Tutor/s

Dr. Oscar Núñez Burcio
Departament d'Enginyeria Química i Química Analítica

Treball Final de Grau

The Role of LC-MS and LC-HRMS in the Characterization and Determination of Polyphenols and Phenolic Acids in Food.
El Paper de la LC-MS i la LC-HRMS en la Caracterització i Determinació de Polifenols i Àcids Fenòlics als Aliments.

Ivan Ruiz Gatell

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Vull agrair al Dr. Oscar Núñez com a tutor del treball, l'acompanyament, la comprensió i els bons consells que ha sabut donar-me, i a la meva tutora de grau, la Dra. Maria Sarret, per donar-me confiança i creure en les segones oportunitats.

Als meus fills i la meva dona per les hores que no he estat amb ells, però que de ben segur recuperarem.

REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDGS)

1. *Ensuring healthy lives and promoting well-being at all ages is essential to sustainable development.*
2. *Sustainable growth and development require minimizing the natural resources and toxic materials used, and the waste and pollutants generated, throughout the entire production and consumption process.*

These two statements from UN have taken as a guide to include the present work in the development goal of good health and well-being and the goal of responsible consumption and production. This bibliographic report aims to explore the trends in determination of phenolic compounds in food by LC-MS techniques. The way this type of natural occurring compounds is related with healthy promoting diet and how polyphenols are used as natural biocides, among other applications, allows to expect that finding accurate methods to detect polyphenols in foods could help improving the developing of these goals.

Concerning the goal of good health and well-being, there is no exact match in targets proposed by UN, but the World Health Organization estimates that cardiovascular diseases take 17.9 million lives each year caused principally by unhealthy diet, physical inactivity, tobacco use and harmful use of alcohol.

Regarding the targets for the goal of responsible consumption and production, the use of polyphenols as natural antibacterial additives could reduce the consumption of chemicals and increase the shelf life of foods avoiding waste:

. Target 12.2 *By 2030, achieve the sustainable management and efficient use of natural resources*

. Target 12.3 *By 2030, halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses.*

. Target 12.4 *By 2020, achieve the environmentally sound management of chemicals and all wastes throughout their life cycle, in accordance with agreed international frameworks, and significantly reduce their release to air, water and soil in order to minimize their adverse impacts on human health and the environment*

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1. SUMMARY

Polyphenols and phenolics acids are widespread secondary metabolites found in foods derived from plants which have caught the attention of consumers over the last years due to the recognition of their antioxidant properties, their great abundance in our diet, and the probable role that these compounds are playing in the prevention of various diseases and modulating the activity of a wide range of enzymes and cell receptors. Not only cultivar but also growing area, cultivation techniques, soil management and degree of maturation among others parameters determine the variety of polyphenols and their concentration levels in fruit- and vegetable-based foods, so polyphenols can be used as sample chemical descriptors to develop food authentication methods and to prevent food frauds. Furthermore, polyphenols contribute to sensorial properties and color attributes of natural food products.

The complexity of food matrices, the huge variety of chemical compounds and the great diversity of polyphenols that can be present in samples at low concentration levels, which also could differ in polarity and size, turned liquid chromatography into the most widely used separation technique and mass spectrometry as one of the most well balanced and accurate method for the determination of polyphenols in food matrices. Although conventional liquid chromatography and low-resolution mass spectrometry are still widely used for fast and competitive applications, using ultra-high performance liquid chromatography and high-resolution mass spectrometry allow to obtain accurate mass measurements for the characterization, determination and identification of polyphenols in food products.

The fast forward developing of new methods and techniques force researchers to look at the trends in literature to choose the better conditions to achieve their goals. Exploring the role of LC-MS and LC-HRMS in the determination of phenolic compounds in food and the trends in extraction methods, chromatographic and mass spectrometric parameters and data processing are the main purposes of this work.

Keywords: Polyphenols, Phenolic acids, Liquid chromatography, Mass spectrometry, Food

2. RESUM

Els polifenols i àcids fenòlics són metabòlits secundaris àmpliament distribuïts en aliments d'origen vegetal, que durant els últims anys han atret l'atenció dels consumidors pel reconeixement de les seves propietats antioxidants, la gran abundància en la nostra dieta i el més que probable paper que juguen en la prevenció de malalties, així com la modulació de l'activitat d'una àmplia gamma d'enzims i receptors cel·lulars. La diversitat de polifenols i la concentració a la que es troben presents en aliments no tan sols depenen de la varietat de l'espècie, sinó també d'altres paràmetres com l'àrea geogràfica, les tècniques de cultiu, la gestió del terreny i el grau de maduració de la mostra. D'aquesta manera, els polifenols es poden utilitzar com a descriptors químics per desenvolupar mètodes d'autentificació i prevenció de fraus alimentaris. Els polifenols també son determinants en quant a propietats sensorials i atributs de color en aliments naturals. La seva detecció és complexa perquè poden trobar-se a baixes concentracions i formant part d'una matriu composada per un gran nombre de compostos químics. L'elevat nombre de tipus de polifenols i el fet que poden variar en polaritat i mida, han fet de la cromatografia líquida la tècnica de separació més emprada i l'espectrometria de masses un dels mètodes mes equilibrats i precisos per a la detecció de polifenols en aliments. Tot i que la cromatografia líquida convencional i l'espectrometria de masses de baixa resolució s'utilitzen sovint com a mètodes apropiats i competitius per certes aplicacions, l'ús de la cromatografia líquida d'alt rendiment i l'espectrometria de masses d'alta resolució permeten obtenir mesures precises per a la identificació, caracterització i determinació de polifenols en aliments. El ràpid avenç en la creació de nous mètodes i tècniques analítiques obliga als investigadors a estudiar les últimes tendències en les publicacions per triar les condicions d'assaig més adients i assolir els seus objectius. Explorar el paper de LC-MS i LC-HRMS en la determinació de compostos fenòlics en aliments i les tendències en els mètodes d'extracció, els paràmetres cromatogràfics, d'espectrometria de masses i el processament de dades són els principals propòsits d'aquest treball.

Paraules clau: Polifenols, Àcids fenòlics, Cromatografia de líquids, Espectrometria de masses, Aliments

3. INTRODUCTION

There has been a significant change in the general attitude of consumers on the relation between food, diet, and wellbeing. It is not enough for a given food having good organoleptic properties like colour, taste or aroma, or belonging to a well-balanced nutritional diet. People are increasingly more interested in the presence of naturally occurring compounds with direct beneficial health properties in terms of prevention, from what is commonly known as functional foods. Polyphenols are among the most important phytochemicals in nutritional terms and their antioxidant properties has been deeply studied. As a result of technical improvements in analytical techniques such as liquid chromatography coupled with mass spectrometry, scientific community have much deeper knowledge on the polyphenol composition of food, allowing to better understand the subtle difference from thousands of phenolic compounds and their potential function in our diet, but also as a discriminant method for authentication purposes in food and nutraceutical industry.

3.1. POLYPHENOLS

Polyphenols belongs to a wide variety of substances synthetized as secondary metabolites in plants. It means that polyphenols are not essential for vegetal growth and reproduction but they bring plants competitive advantages in nature like pigments, defense against microorganisms and herbivores, protection against UV radiations, along with many others [1].

Polyphenols are molecules with multiple aromatic rings bearing multiple OH-groups. Due to its free electron pairs, OH-groups make aromatic rings electron-rich, and this behavior turns polyphenols into very good nucleophile compounds acting as good antioxidants. Polyphenols are also good chelating molecules with metal ions due to the hydrogen from OH-groups, which is easily removable. The resulting negative charge is stabilized in the aromatic ring through resonance [2], and this confers them properties for free radicals and reactive oxygen species (ROS) scavenging.

More than 8,000 polyphenolic compounds have been identified in various plant species and they can be classified according to their chemical structure on the basis of the number of phenol

rings that they possess and of the elements that link these rings to each other. According to this classification, there are five families in polyphenols: phenolic acids, flavonoids, stilbenes, lignans and tannins [3]. A brief description from the point of view of food is shown below.

3.1.1. Phenolic acids

The structural unit is constituted by one single phenol ring with an organic carboxylic acid function and one or three carbon chain. Depending on the carbon chain exists two phenolic acids families: hydroxybenzoic acids (C6-C1 chain, Figure 3.1) and hydroxycinnamic acids (C6-C3 chain Figure 3.2). Phenolic acids represent 1/3 of the total amount of phenolic compounds found in food, mostly in fruits like cranberry, apple, blueberry, orange, but also in vegetables as lettuce, potato, spinach, and beverages like coffee, tea and cider. Phenolic acids are known to exhibit a strong antioxidant activity, so they are used as a natural preservative in food-based systems. Regarding health benefits, among many others, recent studies showed that regular dietary rich in phenolic acids are significantly inversely associated with impaired cognition [4].

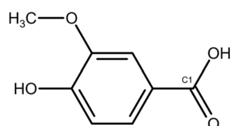

Figure 3.1 Structure of vanillic acid

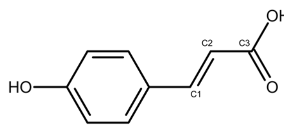

Figure 3.2 Structure of *p*-coumaric acid

3.1.2. Flavonoids

All flavonoids are composed by two benzene rings connected by an oxygenated heterocycle in a fifteen-carbon flavone skeleton (C6-C3-C6 chain). Variations in rings substitution generate six different flavonoid families: flavonols, flavanols, flavones, flavanones, isoflavones and anthocyanidins. Figure 3.3 shows the structure of quercetin, one of the most common flavonols. Flavonoids are known to be used as source of natural pigments for foods, but also as an effective antioxidant to fight virus-induced symptoms such as cellular oxidation in influenza [5].

The principal dietary sources of flavonoids are tea, citrus fruits, blackberries, red wine and soy.

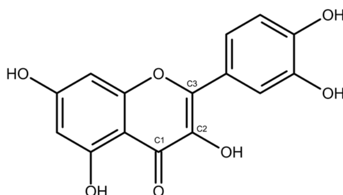

Figure 3.3 Structure of quercetin

3.1.3. Stilbenes

The structural unit is constituted by two phenyl rings linked together by an ethylene bridge (C6-C2-C6 chain) like isorhapontigenin in Figure 3.4. Over a thousand of natural stilbenes have been studied in the literature but only a few numbers of plant families produce these secondary metabolites. The major dietary sources of stilbenes are grape berries and wine. Resveratrol is the most studied stilbene because of his antioxidant properties in red wine but other derivate compounds such as isorhapontigenin, also found in grapes, are providing better results in terms of pharmacokinetic and bioavailability [6].

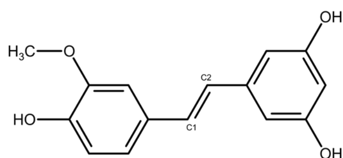

Figure 3.4 Structure of isorhapontigenin

3.1.4. Lignans

The lignans structure is based on two C6–C3 units linked by a bond between positions 8 and 8'. Secoisolariciresinol is a good example in Figure 3.5. The major dietary sources of lignans are oilseeds like sunflower, flaxseed and sesame. Lignans have been widely studied due to their similar steroid chemical structure, being known as phytoestrogens and their use as modulators of gut microbiota-brain axis against neuronal diseases [7].

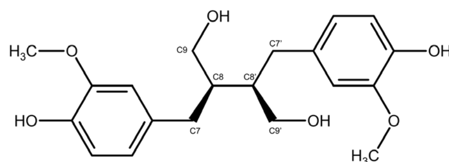

Figure 3.5 Structure of secoisolariciresinol

3.1.5. Tannins

Tannins are known to belong to complex large biomolecules of polyphenolic nature. Their building blocks are always derived from simpler polyphenols like flavan-3-ol. These subunits are linked through C4-C8' or C4-C6' bonds. There are two groups of tannins: hydrolyzable tannins

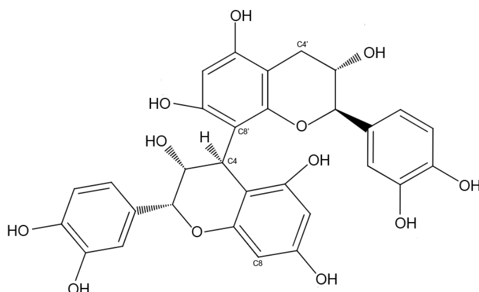

Figure 3.6 Structure of proanthocyanidin B1

and condensed (non-hydrolyzable) tannins. Figure 3.6 shows the structure of proanthocyanidin B1 as an example of condensed tannin with a C4-C8' bond. Tannins have the ability to interact and precipitate proteins like collagen, so leather industry used them widely to tan skins. But tannins also interact with salivary proteins contributing directly to sensorial properties such as astringency and bitter taste [8], and this is also widely used by winemakers.

3.2. POLYPHENOLS IN FOOD

A few examples of interests from different polyphenols families in food have already been shown. The antioxidant character of polyphenols is the main benefit for their interest in the society, the food industry, and many other application fields, but there are so many other properties to take advantages from:

(a) Polyphenols contribute to bitter and astringent flavors to a variety of foods including beer, wine, tree nuts, chocolate, coffee, tea, fruit-based products, and soy products [9].

(b) Phenolic antioxidants led to the reduction of oxidative stress not only by scavenging the excess of reactive oxygen species (ROS), but mostly by genetic modulation. ROS are byproducts of aerobic metabolism, crucial in physiological processes, but overexpression would induce inflammatory responses. The health benefits of polyphenols range from antioxidant and free radicals scavenging effects to anti-inflammatory, anticarcinogenic or cardioprotective properties. However, it depends on the bioavailability of polyphenols, which are known to be poorly absorbed and extensively metabolized by phase enzymatic reactions reaching the target organs in very small concentrations. To improve bioavailability, several promising advances are obtained with encapsulation of polyphenols in nanocarriers or micro/nano emulsions in, for example, pharmaceutical industries [9].

(c) The preference from consumers for natural food additives has encouraged the food industry to consider the use of plant polyphenol-rich extracts as alternatives to synthetic antimicrobials. The antibacterial ability of polyphenols is related to their ability to chelate metals, especially iron, which is vital for the survival of almost all bacteria. Other mechanism works from the interaction of the OH-group in phenolics with the cellular membranes of bacteria, disrupting their membrane structures [10].

(d) The polyphenolic composition content is somehow an identity card for every type and every cultivar of food, and also the contents may differ depending on the region where the food come from. Thus, polyphenols can be used as sample chemical descriptors to develop food authentication methods and to prevent food frauds [11].

The easiest way to determine polyphenols in food is using methodologies that determine the total polyphenols content (TPC) like the colorimetric Folin-Ciocalteu method, based on a redox reaction between the phenolic groups from polyphenols and the reagent which turns from yellow

to blue color. The TCP is measured proportionally to the absorbance of UV-visible at 765 nm [12]. Alternative non-destructive and easy techniques like near-infrared (NIR) spectroscopy are used combined with a proper chemometric analysis to determine directly or indirectly the TCP in some food like whole wheat flour, wines or oils [13]. These methods are useful for determining the antioxidant activity, but if characterization is needed, the use of separation methods like chromatographic or capillary electrophoresis techniques will improve the determination with UV-vis or mass spectrometry (MS) detection techniques.

Regarding the separation methods, although liquid chromatography (LC) is the most used one, for specific uses, like flavonoid aglycones determination, gas chromatography (GC) is a fast technique when a derivatization reaction has been previously done in order to improve volatility [14]. In capillary electrophoresis, separation is carried out applying an electric field within the confines of narrow bore capillaries resulting in short analysis times and high efficiency and resolution with reduced solvents consumption, thus are more sustainable methods than other procedures when working with charged polyphenols like anthocyanins [15]. Regarding detection, photodiode array detector DAD (UV-vis) is the most recurrent used for its simplicity but standards need to be used to correctly identify the compounds, besides for complex samples it suffers from low detection and quantification limits [16].

Alternatively, mass spectrometry (MS) analyses can be done discovering new compounds or distinguishing between polyphenols with the same nominal mass but different elemental compositions when high-resolution MS (HRMS) is used.

This work focusses on the trends using liquid chromatography coupled to mass spectrometry techniques (LRMS and HRMS) for the determination of polyphenols in food.

4. OBJECTIVES

The objective of this work is to carry out a bibliographic analysis on the last advances within the research community for the determination of polyphenols in food by liquid chromatography coupled to mass spectrometry techniques. The analysis focusses on the following points to achieve the aforementioned aim for conventional and high-resolution mass spectrometry:

1. Type of sample and its previous treatments for polyphenols extraction
2. Liquid chromatography-mass spectrometry techniques and set-ups
3. MS ionization techniques, instruments and acquisition modes
4. Statistical treatments employed for the acquired data
5. Purpose for determining polyphenols in the selected publications

5. METHODS

The method used in the present bibliographic research is based on using the SciFinder® database from Chemical Abstracts Service (CAS) looking for references published between 2016 and 2022 and taking advantage from its filtering system with the keywords *polyphenols*, *food*, *liquid chromatography* (LC) and *mass spectrometry* (MS). As the main purpose of this work is to study the determination of polyphenols in all the types of food, *food* as a keyword end up being too much inaccurate, so searches with key words like *fruits, nuts, oils, beverages, juices, coffee, tea, wine, beer and chocolate,* using Boolean operators like *and/or*, were also employed. As a result, a higher number of references was obtained. Then, the most relevant articles were selected and classified in four categories depending on the type of MS technique and article type: low resolution MS (LRMS), high-resolution MS (HRMS), review articles, and other mixed technics with LC and MS. Then, summary tables for some of these categories were prepared for further discussion.

With the purpose of showing the relevance of polyphenols in the food field in the scientific community is interesting to look at the number of publications progress throughout years. Specially we are going to focus on the period from 2000, when an exponential behavior started, and the number of publications per year is shown in Figure 5.1.

As can be seen, there is a paired trend between the publications addressing polyphenols and those addressing polyphenols in food. Since 2016, more than 16.000 articles per year about polyphenols are published, with the third part of them focusing on their determination in food. A little drop in the number of articles published in 2020 is observed, probably due to the pandemic situation, but this decrease seems to be restored in 2021. At the moment of performing this bibliographic search, only two months from 2022 has passed, reason for the low number of publications, but if data is extrapolated considering only these two months, 2022 seems to show the same trend regarding the number of publications as the previous three years.

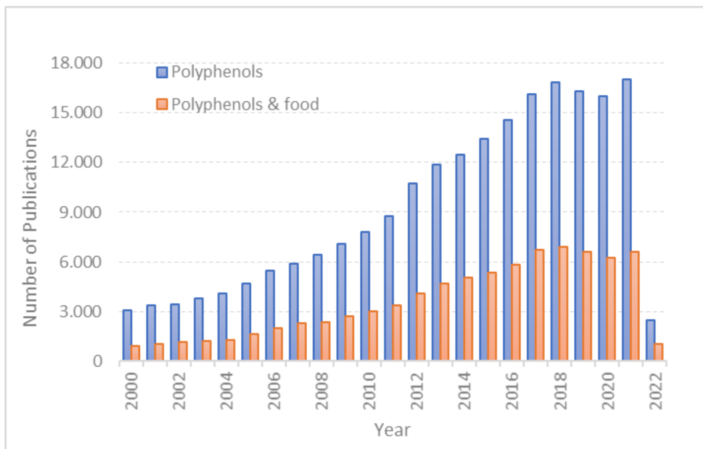

Figure 5.1 Number of publications when searching by *polyphenols* vs. *polyphenols & food*

With a different combination of keywords and Boolean operators in SciFinder® it is possible to find an approximately distribution of the type of food that are more studied regarding polyphenols, and this information is summarized in Figure 5.2. As can be seen, almost 40% of the studies carried out are from beverages, mainly wine and teas. Polyphenols in oils are also widely studied (23% of the publications).

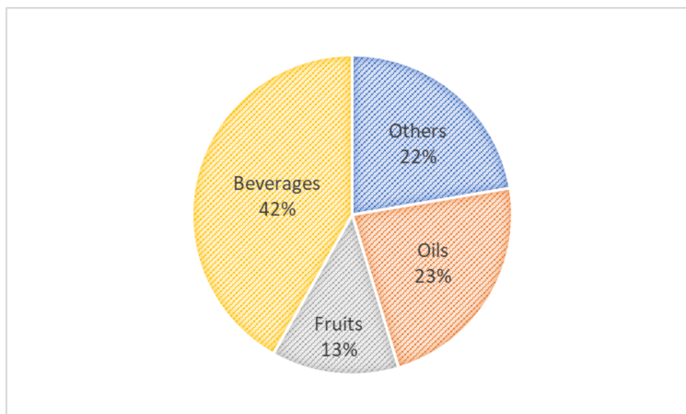

Figure 5.2 Approximate distribution of the type of food studied regarding polyphenols

Still on the subject of showing the relevance on the determination of polyphenols in food, and to focus on the aim of the present project, a study about the number of publications since 2000 dealing with the use of liquid chromatography and mass spectrometry was also performed, and the results are summarized in Figure 5.3

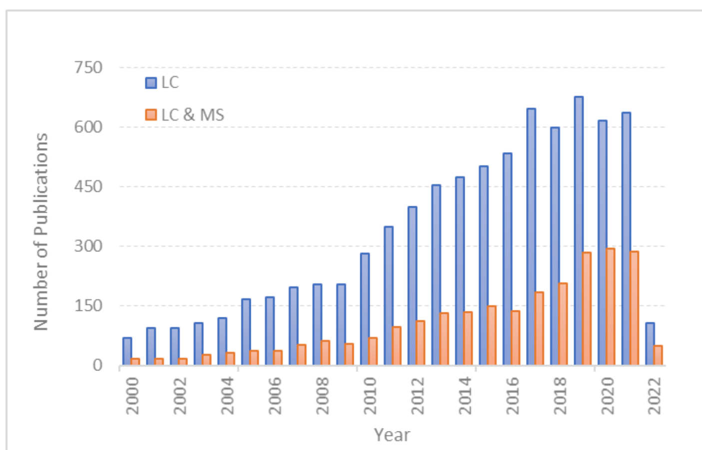

Figure 5.3 Number of publications dealing with the determination of polyphenols in food when searching by *liquid chromatography* vs. *liquid chromatography* and *mass spectrometry*

The principal method for separating polyphenols is liquid chromatography. As Figure 5.3 shows, since 2019 the MS detection technic is employed in almost half of the publications dealing with LC, demonstrating that liquid chromatography coupled to mass spectrometry is nowadays one of the most important methodology for the determination of polyphenols in food.

6. LC-MS METHODS FOR THE DETERMINATION OF POLYPHENOLS IN FOOD

The analysis of polyphenols in food samples is relatively complex due to the wide variety of compounds that can be present, which differ in polarity and size (from simple phenolic acids to oligomers such condensed tannins), but also because many of these compounds in food products are found at low concentration levels, or bounded to other chemical compounds being present in various and complexes food matrices, understanding the concept of food matrix as a part of the microstructure of foods that contains, interacts or gives particular functionalities to a specific constituent of the food. Because of all these reasons, liquid chromatography coupled to mass spectrometry (LC-MS) techniques are widely employed for the determination of polyphenolic compounds in food products, and some selected LC-MS methods published in the literature within the last seven years are summarized in Table 6.1.

Sample extraction is one of the most important aspects of sample preparation before the analysis of complex matrices such as food products. The selection and the correct application of sample extraction procedures will increase method selectivity by means of removing sample interferences. Moreover, sample extraction can also be employed as a concentration step, providing a good chance to improve method sensitivity. Liquid-solid extraction (LSE) and liquid-liquid extraction (LLE) are the most widely used methodologies for the extraction of polyphenols in samples of different nature. As can be seen in Table 6.1, for liquid nature samples most of the authors used LLE procedures [11,17,18], while Petrucci et. al [19] and Royo et. al [20] used direct analysis for fast polyphenol determination in craft beers and wines, respectively. LLE from oil samples [11,17] needed intermediate clean-up steps, mainly with hexane liquid-liquid extraction, to remove fat content that can be problematic when working with LC methodologies. The majority of the sample preparation extractions on solid nature samples were made by LSE [20–32].

Table 6.1 Selected LC-MS methodologies for the determination of polyphenols in food

Sample (compounds)	Sample extraction	LC-MS(/MS) conditions	Application	Data Analysis	Ref.
Cranberry-based pharmaceuticals and natural extracts (29 polyphenols)	Liquid-Solid Extraction (LSE) 0.1 g sample with 10 mL acetone / water / hydrochloric acid (70:29.9:0.1 v/v/v)	*UHPLC-APPI-MS/MS* LC conditions: Syncronis C18 (100×2.1 mm, 1.7 µm) and Hypersil Gold C18 (50×2.1 mm, 1.9 µm) columns. Gradient elution: (A) 0.1% formic acid aqueous solution and (B) methanol; Flow-rate: 0.285 mL/min (Syncronis C18) and 0.319 mL/min (Hypersil Gold C18) MS conditions: Triple quadrupole (QqQ) mass analyzer APPI: acetone dopant-assisted (40 µL/min), 10 µA discharge current H-ESI: -2.5 kV Full scan MS (*m/z* 50-1000) and SRM acquisition modes	Characterization Classification Authentication	Target profiling PCA	[21]
Passion fruit pulp (fresh and dried samples) (15 polyphenols)	Liquid-Solid Extraction (LSE) 2 g sample with 15 mL ethanol 70% solution	*UHPLC-ESI- MS/MS* LC conditions: Ascentis Express F5 (150×2.1mm, 2.7 µm) column. Gradient elution: (A) 0.1% formic acid aqueous solution and (B) acetonitrile; Flow-rate: 0.2 mL/min MS conditions: Triple quadrupole (QqQ) mass analyzer ESI: +3.5 kV SRM acquisition mode	Characterization Quantitation	Target profiling	[25]
Mixed fruit/vegetable juices and smoothies (20 polyphenols)	Liquid-Solid Extraction (LSE) 0.5g residue (after 10 mL sample centrifugation) with 5 mL methanol Dispersive solid-phase extraction (dSPE) 5 mL extract (aqueous phase/methanol 50:50, v/v) with 50 mg of HMS-C18 Separation of the HMS-C18 sorbent and elution with 2x3 mL methanol/water (95:5 v/v pH 2)	*UHPLC-ESI-MS/MS* LC conditions: ACE Excel 2 C18-PFP (100×2.1 mm, 2 µm) column. Gradient elution: (A) methanol and (B) Milli-Q water (both A, B containing 2 mM ammonium acetate and 0.1% formic acid); Flow-rate: 0.25 mL/min MS conditions: Ion-trap mass analyser ESI: -4.5 kV MRM acquisition mode	Characterization Quantitation	Target profiling	[33]
Sweet cherries peel and flesh (9 phenolic acids and 9 flavonoids)	Liquid-Solid Extraction (LSE) Phenolic acids: 1g sample with 20 mL 80% methanol containing 0.5% hydrochloric acid. 3x15 mL extraction with diethyl ether/ethyl acetate 1:1 (v/v) Flavonoids: 2g sample with 30 mL methanol containing 0.5% hydrochloric acid	*HPLC-ESI- MS/MS* LC conditions: Waters ACQUITY HSS C18 (150×2.1 mm, 1.8 µm) column. Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid in acetonitrile solution. Flow-rate: 0.3 mL/min MS conditions: Triple quadrupole (QqQ) mass analyser ESI: +2.5 kV ESI: −1.0 kV MRM acquisition mode	Characterization Quantitation Classification	Target profiling PCA	[26]
Sweet orange pulp powder (34 polyphenols)	Liquid-Solid Extraction (LSE) 20 mL (or g) with 1 mL methanol 90% and 1% formic acid	*HPLC-ESI-MS/MS* LC conditions: ZORBAX Eclipse XDB-C18 (150×2.1 mm, 5 µm) column. Gradient elution: (A) 0.25% acetic acid in water and (B) acetonitrile. Flow-rate: 0.4 mL/min MS conditions: Triple quadrupole (QqQ) mass analyser ESI: −3.0 kV MRM acquisition mode	Characterization Classification Authentication	Target profiling	[27]
Extra-virgin olive oil (29 polyphenols)	Liquid–liquid extraction (LLE) 1g sample solved with hexane (1:1, v v), then extracted with 2 ml methanol Dispersive solid-phase extraction (dSPE) for clean-up 5 mL methanol extract with 50 mg of C18	*HPLC-ESI- MS/MS* LC conditions: Acquity BEH C18 (50×2.1 mm, 1.7 µm) column. Gradient elution: (A) 0.2% acetic acid in water and (B) acetonitrile. Flow-rate: 0.4 mL/min MS conditions: Triple quadrupole (QqQ) mass analyser ESI: −3.5 kV MRM acquisition mode	Characterization	Target profiling	[17]
Ground cocoa beans (30 polyphenols, Fingerprinting)	Liquid-Solid Extraction (LSE) 0.015 g defatted cocoa powder with 75 µL of methanol/acetic acid (98:2,v/v) and 900 µL of acetone/water/acetic acid (70:28:2, v/v/v)	*UHPLC-ESI-MS/MS* LC conditions: BEH C18 (150×1 mm, 1.7 µm) column. Gradient elution: (A) 1% formic acid aqueous solution and (B) 1% formic acid in methanol solution. Flow-rate: 0.08 mL/min. MS conditions: Ion trap mass analyser ESI: −4.5 kV Full scan MS (*m/z* 100-2000) acquisition mode	Characterization Classification Authentication	Fingerprint PCA PLS-DA	[28]

***Sample* (compounds)**	**Sample extraction**	**LC-MS(/MS) conditions**	**Application**	**Data Analysis**	**Ref.**
Gluten free pasta with chestnut flour (13 phenolic acids)	Liquid-Solid Extraction (LSE) 2 g sample with 40 mL ethanol	*UHPLC-ESI-MS/MS* LC conditions: Zorbax SB-C18 (100×2.1 mm, 1.8 µm) column. Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid in acetonitrile solution. Flow-rate: 0.25 mL/min. MS conditions: QTrap mass analyser ESI: −4.5 kV MRM acquisition mode	Characterization Quantitation	Target profiling	[29]
Litchi pericarp (Procyanidins)	Liquid-Solid Extraction (LSE) 3 g sample with 15 mL methanol and 15 mL aqueous acetone solution (70%, v/v)	*HPLC-ESI-MS/MS* LC conditions: Denali C18 (150×2 mm, 5 µm) column. Gradient elution: (A) acetonitrile and (B) 3% acetic acid aqueous solution. Flow-rate: 0.3 mL/min. MS conditions: Ion trap mass analyser ESI: −5 kV Full scan MS (m/z 50-2000) acquisition mode	Characterization	Target profiling	[30]
Sambucus ebulus berry extracts (7 polyphenols)	Liquid-Solid Extraction (LSE) 0.5 g sample with 5 mL of acetone aqueous solution (70%, v/v) acidified with 0.01% hydrochloric acid	*HPLC-ESI-MS* LC conditions: Cortecs UPLC C18 (50×2.1 mm, 1.6 µm) column. Gradient elution: (A) 0.2% formic acid in methanol solution and (B) 0,1% formic acid aqueous solution. Flow-rate: 0.3 mL/min. MS conditions: Single quadrupole (Q) mass analyzer ESI: −15 kV Selected Ion Monitoring (SIM) acquisition mode	Characterization Quantitation	Target profiling	[31]
Fresh and frozen spinach leaves (8 flavonoids, 2 phenolic acids)	Liquid-Solid Extraction (LSE) 2 g sample with 5 mL of methanol aqueous solution (70%, v/v) acidified with 0.1% formic acid	*HPLC-PDA-QqQ- MS/MS* LC conditions: Waters Acquity HSS T3 C18 (100×2.1 mm, 1.8 µm) column. Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid in acetonitrile solution. Flow-rate: 0.6 mL/min. MS conditions: Triple quadrupole (QqQ) mass analyzer ESI: +3.5 kV (flavonoids) ESI: −3.5 kV (phenolic acids) Full scan MS (*m/z* 100-1000) and MRM acquisition modes	Characterization Identification	Target profiling	[32]
Untreated Craft Beer (9 polyphenols)	Direct analysis (dilution 1:100 with the mobile phase)	*HPLC-PDA-ESI-MS/MS* LC conditions: Waters XBridge C18 (150×2.1 mm, 5 µm) column. Gradient elution: (A) 0.02% formic acid aqueous solution and (B) 0.02% formic acid in acetonitrile solution. Flow-rate: 0.2 mL/min. MS conditions: Triple quadrupole (QqQ) mass analyzer ESI: −2.7 kV ESI: +3 kV Selected Ion Recording (SIR) acquisition mode	Characterization Quantitation	Target profiling	[19]
Extra-virgin and refined pomace olive oils (23 polyphenols (60% secoiridoids))	Liquid–liquid extraction (LLE) 4 g sample with 2 mL n-hexane (clean-up), and 4 mL methanol aqueous solution (70:30, v/v)	*HPLC-DAD-ESI–MS/MS* LC conditions: Phenomenex C18 (250×4.6 mm, 5 µm) column (no mention about elution) MS conditions: Triple quadrupole (QqQ) mass analyzer ESI: Negative mode MRM acquisition mode	Characterization Classification Authentication	Target profiling PCA HCA	[11]
Table olives (17 polyphenols)	Liquid-Solid Extraction (LSE) 1 g sample (grinded olive pulp) with 6 mL of ethanol/methanol (1:1; v/v)	*HPLC-ESI-QqQ- MS/MS* LC conditions: Zorbax Eclipse-XDB-C18 (150×4.6 mm, 5 µm) column. Gradient elution: (A) 0.025% acetic acid aqueous solution and (B) 5% acetone in acetonitrile solution. Flow-rate: 0.8 mL/min. MS conditions: Triple quadrupole (QqQ) mass analyzer ESI: −4.2 kV MRM acquisition mode	Characterization Quantitation	Target profiling	[22]
Ground cocoa beans and chocolate (34 Flavan-3-ols)	Liquid-Solid Extraction (LSE) 0.015 g defatted cocoa powder with 75 µL of methanol/acetic acid (98:2, v/v) and 900 µL of acetone/water/acetic acid (70:28:2, v/v/v)	*HPLC-ESI-TQ-MS* LC conditions: Acquity HSS T3 (100×1 mm, 1.8 µm) column. Gradient elution: (A) 1% formic acid aqueous solution and (B) 1% formic acid in methanol solution. Flow-rate: 0.17 mL/min. MS conditions: Triple quadrupole (QqQ) mass analyzer ESI: −2.8 kV MRM acquisition mode	Characterization Quantitation	Target profiling PCA CCSWA	[23]

Sample (compounds)	Sample extraction	LC-MS(/MS) conditions	Application	Data Analysis	Ref.
Lemon juice (6 phenolic acids)	Liquid–liquid extraction (LLE) Sample mixed with 0.1% formic acid in 70% methanol solution (1:1)	*UHPLC-QqQ-MS/MS* LC conditions: Prontosil C18 AQ (100×2.1 mm, 3 μm) column. Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid in acetonitrile solution. Flow-rate: 0.5 mL/min. MS conditions: Triple quadrupole (QqQ) mass analyzer ESI MRM acquisition mode	Characterization Classification Authentication	Target profiling PCA LDA SVM	[18]
Wine, grape berry seed and skin (50 Anthocyanins and non-colored phenols)	Liquid-Solid Extraction (LSE) (grape berry seed and skin) 100 mg sample with 10 mL of methanol / Milli-Q water / formic acid (79:20:1, v/v/v) Direct analysis for wine samples	*UHPLC-QqQ-MS/MS* LC conditions: Waters Acquity BEH C18 (100×2.1 mm, 1.7 μm) column. Gradient elution: (A) 0.1% (2% for anthocyanins) formic acid aqueous solution and (B) 0.1% (2% for anthocyanins) formic acid in acetonitrile solution. Flow-rate: 0.45 mL/min. MS conditions: QTRAP mass analyzer ESI: +4.5 kV (anthocyanins) ESI: −4.5 kV (other phenolics) MRM acquisition mode	Characterization Quantitation	Target profiling	[20]
Blueberry and strawberry fruits and jam (36 polyphenols)	Liquid-Solid Extraction (LSE) 2 g sample with 10 mL of ethanol: water mixture (70:30, v/v) acidified with HCl (1.5%)	*HPLC-ESI-MS/MS* LC conditions: Sinergy Polar–RP C18 (250×4.6 mm, 4 μm) column. Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid in methanol solution. Flow-rate: 0.8 mL/min. MS conditions: Triple quadrupole (QqQ) mass analyzer ESI: +4 kV ESI: −4 kV MRM acquisition mode	Characterization Quantitation	Target profiling	[24]

Regarding the extraction solvents employed, methanol, ethanol and acetone aqueous solutions were used and the half of them has been acidified with hydrochloric, formic or acetic acids.

Other sample treatment methodologies such as dispersive solid phase extraction (dSPE) were also employed either for sample extraction or for clean-up purposes. As an example, Casado et al. [33] proposed the use of dSPE for the extraction of polyphenols in complex matrices like fruit/vegetables smoothies and juices. In this publication, mesostructured silicas are used as new sorbent materials in food sample preparation to avoid interferences into the LC-MS system such as ion suppression or ion enhancement made by proteins, fats, salts, sugars, and others components that are included in high complex food matrices. In this extraction technique, the sorbent material is directly added into the sample solution, increasing the interaction area between the sorbent and the analytes, allowing to use less sorbents and solvents and to reduce time and labor. A synthesized hybrid mesostructured silica (HMS) with wormlike pores modified with chloro-(dimethyl)-octadecylsilane were used as sorbent material to retain polyphenols.

A proposed absorption mechanism is shown in Figure 6.1 where the large pore volume, well-defined pore size distribution and the high surface area improves the interaction with phenolic compounds. In the proposed application, the first step in the extraction was a sample LSE with methanol, and then the resultant solution was mixed with HMS-C18 for dSPE. After filtering the

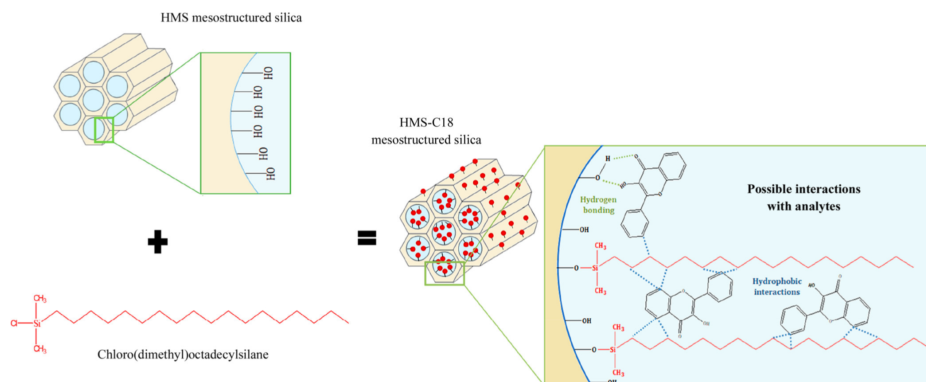

Figure 6.1 Possible interaction mechanism by HMS-C18. Reproduced with permission from reference [33]. Copyright (2018) American Chemical Society.

HMS-C18, the extraction finished with an LSE of the HMS-C18 sorbent with an acidic methanol solution. Figure 6.2 shows the recovery % for different polyphenols depending on the sorbent used (authors compared three sorbents, HMS alone, HMS-C18, and a commercial C18 amorphous silica). HMS-C18 really improved the retention of analytes providing the highest recoveries for most of the studied polyphenols, being the one finally proposed by the authors.

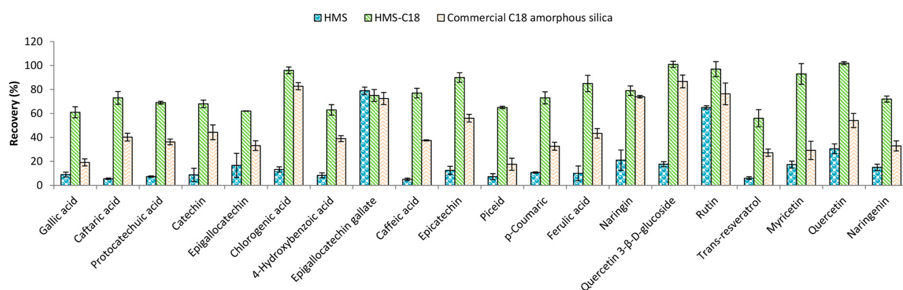

Figure 6.2 Comparison of the recovery percentages obtained for several polyphenols from the analysis of smoothie samples extracted by the optimized dSPE method using different types of sorbents. Adapted with permission from reference [33]. Copyright (2018) American Chemical Society.

As previously commented, dSPE is also proposed for clean-up purposes. For example, López-Yerena et al. [34] recovered and cleaned up methanolic extracts from olive oil by dispersing 50 mg of C18 to eliminate residual non-polar matrix compounds.

In the case of liquid samples, as already noted, direct analysis is frequently proposed. In direct analysis there is no polyphenol extraction but samples must be slightly treated before their introduction into the LC-MS system. For example, Petrucci et. al [19] degassed and filtered craft beers samples to eliminate solid residues and then diluted them 1:100 with the mobile phase in order to obtain the right intensity of the ESI-MS/MS spectrum inside the linearity range of the calibration curve. Concerning the liquid chromatographic techniques, about 60% of authors in the publications shown in Table 6.1 use the classic high-performance liquid chromatography (HPLC) instead of ultra-high pressure liquid chromatography (UHPLC) technique which gives narrower chromatographic peaks and higher sample throughput, but at expensive costs because of the highest pressure needed as a result of the filled column particle size under 2 µm. The majority of the publications used C18 reversed phase columns as stationary phase. Figure 6.3 shows, as an example, the LC-MS chromatographic extracted ions for the separation of polyphenols in a craft beer. In contrast, Shanmugam et al. [25] used pentafluoro phenylpropyl (PFP) superficially porous particles as stationary phase. Fluorinated phases offer better performance with enantiomeric separation of isomers and the superficially porous particles, also known as fused-core or core-shell particles, unlike full porous particles like C18, provides the same efficient separations as the sub-2 µm particles that are used in UHPLC without the problem of the high pressure needed.

Different commercial modified versions of the classic C18 column are used in some publications to improve performance for highly aqueous mobile phases and high polar compounds such as the C18 AQ column [18] or the *C18 T3 column* [23,32], or to enhance mechanical properties like the *C18 HSS* column [23,26,32]. Finally, the proposal of a mixed *C18-PFP* column to take advantage of the extra selectivity provided by the pentafluoro-phenyl phase for the determination of polyphenols in juices and smoothies samples [33].

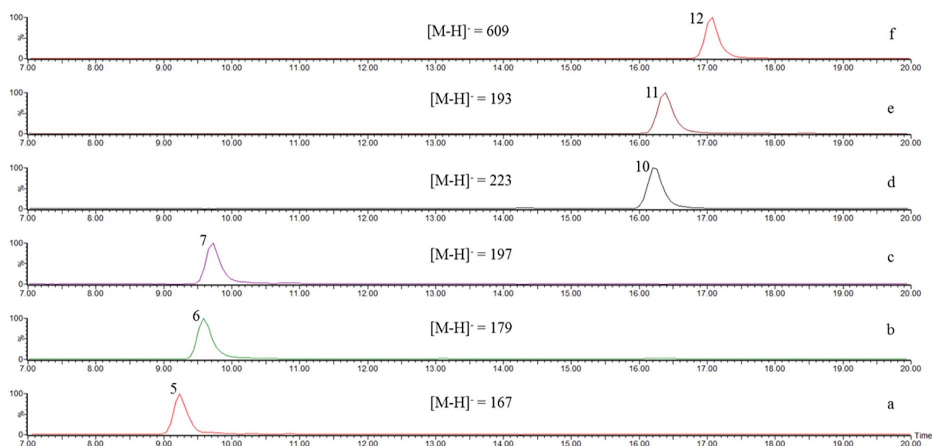

Figure 6.3 LC-MS chromatograms of selected deprotonated *m/z* values of vanillic acid (5), caffeic acid (6), syringic acid (7), sinapic acid (10), ferulic acid (11) and rutin (12). Reproduced with permission from reference [19]. Copyright (2020) American Chemical Society.

As for the mobile phases, all publications worked with binary gradient elution separations. Most of them use formic acid aqueous solution and formic acid acetonitrile solution as eluents A and B, respectively. Other authors used formic acid methanol solution or acetic acid aqueous solutions. Formic acid is mainly used to adjust and acidic pH in the aqueous mobile phase helping to protonate polyphenols for improving their separation by reversed-phase chromatography. Different percentages of acid were used depending on the sample, being 0,1% the most common. However, for example, Royo et. al [20] used higher percentages of acid (2%) for determining anthocyanins while kept 0,1% for the rest of polyphenols. Other solvents have also been proposed for the separation of polyphenols. For example, in their analysis in oil samples, Moreno et. al [22] employed 5% acetone in an acetonitrile solution.

Regarding the mass spectrometry conditions, practically all the authors employed electrospray (ESI) as ionization technique, due to the ease of ionizing polyphenols, especially polyphenolic acids. More compounds are expected to ionize in positive ion mode (ESI+) but negative ion mode (ESI–) is a better option owing to its improved ionization efficiency which gives better sensitivity and potential for lower detection limits with lower background noise [35]. For these reasons, ESI is used in negative mode in the majority of the publications, although both positive and negative ionization modes are proposed for some authors [19,20,24–26,32] because some polyphenols such as anthocyanins are usually detected in the positive ion mode as their native form generating a positive flavylium cation.

Other ionization techniques have also been employed for the LC-MS determination of polyphenols. For instance, Parets et. al [21] proposed the use of atmospheric pressure photoionization (APPI) for the determination of polyphenols in cranberry and grape products, including berries, juices and pharmaceuticals. APPI is used to expand the application of LC-MS techniques to non-polar compounds and compounds which are difficult to ionize by ESI but also is used to reduce matrix effects influence on the polyphenolic characterization. Matrix effects such as ion suppression produced by co-eluted compounds influences signal intensity due to the competition for the available charges. APPI usually generates more reproducible signals and its ionization is less susceptible to matrix effect because many matrix compounds are not often ionizable by photons at only 10 eV, which is the energy frequently employed when working with APPI and an UV lamp source [36].

In previous studies, Parets et al. [21] observed a high polyphenol overlapping when using fast chromatographic methods due to reduction on the total chromatographic elution time and the increase of matrix effects. Thus, they compared several atmospheric pressure ionization (API) sources, such as heated electrospray (H-ESI), atmospheric pressure chemical ionization (APCI) and photoionization (APPI), being the last one the best for a fast and reliable characterization and authentication of grape- and cranberry-based products according to the type of fruit.

Concerning the mass analyzers employed in LC–MS methods, most of the publications used triple quadrupole (QqQ) instruments in multiple reaction monitoring (MRM) acquisition mode, a tandem MS method in which the first and third quadrupoles act as mass filters and the second causes fragmentation of the analyte through interaction with a collision gas. Besides QqQ instruments, several authors used ion trap (IT) [28,30,33] in full scan mode (m/z 50-2000) which gives better sensitivity but worst performance. Other authors employed hybrid quadrupole-ion trap (QTrap) instruments [20,37], working in similar conditions than QqQ instruments, the first quadrupole acting as a mass filter, and the ion-trap allowing to obtain fragmentation spectra.

Regarding LC-MS applications, the main purpose of most of the publications found in the literature is the characterization and determination of polyphenols in a certain food. For example, Oniszczuk et al. [29] determined and quantified polyphenols in gluten-free pasta enriched with chestnut flour as a source of nutritionally valuable food. Kamiloglu [32] determined the changes in polyphenols content of spinach taken from different production steps of the industrial freezing process.

LC-MS methodologies provide huge amounts of data and proper statistical treatment could be as important as the determination techniques. In this sense, several publications [11,18,21,23,26,28] took advantage from chemometric methods for classification and authentication purposes. Principal component analysis (PCA) is the most frequently used multivariate chemometric method as long as it is very useful for reducing the number of variables and highlighting relevant information. Parets et al. [21] used PCA for discriminating grapes and cranberries-based products. Figure 6.4 shows good differentiation between fruit source but also points the similarity in polyphenol content (compounds employed as sample chemical descriptors) between natural cranberries samples like juices or sachets and cranberries-based pharmaceutical products like, syrups, capsules or extracts (bottom left corner). Behavior from samples in the top left corner were explained by differences in polyphenol concentrations because of the employed raw materials or the manufacturing pharmaceutical processes. In the end, PCA results suggested that all the pharmaceutical products were reasonably considered cranberry-based genuine samples.

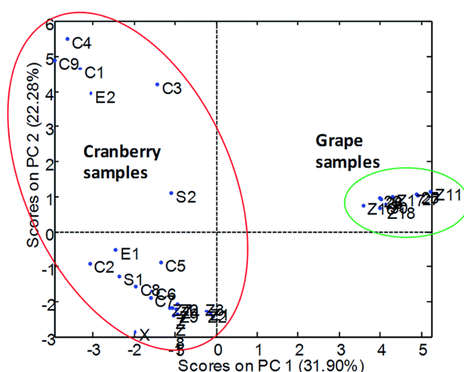

Figure 6.4 PCA results (scatter plots of scores of PC1 and PC2) using the normalized peak areas of the polyphenolic profile data from: juices (Z), capsules (C), sachets (S), extracts (E) and syrup (X). Adapted with permission from reference [21]. Copyright (2016) The Royal Society of Chemistry.

Other chemometric method reported in the literature is partial least-squares discriminant analysis (PLS-DA), which helps finding the components variable which discriminate as much as possible between two or more different groups of samples. For example, Fayeulle et al. [28] proposed a method to sort and classify cocoa beans according to phenolic composition and chocolate sensory properties like bitterness, sourness or astringency, enabling prediction of quality and avoiding time and money-consuming steps to chocolate producers. A fingerprint of cocoa bean polyphenols was obtained, and then related to the sensory-group distribution using chemometrics. Figure 6.5 shows the distribution into the four sensory groups (poles) in PLS-DA performed on the cocoa samples, using the mass-signal intensities of the *m/z* values of the spectra. Poles 1 and 2 were overlapping but still separated from poles 3 and 4. Combining with PCA methods, they concluded that only 5% of the variables were needed to explain the sensory-poles grouping and procyanidins molecules were the variables with the highest rank for discrimination, confirming the impact of flavan-3-ols on sensory-pole separation and suggesting that they may contribute to chocolate taste characteristics, either directly or as precursors of other flavor compounds formed during processing.

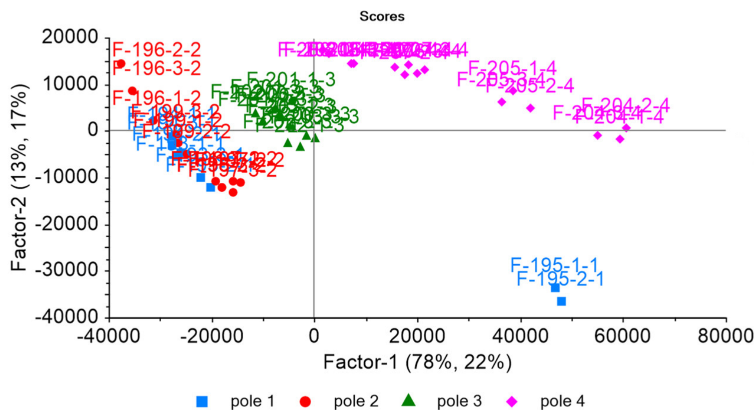

Figure 6.5 Scores obtained after PLS-DA of cocoa bean polyphenol extracts and how they belong to specific sensory poles. Adapted with permission from reference [28]. Copyright (2019) American Chemical Society.

Hierarchical cluster analysis (HCA), a classification chemometric technique that evaluates the distance between the samples and groups them according to their similarities, has also been described in the literature by employing LC-MS methodologies in the analysis of polyphenols. For example, Drira et al. [11] used both PCA and HCA methods for the detection of adulterations in extra virgin olive with refined pomace olive oil by targeting 23 polyphenolic compounds.

7. LC-HRMS METHODS FOR THE DETERMINATION OF POLYPHENOLS IN FOOD

High-resolution mass spectrometry offers accurate mass determination of chemical compounds (down to 1-5 ppm mass errors) that helps in identifying polyphenols to a greater certainty level of confidence. Resolution is commonly expressed in full-width at half-maximum (fwhm) units and it is calculated as the relation between the nominal mass for a particular peak in the mass spectrum, and the peak width at 50% of the peak height. An example of huge difference between MS and HRMS is shown in Figure 7.1.

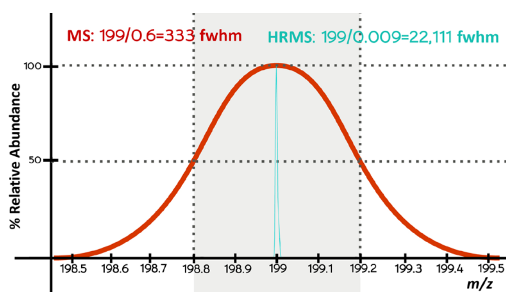

Figure 7.1 Schematic comparison between resolution of MS (red) and HRMS (green) overlapped spectrum peaks for a given compound

Most of the reviewed publications where LC-HRMS methods are used had the main purpose of getting the polyphenolic characterization of samples. Instead of analyze specific, targeted polyphenol compounds, authors followed an untargeted analysis strategy taking advantage of the high resolution and mass accuracy provided by HRMS. Some selected LC-HRMS methods for the determination of polyphenols in food published in the literature within the last seven

years are summarized in Table 7.1 focusing on the sample extraction procedure, LC and MS conditions, analysis for the acquired data and applications. As previously mentioned in LC-MS methods, most of the publications analysing solid samples use LSE techniques [38,39] combined with solid-phase extraction (SPE) to perform further purification and preconcentration processes. For those working with liquid samples [40–42], LLE and SPE techniques were used. As an example, Mathon et al. [40] extracted pomegranate juice samples by employing C18 SPE cartridges to limit the content of ionic compounds and polar compounds such sugars, reducing the MS contaminants and possible matrix effects due to ionization competition in the source. In the same way as with LC-MS methodologies, most of the extraction solvents employed were methanol, ethanol and acetone aqueous solutions acidified with formic or acetic acids. Renai et al. [43] and Bashmil et al. [44] added reagents like sodium fluoride or potassium metabisulfite to the employed solvents to avoid enzymatic degradation of polyphenols (browning reactions) in the analysis of berries and banana samples, respectively.

Table 7.1. Selected LC-HRMS methodologies *for the determination of polyphenols in food*

Sample (compounds)	Sample extraction	LC-HRMS conditions	Application	Data Analysis	Ref.
Apricot, peach and mixed purées (28 polyphenols)	Liquid-Solid Extraction (LSE). 10 g of sample in 100 mL of methanol aqueous solution (50:50 v/v)	UHPLC-ESI-HRMS/MS *LC conditions:* Hypersil Gold C18 column (100×2.1 mm, 1.9 µm) Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid in acetonitrile solution. Flow-rate 0.3 mL/min *MS conditions:* Q-Orbitrap H-ESI: -3.0 kV Full scan MS (*m/z* 100-950) acquisition. Resolution 70,000 fwhm	Characterization Authentication	Target profiling PCA	[45]
Strawberries (untargeted analysis, 18 polyphenols identified, 113 tentatively identified)	Liquid-Solid Extraction (LSE) 300 mg in 9 mL acetone: water: acetic acid solution (70:29.5:0.5 v/v/v).	UHPLC-ESI-HRMS/MS *LC conditions:* Kinetex core–shell C18 column (100×2.1 mm, 2.6 µm); Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid in acetonitrile solution. Flow-rate 0.6 mL/min *MS conditions:* Q-Orbitrap H-ESI: -3.5 kV H-ESI: +2.5 kV DDA mode (*m/z* 150–1000). Resolution 35,000 fwhm	Characterization	Untargeted profiling	[42]
Cranberries, grape and blueberries-based natural products (juices, fruits and raisins) and cranberry-based pharmaceuticals (capsules, syrups and sachets) (53 polyphenols)	Liquid-Solid Extraction (LSE) and Liquid–liquid extraction (LLE) 0.1 g sample with 10 mL of acetone: water: hydrochloric acid (70:29.9:0.1 v/v/v) solution	UHPLC-ESI-HRMS/MS *LC conditions:* Ascentis Express C18 porous-shell column (150×2.1 mm, 2.7 µm); Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid in acetonitrile solution Flow-rate 0.3 mL/min *MS conditions:* Q-Orbitrap H-ESI: -2.5 kV Full MS scan mode (*m/z* 100–1500). Resolution 70,000 fwhm	Characterization Classification Authentication	Target profiling PCA PLS	[46]
Quince fresh, cooked and dried purees (11 polyphenols)	Liquid-Solid Extraction (LSE) 350 mg sample with 5 mL of 75% methanol solution. Solid phase extraction (SPE), C18 extraction cartridges (Agilent Bond-elut) eluted with propanol	UHPLC-ESI-HRMS/MS *LC conditions:* Hypersil Gold C18 column (50×2.1 mm, 1.9 µm) Gradient elution: (A) 0.5% formic acid acetonitrile and water solution (75:24,5 v:v) and (B) 0.5% formic acid water and acetonitrile solution (95:4,5 v:v). Flow-rate 0.3mL/min *MS conditions:* Orbitrap ESI: +2.5 kV	Characterization Classification Quantitation	Target profiling PLS-DA	[38]

Sample (compounds)	Sample extraction	LC-HRMS conditions	Application	Data Analysis	Ref.
Pomegranate natural and commercial juices (punicalagins)	Solid phase extraction (SPE), 2ml sample in C18 cartridges with 1 ml methanol–water (75:25, v/v) solution	HPLC-ESI-HRMS/MS LC conditions: HSS T3 C18 column (100×2.1 mm, 1.8 µm); Gradient elution: 0.1% trifluoroacetic acid in (A) water solution and (B) acetonitrile solution Flow-rate 0.5mL/min MS conditions: Q-TOF ESI: -2.7 kV Full MS scan mode (*m/z* 240–1200).	Characterization Authentication		[40]
Strawberries (57 polyphenols, including isomers)	Liquid-Solid Extraction (LSE) Fruit sample with methanol aqueous solution 70% with 1.5% formic acid	UHPLC-ESI-HRMS/MS LC conditions: Acquity BEH C18-column (100×2.1 mm, 1.7 µm); Gradient elution (A) 4,5% formic acid aqueous solution and (B) acetonitrile. Flow-rate 0.45mL/min MS conditions: Q-TOF ESI: -2 kV ESI: +2 kV (anthocyanins) Full MS scan mode (*m/z* 100–2000).	Characterization Identification	Target profiling PCA	[47]
*Pepper flour (*42 polyphenols)	Liquid-Solid Extraction (LSE) 2 g sample with ethanol aqueous solution (50:50, v/v) and 2 g sample with butanol aqueous solution (50:50, v/v)	UHPLC-ESI-HRMS/MS LC conditions: HSS T3 C18 column (100×2.1 mm, 1.8 µm); Gradient elution: (A) 0.3% formic acid aqueous solution and (B) 0.3% formic acid acetonitrile solution with 5 mM ammonium formate. Flow-rate 0.6mL/min MS conditions: Q-TOF ESI: -2 kV Independent data acquisition (IDA) and low and high energy fragmentation (MSE) acquisition modes (*m/z* 50–1000). Resolution 30,000 fwhm	Characterization Identification	Target profiling	[48]
Fruits and vegetables (phenolic acids and flavonoids)	Liquid-Solid Extraction (LSE) 1 g sample in 10 mL 30% ethanol aqueous solution	HPLC-ESI-HRMS/MS LC conditions: Synergi Hydro-RP C18 column, (250×4.6 mm, 4.0 µm); Gradient elution: (A) 2% acetic acid in aqueous solution and (B) 0,5% acetic acid in acetonitrile aqueous solution (50:50, v/v). Flow-rate 0.8 mL/min MS conditions: Q-TOF ESI: -3.5 kV ESI: +3.5 kV Full MS scan mode (*m/z* 50–1300).	Characterization Identification	Untargeted profiling Factor analysis (FA)	[49]
Walnut septum (75 polyphenols)	Liquid-Solid Extraction (LSE) 1 g sample in 15 mL 70% methanol aqueous solution	UHPLC-ESI-HRMS/MS LC conditions: C18 column (100×2.1 mm, 2.6 µm) Gradient elution: (A) 1% formic acid aqueous solution and (B) acetonitrile Flow-rate 0.5 mL/min MS conditions: Orbitrap H-ESI: -3 kV H-ESI: +3.5 kV Full MS scan mode (*m/z* 100–1000).	Characterization Identification	Untargeted profiling	[50]
Fresh and dried cranberries and lingonberries (14 polyphenols)	Liquid-Solid Extraction (LSE) 1 g fresh sample with 5 mL methanol 1 g dried sample with 0.9 mL water and then 9.5 mL methanol	UHPLC-ESI-HRMS/MS LC conditions: HSS T3 C18 column (100×2.1 mm, 1.8 µm); Gradient elution: (A) 0.1% formic acid aqueous solution with 5mM ammonium formate and (B) 0.1% formic acid methanol solution with 5mM ammonium formate. Flow-rate 0.4 mL/min MS conditions: Q-TOF ESI: -4.5 kV ESI: +5 kV Full MS scan mode (*m/z* 100–1200). Resolution: 40,000 fwhm	Characterization Classification Authentication	Target profiling PCA	[51]
Fresh and high pressure processed carrot juices (25 polyphenols)	Liquid–liquid extraction (LLE) 5 mL sample with 5ml 80% methanol aqueous solution acidified with 0.1% HCl	UHPLC-ESI-HRMS/MS LC conditions: C18 column (50×2.1 mm, 1.7 µm); Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid methanol solution. Flow-rate 0.4 mL/min MS conditions: Q-TOF ESI: -4.5 kV Independent data analysis (IDA) mode (*m/z* 80–1200).	Characterization	Untargeted profiling	[41]
Paprika powder (53 polyphenols)	Liquid-Solid Extraction (LSE) 0.3 mg sample with 3mL of 80% acetonitrile aqueous solution	UHPLC-ESI-HRMS/MS LC conditions: Ascentis Express C18 porous shell column (150×2.1 mm, 2.7 µm); Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid acetonitrile solution. Flow-rate 0.3 mL/min MS conditions: Q-Orbitrap H-ESI:-2.5 kV Full MS scan mode (*m/z* 100–1500). Resolution: 70,000 fwhm	Classification Authentication	Targeted profiling PCA PLS-DA	[52]

Sample (compounds)	Sample extraction	LC-HRMS conditions	Application	Data Analysis	Ref.
Phalsa fruit pulp (50 polyphenols)	Liquid-Solid Extraction (LSE) 5 g sample with 20 mL of 1% formic acid methanol solution. Then, solid phase extraction (SPE) with 5 mL of 1% formic acid methanol solution.	UHPLC-ESI-HRMS/MS *LC conditions:* Acquity BEH C18 column (100×2.1 mm, 1.8 μm); Gradient elution: (A) 10% methanol aqueous solution and (B) 0.1% formic acid methanol aqueous solution (90:10, v/v). Flow-rate 0.4 mL/min *MS conditions:* Q-TOF ESI: +3.5 kV Full MS scan mode (*m/z* 100–1500). Resolution: 20,000 fwhm	Characterization Identification	Untargeted profiling	[39]
Hazelnuts (462 polyphenols tentatively identified)	Liquid-Solid Extraction (LSE) 1 g sample in 10 mL of 70% methanolic solution with 0.1% formic acid	UHPLC-ESI-HRMS/MS *LC conditions:* Zorbax Extend-C18 (75×2.1 mm, 1.8 μm); Gradient elution: (A) water and (B) methanol. *MS conditions:* Q-TOF ESI: -3.5 kV Full MS scan mode (*m/z* 100–1000).	Characterization Classification Authentication	Untargeted profiling HCA OPLS-DA	[53]
Hemp (147 polyphenols)	Liquid-Solid Extraction (LSE) Clean-up 0.25 g sample with n-hexane, extraction with 10 mL of 70% acetone aqueous solution acidified with 0,5% acetic acid	UHPLC-ESI-HRMS/MS *LC conditions:* Kinetex core-shell C18 column (100×2.1 mm, 2.6 μm); Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid acetonitrile solution. Flow-rate 0.6 mL/min *MS conditions:* Q-Orbitrap H-ESI: -2.5 kV H-ESI: +3.5 kV Full MS scan mode: Flavonoids/phenolic acids (*m/z* 150–1000) Tannins:(*m/z* 300–2000). Top 5 DDA acquisition mode Resolution: 70,000 fwhm	Characterization Identification	Untargeted profiling	[54]
Peanuts (58 polyphenols)	Liquid-Solid Extraction (LSE) 0.5 g sample with 4 mL 60% acetone aqueous solution	HPLC-ESI-HRMS/MS *LC conditions:* Atlantis T3 column (100×2.1 mm, 3 μm); Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid acetonitrile solution. Flow-rate 0.35 mL/min *MS conditions:* LTQ-Orbitrap ESI negative mode Full MS scan mode (*m/z* 100–1000). Resolution: 30,000 fwhm	Characterization Classification Authentication	Untargeted profiling HCA PCA	[55]
Custard Apple peel, seed and pulp fruit (85 polyphenols)	Liquid-Solid Extraction (LSE) 5 g sample with 15 mL 80% ethanol solution	HPLC-ESI-HRMS/MS *LC conditions:* Synergi Hydro-RP C18 column (250×4.6 mm, 4 μm); Gradient elution: (A) 0.5% acetic acid aqueous solution and (B) 0.5% acetic acid acetonitrile aqueous solution. (50:49.5 v/v) Flow-rate 0.8 mL/min *MS conditions:* Q-TOF ESI: -3.5 kV ESI: +3.5 kV Full MS scan mode (*m/z* 50–1300).	Characterization Classification	Untargeted profiling HCA	[56]
Raspberries and black-raspberries (68 polyphenols)	Liquid-Solid Extraction (LSE) 500 mg sample with 5 mL of acetone/10 mM sodium fluoride water/acetic acid (70:29.7:0.3, v/v/v)	UHPLC-ESI-HRMS/MS *LC conditions:* Acquity BEH C18 column (150×2.1 mm, 1.7 μm); Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 5% formic acid methanol solution. Flow-rate 0.45 mL/min *MS conditions:* Q-TOF ESI: -4.5 kV ESI: +5 kV Full MS scan mode (*m/z* 100–1000)	Characterization Classification Authentication	Untargeted profiling PCA	[43]
Tomato pomace, peel and seeds (40 polyphenols)	Liquid-Solid Extraction (LSE) 1 g sample with 30 mL of 80% methanol solution	UHPLC-ESI-HRMS/MS *LC conditions:* EC Poroshell 120 C18 column (150×3 mm, 2.7 μm); Gradient elution: (A) 0.5% formic acid aqueous solution and (B) 50% acetonitrile and methanol solution. Flow-rate 0.5 mL/min *MS conditions:* Q-TOF ESI: -2.5kV Full MS scan mode (*m/z* 40–1000)	Characterization Identification	Untargeted profiling	[57]
Banana pulp and peel (24 polyphenols)	Liquid-Solid Extraction (LSE) 10 g sample with 30mL of 70% ethanol aqueous solution and potassium metabisulfite (to stop enzymatic browning reactions)	HPLC-ESI-HRMS/MS *LC conditions:* Synergi Hydro-RP C18 column, (250×4.6 mm, 4.0 μm); Gradient elution: (A) 0.1% formic acid aqueous solution and (B) 0.1% formic acid acetonitrile aqueous solution. (95:5 v/v) Flow-rate 0.6 mL/min *MS conditions:* Q-TOF ESI: Negative and positive modes Full MS scan mode (*m/z* 50–900).	Characterization Classification	Untargeted profiling PCA	[44]

Regarding the employed liquid chromatographic techniques, the percentage of publications using ultra-high pressure liquid chromatography methods in combination with high-resolution mass spectrometry (UHPLC-HRMS) reached 75%, somehow expected in order to get fully advantage of the HRMS detection by also improving separation column efficiency with UHPLC. Following the same trend that with LC-MS methodologies, the use of reversed-phase separations with C18 columns continues being the preferred LC mode, but the use of columns with fused-core particles increased noticeably [42,46,52,54,57], allowing to obtain a performance similar to the UHPLC but at lower pressures. The other publications reporting the use of conventional HPLC systems, addressed the separation of polyphenols with improved high-polar C18 columns like T3 [40,55] or Hydro-RP [44,49,56]. As can be seen in Table 7.1, the mobile phases employed with LC-HRMS methodologies are almost the same as the previously ones described for LC-MS, acidified aqueous or buffer aqueous solutions and methanol or acetonitrile as the organic component.

As for the the mass spectrometry conditions, electrospray ionization (ESI) is the major ionization method as expected, but most of the authors used the variant heated-electrospray H-ESI, which has better ionization efficiency because of the increased temperature in the ionization source [42,45,46,50,52,54]. Both, positive and negative ionization mode are also described, especially in the works that untargeted polyphenolic profiling is addressed. Concerning the mass analyzers employed in LC–HRMS methods, basically authors used Orbitrap [38,50] or Time-of-Flight (TOF) instruments, as well as their hybrid quadrupole variations Q-Orbitrap [42,45,46,52,54] and Q-TOF, respectively. In the case of the Orbitrap, a hybrid configuration combining an ion-trap analyser with the Orbitrap is also available (the LTQ-Orbitrap), which has also been described for the determination of polyphenols in food products. Juliano et al. [55] used LTQ-Orbitrap to find out that those peanut cultivars with better drought tolerance have higher content of flavonoids than other peanuts genotypes. In general, analyses are performed in full scan mode by employing high-resolution, with normally is in the range of 10,000-40,000 fwhm when TOF instruments are employed, and higher (30,000 to 70,000 fwhm) when Orbitrap instruments are used. Koley et al. [39] by employing Q-TOF at resolution of 20,000 fwhm detected 50 polyphenols in phalsa fruits, much more than detected in previous studies using LC-MS methods, allowing to consider this fruit as a good source for nutraceuticals and not just for pigments.

Other acquisition conditions designed for fragmentation studies useful for the non-targeted identification of polyphenols are also proposed such as data dependent analysis (DDA) [42,54] or independent data analysis (IDA) [41,48]. However, the fundamentals of these HRMS fragmentation acquisition modes will not be addressed in this work. Cerrato et al. [54] worked with full scan mode to find the five most intense mass signals and then applied DDA to obtain fragmentation patterns in order to discover untargeted polyphenols in hemp samples.

Finally, regarding LC-HRMS applications, as previously mentioned in LC-MS, most of the proposed methodologies requires of the use of chemometric techniques in order to process the huge amount of data obtained when dealing with this kind of methodologies. Among the most frequently employed chemometric methodologies, once more can be found principal component analysis (PCA), hierarchical cluster analysis (HCA) and partial least squares regression-discriminant analysis (PLS-DA) or OPLS-DA (orthogonal projections to latent structures discriminant analysis) methods for characterization, classification or authentication purposes. As an example, Barbosa et al. [58] classified paprika from different protected designation of origins (PDO). While previous studies using LC-MS methods were not good enough to discriminate La Vera POD, LC-HRMS with PLS-DA analysis allowed an acceptable classification. Figure 7.2 shows the validation for the proposed method classifying 120 paprika samples from three regions. In each graphic there is a different target of paprika class against the other two. The

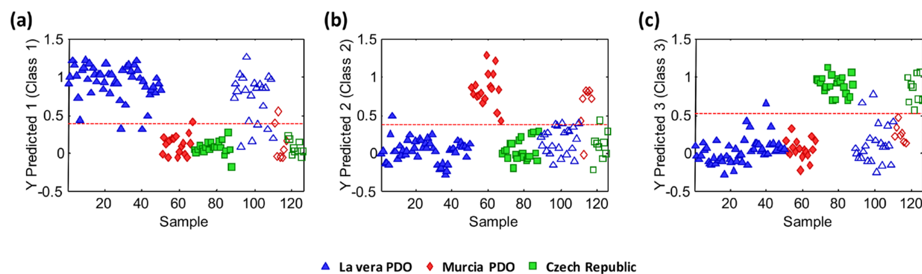

Figure 7.2 PLS-DA classification plots according to the production region. (a) La Vera PDO vs. other classes; (b) Murcia PDO vs. other classes; (c) the Czech Republic vs. other classes. Reproduced from Open Access reference [58].

dashed line defines the classification boundary, so the samples matching the targeted class were located at the top, and those belonging to the other types were at the bottom. Samples used for prediction are drawn with unfilled symbols while samples used for calibration are the

filled ones. Czech samples are 100% classified (all prediction samples matching the targeted class) while La vera drops to 82%, but considering the type and the variability of samples this classification rate was considered acceptable.

Hurkova et al. [51] used LC-HRMS for polyphenol fingerprinting and handled data combining PCA and PLS-DA methods to find selective markers which allows discriminating cranberries in valuable lingonberries mixtures. In Figure 7.3, PCA showed that the two groups of vaccinium species, lingonberries and cranberries, were evidently separated in the scores plot. Then, a supervised analysis by PLS-DA was arranged to create a statistical model to allow classification of vaccinium samples. PLS-DA in Figure 7.3 provided enough discrimination between cranberries and lingonberries and helped finding two glycosylated flavonoids only present at cranberries but not at lingonberries, thus their screening allowed to detect the addition of cranberries to lingonberries down to 1% (w/w). The variability in cranberries plot is thought to be because of differences in cranberries cultivars.

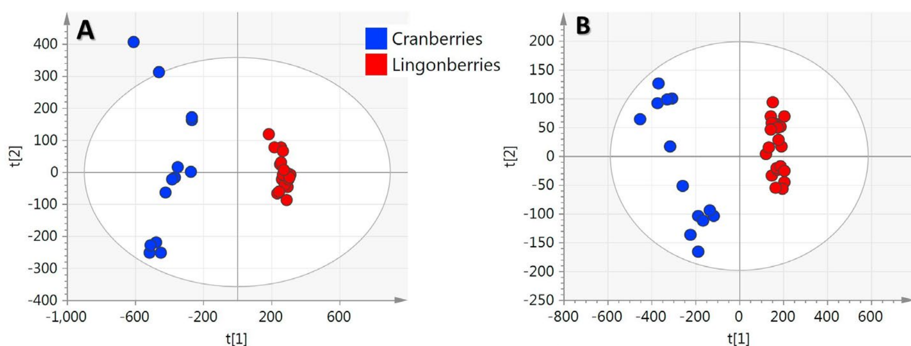

Figure 7.3 Scores in positive ionization mode for plot PCA (A) and PLS-DA (B). Adapted with permission from reference [51]. Copyright (2019) Elsevier Ltd.

8. CONCLUSIONS

In this work, the role of liquid chromatography and mass spectrometry techniques in the characterization and determination of phenolic compounds in food has been addressed through a selection of studies published in the last seven years. Literature research has been carried out focusing on conventional and high-resolution mass spectrometry methods and results were summarized in tables by highlighting type of samples, instrumental separation and detection conditions, the applications and the data analysis.

Regarding the type of samples and polyphenols extraction, for liquid nature samples, LLE and direct analysis methods were used while solid nature samples were extracted by LSE. For both liquid and solid samples SPE and dSPE methods were used as a cleaning-up, concentration or phenolic extraction purposes. Concerning liquid chromatography, authors using LC-MS methods have chosen HPLC while those using LC-HRMS mostly worked with UHPLC. Both methods used C18 columns as a stationary phase and its commercial improved versions, but in LC-HRMS the use of superficially porous particles instead of conventional full porous increased significantly. Principal mobile phase solvents were formic acid aqueous solution and formic acid acetonitrile solution as eluents A and B, respectively. In terms of MS conditions, ESI and H-ESI are the preferred ionization techniques, in negative mode for MS but both negative and positive modes for HRMS. QqQ instrument is the most used in conventional MS but also IT and QTrap, all in MRM acquisition mode. For HRMS, Orbitrap, TOF and their hybrid quadrupole versions are the instruments selected working in full scan acquisition mode. More specific acquisition modes like IDA and DDA were also described. Lastly, characterization and determination with targeted polyphenols are used in MS publications while untargeted analysis were carried out in HRMS. Both techniques used chemometric methods like PCA, HCA and PLS-DA to classify and authenticate samples.

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10. ACRONYMS

API	Atmospheric Pressure Ionization
APCI	Atmospheric Pressure Chemical Ionization
APPI	Atmospheric Pressure Photo Ionization
C18	Chloro-(dimethyl)-octadecylsilane
CAS	Chemical Abstracts Service
DAD	Diode Array Detector
DDA	Data Dependent Analysis
ESI	Electrospray Ionization
dSPE	Dispersive Solid Phase Extraction
FWHM	Full-Width at Half-Maximum
GC	Gas Chromatography
HCA	Hierarchical Cluster Analysis
H-ESI	Heated Electrospray Ionization
HMS	Hybrid Mesostructured Silica
HRMS	High-Resolution Mass Spectrometry
HPLC	High-performance Liquid Chromatography
IDA	Independent Data Analysis
IT	Ion-Trap
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LRMS	Low-Resolution Mass spectrometry
LLE	Liquid-Liquid Extraction

LSE	Liquid-Solid Extraction
LTQ-Orbitrap	Linear Trap hybrid Quadrupole-Orbitrap
MRM	Multiple Reaction Monitoring
NIR	Near-Infrared Radiation
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
PCA	Principal Component Analysis
PDO	Protected Designation of Origins
PFP	Penta-Fluoro Phenylpropyl
PLS-DA	Partial Least Squares regression Discriminant Analysis
Q-Orbitrap	Hybrid Quadrupole-Orbitrap
Q Trap	Hybrid Quadrupole with Linear Ion Trap
QqQ	Triple Quadrupole
Q-TOF	Hybrid Quadrupole- Time-of-Flight
ROS	Reactive Oxygen Species
SDG	Sustainable Development Goals
SPE	Solid Phase Extraction
SRM	Selective Reaction Monitoring
TOF	Time-of-Flight
TPC	Total Polyphenol Content
UHPLC	Ultra-High Performance Liquid Chromatography
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet