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"Environmental, Biochemical and Foodstuff Analytical Control"

Functional Food Research and Development Center (CIDAF)

DOCTORAL THESIS

CHARACTERIZATION OF PHENOLIC COMPOUNDS USING SEPARATION TECHNIQUES COUPLED TO MASS SPECTROMETRY OF PLANT EXTRACTS WITH DEMONSTRATED BIOACTIVITY

CARACTERIZACIÓN DE COMPUESTOS FENÓLICOS MEDIANTE TÉCNICAS SEPARATIVAS ACOPLADAS A ESPECTROMETRÍA DE MASAS DE EXTRACTOS VEGETALES CON BIOACTIVIDAD DEMOSTRADA

Presented by

IHSAN ISWALDI

Submitted for a Doctoral degree in Chemistry

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CHARACTERIZATION OF PHENOLIC COMPOUNDS USING SEPARATION TECHNIQUES COUPLED TO MASS SPECTROMETRY OF PLANT EXTRACTS WITH DEMONSTRATED BIOACTIVITY

by

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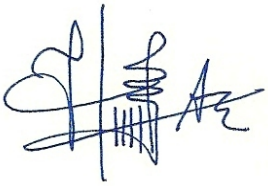

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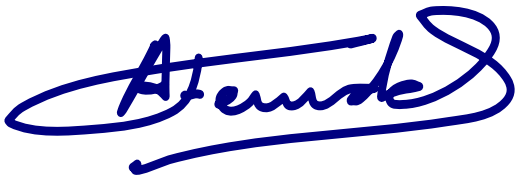
ALBERTO FERNÁNDEZ GUTIÉRREZ, Full Professor of University of Granada, Department of Analytical Chemistry, Head of Research Group FQM-297 "Environmental, Biochemical, and Foodstuff Analytical Control" and Director of CIDAF,

Certifies:

That the work presented in this doctoral thesis entitled "**CHARACTERIZATION OF PHENOLIC COMPOUNDS USING SEPARATION TECHNIQUES COUPLED TO MASS SPECTROMETRY OF PLANT EXTRACTS WITH DEMONSTRATED BIOACTIVITY**" has been carried out under my direction and also under the supervision of both Dr. Antonio Segura Carretero and Dr. David Arráez-Román in the laboratories of the Department of Analytical Chemistry and Functional Food Research and Development Center (CIDAF). He demonstrates all requirements of eligibility to obtain the Doctoral Degree in Chemistry from the University of Granada.

Granada, June 2012



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Summary

This doctoral thesis, entitled "Characterization of phenolic compounds using separation techniques coupled to mass spectrometry of plant extracts with demonstrated bioactivity", consists of two main sections: the Introduction and the Experimental Section.

The first section describes an overview of traditional medicine and functional food, plant foods as natural source of bioactive compounds, polyphenols and bioactivity and the analytical tools used to identify polyphenols such as high-performance liquid chromatography (HPLC) and ultraperformance liquid chromatography (UPLC) coupled to different detectors such as UV-visible (UV-vis) and mass spectrometry (MS) with ion-trap (IT), time-of-flight (TOF), and quadrupole time-of-flight (Q-TOF) analyzers.

The experimental section is divided in several chapters as follows:

Chapter 1 provides an overview of the uses of different powerful analytical tools such as HPLC and capillary electrophoresis (CE) coupled to different detection systems for the characterization of anthocyanins in fruits and vegetables, discussing the fundamentals of sample extraction and separation.

Chapter 2 shows the characterization of phenolic and other polar compounds in whole zucchini (*Cucurbita pepo* L.) fruit. An extraction method has been applied in order to extract the polar fraction from the samples studied, followed by HPLC separation assisted by diode array detector (DAD) and Q-TOF-MS. To the best of our knowledge, 10 phenolic acids, 16 flavonoids, and 17 other polar compounds with their derivatives are reported here in the whole zucchini fruit for the first time.

Chapter 3 demonstrates a powerful analytical method to separate and characterize phenolic compounds in aqueous and ethanolic rooibos (*Aspalathus linearis*) extracts, as rooibos is a rich source of

polyphenols. This plant is used to make a mild-tasting caffeine-free tea that is low in tannins compared to green or black teas and has antioxidant and antimutagenic/antitumoral properties. The method, combining HPLC-ESI-TOF-MS and HPLC-ESI-IT-MS^2 experiments confirmed the presence of 25 and 30 phenolic compounds in the aqueous and ethanolic extracts, of which 11 are reported here for the first time in rooibos. In addition, the rooibos tea extract showed its highest bioactivity in the groups fed with the high-fat high-cholesterol diet (HF), lowering serum cholesterol and triglyceride concentrations compared to control.

Finally, the last chapter is divided into two subchapters. The first subchapter 4A concerns the technique of HPLC-DAD-TOF-MS, using negative and positive ionization modes, to characterize commercial cranberry syrup used to prevent urinary-tract diseases. It was thus possible to identify 34 different compounds, 9 of which have been tentatively characterized for the first time in cranberry syrup. With regard to antibacterial activity, it was possible to demonstrate that very low concentrations of cranberry extract have can modify the non-specific adherence properties of *E. coli*, reducing surface hydrophobicity. Following the approach of this subchapter, subchapter 4B shows the powerful use of UPLC coupled to Q-TOF MS, for identifying polyphenols and their metabolites in human urine after a single dose of cranberry syrup. 9 native phenolics together with 23 conjugated metabolites, including isomers, were identified in urine using this optimized method.

Resumen

La tesis doctoral titulada "Caracterización de compuestos fenólicos mediante técnicas separativas acopladas a espectrometría de masas de extractos vegetales con bioactividad demostrada", consta de dos partes principales: introducción y parte experimental.

En la primera de ellas, se lleva a cabo un resumen acerca de la medicina tradicional, alimentos de origen vegetal como fuente natural de compuestos bioactivos, polifenoles como familia de compuestos bioactivos (estructura, biosíntesis y bioactividad), así como de las técnicas analíticas utilizadas en esta memoria para la identificación de estos compuestos: la cromatografía líquida de alta resolución (HPLC) y la cromatografía líquida de ultra alta resolución (UPLC) acopladas a diferentes sistemas de detección como la espectrofotometría UV-visible (UV-vis) y la espectrometría de masas (MS) empleando analizadores de trampa de iones (IT), tiempo de vuelo (TOF) y cuadrupolo-tiempo de vuelo (Q-TOF).

En cuanto a la parte experimental consta de varios capítulos:

En el capítulo 1 se lleva a cabo una revisión bibliográfica sobre los sistemas de extracción así como las técnicas analíticas empleadas para la caracterización de antocianinas en frutas y verduras.

En el capítulo 2 versa sobre la caracterización de compuestos fenólicos y otros compuestos polares en calabacín (*Cucurbita pepo* L.). Para ello, se llevó a cabo una extracción de la fracción polar, seguida de una separación por HPLC acoplada a detectores de UV-vis y Q-TOF-MS logrando con ello la caracterización de un buen número de compuestos, entre ellos 10 ácidos fenólicos, 16 flavonoides así como otros 17 compuestos polares y derivados que han sido caracterizados por primera vez en esta matriz.

En el capítulo 3 se lleva a cabo la puesta a punto de un nuevo método de análisis, mediante la combinación de HPLC-TOF-MS y HPLC-IT-MS^2, el cual permitió separar y caracterizar 25 y 30 compuestos fenólicos presentes en un

extracto acuoso y etanólico de rooibos (*Aspalathus linearis*) respectivamente. De estos compuestos cabe notar que mediante las técnicas analíticas empleadas 11 de ellos han sido caracterizados en esta matriz por primera vez. El interés del rooibos se debe a que éste está considerado como una fuente rica en polifenoles, con propiedades antioxidantes, antimutagénicas y antitumorales, además de ser muy utilizado como té, exento de cafeína, con una baja composición en taninos en comparación con el té verde o negro. Por otro lado, el extracto de rooibos mostró bioactividad en ratones alimentados con una dieta rica en grasa y en colesterol (HF), en donde se observó una reducción en las concentraciones de colesterol sérico y triglicéridos en comparación con el control.

El último capítulo está dividido en dos subcapítulos. El subcapítulo 4A trata sobre el uso de HPLC-DAD-TOF-MS, utilizando los modos de ionización negativa y positiva, para la caracterización de un jarabe de arándano comercial utilizado en la prevención de enfermedades infecciosas urinarias. Así fue posible la caracterización de 34 compuestos, siendo 9 de ellos caracterizados por primera vez en esta matriz. Este jarabe demostró una potencial actividad antibacteriana a bajas concentración capaz de modificar propiedades de adherencia no específicas de la bacteria *E. coli*. Dado el interés bioactivo de este jarabe, en el subcapítulo 4B se llevó a cabo un estudio de metabolómica, mediante la puesta a punto de un protocolo de extracción y posterior análisis por UPLC-Q-TOF-MS, para la identificación de polifenoles y sus metabolitos en muestras de orina humana de pacientes tratados con este jarabe. Así, se han podido caracterizar 9 compuestos fenólicos junto con otros 23 metabolitos y sus isómeros en estas muestras biológicas.

Objectives

In recent years there has been growing interest in the presence of certain compounds in foods and in increasing the understanding of their potential role in health. In plant-derived foods, these naturally occurring compounds form part of the secondary metabolism of many kinds of fruit and vegetable products, such as polyphenols. The daily intake of polyphenols has received much attention due to the health benefits of their antioxidant/anti-radical, anti-carcinogenic, anti-inflammatory, antiviral, and antimicrobial activities. This group of chemical compounds is widely distributed in plants and plant-derived foods and beverages, which can be divided based on their chemical structure, ranging from simple molecules such as phenolic acids, to highly polymerized compounds such as tannins.

In this sense, the aim of this doctoral thesis was to characterize polyphenolic compounds in plant matrices such as zucchini, rooibos, and cranberry syrup as well as in biological samples such as human urine, using liquid chromatography coupled with two different detection systems: UV-visible and mass spectrometry (ion-trap, time-of-flight, and quadrupole time-of-flight analyzers).

The UV-visible range has proved valuable for identifying the family of these phenolic compounds. TOF analyzer provides excellent mass accuracy over a wide, dynamic range and enables measurements of the isotopic pattern, providing important additional information for the determination of the elemental composition. Also, IT-MS can be used to obtain fragmentation ions of structural relevance for identifying target compounds in a highly complex matrix. Finally, the Q-TOF hybrid analyzer is able to provide information both MS and MS/MS analyses which combine the advantages of both IT and TOF.

With regard to bioactivity studies, anti-hyperlipidemic and antibacterial activities of rooibos extract and cranberry syrup, respectively, were analyzed.

Objetivos

En los últimos años ha habido un creciente interés sobre la presencia de ciertos compuestos en los alimentos así como el papel que juegan en cuanto a la salud. En los alimentos derivados de plantas (frutas y hortalizas), estos compuestos naturales forman parte de su metabolismo secundario y una importante familia la constituye los polifenoles. La ingesta diaria de polifenoles ha recibido mucha atención ya que diferentes estudios demuestran que éstos poseen propiedades antioxidantes, anticancerígenas, antiinflamatorias, antivirales y antibióticas. Este grupo de compuestos químicos están ampliamente distribuidos en las plantas y en los alimentos derivados de las mismas y pueden clasificarse en base a su estructura química, que van desde moléculas simples, como ácidos fenólicos, a compuestos altamente polimerizados como taninos.

Por todo ello, el objetivo principal de la presente tesis doctoral ha sido la caracterización de compuestos fenólicos y sus metabolitos en diferentes matrices de interés, tanto naturales (calabacín, rooibos y jarabe de arándano) como biológicas (orina humana) empleando para este fin técnicas analíticas avanzadas como la cromatografía líquida acoplada a diferentes sistemas de detección: UV-visible y espectrometría de masas (analizadores de trampa de iones (IT), tiempo de vuelo (TOF) y cuadrupolo-tiempo de vuelo (Q-TOF)).

La detección por UV-visible puede ser de gran valía para la identificación de las diferentes familias en función del espectro de absorción mientras que la espectrometría de masas proporcionará una importante información estructural sobre los compuestos de interés. Así, el analizador de TOF proporciona información de masa exacta y permite medidas de relación isotópica, proporcionando una información importante para la determinación de la composición elemental. Por otro lado, la IT-MS puede ser utilizada para obtener iones de fragmentación (MS/MS) de los compuestos de interés en una matriz muy compleja. Por último, el analizador híbrido Q-TOF-MS es capaz de proporcionar información tanto de MS como de MS/MS en un mismo análisis aunando las ventajas tanto del TOF como de la IT.

Por otro lado, se pretenden llevar a cabo estudios antihiperlipidémicos y antibacterianos del extracto de rooibos y jarabe de arándano demonstrando con ello su potencial bioactivo.

Introduction

List of figures and tables

1. An overview of traditional medicine and functional foods

The World Health Organization (WHO) defines traditional medicine as the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses.[1] It has been used in some communities for thousands of years and covers a wide variety of therapies and practices which vary from country to country and region to region as they are influenced by factors such as environment, culture, history, personal attitudes, and philosophy with great contributions made by practitioners to human health, particularly as primary health-care providers at the community level.

Most populations in developing countries around the world use traditional medicine for primary health care. In industrialized countries, the population has used some form of alternative or complementary medicine. Scientific research is needed to compile additional evidence to ascertain its safety and efficacy, since unregulated or inappropriate use of traditional medicines and practices can have negative or dangerous consequences for human health.

"Let food be thy medicine and medicine be thy food," espoused Hippocrates, unaware of how pertinent his tenet in 460 BC would remain. Currently, marketing "healthy" foods to otherwise healthy people has met with unprecedented success. The realization that attention to diet as part of a healthy lifestyle can considerably reduce the risk of disease and promote health has created a lucrative market for a whole range of new products called "functional foods," "nutraceuticals," or "novel foods."

The term "functional foods" was first introduced in Japan in the late 1980s, a government approval process from Ministry of Health and Welfare

1 World Health Organization (2008) Traditional medicine. URL http://www.who.int/mediacentre/factsheets/fs134/en/. Accessed 1 May 2012

covers functional foods called Foods for Specified Health Use (FOSHU).[2] Many definitions exist worldwide for functional foods, but there is no official or commonly accepted definition.

There are several ways of classifying functional foods. One way is according to the type of food, as shown in **Table 1**.[3]

Table 1. Categories of functional foods and nutraceuticals.

Category	Example
Basic foods	Carrots (containing the anti-oxidant beta-carotene)
Processed foods	Oat bran cereal
Processed foods with added ingredients	Calcium-enriched fruit juice
Food enhanced to have more of a functional component (via) traditional breeding, special livestock feeding or engineering beta	Tomatoes with higher levels of lycopene (an antioxidant carotenoid) Oat bran with higher levels of genetic glucan
Isolated, purified preparations of active food ingredients (dosage form)	Isoflavones from soy Beta glucan from oat bran

The definition of functional foods varies among countries for reasons that are historical, cultural, and regulatory. In its broadest use, functional foods are food-derived products that, in addition to their nutritional value, enhance normal physiological or cognitive functions or prevent the abnormality that underlies disease.[4] Internationally, the concept of functional foods seems to be generally agreed upon. A functional food should provide health benefits over and above its normal nutritional values and preference.

2 Ministry of Health, Labour and Welfare of Japan. Food for Specified Health Uses (FOSHU). URL http://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html. Accessed 1 May 2012

3 Arvanitoyannis IS, Van Houwelingen-Koukaliaroglou M (2005) Functional foods: a survey ofhealth claims, pros and cons, and current legislation. *Critic Rev Food Sci Nutr* 45:385–404

4 Galland L (2007) Functional Foods, Heaath effects and clinical applications. In Caballero B, Allen L, Prentice A (Eds.), Encyclopedia of human nutrition, 2ed Ed., Elsevier Academic Press, UK. Vol. 2, pp. 360-366

It is a food in the same form as ordinary food and should be consumed within daily dietary patterns.[5]

In general context, the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) coordinated by ILSI Europe developed and reached a consensus on scientific concepts of functional foods that was published in 1999.[6] This European consensus document proposed a working definition of functional food: "A food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern. It is not a pill, a capsule, or any form of dietary supplement".

From a practical stand point, a functional food can be:

a. a natural food such as fruit or grain which may or may not be modified by plant breeding or other technologies (e.g. vitamin-E-enriched vegetable oils);
b. a food to which a component has been added (e.g. a spread with added phytosterols);
c. a food from which a component has been removed or reduced (e.g. yogurt with reduced fat);
d. a food in which one or several components have been modified, replaced or enhanced to improve health properties and the bioavailability of one or more components has been modified (e.g. a yogurt with added prebiotic or probiotic); or
e. any combination of these possibilities.[7,8]

There are a number of legal terms already used which have some overlap within the scope of functional foods expressed above. These include foods for

5 Kwak NS, Jukes DJ (2000) Functional foods. Part 1: the development of a regulatory concept. *Food Control* 12:99-107
6 Diplock AT, Aggett PJ, Ashwell M, Bornet F, Fern EB, Roberfroid MB (1999) Scientific concepts of functional foods in Europe: consensus document. *Brit J Nutr* 81 suppl 1:S1–S28
7 Roberfroid, MB (2002) Global view on functional foods: European perspectives. *Brit J Nutr* 88 suppl. 2:S133–S138
8 European Commission, Directorate General for Research (2010) Functional foods. URL ftp://ftp.cordis.europa.eu/pub/fp7/kbbe/docs/functional-foods_en.pdf. Accessed 15 May 2012

special dietary use, medical foods, and dietary supplements. Consideration of the concept and the definitions of these other terms will clarify what functional foods are and where functional foods may already be controlled by food regulations. The relationship of functional foods, broken down into fortified foods, foods for special dietary uses, medical foods, and dietary supplements, is represented schematically in the **Figure 1**.[9]

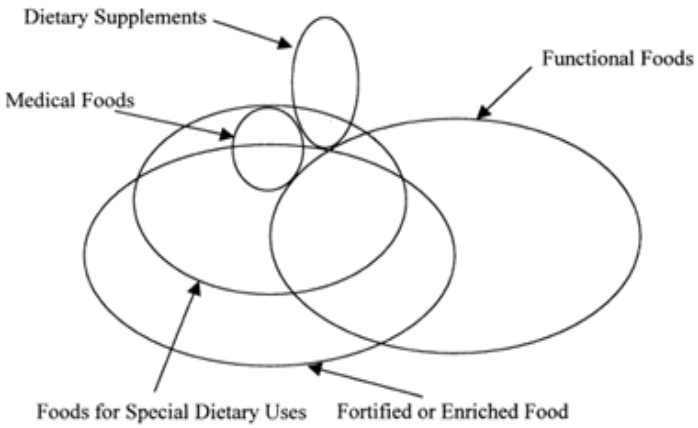


Figure 1. Relationship among the several terms.

Foods for special dietary uses overlap functional foods if they are specially formulated and/or provide a certain population health benefits over and above their normal nutritional values. Medical foods are clearly distinguished from functional foods in that medical claims such as those referring to a specific disease or disorder are permitted for medical foods. Fortified foods can be considered functional foods if essential nutrients are added to ordinary foods for health benefits over and above their normal nutritional values. Dietary supplements are clearly distinguished from functional foods since dietary supplements cannot replace a daily diet and their forms resemble medicines more than they do ordinary foods.

A number of marketing terms such as health food and nutraceutical also have some implications for functional foods. Health food can be concisely defined as food that is claimed or recognized to have healthy properties. In

9 Kwak, NS, Jukes DJ (2001) Functional foods. Part 2: The impact on current regulatory terminology. *Food Control* 12:109-117

this regard functional foods can be considered as a category within health foods. Nutraceutical involves functional food as well as dietary supplement and is the term used from the perspective of the pharmaceutical or medical industry. It could be regarded as designed health foods.

2. Plant foods as natural source of bioactive compounds

Bioactive compounds are extranutritional constituents that typically occur in small quantities in foods eliciting pharmacological or toxicological effects in men and animals[10, 11]. They are knows as secondary plant metabolites that do not participate directly in growth and development and often differentially distributed among taxonomic groups within plant kingdom.[12]

2.1. Zucchini (*Cucurbita pepo* L.)

The zucchini (from the Italian word) or courgette (from the French word), *Cucurbita pepo* L. (Cucurbitaceae family), is low in calories and contains a high percentage of water (over 95%)[13], as well as high nutritional [14], and medical value.[15,16] These fruits can be found in many shapes, from spherical

10 Kris-Etherton P M, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD (2002) Bioactive compounds in foods: Their role in the prevention of cardiovascular disease and cancer. *Am J Med* 113:71S-88S

11 Bernhoft A (2010) A brief review on bioactive compounds in plants, in Bernhoft A (Ed.) Bioactive compounds in plants – benefits and risks for man and animals.. The Norwegian Academy of Science and Letters. Oslo pp 11-17

12 Hounsome N, Hounsome B, Tomos D, Edwards-Jones G (2008) Plant metabolites and nutritional quality of vegetables. *J Food Sci* 73:R48

13 Botanical-online, SL (1999) Courgettes. URL http://www.botanical-online.com/english/courgettes.htm. Accessed 1 May 2012

14 National Food Institute, TU Denmark (2009) Danish Food Composition Databank. URL http://www.foodcomp.dk/v7/fcdb_details.asp?FoodId=0402. Accessed 13 January 2012

15 Menéndez AB, Capó JT, Menéndez-Castillo RA, González OL, Domínguez CC, Sanabria MLG (2006) Evaluation of *Cucurbita pepo* L. lipophilic extract on androgen-induced prostatic hyperplasia. *Revista Cubana de Plantas Medicinales* 11(2)

16 Shokrzadeh M, Azadbakht M, Ahangar N, Hashemi A, Saeedi Saravi SS (2010) Cytotoxicity of hydro-alcoholic extracts of *Cucurbita pepo* and *Solanum nigrum* on HepG2 and CT26 cancer cell lines. *Phcog Mag* 6:176–179.

to elongated, and they vary in skin colors from dark to light green, sometimes with fine whitish mottling or stripes (**Figure 2**).[17]

Zucchini, technically a fruit, is usually treated as vegetable, often being included in cooked dishes. Zucchini can be eaten raw, sliced or shredded in a cold salad, as well as hot and barely cooked in hot salads. When used for food, zucchini are usually picked when under 20 cm (8 in.) in length and the seeds are soft and immature. Mature zucchini can be as much as three feet long, but are often fibrous and not appetizing to eat. It is available all year.

Most of the data reported in this sphere concern the composition of pumpkin seeds. Adlercreutz and Mazur (1997) have found isoflavones such as daidzein and genistein in the pumpkin seeds in addition to secoisolariciresinol.[18] Large amounts of two triterpenes esterifed with *p*-aminobenzoic acid have also reported by Appendino *et al*. (1999).[19] Later, Sicilia *et al*. (2003) found lariciresinol as the second lignin in this kind of sample.[20] Cucurbitosides A to E and cucurbitosides F to M as acylated phenolic glycocides have been identified in the seed of squash *C. moschata* [21] and zucchini[22], respectively.

17 Comisión Nacional para el Conocimiento y Uso de la Biodiversidad. *Cucurbita pepo pepo*. URL http://www.conabio.gob.mx/conocimiento/bioseguridad/pdf/20870_sg7.pdf. Accessed 1 May 2012

18 Adlercreutz H, Mazur W (1997) Phyto-oestrogens and western diseases. *Annals Med* 29:95–120

19 Appendino G, Jakupovic J, Belloro E, Marchesini A (1999) Multiflorane triterpenoid esters from pumpkin. An unexpected extrafolic source of PABA. *Phytochem* 51:1021–1026

20 Sicilia T, Niemeyer HB, Honig DM, Metzler M (2003) Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. *J Agric Food Chem* 51:1181–1188

21 Koike K, Li W, Liu L, Hata E, Nikaido T (2005) New phenolic glycosides from the seeds of *Cucurbita moschata*. *Chem Pharm Bull* 53:225–228

22 Li F-S, Dou D-Q, Xu L, Chi X-F, Kang T-G, Kuang H-X (2009) *J Asian Nat Prod Res* 11:639–642

Figure 2. Zucchini (*Cucurbita pepo* L.) fruit.

2.2. Rooibos (*Aspalathus linearis*)

Throughout the world, tea is one of the most consumed beverages, second only to water. One reason for the popularity of tea consumption is the interest of consumers to improve their health. Although tea is a poor source of most classically defined essential nutrients, it is a good and important source of many phytochemicals that have been presumptively linked to reduction of risk of cancer, cardiovascular disease, and diabetes. Many of these phytochemicals have strong antioxidant properties, and presumably these antioxidant properties are associated with improved health.[23]

The rooibos plant, often called rooibos tea, is a shrubby legume indigenous to the mountains of South Africa's Western Cape (shown in **Figure 3**). A flowering shrub, it is used to make a mild-tasting tea (technically an infusion) that has no caffeine, very little tannin, and significant amounts of polyphenol antioxidants. There are two types of rooibos, i.e. unfermented and fermented (meant to be similar to the tea-fermenting process). The unfermented product remains green in color and is referred to as green rooibos. During fermentation, the color changes from green to red with oxidation of the constituent polyphenols, so the final product is often referred to as red tea or red bush tea.[24] The most important characteristics of rooibos are related to its antioxidant and antimutagenic/anticancer properties.[25-28]

23 Baer DJ, Chen SC (2007) Tea. . In Caballero B, Allen L, Prentice A (Eds.), Encyclopedia of human nutrition, 2ed Ed., Elsevier Academic Press, UK. vol. 4, pp. 258-262

24 Erickson L (2003) Rooibos tea: Research in antioxidant and antimutagenic properties. HerbalGram, *J Am Bot Counc* 59:34–45

25 Von Gadow A, Joubert E, Hansmam CF (1997) Comparison of the antioxidant activity of rooibos tea *(Aspalathus linearis)* with green, oolong and black tea. *Food Chem* 60:73-77

26 Von Gadow A, Joubert E, Hansmam CF (1997) Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea *(Aspalathus linearis)*, α-tocopherol, BHT, and BHA. *J Agric Food Chem* 45:632-638

27 McKay DL, Blumberg JB (2007) A review of the bioactivity of South African herbal teas: Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), *Phytother Res* 21:1–16

28 Joubert E, Gelderblom WCA, Louw A, de Beera D (2008) South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*—A review, *J Ethnopharmacol* 119:376–412

Figure 3. Rooibos (*Aspalathus linearis*) plant.

Koeppen *et al.,* who led research on determining the phenolic ingredients in rooibos tea, identified the presence of isoquercitrin, rutin[29], orientin, isoorientin [30], and aspalathin[31] as the major flavonoid constituents of this plant. The presence of several other flavonoids such as vitexin, isovitexin, luteolin, chrysoeriol, quercetin, and isoquercetrin has been reported.[32] The asplathin and nothofagin contents were found to decrease with processing and depend on the degree of oxidation of the tea.

A recent analysis of fermented rooibos measured the levels of all the polyphenols. Of 10 polyphenols measured, the three that occurred in largest amounts were aspalathin, rutin, and orientin, followed by isoorientin and isoquercitrin. Nothofagin was identified by mass spectrometry but was not quantified because a standard was not available. The amount of nothofagin in

29 Koeppen BH, Smit CJB, Roux DG (1962) The flavone *C*-glycosides and flavonol *O*-glycosides of A*spalathus acuminatus* (rooibos tea). *Biochem J* 83:507-511

30 Koeppen BH, Roux DG (1965a) *C*-Glycosylflavonoids. The chemistry of orientin and iso-orientin, *Biochem J* 97:444-448

31 Koeppen BH, Roux DG (1965b) Aspalathin: a novel *C*-glycosylflavonoid from A*spalathus linearis*. *Tetrahedron Lett* 39:3497-3503

32 Rabe C, Steenkamp JA, Joubert E, Burger JFW, Ferreira D (1994) Phenolic metabolites from rooibos tea (*Aspalathus linearis*). *Phytochem* 35:1559–1565

fermented and unfermented rooibos was estimated to be about three times less than aspalathin in one study. Aspalathin and nothofagin are present in relatively large amounts in unfermented rooibos tea, but some of the aspalathin and nothofagin oxidizes to other substances during fermentation; thus, fermented rooibos contains less aspalathin and nothofagin than does unfermented rooibos. The change in polyphenol composition is why the tea changes color with fermentation.

2.3. Cranberry (*Vaccinium macrocarpon*)

The cranberry plant (*Vaccinium macrocarpon*), of the family Ericaceae, is a low-growing, viny, woody perennial plant with small, alternate, ovate leaves. The plant produces small, red, strongly flavored tart fruits that are used primarily for sauces and juices (**Figure 4**).[33]

Cranberry was recognized as being effective for treating urinary-tract infections ever since Blatherwick (1914) reported that this fruit, rich in benzoic acid, acidified the urine. Recent investigations have focused on the ability of cranberry to inhibit the adherence of *Escherichia coli* to uroepithelial cells. This phenomenon has been attributed to two compounds: fructose and a nondialyzable polymeric compound. The latter compound, subsequently isolated from cranberry and blueberry juices was found to inhibit adhesins present on the pili of the surface of certain pathogenic *E. coli*.

Cranberries are a rich source of dietary flavonoids and other phenolic acids that research has shown may provide a variety of health benefits such as antiproliferative, antioxidant[34], anti-inflammatory and antimicrobial properties, which inhibit the growth of pathogenic bacteria such as *E. coli* and *Helicobacter pylori*.[35,36] It has traditionally been used to treat and prevent

33 The Cranberry Institute (2003) About cranberries. URL http://www.cranberryinstitute.org/about_cranberry.htm. Accessed 1 May 2012

34 He X, Rui HL (2006) Cranberry phytochemicals: isolation, structure elucidation, and their antiproliferative and antioxidant activities, *J Agric Food Chem* 54:7069-7074

35 Lin YT, Kwon YI, Labbe RG, Shetty K (2005) Inhibition of *Helicobacter pylori* and associated uruase by oregano and cranberry phytochemical synergies. *Appl Environ Microbiol* 71:8558-8564

urinary-tract infections in women and also digestive-tract ailments. The antitumoral properties of cranberries have made them a popular dietary component to prevent neoplastic diseases.[37] A growing body of evidence has linked the phytonutrients found in cranberries to a number of conditions. The phenolic compounds found in cranberries are believed to be the principal ingredients responsible for these beneficial effects. Cranberries are known for their high concentration of anthocyanins, as well as their significant contents of flavonols, flavan-3-ols, tannins (ellagitannins and proanthocyanidins) and phenolic-acid derivatives.[38]

Figure 4. Cranberry (*Vaccinium macrocarpon)* fruit and syrup.

36 Foo LY, Lu Y, Howell AB, Vorsa N (2000) The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli* in vitro. *Phytochem* 54:173-181

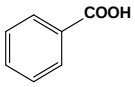
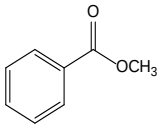
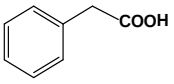
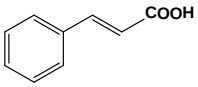
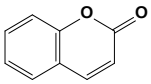
37 Seeram NS, Adams LS, Zhang Y, Lee R, Sand D, Sheuller HS, Heber D (2006) Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J Agric Food Chem* 54:9329-9339

38 Cote J, Caillet S, Doyon G, Sylvain J-F, Lacroix M (2010) Analyzing cranberry bioactive compounds. *Crit Rev Food Sci Nutr* 50:872-888

3. Polyphenolic compounds: Structure and classification

Polyphenols constitute one of the most widely distributed groups of substances in the plant kingdom and it is estimated that more than 10,000 phenolic structures are currently known[39], with more than 500 phenolic compounds reported in foods.[40] They can be divided into at least 10 different classes based upon their chemical structure, ranging from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins.[39,41] Also, they can be classified based on the number and arrangement of their carbon atoms (**Table 2**) and are commonly found conjugated to sugars and organic acids.[40,42]

Table 2. Structural skeletons of phenolic compounds (hydroxyl groups not shown).

Classification	Skeleton	Example	Basic structure
Phenolic acids	C_6-C_1	Gallic acid	
Acetophenones	C_6-C_2	Gallacetophenone	
Phenyacetic acid	C_6-C_2	*p*-Hydroxyphenyl-acetic acid	
Hydroxycinnamic acids	C_6-C_3	*p*-Coumaric acid	
Coumarins	C_6-C_3	Esculetin	

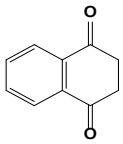
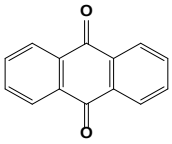
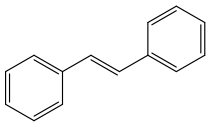
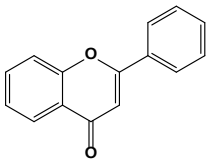
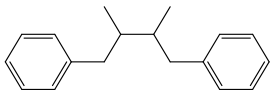
39 Pietta P, Minoggio M, Bramati L (2003) Plant polyphenols: structure, occurrence and bioactivity, Atta-ur-Rahman (Ed.) *Studies in Natural Products Chemistry*, Elsevier Science B.V 28:257-312

40 Crozier A, Clifford MN, Ashihara H (2006) Plant secondary metabolite: Occurrence, structure and role in the human diet. Blackwell Publishing. Oxford, UK

41 Manach C, Williamson G, Morand C, Scalbert A, Rémésy C (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 81:230S

42 Crozier A, Jaganath IB, Clifford MN (2009) Dietary phenolics: chemistry, bioavailability and effects on health. *Nat Prod Rep* 26:1001-1043

Table 2. (Continued).

Naphthoquinones	C_6-C_4	Juglone	
Xanthones	C_6-C_1-C_6	Mangiferin	
Stilbenes	C_6-C_2-C_6	Resveratol	
Flavonoids	C_6-C_3-C_6	Naringenin	
Lignans	$(C_6$-$C_3)_2$	Syringaresinol	

Flavonoids are a chemically defined family of polyphenols that includes several thousand compounds. These have a C_6–C_3–C_6 basic structure (**Figure 5**), and several subclasses of flavonoids which share a common structure consisting of 2 aromatic rings (A and B) bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C) may themselves be divided into 6 subclasses according to the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins) (**Figure 6**).[43,44]

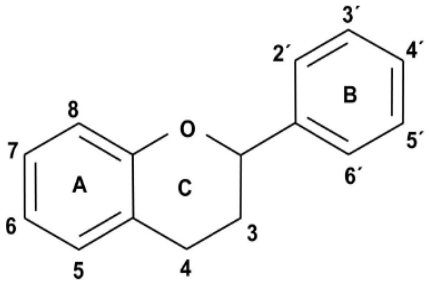
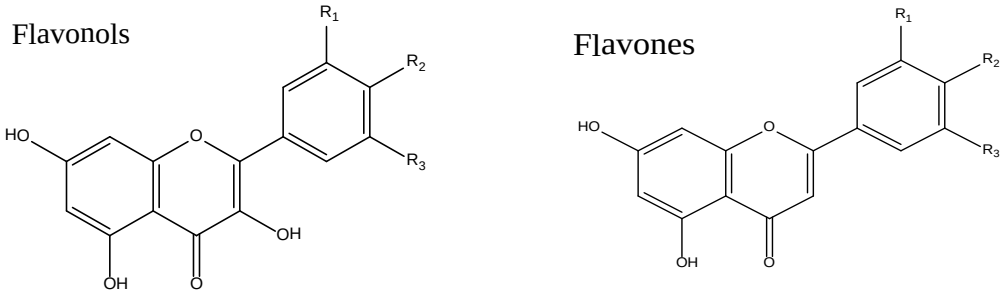


Figure 5. Basic structure of flavonoids indicating rings (A, C, and B) and substitution numbers.

43 Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79:727–747

44 Iwashina T (2000) The structure and distribution of the flavonoids in plants. *J Plant Res* 113:287-299

Flavonoids are often present in glycoside forms which have effects to render the flavonoid less reactive and more water soluble and allowing their storage safely in the cell vacuole.[45] There are two types of glycosidic bonds i.e. *O*- and *C*-glycosides. The former site is often found in the phenolic hydroxyl group of the aglycone with formation of a glycosidic O–C bond. In theory, any of the hydroxyl groups can be glycosilated but certain positions are favored such as the 7-hydroxyl group in flavones, flavanones and isoflavones, the 3- and 7-hydroxyls in flavonols and flavanols and the 3- and 5-hydroxyls in anthocyanidins (see **Figure 5** and **6**). The latter site which takes place by direct linkage of sugar to the the flavonoid's basic nucleus via an acid-resistant C—C bond, has only been found at the *C*-6 or *C*-8 position of the aglycone to date.[46,47] Flavonoid *C*-glycosides are commonly further divided into mono-*C*-glycosylflavonoids, di-*C*-glycosylflavonoids and *C*-lycosylflavonoid-*O*-glycosides.



R_2 = OH; R_1 = R_3 = H : Kaempferol
R_1 = R_2 = OH; R_3 = H : Quercetin
R_1 = R_2 = R_3 = OH : Myricetin

R_1 = H; R_2 = OH : Apigenin
R_1 = R_2 = OH : Luteolin

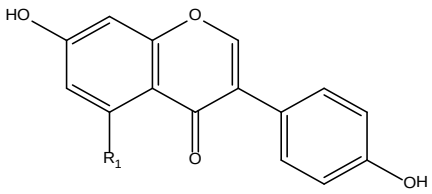
Figure 6. Chemical structures of flavonoids.

45 Harborne JB, Mabry TJ (1982) The Flavonoids: Advances in Research. Chapman and Hall. London-New York

46 Becchi M, Fraisse D (1989) Fast atom bombardment and fast atom bombardment collision-activated dissociation/mass-analysed ion kinetic energy analysis of *C*-glycosidic flavonoids. *Biomed Environ Mass Spectrom* 18:122–130

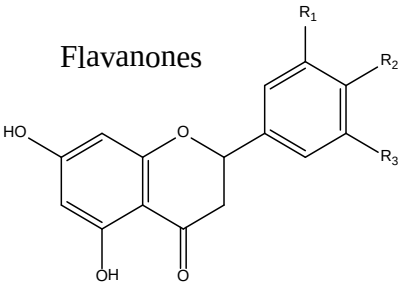
47 Pereira CAM, Yariwake JH, McCullagh M (2005) Distinction of the *C*-glycosylfavone isomer pairs orientin/isoorientin and vitexin/isovitexin using HPLC-MS exact mass measurement and in-source CID. *Phytochem Anal* 16:295

Isoflavones



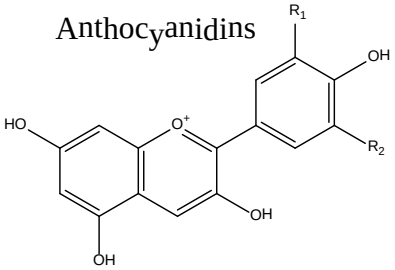
R_1 = H : Daidzein
R_1 = OH : Genistein

Flavanones



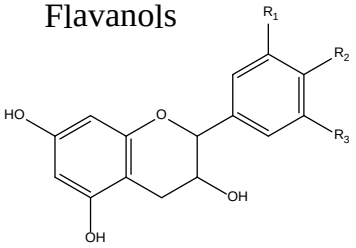
R_1 = H; R_2 = OH : Naringenin
R_1 = R_2 = OH; Eriodictyol
R_1 = OH; R_2 = OCH_3 : Hesperetin

Anthocyanidins

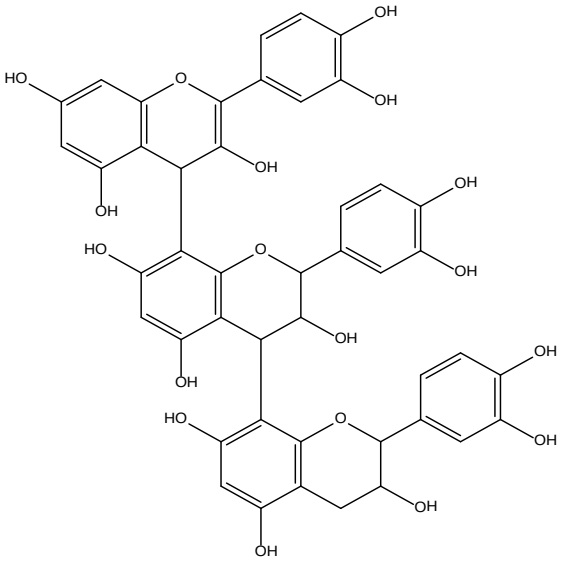


R_1 = R_2 = H : Pelargonidin
R_1 = OH; R_2 = H : Cyanidin
R_1 = R_2 = OH : Delphinidin
R_1 = OCH_3; R_2 = OH : Petunidin
R_1 = R_2 = OCH_3 : Malvidin

Flavanols



R_1 = R_2 = OH; R3 = H : Catechins
R_1 = R_2 = R3 = OH : Gallocatechin



Trimeric procyanidin

Figure 6. (Continued).

Phenolics are not uniformly distributed in plants at the tissue, cellular, and subcellular levels. Insoluble phenolics are the components of cell walls, while soluble phenolics are compartmentalized within the plant-cell vacuoles.

At the tissue level, the outer layers of plants contain higher levels of phenolics than do inner layers.[48]

4. Polyphenols and bioactivity

Polyphenols are endowed with different biological activities, including antioxidant/anti-radical activity[49,50] and chelation of metal ions, modulation of the activity of certain enzymes, anti-carcinogenic activity, anti-atherosclerotic activity, anti-inflammatory activity, inhibition of histamine release and spasmolytic activity, hepatoprotective activity, antiviral and antimicrobial activity, and oestrogenic activity.[39]

Beyond the capacity of polyphenols to scavenge free radicals and/or to chelate metals, other biochemical mechanisms can explain their *in vitro* and *in vivo* antioxidant effects (**Figure 7**). These mechanisms are related to polyphenol-lipid and polyphenol-protein interactions, and are based on the presence of hydroxyl groups that give polyphenols high possibilities for physical and chemical interactions. These mechanisms do not preclude a direct free-radical or metal-chelating action, but are more consistent with *in vivo* polyphenol levels observed in human and animals. Furthermore, the actual occurrence of these potential actions *in vivo* should be considered when linking an antioxidant action to a polyphenol, or to any other molecule, for which metabolic fate and biological actions are not firmly established.[51]

48 Naczk M, Shahidi F (2006) Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *J Pharm Biomed Anal* 41:1523-1542

49 Stevenson DE, Hurst RD (2007) Polyphenolic phytochemicals - Just antioxidants or much more? *Cell Mol Life Sci* 64:2900

50 Pereira DM, Valentão P, Pereira JA, Andrade PB (2009) Phenolics: From chemistry to biology. *Molecules* 14:2202

51 Fraga CG (2007) Critical review plant polyphenols: How to translate their in vitro antioxidant actions to in vivo conditions. *Life* 59:308–315

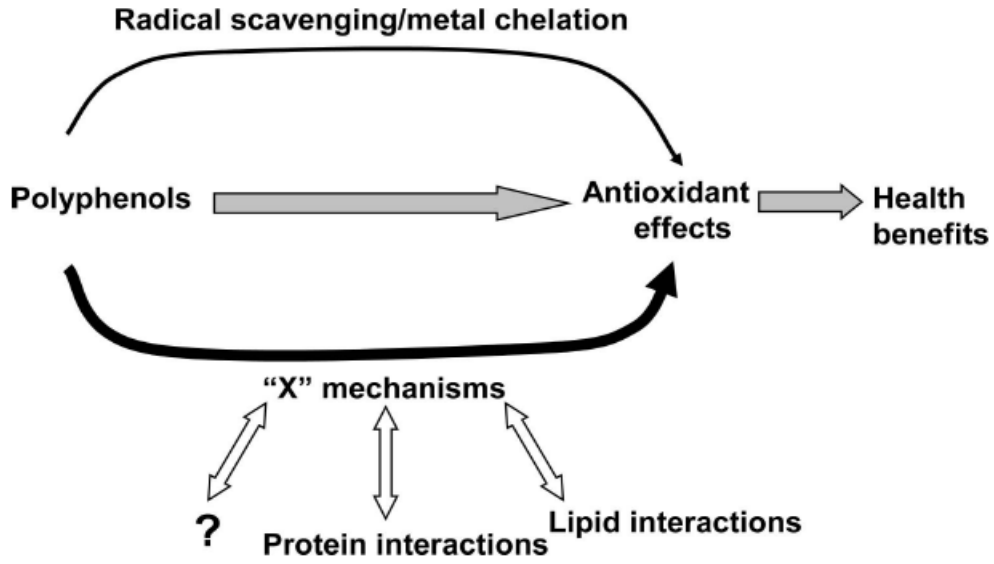


Figure 7. Scheme relating polyphenols with health benefits, through their observed antioxidant effects. The body of the black arrows indicates the 'x' mechanism with free-radical scavenging or metal-chelating capacity of polyphenols.

4.1. Polyphenols and obesity

Obesity has become a global public health issue. Obesity or being overweight is defined as abnormal or excessive fat accumulation that presents a risk to health. This disorder includes liver steatosis, type-2 diabetes, dyslipidemia, and probably cancer, which exhibits increases in morbidity and mortality rates not only in Western societies but also throughout the world.[52]

Caloric intake is a major contributor to these diseases, but the type of diet consumed also plays a role. It has been known for decades that a diet rich in fruits, vegetables and olive oil may help to reduce cardiovascular and metabolic complications, but the components of these foods that confer health benefits are still unknown. Recent interest has been focused on the biological activities of plant-derived phenolic compounds, but safety and efficacy issues remain unresolved in humans. For instance, it has been documented that polyphenols from green tea or wine may confer a protective cardiovascular effect, and other medicinal plants are currently being considered for anti-inflammatory purposes.

52 WHO (World Health Organization) (2012) Obesity and overweight, Fact sheet N°311. URL http://www.cdc.gov. Accessed 15 May 2012

Rooibos (*Aspalathus linearis*) is widely used to prepare infusions that represent a rich source of polyphenols without caloric value. Moreover, *in vitro* and *in vivo* studies have demonstrated that rooibos has significant bioactivity, preventing lipid oxidation and oxidative stress, inflammation, hyperglycemia, and chemically induced liver damage.

4.2. Polyphenols and urinary tract infection (UTI)

Clinical evidence supports the role of cranberry in the prevention of urinary-tract infections (UTIs). Adhesion of bacteria to the uroepithelium is the initial step in development of mammalian UTI. Cranberry may act by inhibiting the adhesion of P-fimbriated uropathogenic strains of *Escherichia coli* to uroepithelial cells. *E. coli* strains that express P-fimbriae are associated with both cystitis and pyelonephritis.[53]

Traditionally, cranberries have been used to treat urinary-tract infections, but the mechanisms of action are still unknown. At first, the anti-infective quality of cranberries had been attributed to their content in organic acids. The antiseptic and antibacterial action of the cranberry was attributed to its capacity to acidify the urine owing to its vitamin C content and its capacity to metabolize benzoic acid into hippuric acid with antibacterial properties[54], but it has been demonstrated that the hypothesis that cranberry works via urinary acidification (due to enhanced salicylate excretion and the metabolism of quinic and benzoic acids to hippuric acid *in vivo*) is not tenable because individuals cannot ingest sufficient cranberry juice to generate sufficient hippuric acid to prompt a bacteriostatic effect.[55]

Later, it was discovered that cranberry-juice works primarily by inhibiting the adhesion of ubiquitous type-1 fimbriated *E. coli*, as well as of uropathogenic p-fimbriated strains to uroepithelia.[56] The pathogenicity of

53 Howell AB (2002) Cranberry proanthocyanidins and the maintenance of urinary tract health. *Crit Rev Food Sci Nutr* 42(suppl):273-278
54 Dessì A, Atzei A, Fanos V (2011) Cranberry in children: prevention of recurrent urinary tract infections and review of the literature. *Braz J Pharmacogn* 21: 807-813
55 Guay DR (2009) Cranberry and urinary tract infections. *Drugs* 69:775-807
56 Nowack R (2007) Cranberry juice – a well-characterized folk-remedy against bacterial urinary tract infection. *Wien Med Wochenschr* 157:325–330

these bacteria is connected to the presence of fimbriae at the extremities of which there are protein structures called adhesins, which make it possible for the pathogens to adhere to the membrane of uroepthelial cells. Adhesion involves the binding of lectins exposed on the surface of these fimbriae to complementary carbohydrates of the host tissue. Fimbrial adhesion is a key event in UTI following bacterial colonization of the host tissue.

The action mechanism by which cranberry appears to prevent UTIs involves the inhibition of the binding of the P-fimbriae of uropathogens via mannose-specific, lectin-like structures to mannose-like residues on mucosal cells. Studies have established that cranberry reduces the strength of the binding between these two moieties and also alters the conformation of surface macromolecules.

Type A proanthocyanidins appear to be those mainly responsible for the antiseptic and antibacterial action. In a study by Howell and Foxman (2002)[57], the anti-adhesive activity of cranberry against type-B uropathogen fimbriated *E. coli* was studied *in vitro* by examining proanthocyanidin A, which has been shown to inhibit adhesion.

Proanthocyanidins, by acting as receptor analogues and adhering to the extremities of the fimbriae, competitively inhibit the adhesion of *E. coli*, thus causing an elongated deformation of the bacterium cell bodies, a reduction in the length and density of the P-fimbriae and finally an inhibition of their synthesis with the total disappearance of the adhesins. The anti-adhesive activities of the cranberry help to prevent UTI also indirectly by selecting at the intestinal level less adhesive uropathogenic bacteria.

The inhibition of P-fimbriae is thought to be irreversible. A cochrane meta-analysis in 244 females with symptomatic UTI suggests that the effect was more pronounced in women with recurrent UTIs than in elderly males and females or subjects requiring catheterization. A first head-to-head trial in older females has been published comparing effectiveness of a low-dose

57 Howell AB, Foxman B (2002) Cranberry juice and adhesion of antibiotic-resistant uropathogens. *JAMA* 287:3082-3083

antibiotic vs. cranberry in which investigators suggest that cranberry products may have a role in older females with recurrent UTI. Still with regard to antibiotic treatment in women, a recently published study investigated also the potential cranberry juice interaction with beta-lactam antibiotics, supporting the hypothesis that cranberry juice in usual quantities as prophylaxis for UTI is not likely to alter the pharmacokinetics of these oral antibiotics.[58] According to this, cranberry treatment is a safe, well-tolerated supplement that does not have significant drug interactions.[59]

5. Analytical tools for the extraction and identification of polyphenols

Phenolic compounds are ubiquitous in the plant kingdom. More than 10,000 phenolic compounds have been isolated in a wide variety of forms. They can be combined with five sugars in various positions. Consequently, the identification of phenolic compounds present in a matrix of plant origin is an extremely complex task that requires the combination use of sophisticated analytical techniques. In general, a procedure to analyze phenolic compounds in plant matrices involves two basic steps: 1) extraction of the sample and 2) the determination of phenolic compounds. The latter in turn consists of: (i) quantification of total phenolic contents, and (ii) detailed determination of each phenolic compound qualitatively and quantitatively by using a separation technique. These steps will be discussed briefly below.

5.1. Polyphenols extraction

The first step is the extraction of the analytes from the sample matrix. The plant matrices are usually very complex, so it is not possible to address the direct analysis of the matrix. This step aims to release the components of interest, in this case phenolic compounds which are not uniformly distributed in plants at the tissue, cellular, or subcellurar levels. Soluble phenolics are

58 Rossi R, Porta S, Canovi B (2010) Overview on cranberry and urinary tract infections in females. *J Clin Gastroenterol* 44:S61-62

59 Pérez-López FR, Haya J, Chedraui PJ (2009) *Vaccinium macrocarpon*: an interesting option for women with recurrent urinary tract infections and other health benefits. *Obstet Gynaecol Res* 35:630-639

compartmentalized within the plant-cell vacuoles, while insoluble phenolics are the component of cell walls.[60-63] At the tissue level, the outer layers of plants contain higher levels of phenolics than those located in their inner parts.[61,64,65]

Phenolic compounds can be extracted from fresh, frozen or dried plant matrices. Usually, before extraction, plant samples are treated by milling, grinding, and homogenization, which may be preceded by air-drying or freeze-drying. The type of extraction plays a crucial role in solubility of phenolic compounds in the solvents used. Their solubility depends on the chemical nature of the compound, which in the case of polyphenols may vary according to their family, as well as the polarity of the solvents used. It also should be taken into account the possible association with other matrix components such as carbohydrates or proteins. For this reason, it is not possible to carry out a general extraction procedure. The choice of a particular procedure depends on the matrix, the compounds which are determined, and the type of information sought (qualitative or quantitative).[66,67]

This step in polyphenol analysis is especially critical in the case of quantitative analysis. Different classes of polyphenols may require different extraction methods. Many studies have described three main techniques to extract polyphenol from plant matrices: (1) solvent extraction, (2) solid-phase

60 Beckman CH (2000) Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants?. *Physiol Mol Plant Pathol* 57:101-110

61 Bengoechea ML, Sancho AI, Bartolomé B, Estrella I, Gómez-Cordovés C, Hernández MT (1997) Phenolic composition of industrially manufactured purées and concentrates from peach and apple fruits. *J Agric Food Chem* 45:4071-4075

62 Wink M (1997) Compartmentation of secondary metabolites and xenobiotics in plant vacuoles. *Adv Bot Res* 25:141-169

63 Naczk M, Shahidi F (2004) Extraction and analysis of phenolics in food. *J Chromatogr A* 1054:95-111

64 FJ Pérez-Ilzarbe, Martinez V, Hernández T, Estrella I (1991) Liquid chromatographic determination of apple pulp procyanidins. *J Liq Chromatogr* 15:637-646

65 Fernández de Simón B, Pérez-Ilzarbe J, Hernández T, Gómez-Cordovés C, Estrella I (1992) Importance of phenolic compounds for the characterization of fruit juices. *J Agric Food Sci* 40:1531-1535

66 Dai J, Mumper RJ (2010) Plant Phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules* 15:7313-7352

67 Antolovich M, Prenzler P, Robards K, Ryan D (2000) Sample preparation in the determination of phenolic compounds in fruits. *Analyst* 125:989

extraction, and (3) supercritical liquid extraction. Among them, solvent extraction is the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability.[68],[69] The extraction method for anthocyanins is discussed in depth in Chapter 1 of this thesis.

5.2. Separation technique

Many analytical techniques based on differences in physical-chemical properties of the various components of a sample are used for separation. Continuous techniques are generally used, i.e. in which the analytes are detected continuously (on-line) after separation. One of the most widely used of these techniques is liquid chromatography.

Liquid chromatography (LC)

LC is a separation technique by which the components of the sample are distributed between a mobile phase (liquid) and a stationary phase (typically consisting of small porous particles with a large surface area). Sample molecules travel through the chromatography system and interact with the surface of the stationary phase. Sample molecules that interact strongly with the stationary phase will take a longer time to travel through the chromatography system, i.e. will have longer retention times.

In LC separation, the mobile phase is aspirated by the pump through the chromatographic column. Upon entering the sample in the column, its components interact with variable length in the stationary phase and it is distributed differently between the two phases. On being eluted from the end of the column, each analyte enters the detector and produces a signal that is measurable in some way. The intensity and duration of the signal is related to the amount or nature of the analyte. Generally, the signal is amplified and recorded by an electronic integrator, a computer or other

68 Harnly JM, Bhagwat S, Lin L Z (2007) Profiling methods for the determination of phenolic compounds in foods and dietary supplements. *Anal Bioanal Chem* 389:47-61

69 Garcia-Salas P, Morales-Soto A, Segura-Carretero A, Fernández-Gutiérrez A (2010) Phenolic-compound-extraction systems for fruit and vegetable samples. *Molecules* 15:8813-8826

means of producing the chromatogram in order to identify and quantify the analyte.

LC equipment consists of a series of building blocks that are common to all models: pump, injector, chromatographic column, thermostatically controlled oven, detector, and data-acquisition system. **Figure 8** below presents the basic scheme of commercial LC equipment, showing its commercial appearance.

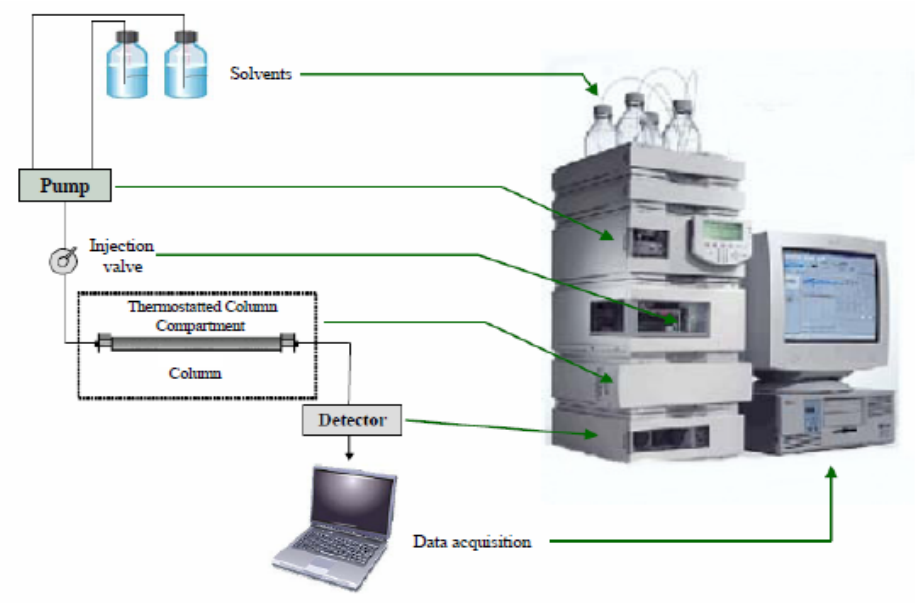


Figure 8. Simplified outline of a commercial LC.

Traditionally, LC columns (the stationary phase) are highly polar, e.g. silica, and the mobile phases used relatively nonpolar, e.g. hexane. This mode of LC is called normal-phase chromatography. The retention time of the analyte lengthens with increasing polarity. If the polarity of the mobile phase is increased, the retention time can be decreased. This mode of chromatography can be useful for separation on nonpolar or weakly polar flavonoid aglycones.

More common today is "reversed-phase" chromatography for the analytical and preparative separations of analytes. This term derives from the fact that the mobile phase is more polar than the stationary phase and that it reverses the order of elution of the analytes. The retention time, which is

longer for more hydrophobic molecules, can be decreased by the addition of nonpolar solvent to the mobile phase. The hydrophobicity of the sample molecules is pH dependent, and therefore an organic acid such as formic acid, acetic acid, or trifluoroacetic acid, is often added to mobile phase. The effect varies depending on application but generally improves the chromatography.[70-72]

The quality of any chromatography separation depends on the characteristics of the compounds to be separated and their interactions with the column and the solvent. A profiling method for phenolic compounds requires uniform separation across the range of polarities; from the most polar (hydroxybenzoic and hydroxycinnamic acids) to the least polar (aglycones and polymethoxylated flavonoids) compounds.[73]

Phenolic compounds, because of their polar character, are separated mainly by partition chromatography on reversed phase, using columns filled with hydrocarbons such as C_8 (n-octyl) or more commonly, C_{18} (n-octadecyl). The elution was performed with a mobile phase of high polarity such as aqueous solutions which may contain a certain percentage of polar organic solvents such as acetonitrile or methanol.

Only reversed-phase columns were used, since phenolic compounds are weak acids that can be separated as neutral, relatively hydrophobic compounds in a weak acid matrix. Normal-phase columns have been used for proanthocyanidins in order to provide separation of the monomers through the decamers and a broad peak for the higher polymers.[74]

In liquid chromatography (LC), formerly *high-performance liquid chromatography* (HPLC), a combination of packing materials with smaller and

70 Lough WJ, Wainer IW (1992) High performance liquid chromatography, fundamental principles and practice. Blackie Academic & Professional. London, UK
71 McMahon G (2007) Analytical Instrumentation. John Wiley & Son. Chichester, UK
72 Meyer VR (2010) Practical high-performance liquid chromatography. John Wiley & Son. Chichester, UK
73 Harnly JM, Bhagwat S, Lin L-Z (2007) Profiling methods for the determination of phenolic compounds in foods and dietary supplements. *Anal Bioanal Chem* 389:47–61
74 Prior R, Gu L (2005) Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochem* 66:2264–2280

more uniform particles sizes, greater selectivity, and increased flow pressure yields both higher resolving power and faster analyses. Martin and Synge (1941), the inventors of modern chromatography, were aware that in theory the stationary phase requires very small particles, and therefore a high pressure was used to generate the flow required for LC in packed columns. Hence, HPLC was sometimes referred to as *high-pressure liquid chromatography*.[75]

Recently, there have been improvements in the LC technique with the development of ultra-performance liquid chromatography (UPLC) (**Figure 9**). This technique takes full advantage of chromatographic principles to perform separations using columns packed with smaller-sized particles, i.e. less than 2 μm, and a chromatographic system operating at very high pressures. This leads to a shorter analysis time, higher peak efficiency, and higher resolution.[76]

Figure 9. Simplified outline of a commercial UPLC.

75 Martin AJP, Synge RLM (1941) A new form of chromatogram employing two liquid phases. *Biochem J* 35:1358-1368

76 Wilson ID, Nicholson JK, Castro-Perez J, Granger JH, Johnson KA, Smith BW, Plumb RS (2005) High resolution "Ultra Performance" Liquid Chromatography coupled to oa-TOF mass spectrometry as a tool for differential metabolic pathway profiling in functional genomic studies. *J Proteome Res* 4:591-598.

5.3. Detectors coupled to LC

Different types of detectors can be coupled to liquid chromatography. The choice of the ideal detector for each application is based on the nature and properties of the analytes to be determined and the sensitivity required and what information is sought (structural, quantitative, etc.).

For the detection of phenolic compounds, two detection systems are widely used i.e. spectroscopic UV-visible and mass spectrometry. For the identification of compounds for which the standard is not available, nuclear magnetic resonance is very useful.

5.3.1. UV-visible absorbance

Polyphenols absorb in the ultraviolet (UV) region. Two absorption bands are characteristic of flavonoids. Band II, with a maximum in the 240-285 nm range, is believed to arise from the A-ring. Band I, with a maximum in the 300-550 nm range, presumably arises from the B-ring (**Figure 10**).[77,78] Among the flavonoids, anthocyanins are the most unique subclasses because they absorb visible light near 520 nm when the molecules are in the flavylium cation status (when pH is low).

The detector itself consists of a small liquid flow cell through which the eluent from the column flows. UV light passes through the cell and hits the UV photodetector. UV absorbance detectors are not destructive and respond only to substances that absorb radiation at the wavelength of the source light. There are two kinds of wavelength detectors: fixed and multiple wavelength detectors. The former does not allow changing the wavelength of the radiation, meanwhile the latter utilizes a narrow range of wavelengths to detect the solute. Its sensitivity is not as good as the fixed wavelength but it has advantage of flexibility. There are two main types of multiple wavelength detectors: the dispersion detector (variable wavelength detectors) that monitors the eluent at one wavelength only, and the diode array detector

77 Merken HM, Beecher GR (2000) Measurement of food flavonoids by high-performance liquid chromatography: A review. *J Agric Food Chem* 48:577

78 Robards K, Antolovich M (1997) Analytical chemistry of fruit bioflavonoids. *Analyst* 122:11R-34R

(DAD) that monitors the eluted solute over a range of wavelengths simultaneously. The second type has two major advantages, firstly it allows for the best wavelength(s) to be selected for analysis and secondly it allows all the spectra under a peak to be obtained and its purity to be determined.[71,79]

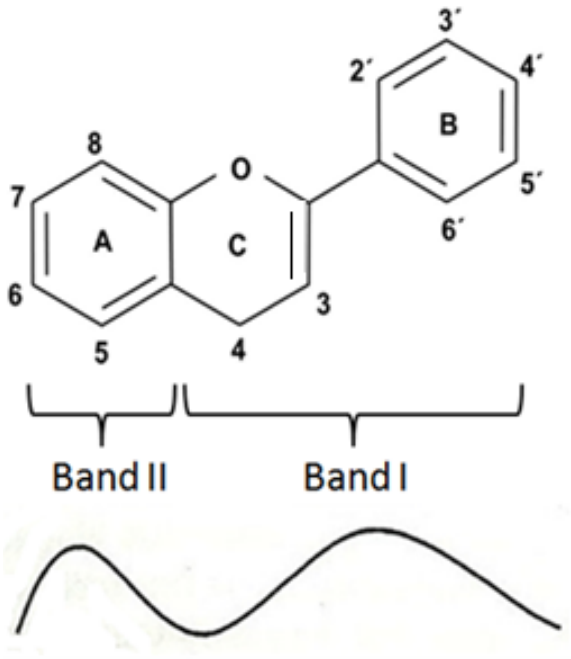


Figure 10. Basic structure of flavonoids and spectrum.

There is also combination of UV-vis spectrometric detector which is the most commonly used detector for HPLC. In terms of the electromagnetic spectrum, UV radiation covers the region from 190-350 nm, visible radiation covers the region 350-800 nm. Detection system based on the absorption of UV-visible is the most common in commercial HPLC equipment. The detection of analytes is based on the interaction between the UV-visible and the material that gives rise to absorption of certain wavelengths of radiation from such compounds. The multiple links combined in the compounds into the phenolic chromophore showing characteristic absorption bands in the UV region and visible (see **Table 3**).

79 Morgan NY, Smith PD (2011) HPLC detectors, in: Corradini (Ed.) Handbook of HPLC 2nd Ed. CRC Press, FL

Table 3. Absorption bands of each family of polyphenols.

Polyphenol classes	UV max (nm)	
Phenolic acids	270-280	
Hidroxycinamic acids	305-325	
Flavonoids		
Anthocyanidins	240-280	450-560
Aurones	240-270	340-370
Chalcones	220-270	340-390
Dihydrochalcones	~ 220	~ 280
Flavones	250-270	330-350
Flavonols	250-270	350-380
Flavanones	270-295	
Flavanols	270-280	
Proanthocyanidins	~ 280	
Isoflavonoids	245-270	300-340
Other polyphenols		
Coumarins	220-230	310-350

Many compounds in solution can absorb radiation in this region of the electromagnetic spectrum and, therefore, this detector can be considered universal. This behavior has the advantage that it can be used to solve a large number of analytical problems but may also be a drawback in cases requiring selectivity. The detection of UV-visible is very robust, making it one of the best ways to perform quantifications. While spectroscopy UV-visible gives no structural information and cannot unequivocally identify compounds when there is no pattern, it can be useful in determining phenolic compounds in order to limit the family to which the analytes belong, as every family has

certain characteristic absorption bands. Therefore, other techniques such as mass spectrometry are often necessary.

5.3.2. Mass spectrometry (MS)

Mass spectrometry is a microanalytical technique that can be used for the structural elucidation or confirmation of organic, bioorganic and organometallic compounds, and for quantitative analysis in environmental, pharmaceutical, forensic, food, and other sciences. The first step in MS measurement is the conversion of neutral molecules to charged species (i.e. ions), which are then separated according to their mass-to-charge (*m*/*z*) ratio in a mass analyzer. It is a fundamental requirement of mass spectrometry that the ions be in the gas phase before they can be separated according to their individual *m*/*z* values and detected. Any particles that are not ionic (molecules or radicals) are removed from the mass spectrometer by the continuous pumping that maintains the vacuum.

The relative abundances of individual *m*/*z* values are recorded by a suitable detector to produce what is known as a mass spectrum (MS). MS can be coupled to both gas-phase and liquid-phase separation techniques, enabling the structural analysis of complex mixtures after their chromatographic separation without time-consuming off-line isolation. [80-82] The unit of measure has become the dalton (Da), displacing other terms such as amu. 1 Da = 1/12 of the mass of a single atom of the isotope of carbon-12 (^{12}C).[83]

A mass spectrometry should always perform the following processes:

1. Produce ions from the sample in the ionization source.
2. Separate these ions according to their mass-to-charge ratio in the mass analyzer.

80 Ekman R, Sillberring J, Westman-Brinkmalm A, Kraj A, Desiderio DM, Nibbering NM (2009). Mass Spectrometry Instrument Interpretation, and Applications. John Wiley & Sons. New York, US

81 Miller JM (2005) Chromatography concepts & contrasts. 2nd Ed. Wiley & Sons

82 Watson JT, Sparkman OD (2007) Introduction to Mass Spectrometry. 4th ed. John Wiley & Sons. Chichester, UK

83 Balogh MP (2008) A Mass Spectrometry Primer: Part I. URL http://www.chromatographyonline.com/lcgc/article/articleDetail.jsp?id=542753. Accessed 10 May 2012

3. Eventually fragment the selected ions and analyze the fragments in a second analyzer.
4. Detect the ions emerging from the last analyzer and measure their abundance with the detector that converts the ions into electrical signals.
5. Process the signals from detector that are transmitted to the computer and control the instrument through feedback.[84]

Generally, the mass spectrometer consists of three main parts: the ion source, the mass analyzer, and the detector.

Ion source

In this part, analyte atoms, molecules, or clusters are transferred into gas phase and ionized neutral species into charged particles. The sample solution is introduced to the source by a syringe pump (direct infusion) or as eluent from a separation technique such as LC.

The main ion sources used to separate and determine phenolic compounds are electrospray ionization (ESI)[85-88], atmospheric-pressure chemical ionization (APCI)[89-91], fast-atom bombardment (FAB)[92-95], and

84 De Hoffmann E, Stroobant V (2007) Mass spectrometry: principles and applications, 3rd Ed. John Wiley & Sons. West Sussex, England

85 Waridel P, Wolfender J-L, Ndjoko K, Hobby KR, Major HJ, Hostettmann K (2001) Evaluation of quadrupole time-of-flight tandem mass spectrometry and ion-trap multiple-stage mass pectrometry for the differentiation of *C*-glycosidic flavonoid isomers. *J Chromatogr A* 926:29–41

86 Ferreres F, Silva BM, Andrade PB, Seabra RM, Ferreira MA (2003) Approach to the study of *C*-glycosyl flavones by ion trap HPLC–PAD–ESI/MS/MS: application to seeds of quince (*Cydonia oblonga*) *Phytochem Anal* 14:352–359

87 Hvattum E, Ekeberg D (2003) Study of the collision-induced radical cleavage of flavonoid glycosides using negative electrospray ionization tandem quadrupole mass spectrometry. *J Mass Spectrom* 38:43–49

88 Parejo I, Jáuregui O, Viladomat F, Bastida J, Codina C (2004) Characterization of acylated flavonoid-*O*-glycosides and methoxylated flavonoid from*Tagetes maxima* by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* 18:2801–2810

89 Barnes S, Kirk M, Coward L (1994) Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry. *J Agric Food Chem* 42:2466-2474

90 Wolfender JL, Waridel P, Ndjoko K, Hobby KR, Major HJ, Hostettmann K (2000) Evaluation of Q-TOF-MS/MS and multiple stage IT – MS^n for the dereplication of flavonoids and related compounds in crude plant extracts. *Analusis* 28: 895-906

91 De Rijke E, Zappey H, Ariese F, Gooijer C, Brinkman UATh (2003) Liquid chromatography with atmospheric pressure chemical ionization and electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments. *J Chromatogr A* 984:45-58

matrix-assisted laser-desorption ionization (MALDI).[96 - 98] These interphases supply energy to the solid sample or liquid in different ways, so that gaseous ions are directly formed while avoiding volatilization, thereby allowing the subsequent ionization of the compounds. APCI is commonly used for detecting low-molecular-weight polar and nonpolar compounds. MALDI, as a special soft ionization technique is applied mostly to the analysis of large organic molecules such as proteins, peptides, and polysaccharides. ESI is one of the most versatile ionization sources that provides a simple, real-time means of analyzing a wide range of polar molecules (100-200,000 Dalton range). It becomes the preferred choice for detecting polar compounds separated by liquid chromatography and capillary electrophoresis (**Figure 11**).

92 M. Becchi, D. Fraisse (1989) Fast atom bombardment and fast atom bombardment collision-activated dissociation/mass-analysed ion kinetic energy analysis of *C*-glycosidic flavonoids. *Biomed Environ Mass Spectrom* 18:122–130

93 Li QM, Claeys M (1994) Characterization and differentiation of diglycosyl flavonoids by positive ion fast atom bombardment and tandem mass spectrometry. *Biol Mass Spectrom* 23:406–416

94 Ma Y-L, Cuyckens F, Van den Heuvel H, Claeys M (2001) Mass spectrometric methods for the characterisation and differentiation of isomeric *O*-diglycosyl flavonoids. *Phytochem Anal* 12:159–165

95 Cuyckens F, Shahat AA, Pieters L, Claeys M (2002) Direct stereochemical assignment of hexose and pentose residues in flavonoid *O*-glycosides by fast atom bombardment and electrospray ionization mass spectrometry. *J Mass Spectrom* 37:1272–1279

96 Ramirez-Coronel MA, Marnet N, Kolli VSK, Roussos S, Guyot S, Augur C (2004) Characterization and estimation of proanthocyanidins and other phenolics in coffee pulp (*Coffea arabica*) by thiolysis-high-performance liquid chromatography. *J Agric Food Chem* 52:1344-1349

97 Monagas M, Garrido I, Lebrón-Aguilar R, Bartolome B, Gómez-Cordovés C (2007) Almond (*Prunus dulcis* (Mill.) D.A. Webb) skins as a potential source of bioactive polyphenols. *J Agric Food Chem* 55:8498-8507

98 Fulcrand H, Mané C, Preys S, Mazerolles G, Bouchut C, Mazauric J-P, Souquet J-M, Meudec E, Li Y, Cole RB, Cheynier V (2008) Direct mass spectrometry approaches to characterize polyphenol composition of complex samples. *Phytochem* 69:3131-3138

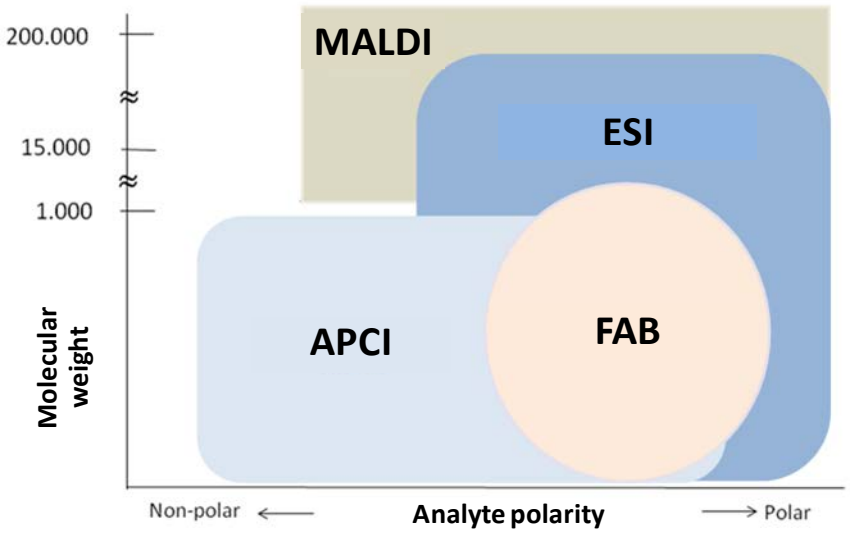


Figure 11. The application spectrum of different ion-source techniques.

In ESI, the sample is dissolved in a polar, volatile solvent and passed through the electrospray needle, which has a high potential difference (with respect to the counter electrode) applied to it. This high voltage between the needle and nozzle causes the fluid to form a Taylor cone, which is enriched with negative or positive ions at the tip. A spray of charged droplets is ejected from the Taylor cone by the electric field. The droplets shrink through evaporation, assisted by a warm flow of nitrogen gas passing across the front of the ionization source. Ions are formed at atmospheric pressure and pass through a small aperture into the high vacuum at the mass analyzer. The sample arrives to the spray chamber as a fine mist of droplets or spray. A drying gas, e.g. N_2, at a fairly high temperature causes the evaporation of any solvent from the droplets. A voltage gradient between the tip of the spray needle and the entrance to the transfer capillary, as well as a pressure difference from atmospheric pressure to vacuum, encourages appropriately charged ions to move into the capillary and on towards the skimmers.

This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle. The droplets are repelled from the needle towards the source-sampling cone on the counter electrode (shown in blue). As the droplets traverse the space between the needle tip and the cone, any solvent evaporates. This is circled

on the **Figure 12** and enlarged upon in **Figure 13**. As the solvent evaporates, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a "Coulombic explosion" occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the process as well as naked charged analyte molecules.

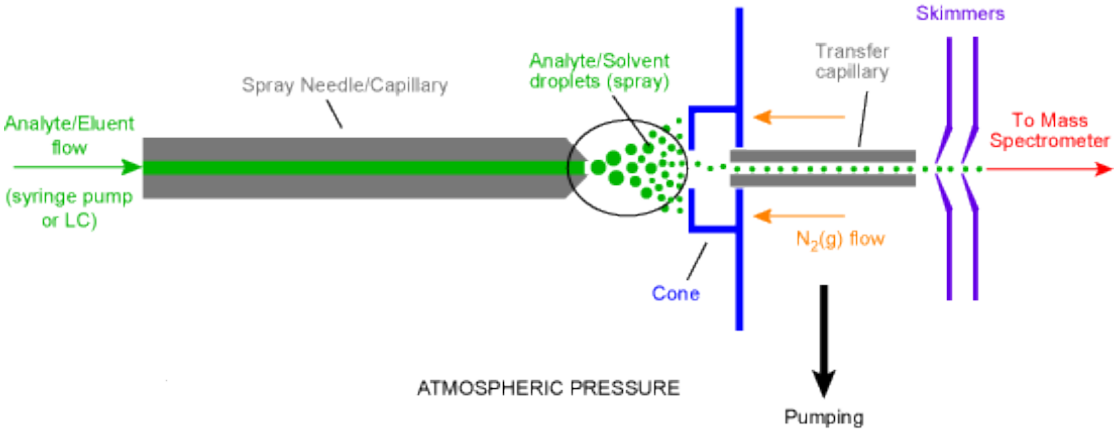


Figure 12. A schematic of an ESI interface.

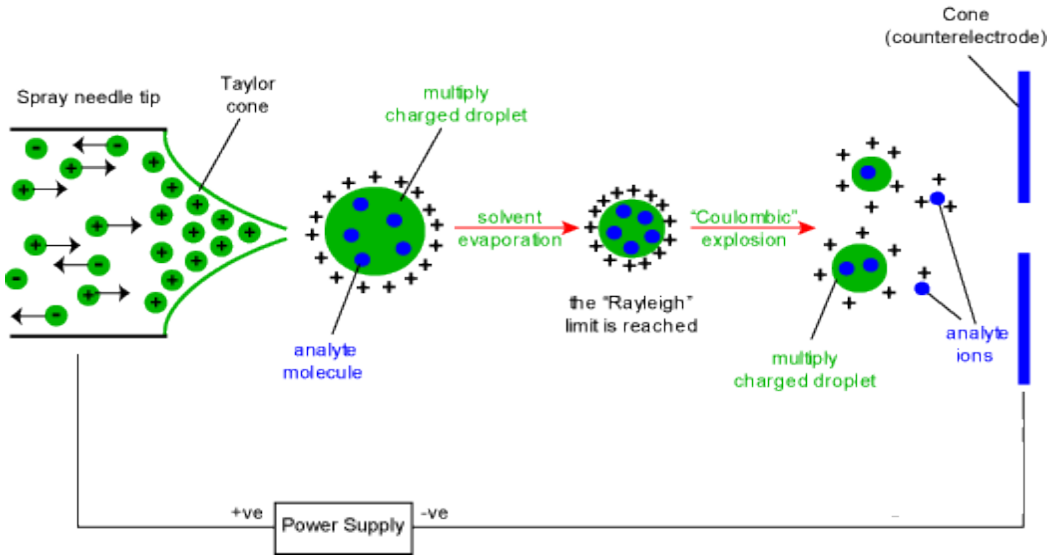


Figure 13. A schematic of the ion-formation mechanism.

These charged analyte molecules (which are not strictly ions) can be singly or multiply charged. This is a very soft method of ionization as very little residual energy is retained by the analyte upon ionization. It is the

generation of multiply charged molecules that enables high-molecular-weight components such as proteins to be analyzed, since the mass range of the mass spectrometer is greatly increased, as it actually measures the *mass-to-charge ratio* rather than mass *per se*. The major disadvantage of the technique is that very little (usually no) fragmentation occurs although this may be overcome through the use of tandem mass spectrometric techniques such as MS/MS or MS^n.[99,100]

A trace of formic acid or acetic acid is often added to aid protonation of the analyte molecules in the negative and positive ionization mode. In negative ionization mode, ammonia solution or a volatile amine is added to aid the deprotonation of the analyte molecules.

Mass analyzer

This is a device that can separate and sort ions coming from the ion source according to their *m/z* values. Therefore, an ion source has to be coupled to mass analyzer as the "heart" of the mass spectrometer.

The analyzer uses electrical or magnetic fields, or a combination of the two, to move the ions from the region where they are produced, to a detector, where they produce a signal which is amplified. Since the motion and separation of ions is based on electrical or magnetic fields, the mass-to-charge ratio, and not only the mass, is important. The analyzer is operated under high vacuum, so that the ions can travel to the detector with a sufficient yield.

Ion-trap (IT)

The principle of the trap is to store the ions in a device consisting of a ring electrode and two-end cap electrodes. The ions are stabilized in the trap by applying a RF (radio frequency) voltage on the ring electrode. For maximum efficiency, the ions must be focused near the center where the

99 Bristol Biogeochemistry Research Centre, University of Bristol (2005) High performance liquid chromatography mass spectrometry (HPLC/MS). URL http://www.bris.ac.uk/nerclsmsf/techniques/hplcms.html. Accessed 27 April 2005

100 Anon (2007) LC/MS applications guide. Dionex Corporation

trapping fields are closest to the ideal and the least-distorted, maximizing resolution and sensitivity. This is achieved by introducing a damping gas (99.998% helium) that by collision cools injected ions, damping down their oscillations until they stabilize (see **Figure 14**).

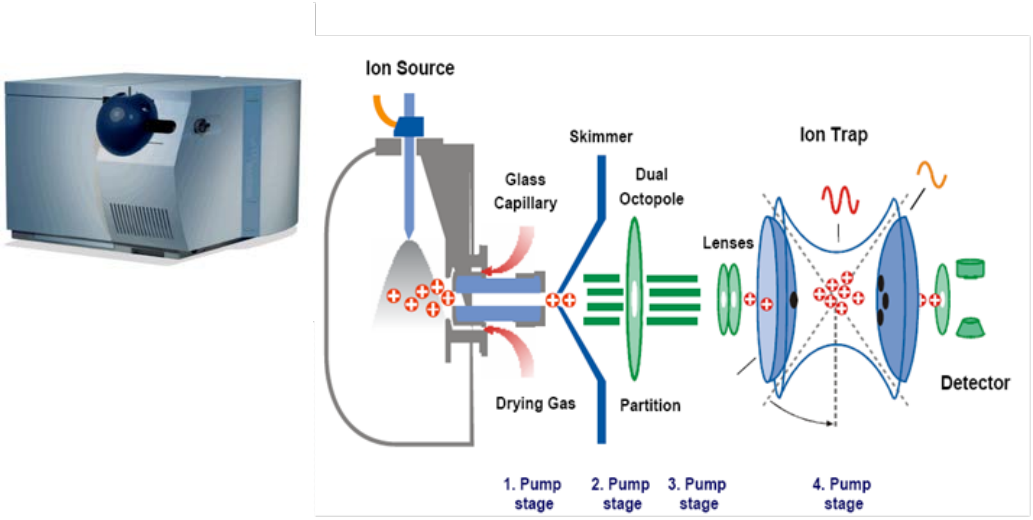


Figure14. A schematic presentation of ion transmission from the atmosphere to the ion-trap and detection. Copyright 2000 Bruker Daltonik GmbH.

By ramping the RF voltage, or by applying supplementary voltages on the end cap electrodes, or by a combination of the two, it is possible to:

a. Destabilize the ions, and eject them progressively from the trap
b. Keep only one ion of a given *m/z* value in the trap, and then eject it to observe it specifically
c. Or keep only one ion in the trap, fragment it by inducing vibrations, and observe the fragments. This is MS/MS. Since everything takes place in the same place, but at a different time, this approach is called MS/MS "in time"
d. Repeat the last operation a few times to progressively fragment the ions. That is MS/MS^n with a very high conversion efficiency for each fragmentation step at the the same time.

Briefly, steps of IT MS/MS described in **Figure 15** as follows: (1) Accumulation of "all" ions; (2) Isolation: only ions with a defined *m/z* value remain in the trap; (3,4) Excitation and fragmentation: acceleration of the ions within the trap. Ions undergo fragmentation due to collisions with background gas (He; "collision induced dissociation": CID). Only the ions of interest are fragmented; once fragments are produced they are not further

excited; (5) Fragment accumulation: without further acceleration fragments are “cooled“ by collisions with He. (6) Scan of fragment ions and acquisition of data; Instead of step (6), also a subsequent MS^3 or any further MS^n step (back to step 2) can be applied.[101]

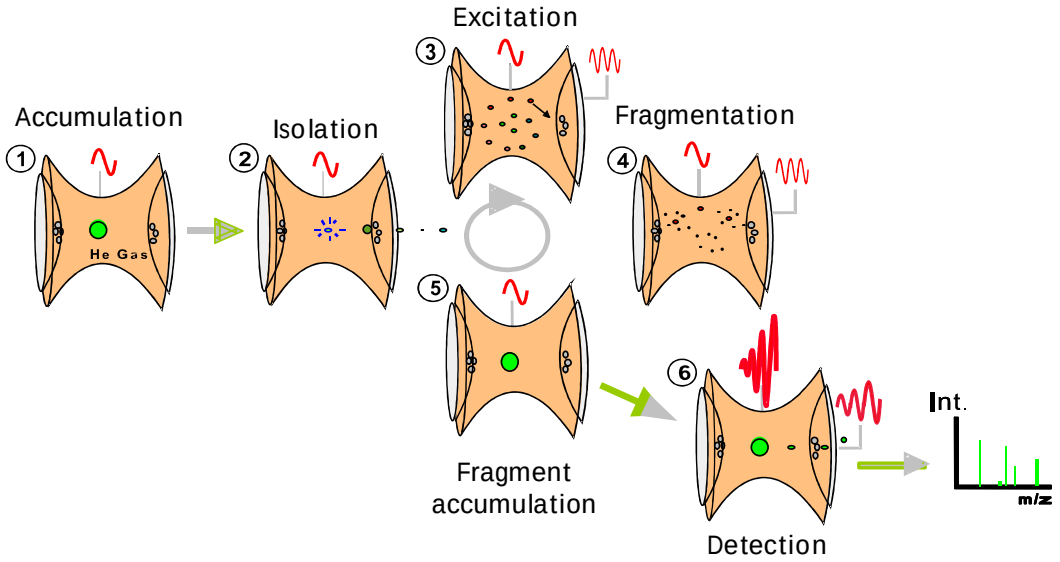


Figure15. Schematic representation of the IT process.

Resolution and performance in an ion-trap depend on the charge density of ions in the trap. If too many ions are present at the same time in the trap, the electrical fields become distorted. Also, collisions between the ions may occur, leading to unexpected dissociation or chemical reactions. In this case, the spectra and the quantification will be impacted.

Time-of-flight (TOF)

Time-of-flight mass analyzer separates ions according to the time difference between a start signal and the pulse generated when an ion hits the detector, i.e. time of flight. The faster or lighter the ion, the shorter the time of flight and the resulting time-of-flight spectrum can be converted into a mass spectrum. There is no need to know the exact potentials and distances of the spectrometer, as the time/mass conversion is made by calibration with ions of known masses. ESI can be connected to the TOF analyzers through

101 Bruker Daltonics Application Note #LCMS-18 (2001) Structural characterization of oligosaccharide derivatives using LC-ESI ion trap MS

orthogonal acceleration where the ions generated by the ion source enter the TPF analyzer perpendicular to its main axis.

In the time-of-flight (TOF) analyzer, ions formed in an ion source are extracted and accelerated to a high velocity by an electric field into an analyzer consisting of a long straight 'drift tube'. The ions pass along the tube until they reach a detector. After the initial acceleration phase, the velocity reached by an ion is inversely proportional to its mass (strictly, inversely proportional to the square root of its *m/z* value). Since the distance from the ion origin to the detector is fixed, the time taken for an ion to traverse the analyzer in a straight line is inversely proportional to its velocity and hence proportional to its mass (strictly, proportional to the square root of its *m/z* value). Thus, each *m/z* value has its characteristic time of flight from the source to the detector.[102,103]

Off-axis or orthogonal acceleration TOF-MS of ions from continuous ion beams has been known since the 1960s. However, it was only in the late 1980s and early 1990s that the current range of TOF-MS instruments with greatly improved resolving power and mass accuracy was developed. The key features enabling accurate mass measurement include high efficiency in gating ions from an external continuous source (e.g. ESI, APCI), simultaneous correction of velocity and spatial dispersion, and increased mass resolving power. The digital electronics revolution has supported TOF-MS more than MS technologies more heavily reliant on analog-signal processing. In this case, TOF-MS refers to orthogonal acceleration technology unless otherwise stated. A schematic presentation of orthogonal acceleration TOF-MS is shown in **Figure 16**.[104]

TOF-MS uses the differences in transit time through a flight/drift zone to separate ions of different masses. The principle is that smaller ions, being

102 Watson, JT, Sparkman OD (2007) Introduction to Mass Spectrometry, 4th ed. John Wiley & Sons
103 Anon., LC/MS booklet, Waters, France
104 Ojanperä S (2009) Drug analysis without primary reference standards: Application of LC-TOFMS and LC-CLND to Biofl uids and Seized Material, Dissertation. University of Helsinki Finland

lighter, will reach the detector faster than heavier ions will. It operates in a pulsed mode so that ions must be produced or extracted in pulses. An electric field is used to accelerate all the ions into the field-free drift zone where lighter ions have a higher velocity than heavier ones and reach the detector sooner.

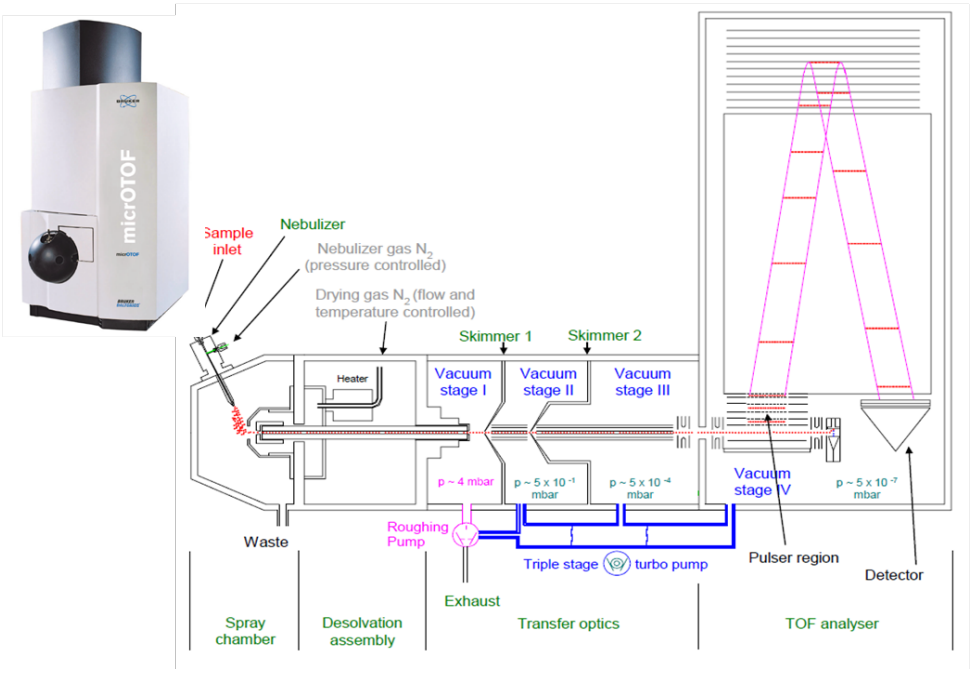


Figure16. A schematic presentation of an orthogonal acceleration (oa) time-of-flight mass spectrometer (TOF-MS). Copyright 2007 Bruker Daltonik GmbH.

Quadrupole-time-of-flight (Q-TOF)

The term "hybrid" applies to various mass spectrometer designs that are composites of existing technologies such as double-focusing, magnetic sectors, and more recently, ion-traps that "front" cyclotrons. One of the most interesting designs, the quadrupole time-of-flight (Q-TOF) mass spectrometer, couples a TOF instrument with a quadrupole instrument. This pairing results in the best combination of several performance characteristics: accurate mass measurement, the ability to carry out fragmentation experiments, and high-quality quantitation.

The development of quadrupole–time-of-flight (TOF) mass spectrometers have rapidly been embraced by the analytical community as powerful and robust instruments with unique capabilities. In particular, they combine the

high-performance of time-of-flight analysis in both the mass spectrometry (MS) mode as precursor and tandem MS (MS/MS) mode as product ion, with the well-accepted and widely used techniques of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). They are now applied to problems which range from nanospray analysis of biological samples to liquid chromatography (LC)/MS/MS of pharmaceutical preparations at high flow rates. Their rapid acceptance is due not only to the attractive combination of high sensitivity and high mass accuracy for both precursor and product ions, but also to the simplicity of operation for those already familiar with LC/MS analysis on quadrupole and triple quadrupole instruments.[105]

The tandem transmission Q-TOF mass spectrometer has the advantage of mass accuracy to the nearest 0.1 millimass unit. Tandem Mass Spectrometry (MS/MS) is a technique where structural information on sample molecules is obtained by using multiple stages of mass selection and mass separation. Meanwhile, hybrid mass spectrometers are instruments equipped with two or more different types of mass analyzers coupled together.

The high-mass accuracy results from the orthogonal arrangement between the longitudinal axis of the ion beam coming from the quadrupole and the direction in which the ion beam is extracted into the TOF instrument (**Figure 17**).

105 Chernushevich IV, Loboda AV, Thomson BA (2001) An introduction to quadrupole–time-of-flight mass spectrometry. *J Mass Spectrom* 36:849–865

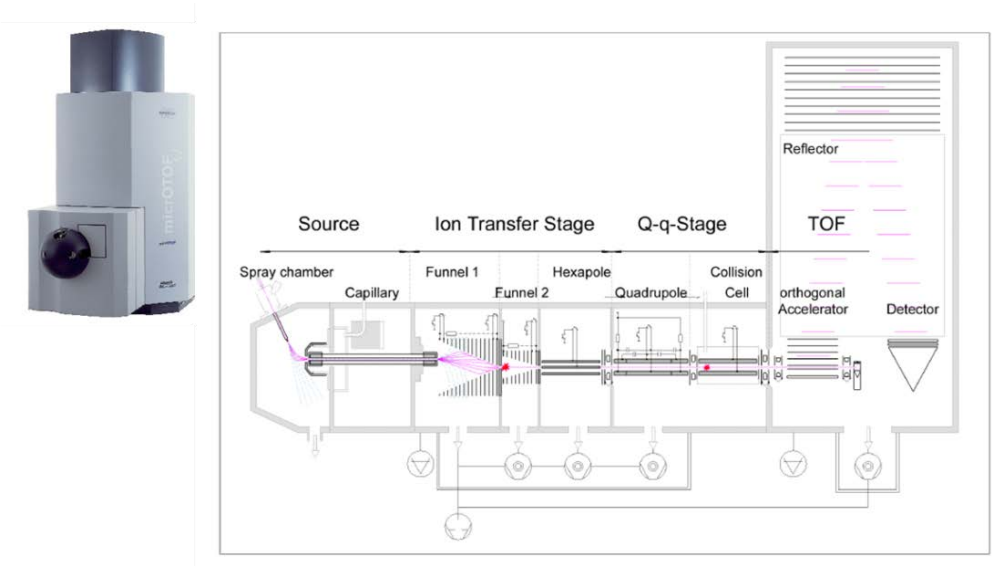


Figure17. A schematic presentation of an orthogonal acceleration (oa) quadrupole-time-of-flight mass spectrometer (Q-TOF-MS). Copyright 2007 Bruker Daltonik GmbH.

Detector

The role of the detector is to convert the energy of incoming particles into a current signal that is registered by the electronic devices and transferred to the computer of the acquisition system of mass spectrometer. The detector records the relative abundances of individual *m/z* values.[106] Since the mass analyzer and the detector require low pressure for operation the instrument also needs a pumping system.

Relative ion current (signal) is plotted versus *m/z*, producing mass spectrum. The peak at the highest *m/z* ratio is not always the molecular ion species ($[M–H]^-$ in the negative mode and $[M+H]^+$ in the positive mode), because adducts with solvent and/or acid molecules and also molecular complexes ($[2M–H]^-$ or $[2M+H]^+$) can be generated.[107,108]

106 Holčapek M (2009) A liquid chromatographer's introduction to mass spectrometry, *Sep sci* 1:19-23

107 Barnes S, Kirk M, Coward L (1994) Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry. *J Agric Food Chem* 42:2466-2474

108 Tian Q, Li D, Patil BS (2002) Identification and determination of flavonoids in buckwheat (*Fagopyrum esculentum* Moench, Polygonaceae) by high-performance liquid chromatography with electrospray ionisation mass spectrometry and photodiode array ultraviolet detection. *Phytochem Anal* 13:251-256

Experimental Section

Chapter 1

Advanced analytical techniques for the characterization of anthocyanins in fruits and vegetables

This work has been accepted as book chapter.

Chapter 8: Advanced Analytical Techniques for Characterization of Anthocyanins in Fruits and Vegetables in N. Motohashi (ed.) “Anthocyanins: Structure, Biosynthesis and Health Benefits”. Nova Science Publishers, Nova Science Publisher, NY. ISBN: 978-1-62257-329-5. *In press.*

Advanced Analytical Techniques for the Characterization of Anthocyanins in Fruits and Vegetables

Abstract

Over 600 anthocyanins have been identified to date. These structures have been determined by high-performance liquid chromatography (HPLC) coupled to UV-Vis (UV) detector, diode-array detector (DAD), and different mass spectrometry (MS) analyzers, such as ion-trap (IT), quadrupole (Q), time-of-flight (TOF) and hybrid analyzers Q-TOF. MS has generally been coupled to different ion sources such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Moreover, capillary electrophoresis (CE) coupled to DAD and MS detectors has also been used to characterize anthocyanins.

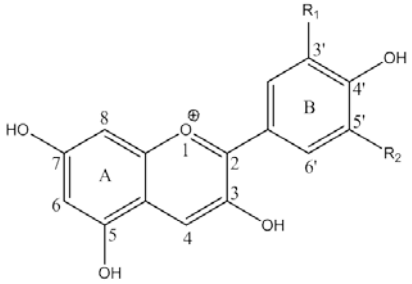
Among these analytical methods, the UV-Vis spectra represents a valuable analytical tool for identifying anthocyanins because they have a specific and intense absorbance band in the range of 520 to 560 nm; meanwhile, MS is a powerful tool which can provide information on molecular mass and also structural features of the different constituents.

This chapter provides an overview of the uses of HPLC and CE coupled to different detection systems, discussing the fundamentals of sample extraction and separation.

Introduction

Anthocyanins, water-soluble pigments found in flowers, fruits, leaves, grains, and other plant organs, are responsible for bright colors such as orange, red, purple, and blue. These pigments are found in the vacuoles and at the surface of the fruit and vegetable skin. Meanwhile, in flowers, they are located in epidermal cells and rarely in the mesophyll. Because anthocyanins act as pigments in a variety of plant organs, the color intensity, hue, and stability are key properties which are highly influenced by structure, pH, oxygen, and a large number of other factors.[1,2]

The basic chemical structure of an anthocyanin is an aglycone (anthocyanidin), with one or more sugars attached at the 3, 5, or 7 positions and possibly esterification on the sugars. The six most common anthocyanidins occurring in nature include delphinidin, cyanidin, pelargonidin, petunidin, peonidin, and malvidin (**Figure 1**) while the most common sugars in natural anthocyanins are glucose, galactose (hexoses), rhamnose, xylose, and arabinose (pentoses). Many anthocyanins have been found to be acylated by aliphatic or aromatic acids. The most frequent acylations are those with acetic, malonic, *p*-coumaric, and caffeic acids, whereas acylations with ferulic, *p*-hydroxybenzoic, succinic, oxalic, gallic, vanillic, syringic, protocatechuic, and malic acids are more restricted.[1,3] There are over 600 anthocyanins which have been identified from natural sources considering all these factors.



Anthocyanidins	R1	R2	Substitution pattern	MW
Pelargonidin (Pg)	H	H	3,5,7,4'-OH	271
Cyanidin (Cy)	OH	H	3,5,7,3',4'-OH	287
Delphinidin (Dp)	OH	OH	3,5,7,3',4',5'-OH	303
Peonidin (Pn)	OMe	H	3,5,7,4'-OH; 3'-OMe	301
Petunidin (Pt)	OMe	OH	3,5,7,4',5'-OH; 3'-OMe	317
Malvidin (Mv)	OMe	OMe	3,5,7,4'-OH; 3',5'-OMe	331

Figure 1. Chemical structures of six common anthocyanidins.

In aqueous media, anthocyanins undergo structural transformations with a change in pH in four basic structures (**Figure 2**), where the colored oxonium form predominates at a pH of approximately 2.0 or lower, which is orange to purple, and the colorless carbinol pseudo-base form at pH 4.5. When the pH rises, most of flavylium cation changes to other anthocyanin forms. The first reaction gives a colorless carbinol pseudo-base, which can undergo ring opening to a yellow chalcone, and later reactions give rise to a quinonoidal base with the formation of purplish-blue resonance-stabilized quinonoid anions.[4]

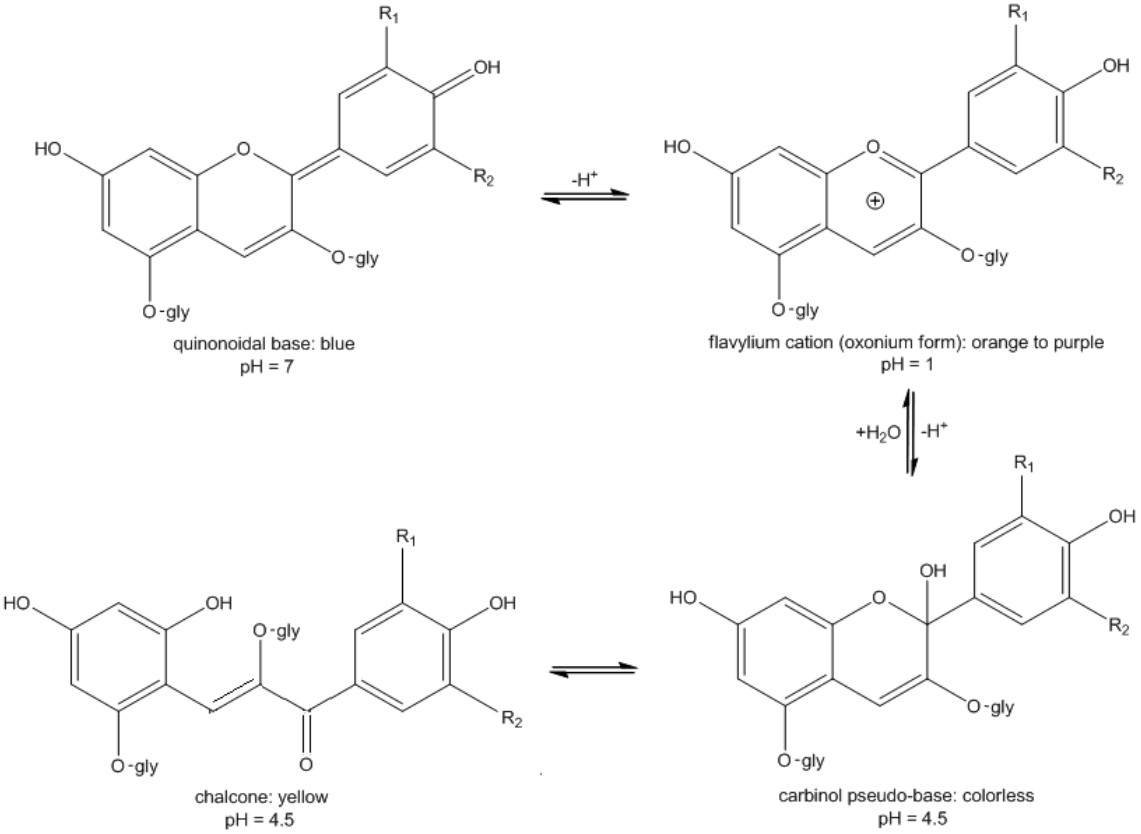


Figure 2. Structural transformations of cyanidins with change in pH.

Anthocyanins are highly reactive and easily degraded. Other factors affecting the anthocyanin stability, besides chemical structure and pH, include storage temperature, concentration, light, oxygen, solvents, enzymes, flavonoids, proteins, and metal ions.[5] Turker *et al.*, studying temperature in relation to the stability of shalgam anthocyanins, found that the monomeric

anthocyanin content and color density decreased with time as a function of storage temperature whereas the percentage of polymeric color and browning increased.[6] Furthermore, the effect of light, temperature, pH, and species on the stability of anthocyanin pigments in four *Berberis* species has also been investigated, revealing that increased pH, temperature or light exposure can degrade the anthocyanin molecule.[7]

The great interest in anthocyanins emerged because of their possible human-health benefits such as antiartherogenic, anticancer, antidiabetic, anti-inflammatory and antioxidant activities.[8-10] Recently, studying the red petals of *Rosa hybrida*, Lee *et al.* have reported that cyanidin 3,5-di-*O*-glucoside, the predominant constituent, showed high antioxidant activity, while pelargonidin 3,5-di-*O*-glucoside exhibited potent anticancer effects against cell cultures of LNCap (a human prostate cell line), ACHN (a human renal cell line), and MOLT-4F (a human leukemia cell line).[11]

Sample extraction methods

Plant matrices are usually so complex that direct analysis of the matrix is not possible. This makes it necessary to extract the components of interest —in this case, phenolic compounds. This first step is especially critical in the case of quantitative analysis. Many procedures for the polyphenol extraction from plant matrices have been described, most using liquid-liquid extraction (LLE) or solid-phase extraction (SPE).[12]

Whichever method is preferred, SPE or LLE, it must allow the recovery of the anthocyanins without any chemical modification. Anthocyanins are commonly extracted by organic solvents such as ethanol, methanol or acetone due to their property as polar molecules. The solvent destroys the cell membranes and simultaneously dissolves the anthocyanins and stabilizes them.[13] The extracting solution should be slightly acidic to maintain the flavylium cation form, which is red and stable in a highly acidic medium. The most common acids used for the extraction are hydrochloric acid or formic acid. However, the use of strong acid media should be avoided because the acyl moieties in acylated anthocyanins might be degraded by hydrolysis.[14]

Acetone is normally used for LLE, offering more efficient and reproducible results than using methanol or ethanol. This method works well because it can be performed at lower temperatures and, furthermore, it can avoid trouble with the presence of pectins found in the walls of some anthocyanins such as in berries. In acidic extraction environments, they form a turbid extract that slows the process. However, acetone contains pectin-clotting properties and rapidly produces a clean anthocyanin extract. For SPE, popular methods utilize C_{18} cartridges or Sephadex LH-20 for the initial separation of the anthocyanin extracts from the crude plant sample. The polar anthocyanins bind to these adsorbents through unsubstituted hydroxyl groups and are separated from other plant compounds using solvents of increasing polarity. Studies have shown that SPE is fast and highly efficient for separating crude anthocyanin solutions from plant material. With the collection of the anthocyanin extract, the next step is to remove the solvent to a known amount and continue with the analysis. Samples which need to be stored are generally stored as dried samples, evaporated and lyophilized, and storage in cold and dark environments to avoid decomposition of the anthocyanins.

Preliminary separation

After an anthocyanin extract is separated from plant tissue, it is necessary to evaluate whether purification is required. Generally, additional separation methods are followed to purify the sample before analysis because the extraction methods are not selective for anthocyanins and give crude anthocyanin extracts that might be co-extracted with sugars or organic acid. The appropriate techniques for a preliminary separation include large-scale chromatographic techniques, such as normal-phase column chromatography, counter-current chromatography (CCC), and preparative-HPLC.

The classic column chromatography, originally grew in popularity because of the need to obtain pure anthocyanin compounds in sufficient quantities for comprehensive characterization and to be used as reference standards. Normal-phase chromatography is normally used to separate non-ionic compounds with moderate molecular weight. Separation by CCC is based

on the partition of an analyte between two immiscible liquids by vigorous mixing in a separatory funnel, where one liquid works as the stationary phase and the other as the mobile phase. This chromatographic technique allows non-destructive isolation of the anthocyanin compounds. High-speed counter-current chromatography (HSCCC), a relatively new technique and the most advanced CCC, requires the use of two immiscible solvents, generally an organic and an aqueous phase, one being used as the stationary phase and the other as the mobile phase. HSCCC does not use a solid support as the stationary phase and therefore has several advantages over conventional chromatography: (1) the elimination of sample losses caused by irrecoverable adsorption to the solid support matrix; (2) easy scale-up to a larger fractionation system by simply changing the Teflon tubing coil (column) to larger sizes; (3) low-cost for not using expensive absorbents and columns; and (4) reflecting the real distribution profile of phytochemicals in a sample.[15]

A common multisolvent system composed of *tert*-butyl methyl ether (TBME)/*n*-butanol/acetonitrile/water acidified with 0.1% (v/v) trifluoroacetic acid (TFA) has been proven to be efficient regarding anthocyanin fractionation from a number of different plant matrices by using CCC.[16] A similar solvent system containing less TFA (0.01% v/v) can be used to avoid degradation of the labile malonylated anthocyanins.[17] Hillebrand *et al*. have used HSCCC and low-speed rotary CCC for the preparative isolation of anthovyanins in blue-fleshed potatoes. The common multisolvent system above was used. By means of HSCCC, it was possible to isolate the major pigment in large yields and good purity. Further isolation by using preparative-HPLC (described below) made it possible to isolate minor pigments from different CCC fractions.[18]

Preparative HPLC is used to isolate and/or purify compounds. For identification and structure elucidation of unknown compounds in crude plant extracts it is necessary to obtain pure compounds in amounts ranging from one to a few milligrams. This approach makes it possible to apply a large amount of sample to the stationary phase. The columns that are used can be constituted by the same packing material, but with a larger dimension, as the material used for the analytical HPLC methods and the same mobile phases

with simple gradient or isocratic system. The collected fractions from preparative HPLC can be monitored by TLC and the similar TLC plates will correspond to the fractions that can be combined, concentrated, and used to proceed with isolation.[19]

Separation methods

The analysis of anthocyanins starts with the extraction of anthocyanins from plant tissues, followed by their isolation and purification from crude plant extract and, ultimately, the identification of each anthocyanin. This analysis is quite complex because of possible structural transformations and complexation reactions. These compounds are difficult to measure separately from other flavonoids because they have similar structures and reactivity characteristics. Finally, pure anthocyanin standards, which are integral for accurate quantification purposes, are not readily available for purchase and are not easily purified from plant sources for use in research, partly due to the aforementioned instability issues.

High-performance liquid chromatography (HPLC)

HPLC methods tend to utilize gradient solvent systems of acetonitrile water or methanol water with a small amount of acid to lower the solution pH and bolster the stability of the anthocyanins. These solvents are commonly used due to their compatibility with gradient methods for isolation and the various detection systems used for identification that are able to be coupled to the HPLC. For reproducible results using this instrumentation, the pH of the mobile phase and temperature of the column must be controlled due to the instability of anthocyanin compounds in changing pH and temperature environments.

In reverse-phase HPLC, the most common mode for separating anthocyanins, the order of elution is delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin derivatives, consecutively. This can be explained by the fact that the retention time decreases with increasing polarity signed by raising the number of hydroxyl moieties in the flavylium

ion. On the contrary, acylation and the presence of sugars lengthens the retention time where diglycosides elute before monoglycosylated anthocyanins.[20] Normal-phase systems, or those using unmodified silica gel, are not effective for separating anthocyanins due to the polarity of these compounds.

The analytical separation and identification of anthocyanins have been summarized in recent review articles,[4,21-24] reflecting that liquid chromatography occupies a primary position among the other analytical techniques. In general, HPLC separations are performed by using C_{18} reverse-phase columns and a binary solvent gradient. The mobile phases used consist of an acidified aqueous solution and an organic solvent such as acetonitrile and methanol. HPLC is most frequently coupled to simple ultraviolet (UV), photodiode-array detection (DAD) and MS detector. This technique has proven to be a powerful analytical tool for identifying anthocyanins, particularly. **Table 1** lists the most representative references on anthocyanin analysis by HPLC.

Table 1. Recent reports on the analysis of anthocyanins by HPLC.

Sample	Sample preparation and extraction conditions	Separation techniques and detector	Stationary phase	Mobile phase	Refer-ence
Vitis vinifera L.	Extraction with MeOH:HCl (1000:1 v/v) centrifuged, filtered	HPLC–DAD–ESI-Q-MS	Waters Novapack C_{18} (150 × 3.9 mm, 4 µm)	A: water/formic acid, 90:10 B: water/MeOH/ formic acid, 45/45/10, v/v	25
Blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry	Extraction with MeOH containing 0.1% HCl	HPLC–DAD–ESI-IT-MS	Symmetry C_{18} (250 × 4.6 mm, 5 µm)	A: water, 1% formic acid B: ACN	26
Grape pomace	Extraction with MeOH with 1% 1N HCl and followed by	HPLC–UV-DAD HPLC–ESI-MS	Phenomenex Luna C_{18} (250 × 4.6	A: water/formic acid, 9:1 (v/v)	27

Table 1. (Continued).

	hexane		mm, 5 µm)	B: ACN/formic acid, 9:1 (v/v)	
Strawberry fruit (*Fragaria ananassa*)	Liquid-liquid extraction with MeOH:0.1% HCl (99:1) followed by removal of lipids by *n*-hexane. Lipid-free extract was subjected to silica-gel-column chromatography to separate sugars and acid; whereas carotenoids were isolated from anthocyanins using TLC	HPLC-UV-Vis	Particil C_{18} (250 x 4.6 mm, 5 µm)	A: 0.1% TFA in water B: ACN	28
Commercial fruit juices and fruit drinks	Samples were centrifuged, filtered and injected directly to HPLC system	HPLC-PDA-MS^2	Synergi Max-RP column (250 x 4.6 mm i.d., 4 µm)	A: 1% formic acid B: ACN	29
Bilberry (*Vaccinium myrtillus* L.)	Extraction with solvent A-B (10:90 v/v); ACN-MeOH (85:15 v/v) (solvent A) and 8.5% aqueous formic acid (solvent B)	HPLC-DAD	Phenomenex Gemini C_{18} (150 x 4.6 mm i.d., 5 µm) equipped with 4 x 3 mm C_{18} precolumn	A: ACN-MeOH (85:15 v/v) B: 8.5% aqueous formic acid	30
Delonix regia flowers	Extraction with deionized tap water acidified with citric acid (0.05 N), filtration	HPLC-DAD-ESI-MS^2	HPLC-DAD: RP 18 Satisfaction column, (250 x 4.6 mm, 0.45 µm) HPLC-MS: Merck LiChrospher 100-RP 18 (column 250 x 2 mm, 5 µm)	HPLC-DAD A: 10:90 formic acid/water B: 10:90 formic acid/ACN HPLC-MS A: water and formic acid (98:2,v/v) B: water:ACN: formic acid (18:80:2, v/v)	31
Vegetables, nuts, and grains	Extraction with MeOH:water:acetic acid (85:15:0.5; v/v, MeOH:H_2O:AcAc), dilution with acidic MeOH	HPLC-DAD-ESI-MS^2	Zorbax Stablebond Analytical SB-C_{18} column (4.6 x 250 mm, 5 µm)	A: 5% formic acid aqueous solution) B: MeOH	32
Blue-fleshed potatoes	Extraction with water/HCl (19/1, v/v)	Preliminary separation: 1.	Luna RP-18 column	A: water/ACN/ formic acid:	18

Table 1. (Continued).

(*Solanum tuberosum* L.)		Amberlite XAD-7 column. The column was washed with water and the anthocyanins were eluted with a mixture of MeOH/acetic acid (19:1, v/v). 2. Countercurrent Chromatography (CCC): High-Speed CCC and Low Speed Rotary CCC. 3. Preparative HPLC HPLC-DAD, ESI-MSn and NMR	(250 x 4.6 mm, 5 µm)	87/3/10 v/v B: water/ACN/ formic acid: 40/50/10 v/v	
Muscadine grapes	Extracted with 80:20 (v/v) MeOH–water solution containing 0.1 mL/L HCl. The suspension was loaded to Waters C_{18} Sep-Pak SPE and eluted with MeOH	HPLC–DAD–ESI-Q-MS	Symmetry C_{18} (150 × 3.0 mm, 5 µm)	A: water/formic acid, 95:5 B: MeOH/formic acid, 95:5	33
Purple rice bran	Extraction of lipophilic sample with hexane. Extraction of hydrophilic sample with MeOH	HPLC-PDA	Discovery C_{18} column (3 mm i.d. × 25 cm)	A: 0.4% TFA in water B: ACN	34
Organic commercial juices	Samples were filtered, diluted, and injected directly into the HPLC system	LC-DAD-ESI-Qq-TOF-MS	Phenomenex Synergy Fusion RP100A (50 × 2 mm, 2.5 µm)	A: water B: ACN, both with 0.1% (v/v) formic acid	35
Blueberry	Extraction with a mixture of MeOH:water:TFA (70:30:1, v/v/v)	HPLC-UV HPLC-ESI-IT-TOF-MS	Method 1: ODS Hypersil column (200 × 4.6 mm, 5 µm) Method 2: Prodigy 5 mm ODS3 reversed phase silica (250 x 4.6 mm i.d.) and followed by	Method 1 and 2: A: water:THF: TFA (98:2:0.1, v/v/v) B: ACN A: 10% formic acid in water B: ACN	36

Table 1. (Continued).

			Zorbax SB-C_{18} column (150 mm × 4.6 mm, 3.5 µm)		
Caucasian blueberries (*Vaccinium arctostaphylos* L.)	Extraction with solvent A-B (10:90 v/v); ACN-MeOH (85:15 v/v) (solvent A) and 8.5% aqueous formic acid (solvent B)	HPLC-DAD-ESI-MS^3	Gemini C_{18} (150 x 4.6 mm i.d., 5 µm) fitted with 4 x 3 mm i.d. C_{18} guard column	A: MeCN/MeOH (85:15 v/v) B: aqueous 8.5% formic acid	37
Hibiscus sabdariffa	The powder was mixed with water and injected directly to HPLC system	HPLC-DAD-ESI-TOF/IT-MS	Zorbax Eclipse Plus C_{18} (4.6 x 150 mm, 1.8 µm)	A: 10% formic acid B: ACN	38
Chinese sugarcane (*Saccharum sinensis* Roxb.) tips, stems, roots and leaves	Sugarcane tips, stems, roots: Ultrasonic maceration with MeOH/water (6:4, v/v) Sugarcane leaves: washing with petroleum ether to remove chlorophyll and ultrasonic maceration with MeOH/water (6:4, v/v). TLC analysis using n-BuOH/EtOH/water/ formic acid (16:4:8:1, v/v)	HPLC-UV DAD HPLC-IT-MS/MS	Waters C_{18} Symmetry column (150 x 4.6 mm i.d., 4.6 µm)	A: 0.1% formic acid in water B: MeOH	39
Fruit peel of *Vitis coignetiae* Pulliat (meoru)	Extraction with MeOH containing 0.1% HCl. The solid residue was washed with MeOH. The extract was loaded onto an Amberlite XAD-7 column and washed with water and ethyl acetate. The anthocyanin pigments were eluted with MeOH containing 1% aqueous HCl.	HPLC-PAD and ESI-IT-MS	Zorbax StableBond Analytical SB-C_{18} column (4.6 x 250 mm, 5 µm)	A: aqueous 0.5% v/v formic acid B: MeOH	40
Mulberry Fruit (*Morus alba* L.)	Ectraction with 95% alcohol/0.1% HC l (1:1, ratio) and	HPLC-PAD-ESI-IT-MS and ^1H NMR	Waters RP C_{18} column (250 x 4.6 mm i.d., 5	A: water containing 0.1% TFA	41

Table 1. (Continued).

	isolation using C-18Sep-Pak cartridge		µm)	B: ACN containing 0.1% TFA	
Purple-Fleshed Sweetpotatoes	Extraction with 7% acetic acid in 80% MeOH	HPLC-DAD/ESI-MS/MS	Synergi Polar-RP C_{18} (250 x 2 mm i.d., 4 µm) equipped with a guard column of 7.5 x 4.6 mm i.d., Allsphere Phenyl 5 µm.	A: 1% v/v formic acid in water B: ACN containing 1% formic acid)	42
Berberis boliviana Lechler	Extraction with aqueous acetone/water (30:70 v/v), filtration	Anthocyanin purification: The aqueous extract was passed through a C-18 mini column and eluted with 0.01% aqueous HCl. HPLC-PDA, HPLC- triple quadrupole - MSMS	Sample was semi-purified with a C_{18} cartridge and the phenolic fraction (containing anthocyanins) was eluted with 1% HCl-acidified MeOH. Waters Symmetry C_{18} column (4.6 x 150 mm, 3.5 µm)	A: 10% formic acid in water B: ACN	43
Waxberry (*Myrica rubra* Sieb. et Zucc.)	Extraction with 0.1% HCl in EtOH	(HPLC) with photodiode-array detection (PAD) and (MALDI-TOF-MS) ^{1}H NMR	Preparative HPLC (separation and isolation): (250 × 21.2 mm i.d., 7 µm) Zorbax Stable Bond-C_{18} preparative column, coupled to a (50 × 21.2 mm i.d., 5 µm). Zorbax Stable Bond-C_{18} guard column. The crude anthocyanin extract was purified by C_{18} Sep-Pak cartridge	Preparative HPLC:formic acid/water/ MeOH (10:75:15 v/v). Analytical HPLC-PAD: A: 0.1% TFA in water B: 0.1% TFA in ACN	44

Table 1. (Continued).

			open-column chromatography. Analytical HPLC-PAD: Kromasil C_{18} (250 × 4.6 mm i.d., 5 µm) with (4 × 3 mm i.d.) Phenomenex C_{18} guard cartridge		
Skin extracts of red grape (*Vitis vinifera* and *Vitis labrusca*) pomace	Extraction with 50 mL of a solvent mixture comprising MeOH/ water/acetic acid (80:20:5). Extract was added to polyamide SC6 column and eluted with MeOH	HPLC-DAD	Prodigy ODS3 reversed-phase silica column (250 × 4.6 mm i.d., 5 µm)	A: water:THF: TFA (98:2:0.1) B: MeOH:THF: TFA (98:2:0.1)	45
Purple Bordeaux Radish (*Raphanus sativus* L.)	Extraction with MeOH/water/HCl (60:40-0,03, v/v). The residue was mixed with 2 N NaOH and HCl (37%) was added to the reaction mixture and passed through a Waters OASIS HLB cartridge and washed with water and then with MeOH-1% HCl	LC-PDA-ESI/MS^n For accurate mass measurements: UPLC-HRMS	Symmetry 100 RP-18 (150 x 2.1 mm i.d., 3.5 µm) with (10 mm x 2.1 mm i.d.) guard column of the same material. For accurate mass measure-ments: column (200 x 2.1 mm i.d., 1.9 mm) Thermo Hypersil Gold AQ RP-C_{18} with an HPLC/UHPLC precolumn filter	A: 0.1% formic acid in water B: 0.1% formic acid in ACN	46
Strawberry and elderberry extracts	Extraction with MeOH containing 0.1% HCl	RP-HPLC at pH 1.8, and compared with separations achieved by capillary zone electrophoresis	ODS-Hypersil reversed phase column (200 x 2.1 mm, 5 pm)	*Strawberry* A: aqueous 0.6% $HC1O_4$ B: MeOH, *Elderberry* A: aqueous 0.6% $HC1O_4$	47

Table 1. (Continued).

		using a standard silica capillary and pH 8.0 running buffer		B: THF CZE: The fused silica capillary was 57 cm total length (50 cm to detector) x 75 pm i.d., with 150 mM sodium borate buffer (pH 8.0)	

* This table contains a representative selection of references. Additional references are available in the references cited.

Capillary electrophoresis (CE)

Another analytical technique such as capillary electrophoresis (CE), has proven to be an advantageous alternative to HPLC in anthocyanin analysis for a very small sample size, low operation cost, high efficiency, shorter analytical time, and reagent use. CE separates compounds based on differences in their electrophoretic mobility and offers excellent mass sensitivity, high resolution, low sample consumption, and minimal generation of solvent waste. In fact, for anthocyanins, CE can be highly efficient separation technique and ideal for charged compounds such as anthocyanins (**Table 2**).

There are different modes in CE separations. Capillary-zone electrophoresis (CZE) is the most common mode used for separating anthocyanins, the migration of a particular compound depending on its charge-to-size ratio. That is, the total migration time for positively charged smaller molecules is longer than that for molecules of lesser charge and/or larger size. The separation is usually performed in the positive polarity mode followed by a positive electroosmotic flow with migration of the compounds from anode to cathode. The applicability of CZE in basic media is limited by the instability of anthocyanins in basic environments; therefore, the separation of anthocyanins from plant extracts by CZE use acidic media and configure the system to run from cathode to anode. The acidic media will

ensure that the anthocyanins remain in the protonated flavylium form and the migration of anthocyanins will be towards the anode, reversing the electroosmotic flow. The speed by which they are drawn to the anode depends on their charge-to-size ratio, as seen with normal electroosmotic flow. With acidic media and the acidic form of anthocyanins, another problem arises with the formation of ionic interactions between the flavylium cation and the anionic silanol groups covering the capillary tube.

Table 2. Recent reports on the analysis of anthocyanins by capillary electrophoresis.

Sample matrix	Sample preparation and extraction condition	Separation conditions	Reference
Wine and wine must	Extraction with SP (polymer phase, Strata SDB-L) and elute with 0.1% (v/v) formic acid in MeOH	CE/MS^n Fused-silica separation capillary without internal coating (60 cm x 50 µm i.d.) Buffer: 1) acidic one (chloroacetate ammonium, pH 2.0) and 2) basic one with high selectivity towards derivatives containing vicinal hydroxyl groups (borate ammonium, pH 9.0)	48
Red wines	Fractionation of sample by Gel Permiation Chromatography (GPS) TSK Toyopearl gel HW-50F, column (120mm×12mm i.d.). First fraction (F1) was eluted with EtOH/water/TFA (55:45:0.05, v/v/v) and second fraction (F2) with acetone/water (60:40, v/v). Sample fractions were dissolved in synthetic wine (12% (v/v) EtOH in aqueous solution containing 6 g L^{-1} tartaric acid, pH 3.5)	CZE-DAD Uncoated fused-silica capillary Buffer: Sodium tetraborate buffer solutions (50 mM) of pH 9.4 with 10% MeOH (v/v) content, and 56 cm (effective length) capillary	49
Hibiscus sabdariffa L.	Extraction A: with MeOH/HCl (99:1 v/v) for 4 h. Extraction B: with MeOH/HCl (99:1 v/v) by sonication for 30 min Extraction C: with acetic acid (15% v/v) for 48 h. Filtrate was mixed with Amberlite XAD-2, packed into a glass column and eluted with EtOH (70% v/v)–acetic acid (1% v/v)	CE-TOF/IT-MS Fused-silica capillaries with 50 µm i.d. x 80 cm Buffer: 200 mM boric acid (pH 9.0)	50

Table 2. (Continued).

Strawberry	Extraction with MeOH, followed with 95% aqueous MeOH. The extract was dissolved in acidified water (3% formic acid) and passed through an SPE Strata C18-E 55 µm 70 A cartridge (500 33333333mg/3 mL) (Phenomenex) and recovered with MeOH containing 3% formic acid	CZE UV–vis (DAD) Capillary cartridge: polyimide-coated fused-silica tube (375 µm o.d., 50 µm i.d.) Total capillary Length: 57 cm with effective length: 50 cm. Buffer: 250 mM monobasic sodium phosphate containing 30% v/v ACN (pH 1.4)	51
Red onions (*Allium cepa* L.)	Extraction with (EtOH and formic acid 99:1 v/v)	CZE -ESI-TOF-MS Bare and poly-LA 313-coated fused-silica capillaries with 50 µm i.d. x 365 mm o.d. and 57 cm length. Buffers: ammonium acetate, ammonium formate, borate, acetic acid, formic acid and mixtures of formic acid and hydrochloric acid, with ionic strength (IS) in the range 1–75 mM and with pH in the range 1.6–11.0	52
Grape (*Vitis vinifera* L. cv. Malbec)	Extraction with synthetic solution (EtOH, 12%; tartaric acid, 6 mg/mL; SO2, 100 µg/mL; pH 3.2) and diluted 1:50 (v/v) with acidified distilled water (1% v/v HCl)	CZE-UV fused-silica capillary, 67 cm full length, 50 cm effective length, 75 µm i.d., 375 µm o.d. Buffer: 20 mM sodium tetraborate containing 30% MeOH, pH 9.00	53

Detection and edentification

UV-Vis spectroscopy

Spectral characteristics of anthocyanins can be detected in two distinctive absorption bands, *i.e.* in the UV-region (260-280 nm) and in the visible region (490-550 nm). It should be taken into consideration when comparing the same anthocyanins that they must be in the same solvent because the solvent will affect the position of absorption bands.

Mass spectrometry

Mass spectrometry is a technique that can be used for the structural elucidation or confirmation of organic, bioorganic, and organometallic compounds, as well as for quantitative analysis. The first step in MS

measurement is the conversion of neutral molecules to charged species (*i.e.* ions), which are then separated according to their mass-to-charge (*m*/*z*) ratio in a mass analyzer. The relative abundances of individual *m*/*z* values are recorded by a suitable detector to produce what is known as a mass spectrum. MS can be coupled to both gas-phase and liquid-phase separation techniques, enabling the structural analysis of complex mixtures after their chromatographic separation without time-consuming off-line isolation.

Different MS analyzers have been used in the determination of anthocyanins such as triple quadrupole (QqQ), ion-trap (IT) and time-of-flight (TOF). The QqQ was generally employed to search for anthocyanins with experiments such as neutral loss scan and product ion scanning, while IT allows structure fragmentation of sample by MS^n. Both analyzers, however, provide nominal mass accuracy alone and may not be well applied in untargeted phenolic compounds. Orthogonal acceleration TOF-MS provides much better accuracy and precision of mass information generated. These accurately measured mass values with a mass error less than 5 ppm can be used to produce candidate empirical formulae and identify the potential substance with elemental composition analysis.[54]

The main ion sources used to separate and determine phenolic compounds are electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), continuous-flow fast-atom bombardment (CF-FAB) and matrix-assisted laser desorption ionization (MALDI) (**Figure 3**). These interphases supply energy to the solid sample or liquid in different ways, so that the direct formation of gaseous ions occurs while avoiding the volatilization, thereby allowing the subsequent ionization of the compounds. APCI is commonly used for detecting low-molecular-weight polar and nonpolar compounds. MALDI, as a special soft ionization technique is applied mostly to the analysis of large organic molecules such as proteins, peptides, and polysaccharides. ESI is one of the most versatile ionization sources that provides a simple, real-time means of analyzing a wide range of polar molecules (100-200,000 Dalton range). It becomes the preferred choice for

detecting polar compounds separated by liquid chromatography and capillary electrophoresis.

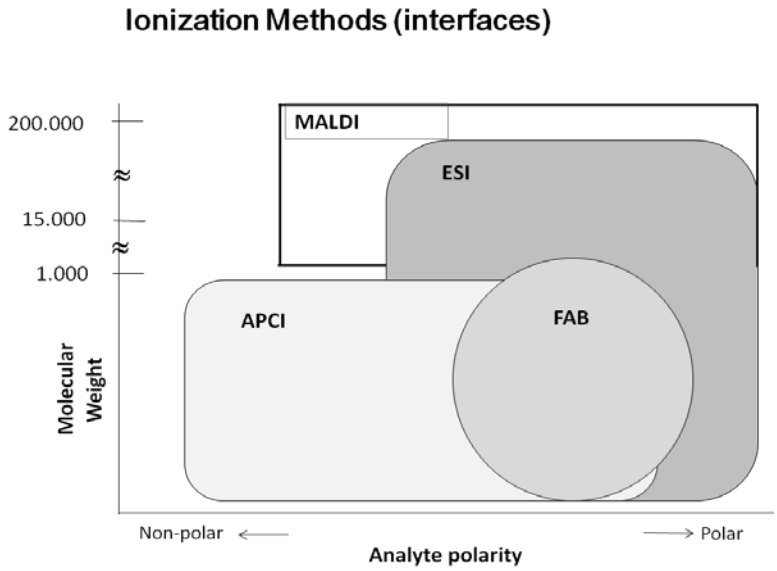


Figure 3. The application spectrum of different ion-source techniques.

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is highly useful for identifying compounds when standards are not available. To facilitate the interpretation of NMR spectra, acidified media such as DCl or CF_3COOD need to be used in order to displace the equilibrium, and therefore only the flavylium cation exists in the solution.

The hyphenated technique of HPLC-NMR is now well established as a powerful technique for the structure elucidation of unknown compounds, including the identification of various forms of anthocyanins in crude plant matrices. The physical interfacing of these two instruments is not difficult. The development of solvent-suppression technique can solve the problems in observing of analyte response in the presence of non-deuterated solvents.

Applications

HPLC

The most frequently used detection system for HPLC is UV/Vis spectrophotometry. This is due to the prevalence of spectrophotometric

instrumentation in laboratories studying anthocyanin chemistry, as well as in the industrial and academic sectors. Anthocyanins have a unique absorption maximum at ~520 nm, which sets these compounds apart from other flavonoids in the plant extract and simplifies the resulting chromatograms for isolation and purification.

HPLC hyphenated to mass spectrometry (MS) is one of the most important analytical techniques used for detecting phenolic compounds. Recently, chromatographic performance has been improved by using columns packed with small particles. The newest column packed with a particle size of less than 2 µm and operated at a pressure of up to 600 bar enables high-resolution analysis in shorter times. The on-line coupling of HPLC with MS using ESI as an interface constitutes a powerful method because of its highly efficient resolution and accurate characterization of a wide range of polar compounds. ESI, one of the most versatile ionization techniques, is preferred for detecting polar compounds separated by liquid chromatography. The advantages of MS detection includes the ability to determine molecular weight and to gain structural information. TOF-MS offers excellent mass accuracy over a wide dynamic range while offering measurements of the isotopic pattern and providing important additional information for determining the elemental composition. Furthermore, TOF-MS permits the rapid and efficient confirmation of the elemental composition of ions in fragmentation studies and also provides high selectivity in the determination of compounds in complex matrices using the extracted ion chromatogram (EIC) mode when there are overlapping peaks. Otherwise, IT-MS can be used to fragment ions of structural relevance for identifying target compounds in a highly complex matrix.

Figure 4 shows an example of the chromatograms resulting from the coupling HPLC-DAD and MS (TOF/IT) to analyze the aqueous extract of *Hibiscus sabdariffa* (roselle). Dry leaves of the plant were manually milled to a fine powder. An amount of *H. sabdariffa* was weighed and mixed with ultrapure water at room temperature. The aqueous extract of *H. sabdariffa* was freshly prepared for each analysis. The aqueous extract was stirred in a

vortex, filtered, and directly injected into the HPLC system. In the positive ionization mode, the two most representative anthocyanins were identified, namely delphinidin 3-sambubioside (peak 16) and cyanidin 3-sambubinoside (peak 17) using the base peak chromatogram (BPC). This was consistent with the UV spectra at 520 nm.

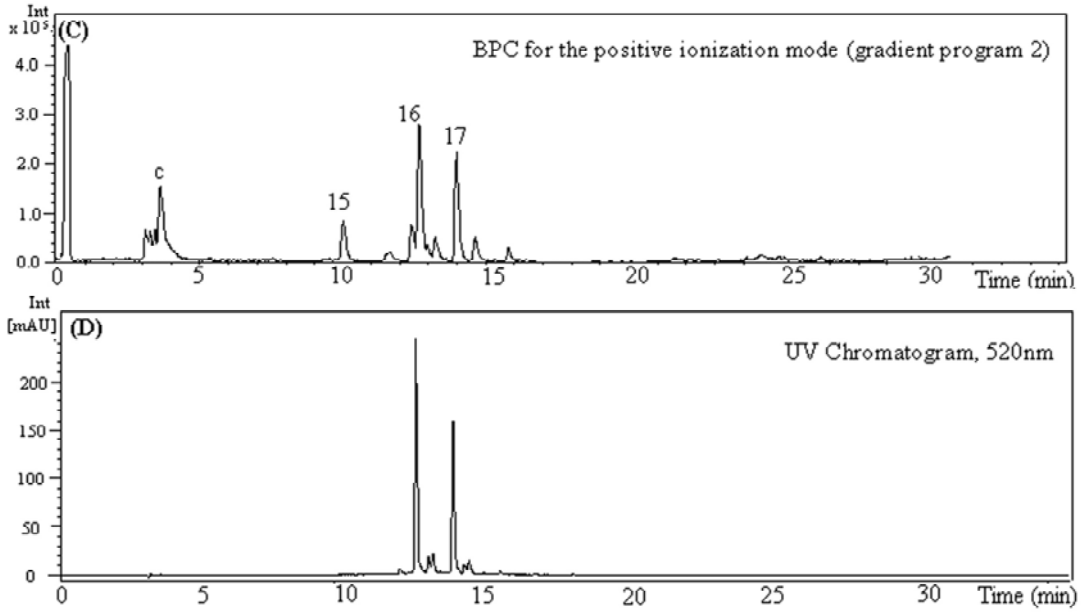


Figure 4. Chromatographic profiles an aqueous extract of *Hibiscus sabdariffa* (C) Base peak chromatogram (BPC) in positive mode. (D) UV chromatogram at 520 nm. Reproduced from Rodríguez-Medina *et al.* (2009)[35] with permission from John Wiley and Sons.

The application of tandem MS (MS-MS or MS^n) can be used to determine individual compounds in a mixture or to identify the structure of compounds by separate ionization and fragmentation steps. An important mass spectrometer for structure elucidation is the triple quadrupole ion-trap because it can serve as an exceptionally high specificity detector due to a mass filter capable of transmitting only the ion of choice. There are three quadrupoles in which the first (Q1) and third (Q3) quadrupoles act as mass filters, and the middle (Q2) quadrupole is employed as a collision cell, where the ionization takes place. This filtering process works by mass to charge ratios (*m/z*) and allows the study of fragments which are crucial in structural elucidation. For example, the Q1 can be set to filter out an ion of a known mass, which is fragmented in Q2, and Q3 can then be set to scan the entire *m/z* range, giving information on the sizes of the fragments made as well as information on one specific ion fragment. Thus, the structure of the original

ion can be deduced. However, as mentioned above, ESI has been an efficient ionization technique for HPLC-MS because it is a soft technique and does not tend to produce many fragments, which are essential for MS/MS. Because of this, HPLC-MS/MS uses two ionization methods: ESI to introduce a parent ion into the mass spectrometer, and then collision-induced dissociation (CID) to enhance the fragmentation of the parent ion.

CE

An example of a complete anthocyanin analysis would be selective extraction, separation, and identification of anthocyanins in roselle using SPE-CE-MS(TOF-IT).[50] Three extraction procedures (extraction procedures A-C) were tested in this study in order to compare the one that showed the highest capacity to extract mainly anthocyanins. With extraction procedure A, the sample was mixed with MeOH/HCl (99:1 v/v) for 4 h at room temperature. During this time the solution was magnetically stirred. In extraction procedure B, the sample was extracted with MeOH/HCl (99:1 v/v) by sonication for 30 min and filtered. Finally, with extraction procedure C, the sample was mixed with acetic acid (15% v/v) for 48 h with magnetic stirring and then filtered. The filtrate was mixed with Amberlite XAD-2 for 40 min. The amberlite particles were then packed into a glass column. The anthocyanins remained absorbed on the column they were eluted with 1 L of EtOH (70% v/v) and acetic acid (1% v/v).

As reflected in **Table 2**, a major number of compounds were extracted by employing procedure C. The analysis by CE-ESI-MS using IT and TOF analyzers showed that anthocyanins glycoside derivatives were present in the methanolic extract of dried roselle calyces. **Figure 5** shows the base peak electropherogram (BPE) and the extracted ion electropherogram (EIE) of the identified compounds of the extract C under CE-ESI-MS conditions, and revealed two main anthocyanins with retention times at 11.8 min (Cy 3-sambubioside) and 11.9 min (Dp 3-sambubioside).

Table 2. The *m/z* ratio found in different roselle extracts. Reproduced from Segura-Carretero *et al.* (2008)[50] with permission from John Wiley and Sons.

Extract A	Extract B	Extract C	Analytes
-	-	465.1	Delphinidin 3-*O*-glucoside
581.2	581.2	581.2	Cyanidin 3-sambubioside
-	-	595.1	Cyanidin 3-rutinoside
597.2	597.2	597.2	Delphinidin 3-sambubioside
-	-	611.2	Cyanidin 3,5-*O*-diglicoside

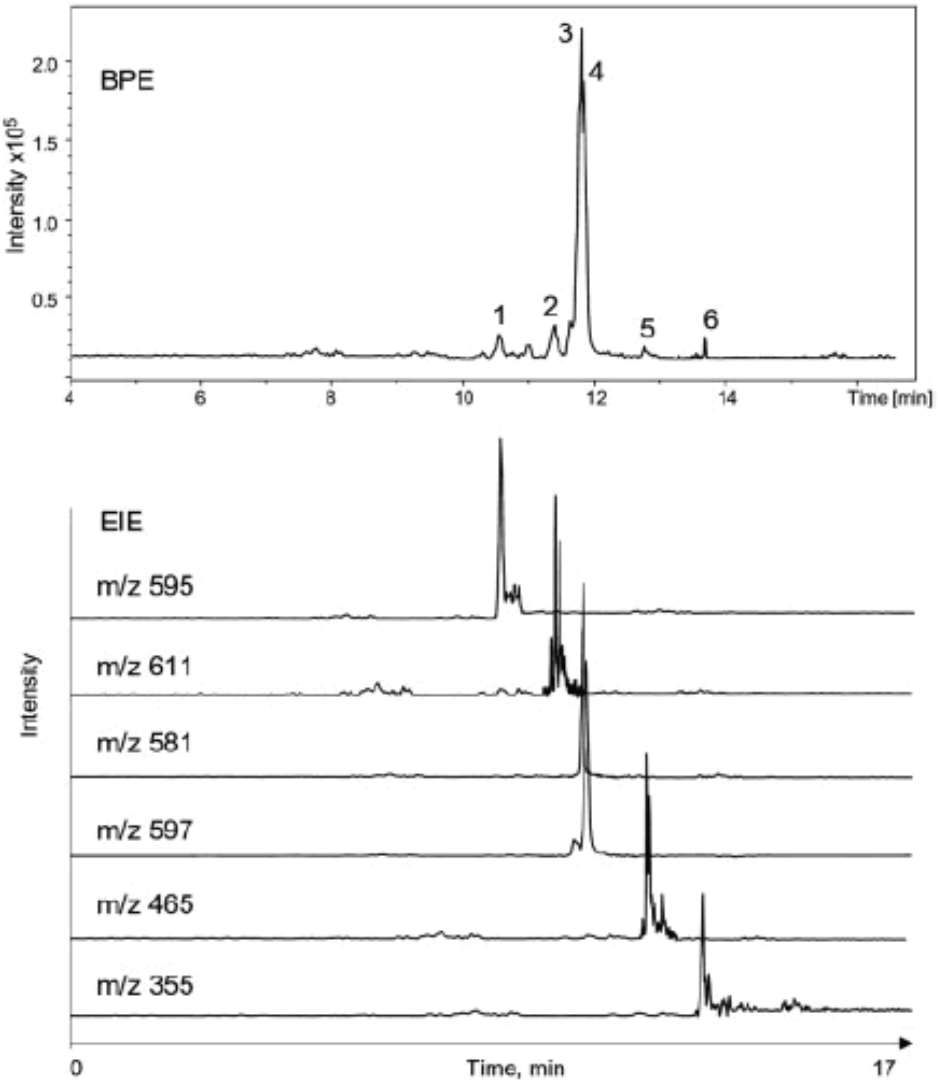


Figure 5. Base-peak electropherogram and extracted ion electropherogram of roselle. Reproduced from Segura-Carretero *et al.* (2008)[50] with permission from John Wiley and Sons.

In **Figure 6**, the MS and MS/MS spectra of the most important compounds in dried roselle flower are shown and resulted in clear and characteristic fragmentation patterns.

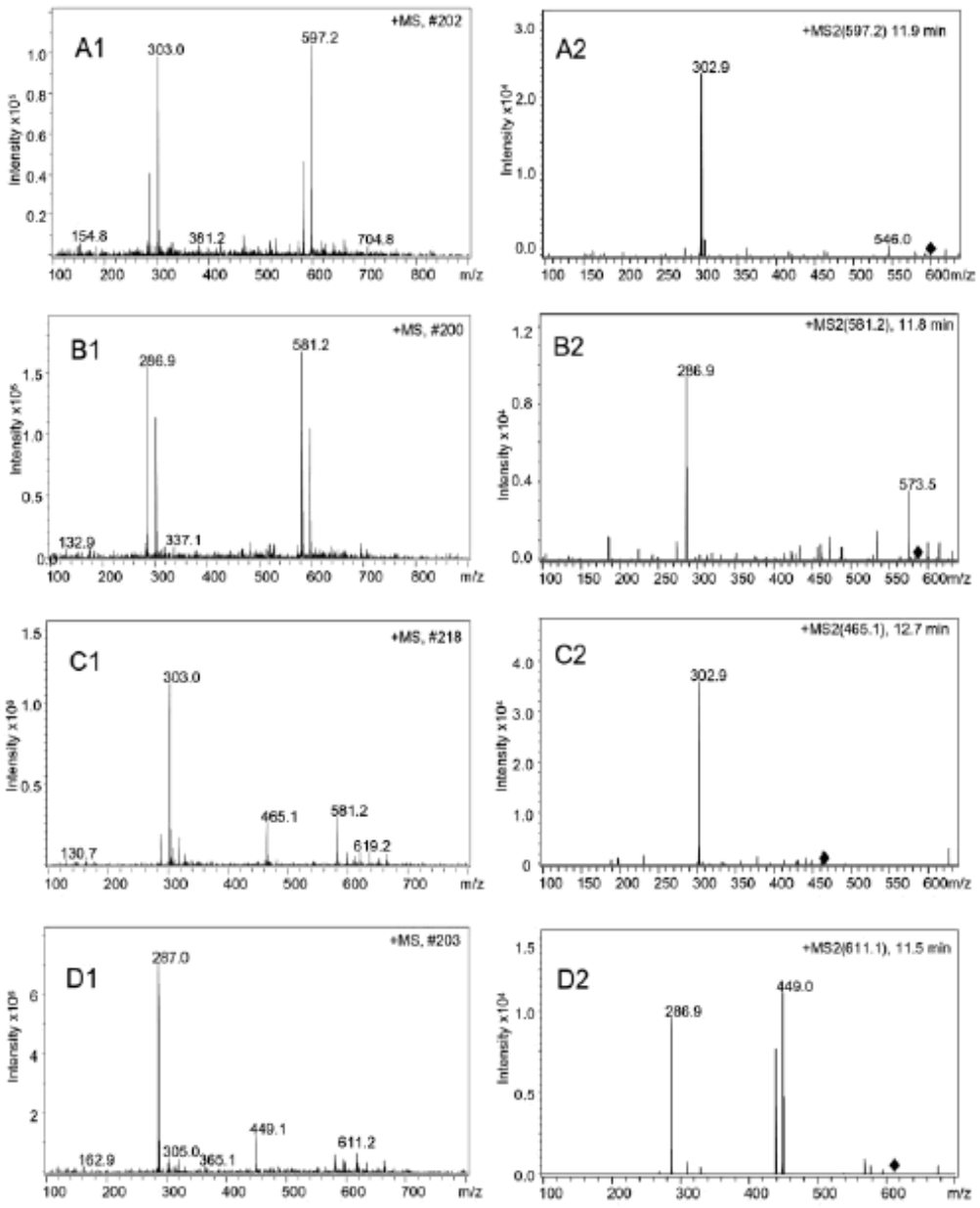


Figure 6. MS (A1, B1, C1, D1) and MS^2 (A2, B2, C2, D2) spectra of the most important dried calyces of roselle compounds. MS/MS of compounds in extract C. (A) Dp-3-sambubioside, (B) Cy-3-ambubioside, (C) Dp-3-glucoside, and (D) Cy-3,5-diglucoside. Reproduced from Segura-Carretero *et al.* (2008)[50] with permission from John Wiley and Sons.

Conclusions

In this chapter, different powerful analytical tools, such as HPLC and CE coupled to DAD, MS and NMR have been described to separate and determine anthocyanins from complex matrices. These techniques have proved very useful for determining most unknown compounds without reference in complex matrices. The difficulty to obtain reference compounds and the

spectral similarities of the anthocyanins represents a major drawback. Therefore, MS and NMR have become the preferred techniques for anthocyanin identification.

Given the beneficial effect for the health of anthocyanins such as anti-artherogenic, anticancer, antidiabetic, anti-inflammatory and antioxidant activities, and their use as a natural colorant of processed foods, the interest in anthocyanins continues to grow. The implementation of better extraction, purification, and identification methodologies will have an impact in the establishment of new tools for the food-product authentication.

The analytical methodologies described here represent the current and foremost methodologies for the complete analyses of anthocyanins. As we look forward to the development of HPLC/CE-MS, the coupling with other efficient structural analysis techniques, especially NMR, can offer advantages, thanks to several improvements that have been made to increase the sensitivity of NMR, bringing it more into line with MS sensitivity and enabling the analysis of anthocyanins at low concentration.

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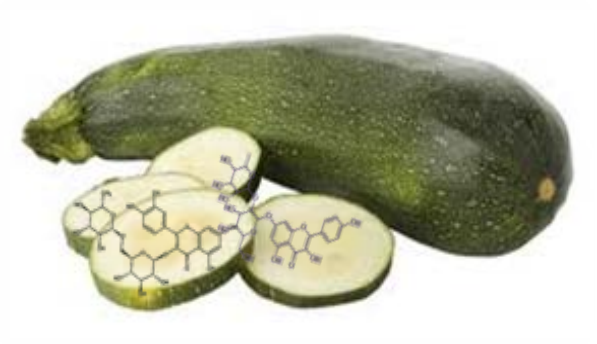

Chapter 2

Profiling of phenolic and other polar compounds in zucchini (*Cucurbita pepo* L.) by reverse-phase high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

This work has been submitted to *Food Research International.*

Profiling of phenolic and other polar compounds in zucchini (*Cucurbita pepo* L.) by reverse-phase high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

Abstract

Polyphenolic compounds are widely distributed in vegetables. Zucchini (*Cucurbita pepo* L.) has a high nutritional value and a low amount of calories. Nevertheless, few studies in the literature focus on the separation and identification of phenolic and other polar compounds in whole zucchini fruit. Therefore, the present study characterizes phenolic and other polar compounds in three kinds of zucchini (Verde, Redondo, and organic Verde) bought in Almería (Spain). For this purpose, an extraction method using methanol:water (80:20 w/w) has been applied in order to extract the polar fraction from the samples studied. Afterwards, the extracts were injected into the RP-HPLC system and separated by a C_{18} column. Q-TOF-MS detection was conducted using a micrOTOF-Q equipped with an electrospray ionization (ESI) interface. The combined use of RP-HPLC coupled to DAD and Q-TOF-MS has proved useful for characterizing numerous phenolic and other polar compounds in zucchini. A total of 57 compounds were identified with the present methodology. In fact, to the best of our knowledge, 41 out of 57 identified compounds are being reported here for the first time in whole zucchini fruit.

Keywords: Cucurbita pepo L.; zucchini; phenolic compounds; polar compounds; HPLC; Q-TOF-MS

Introduction

The daily intake of polyphenols has received much attention due to the health benefits of their antioxidant/anti-radical, anti-carcinogenic, anti-inflammatory, antiviral, and antimicrobial activities. This group of chemical compounds is widely distributed in vegetables and can be divided based on their chemical structure, ranging from simple molecules such as phenolic acids, to highly polymerized compounds such as tannins.[1]

The zucchini (*Cucurbita pepo* L.), of the Cucurbitaceae family, is low in calories and contains high nutritional[2] and medical value.[3,4] These fruits can be found in many shapes, from spherical to elongated, and they vary in skin colors from dark to light green, sometimes with fine whitish mottling or stripes.

Most of the data reported in this sphere concern the composition of pumpkin (*C. moschata*) seeds. Appendino *et al*. have reported large amounts of two triterpenes esterifed with *p*-aminobenzoic acid.[5] Isoflavones such as daidzein and genistein have been found in the pumpkin seeds by Adlercreutz and Mazur[6] who also found secoisolariciresinol. Later, Sicilia *et al*. found lariciresinol as the second lignin in this kind of sample.[7] Cucurbitosides A to E and cucurbitosides F to M as acylated phenolic glycocides have been identified in the seed of *C. moschata*[8] and *C. pepo*[9], respectively. Furthermore, pumpkin-seed oil has been demonstrated to possess high antioxidant capacity in methanol-water extract due to its content in polar phenolic compounds[10,11], but the compounds which are responsible of this antioxidant capacity have not been identified.

Little knowledge is available on the phenolic compounds present in the extract of whole zucchini fruit. Therefore, in the present study, polyphenolic compounds in zucchini extract were studied using HPLC coupled with two different detection systems: diode array (DAD) and quadrupole time-of-flight (Q-TOF) mass spectrometry. The UV/visible range has proved valuable for identifying the family of these phenolic compounds while MS and MS/MS has enabled accurate mass measurements and complementary structural

information. Other polar compounds that could be characterized using this method have also been reported.

Materials and methods

Zucchini samples

Two zucchini varieties were analyzed: “Verde” (elongated in shape with light-green skin and light-grayish mottling) and “Redondo” (more spherical and dark-green skin with sharp longitudinal stripes and whitish speckles). Also, the organically grown “organic Verde” (cultivated chemical free) was studied. These were bought at local markets and stored at 4 ºC until used.

Sample preparation

Each sample (whole fruit) was ground, frozen at -20 ºC, and then placed on a lyophilizer (Christ Alpha 1-2 LD Freeze dryer, Shropshire, UK) shelf, which had been pre-cooled to -50ºC at 1 mbar for 1 h. Afterwards, 500 mg of lyophilized zucchini were extracted using 16 mL of 80:20 (v/v) methanol/H_2O and sonicated at room temperature for 30 min. The mixture was centrifuged at 4000 rpm for 15 min and the supernatant was collected. After centrifugation, the solvent was evaporated to dryness using a rotary evaporator under vacuum, and the residue was resolved in 500 μL of 80:20 (v/v) methanol/H_2O. Finally, the supernatant was filtered with a polytetrafluoroethylene (PTFE) syringe filter (0.2 μm pore size) and stored at -20C until analyzed.[12]

Chemicals

Acetic acid was from Fluka, Sigma-Aldrich (Steinheim, Germany) and the organic solvents methanol and acetonitrile were from Lab-Scan (Gliwice, Sowinskiego, Poland). Distilled water with a resistance of 18.2 MΩ was deionized using a Milli-Q system (Millipore, Bedford, MA, USA). A G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA) was used as a vortex mixer.

HPLC conditions

LC analyses were made with an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode array detector (DAD). Separation was carried out with a Zorbax Eclipse Plus C_{18} analytical column (150 mm x 4.6 mm, 1.8 µm particle size).

Gradient elution was conducted with two mobile phases consisting of acidified water (0.5% acetic acid v/v) (phase A) and acetonitrile (phase B) according to gradient methods described previously[12] with some modifications: 0-20 min, linear gradient from 0% B to 20% B; 20-30 min from 20% B to 30% B; and 30-35 min from 30% B to 50% B, 35-45 min from 50% B to100% B. Finally, the initial conditions were maintained for 10 min. The flow rate used was set at 0.80 mL/min. The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio=1:4). Thus, in this study the flow which arrived into the ESI-Q-TOF-MS detector was 0.2 mL/min. The column temperature was maintained at 25°C and the injection volume was 10 µL. UV data were collected using DAD set at 240 and 280 nm.

ESI-Q-TOF-MS conditions

Q-TOF-MS was conducted using a micrOTOF-Q™, Bruker Daltonics GmbH, Bremen, Germany) an orthogonal-accelerated Q-TOF mass spectrometer equipped with an electrospray ionization source (ESI). Analysis parameters were set using a negative ion mode with spectra acquired over a mass range of 50-1,100 *m/z*. The optimum values of ESI-MS parameters were: capillary voltage, +4,000 V; dry gas temperature, 190 ºC; dry gas flow, 9.0 L/min; nebulizer pressure, 2.0 bar; and spectra rate 1 Hz. Moreover, automatic MS/MS experiments were performed adjusting the collision energy values as follows: *m/z* 100, 20 eV; *m/z* 500, 30 eV; *m/z* 1000, 35 eV, and using nitrogen as a collision gas.

The accurate mass data of the molecular ions were processed through the software Data Analysis 4.0 (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formula by using the Smart Formula Editor. During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, passing a solution of sodium acetate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% acetic acid in water/isopropanol 1:1 (v/v). With this method, an exact calibration curve was drawn based on numerous cluster masses, each differing by 82 Da ($NaC_2H_3O_2$). Due to the compensation of temperature drift in the micrOTOF-Q, this external calibration provided accurate mass values (better 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

Results and discussion

Chromatographic profile and compound characterization

Metabolite profiling compounds were successfully separated and characterized using RP-HPLC-ESI-DAD-Q-TOF-MS. The Q-TOF-MS base peak chromatogram (BPC) of "Verde", "Redondo", and "organic Verde" zucchini extracts are depicted in **Figure 1** (**a**, **b**, **c**, respectively), where the compounds are identified by number from 1 to 62. In the variety Verde, 48 compounds were identified and most were also identified in both Redondo and organic Verde zucchini. Compounds which were not identified in Verde zucchini were subsequently numbered in the two other kinds of zucchini analyzed. The metabolite assignments were identified by interpreting the mass spectra determined via their MS and MS/MS (Q-TOF), taking into account all the data provided by the literature. **Table 1** provides a summary of all the compounds studied, including retention times, experimental and calculated *m/z*, molecular formula, error (deviation between the measured mass and theoretical mass), sigma value (an exact numerical comparison between the theoretical and measured isotope patterns), MS/MS fragments, as well as

putative compounds.

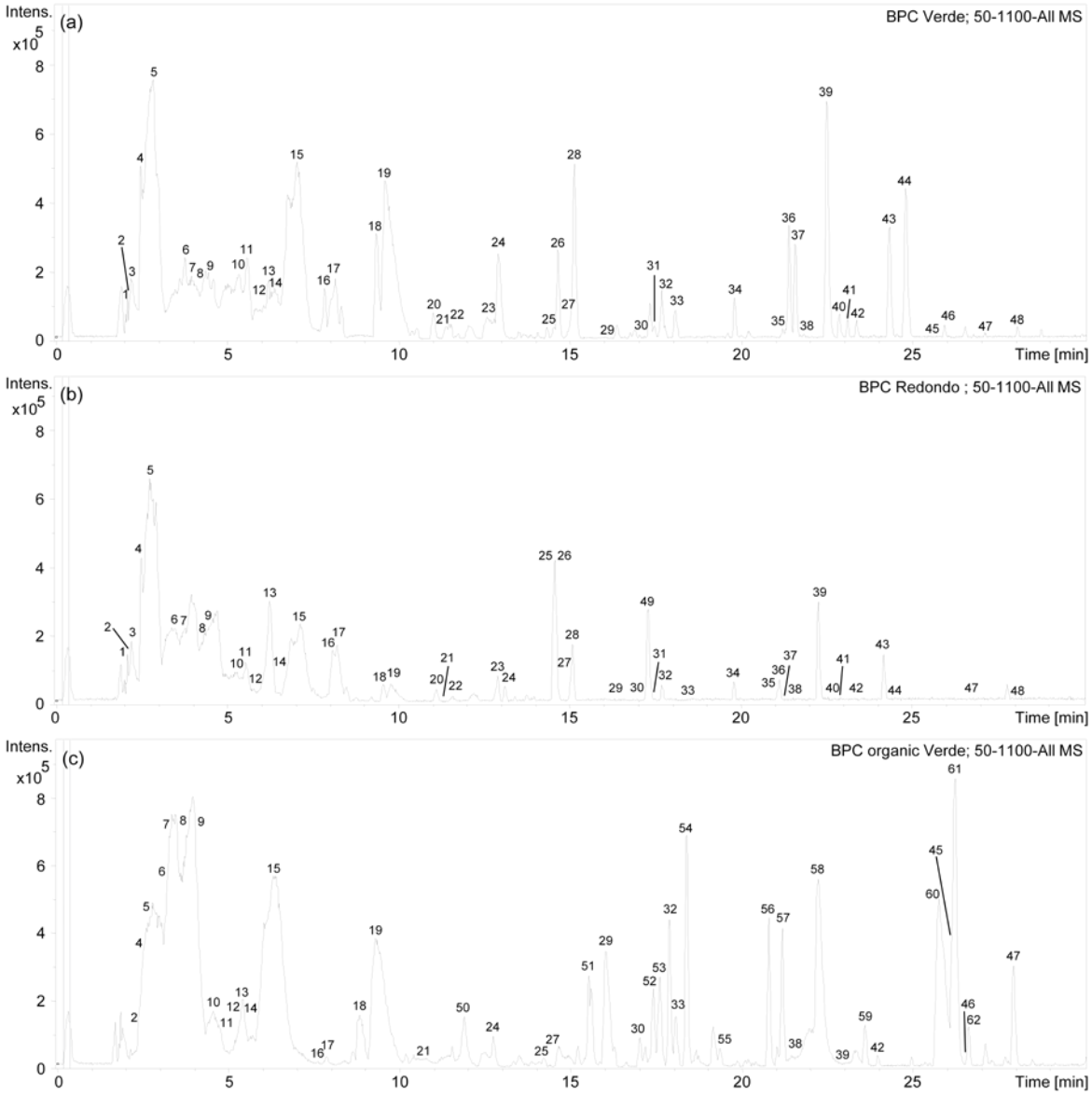

Figure 1. Base peak chromatograms of Verde (a), Redondo (b) and organic Verde (c) zucchinis.

Phenolic acids and derivatives

Peak **14** (RT 6.28 min) with a precursor ion at *m/z* 163.0401 presented a daughter ion at *m/z* 119.0501 $[M\text{-}H\text{-}CO_2]^-$. This ion was assigned tentatively to *p*-coumaric acid, which has previously been reported in pumpkin.[13,14] Ferulic acid is the other hydroxycinnamic acids detected in the three zucchini studies.

Table 1. Phenolic and other polar compounds characterized in three zucchini samples by HPLC-ESI-Q-TOF-MS

Peak	RT (min)	Selected ion	Molecular Formula	*m/z* experimental	*m/z* calculated	Error (ppm)	mSigma value	Fragments	Proposed compound	Verde	Redondo	organic Verde
Phenolic acids and derivatives												
Hydroxycinnamic acids and derivatives												
14	6.28	$[M-H]^-$	$C_9H_7O_3$	163.0401[a]	163.0401	0.0	7.1	119.0501	*p*-coumaric acid	+	+	+
38	21.80	$[M-H]^-$	$C_{10}H_9O_4$	193.0506[a]	193.0506	0.1	8.4	178.0268; 134.0372	ferulic acid	+	+	+
50	11.90	$[M-H]^-$	$C_{13}H_{11}O_9$	311.0417[c]	311.0409	2.7	1.4	135.0456; 149.0098; 179.0353	caftaric acid	-	-	+
51	15.52	$[M-H]^-$	$C_{16}H_{17}O_9$	353.0891[c]	353.0878	3.7	5.1	191.0575	chlorogenic acid	-	-	+
52	17.38	$[M-H]^-$	$C_9H_7O_4$	179.0363[c]	179.0350	7.5	1.1	135.0455	caffeic acid	-	-	+
55	19.35	$[M-H]^-$	$C_{13}H_{11}O_8$	295.0476[c]	295.0459	5.5	4.0	179,0355; 133.0173	2-*O*-caffeoylmalic acid	-	-	+
58	22.23	$[M-H]^-$	$C_{22}H_{17}O_{12}$	473.0743[c]	473.0725	3.7	23.6	311.0407; 293.0315; 179.0360; 149.0099	chicoric acid	-	-	+
60	25.73	$[M-H]^-$	$C_{18}H_{13}O_7$	341.0720[c]	341.0667	15.7	18.7	281.2489; 179.0387; 161.0270	dicaffeic acid	-	-	+
31	17.43	$[M-H]^-$	$C_{17}H_{21}O_{10}$	385.1157[a]	385.1140	4.4	33.6	223.0564	sinapic acid hexoside	+	+	-
Hydroxybenzoic acids and derivatives												
21	11.30	$[M-H]^-$	$C_7H_5O_4$	153.0201[a]	153.0193	5.0	21.4	109.0302	protocatechuic acid	+	+	+
28	15.12	$[M-H]^-$	$C_{26}H_{47}O_{18}$	647.2772[a]	647.2768	0.6	6.8	323.1370	3,4,5 tri-*O*-galloylquinic acid	+	+	-
27	15.00	$[M-H]^-$	$C_7H_5O_3$	137.0239[a]	137.0244	4.1	43.9	–	*p*-hydroxybenzoic acid	+	+	+
29	16.15	$[M-H]^-$	$C_7H_5O_2$	121.0286[a]	121.0295	7.1	8.3	–	*p*-hydroxybenzaldehyde	+	+	+
33	18.11	$[M-H]^-$	$C_7H_5O_2$	121.0288[a]	121.0295	6.1	3.0	–	benzoic acid	+	+	+
30	17.01	$[M-H]^-$	$C_8H_7O_4$	167.0338[a]	167.0350	6.9	7.3	–	vanillic acid	+	+	+
20	11.00	$[M-H]^-$	$C_{14}H_{17}O_9$	329.0874[a]	329.0878	1.2	20.0	167.0353	vanillic acid glycoside (isomer 1)	+	+	-
23	12.81	$[M-H]^-$	$C_{14}H_{17}O_9$	329.0882[a]	329.0878	1.2	10.9	167.0335	vanillic acid glycoside (isomer 2)	+	+	-
22	11.47	$[M-H]^-$	$C_{13}H_{15}O_8$	299.0768[a]	299.0772	1.4	13.0	137.0272	hydroxybenzoic acid hexose	+	+	-
Flavonoids												
Flavones and glycosides												
62	26.50	$[M-H]^-$	$C_{21}H_{19}O_{11}$	447.0947[c]	447.0933	3.1	22.2	285.0418	luteolin *O*-glucoside	-	-	+
Flavonols and glycosides												
34	19.75	$[M-H]^-$	$C_{33}H_{39}O_{20}$	755.2065[a]	755.2040	3.3	2.4	301.0330	quercetin 3-*O*-rhamnosyl-rhamnosyl-glucoside	+	+	-
39	22.45	$[M-H]^-$	$C_{27}H_{29}O_{16}$	609.1493[a]	609.1461	5.2	13.1	301.0353	quercetin 3-rutinoside	+	+	+
42	23.35	$[M-H]^-$	$C_{21}H_{19}O_{12}$	463.0886[a]	463.0882	0.9	7.1	301.0342	quercetin *O*-glucoside	+	+	+
37	21.53	$[M-H]^-$	$C_{34}H_{42}O_{20}$	769.2237[a]	769.2197	5.2	11.8	315.0501; 623.1567	isorhamnetin 3-rutinoside-7-rhamnoside	+	+	-
41	23.03	$[M-H]^-$	$C_{28}H_{31}O_{16}$	623.1630[a]	623.1618	2.0	7.7	315.0509	isorhamnetin *O*-rutinoside (isomer 1)	+	+	-
44	24.76	$[M-H]^-$	$C_{28}H_{31}O_{16}$	623.1665[a]	623.1618	6.1	11.8	315.0497	isorhamnetin *O*-rutinoside (isomer 2)	+	+	-
36	21.34	$[M-H]^-$	$C_{33}H_{39}O_{19}$	739.2122[a]	739.2091	4.2	11.2	285.0387	robinin	+	+	-
40	22.83	$[M-H]^-$	$C_{27}H_{29}O_{15}$	593.1517[a]	593.1512	0.9	5.8	285.0381	kaempferol rutinoside (isomer 1)	+	+	-
43	24.27	$[M-H]^-$	$C_{27}H_{29}O_{15}$	593.1534[a]	593.1512	3.7	7.3	285.0415	kaempferol rutinoside (isomer 2)	+	+	-
45	25.56	$[M-H]^-$	$C_{21}H_{19}O_{11}$	447.0942[a]	447.0933	2.0	16.9	285.0404	kaempferol *O*-glycoside (isomer 1)	+	-	+
46	26.00	$[M-H]^-$	$C_{21}H_{19}O_{11}$	447.0923[a]	447.0933	2.2	5.8	285.0404	kaempferol *O*-glycoside (isomer 2)	+	-	+
47	27.10	$[M-H]^-$	$C_{23}H_{21}O_{12}$	489.1017[a]	489.1038	4.5	15.8	285.0370	kaempferol *O*-(6"-*O*-acetyl-glucoside	+	+	+

Table 1. (Continued).

Peak	RT (min)	Selected ion	Molecular Formula	*m/z* experimental	*m/z* calculated	Error (ppm)	mSigma value	Fragments	Proposed compound	Verde	Redondo	organic Verde
48	28.09	$[M-H]^-$	$C_{21}H_{19}O_{10}$	431.0992[a]	431.0984	1.8	28.0	285.0397	kaempferol *O*-α-L-rhamnoside	+	+	-
59	23.57	$[M-H]^-$	$C_{26}H_{27}O_{15}$	579.1361[c]	579.1355	0.9	12.9	447.0938; 285.0411	kaempferol O-sambubioside	-	-	+
61	26.18	$[M-H]^-$	$C_{21}H_{17}O_{12}$	461.0741[c]	461.0725	3.4	13.7	285.0412	kaempferol *O*-glucuronide	-	-	+
Organic acids and derivatives												
35	21.21	$[M-H]^-$	$C_6H_5O_6$	173.0100[a]	173.0092	4.8	7.3	–	t-aconitic acid	+	+	-
2	2.16	$[M-H]^-$	$C_6H_{11}O_7$	195.0513[a]	195.0510	1.4	6.2	–	gluconic/galactonic acid	+	+	+
4	2.51	$[M-H]^-$	$C_4H_3O_4$	115.0038[a]	115.0037	1.4	2.6	–	fumaric acid	+	+	+
5	2.88	$[M-H]^-$	$C_4H_5O_5$	133.0149[a]	133.0149	5.0	5.2	115.0034	malic acid	+	+	+
7	3.90	$[M-H]^-$	$C_6H_7O_7$	191.0198[a]	191.0197	0.4	11.9	173.0111, 111.0102	isocitric acid	+	+	+
8	4.25	$[M-H]^-$	$C_6H_7O_7$	191.0197[a]	191.0197	0.3	3.7	173.0122; 111.0081	citric acid	+	+	+
10	5.48	$[M-H]^-$	$C_4H_5O_4$	117.0191[a]	117.0193	2.3	2.5	–	succinic acid	+	+	+
11	5.61	$[M-H]^-$	$C_5H_7O_5$	147.0305[a]	147.0299	4.2	9.6	–	citramalic acid	+	+	+
25	14.53	$[M-H]^-$	$C_7H_{11}O_5$	175.0616[a]	175.0616	2.5	5.7	115.0400	isopropylmalic acid	+	+	+
Aminoacids and derivatives												
1	2.11	$[M-H]^-$	$C_4H_6NO_4$	132.0297[a]	132.0302	3.7	4.2	–	aspartic acid	+	+	-
3	2.20	$[M-H]^-$	$C_3H_6NO_3$	104.0339[a]	104.0353	13.8	9.2	–	serine	+	+	-
6	3.82	$[M-H]^-$	$C_5H_{10}NO_2$	116.0713[a]	116.0717	3.6	9.8	–	valine	+	+	+
12	6.13	$[M-H]^-$	$C_9H_{10}NO_3$	180.0664[a]	180.0666	1.2	8.5	163.0398	tyrosine	+	+	+
18	9.36	$[M-H]^-$	$C_9H_{10}NO_2$	164.0721[a]	164.0717	2.7	1.2	147.0447	phenylalanine	+	+	+
24	12.92	$[M-H]^-$	$C_{11}H_{11}N_2O_2$	203.0832[a]	203.0826	2.8	7.7	–	tryptophan	+	+	+
9	4.42	$[M-H]^-$	$C_5H_6NO_3$	128.0350[a]	128.0353	2.7	31.9	–	pyroglutamic acid	+	+	+
15	7.05	$[M-H]^-$	$C_{12}H_{22}NO_7$	292.1416[a]	292.1402	4.8	11.6	202.1095; 130.0879	fructosyl leucine/isoleusin	+	+	+
19	9.61	$[M-H]^-$	$C_{15}H_{20}NO_7$	326.1256[a]	326.1245	3.3	6.9	236.0940; 164.0725	fructosyl phenylalanine	+	+	+
Nucleosides												
13	6.25	$[M-H]^-$	$C_9H_{11}N_2O_6$	243.0630[a]	243.0623	2.8	2.4	200.0572; 111.0207	uridine	+	+	+
16	7.85	$[M-H]^-$	$C_{10}H_{12}N_5O_4$	266.0904[a]	266.0895	3.3	7.8	134.0470	adenosine	+	+	+
17	8.07	$[M-H]^-$	$C_{10}H_{12}N_5O_5$	282.0854[a]	282.0844	3.4	3.6	150.0420	guanosine	+	+	+
Other metabolites												
32	17.66	$[M-H]^-$	$C_{10}H_{12}NO_4$	210.0777[a]	210.0772	2.5	4.3	124.0393	3,4-dihydroxyphenyl-1-methyl ester-carbamic acid	+	+	+
53	17.56	$[M-H]^-$	$C_6H_{11}O_3$	131.0719[c]	131.0714	4.1	3.8	–	ethyl 3-hydrobutanoate	-	-	+
Unknown compounds												
26	14.63	$[M-H]^-$	$C_{20}H_{29}O_{12}$	461.1667[a]	461.1664	0.6	3.8	–		+	+	-
49	17.28	$[M-H]^-$	$C_{21}H_{31}O_{12}$	475.1829[b]	475.1821	1.7	7.4	–		-	+	-
54	18.36	$[M-H]^-$	$C_{17}H_{21}O_7$	337.1313[c]	337.1293	6.0	3.9	215.1081		-	-	+
56	20.76	$[M-H]^-$	$C_{21}H_{31}O_9$	427.2025[c]	427.1974	12.0	16.5	202.1095		-	-	+
57	21.14	$[M-H]^-$	$C_{29}H_{13}O_7$	473.0724[c]	473.0667	12.0	23.9	202.1096		-	-	+

[a]*m/z* experimental and calculated, sigma value and error were from Verde sample; [b]*m/z;* experimental and calculated, sigma value and error were from Redondo sample; [c]*m/z* experimental and calculated, sigma value and error were from organic Verde sample;
sh=shoulder.

It was eluted at RT 21.80 min (peak **38**) with *m/z* 193.0506 and presented two product ions at *m/z* 178.0268 and *m/z* 134.0372, which corresponded to the loss of CH_3 and was followed by CO_2, respectively. This compound has been previously reported in hull-less pumpkin seed, skin, and oil-cake meal, as well as in the dehulled kernel and the hull itself.[14] Another six hydroxycinnamic acids were identified but only in "organic Verde" zucchini. The ion found at *m/z* 311.0417 was assigned to caftaric acid or *O*-caffeoyltartaric acid (peak **50**) (RT 11.90 min), which presented a product ion at *m/z* 149.0098 corresponding to the loss of caffeoyl acid moiety (162 Da). Two other fragment ions at *m/z* 179.0353 and 135.0456 were caffeic acid derived. These results are consistent with the findings of the previous study in lettuce varieties and escarole.[15]

MS spectrum showed a molecular mass of *m/z* 353.0891 for peak **51** (RT 15.52 min), which was assigned to chlorogenic acid (3-*O*-caffeoylquinic acid) with the fragmentation pattern at *m/z* 191.0575, corresponding to the loss of the caffeoyl acid moiety (162 Da) from the main fragment.[12,13] Caffeic acid (peak **52**) was identified at RT 17.38 min (*m/z* 179.0363) with a product ion at *m/z* 135.0455 $[M\text{-}H\text{-}CO_2]^-$. This compound has been previously mentioned in pumpkin.[13,14] The presence of 2-*O*-caffeoylmalic acid (peak **55**) was identified at RT 19.35 min with *m/z* 295.0476 and two product ions at *m/z* 179.0355 and 133.0173, which corresponded to the $[\text{caffeic acid–H}]^-$ and $[\text{malic acid–H}]^-$ fragment ions, respectively, indicating the presence of caffeic and malic acids in its structure. The presence of this compound has previously been mentioned in lettuce (*Lactuca sativa* L.) by Ribas-Agustí *et al*.[16] Peak **58** (RT 22.23 min) showed a parent ion at *m/z* 473.0743. It presented predominant product ions at *m/z* 311.0407 $[\text{M–H–caffeoyl moiety}]^-$, *m/z* 149.0099 $[M\text{-}H\text{-}162\text{-}162]^-$ (loss of a second caffeic acid moiety), *m/z* 293.0315 and *m/z* 179.0360. This compound was tentatively proposed as chicoric acid (dicaffeoyltartaric acid) based on the fragment patterns and identical UV spectra at 244, 305(sh: shoulder) and 328 by Schütz *et al*.[17] Peak **60** (RT 25.73) with precursor ion at *m/z* 341.0720 was assigned to dicaffeic acid. It gave product ions at *m/z* 281.2489, 179.0387, and 161.0270, corresponding to the loss of acetic acid (CH_3COOH) group, and deprotonated and dehydrated

caffeic acid, respectively.[18] Another phenolic acid with its glycoside conjugate was identified at RT 17.43 min (peak **31**) with *m/z* 385.1157 and a product ion at *m/z* 223.0564, corresponding to the loss of hexose moiety from the precursor ion. This was tentatively assigned to sinapic acid hexoside.[12]

Nine hydroxybenzoic acids and derivatives were identified in both of Verde and Redondo zucchini, and five were identified in all the zucchini analyzed. A signal at *m/z* 153.0201 (peak **21**) was detected at 11.30 min. This compound, with a molecular formula of $C_7H_5O_4$ and fragments at *m/z* 109.0302, corresponding to the loss of CO_2, was tentatively identified as protocatechuic acid.[14] The ion found at RT 15.12 min (peak **28)** was suggested to be 3,4,5 tri-*O*-galloylquinic acid with the precursor and product ions at *m/z* 647.2772 and 323.1370 $[M-2H]^{2-}$, respectively. Galloylquinic acid derivatives have been identified in other plants.[19] At RT 15.00 min, a signal with *m/z* 137.0239 (peak **27)** was tentatively identified as *p*-hydroxybenzoic acid, which has previously been reported in pumpkin seed.[14] Peaks **29** (RT 16.15 min) and **33** (RT 18.11 min) showed the same parent ions at *m/z* 121.0286/121.0288 and gave the same molecular formula according to Smart Formula Editor. Based on the literature and using reversed-phase liquid chromatography, *p*-hydroxybenzaldehyde was eluted just before benzoic acid. Therefore, these compounds were tentatively identified as *p*-hydroxybenzaldehyde[14] and benzoic acid[20], consecutively. Peak **30** (RT 17.01 min), with precursor ion at *m/z* 167.0350, corresponded to vanillic acid.[13,14] Meanwhile, vanillic acid glycoside isomers (peaks **20** and **23**) were identified at RT 11.00 and 12.81 min. Found at *m/z* 329.0874 and 329.0882, they presented the same two product ions at *m/z* 167.0353 and 167.0335, respectively, corresponding to the loss of the sugar moiety. This compound has been identified in cucumber.[12] The presence of hydroxybenzoic acid hexose, identified at RT 11.47 min (peak **22**) with ion found at *m/z* 299.0768, presented one main product ion at *m/z* 137.0272, corresponding to the loss of hexose moiety.[20]

Flavonoids

Peak **62** (RT 26.50 min and m/z 477.0947) was tentatively identified as luteolin *O*-glucoside, showing a fragment at 285.0414, which is the characteristic fragment mass of luteolin. This compound, the only flavone identified in "organic Verde" zucchini, has been isolated from other plant family Cucurbitaceae, as reported by Mallavarapu and Row.[21]

The ion found at *m/z* 755.2065 was assigned to quercetin 3-*O*-rhamnosyl-rhamnosyl-glucoside (peak **34**) (RT 19.75 min), which presented a product ion at *m/z* 301.0330 corresponding to quercetin (**Figure 2a**). Two other quercetin-sugar conjugates identified in zucchini (peak **39** and **42**) proved identical to those reported previously in Cucurbitaceae.[12] Peak **39** (RT 22.45 min) and **42** (RT 23.35 min), with ions found at *m/z* 609.1493 and *m/z* 463.0886, both presented the same fragment at *m/z* 301.0353 and 301.0342, respectively, corresponding to the loss of the sugar moieties, rutinoside and glucose, respectively. Therefore, these peaks were suggested as quercetin rutinoside (**Figure 2b**) and glucoside, respectively.

Peak **37** gave a molecular mass at *m/z* 769.2226 and a product ion at *m/z* 315.0501 [M-H-146-146-162], which corresponded to a complete loss of the sugar moieties. The fragment ion at *m/z* 623.1567 $[M-H-146]^-$ was also detected but with very low relative intensity (below 5%), indicating a loss of rhamnose from C-7. Since this fragmentation behavior is consistent with a previous report[22] and was corroborated with the same UV absorbance spectra at 255, 266 (sh) and 354 nm, on the basis of such features, it was assigned to isorhamnetin 3-rutinoside-7-rhamnoside (**Figure 2c**). Peak **41** (RT 23.03 min) and **44** (RT 24.76) gave the same molecular formula $C_{27}H_{29}O_{15}$ and *m/z* 623.1630 and 623.1665, respectively. Both of them presented the same product ion at *m/z* 315.0509 and 315.0497, which corresponds to isorhamnetin after the loss of the rutinose sugar moiety. The UV absorbances of both compounds are the same at 264, 303 (sh) and 350 nm. Therefore, we could not distinguish between them and tentatively proposed as isorhamnetin *O*-rutinoside isomers.

Nine kaempferol glycosides (peak **36**, **40**, **43**, **45**, **46**, **47**, **48**, **59** and **61**) were tentatively identified for the first time in zucchini. They were corroborated by their UV spectra at 264, 300 (sh) and 350 nm, which are specific for flavonol glycosides. All the kaempferol-sugar conjugates showed product ions at *m/z* 285 corresponding to kaempferol. Peak **36,** presenting a

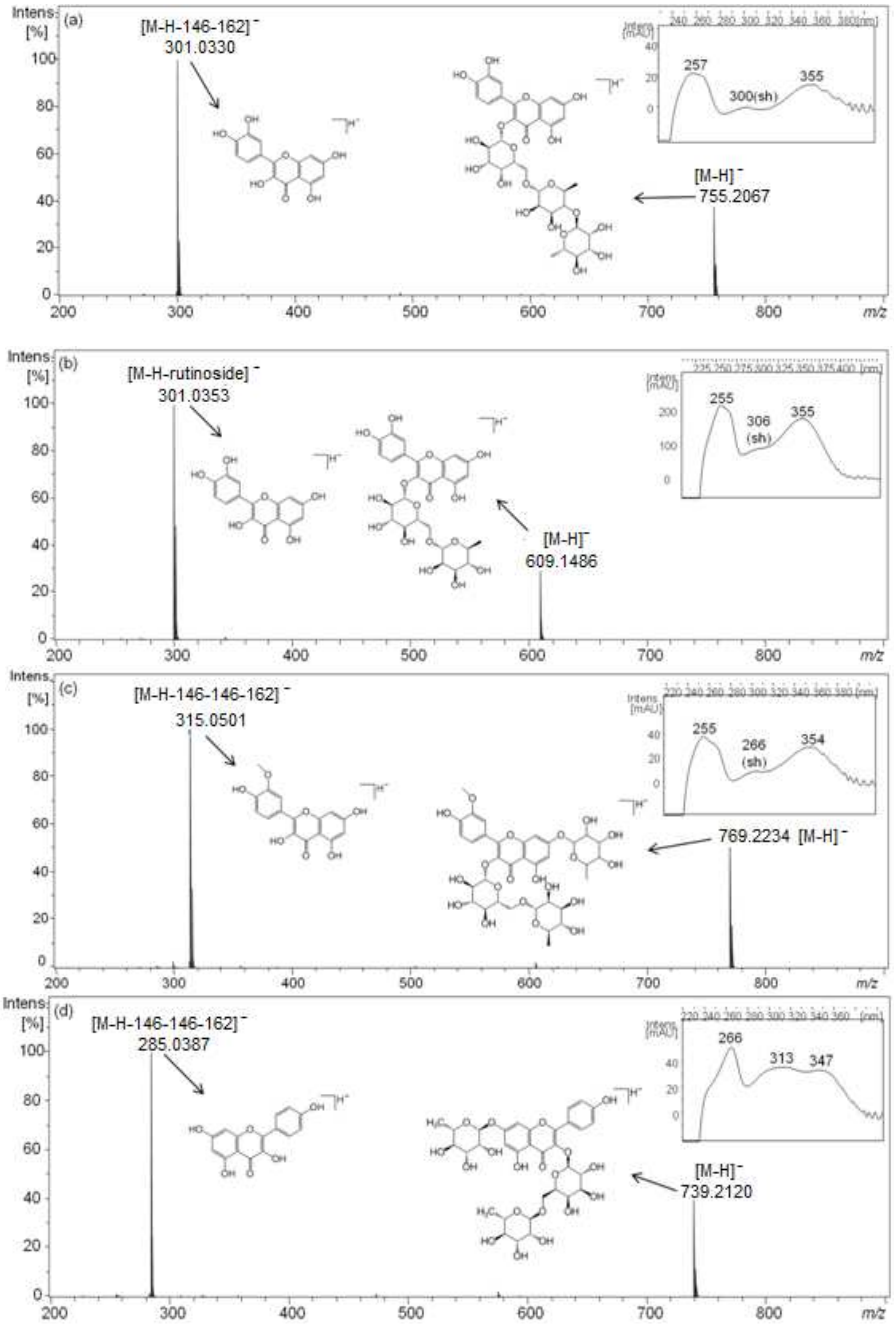

Figure 2. MS/MS and UV spectra of the most representative flavonoids: quercetin 3-*O*-rhamnosyl-rhamnosyl-glucoside, peak **34** (a), quercetin 3-rutinoside, peak **39** (b), isorhamnetin 3-rutinoside-7-rhamnoside, peak **37** (c), robinin, peak **36** (d), kaempferol rutinoside (isomer 2), peak **43** (e), kaempferol *O*-glycoside (isomer 2), peak **46** (f).

peak **43** (e), kaempferol *O*-glycoside (isomer 2), peak **46** (f).

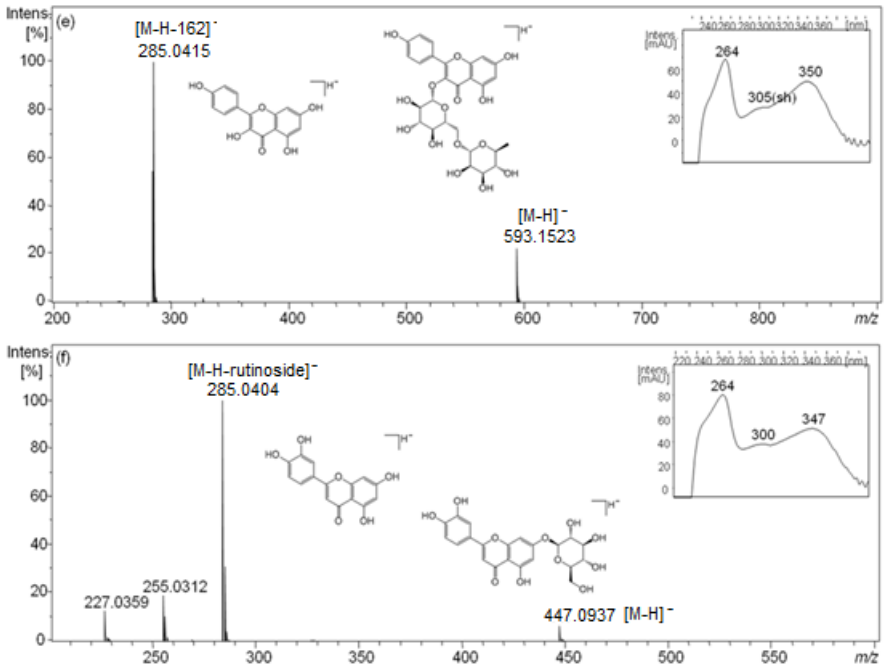

Figure 2. (Continued).

precursor ion at *m/z* 739.2122 with molecular formula $C_{33}H_{39}O_{19}$, was tentatively identified as robinin (kaempferol-3-*O*-robinoside-7-*O*-rhamnoside). It has been previously reported in fruit juices by Abad-García *et al*.[23] (**Figure 2d**). The identities of the peaks **39**, **40**, **59**, **42**, **43** (**Figure 2e**), **45**, **46** (**Figure 2f**) and **47** of this study agree with the findings of Abu Reidah *et al*.[12] in cucumber whole-fruit extract. Peak **48** has been previously reported in endive (*Cichorium endivia*), as well as peak **61** which was the predominant form in that extract.[24] Although all flavonols and glycosides above are known, as far as we are aware this is the first time that they are reported in whole zucchini fruit.

Other polar compounds

Besides polyphenolic compounds, other polar compounds could also be characterized in the whole zucchini fruit. Organic acids, amino acids and their derivatives eluted as a large peak with signals overlapping. It is due to the very polar compounds that could not be properly separated by C_{18} reversed-phase chromatography.[20]

Eight organic acids and derivatives were tentatively identified in all zucchini with the exception of peak **35** (RT 21.21 min). This peak with an ion found at *m/z* 173.0100 was tentatively identified as t-aconitic acid. Peak **2** (RT 2.16 min), which gave a molecular mass of *m/z* 195.0513, was tentatively assigned to gluconic/galactonic acid according to the molecular formula provided for its accurate mass $C_6H_{11}O_7$. Fumaric acid with molecular mass at *m/z* 115.0038 was identified at RT 2.51 (peak **4**). Peak **5** (RT 2.88 min) with *m/z* 133.0149 was suggested as malic acid and presented fragment ion at *m/z* 115.0034 corresponding to the loss of a molecule of water from the precursor ion.[25] Peak **7** and **8** presented isocitric acid and citric acid, with an ion found at *m/z* 191.0198 and 191.0187, respectively. Both isomers showed the same fragmentation pattern at *m/z* 173.0111 and 173.0122, which correspond to $[M\text{-}H\text{-}H_2O]^-$, while 111.0102 and 111.0081 were related to the loss of $[M\text{-}H\text{-}CO_2\text{-}2H_2O]^-$.[20,25] Succinic and citramalic acids were identified at RT 5.48 and 5.61 min (peak **10** and **11**) and with precursor ions at *m/z* 117.0191 and *m/z* 147.0305, respectively.[20,25] At RT 14.53 min (peak **25**), the ion found at *m/z* 175.0616 presented a product ion at *m/z* 115.0400 $[M\text{-}H\text{-}2CHOH]^-$, which was tentatively proposed as isopropylmalic acid.[20,25]

With regard to amino acids and derivatives, these were detected at RT from 2.11 to 12.92 min. Six amino acids namely aspartic acid (peak **1**), serine (peak **3**), valine (peak **6**), tyrosine (peak **12**), phenylalanine (peak **18**), and tryptophan (peak **24**) which have been previously found in zucchini by the National Food Institute,[2] were also identified in the extracts analyzed. Only peak **9** (RT 4.42 min) was proposed as pyroglutamic acid with a molecular mass at *m/z* 128.0350, the only one that has not been reported in this database.

Amadori compounds, derived from the condensation of amino acids with reducing sugars formed in food as part of the Maillard reactions, were identified in all zucchini varieties at *m/z* 292.1402 (peak **15**) and *m/z* 326.1245 (peak **19**). Peak **15** (RT 7.05 min) gave product ions at *m/z* 202.1095 and *m/z* 130.0879 corresponding to the loss of 90 Da and the hexose moiety, respectively. This peak was tentatively identified as fructosyl

leucine/isoleusin. Peak **19** (RT 9.61 min) showed a MS/MS spectrum with fragments at *m/z* 236.0904 and 164.0725, corresponding to the same losses of peak **15,** which was assigned to fructosyl phenylalanine.[26] These fragment ions were also in agreement with the findings of amadori compounds in tomato fruit by Gómez-Romero *et al*.[20]

Several nucleosides were also found in all zucchini. Peak **13**, with ion found at *m/z* 243.0630, was assigned to uridine. It yielded two fragment ions at *m/z* 200.0572 and *m/z* 111.0207. The former corresponded to the loss of the $[M-H-CHNO]^-$ ion and the latter corresponded to the loss of the pentose moiety from the precursor ion. Other nucleosides were detected at RT 7.85 min (peak **16**) and RT 8.07 min (peak **17**). Peak **16** gave a molecular mass at *m/z* 266.0904 and a main fragment at *m/z* 134.0470. Moreover, peak **17** presented a precursor ion at *m/z* 282.0854 with a main fragment at *m/z* 150.0420. The difference between precursor and fragment ions for both of these compounds is the same and they were assigned to losses of pentose moieties. These nucleosides have been previously reported in tomato.[20]

Two other compounds were tentatively proposed in the methanol-water extract of zucchini. A signal at *m/z* 210.0777 (peak **32**) was observed at 17.66 min with molecular formula $C_{10}H_{12}NO_4$. It presented a fragment ion at *m/z* 124.0393, corresponding to the loss of CO_2 and C_2H_2O groups from the parent ion. It was tentatively identified as 3,4-dihydroxyphenyl-1-methyl ester-carbamic acid.[20] Peak **53** (RT. 17.56 min) was assigned to ethyl 3-hydrobutanoate which has been identified previously in melon.[27]

Finally, peaks **26**, **49**, **54**, **56,** and **57** could not be identified, not even through their MS/MS spectral analyses, and therefore they were classified as unknown compounds.

Conclusions

A powerful analytical method to separate and characterize polyphenolic and other polar compounds in three zucchini samples has been followed using reversed-phase LC coupled to DAD and Q-TOF mass spectrometry. The use of

LC separation assisted by UV-Vis and mass spectrometric detection has proved to be a useful tool for identifying 34 polyphenolic compounds and 23 other polar compounds in these extracts in less than 29 min. It is also important to highlight that, to our knowledge, 10 phenolic acids, 16 flavonoids, and 17 other polar compounds with their derivatives are reported here in the whole zucchini fruit for the first time. Further research will be devoted to quantifying the compounds identified and evaluating their biological activity.

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Chapter 3

Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT) and their antihyperlipidemic activity in LDLr deficient mice

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ORIGINAL PAPER

Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT)

(ii) Phytomedicine

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Continuous administration of polyphenols from aqueous rooibos (*Aspalathus linearis*) extract ameliorates dietary-induced metabolic disturbances in hyperlipidemic mice

Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT) and their antihyperlipidemic activity in LDLr deficient mice

Abstract

Rooibos (*Aspalathus linearis*) is a rich source of polyphenols and used to make a mild-tasting tea containing no caffeine, low in tannins compared to green or black teas and has antioxidant and antimutagenic/antitumoral properties. *In vivo* results show that rooibos has beneficial effects upon the lipid profile by decreasing serum triglycerides and cholesterol. In this sense, we have developed a simple and rapid method to separate and characterize simultaneously the polyphenolic compounds in aqueous and ethanolic rooibos extract using high-performance liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (HPLC-ESI-TOF-MS) and ion-trap multiple mass spectrometry (HPLC-ESI-IT-MS^2). The phenolic compounds were separated on a C_{18} column (4.6 x 150 mm, 1.8 μm) with 1 % formic acid in water/acetonitrile 90:10 v/v and acetonitrile as mobile phases. The accuracy mass data generated by TOF-MS together with the fragmentation pattern obtained by IT-MS^2 experiments confirmed the presence of 25 and 30 phenolic compounds in the aqueous and ethanolic extracts respectively. Aqueous rooibos extract containing approximately 25% w/w total polyphenol content showed its highest bioactivity in assays with male $LDLr^{-/-}$ mice. The group fed on a high-fat high-cholesterol diet (HF, 20% fat and 0.25% cholesterol w/w) plus rooibos tea extract (10 g/L) in place of pure drinking water was found to have significantly reduced serum cholesterol and triglyceride concentrations compared to the control group.

Keywords: Rooibos tea; Polyphenols; High-performance liquid chromatography (HPLC); Mass spectrometry (MS)

Introduction

Polyphenols are widely distributed throughout the plant kingdom and represent an abundant antioxidant component of the human diet. These compounds are generally classified into flavonoids, phenolic acids, lignans and stilbenes. During the past decade interest in the possible health benefits of polyphenols has increased due to their antioxidant capacity and many polyphenols, particularly the flavonoids, have been found to possess relatively potent antioxidant, antiatherosclerotic, anti-inflammatory, antitumoral, antithrombogenic, antiosteoporotic and antiviral activities.[1-3]

Aspalathus linearis (*Fabaceae*), is a flowering shrub-like leguminous bush native to the Cedarberg Mountains in South Africa's Western Cape and used to make a mild-tasting tea, known as rooibos, containing no caffeine and very little tannin but significant amounts of polyphenolic antioxidants. There are two types of rooibos tea, unfermented and fermented. The unfermented product remains green in colour and is referred to as green rooibos. During fermentation the colour changes from green to red, with oxidation of the constituent polyphenols, so the final product is often referred to as red tea or red bush tea.[4] The most important characteristics of rooibos are its antioxidant[5,6] and antimutagenic/antitumoral properties.[7]

Koeppen *et al.* were the first chemists to attempt to determine phenolic compounds in rooibos tea and they were able to identify the presence of orientin, iso-orientin (formerly known as homo-orientin), isoquercitrin, rutin[8], orientin, iso-orientin[9] and aspalathin[10]. Hydroxylated benzoic and cinnamic acids, luteolin, chrysoeriol, quercetin, isoquercitrin, the C-C linked β-D-glucopyranosides have also been isolated by various extraction methods.[11] Gradient separation of the C-glucoside dihydrochalcones, aspalathin and nothofagin, was later achieved on a reversed-phase C_{18} column.[7] Multilayer counter-current chromatography and preparative HPLC have recently been used to isolate the polyphenols present in rooibos.[12] *In vivo* studies have shown that aspalathin has hypoglycaemic effects in a diabetic mouse model.[13] Other studies conducted in rats have revealed that the whole extract of

rooibos tea had hepatoprotective effects on CCl_4–induced liver damage, partially prevented oxidative stress in streptozotocin-induced diabetes and increased serum SOD activity.[14-16]

High-performance liquid chromatography (HPLC) hyphenated to mass spectrometry (MS) detection is one of the most important analytical techniques used for the analysis of phenolic compounds. Recently, an improvement in chromatographic performance has been achieved by using columns packed with small particles. The newest column packed with particle size less than 2 μm, operated at a pressure up to 600 bar, thus allowing high resolution analysis in shorter times.[17] The on-line coupling of HPLC with MS using electrospray ionization (ESI) as an interface yields a powerful method because of its high efficient resolution and characterization of a wide range of polar compounds. ESI is one of the most versatile ionization techniques, and is the preferred one for detection of polar compounds separated by liquid chromatography. The advantages of MS detection include the ability to determine the molecular weight and to obtain structural information. TOF-MS can provide excellent mass accuracy over a wide dynamic range and allow measurements of the isotopic pattern, providing important additional information for the determination of the elemental composition. Futhermore, TOF-MS permits the rapid and efficient confirmation of the elemental composition of ions when carrying out fragmentation studies and also provides high selectivity in the determination of compounds in complex matrices using the extracted ion chromatogram (EIC) mode when there are overlapping peaks. Otherwise, IT-MS can be used to obtain fragmentation ions of structural relevance for identifying target compounds in a highly complex matrix.

The aim of our study has been to contribute to the phenolic profile of rooibos and to characterize polyphenolic compounds in rooibos extracts (aqueous and ethanolic) using HPLC coupled with two different detection systems: diode array (DAD) and mass spectrometry with TOF and IT analyzers. UV/visible has proved to be a valuable tool for identifying the family of these phenolic compounds whilst TOF-MS and IT-MS allows accurate mass measurements and complementary structural information.

Experimental

Chemicals

All chemicals were analytical HPLC reagent grade and used as received. Formic acid and acetonitrile used for preparing mobile phases were from Fluka, Sigma-Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland) respectively. Solvents were filtered before use with a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). The tuning mix solution to optimize the TOF parameters was from Agilent Technologies (Palo Alto, CA, USA). A G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA) was used as a vortex mixer. Distilled water with a resistance of 18.2 MΩ was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA).

Sample preparation

Fermented rooibos plant material (red-brownish dry leaves) was provided by Arend Redelinghuys (Rooibos BPK Ltd., South Africa). The aqueous extract was prepared in aqueous solution at a concentration of 1000 ppm, filtered on a polytetrafluoroethylene (PTFE) syringe filter (0.2 μm pore size) and injected directly into the HPLC system. The ethanolic extract was prepared by boiling 20 g of rooibos leaves in 100 mL of distilled H_2O for 5 minutes. Subsequently, extract was brought down to room temperature and filtered. 40 mL of the extract was collected and to precipitate the polysaccharides, 160 mL of ethanol (80% v/v) was added. The extract was conserved at 4 ºC over night, afterwards, the extract was centrifuged at 3000 rpm for 5 min to remove the precipitate and evaporated at 40 °C to recover 40 mL. The extract was aliquotted and lyophilized. Same as preparing aqueous extract, the ethanolic extract sample was prepared in aqueous solution at concentration 1000 ppm, filtered on a polytetrafluoroethylene (PTFE) syringe filter (0.2 μm pore size) and injected directly into the HPLC system.

Determination of total phenols

The total phenolic content of rooibos tea was determined in triplicate by colorimetric assay using Folin-Ciocalteu reagent.[17] The absorbance of the solution was measured after incubation for 2 h against caffeic acid concentration at a wavelength of 725 nm in a Spectronic Genesys™ 5 spectrophotometer. The results are expressed as the equivalent of caffeic acid. The absorbance curve versus concentrations for the caffeic acid is described by the equation $y = 0.027x–0.0373$ ($R^2=0.995$).

HPLC conditions

LC analyses were made with an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode array detector (DAD). Separation was carried out with a Zorbax Eclipse Plus C_{18} analytical column (150 mm x 4.6 mm, 1.8 µm particle size). Gradient elution was conducted with two mobile phases consisting of 1 % formic acid in water/acetonitrile 90:10 v/v (phase A) and acetonitrile (phase B) using the following gradient: 0-20 min, linear gradient from 5% B to 20% B; 20-25 min, linear gradient from 20% B to 40% B; 25-30 min, linear gradient from 40% B to 5% B; and 30-35 min, isocratic of 5% B. The flow rate used was set at 0.50 mL/min throughout the gradient. The effluent from the HPLC column was splitted using a T-type phase separator before being introduced into the mass spectrometer (split ratio=1:3). Thus, in this study the flow which arrived into the ESI-TOF-MS and ESI-IT-MS^2 detector was 0.2 mL/min. The column temperature was maintained at 25 °C and the injection volume was 10 µL.

TOF-MS conditions

TOF-MS was conducted using a microTOF™ (Bruker Daltonics, Bremen, Germany) orthogonal-accelerated TOF mass spectrometer equipped with an electrosprayionization (ESI) interface. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range of 50–1,000 *m/z*. The other optimum values of the ESI-MS parameters were: capillary voltage,

+4.5 kV; dry gas temperature, 200 ^{o}C; dry gas flow, 7.0 L/min; nebulizer pressure, 1.5 bar; and spectra rate 1 Hz.

The accurate mass data of the molecular ions were processed through the software Data Analysis 3.4 (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formula by using the Smart Formula Editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm.

During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, passing a solution of sodium formate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 (v/v). Using this method, an exact calibration curve based on numerous cluster masses each differing by 68 Da ($NaCHO_2$) was obtained. Due to the compensation of temperature drift in the micrOTOF, this external calibration provided accurate mass values (better 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

IT-MS conditions

The identical HPLC system was coupled to a Bruker Daltonics Esquire 2000^{TM} ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray interface (Agilent Technologies, CA, USA) in negative ion mode. The ion-trap scanned at 50-1,000 *m/z* range at 13,000 u/s during the separation and detection. The maximum accumulation time for the ion-trap was set at 200 ms, the target count at 20,000 and compound stability was set at 50%. The optimum values of the ESI-MS parameters were: capillary voltage, +3.0 kV; drying gas temperature, 300 °C; drying gas flow, 7.0 L/min;

and nebulizing gas pressure, 1.5 bar. The instrument was controlled by Esquire NT software from Bruker Daltonics.

Experimental mouse design

Male LDLr$^{-/-}$ mice (n=32) were from the Jackson Laboratory (Bar Harbor, ME, USA). They were housed under standard conditions and fed on a commercial mouse diet (14% Protein Rodent Maintenance diet, Harlan, Barcelona, Spain) in accordance with our institutional guidelines. At 10 weeks of age they were divided into two experimental dietary groups (n=16/group), one fed on the same maintenance diet (chow diet, 3% fat and 0.03% cholesterol w/w) and the other group fed on a Western-type diet (high-fat diet, 20% fat and 0.25% cholesterol w/w). Each dietary group was divided into two subgroups (n=8), one of which consumed water during the study whilst the second group consumed only rooibos-tea extract at a final concentration of 10 g/L. Either water or tea extract (freshly prepared every two days) were provided to all four groups *ad libitum*. The size of the experiment was planned according to data published elsewhere[18] and the mice were assigned randomly to each experimental group. Cholesterol and triglyceride serum concentrations were determined using a Synchron LXi 725 autoanalizer system (Beckman Coulter, IZASA, Barcelona). The differences were analized using the U Mann-Whitney test with the level of significance set at $p<0.05$. SPSS/PC + 12.0 (SPSS, Chicago, IL) software was used.

Results and discussion

Total polyphenolic contents

The Folin-Ciocalteu test is an established method to give a rough estimate of the total polyphenolic content. The calibration curve showed good linearity between caffeic acid concentration and absorbance. The average total polyphenol content in the aqueous and ethanolic rooibos extracts, as evaluated by the Folin-Ciocalteu assay, were 252.07 ± 8.01 mg/g and 233.80 ± 7.71, respectively. The concentrations observed were multiplied by dilution factor of the original sample. The Folin-Ciocalteu reagent is

nonspesific for any phenolics and the colour yielded depends on hydroxyl groups and their place in molecules. Despite the fact that phenolic reagents are unspesific, in certain circumstances (e.g. absence of interfering substances) we may get only relative results for phenolics. Therefore, by the Folin-Ciocalteu test we can observe that the total polyphenolic content in fermented rooibos (*Aspalathus linearis*) is higher than in the fermented *Cyclopia* species.

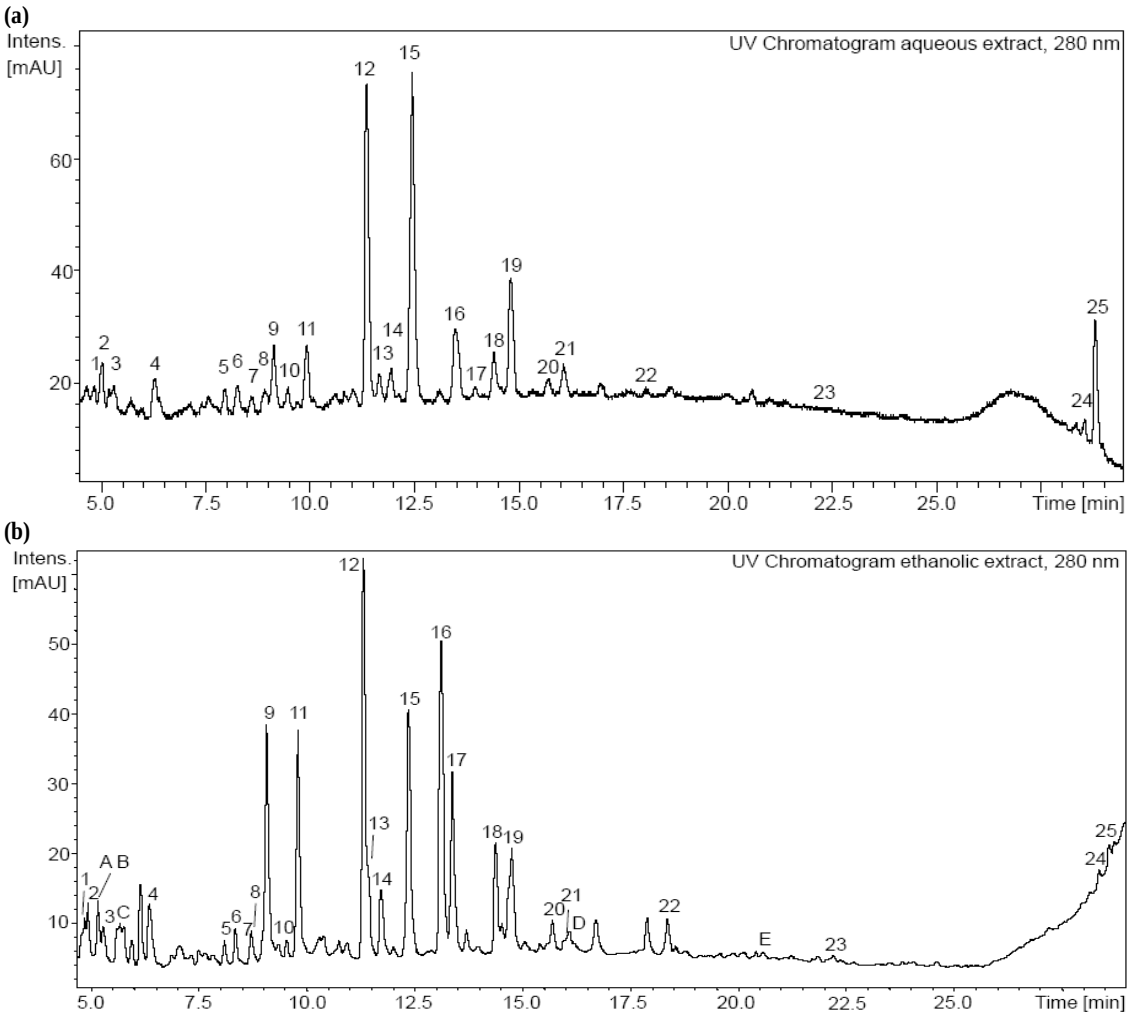

Figure 1. UV chromatograms of aqueous (a) and ethanolic (b) rooibos extracts at 280 nm. Base peak chromatograms of aqueous (c) and ethanolic (d) rooibos extracts.

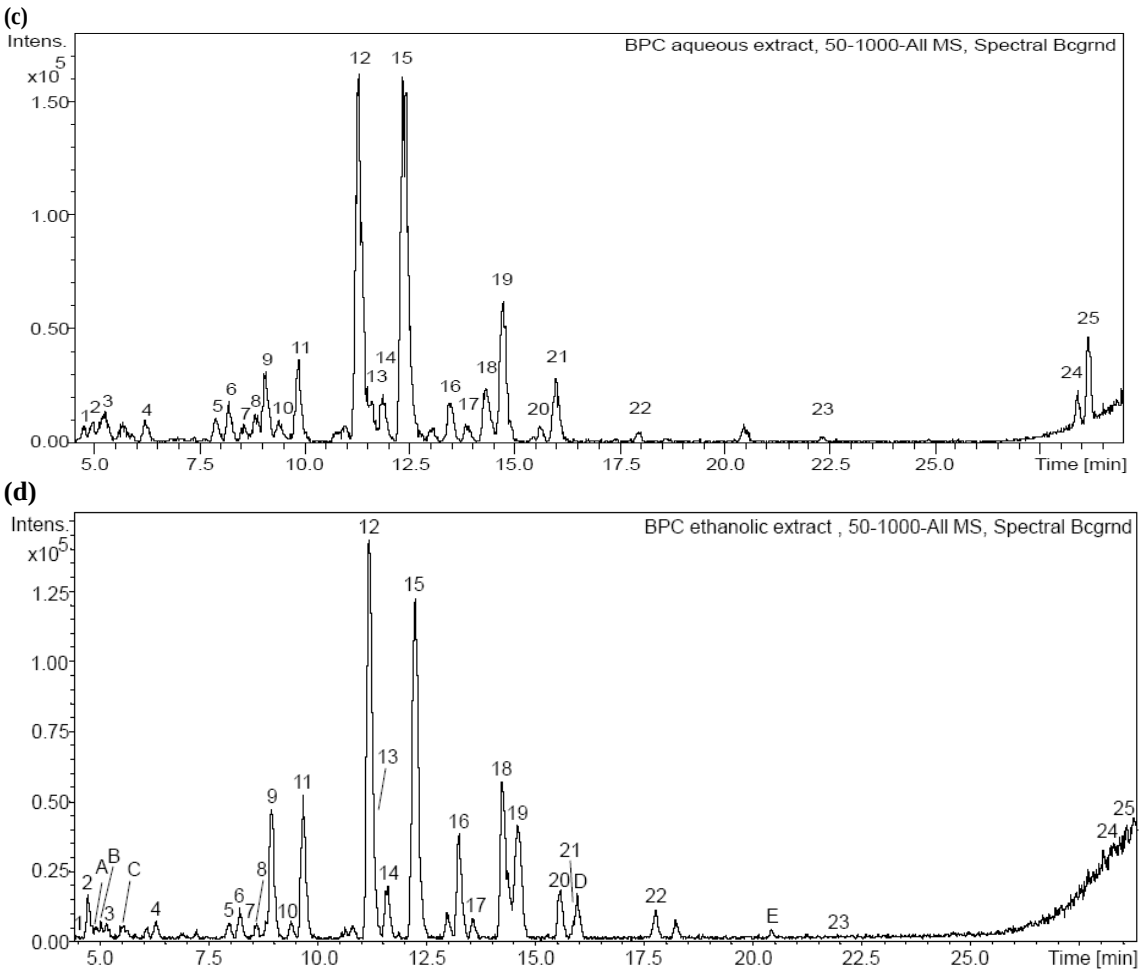

Figure 1. (continued)

Profile and compound characterization

Polyphenolic compounds were successfully separated and identified using HPLC-ESI-MS (TOF/IT). The UV chromatogram at 280 nm and the TOF-MS base peak chromatogram (BPC) of aqueous and ethanolic rooibos extracts are shown in **Figure 1a-b** and **1c-d** respectively. The phenolic compounds were identified by interpreting their mass spectra obtained via their MS (TOF), MS/MS (IT) and UV spectra, taking into account all the data provided by the literature. **Table 1** provides a summary of all the compounds studied, including retention times, experimental and calculated *m/z*, molecular formula, error, sigma value, MS/MS fragments, UV bands as well as their proposed identities. It must be emphasised that the names attributed to the polyphenolic compounds are only proposed ones after the information provided by TOF-MS, the MS/MS fragmentation by IT-MS and bearing in mind

all the data reported in the literature. This also applies to the isomers of these compounds since it was impossible to distinguish between them. **Figure 2** shows the proposed structures of the compounds identified (by number).

The fragmentation observed for each ion was useful in order to confirm the proposed structures. Thus, losses of 120 and 90u were observed, corresponding to cross-ring cleavages in the sugar moiety. **Figure 3** illustrates the theoretical fragmentation pattern of a glycosyl flavonoid. The description of the cleavage and the nomenclature follow the system adopted by Waridel *et al*.[20]

Peak **1** (RT 4.60 min) gave a molecular mass of *m/z* 493. It was tentatively assigned to patuletin 7-glucoside according to the molecular formula provided for its accurate mass and corroborated by the HPLC profile of this compound, in this case with RT at 4.40 min. This compound has been encountered previously in other species such as *Centaurea ruthenica*.[21] Its MS/MS spectrum showed fragments at *m/z* 475, 403, 385, 373 and 355, corresponding to the loss of water (475), the $[M-H-90]^-$ (403) and $[M-H-90-H_2O]^-$ (385) ions as well as the successive loss of the $[M-H-120]^-$ ion (373) and water (355) from the main fragment.

Peak **2** (RT 4.77 min) had $[M-H]^-$ at *m/z* 339, which gave daughter ions at *m/z* 249 $[M-H-90]^-$ and 219 $[M-H-120]^-$. These fragmented ions were also derived from cross-ring cleavage in its sugar moiety. It was assigned tentatively to esculin, which has been previously reported in other sweet grass species such as Cortex fraxini and corroborated with the HPLC profile of this compound at RT 5.89 min.[22]

The ion found at *m/z* 611 was assigned to safflomin A (peak **3**) (RT 5.19 min), which presented four product ions at *m/z* 521, 491, 401 and 371. The first one, at *m/z* 521, corresponded to the loss of the $[M-H-90]^-$ ion and the others corresponded to the loss of the $[M-H-120]^-$ (491), $[M-H-120-90]^-$ (401) and $[M-H-120-120]^-$ (371) ions. This compound has been reported elsewhere in *Carthamus tinctorius* L.[23]

Table 1. Phenolic compounds characterized in rooibos extract by HPLC-ESI-MS (TOF/IT)

Peak	RT (min)	*m/z* experimental	*m/z* calculated	Error (ppm)	mSigma value	Formula $[M-H]^-$	λ^{max} (nm)	MS/MS fragments	Proposed compound
1	4.60	493.0990	493.0988	0.4	42.1	$C_{22}H_{21}O_{13}$	278	475, 403, 373, 385, 355	patuletin 7-glucoside
2	4.77	339.0717	339.0722	-1.4	7.1	$C_{15}H_{15}O_9$	257, 284, 314(sh)	249, 219	esculin
3	5.19	611.1620	611.1618	0.3	22.7	$C_{27}H_{31}O_{16}$	284	521, 491, 401, 371	safflomin A
4	6.32	609.1506	609.1461	7.3	12.6	$C_{27}H_{29}O_{16}$	261, 341	519, 489	quercetin-3-*O*-robinobioside
5	7.98	579.1336	579.1355	-3.3	37.9	$C_{26}H_{27}O_{15}$	271, 341	561, 519, 489, 459, 441, 399, 369	carlinoside or isocarlinoside or neocarlinoside or 2''-*O*-*β*-arabinopyranosylorientin
6	8.23	593.1482	593.1512	-5.1	24.1	$C_{27}H_{29}O_{15}$	271, 333	575, 503, 473, 413, 383, 353	vicenin-2
7	8.61	579.1334	579.1355	-2.0	33.9	$C_{26}H_{27}O_{15}$	271, 341	561, 519, 489, 459, 429, 399, 369	carlinoside or isocarlinoside or neocarlinoside 2''-*O*-*β*-arabinopyranosylorientin
8	8.83	579.1355	579.1355	0.1	41.6	$C_{26}H_{27}O_{15}$	271, 341	559, 519, 489, 459, 399, 369	carlinoside or isocarlinoside or neocarlinoside or 2''-*O*-*β*-arabinopyranosylorientin
9	8.97	449.1087	449.1089	-0.5	20.0	$C_{21}H_{21}O_{11}$	287	359, 329	(*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside
10	9.40	579.1335	579.1355	-3.5	30.1	$C_{26}H_{27}O_{15}$	271, 341	561, 519, 489, 459, 429, 399, 369	carlinoside or isocarlinoside or neocarlinoside or 2''-*O*-*β*-arabinopyranosylorientin
11	9.68	449.1082	449.1089	-1.7	15.4	$C_{21}H_{21}O_{11}$	287	359, 329	(*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside
12	11.21	447.0935	447.0933	0.5	11.7	$C_{21}H_{19}O_{11}$	269, 348	429, 411, 357, 327	iso-orientin
13	11.34	449.1020	449.1089	-1.2	18.2	$C_{21}H_{21}O_{11}$	285	359, 329	(*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside
14	11.62	449.1084	449.1089	-0.2	43.8	$C_{21}H_{21}O_{11}$	286	359, 329	(*R*)-eriodictyol-8-*C*-*β*-D-glucopyranoside

Table 1. (Continued).

Peak	RT (min)	*m/z* experimental	*m/z* calculated	Error (ppm)	mSigma value	Formula [M-H]$^-$	λmax (nm)	MS/MS fragments	Proposed compound
15	12.26	447.0930	447.0933	-0.7	6.6	$C_{21}H_{19}O_{11}$	254, 267(sh), 349	357, 327	orientin
16	13.26	451.1253	451.1246	1.5	1.6	$C_{21}H_{23}O_{11}$	280	361, 331	aspalathin
17	13.53	449.1082	449.1089	-1.6	34.3	$C_{21}H_{21}O_{11}$	282	359, 329, 285	aspalalinin
18	14.32	609.1452	609.1461	-1.5	15.2	$C_{27}H_{29}O_{16}$	267, 348	301	rutin
19	14.58	431.0991	431.0984	1.8	7.2	$C_{21}H_{19}O_{10}$	270, 336	413, 341, 311	isovitexin
20	15.57	463.0880	463.0882	-0.5	24.5	$C_{21}H_{19}O_{12}$	276	301, 343, 373	quercetin-3-*O*-glucoside/galactoside
21	15.89	447.0932	447.0933	-0.2	22.3	$C_{21}H_{19}O_{11}$	270, 342	285	luteolin-7-*O*-glucoside
22	17.76	435.1309	435.1297	2.8	39.8	$C_{21}H_{23}O_{10}$	283	345, 315	nothofagin
23	22.01	361.1644	361.1657	-3.4	88.3	$C_{20}H_{25}O_6$	280	346, 331	secoisolariciresinol
24	28.20	285.0348	285.0405	-2.0	45.5	$C_{15}H_9O_6$	241, 269, 335	151, 107	luteolin
25	28.41	301.0292	301.0354	-6.2	24.8	$C_{15}H_9O_7$	254, 271(sh), 371	179, 151	quercetin
A	4.94	339.0705	339.0722	-3.0	34.9	$C_{15}H_{15}O_9$	235, 284	-	5,7,dihydroxy-6-*C*-glucosyl-chromone
B	5.05	611.1600	611.1618	-4.9	53.3	$C_{27}H_{31}O_{16}$	235, 284	449, 287	eriodictyol 5,3'di-*O*-glucoside
C	5.50	595.1285	595.1305	-3.3	60.5	$C_{26}H_{27}O_{16}$	235, 274	301	quercetin-3-*O*-arabinoglucoside
D	15.97	463.0891	463.0882	1.9	17.0	$C_{21}H_{19}O_{12}$	245, 264(sh), 284(sh), 349(sh)	301	isoquercitrin
E	20.40	461.1070	461.1089	-4.2	53.9	$C_{22}H_{21}O_{11}$	247, 286	371, 341	scoparin

Note: sh = shoulder.

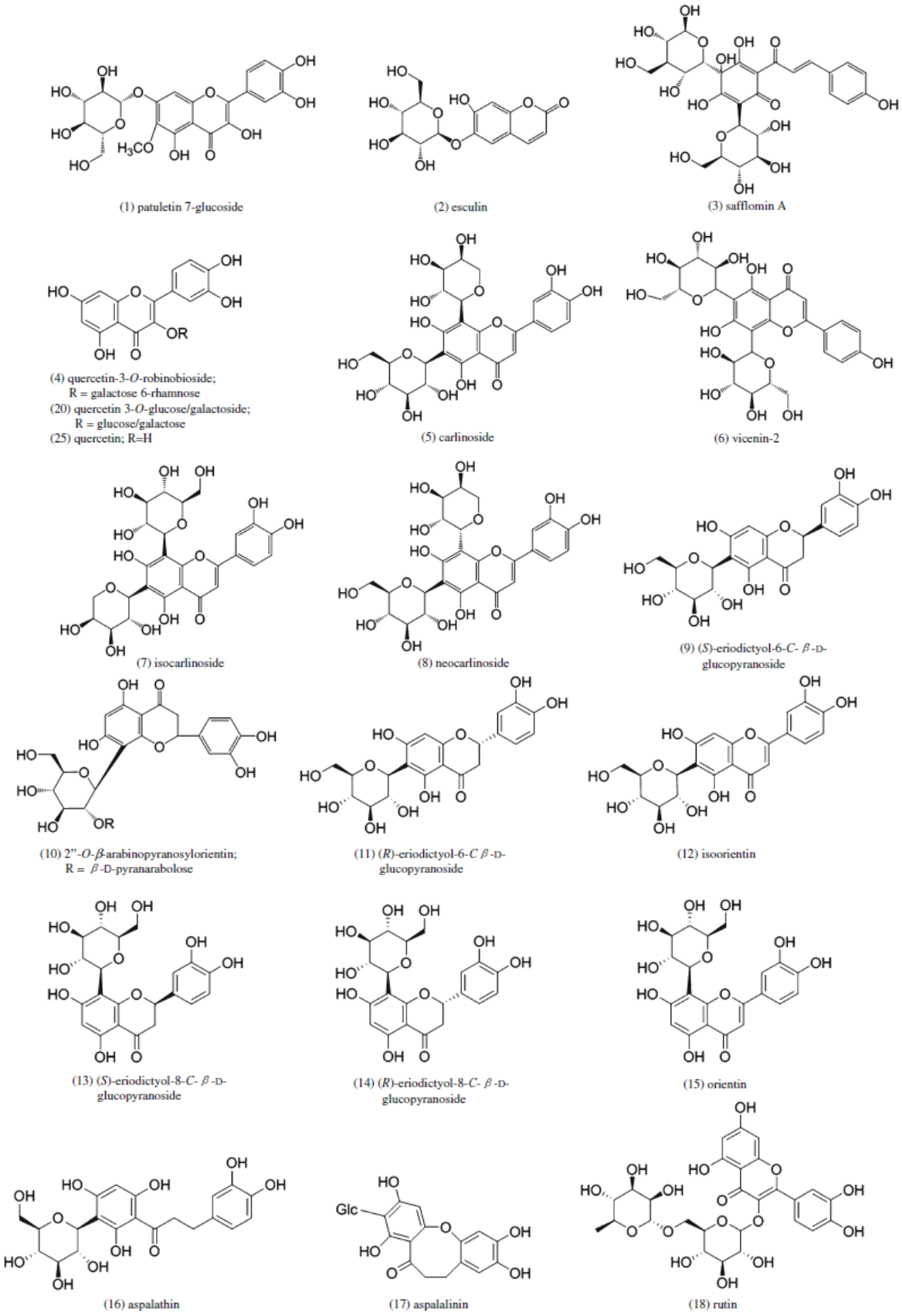

Figure 2. Chemical structures of the proposed compounds.

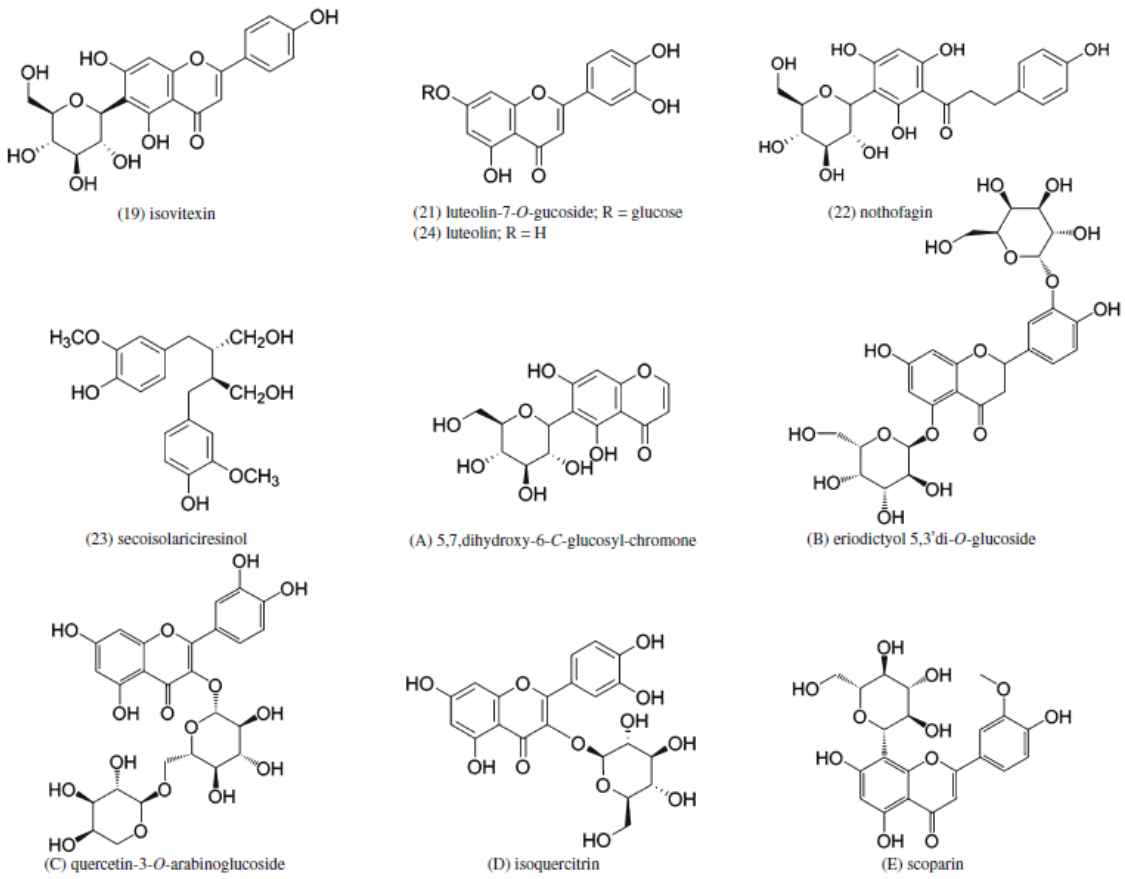

Figure 2. (Continued).

MS/MS delivered a molecular mass of *m/z* 609 for peak **4** (RT 6.32 min), which we assigned to quercetin-3-*O*-robinobioside with the fragmentation pattern at *m/z* 519 and 489.[24,25] The former corresponded to the loss of [M-H-90]$^-$ and the latter to the loss of [M-H-120]$^-$.

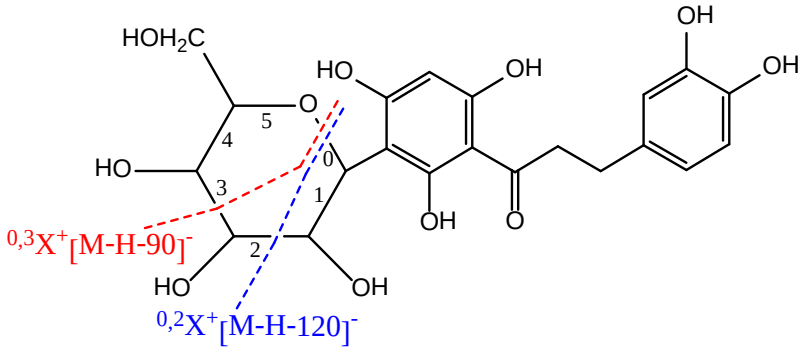

Figure 3. Fragmentation of a glycosil flavonoid illustrated for aspalathin.

Peaks **5**, **7**, **8** and **10**, with *m/z* 579, were detected at RT 7.98, 8.61, 8.83 and 9.40 min and showed the same molecular formula according to Smart Formula Editor. All these compounds responded in a similar way to UV at 271 and 341 nm as their rutinoside counterparts, which were tentatively proposed as being carlinoside,[26] isocarlinoside,[26,27] neocarlinoside[28] and/or 2''-*O*-*β*-arabinopyranosyl-orientin.[29] In the absence of standards it was impossible to distinguish between isomers and to ascertain the position of conjugating groups on the flavonoid skeleton. Thus, they could only be partially identified on the basis of their MS fragmentation pattern. The fragment at *m/z* 561 represented the loss of water. The fragments at *m/z* 489 and 459 were consistent with the loss of the $[M-H-90]^-$ and $[M-H-120]^-$ ions respectively from the precursor. The fragments at *m/z* 441 and 399 corresponded to the loss of the $[M-H-18-120]^-$ and $[M-H-90-90]^-$ ions respectively.

The ion found at *m/z* 593 (peak **6**; RT 8.23) was tentatively assigned to vicenin-2, which was reinforced by its fragmentation pattern. This compound has previously been found in *Lychnophora ericoides* Mart.[30] It presented fragments at *m/z* 575, 503, 473, 413 and 383, corresponding to a loss of water from the precursor followed by a loss of the $[M-H-90]^-$, $[M-H-120]^-$, $[M-H-90-90]^-$ and $[M-H-90-120]^-$ ions.

The Smart Formula Editor provided the same molecular formula for peaks **9**, **11**, **13** and **14** (RT 8.97, 9.68, 11.34 and 11.62 min respectively) with *m/z* 449. These compounds showed the same UV absorbance band at 287 nm, thus revealing that they have a similar structure and the same substituents, which we tentatively identified as (*R*/*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside and (*R*/*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside. Based on literatures,[24,31] by using reversed-phase chromatography, compound eriodictyol-6-*C*-*β*-D-glucopyranoside was eluted earlier than eriodictyol-8-*C*-*β*-D-glucopyranoside. Krafczyk and Glomb have studied four flavanone-*C*-glycosides which gave different retention times in HPLC analysis.[12] Although the absolute stereochemistry was not determined, the peak eluting first was tentatively assigned to S-isomer the basis of Philbin and Schwartz.[32] In addition, during

the process (*R*)/(*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside were the major products coinciding with minor concentrations of (*R*)/(*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside.[12] Hence, we tentatively identified peaks **9**, **11**, **13** and **14** as (*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside, followed by (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside, (*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside and (*R*)-eriodictyol-8-*C*-*β*-D-glucopyranoside, respectively. Each of them gave the same fragments at *m/z* 359 and 329, which represented a loss of the $[M-H-90]^-$ and $[M-H-120]^-$ ions respectively from the precursor.

Peaks **12** and **15** (RT 11.21 and 12.26 min) presented iso-orientin and orientin, with an ion found at *m/z* 447, and showed the same fragmentation pattern at *m/z* 357 and 327, corresponding to $[M-H-90]^-$ and $[M-H-120]^-$ respectively from the main fragment. In addition, peak **12** showed an ion at *m/z* 429 and 411, which is consistent with the successive loss of a molecule of water and two molecules of water (**Figure 4a**). Waridel *et al.* have studied in more detail for the differentiation of isomer pairs orientin/iso-orientin. In the negative mode, a clear loss of water (*m/z* 429) and a low abundant of *m/z* 411 were specific to 6-*C* glycosides and can be used for discrimination.[20] These losses were not recorded for the 8-*C* isomer orientin (**Figure 4b**). It was possible as well to distinguish them by comparing UV absorbance where only peak **15** showed UV absorbance spectrum at 267 nm (shoulder) which corresponded to orientin. These compounds have also been described elsewhere in the literature.[8-12,24,31] Snijman *et al.* have studied the antimutagenic acitivity of orientin and iso-orientin, containing the catechol moiety, were more effective inhibitors of 2-AAF-induced mutagenesis than the C4-monohydroxylated flavonoids.[33]

Peak **16** (RT 13.26 min) was assigned to aspalathin[10-12,24,31] with an ion found at *m/z* 451. It presented fragments at *m/z* 361 and 331, corresponding to the $[M-H-90]^-$ and $[M-H-120]^-$ ions respectively from the main fragment (**Figure 4c**).

Peak **17** (RT 13.53 min) had a $[M-H]^-$ ion at *m/z* 449 and was assigned to aspalalinin.[24] It presented daughter ions at *m/z* 359, 329 at 285. The first two

fragments corresponded to the $[M-H-90]^-$ and $[M-H-120]^-$ ions respectively from the main fragment, and *m/z* 285 corresponded to the loss of CO_2.

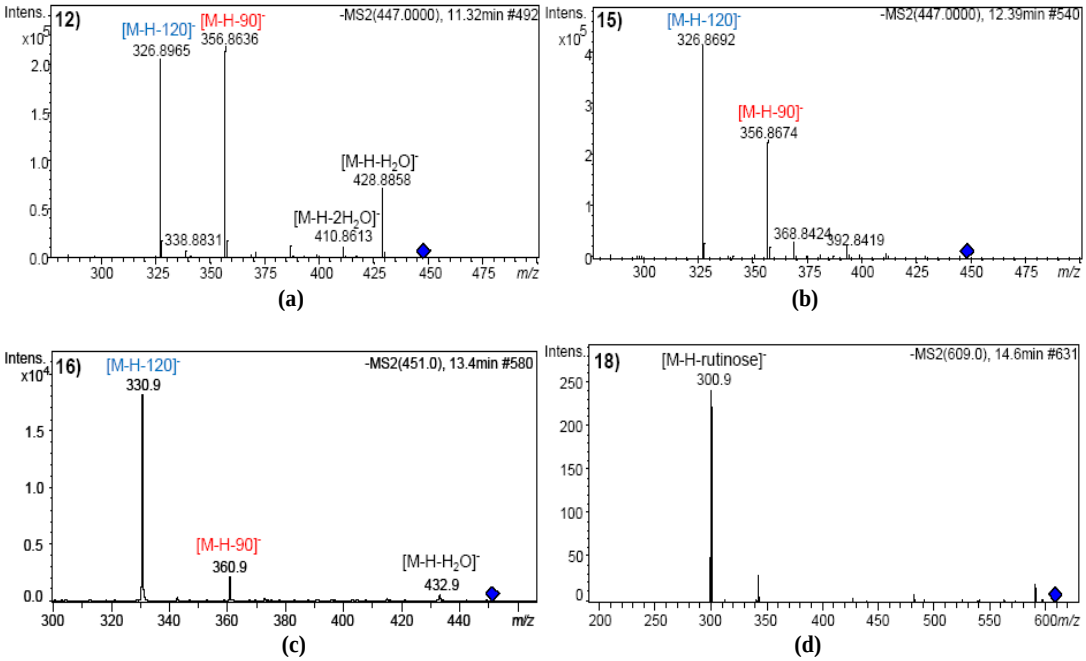

Figure 4. MS/MS spectra of the most representative phenolic compounds: iso-orientin (a), orientin (b), asphalatin (c), rutin (d).

Peak **18** (RT 14.23 min), with precursor and product ions at *m/z* 609 and 301 respectively, corresponds to the characteristic fragment mass of *O*-diglycoside, which is neutral loss of rutinose (**Figure 4d**). It was identified as rutin.[8,12,31]

Peak **19** (RT 14.58 min), with an ion at *m/z* 431, was assigned to isovitexin.[8,10,11,24,31] It presented fragments at *m/z* 413, 341 and 311, corresponding to a successive loss of water and the $[M-H-90]^-$ and $[M-H-120]^-$ ions. It was corroborated by its UV absorbance spectrum at 272 and 337 nm which distinguish it from vitexin.

Peak **20** (RT 15.57 min), with an ion found at *m/z* 463, showed fragments at *m/z* 373, 343, and 301 corresponding to the $[M-H-90]^-$ and $[M-H-120]^-$ ions, and the loss of sugar moiety. We assigned it to either quercetin-3-*O*-glucoside or quercetin-3-*O*-galactoside.[8,11,12,24,31]

The ion found at peak **21** (RT 15.89 min) corresponded to luteolin-7-*O*-glucoside[34] with the precursor and product ions at *m/z* 447 and 285

respectively, which is the characteristic fragment mass of *O*-monoglycoside, representing luteolin.

The presence of nothofagin[12,24,31] was identified at peak **22** (RT 17.76 min) and the ion was found at *m/z* 435. It presented two main product ions at *m/z* 345 (loss of 90 u) and *m/z* 315 (loss of 120 u) from the main fragment.

Peak **23** (RT 22.01 min), with an ion found at *m/z* 361, corresponded to secoisolariciresinol.[24] It presented two main product ions at *m/z* 346 [M-H-CH_3]$^-$ and *m/z* 331 [M-H-OCH_3]$^-$ from the main fragment.

Peaks **24** and **25** were assigned to aglycone. The fragmentation pattern of the [M-H]$^-$ ion at *m/z* 285 (peak 24) (RT 28.20 min) produced MS/MS fragments at *m/z* 151 and 107, corresponding to an A^- ring fragment released after RDA fission followed by the loss of CO_2. It was assigned to luteolin.[11,12,24,31] The ion found at *m/z* 301 (peak 25) (RT 28.41 min) was assigned to quercetin[11,12,24,31], which presented fragments at *m/z* 151 and 179, corresponding to an A^- ring fragment released after RDA fission and retrocyclization after fission on bonds 1 and 2 (**Figure 5**).[35-37]

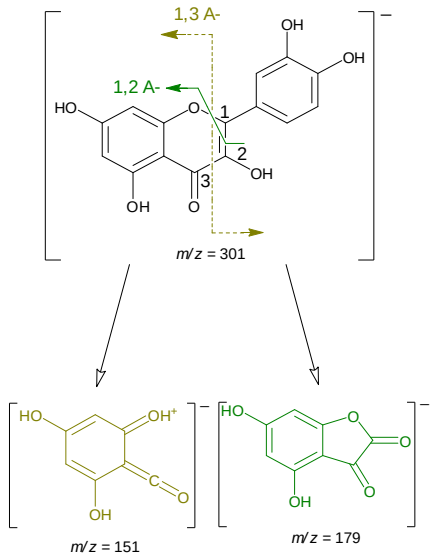

Figure 5. Proposed fragmentation pathway of quercetin.

Another five compounds were tentatively identified in ethanolic extract which were not detected in aqueous rooibos extract. Peak **A** (RT. 4.94 min)

and peak **D** (RT. 15.97 min) were assigned to 5,7,dihydroxy-6-*C*-glucosyl-chromone[38] and isoquercitrin[8,11,12,24,31], respectively. These compounds have been also described previously in rooibos. A signal at *m/z* 611 (peak **B**) was observed at 5.05 min. This compound showed a molecular formula $C_{27}H_{32}O_{16}$. It presented fragments at *m/z* 449 and 287, corresponding to the loss of a sugar moiety and two sugar moiety consecutively from eriodictyol (aglycone). It was tentatively identifed as eriodictyol 5,3'di-*O*-glucoside. At a retention time of 5.50 min, a signal with *m/z* 595.1285 (peak **C)** was detected as quercetin-3-*O*-arabinoglucoside corroborated with its fragment ion at *m/z* 301 corresponded to quercetin.[40] Finally, peak **E** (RT. 20.40 min) with molecular formula $C_{22}H_{22}O_{11}$ was tentatively identified as scoparin.[41] It presented two main product ions at *m/z* 371 $[M-H-90]^-$ and *m/z* 341 $[M-H-120]^-$ from the main fragment.

Effects on lipid metabolism

We assessed the effects of rooibos tea extract on cholesterol and triglyceride serum concentrations in a hypercholesterolemic LDLr knock-out mouse model. Rooibos tea extract did not change the serum concentration of these metabolites when the mice were fed on the chow diet (3% fat and 0.03% cholesterol w/w), there being no significant differences between the control and the rooibos groups. Rooibos tea extract did however show high bioactivity in those groups fed on the high-fat high-cholesterol diet (20% fat and 0.25% cholesterol w/w), in which it lowered serum cholesterol and triglyceride concentrations compared to the control. These *in vivo* results suggest that rooibos tea may exert beneficial effects upon the lipid profile by decreasing serum triglycerides and cholesterol, thus protecting the mice against harm caused by LDL cholesterol.

Conclusions

In this work, a powerful analytical method has been used to separate and characterize phenolic compounds in aqueous and ethanolic rooibos extracts by combining HPLC-ESI-TOF-MS and HPLC-ESI-IT-MS^2. Identification

was accomplished with the determination of accurate mass of the deprotonated molecules in the studied phenolic compounds by TOF-MS and confirmation of their fragmentation ions by IT-MS^2 further. This analytical tecnique has proved to be a useful tool for the identification of 25 major compounds in both extracts and five more compounds in ethanolic extract for HPLC runs for less than 30 min. Orientin and iso-orientin with their antimutagenic activity were found as two predominant compounds in both extracts. It is also important to highlight that, to our knowledge, the compounds patuletin 7-glucoside, esculin, safflomin A, carlinoside, isocarlinoside, neocarlinoside, 2''-*O*-β-arabinopyranosylorientin and vicenin-2, eriodictyol 5,3'di-*O*-glucoside, quercetin-3-*O*-arabinoglucoside, scoparin are reported here for the first time in rooibos. The Folin-Ciocalteu assay was used to determine the total polyphenolic contents as 25% and 23% w/w of aqueous and ethanolic extracts, respectively. In addition, the extract showed its highest bioactivity in the groups fed with the high-fat high-cholesterol diet (HF), lowering serum cholesterol and triglyceride concentrations compared to the control.

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Chapter 4A

Characterization by high-performance liquid chromatography with diode-array detection coupled to time-of-flight mass spectrometry of the phenolic fraction in cranberry syrup used to prevent urinary tract diseases, together with a study of its antibacterial activity

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ORIGINAL RESEARCH

Cranberry (Vaccinium macrocarpon) changes the surface hydrophobicity and biofilm formation of *E. coli*

Characterization by high-performance liquid chromatography with diode-array detection coupled to time-of-flight mass spectrometry of the phenolic fraction in cranberry syrup used to prevent urinary tract diseases, together with a study of its antibacterial activity

Abstract

The phenolic fraction of commercial cranberry syrup, which is purported to have good properties for the prevention of urinary diseases, has been thoroughly characterized using HPLC-DAD-TOF-MS. A study of its antibacterial activity has also been carried out. For this purpose a new HPLC-DAD-TOF-MS method using negative and positive ionization modes was developed and it was thus possible to identify 34 different compounds, nine of which have been tentatively characterized for the first time in cranberry syrup. It is also important to highlight that different coumarins in this matrix were also determined, which, to our knowledge, have not been found previously in the cranberry. The phenolic fraction obtained by HPLC-DAD was found to be 5.47 mg/mL. Catechin and procyanidins belonging to flavanols were the family of compounds found at the highest concentrations (2.37 mg/mL); flavonols were at a concentration of 1.90 mg/mL and phenolic-acid derivatives were found at the lowest concentration (0.28 mg/mL). With regard to antibacterial activity, the incubation of *E. coli* with cranberry syrup was found to reduce surface hydrophobicity as a function of the concentration of the extract.

Keywords: cranberry syrup; phenolic compounds; high-performance liquid chromatography (HPLC); mass spectrometry (MS); anti-adhesion activity.

Introduction

In recent years there has been ever increasing interest in the presence of certain compounds in foods that are beneficial to human health. In plant-derived foods these naturally occurring compounds form part of the secondary metabolism of many kinds of fruit and vegetable products and are known as phytochemicals. The antioxidant capacity of phytochemicals, as well as their health-promoting and/or disease-preventing properties, are currently the subject of intense study by the scientific community.

Berries, including raspberries, blueberries, black currants, red currants, and cranberries, are a rich source of these dietary antioxidants.[1] The American cranberry (*Vaccinium macrocarpon*) in particular is a rich source of bioactive compounds with antiproliferative, antioxidant[2], anti-inflammatory and antimicrobial properties, which inhibit the growth of pathogenic bacteria such as *Escherichia coli* and *Helicobacter pylori* for example.[3,4] It has traditionally been used in the treatment and prevention of urinary-tract infections in women and also in digestive-tract ailments. The antitumoral properties of cranberries have made them a popular diet component with an eye to the prevention of neoplastic diseases.[5] The phenolic compounds found in cranberries are believed to be the principal ingredients responsible for these beneficial effects. Cranberries are known for their high concentration of anthocyanins, as well as their significant contents of flavonols, flavan-3-ols, tannins (ellagitannins and proanthocyanidins) and phenolic-acid derivatives.[6]

One out of two women experience some sort of urinary tract disorder during their lifetime, which often reoccurs. As has been observed, the consumption of cranberries has a significant influence on lowering the incidence of urinary diseases.[7,8] Nowadays therefore, some dietary supplements containing cranberry extracts are being developed.

The aim of this work was to characterize the phenolic fraction contained in cranberry syrup, made up of glucose, sodium benzoate, potassium sorbate and American cranberry (*Vaccinium macrocarpon*), using HPLC-DAD-TOF-MS, and also to study its antibacterial activity. This study is a preliminary step in

our thorough research into the composition of cranberry syrup. The syrup will be then be used for *in vivo* analyses to study the metabolites of these phenolic compounds in urine and evaluate the incidence of urinary disorders in its consumers.

Materials and methods

Materials

Standards of myricetin, *p*-coumaric acid, 7-hydroxycoumarin, and proanthocyanidin A2 were from Extrasynthese (Genay, France). Folin-Ciocalteu phenol reagent was from Fluka, Sigma-Aldrich (Steinheim, Germany). Formic acid and acetonitrile used for preparing mobile phases were from Fluka, Sigma-Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland) respectively. Distilled water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Millipore, Bedford, MA, USA). Solvents were filtered before use with a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA).

Sample preparation

200 µL of cranberry syrup, bought in a local pharmacy, was dissolved in 4 mL methanol, vortexed for 2 min in a G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA), filtered with a polytetrafluoroethylene (PTFE) syringe filter (0.2 µm pore size) and injected directly into the HPLC system.

Measurement of total polyphenols, proanthocyanidins and anthocyanins

To quantify the total phenolic content in cranberry syrup, the Folin-Ciocalteu method was used.[9] The proanthocyanidin (condensed tannins) and total anthocyanin contents were determined according to the vanillin-HCl method[10] and the methods described by Fuleki and Francis respectively.[11]

Chromatographic separation

HPLC analyses were made with an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode-array detector (DAD). Separation was carried out with a Zorbax Eclipse Plus C_{18} analytical column (150 mm x 4.6 mm, 1.8 µm particle size). Gradient elution was conducted using two different programs. Gradient program 1 was used for the MS negative ionization mode consisting of 1% formic acid in water-acetonitrile (90:10 v/v) (phase A) and acetonitrile (phase B) at a constant flow rate of 0.5 mL/min using the following gradient: 0-20 min, linear gradient from 5% B to 20% B; 20-25 min, linear gradient from 20% B to 40% B; 25-30 min, linear gradient from 40% B to 5% B; and 30-35 min, isocratic of 5% B. Subsequently a different chromatographic method (gradient program 2) was used for the MS positive ionization mode. Due to their acid–base equilibrium, anthocyanins need a more acidic pH to be resolved and so the gradient was modified as follows: water-formic acid (90:10 v/v) (phase A) and acetonitrile (phase B) at a constant flow rate of 0.5 mL/min using the following gradient: 0-13 min, linear gradient from 0% B to 20% B; 13-20 min, linear gradient from 20% B to 30% B; 20-25 min, linear gradient from 30% B to 80% B; and 25-30 min, linear gradient from 80% B to 0% B, and 30-35 min, isocratic of 0% B. The addition of formic acid gave better results for the ionization of the compounds in positive mode. The injection volume was 10 µL for both gradient elution programs. The two different methods were chosen as they both afforded short analysis times and good chromatographic separations. UV data were collected using DAD set at 280, 320, 360, and 520 nm.

ESI-TOF-MS conditions

TOF-MS was conducted using a microTOF^{TM} (Bruker Daltonics, Bremen, Germany) orthogonal-accelerated TOF mass spectrometer equipped with an electrospray ionization (ESI) interface. The parameters for analysis were set using both negative and positive ion modes with spectra acquired over a mass range of 50–1000 *m/z*. The other optimum values of the ESI-MS parameters

were: capillary voltage, 4500 V; dry gas temperature, 190 ^{o}C; dry gas flow, 9.0 L/min; nebulizer pressure, 2.0 bar; and spectra rate 1 Hz. The flow delivered into the MS detector from HPLC was split using a flow splitter (1:2) to achieve stable electrospray ionization and obtain reproducible results. The calibrant was a sodium-formate cluster containing 5 mM sodium hydroxide and 0.2% formic acid in water-isopropanol (1:1 v/v), injected at the beginning of each run with a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface. All the spectra were calibrated prior to compound identification. All operations were controlled by DataAnalysis 3.4 software (Bruker Daltonik), which provided a list of possible elemental formulas by using the GenerateMolecularFormula™ Editor.

Assessment of the method

Quantification was made according to the linear calibration curves of standard compounds. Four calibration curves were prepared using the following standards: myricetin, *p*-coumaric acid, 7-hydroxycoumarin and procyanidin A2. The different parameters of each standard compound are summarized in **Table 1**. All calibration curves show good linearity between different concentrations depending upon the analytes in question. The calibration plots reveal good correlation between peak areas and analyte concentrations, and the regression coefficients were always higher than 0.995. LOD was found to be within the range 0.053-0.233 µg/mL whilst LOQ was within 0.175-0.679 µg/mL.

Table 1. Analytical parameters of the method.

Analyte	RSD	LOD (µg/mL)	LOQ (µg/mL)	Calibration range (µg/mL)	Calibration equations	r^2	Accuracy
Myricetin	0.23	0.053	0.175	LOQ-25	y = 22.852x + 21.117	0.996	98.7
***p*-Coumaric acid**	0.31	0.204	0.679	LOQ-250	y = 127.13x + 7.2384	0.999	99.2
Procyanidin A2	0.36	0.152	0.287	LOQ-100	y = 5.8648x + 3.8544	0.997	101.3
7-Hydroxycoumarin	0.27	0.233	0.656	LOQ-50	y = 37.724x + 12.555	0.998	100.8

Intraday and interday precisions were developed to assess the repeatability of the method. A syrup extract was injected (n = 6) during the same day (intraday precision) for 3 consecutive days (interday precision, n = 18). The relative standard deviations (RSDs) of analysis time and peak area

were determined. The intraday repeatability of the peak area, expressed by the RSD, was 1.2%, whereas interday repeatability was 3.8%.

The accuracy of the assay can be determined by the closeness of the test value to the nominal value and was evaluated with separately prepared individual primary stock solutions, mixtures and working solutions of all standards. It was calculated over the linear dynamic range at three concentration levels: low (LOQ), medium (intermediate concentration value of the linear calibration range), high (highest concentration value of the linear calibration range) via three assays per concentration on different days. The analyte concentrations were calculated from calibration curves and accuracy was calculated by the ratio of this calculated concentration versus the theoretical (spiked) one.

Bacteria and cultures

Nine strains of uropathogenic *E. coli* (695, 787, 471, 472, 593, 595, 760, 629 and 607) were obtained from patients with acute pyelonephritis, together with 4 strains of *E. coli* from the Spanish Type Culture Collection (CECT): CECT 424 (F- thr- leu- lacY mtl- thi- ara gal ton 2 malA xyl, resistant to phages T1, T2 and T6.); CECT 4076 (Serovar. O157:H7, originally isolated from haemorrhagic colitis); CECT 417 (SupE44[am]. mutant tRNA); and CECT 743 (Serovar. O142 K86B:H6, isolated from children with diarrhoea). To enhance the activity of the Type 1 fimbriae[12] the strains were grown in TSB culture medium at 37 ºC for 48 h and then centrifuged at 2000 g for 10 min. The supernatant was then discarded, and the strains resuspended in PBS (pH 7.4). This washing process was performed twice. Finally, the bacterial suspension was adjusted to 10^9 bacteria/mL (OD of 1.0 to 542 nm). To enhance the activity of the P type fimbriae, the strains were incubated for 16 h on CFA agar[13], extracted from the surface of the agar after washing with 5 mL PBS, and then centrifuged at 2000 g for 10 min.

Ammonium sulphate aggregation test

The technique used was that described by Lindahl *et al*.[14] Briefly, solutions of ammonium sulphate were prepared, with osmolarities ranging from 0.2 M to 4 M, using sodium phosphate as dilutant. Taking 20 µL of bacterial suspension, an equal volume of ammonium sulphate solution was added and then gently mixed. The presence of aggregation was observed after 30 seconds' gentle manual rotation at room temperature over a glass slide, and the lowest concentration of ammonium sulphate that produced visible aggregation was noted. Aggregation with the 4 M solution was interpreted as a hydrophobicity of 0%, whilst aggregation with the 0.2 M solution was interpreted as 95% hydrophobicity.

Results and discussion

Chromatographic profile and compound identification

The base-peak chromatograms (BPC) of a cranberry syrup, obtained using both negative and positive ionization modes, are set out in **Figure 1a** and **b**. The tentatively identified phenolic compounds are summarized in **Tables 2** and **3** (negative and positive ionization modes respectively), including retention times, experimental and calculated *m/z*, molecular formula, error, sigma values (comparison of theoretical with measured isotope patterns), together with their proposed identities.

Phenolic compounds were successfully separated and identified with a gradient optimized for negative and positive ionization modes. The compounds were identified by interpreting their mass spectra obtained via TOF-MS, taking into account all the data reported in the literature. All these facts were also complemented with the UV spectra provided by DAD, which gave additional information about the family of compounds as far as the absorbance bands are concerned.

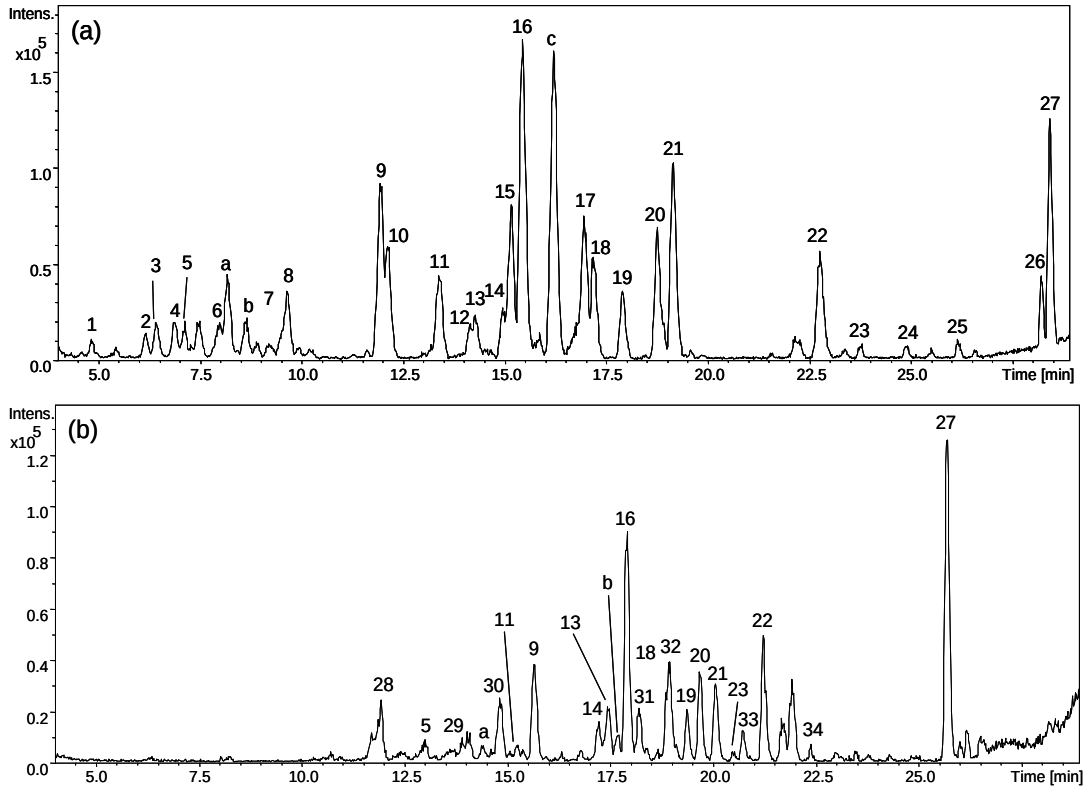

Figure 1. Chromatographic profiles using the gradient programs: (a) Base peak chromatogram (BPC) for the gradient program 1, negative mode; (b) Base peak chromatogram (BPC) for the gradient program 2, positive mode.

Compounds identified in negative ionization mode

Twenty-seven phenolic compounds were identified in negative ionization mode, including 6 new proposed compounds reported for the first time in the American cranberry (*Vaccinium macrocarpon*). **Table 2** and **Figure 1a** show the base-peak chromatogram (BPC) in negative mode of an extract of cranberry syrup.

Phenolic-acid derivatives

The first group of peaks migrated between 4.85 and 8.92 min and the compounds were related to the phenolic-acid family. Peak **1** (RT 4.85 min) gave a molecular mass of *m/z* 325.0929, which was tentatively identified as coumaroyl-hexose according to the molecular formula provided for its mass and corroborated by its fragment ion at *m/z* 163.0406, corresponding to a loss of the sugar moeity (162 Da).[6] Peak **2** (RT 6.19 min), which showed an ion at

Table 2. Phenolic compounds in cranberry syrup characterized by HPLC-DAD-TOF-MS in negative ionization mode.

Peak number	Class/phenolic compounds	RT(min)	Selected ion	*m/z* experimental	*m/z* calculated	Fragments	Error (ppm)	Sigma	Molecular formula
	Phenolic acid derivatives								
1	Coumaroyl-hexose	4.85	$[M-H]^-$	325.0929	325.0929	163.0406	0.0	0.0213	$C_{15}H_{18}O_8$
2	Sinapoyl-hexose	6.19	$[M-H]^-$	385.1127	385.1140	223.0596	3.3	0.0176	$C_{17}H_{22}O_{10}$
3	Caffeoyl glucose	6.44	$[M-H]^-$	341.0888	341.0878	179.0351	2.9	0.0107	$C_{15}H_{18}O_9$
4	Chlorogenic acid	6.89	$[M-H]^-$	353.0867	353.0878	191.0558	3.0	0.0561	$C_{16}H_{18}O_9$
5	Coumaroyl-hexose	7.16	$[M-H]^-$	325.0918	325.0929	163.0398	3.4	0.0298	$C_{15}H_{18}O_8$
7	Canthoside A	8.92	$[M-H]^-$	445.1355	445.1351		0.8	0.0238	$C_{19}H_{26}O_{12}$
10	Dihydroferulic acid 4-*O*-β-D-glucuronide	12.16	$[M-H]^-$	371.0989	371.0984	175.0283	1.5	0.0131	$C_{16}H_{20}O_{10}$
	Flavonols								
9	Myricetin 3-*O*- hexose	11.94	$[M-H]^-$	479.0835	479.0831	317.0301	0.8	0.0204	$C_{21}H_{20}O_{13}$
14	Myricetin 3-*O*-arabinoside	14.94	$[M-H]^-$	449.0739	449.0725	317.0286	2.9	0.0217	$C_{20}H_{18}O_{12}$
15	Caviunin glucoside	15.15	$[M-H]^-$	535.1482	535.1457	373.0907	4.7	0.0109	$C_{25}H_{28}O_{13}$
16	Quercetin 3-*O*- hexose	15.44	$[M-H]^-$	463.0894	463.0882	301.0293	2.5	0.0107	$C_{21}H_{20}O_{12}$
18	Quercetin 3-*O*-xylopyranoside	17.14	$[M-H]^-$	433.0784	433.0776	301.0350	1.9	0.0113	$C_{20}H_{18}O_{11}$
19	Quercetin 3-*O*-arabinopyranoside	17.88	$[M-H]^-$	433.0797	433.0776	301.0302	4.8	0.0238	$C_{20}H_{18}O_{11}$
20	Quercetin 3-*O*-arabinofuranoside	18.75	$[M-H]^-$	433.0781	433.0776	301.0332	1.0	0.0132	$C_{20}H_{18}O_{11}$
21	Quercetin 3-*O*-rhamnoside	19.13	$[M-H]^-$	447.0937	447.0933	301.0325	0.9	0.0021	$C_{21}H_{20}O_{11}$
22	Myricetin	22.73	$[M-H]^-$	317.0292	317.0303		3.6	0.0178	$C_{15}H_{10}O_8$
23	Methoxyquercetin 3-*O*-galactoside	23.75	$[M-H]^-$	477.1033	477.1038		1.2	0.0271	$C_{22}H_{22}O_{12}$
26	Kaempferol 3-*O*-β-D-(6''-*p*-hydroxybenzoyl)-galactopyranoside	28.15	$[M-H]^-$	567.1143	567.1144		0.2	0.0104	$C_{28}H_{24}O_{13}$
27	Quercetin	28.36	$[M-H]^-$	301.0337	301.0354		5.5	0.0196	$C_{15}H_{10}O_7$

Table 2. (Continued).

Peak number	Class/phenolic compounds	RT(min)	Selected ion	*m/z* experimental	*m/z* calculated	Fragments	Error (ppm)	Sigma	Molecular formula
	Flavanols								
6	Procyanidin B type isomer 1	8.00	$[M-H]^-$	577.1328	577.1351	289.0687	4.1	0.0405	$C_{30}H_{26}O_{12}$
8	(+)-Catechin	9.65	$[M-H]^-$	289.0714	289.0718		1.3	0.0033	$C_{15}H_{14}O_6$
11	Proacyanidin A2 type isomer 1	13.35	$[M-H]^-$	575.1211	575.1195	423.0631	3.0	0.0156	$C_{30}H_{24}O_{12}$
12	Procyanidin B type isomer 2	14.15	$[M-H]^-$	577.1306	577.1351	425.0761	5.5	0.0263	$C_{30}H_{26}O_{12}$
13	Proacyanidin A2 type isomer 2	14.28	$[M-H]^-$	575.1216	575.1195	423.0631	3.7	0.0476	$C_{30}H_{24}O_{12}$
17	Procyanidin A2 type isomer 3	16.94	$[M-H]^-$	575.1218	575.1195	423.0733	4.0	0.0290	$C_{30}H_{24}O_{12}$
25	Prodelphinidin B4	26.11	$[M-H]^-$	609.1238	609.1250		1.9	0.0355	$C_{30}H_{26}O_{14}$
	Isoflavonoids								
24	Biochanin A-7-*O*-glucoside	24.82	$[M-H]^-$	445.1156	445.1140		3.5	0.0284	$C_{22}H_{22}O_{10}$
	Unknown compounds								
a		8.20	$[M-H]^-$	431.1554	431.1559		1.1	0.0138	$C_{19}H_{28}O_{11}$
b		8.67	$[M-H]^-$	431.1923	431.1923		0.1	0.0247	$C_{20}H_{32}O_{10}$
c		16.19	$[M-H]^-$	537.1641	537.1614		5.2	0.0109	$C_{25}H_{30}O_{13}$

m/z 385.1127, gave a fragment at *m/z* 223.0596, corresponding to a loss of sugar moiety; thus, it was identified as sinapoyl-hexose.[15] The ion at RT 6.44 min corresponds to caffeoyl glucose with the precursor and fragment ions at *m/z* 341.0888 and 179.0351 respectively, indicating the loss of a sugar moeity. Peak **4** (RT 6.89 min) was assigned to chlorogenic acid[16], showing a fragment at *m/z* 191.0558, corresponding to the quinic-acid moiety previously reported. The presence of another isomeric form of coumaroyl-hexose was tentatively identified in peak **5** (RT 7.16 min). Peak **7** (RT 8.92 min) was tentatively identified as canthoside A[17], this apparently being the first time that this compound has been found in the cranberry. Dihydroferulic acid 4-*O*-β-D-glucuronide (peak **10**) was tentatively identified at *m/z* 371.0989. It presented a fragment at a *m/z* of 175.0283 which corresponds to the glucuronide moiety after the fragmentation of the dihydroferulic acid.

Flavonoids

The HPLC-DAD-TOF-MS analysis of the cranberry syrup extract revealed a total of 20 flavonoids (summarized in **Table 2**). For most flavonoids, the negative ionization mode provided the highest sensitivity and selectivity.[18] The following flavonols already found in cranberry were confirmed in our sample: myricetin 3-*O*-hexose (peak **9**)[19,20], myricetin 3-*O*-arabinoside (peak **14**)[21], quercetin 3-*O*- hexose (peak **16**)[19], quercetin 3-*O*-xylopyranoside (peak 18)[19] quercetin 3-*O*-arabinopyranoside (peak **19**)[19,21], quercetin 3-*O*-arabinofuranoside (peak **20**)[19,21], quercetin 3-*O*-rhamnoside (peak **21**)[20,21], myricetin (peak **22**)[21], methoxyquercetin 3-*O*-galactoside (peak **23**)[19] and quercetin (peak **27**).[16,21] As can be see in **Table 2**, quercetin and derivates with sugar bonds gave the fragment ion at *m/z* 301, corresponding to the loss of a sugar moiety.

Three different isomers of A-type procyanidin were identified in cranberry syrup at times 13.35, 14.28, 16.94 min with a *m/z* of 575.12 (peaks **11**, **13** and **17**), showing a typical fragment at *m/z* 423. Two B-type procyanidin isomers with a *m/z* of 577.13 eluted at 8.00 and 14.15 min (peaks **6** and **12**) and their fragments at 425 and 289 were also detected.[22] Peak **8**,

with a *m/z* of 289.0714 and a retention time of 9.65 min, was identified as (+)-catechin.

Caviunin glucoside (peak **15**), biochanin A-7-*O*-glucoside (peak **24**), prodelphinidin B4 (peak **25**) and kaempferol 3-*O*-*β*-D-(6''-*p*-hydroxybenzoyl)-galactopyranoside (peak **26**) were identified using mass spectra, UV spectra and the information provided by the GenerateMolecularFormula™ Editor. As far as we know, this is the first time that these compounds have been reported in the cranberry.

Cavinium glucoside at *m/z* 535.1482 showed a fragment at *m/z* 373.0907, indicating the loss of the sugar moiety. **Figure 2** shows the structures of the newly identified compounds in cranberry syrup. Additional unidentified compounds have been included in **Table 2** as they form an important part of the polar fraction of cranberry syrup.

Compounds identified in positive ionization mode

The presence of anthocyanins in cranberries has been reported in the literature, and since anthocyanins have maximum sensitivity in positive mode due to their inherent positive charge[18] the extract was also characterized in positive ionization mode**. Figure 1b** shows the BPC of an extract of cranberry syrup in positive ionization mode. Thus, by using the proposed method 20 phenolic compounds were identified in positive ionization mode in the cranberry-syrup extract. Despite the fact that several anthocyanidins have been previously described in the cranberry, only petunidin (peak **34**)[23] has so far been identified in the syrup.

In addition, several phenolic acids, flavonols, flavanols and hydroxycoumarins were identified in positive ionization mode. It is important to note that some of them were also found in the analysis carried out in negative ionization mode, but most of them could only be identified thanks to this positive ionization mode.

Table 3. Phenolic compounds in cranberry syrup characterized by HPLC-DAD-TOF-MS in positive ionization mode.

Peak number	Class/phenolic compounds	RT (min)	Selected ion	*m/z* experimental	*m/z* calculated	Fragments	Error (ppm)	Sigma	Molecular formula
	Phenolic acid derivatives								
5	Coumaroyl-hexose	13.01	$[M+K]^+$	365.0575	365.0633	203.0028	15	0.0225	$C_{15}H_{18}O_8$
29	2-Hydroxybenzoic acid	13.90	$[M+H]^+$	139.0401	139.0390		7.8	0.0113	$C_7H_6O_3$
30	Digallic acid	14.82	$[M+H]^+$	323.0438	323.0398	140.9858	12.4	0.0131	$C_{14}H_{10}O_9$
	Flavonols								
9	Myricetin 3-*O*- hexose	15.66	$[M+H]^+$	481.0973	481.0977	319.0446	0.9	0.0293	$C_{21}H_{20}O_{13}$
14	Mryicetin 3-*O*-arabinoside	17.19	$[M+H]^+$	451.0872	451.0871	319.0439	0.2	0.0352	$C_{20}H_{18}O_{12}$
16	Quercetin 3-*O*- hexose	17.91	$[M+H]^+$	465.1023	465.1028	303.0491	1.0	0.0294	$C_{21}H_{20}O_{12}$
18	Quercetin 3-*O*-xylopiranoside	18.85	$[M+H]^+$	435.0933	435.0922	303. 0485	2.5	0.0351	$C_{20}H_{18}O_{11}$
19	Quercetin 3-*O*-arabinopyranoside	19.33	$[M+H]^+$	435.0936	435.0922	303.0509	3.4	0.0440	$C_{21}H_{18}O_{11}$
20	Quercetin 3-*O*-arabinofuranoside	19.67	$[M+H]^+$	435.0934	435.0922	303.0494	2.7	0.0355	$C_{20}H_{18}O_{11}$
21	Quercetin 3-*O*-rhamnoside	20.04	$[M+H]^+$	449.1063	449.1078	303.0495	3.4	0.0340	$C_{21}H_{20}O_{11}$
23	Methoxyquercetin 3-*O*-galactoside	20.45	$[M+H]^+$	479.1191	479.1184	317.0663	2.1	0.0773	$C_{22}H_{22}O_{12}$
33	Syringetin (3',5'-*O*-Dimethylmyricetin)	20.69	$[M+H]^+$	347.0765	347.0761		1.1	0.0214	$C_{17}H_{14}O_8$
22	Myricetin	21.19	$[M+H]^+$	319.0445	319.0448		1.2	0.0123	$C_{15}H_{10}O_8$
27	Quercetin	25.66	$[M+H]^+$	303.0500	303.0499		0.2	0.0078	$C_{15}H_{10}O_7$
	Flavanols								
11	Procyanidin A2-type isomer 1	15.20	$[M+H]^+$	577.1342	577.1341	425.0875/ 287.0542	0.2	0.0713	$C_{30}H_{24}O_{12}$
13	Procyanidin A2-type isomer 2	17.41	$[M+H]^+$	577.1336	577.1341	425.0858/ 287.0532	0.9	0.0127	$C_{30}H_{24}O_{12}$
	Coumarins								
28	7-Hydroxycoumarin	11.94	$[M+H]^+$	163.0395	163.0390		3.1	0.0168	$C_9H_6O_3$
31	Coumarin	18.21	$[M+H]^+$	147.0447	147.0441		4.6	0.0058	$C_9H_6O_2$
32	Scopoletin	18.90	$[M+H]^+$	193.0415	193.0417	177.0538/ 147.0446	0.4	0.0271	$C_{10}H_8O_4$
	Anthocyanin								
34	Petunidin	22.34	$[M+H]^+$	317.0629	317.0656		8.5	0.0252	$C_{16}H_{14}O_7$
	Unknown compounds								
a		14.37	$[M+H]^+$	441.1104	441.1086		4.1	0.0258	$C_{30}H_{16}O_4$
b		17.68	$[M+H]^+$	397.1375	397.1341		8.7	0.0248	$C_{15}H_{24}O_{12}$

Table 4. Phenolic compounds in cranberry syrup expressed in μg/mL of syrup (n=5).

Class/phenolic compounds	μg/mL cranberry syrup
Phenolic-acid derivatives	
Coumaroyl-hexose	56.70±2.05
Sinapoyl-hexose	10.72±0.37
Caffeoyl glucose	19.63±0.62
Chlorogenic acid	19.35±0.53
Coumaroyl-hexose	40.18±1.15
Canthoside A	1.61±0.04
2-Hydroxybenzoic acid	49.40±1.45
Gallic acid 3-*O*-gallate	58.36±1.63
Dihydroferulic acid 4-*O*-β-D-glucuronide	23.96±1.21
Flavonols	
Myricetin 3-*O*- hexoside	125.87±4.87
Myricetin 3-*O*-arabinoside	226.63±8.83
Caviunin glucoside	297.30±13.43
Quercetin 3-*O*- hexoside	391.13±19.29
Quercetin 3-*O*-xylopiranoside	68.13±2.37
Quercetin 3-*O*-arabinopyranoside	66.39±2.17
Quercetin 3-*O*-arabinofuranoside	120.53±4.31
Quercetin 3-*O*-rhamnoside	130.51±4.23
Myricetin	114.78±3.79
Methoxyquercetin 3-*O*-galactoside	nq*
Kaempferol 3-*O*-β-D-(6''-*p*-hydroxybenzoyl)-galactopyranoside	30.70±1.39
Quercetin	303.27±12.81
Syringetin	24.72±0.83
Flavanols	
Procyanidin B type isomer 1	202.84±7.39
(+)-Catechin	374.31±15.43
Proacyanidin A2 type isomer 1	364.03±14.89
Procyanidin B type isomer 2	215.64±9.10
Proacyanidin A2 type isomer 2	356.99±12.88
Procyanidin A2 type isomer 3	644.37±49.71
Prodelphinidin B4	209.98±7.37
Isoflavonoids	
Biochanin A-7-*O*-glucoside	nq
Coumarins	
7-Hydroxycoumarin	246.91±8.77
Coumarin	174.90±6.21
Scopoletin	449.99±19.82
Anthocyanin	
Petunidin	9.59±2.90
Total	5469.35±194.81

*nq, not quantified

Thus, the phenolic acids identified were: coumaroyl-hexose (peak **5**), which had also been identified in the negative mode; 2-hydroxybenzoic acid (peak 29)[24], which presented a *m/z* of 139.0401 and digallic acid (peak **30**) at *m/z* 323.0438, which showed a fragment at *m/z* 141, corresponding to the loss of a gallate moiety, thus corroborating its identification.[25]

Finally, several coumarins were tentatively identified only in positive ionization mode. Peak **28** presented a *m/z* of 163.0395 and was assigned to 7-hydroxycoumarin, as reported in the bibliography.[26] Peak **31**, with a *m/z* of 147.0447, was tentatively identified as coumarin. Another coumarin, at *m/z* 193.0415 and showing fragments at *m/z* 177 and 147, was identified as scopoletin, according to Chen *et al*.[27] As far as we know, this is the first time that 7-hydroxycoumarin and coumarin have been identified in the cranberry (**Figure 2**) by using HPLC-DAD-TOF-MS in the same run.

Several flavanols that had already been identified in the negative ionization mode were also detected and identified: myricetin 3-*O*-hexose (peak **9**), myricetin 3-*O*-arabinoside (peak **14**), quercetin 3-*O*-hexose (peak **16**), quercetin 3-*O*-xylopiranoside (peak **18**), quercetin 3-*O*-arabinopiranoside (peak **19**), quercetin 3-*O*-arabinofuranoside (peak **20**), quercetin 3-*O*-rhamnoside (peak **21**), methoxyquercetin 3-*O*-galactoside (peak **23**); myricetin (peak **22**) and quercetin (peak **27**). Furthermore, syringetin (peak **33**), a flavonoid that could not be detected in negative ionization mode, was also identified. All the quercetin and myricetin-sugar conjugates showed a fragment ion at *m/z* 303 and 319 repectively, corresponding to the aglycone of quercetin and myricetin. Moreover, two isomers of Type A procyanidin (peaks **11** and **13** respectively) were also detected in positive ionization mode.

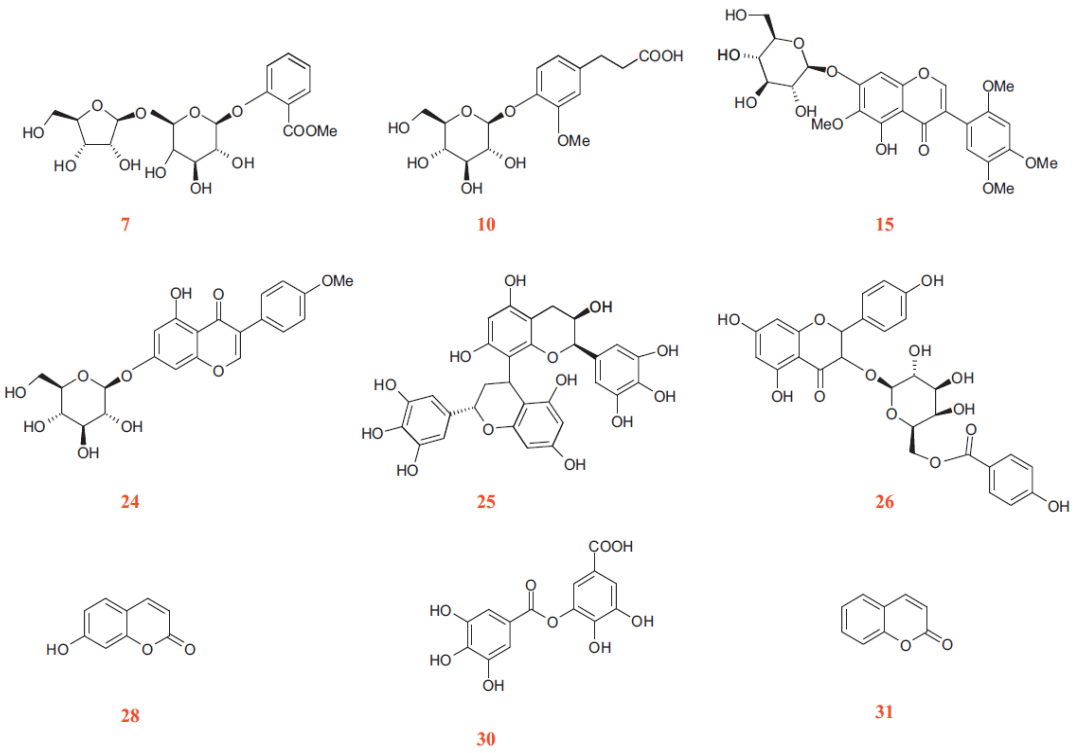

Figure 2. Structures of new compouds identified in cranberry syrup: (**7**) canthoside A, (**10**) dihydroferulic acid 4-*O*-β-D-glucuronide, (**15**) caviunin glucoside, (**24**) biochanin A-7-*O*-glucoside, (**25**) prodelphinidin B4, (**26**) kaempferol 3-*O*-β-D-(6''-*p*-hydroxybenzoyl)-galactopyranoside, (**28**) 7-hydroxycoumarin, (**30**) digallic acid, and (**31**) coumarin.

Quantification

Quantification was done using the calibration curves shown in **Table 1**. The calibration curve of myricetin at λ=280 nm was used to quantify flavonols, whilst phenolic-acid derivatives were quantified with the calibration curve of *p*-coumaric acid at λ=280 nm. Flavanols were quantified using the curve of procyanidin A2 at λ=280 nm and coumarins with the calibration curve of 7-hydroxycoumarin at λ=280 nm. The concentrations of the phenolic compounds identified in cranberry syrup are summarized in **Table 4**. Thus, the overall phenolic content obtained by HPLC-DAD was found to be 5.47 mg/mL. The family of flavanols (catechin and procyanidins) was found at the highest concentration (2.37 mg/mL) followed by flavonols (1.90 mg/mL). Phenolic-acid derivatives were found at the lowest concentration (0.28 mg/mL).

Total polyphenol, proanthocyanidin and anthocyanin contents

Spectrophotometric methods are normally used to measure phenolic contents so various spectrophotometric assays were made to the syrup extracts. To quantify the total phenolic compounds by the Folin Ciocalteu method, a caffeic acid calibration curve was constructed. The calibration curve showed good linearity between the concentration of caffeic acid and absorbance, as described by the equation $y = 0.0149x–0.0206$ ($r^2 = 0.995$). The total polyphenol content was 15.26 ± 0.08 mg/mL of cranberry syrup.

The total proanthocyanidins expressed as catechin equivalents was 9.9 ± 0.1 mg/mL of cranberry syrup, as described by the equation $y = 0.0098x–0.0153$ ($r^2 = 0.991$). The anthocyanin content found in cranberry syrup was 1.35 ± 0.04 mg/mL. The results obtained by these spectrophotometric analyses were higher than those obtained by HPLC-DAD. This can be put down to the interference of compounds such as sugars, which can cause an overestimation of the results, and cranberry syrup does in fact, contain a high quantity of glucose.

Antibacterial activity evaluation

Previous studies[28] have reported the beneficial effect of cranberry syrup in preventing urinary tract infection (UTI) among women, achieving a reduction in the absolute risk of UTI infection compared to placebo treatment. This effect has been explained in terms of the anti-adherent effect of cranberry on *E. Coli*. Ferrara *et al*.[29] in a controlled clinical trial including placebo treatment in children aged over 3 years, showed that cranberry syrup prevents the recurrence of symptomatic UTI.

The first step in the colonisation of the epithelium by *E. coli* is determined by its capacity to adhere to the host cells. Initially, this capacity is determined by the micro-organism's electric surface charge and surface hydrophobicity, and subsequently by other factors such as the formation of diverse types of fimbriae and of specific adhesins. Other authors have shown that fimbriae Types 1, P and S are not essential factors in the adhesion and

subsequent colonisation of the urogenital epithelium by *E. coli*. These adhesions may, in general, be considered features of the virulence of extra-intestinal *E. coli*, but they are not essential for *E. coli* to become uropathogenic, all of which accounts for the current research interest in the extent to which cranberry extract may affect the non-specific adherence properties of *E. coli*.

In fact, no differences were observed in the surface hydrophobicity of *E. coli* following its growth in TSB culture medium to enhance the expression of Type 1 fimbriae (Z=0.35; pNS). Nevertheless, the incubation of the bacterial suspension with cranberry syrup at final concentrations of either 1:1000 or 1:100 resulted in significant reductions in surface hydrophobicity, depending upon the concentration of cranberry, both after growth in TSB medium and on CFA agar.

Incubation of *E. coli* with cranberry syrup resulted in a reduction in its surface hydrophobicity and did not depend upon the quantities of Type 1 or Type P fimbriae expressed. In earlier studies[30] it was reported that the incubation of *E. coli* with certain anti-oxidants, such as vitamin E, produced similar reductions in surface hydrophobicity. As far as surface hydrophobicity is concerned, however, the incubation of *E. coli* with 1:1000 dilutions of cranberry extract (which do not affect the haemagglutination mediated by Type P fimbriae) did produce significant reductions in surface hydrophobicity, thus showing that extremely low levels of this extract are capable of modifying the non-specific adherence properties of *E. coli*.

The A-linkage in cranberry procyanidins may represent an important structural feature for anti-adhesive activity in bacteria. It has been demonstrated that Type A cranberry procyanidins hinder the adhesion of P-fimbriated uropathogenic *E. coli* to uroepithelial cells *in vitro*[8] and it has also been found that trimeric proanthocyanidins and Type A dimeric procyanidins are responsible for the anti-adhesive effect of cranberry. The composition of the cranberry syrup used in our study, which was rich in type A procyanidins, may account for the reductions in surface hydrophobicity.

Conclusions

The powerful analytical method HPLC-DAD-TOF-MS was used to characterize a commercial cranberry syrup used to prevent urinary tract diseases. It was possible to identify a total of 34 compounds in the sample in less than 29 min using positive and negative ionization modes. To our knowledge, nine of these compounds are tentatively identified in cranberry for the first time. Different coumarins were also found in cranberry for the first time when analyzing its whole phenolic fraction by HPLC-DAD-TOF-MS. This finding was possible thanks to the mass accuracy and sensitivity provided by TOF-MS. Antibacterial activity was investigated further and it was possible to prove that very low concentrations of cranberry extract have the capacity to modify the non-specific adherence properties of *E. coli*, producing a reduction in surface hydrophobicity.

This study is of great significance for understanding the beneficial effects of cranberry syrup on health. Further analysis will be required to find out more about the activity leading to the preventative function of these compounds.

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Chapter 4B

Identification of polyphenols and their metabolites in human urinary excretion after consumption of cranberry syrup

This work has been submitted to *Analytical and Bioanalytical Chemistry.*

Identification of polyphenols and their metabolites in human urinary excretion after consumption of cranberry syrup

Abstract

The beneficial effects of American cranberry might be in part attributed to its phenolic composition; thus, the evaluation of the physiological behaviour of this fraction is crucial. A rapid and sensitive method by ultra performance liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS) has been used to identify phenolic metabolites in human urine after a single dose of cranberry syrup. Prior to the analysis, metabolites were extracted using an optimized solid phase extraction procedure. All possible metabolites were carefully investigated based on retention time, accurate mass data and isotope and fragmentation patterns. Free coumaroyl hexose (isomer 1 and 2), dihydroxybenzoic acid, caffeoyl glucose, dihydroferulic acid 4-*O*-β-D-glucuronide, methoxyquercetin 3-*O*-galactoside, scopoletin, myricetin and quercetin, together with other 23 phase I and phase II metabolites, including various isomers, could be tentatively identified in urine. After that, the metabolites were simultaneously screened in urine of different subjects at 0, 2, 4 and 6 h after the ingestion of cranberry syrup by Target Analysis™ software.

Keywords: Cranberry; polyphenols; urinary metabolites; ultraperformance liquid chromatography; mass spectrometry

Introduction

American cranberry (*Vaccinium macrocarpon*) is a rich source of bioactive phenolic compounds with antiproliferative, antioxidant[1], antimicrobial[2], anti-inflammatory and anticancinogenic activities.[3] It contains large amounts of sugars, proanthocyanidins, flavonoids, organic acids and also anthocyanins. In a previous work it was showed that cranberry changes the surface hydrophobicity and biofilm formation of P-fimbriated *Escherichia coli* that is related to urinary tract infection (UTI).[4]

Due to the interest on this fruit, research should be directed not only towards the characterization and study of its bioactivity, but also it is important to reveal the bioavailability of the phenolic components and to get new insight of their physiological behaviour *in vivo*. In this sphere, Ohnishi *et al.* (2006)[5] have identified six anthocyanins in human urine by HPLC-MS/MS in multiple reaction mode (MRM) in which peonidin 3-*O*-galactoside was the most plentiful compound found after cranberry juice ingestion. The recovery of total anthocyanins in the urine over 24 h was estimated to be 5.0% of the amount consumed. In the same manner, quercetin and anthocyanins such as peonidin 3-*O*-galactoside and cyanidin 3-*O*-galactoside were detected in the rat urine after cranberry administration.[6]

The absorption and excretion of twenty cranberry-derived phenolics in plasma and urine have been studied by using GC-MS in selective ion monitoring (SIM) mode after consumption of cranberry juice, sauces, and fruits.[7] These studies performed a targeted analysis to detect cranberry metabolites using MRM or SIM detection mode that represent only a selected part of the urinary spectra of cranberry. In other study, using microLC-MS, Valentová *et al.* (2007)[8] evaluated the effect of consumption of cranberry to prevent oxidative stress and the main metabolites identified in human urine were isomers of salicyluric and dihydroxybenzoic acids, hippuric acid, and quercetin glucuronide. None of these studies paid attention to the phase I metabolism of polyphenols. Phase I metabolism involves oxidation, reduction, hydrolysis, among other less frequent reactions and results in small chemical

changes that make a compound more hydrophilic, thus it can be effectively eliminated by the kidneys. These reactions expose or introduce a reactive functional group (–OH, –SH, –NH_2, –NH-, –COOH) in the molecule and usually preceed to phase II metabolism.[9]

Liquid chromatography (LC) coupled to mass spectrometry has become an useful tool for the the separation and identification of metabolites. The analysis of phenolic- derived metabolites by GC-MS requires steps of chemical deconjugation and derivatization to enhance their volatility that increases the total time of analysis. In contrast, LC has lower resolution compared with GC. Q-TOF analyzers can provide excellent mass accuracy over a wide dynamic range and allow measurements of the isotopic pattern, providing important additional information for the determination of the elemental composition. Futhermore, this instrument permits the rapid and efficient confirmation of the structure of metabolites, when fragmentation studies are carried out, and also provides high selectivity using the extracted ion chromatogram (EIC) mode when there are overlapping peaks. In previous study, the phenolic fraction of cranberry syrup with beneficial properties against UTI by HPLC-TOF-MS was determined.[10]

For continuation of the earlier study, the aim of this work was to identify polyphenols from cranberry syrup and their metabolites that appear in human urine after a single dose of cranberry. Urinary metabolites were previously extracted by solid phase extraction (SPE) with several sorbents and analyzed by UPLC-ESI-Q-TOF-MS. Furthermore, a rapid multi-screening method was applied to determine the putative identified metabolites in urine 0, 2, 4 and 6 h after consumption of cranberry syrup.

Materials and methods

Chemicals

All chemicals were analytical HPLC reagent grade and used as received. Formic acid and acetonitrile used for preparing mobile phases were from Fluka (Sigma-Aldrich, Steinheim, Germany) and Lab-Scan (Gliwice,

Sowinskiego, Poland), respectively. A G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA) was used for mixing. Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA). The resisitivity of the deionized water was 18.2 MΩ.

Cranberry syrup

A cranberry syrup was purchased from a local pharmacy. It contained 1.10% (w/v) total phenolics, 0.71% (w/v) proanthocyanidins, and 0.10% (w/v) anthocyanins. The estimation of these amounts and the characterization of the phenolic compounds were according to previous work.[10]

Subjects and study design

Four healthy volunteers (2 male and 2 female), who were non-smokers and not on any medication, participated in the study. They were aged between 25 and 40 years and with a weight between 60 and 80 kg. Subjects were required to follow a low-diet on polyphenols, which excluded fruits and vegetables, high-fiber products, and beverages such as tea, coffee, fruit juice and wine, for two days before the study. After a fasting overnight, early-morning urine samples were collected before (0 h) the ingestion of cranberry syrup and at time points, 2, 4, 6 h after consuming of cranberry syrup with a dose of 0.6 mL/kg. An aliquot of each urine sample was acidified with 5% aqueous TFA solution (1:10, v/v). The urine samples were stored at -80 $^{\circ}$C before analysis. The study protocol was approved by the ethics committe of the University of Granada, Spain.

Extraction of phenolic-derived metabolites from urine samples

Urine samples (4 mL) were diluted with 4 mL of 10 mM of oxalic acid and applied to Supelclean LC-18 (500 mg) (Supelco, Bellefonte, PA, USA), Evolute ABN (200 g) (Biotage, Sweden) and Isolute ENV^{+} (500 mg) (Biotage, Sweden) SPE columns. The columns were previously conditioned with 10 mL of methanol and 30 mL of 10 mM of oxalic acid. Impurities were washed out with 20 mL of 10 mM of oxalic acid and the retained phenolic-derived metabolites were eluted with 25 mL methanol acidified with 0.1% HCl. The flow rate

through the column was controlled by means of a vacuum manifold. The eluate obtained was carefully evaporated to dryness under vacuum (< 40 ºC), redissolved in 200 µL methanol:acetic acid (95:5, v/v), and filtered through 0.22 µm tetrafluoroethylene (PTFE) syringe filters prior to UPLC-Q-TOF-MS analysis.

Chromatographic and MS conditions

LC analyses were made with a Waters Acquity UPLC™ system (Waters Corporation, Milford, MA). Separation was carried out with a Zorbax Eclipse Plus C_{18} analytical column (4.6 × 150 mm, 1.8 µm particle size). Gradient program 1 was used for the MS negative ion mode and consisted of 1% formic acid in water/acetonitrile (90:10, v/v) (phase A) and acetonitrile (phase B) at a constant flow rate of 0.5 mL/min using the following multi-step linear gradient: 0-20 min, from 5% B to 20% B; 20-25 min, from 20% B to 40% B; 25-30 min, from 40% B to 5% B; and 30-35 min, isocratic of 5% B. Subsequently, a different chromatographic method (gradient program 2) was used for the MS positive ion mode and consisted of 10% (v/v) aqueous formic acid (phase A) and acetonitrile (phase B) at a constant flow rate of 0.5 mL/min using the following gradient multi-step linear: 0-13 min, from 0% B to 20% B; 13-20 min, from 20% B to 30% B; 20-25 min, from 30% B to 80% B; and 25-30 min, from 80% B to 0% B; and 30-35 min, isocratic of 0% B. The column was set to room temperature and the injection volume was 1 µL for both gradient elution programs.

The UPLC instrument was coupled to a micrOTOF-Q™ (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface. Parameters for analysis were set using negative and positive ion modes with spectra acquired over a mass range of 50–1000 *m/z*. The other optimum values of the ESI-MS parameters were: capillary voltage, +4000 V; dry gas temperature, 190 ºC; dry gas flow, 9.0 L/min, nebulizer pressure, 2.0 bar; and spectra rate 1 Hz. The calibrant was a sodium formate cluster containing 5 mM sodium hydroxide and 0.2% formic acid in water:isopropanol (1:1, v/v), injected at the beginning of each run with a 74900-00-05 Cole Palmer syringe

pump (Vernon Hills, Illinois, USA). All the spectra were calibrated prior to the compound identification. All operations were controlled by the newest DataAnalysis 4.0 software (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormula™ editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. TargetAnalysis™ 1.2 software (Bruker Daltonik) was used for multi-target screening of metabolites in urine samples.

Results and discussion

Selection of the extraction and analytical conditions for the analysis of urinary phenolic metabolites

In the present study, because of the large number of compounds contained in each human urine sample, the aim was focused on metabolites of polyphenols. Thus, samples were previously extracted by SPE in order to concentrate metabolites of interest, which are generally found in trace levels, and to avoid the contamination of the instrument. Three types of stationary phases, LC-18 (octadecylsilane phase), Evolute ABN (a water-wettable polymer-based sorbent) and Isolute ENV$^+$ (a hydroxylated polystyrene divinylbenzene co-polymer) columns, were tested. As preliminary result, larger number of compounds was found using Isolute ENV$^+$ (data not shown) and, therefore, this cartridge was selected. This type of sorbent was also found appropriate for extracting drugs metabolites from urine and plasma.[11] Then, metabolite compounds were successfully separated with the previoulsy optimized gradients by UPLC and analyzed by coupling with Q-TOF-MS in both negative and positive ionization modes. As example of the chromatographic profiles, the extracted ion chromatograms (EICs) of the metabolites in human urine 4 h after the intake of cranberry syrup and obtained using negative and positive ion modes are set out in **Figure 1a** and **b**, respectively. The proposed

compounds, peak number and retention time according to **Figure 1**, molecular formula, *m/z* experimental, error, sigma value, and MS/MS major fragments are described in **Table 1**.

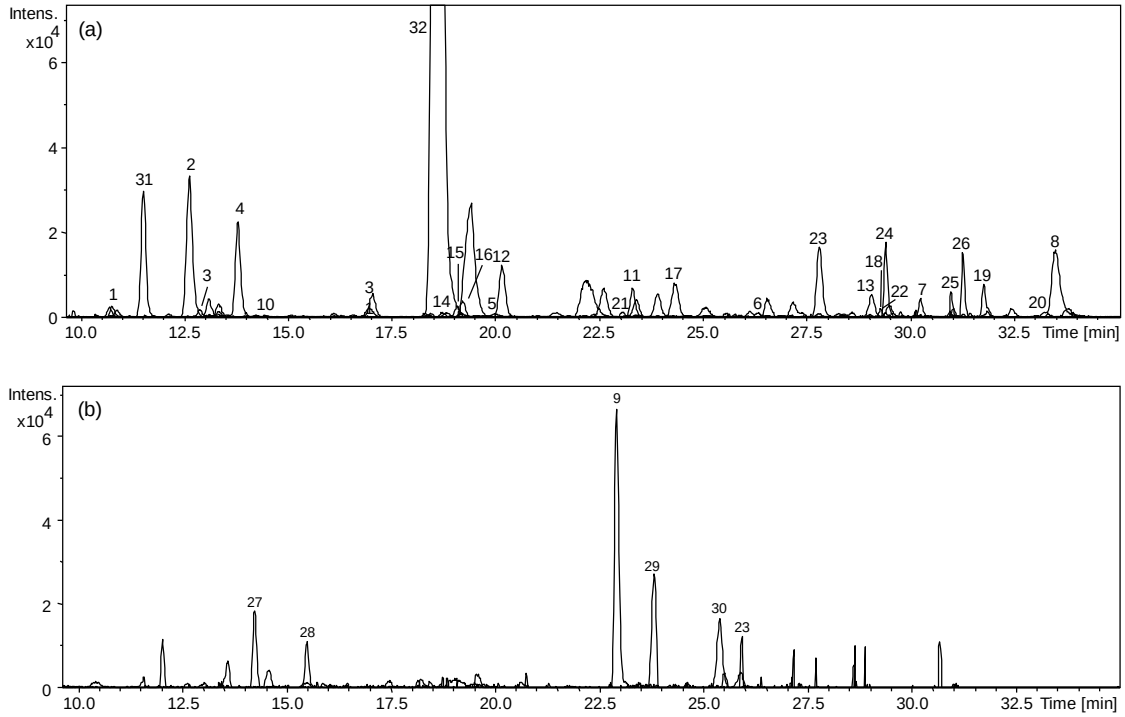

Figure 1. Chromatographic profiles using the gradient programs of urinary metabolites collected 4 h after ingestion of crancerry syrup: (a) Extracted ion chromatograms (EICs) for the gradient program 1, negative mode; (b) Extracted ion chromatograms (EICs) for the gradient program 2, positive mode.

Identification of native polyphenols in urinary excretion

The native phenolic compounds were studied in depth based on the matching of molecular weight (parent and product ions), isotope pattern and retention time with those polyphenols previously characterized in cranberry syrup, and taking into account the polarity of the compounds. As **Figure 2** shows, retention properties of compounds corresponding to various free polyphenols in cranberry syrup and human urine samples were very similar. Once then, it was possible to succesfuly identified ten native forms of polyphenols in urine samples using negative ion mode and one using positive ion mode.

Structure of the metabolites was confirmed by MS^2 analysis. For instance, **Figure 3** shows the MS^2 spectra of some metabolites identified. For glycosylated compounds, it was taken into account that breakage occurred at the most labile linkage of the molecular ion, corresponding to the glycosidic bond, and in agreement with results observed by ion-trap tandem MS and in-source fragmentation by TOF.[10,12] In this manner, neutral loss of monosaccharide residues was established by the mass difference between the glycoside and the aglycone form: a difference of 162 Da for hexose, and 176 Da for glucuronide.

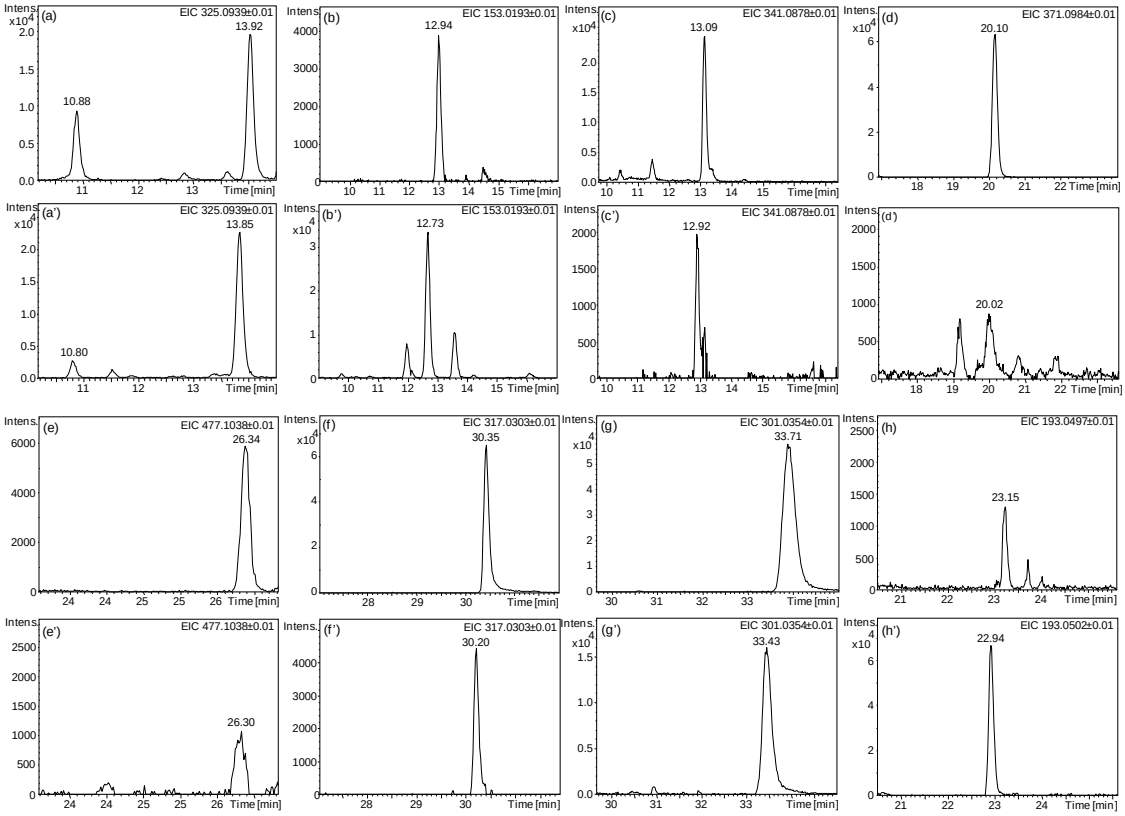

Figure 2. Representative extracted ion chromatograms (EICs) of native polyphenols identified in cranberry syrup (a-h) and compared with those detected in 4 h urine (a'-h') using negative ion mode: (a) coumaroyl-hexose isomer 1 and 2, (b) dihydroxybenzoic acid, (c) caffeoyl glucose, (d) dihydroferulic acid-4-*O*-β-D-glucuronide, (e) methoxyquercetin-3-*O*-galactoside, (f) myricetin, (g) quercetin; and positive ion mode: (h) scopoletin.

Table 1. Free polyphenols, phase I and II metabolites identified in human urine 4 h after intake of cranberry syrup and classified by the type of metabolic reaction.

Conjugation	Peak	Selected ion	RT (min)	Molecular formula	Experimental *m/z*	Error (ppm)	mSigma value	MS/MS fragments	Identification of putative metabolites
Negative ion mode									
					Free polyphenols				
Free polyphenols	1	$[M-H]^-$	10.80	$C_{15}H_{17}O_8$	325.0939	3.1	0.0163	163.0359	Coumaroyl hexose (isomer 1)
	2	$[M-H]^-$	12.68	$C_7H_5O_4$	153.0189	2.8	0.0021		Dihydroxybenzoic acid
	3	$[M-H]^-$	12.92	$C_{15}H_{17}O_9$	341.0864	4.2	0.0038	179.0331	Caffeoyl glucose
	4	$[M-H]^-$	13.85	$C_{15}H_{17}O_8$	325.0927	-0.6	0.0048	163.0392	Coumaroyl hexose (isomer 2)
	5	$[M-H]^-$	20.02	$C_{16}H_{19}O_{10}$	371.0972	-3.2	0.0118	175.0305	Dihydroferulic acid 4-*O*-β-D-glucuronide
	6	$[M-H]^-$	26.30	$C_{22}H_{21}O_{12}$	477.1036	-0.6	0.0433		Methoxyquercetin 3-*O*-galactoside
	7	$[M-H]^-$	30.20	$C_{15}H_9O_8$	317.0306	1.0	0.0206		Myricetin
	8	$[M-H]^-$	33.43	$C_{15}H_9O_7$	301.0349	-1.5	0.0028	178.9988, 151.0028	Quercetin
					Phase I metabolites				
Hydrogenation (+ H_2)	10	$[M-H]^-$	14.50	$C_{19}H_{27}O_{12}$	447.1463	-3.0	0.0304	445.1366	Canthoside A + H_2
	11	$[M-H]^-$	23.29	$C_{25}H_{29}O_{13}$	537.1584	-5.5	0.0044	373.0902	Caviunin glucoside + H_2
Dehydrogenation (- H_2)	12	$[M-H]^-$	20.16	$C_{15}H_{11}O_6$	287.0549	-4.3	0.0030		(+)-Catechin – H_2
	13	$[M-H]^-$	29.03	$C_{15}H_{15}O_9$	339.0709	-3.6	0.0029		Caffeoyl glucose – H_2
Hydroxylation (+ OH)	14	$[M-H]^-$	18.74	$C_{16}H_{17}O_{10}$	369.0827	0.0	0.0156		Chlorogenic acid + OH
	15	$[M-H]^-$	19.09	$C_{22}H_{21}O_{13}$	493.0971	-3.3	0.0278		Methoxyquercetin-3-*O*-galactoside + OH
	16	$[M-H]^-$	19.22	$C_{16}H_{19}O_{11}$	387.0934	0.3	0.0085	175.0221	Dihydroferulic acid 4-*O*-β-D-glucuronide + OH
Hydration (+ H_2O)	17	$[M-H]^-$	24.29	$C_{16}H_{19}O_{10}$	371.0981	-0.8	0.0050	353.0856	Chlorogenic acid + H_2O
					Phase II metabolites				
Glucuronidation (+ $C_6H_8O_6$)	18	$[M-H]^-$	29.20	$C_{21}H_{17}O_{13}$	477.0717	8.9	0.0397	301.0390	Quercetin - glucuronide
Methylation (+ CH_2)	19	$[M-H]^-$	31.71	$C_{16}H_{11}O_7$	315.0506	-1.4	0.0044	301.0389	Quercetin + CH_3
	20	$[M-H]^-$	33.17	$C_{16}H_{11}O_8$	331.0439	-6.3	0.0129	317.0252	Myricetin + CH_3
Dehydrogenation + glucuronidation	21	$[M-H]^-$	23.04	$C_{21}H_{19}O_{12}$	463.0882	0.1	0.0047		(+)-Catechin – H_2-glucuronide
Methylation + glucuronidation	22	$[M-H]^-$	29.23	$C_{22}H_{19}O_{14}$	507.0766	-2.7	0.0245	317.0390	Myricetin + CH_3-glucuronide
	23	$[M-H]^-$	27.76	$C_{22}H_{19}O_{13}$	491.0830	0.3	0.0069	301.0337	Quercetin + CH_3-glucuronide (isomer 1)
	24	$[M-H]^-$	29.37	$C_{22}H_{19}O_{13}$	491.0824	-1.5	0.0019	301.0349, 315.0458	Quercetin + CH_3-glucuronide (isomer 2)
	25	$[M-H]^-$	30.92	$C_{22}H_{19}O_{13}$	491.0847	-3.3	0.0158	301.0349	Quercetin + CH_3-glucuronide (isomer 3)
	26	$[M-H]^-$	31.21	$C_{22}H_{19}O_{13}$	491.0820	2.2	0.0075	301.0389	Quercetin + CH_3-glucuronide (isomer 4)

Table 1 (Continued).

Conjugation	Peak	Selected ion	RT (min)	Molecular formula	Experimental *m/z*	Error (ppm)	mSigma value	MS/MS fragments	Identification of putative metabolites
Positive ion mode									
Free polyphenols	9	$[M+H]^+$	22.92	$C_{10}H_9O_4$	193.0502	3.7	0.0320		Scopoletin
Dehydrogenation	27	$[M+H]^+$	14.23	$C_7H_5O_3$	137.0233	0.0	0.0124		2-Hydroxybenzoic acid – H_2
Hydroxylation + glucuronidation	28	$[M+H]^+$	15.50	$C_{15}H_{15}O_{10}$	355.0641	-5.4	0.0464		7-Hydroxycoumarin + OH-glucuronide
Methylation + glucuronidation	29	$[M+H]^+$	23.82	$C_{16}H_{17}O_9$	353.0865	-0.5	0.0495		7-Hydroxycoumarin + CH_3-glucuronide
Methylation	30	$[M+H]^+$	25.38	$C_{11}H_{11}O_4$	207.0664	5.7	0.0184		Scopoletin + CH_3
Methylation + glucuronidation	23	$[M+H]^+$	25.92	$C_{22}H_{21}O_{13}$	493.0982	1.1	0.0132		Quercetin + CH_3-glucuronide*
					Other metabolites				
Glycine conjugation ($+C_2H_3ON$)	31	$[M-H]^-$	11.57	$C_9H_9NO_4$	194.0459	1.9	0.0047		Salicyluric acid (2-hydroxybenzoic acid-glycine)
	32	$[M-H]^-$	18.62	$C_9H_8NO_3$	178.0512	1.5	0.0009		Hippuric acid

The same peak number corresponds to the same compound in **Figure 1**.
*This compound has also been detected in negative ion mode.

Compounds corresponding to peak **1** (RT 10.80 min) and **4** (RT 13.85 min) produced the same deprotonated molecular ion $[M–H]^-$ at *m/z* 325.0939 and 325.0927, respectively, and one prominent product ion $[M–H–162]^-$ at *m/z* 163.0359 by loss of a sugar moiety (**Figure 3a**). The minor fragment (*m/z* 119.0485) was the subsequent loss of CO_2 (44 Da), suggesting the presence of carboxylic acid in the parent ion. Therefore, these compounds were proposed as coumaroyl hexose (isomers) and this is consistent with a previous study.[10] In this way, it was also tentatively identified caffeoyl glucose, corresponding to peak **3** (RT 12.92 min) with parent ion at *m/z* 341.0864 and dihydroferulic acid 4-*O*-β-D-glucuronide (peak **5**, RT 20.02 min, *m/z* 371.0972). Moreover, the proposed analytical methodology enables to detect dihydroxybenzoic acid at RT 12.68 min (peak **2**) and *m/z* 153.0189. The urinary excretion of dihydroxybenzoic acid after ingestion of cranberry products is in agreement with the results of Valentová *et al.* (2007)[8] and Prior *et al.* (2010).[13]

Three flavonols were tentatively identified in urine samples: methoxyquercetin 3-*O*-galactoside (peak **6** RT 26.30 min, *m/z* 477.1036), myricetin (peak **7**, RT 30.20 min, *m/z* 317.0306) and quercetin (peak **8,** RT 33.43 min, *m/z* 301.0349). Dihydroferulic acid 4-*O*-β-D-glucuronide showed a daughter ion at *m/z* 175.0305, which corresponds to the loss of glucuronide moiety ($[M-H-176]^-$). Quercetin (**Figure 3b**) presented a characteristic fragmentation pattern, with two major fragments at *m/z* 151.0028 and 178.9988, corresponding to an A^- ring fragment released after retro Diels-Alder (RDA) fission and retrocyclization after fission on bonds 1 and 2.[14] The presence of myricetin and quercetin have been previously detected in rat urine by HPLC-MS/MS (triple quadrupole).[6] Quercetin was also detected in plasma and human 24 h urine using HPLC with electrochemical detection, showing that this compound is bioavailable from a diet containing moderate amounts of blue and red berries.[15] In general, flavonoid glycosides are cleaved prior to absorption in different parts of the intestines[16], and in fact, although quercetin and myricetin exists in cranberries as aglycone and glycosidic forms[10], only the aglycone forms were excreted. In contrast, dihydroferulic

acid-4-*O*-β-glucuronide and methoxyquercetin 3-*O*-galactoside were detected in human urine.

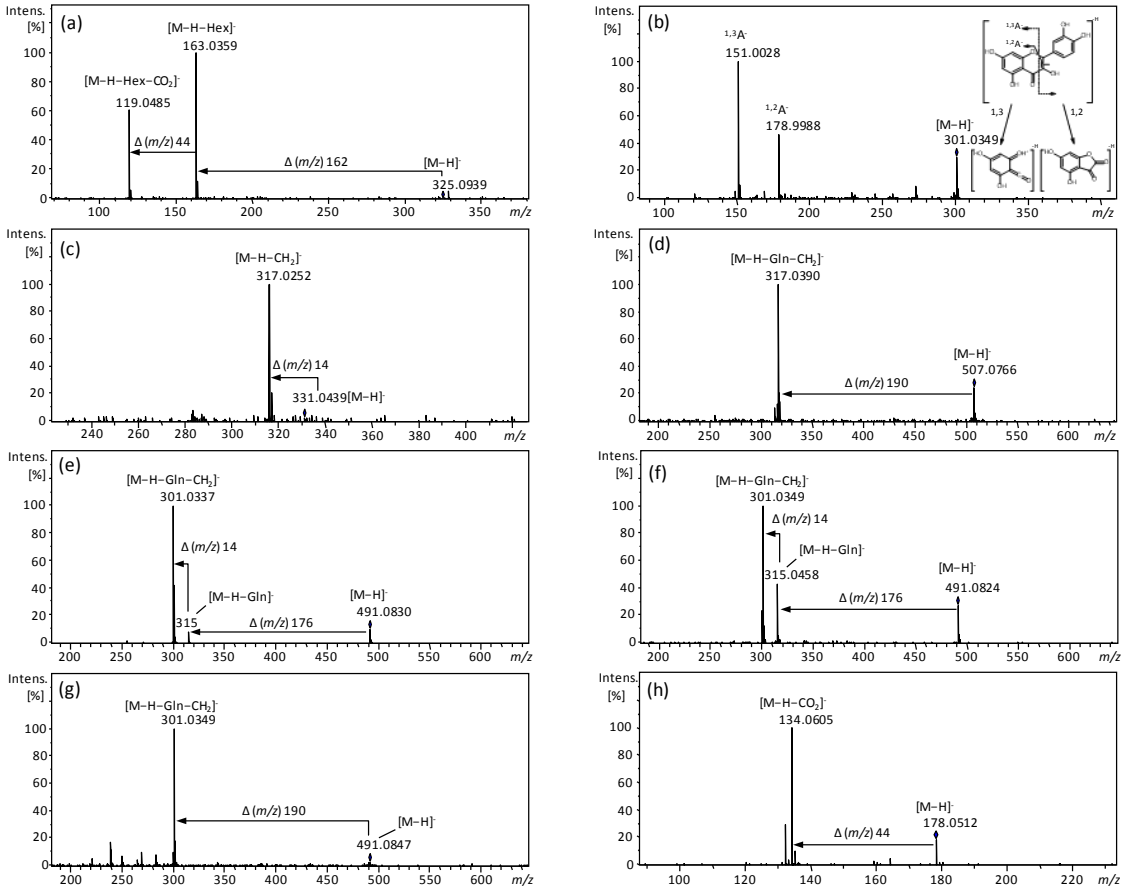

Figure 3. Negative ESI-MS^2 spectra of (a) coumaroyl-hexose isomer 1, (b) quercetin, (c) myricetin + CH_3, (d) myricetin + CH_3-glucuronide, (e-g) quercetin + CH_3-glucuronide isomers 1-3, and (h) hippuric acid. Hex, hexose; Gln, glucuronide.

Using positive ionization mode, scopoletin (peak **9,** RT 22.92 min**,** *m/z* 193.0502) was the only native polyphenol tentatively identified in urine and it was found as one of the main urinary metabolites (**Figure 1b**).

Cranberry procyanidins and anthocyanidins were not found in human urine by the proposed methodology. Similar results were found in rat urine after five days repeated dose of procyanidin dimer B3[17], and very low amounts were found after single dose of grape seed procyanidins B1, B2 and B3 to rats.[18] The metabolism of procyanidins is controversial[19], and a recent study suggest further degradation by the gut microflora before absorption, and therefore, subsequent microbial metabolites could be active forms.[20]

Interestingly, ex vivo urine of humans treated with dried cranberry juice had an inhibitory effect on the adhesion of bacteria relating to UTI, although procyanidins were not detected.[8] In previous work, petunidin was the only anthocyanidin found in cranberry syrup and at low concentration.[10] This fact together with the inestability of this class of compounds and their metabolites[21] make difficult their detection in biological samples, such as in urine.

Identification of phenolic-derived metabolites

As for native polyphenols, similar strategy was followed for tentatively identified phenolic-derived metabolites in urine, and bearing in mind all the data provided by the literature about metabolic reactions occurred *in vivo*. As mentioned above, high-resolution ESI-Q-TOF measurements provide accurate mass and isotope pattern data, which enable to reduce number of possible molecular formulae for putative novel compounds to be matched against available databases. **Table 1** provides a summary of all the putative metabolites found in urine and classified by the type of metabolism reaction. There were three types of phenolic-derived metabolites: (i) compounds found in their native form, which were described above; (ii) phase I metabolites formed by chemical modifications, such as hydrogenation ($M+H_2$), dehydrogenation ($M-H_2$), hydroxylation (M+OH), and hydration ($M+H_2O$); and (iii) phase II metabolites formed by conjugation, such as methylation ($M+CH_3$), glucuronidation ($M+C_6H_8O_6$), and other conjugation reactions.

Nine phase I metabolites were successfully characterized, being the most abundant phenolic acids (canthoside A + H_2; caffeoyl glucose – H_2; chlorogenic acid + OH and + H_2O; dihydroferulic acid 4-*O*-β-D-glucuronide + OH; and 2-hydroxybenzoic – H_2) and flavonols (caviunin glucoside + H_2 and methoxyquercetin 3-*O*-galactoside + OH) that were likely absorbed and metabolized. One monomeric flavanol, (+)-catechin – H_2, was also detected.

Phase I reactions usually precede phase II metabolism, though not necessarily, since, as in our case, parent compounds presents one or more suitable functional groups, concerning to OH and COOH. Hence, it was

possible to identify glucuronoconjugated and methylated forms of polyphenols and phase I metabolites from cranberry, mainly.

In order to confim the structure of phase II metabolites, tandem MS analysis was automatically performed by Q-TOF. The MS^2 spectrum of the compound with *m/z* 477.0717 (peak **18**, RT 29.20 min) gave an abundant daughter ion at *m/z* 301.0390 by neutral loss of 176 Da. Based on the metabolism rule of phase II *in vivo* and the above MS/MS spectrum data, the latter metabolite was identified as the monoglucuronide product of quercetin, which has been previously identified in human urine as well.[8,22-24] Peak **19** (RT 31.71 min) was observed as a deprotonated ion $[M–H]^-$ at *m/z* 315.0506. A product ion at *m/z* 301.0389 suggested the loss of methyl group ($[M-H-14]^-$) to form quercetin aglycone. Therefore, this peak was tentatively identified as quercetin-CH_3. In the same manner, the methylation product of myricetin (myricetin-CH_3) (peak **20**, RT 33.17 min) was observed as an $[M–H]^-$ ion at *m/z* 331.0439 and the major MS/MS fragment was at *m/z* 317.0252 (**Figure 3c**). According to the metabolism rule of phase II *in vivo*, the methylation reactions may occur at the skeleton of myricetin since presents six free OH groups.

Myricetin + CH_3-glucuronide with a *m/z* of 507.0766 (peak **22**, RT 29.23 min) was also detected and it presented a prominent product ion at *m/z* 317.0390, which corresponded to the neutral loss of both the methyl and glucuronide groups (a total of 190 Da) (**Figure 3d**). This is in agreement with previous studies on rats in which glucuronidated and methylated forms were the main conjugated compounds detected.[25] Other phase II metabolites, (+)-catechin — H_2-glucuronide, and several isomers of quercetin + CH_3-glucuronide (for example, **Figure 3e-g**) were found in the urine samples. Similarly, eight *O*-methylated and glucuronidated forms of quercetin were identified after incubation of apple-derived polyphenols with hepatocytes cells, which mimic the metabolism occurring in liver.[26]

Other metabolites were also identified in urine. The presence of hippuric acid (peak **32**) was detected as major compound in the reconstructed chromatogram at *m/z* 178.0512 (**Figure 1a**). The loss of CO_2 resulting in the

formation of the ion *m/z* 134.0605 can be clearly seen in the related collision spectrum collected over the chromatographic peak (**Figure 3h**). The metabolite corresponding to peak **31** (RT 11.57 min) and at *m/z* 194.0450 was tentatively identified as salicyluric acid (also known as 2-hydroxyhippuric acid). This compound is formed by conjugation of salicylic acid (2-hydroxybenzoic acid) with glycine mediated by glycine N-acyltransferase. Urinary excretion of the latter compounds has been previously described by feeding rats with concentrated cranberry powder[15] and after consumption of cranberry juice by humans.[8]

These results suggest that, at least, a fraction of the ingested compounds could be absorbed intact, and act *in vivo* in this form previous to excretion, whereas other fraction were absorbed, metabolized and excreted in urine as phase I and phase II metabolites, being glucuronidation and methylation the more frequents reactions. Further studies of the biological properties of these metabolites are of interest to understand the mechanism of action of cranberry syrup.

Screening of phenolic-derived metabolites during urinary excretion

Urinary metabolites spectra from four subjects were compared before (wash-out period) (0 time point) and after 2, 4, and 6 h of consumption of the cranberry syrup using the novel software TargetAnalysis™ 1.2. This tool allowed performing an automatic multi-screening target analysis of the metabolites based on the information provided by the UPLC-ESI-Q-TOF analysis, regarding the time of retention, accurate *m/z* and isotope pattern, which is matched using stored data run through our in-house database. **Table 2** shows free polyphenols, phase I and phase II metabolites detected in human urine at the studied times 0, 2, 4 and 6 h. Most of the metabolites appeared between 2 and 4 h after consuming cranberry syrup. The urinary excretion of several of the studied metabolites varied between subjects. Such variability in absorption or post-absorptive metabolism could be attributed in part to variability in the expression of metabolizing enzymes and transporters due to genetic variability within the population[27] and in colonic microbiota.[28]

Table 2. Metabolites detected in urine from four human subjects at different times after consumption of cranberry syrup.

Peak	Putative metabolites identified	Time points (hours after ingestion of cranberry syrup)			
		Subject-1	Subject-2	Subject-3	Subject-4
Negative ion mode					
	Free polyphenols				
1	Coumaroyl-hexose (isomer 1)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
2	Dihydroxybenzoic acid (isomer 1)	0, 2, 4, 6	0, 2, 4, 6	2, 4, 6	2, 4, 6
3	Caffeoyl-glucose	2	2, 4	2, 4	2, 4, 6
4	Coumaroyl-hexose (isomer 2)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
5	Dihydroferulic acid 4-*O*-glucuronide	2, 4	2, 4, 6	nd*	2, 4
6	Methoxyquercetin-3-*O*-galactoside	2	6	nd	4
7	Myricetin	2, 4, 6	nd	2, 4, 6	2, 4, 6
8	Quercetin	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
	Phase I metabolites				
10	Canthoside A + H_2	2, 4, 6	4, 6	4, 6	4, 6
11	Caviunin glucoside + H_2	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
12	(+)-Catechin – H_2	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
13	Caffeoyl glucose – H_2	6	nd	4, 6	4, 6
14	Chlorogenic acid + OH	2, 4	2, 4	nd	4
15	Methoxyquercetin-3-*O*-galactoside + OH	2, 4	2, 4	2	2, 4, 6
16	Dihydroferulic acid 4-*O*-glucuronide + OH	2, 4	2, 4, 6	2, 4, 6	2, 4, 6
17	Chlorogenic acid + H_2O	4, 6	6	2, 4, 6	2, 4, 6
	Phase II metabolites				
18	Quercetin glucuronide	2	nd	nd	2, 4
19	Quercetin + CH_3	2, 4, 6	2, 4, 6	2	2, 4, 6
20	Myricetin + CH_3	2, 4, 6	2, 4	2, 4, 6	2, 4, 6
21	(+)-catechin – H_2-glucuronidation	2, 4, 6	2, 4	2, 4, 6	4, 6
22	Myricetin + CH_3-glucuronide	nd	nd	2, 4	2, 4
23	Quercetin + CH_3-glucuronide (isomer 1)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
24	Quercetin + CH_3-glucuronide (isomer 2)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
25	Quercetin + CH_3-glucuronide (isomer 3)	2, 4, 6	2, 4, 6	2	4, 6
26	Quercetin + CH_3-glucuronide (isomer 4)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
Positive ion mode					
9	Scopoletin	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
23	Quercetin + CH_3-glucuronide	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
27	2-Hydroxybenzoic acid – H_2	2, 4	nd	2, 4, 6	2, 4, 6
28	7-Hydroxycoumarin + OH-glucuronide	nd	4	nd	4
29	7-Hydroxycoumarin + CH_3-glucuronide	2, 4, 6	6	nd	4, 6
30	Scopoletin + CH_3	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
	Other metabolites				
31	Salicyluric acid	0, 6	0, 2, 4, 6	0, 2, 4, 6	0, 2, 4
32	Hippuric acid	0, 2, 4, 6	0, 2, 4, 6	0, 2, 4, 6	0, 2, 4, 6

**nd: not detectable.*

Presumably, the studied metabolites should not be present at the 0 time point and should appear at later ones. However, hippuric acid, salicyluric acid, and dihydroxybenzoic acid were detected at 0 time point. The origin of phenolic acids, such of those, is diverse and it can be produced by colonic microflora from a number of different phenolic compounds and also

endogenously from amino acids such as tyrosine and phenylalanine.[13] In this sense, hippuric acid is one of the main urinary endogenous metabolites and an important compound from the route of benzoate elimination. Previous studies have suggested that dihydroxybenzoic acids may be metabolites of cranberry polyphenols[29] and be formed by ring fission of quercetin and derived from anthocyanins.[15] As commented above, our results suggest that dihydroxybenzoic acid may be also excreted as a free native form. At this respect, **Figure 4** shows the time course evolution of this compound and other free polyphenols represented for subject 4 after consumption of cranberry syrup. The urinary estimated amounts of these compounds, expressed as peak area, reached maximum concentrations in human urine 4 h after administration.

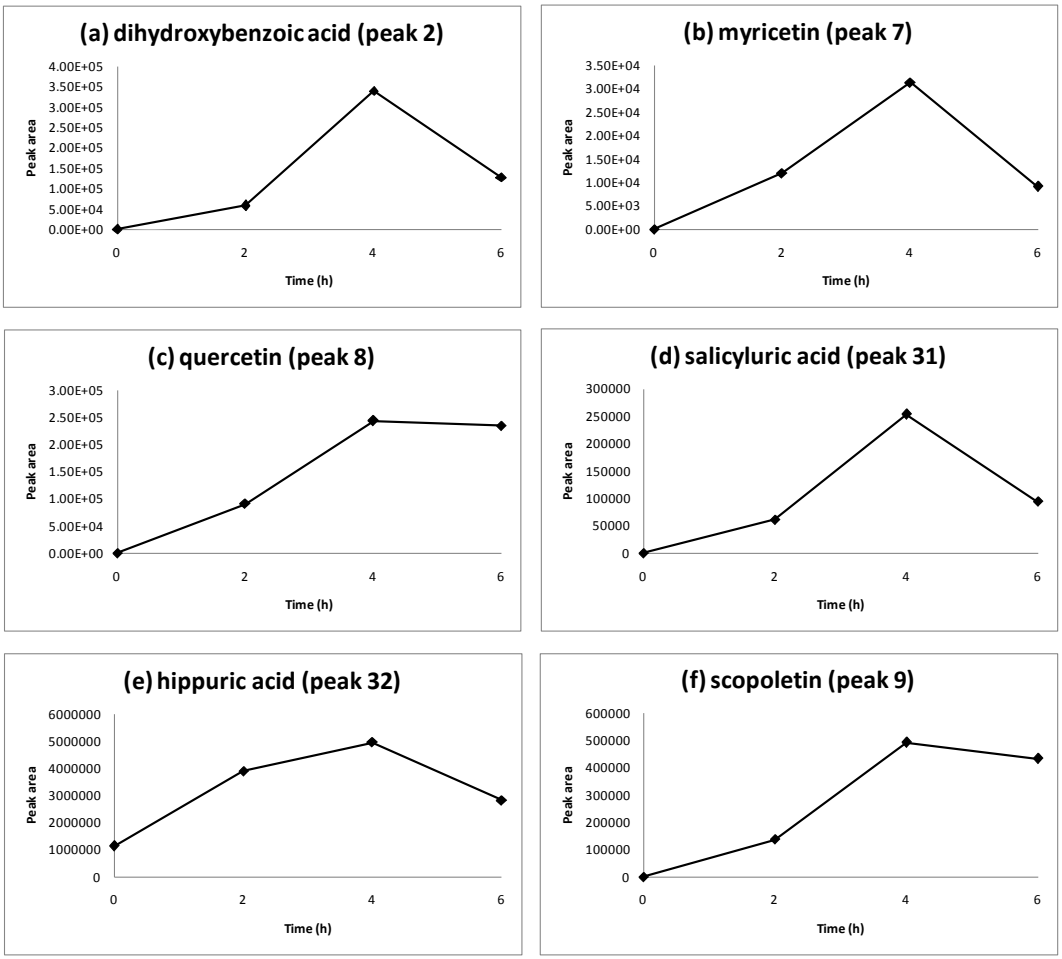

Figure 4. Changes in major urinary metabolites during time after consumption of cranberry syrup: (a) dihydroxybenzoic acid, (b) myricetin, (c) quercetin, (d) salicyluric acid, (e) hippuric acid, (f) scopoletin.

Conclusions

In the present study, human urinary metabolites of polyphenols from cranberry syrup were firstly extracted by ENV$^+$ SPE and then succesfully profiled using UPLC-Q-TOF-MS. In total, 32 metabolites were tentatively identified, including phase I metabolites and phase II metabolites, such as methylated and glucuronide conjugated forms. Among them, free phenolic acid derivatives (coumaroyl hexose, dihydroxybenzoic acid, caffeoyl glucose, and dihydroferulic acid 4-*O*-β-D-glucuronide), flavonols (methoxyquercetin 3-*O*-galactoside, myricetin, and quercetin) and one coumarin (scopoletin) were detected in the human urine. Most of these urinary metabolites have been identified for the first time after cranberry consumption. The proposed analytical technique has proven to be an useful tool to study biological matrices such as urine. In this manner, further estudies are in progress for complementing our knowledge of the bioavalability of cranberry-derived polyphenols, and getting new insights into the active metabolites.

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Conclusions

CONCLUSIONS

1. The analytical methodologies described in the review represent the current and foremost methodologies for the complete analyses of anthocyanins. As we look forward to the development of HPLC/CE-MS, the coupling with other efficient structural analysis techniques, especially NMR, can offer advantages, thanks to several improvements that have been made to increase the sensitivity of NMR, bringing it more into line with MS sensitivity and enabling the analysis of anthocyanins at low concentration. The implementation of better extraction, purification, and identification methodologies will have an impact in the establishment of new tools for the food-product authentication.

2. The development of HPLC-DAD-ESI-TOF/IT-MS method has proved useful for characterizing numerous phenolic and other polar compounds in zucchini. In fact, to the best of our knowledge, 10 phenolic acids, 16 flavonoids, and 17 other polar compounds with their derivatives are reported here in the whole zucchini fruit for the first time.

3. The cracterization of phenolic compounds in rooibos extracts has been performed HPLC-ESI-MS (TOF/IT). It confirmed the presence of 25 and 30 phenolic compounds in the aqueous and ethanolic extracts respectively and 11 of them are reported here for the first time in rooibos. The aqueous extract of rooibos containing approximately 25% w/w total polyphenol content showed its highest bioactivity in assays with male $LDLr^{-/-}$ mice. The group fed on a high-fat high-cholesterol diet (HF, 20% fat and 0.25% cholesterol w/w) plus rooibos tea extract (10 g/L) in place of pure drinking water was found to have significantly reduced serum cholesterol and triglyceride concentrations compared to the control group.

4A. A comprehensive method (including identification, quantification and bioactivity study) has been developed to study polyphenols fraction containing in cranberry syrup. This method allowed the identification of 34 different

compounds, 9 of which have been tentatively characterized for the first time in cranberry syrup. Catechin and procyanidins belonging to flavanols were the family of compounds found at the highest concentrations followed by flavonols and phenolic-acid derivatives were found at the lowest concentration. Antibacterial activity was investigated further and it was possible to prove that very low concentrations of cranberry extract have the capacity to modify the non-specific adherence properties of *E. coli*, producing a reduction in surface hydrophobicity.

4B. The potential of UPLC coupled to Q-TOF MS using negative and positive ion modes has been applied to identify polyphenols and their metabolites in human urine samples after consuming cranberry syrup. Quercetin, myricetin, methoxyquercetin 3-*O*-galactoside, coumaroyl hexose, sinapoyl-hexose, caffeoyl glucose together with 23 conjugated metabolites included isomers were identified. These results demonstrate that the developed method could identify those free polyphenols, phase I and II metabolites in human urine samples.

Conclusiones finales

CONCLUSIONES FINALES

1. Se ha llevado a cabo una revisión bibliográfica sobre las metodologías analíticas más utilizadas para el análisis de una importante familia de compuestos fenólicos como son las antocianinas. En este sentido, se ha podido observar que hoy día las técnicas separativas que se emplean mayoritamente para el análisis de estos compuestos son la cromatografía líquida y la electroforesis capilar acopladas a espectrometría de masas y más recientemente a RMN debido a las ventajas inherentes de dicha técnica, como proporcionar información estructural, así como a los avances tecnológicos que han dado lugar a una mejora de su sensibilidad permitiendo el análisis de antocianinas a baja concentración. La mejora en los procesos de extracción y purificación así como en las metodologías empleadas para la identificación tendrán un gran impacto en el sector alimentario.

2. El acoplamiento de la cromatografía líquida de alta resolución (HPLC) con la espectrometría de masas (TOF/IT-MS) han demostrado ser una herramienta de análisis de gran utilidad para la caracterización de mezclas de compuestos fenólicos y otros compuestos polares en muestras de calabacín. En este sentido, 10 ácidos fenólicos, 16 flavonoides y 17 otros compuestos polares han sido caracterizados por primera vez en esta matriz.

3. Se ha llevado a cabo un análisis del contenido polifenólico de extractos de rooibos mediante HPLC-MS (TOF/IT-MS) en el que se han detectado 25 y 30 compuestos fenólicos en el extracto acuoso y etanólico, respectivamente, siendo 11 de ellos identificados por primera vez en este tipo de matriz. Por otro lado, el extracto de rooibos acuoso, cuyo contenido en polifenoles totales oscila entre el 25% w/w, mostró bioactividad en ensayos con ratones $LDL^{r-/-}$ alimentados con una dieta rica en grasa (HF, 20% de grasa y 0,25% de colesterol w/w) observando una reducción significativa en las concentraciones de colesterol sérico y triglicéridos en comparación con el grupo control.

4A. Se ha puesto a punto un nuevo método para la caracterización y cuantificación del contenido polifenólico en un jarabe de arándano usado para el tratamiento de la infección urinaria. Este método permitió la caracterización de 34 compuestos, 9 de los cuales han sido descritos por primera vez en esta matriz, siendo las catequinas y las procianidinas los flavonoides que se encontraron en mayor concentración seguidos de los flavonoles y derivados del ácido fenólico. En cuanto a los estudios realizados acerca de su actividad antibacteriana se pudo demostrar que concentraciones muy bajas del extracto de arándano tenían la capacidad de modificar las propiedades de adherencia no específicas de la bacteria *E. coli* produciendo una reducción de la hidrofobicidad en la superficie.

4B. El empleo de la técnica UPLC acoplada a Q-TOF-MS ha permitido la caracterización de un buen número de polifenoles y sus metabolitos en muestras de orina de pacientes tratados con jarabe de arándano. Así, quercetina, miricetina, metoxiquercetina 3-*O*-galactósido, cumaroil hexosa, sinapoil-hexosa, cafeoil-glucosa, junto con otros 23 metabolitos conjugados y sus isómeros pudieron ser caracterizados en orina humana. Estos resultados ponen de manifiesto el potencial del método desarrollado para la cararacterización tanto de polifenoles libres como de metabolitos provenientes de la fase I y II.