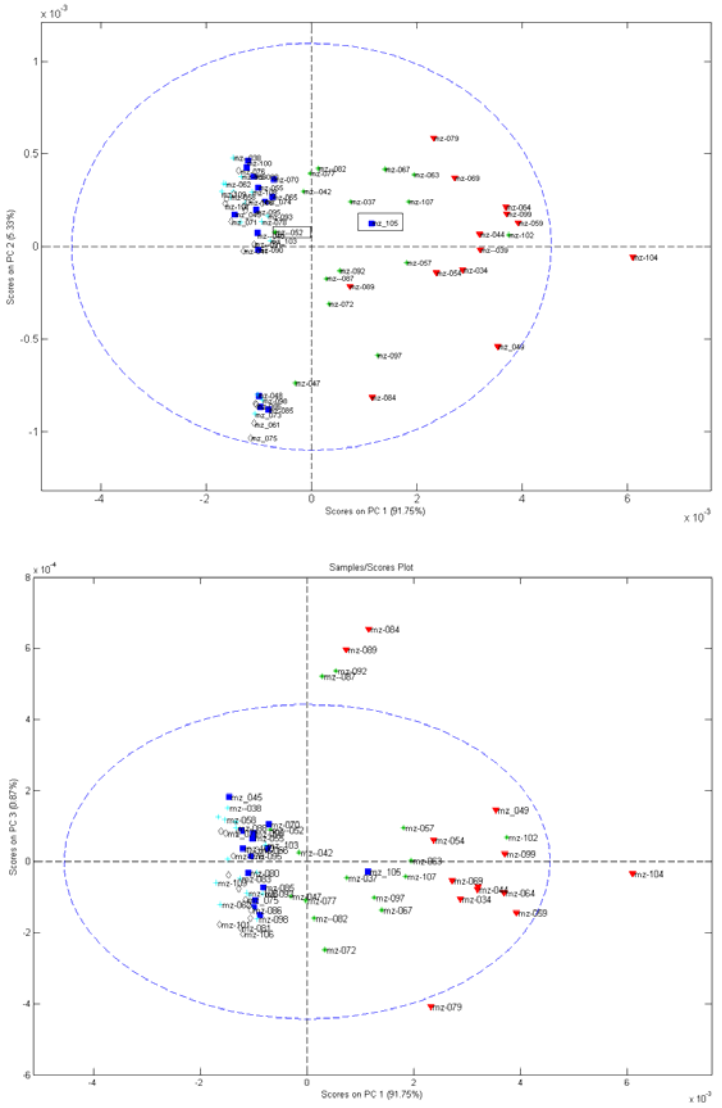

Figure 6. PCA score plots of blend oil samples. Red: 10% olive oil, Green: 30% olive oil, Blue: 50% olive oil, Cyan: 70% olive oil and White: 90% olive oil. 95% confidence ellipse in blue

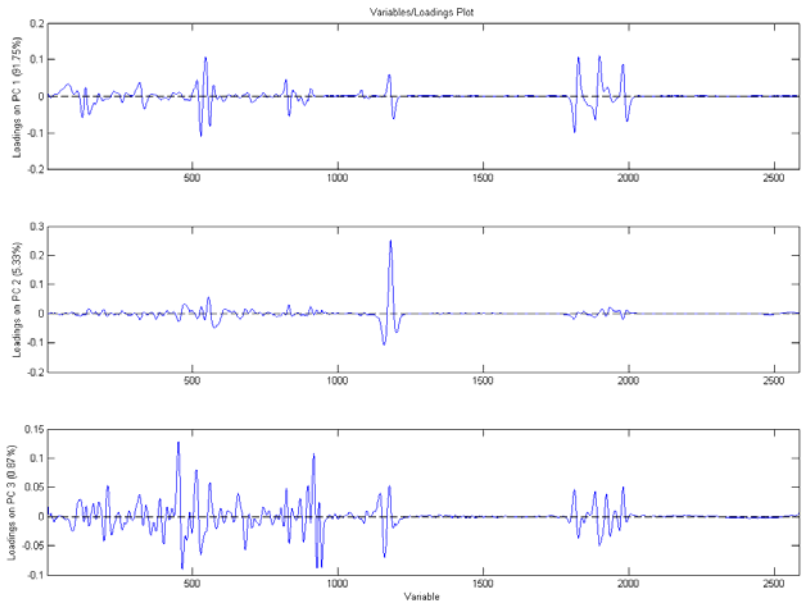

Figure 7. Three first PCA loadings of blend oil samples.

PCA

Exploring the FTIR spectra of blend samples with PCA showed that three principal components explained 97.95% of spectral variance. The first component achieved 91.75% while the second and third component 5.33% and 0.87% respectively. Score and loading plots are shown in figures 6 and 7, respectively. PC1 seems to be related to the amount of olive oil in the blends, samples with low amount of this oil have high positive scores while most of the samples with olive oil content higher than 50%, form a cluster with negative scores. Only two samples (mz_105 and mz_052) are located in an inappropriate group in relation to their olive oil percentage. The corresponding loading is very similar to the above commented loading for PC1 when only pure oils were con-

sidered. This fact indicates that the separation is again due to the unsaturation level of the oil blend.

In general blend samples with olive oil content lower than 50% do not show a clear grouping, whereas, it is interesting to note that PC2 split the samples with higher contents of olive oils in two groups. A major group with positive scores and a minor group with negative scores formed by samples containing olive pomace oil in the blend were observed. The main spectral contribution observed in the loading of this component is in the region of 1740 cm^{-1} and can be attributed to the stretching of C=O groups.

Finally, considering score for PC3, a grouping of samples is also observed with high positive values outside of the confidence ellipse. All these samples contained high amount of sesame oil.

PLS_DA

According to the European Regulation, the classification of blends samples into two groups containing “more than” or “less than” 50% of olive oil would be desirable. Thus, PLS-DA was employed with this objective. The analysis was performed using 65 samples as calibration set. The model was also validated with an external set of 11 samples. Using one latent variable the variance explained for X was 84.77%. Only two samples of the validation set were misclassified in a model with RMSEC of 0.304 and RMSEP 0.350 (Figure 8).

PLS Results

Finally, an attempt to quantify the amount of olive oil in the blends was perfomed using PLS regression. All blend samples containing 10-90 % of olive oil in their composition were analyzed (76 samples). The performance of the model was evaluated with R2, RMSEC and root mean squared error of cross validation (RMSECV).

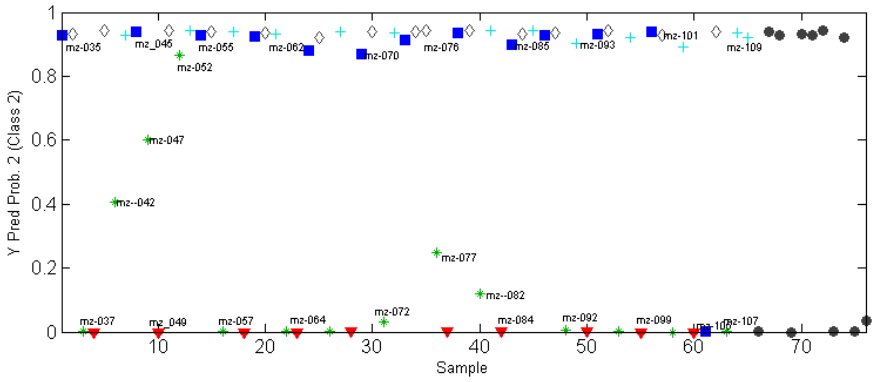

Figure 8. PLSDA of blend oil samples.

$$RMSECV_k = \sqrt{\frac{PRESS_k}{n}} \quad (3)$$

where: k is the number of latent variables; PRESSk is the sum of squared prediction error for the model and n is the number of calibration samples.

This model failed in the quantification for samples containing more than 70 % of olive oil, presenting values for R2 of 0.734 and for RMSECV of 14.39.

This can be explained considering the results of PCA where these samples formed a very tight cluster. To see if there is possible to quantify lower amounts of olive oil, a second model was built with the samples with concentrations of olive oil ranging from 0 to 50% (49 samples). The model presented a better prediction of the samples, obtaining a R2 of 0.79 and an error percentage prediction of the model, RMSECV, of 8.28. Although this model improved the results, they cannot be considered satisfactory. Nevertheless, a semi-quantitative estimation of the amount of olive oil can be obtained in a fast manner.

Conclusions

The information contained in the ATR-FTIR spectra of vegetable oils can be used to distinguish between olive oils and other edible oils. The characteristic spectral bands of triglycerides containing unsaturated fatty acids have been observed to constitute the main basis of the discrimination. Furthermore, the results obtained demonstrated that ATR-FTIR can be a valuable tool for the analytical control of oil blends containing olive oil. Even when only a semi-quantification can be performed the method is suitable for screening of edible oil blends with a view to distinguishing blends with content lower than 50% from those with content higher than 50% which satisfies European legislation requirements.

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References

1 Commision Regulation (EC) No. 1019/2002, on marketing standards for olive oil, Official Journal of the European Communities, L 155/27, 2002

2 COI/T.15/NC No 3/Rev. 4, Trade standard applying to olive oils and olive-pomace oils (2009). International Olive Council, Madrid

3 Commission Regulation (EEC) No 1989/2003 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-pomace oil and on relevant methods of analysis, Off J Eur Commun L295/57

4 AMS-FV-08-0073-0006 (2010). United States Standards for Grades of Olive Oil and Olive-Pomace Oil. US Department of Agriculture, Washington

5 Aparicio R, Aparicio-Ruíz R (2000) J Chromatogr A 881: 93-104

6 E. Cristopoulou, M. Lzaraki, M. Komaitis, K. Kaselimis, Food Chem. 84 (2004) 463-474

7 F. Priego Capote, J. Ruiz Jiménez, M.D. Luque de Castro, Anal. Bioanal. Chem. 388 (2007) 1859-1865

8 Lai Y L., Kemsley E K, Wilson R H (1994) J Agric Food Chem 42:11454-1159

9 Obeidat S W, Khanfar M S, Obeidat W M (2009) Australian J Basic Appl Sci 3:2048-2053

10 Gurdeniz G, Ozen B (2009) Food Chem 116:519-525

11 Maggio R M, Cerretani L, Chiavaro E, Kaufman T, Bendini A (2010) Food Control 21:890-895

12 Lerma-García M J, Ramis-Ramos G, Herrero-Martínez J M, Simó-Alfonso (2010) Food Chemistry 118: 78-83

13 Tay A, Singh R K, Krishnan S S, Gore J P (2002) Lebnsm-Wiss. u. Technol. 35:99-103

14 Varmuza K, Filzmoser P (2009) Introduction to multivariate statistical analysis in chemometrics. CRC, London

15 Wise B.M., Gallagher N.B., Bro R., Shaver J.M., Windig W., Koch R.S., PLS_Toolbox 4.0 Manual for use with MATLABTM, Eigenvector Research, Inc., Wenatchee, WA, USA, 2006

5.3 Discusión

En este capitulo se realizó un estudio con aceite de oliva y aceites vegetales utilizando metodología FTIR-ATR en combinación con herramientas quimiométricas. Se obtuvieron resultados satisfactorios en la clasificación y discriminación de aceite de oliva frente a aceites comestibles vegetales. En este estudio se han considerado diferentes variedades y categorías de aceite de oliva, así como diferentes tipos de aceites vegetales. Teniendo en cuenta esta variabilidad, los resultados obtenidos en la clasificación fueron satisfactorios presentando una correcta clasificación del 90% de las muestras.

Con respecto a la cuantificación de aceite de oliva en mezclas de aceites vegetales, los modelos no tuvieron resultados satisfactorios, ya que presentan variabilidad significativa. Adicionalmente, los modelos no presentan una buena cuantificación a partir de 50% de aceite de oliva. Sin embargo, utilizando el PLS-DA en las mezclas se pudieron clasificar las muestras en dos grupos, mayores y menores de 50% de aceite de oliva. Así mismo, los resultados de la clasificación entre los dos grupos fueron buenos, obteniendo una correcta clasificación en más de un 90% de las muestras. Estos resultados permiten cubrir la necesidad de un método analítico que permite verificar el requisito del Reglamento de la Unión Europea (EC) No. 1019/2002 que establece que se debe declarar el porcentaje del aceite de oliva si su contenido es mayor al 50% en el producto.

CONCLUSIONES GENERALES

En este capítulo se resumen las conclusiones generales del trabajo realizado en la presente tesis doctoral. Las conclusiones específicas de cada objetivo se han establecido en los correspondientes artículos publicados y en un breve apartado de discusión al final de cada uno de los capítulos.

Las conclusiones se discuten a continuación:

1. Se obtuvieron buenos resultados en el procedimiento de certificación de cuatro materiales de referencia. El estudio de la homogeneidad y estabilidad de los materiales puso de manifiesto la adecuación de dichas propiedades para el estudio de certificación. Se ha establecido un periodo de 2 años de estabilidad para los materiales a partir de la fecha de preparación. De las 50 características químico-físicos estudiadas se han certificado 29, estableciéndose intervalos de validez para las restantes. Estos cuatro materiales de referencia certificados son los primeros materiales desarrollados en su campo para el control de calidad y para la validación de métodos analíticos en las medidas de caracterización de aceites de oliva.

2. El método propuesto de HPLC-CAD, para la caracterización y cuantificación de TGs en aceite de oliva, presentó dos principales ventajas frente al método oficial de la Unión Europea: menor tiempo de análisis y mejor sensibilidad. La ausencia de efecto matriz y la existencia comprobada de linealidad en el rango estudiado permitió la cuantificación de los TGs utilizando el factor de respuesta con un patrón interno. El método puede ser aplicado a la determinación de TGs en alimentos que contengan aceite de oliva como patatas fritas, embutidos y productos de panadería.

3. Usando los perfiles cromatográficos completos de los TGs se realizaron estudios quimiométricos satisfactorios. La agrupación y clasificación de los aceites de oliva y aceites comestibles vegetales tuvieron buenos resultados al clasificar todas las muestras en los dos grupos (aceite de oliva y aceites vegetales) correctamente.

4. Se logró cuantificar aceite de oliva en mezclas de aceites vegetales con los cromatogramas completos usando la metodología HPLC-CAD con técnicas quimiométricas. Se

utilizó toda la variabilidad posible en las muestras (diferentes tipos y mezclas de aceites vegetales), obteniendo cuantificaciones válidas desde 10%-90% de aceite de oliva.

5. Se realizó un estudio de aceites de oliva y vegetales con metodología FTIR-ATR, en el infrarrojo medio, y técnicas quimiométricas, obteniendo buenos resultados en la agrupación y clasificación tanto de muestras puras como en mezclas de dichos aceites. Se ha podido establecer un criterio para diferenciar entre porcentajes mayores y menores de 50% de aceite de oliva.

ANEXO

PUBLICACIONES PRESENTADAS EN LA TESIS

L. Cuadros-Rodríguez a, J.M. Bosque-Sendra a, A.P. de la Mata-Espinosa, A. González-Casado, F.P. Rodríguez-García Elaboration of four olive oil certified reference materials: InterOLEO-CRM 2006 certification study, Food Anal. Meth. 1, 259-269 (2008)

J.M. Bosque-Sendra, A.P. de la Mata-Espinosa, L. Cuadros-Rodríguez, A. González-Casado, F.P. Rodríguez-García Stability for olive oil control materials, Food Chem. 125 1418-1422 (2011)

P. de la Mata Espinosa, J.M. Bosque Sendra, L. Cuadros Rodríguez, Quantification of triacylglycerols in olive oils using HPLC-CAD, Food Anal. Meth. (enviado 2010)

P. de la Mata-Espinosa, J.M Bosque-Sendra, R.Bro, L. Cuadros-Rodríguez, Discriminating olive and non-olive oils using HPLC-CAD and chemometrics, Anal. Bioanal. Chem. DOI 10.1007/s00216-010-4366-4

P. de la Mata-Espinosa, J.M Bosque-Sendra, R.Bro, L. Cuadros-Rodríguez, Olive oil quantification of edible vegetable oil blends using triacylglycerols chromatographic fingerprints and chemometrics tools, Talanta (enviado 2010)

P. de la Mata-Espinosa, A. Dominguez-Vidal, J.M Bosque-Sendra, A. Ruiz-Medina, L. Cuadros-Rodríguez, M.J. Ayora- CAñada, Olive oil assessment in edible oil blends by means of infrared spectroscopy and chemometrics, Analytica Chimica Acta (enviado 2011)

OTRAS PUBLICACIONES

C.Ruiz-Samblás, L. Cuadros-Rodríguez , A. González-Casado , F.P. Rodríguez García, P. de la Mata-Espinosa, J. M.Bosque-Sendra, Multivariate analysis of HT/GC-(IT)MS chromatographic profiles of triacylglycerol for classification of olive oil varieties, Anal. Bioanal. Chem. DOI 10.1007/s00216-010-4423-z

Giorgio Tomasi, Francesco Savorani, Paulina de la Mata, Søren B. Engelsen, icoshift as an effective tool for the alignment of chromatographic data, J. of Chromatography A (proceso de envío)

APORTACIONES A REUNIONES CIENTÍFICAS

P. de la Mata Espinosa, L. Cuadros Rodríguez, J.M. Bosque Sendra, F.P. Rodríguez García, Potencialidad del esquema de Youden para la evaluación del desempeño de laboratorios mediante estudios interlaboratorios de aptitud
Comunicación electrónica (en fichero)
Cuarto Congreso Virtual Iberoamericano sobre Gestión de la Calidad en Laboratorios (IV IBEROLAB) 2007

L. Cuadros Rodríguez, P. de la Mata Espinosa, J.M. Bosque Sendra, A. González Casado, F.P. Rodríguez García
InterOLEO-MRC 2006: Elaboration of four certified reference materials of olive oil
Comunicación en cartel
2nd International EuroFIR Congress
Granada (España) 2007

L. Cuadros Rodríguez, J.M. Bosque Sendra, A. González Casado, P. de la Mata Espinosa, F.P. Rodríguez García
Stability study of certified reference materials of olive oil
Comunicación en cartel
VII Scientific Meeting of the Spanish Society of Chromatography and Related Techniques (SECyTA)
Granada (España) 2007

C. Ruiz Samblás, L. Cuadros Rodríguez, P. de la Mata Espinosa
Aplicación de la extracción presurizada con disolventes para la cuantificación del contenido total de grasas en alimentos de panadería
Comunicación en cartel
XI Reunión del Grupo Andaluz de la Soc. Española de Química Analítica (GRASEQA) 2008)
Huelva (España) 2008

P. de la Mata Espinosa, L. Cuadros Rodríguez, J.M. Bosque Sendra, C. Ruiz Samblás
A normalized youden-plot in interlaboratory proficiency tests
Comunicación en cartel
Chemometrics in Analytical Chemistry 2008 (CAC 2008)
Montpellier (Francia) 2008

P. de la Mata Espinosa, L. Cuadros Rodríguez, J.M. Bosque Sendra, C. Ruiz Samblás
Dependencia entre los términos "suave e intenso" del aceite de oliva andaluz y diferentes parámetros fisico-químicos
Comunicación oral
III Workshop de Quimiometría
Burgos (España) 2008

A.P. de la Mata Espinosa, E. Pérez Castaño, C. Ruiz Samblás, L. Cuadros Rodríguez, D. Gázquez Evangelista , A. González Casado
Identification and quantification of TAGs in olive oil by high-temperature GC-FID and GC-IT/MS
Comunicación en cartel
12as Jornadas de Análisis Instrumental (JAI)

Barcelona (España) 2008

P. de la Mata Espinosa, L. Cuadros Rodríguez, J.M Bosque-Sendra, M.G. Bagur-González, F.P. Rodríguez-García
Potencialidad del uso del detector de aerosol de partículas cargadas (CAD) para la caracterización del perfil de triglicéridos en aceites vegetales mediante HPLC
Comunicación oral
XIV Simposium Científico-Técnico de Expoliva 2009
Jaén (España) 2009

P. de la Mata Espinosa, C. Guerrero García, C. Ruiz Samblás, A. González Casado, L. Cuadros Rodríguez
Comunicación en cartel
Extracción (con líquidos presurizados) y caracterización de la fracción grasa de embutidos que contienen aceite de oliva.
EXPOLIVA: XIV Feria Internacional del aceite de oliva e industrias afines.
Jaén (España) 2009

C. Guerrero-García, P. de la Mata Espinosa, C. Ruiz-Samblás, L. Cuadros Rodríguez,
Validación de un método de extracción con líquidos presurizados y cuantificación del contenido de grasa total en patatas fritas envsadas y en embutidos
Comunicación electrónica (en fichero)
Quinto Congreso Virtual Iberoamericano sobre Gestión de la Calidad en Laboratorios (IV IBEROLAB)
red Internet 2009

P. de la Mata Espinosa, L. Cuadros Rodríguez, J.M Bosque-Sendra, F.P. Rodríguez-García
Triacylglycerol compositional profile characterization in food fat extracts, that contains, olive oil, using HPLC coupling to a charged aerosol detector (CAD)
Comunicación oral
Euro Fed Lipid
Graz (Austria) 2009

P. de la Mata Espinosa, R. Bro, J.M. Bosque Sendra, L. Cuadros Rodríguez, F.P Rodríguez García
Olive oil quantification in vegetable oil blends using HPLC-CAD in combination with chemometric tools
Comunicación oral
VII Colloquium Chemiometricum Mediterraneum (CCM VII)
Granada (España) 2010

C. Ruiz Samblás, P. de la Mata Espinosa, L. Cuadros Rodríguez, A. González Casado, F.P. Rodríguez García
Exploratory data analysis of chromatographic profiles of TAGs in varieties of olive oil by using HT/GC-(IT)MS
Comunicación en cartel
VII Colloquium Chemiometricum Mediterraneum (CCM VII)
Granada (España) 2010

P. de la Mata Espinosa, R.Bro, J.M. Bosque Sendra, L. Cuadros Rodríguez, F.P Rodríguez García
Chemometric approach: triacylgycerides profiles in vegetable oils by principal component analysis
Comunicación en cartel
VII Colloquium Chemiometricum Mediterraneum (CCM VII)
Granada (España) 2010

M.J. Ayora Cañada, A. Domínguez Vidal, A.P. de la Mata Espinosa, C. Ruiz Samblás, L. Cuadros Rodríguez
Detección y cuantificación de aceite de oliva en mezclas de aceites empleando espectroscopía infrarroja
Comunicación en cartel
VII Colloquium Chemiometricum Mediterraneum (CCM VII)
Granada (España) 2010

I. García Garrido, P. de la Mata Espinosa, C. Ruiz Samblás, L. Cuadros Rodríguez
Extracción (con líquidos presurizados) y caracterización de la fracción grasa de frutos secos
Comunicación en cartel
XII Reunión del grupo regional andaluz de la sociedad española de Química Analítica, GRASEQA
Córdoba (España) 2010

P. de la Mata-Espinosa, R.Bro, J.M. Bosque-Sendra, L. Cuadros-Rodríguez, F.P. Rodríguez-García
Caracterización y cuantificación del aceite de oliva utilizando HPLC-CAD y Técnicas Quimiométricas
Comunicación oral
XII Reunión del grupo regional andaluz de la sociedad española de Química Analítica, GRASEQA
Córdoba (España) 2010