Advances in the Optimization of Chromatographic Conditions for the Separation of Antioxidants in Functional Foods

Natasa P. Kalogiouri, Victoria F. Samanidou*

ABSTRACT
Antioxidants represent a class of naturally occurring molecules that are responsible for several health properties. Antioxidants are present in various natural products and food matrices, typically known as “functional foods”. Because of their high nutritional value and antioxidant activity, functional foods gain considerable attention, in recent years. Phenolic compounds are the most abundant class of antioxidants and Liquid Chromatography (LC) is the main technique used for their separation. Conventional High-Pressure Liquid Chromatography provides insufficient resolving power and requires long analysis times. New techniques like ultra-high-pressure liquid chromatography (UHPLC), in reversed-phase (RP) or hydrophilic interaction (HILIC) mode, and the development of multidimensional methods prove promising with increasing applications for phenolic analysis. The aim of this review is to provide an updated overview of the applications of recent advances in LC phenolic separation of antioxidants in functional within the period 2015-2019.


1.0 Introduction
Current trends in the food industry focus on the production of innovative functional foods, which possess a significant place in modern diet [1]. Still, there is not an official definition for functional foods, but under convention the term describes foods that have been designed in such a way by the industry, with the contribution of chemistry and nutrition science, to provide health benefits [2,3]. The main objective is, of course, to create innovative products that are distinguished for their appearance, texture and special organoleptic characteristics in order to attract consumers’ interest. There are two categories of functional foods, those that are physically functional and those with added value, formulated from natural constituents in order to target certain physical and biological functions [4]. Food constituents with functional properties are studied and extracted in order to be used as physical additives, providing both advanced technological properties and health benefits [5,6]. Among physically functional, plant-based foods such as seeds, olive drupes and olive oil, honey, nuts, grapes, and berries are rich in antioxidants and have supported clinical health claims [7,8]. Between natural antioxidants, phenolic...
antioxidants gain particular interest since these compounds inhibit free radical formation and/or interrupt propagation of autoxidation [9]. At laboratory scale, different separation techniques have been developed for the determination of phenolic antioxidants from food matrices, commonly known as “bioactive phenolic compounds”. The general term “bioactive” describes the naturally occurring physical constituents of foods presenting antioxidant activity and have the capacity to modulate metabolic processes demonstrating beneficial effects, such as induction and inhibition of enzymes and gene expression, providing protection against many diseases, including cancer [10-12]. Bioactive compounds vary in function and chemical structure and thus, are grouped accordingly. Among bioactive compounds, polyphenols and simple phenols are the most abundant antioxidants and gain particular attention, since they have been associated with numerous nutritional and functional properties [13,14]. They contribute to the organoleptic properties, such as aroma, taste, and color, showing antioxidant activity, as well [15].

The analysis of phenolic antioxidants remains challenging because of their structural diversity. Different analytical techniques have been developed for the separation of phenolic compounds in foodstuffs. Liquid chromatography (LC) is the technique of choice, employed for the separation of phenolic compounds in functional food matrices [16,17]. Because of the complexity of the matrices, many efforts have been made for the optimization of the analytical LC methodologies to achieve separation. Gas chromatography (GC) is widely used in applications in food analysis, as well. This review gathers all the recent advances concerning the analytical separation of phenolic antioxidant compounds in functional foods within the period 2015-2019.

1.1 Search/Selection Criteria
Taking into consideration the emerging need for the development of analytical methodologies that aim at the successful separation of antioxidant compounds, this review summarizes all recent advances in the separation of phenolic antioxidant compounds from food matrices between the years 2015-2019. This time-frame was selected after examining the literature and realizing that Pyrzynska et al. [16] have sum-
marized the analytical developments in the LC separation of phenolic food compounds up to 2015. In the meantime, Capriotti et al. published [18] a review article summarizing all advances in LC and GC methodologies up to 2015. All the LC and GC methodologies that were published after that and have not yet been reviewed, were gathered and discussed in this mini review article. As it is illustrated in Figure 1, the majority of the research papers use LC methodologies for the separation of the phenolic compounds in food matrices, instead of GC. This could be easily explained by the polarity of the phenolic compounds, which need to undergo derivatization before GC analysis, as it is described in the following sections.

2.0 Discussion

2.1 Liquid Chromatography

Low and high molecular weight phenolic compounds can be separated by LC, since it provides a wide number of possible combinations between the stationary and mobile phases [19, 20]. However, conventional chromatographic methods, even if they use High Pressure Liquid Chromatography (HPLC), are considered tedious, time consuming and analysis usu-
ally lasts longer than 30 min. Thus, it is essential to imply a target strategy in order to develop rapid and efficient procedures to perform qualitative and quantitative analysis within reduced analysis times. The use of UHPLC gains interest over conventional HPLC, since the chemistry of HPLC columns and dimensions affect resolution and sensitivity [21]. The employment of sub-2 \( \mu m \) particle stationary phases in UHPLC provides narrower peaks, lower detection limits and rapid analysis times [22,23]. Moreover, it decreases the volumes of solvents and waste. The separation that takes place is based on the interactions of the sample with the stationary and mobile phases. Since many different stationary and mobile phase combinations that can occur for the separation of a mixture, the LC modes that can be applied for the separation of phenolic compounds in functional foods are also many, including HPLC and UHPLC in reversed phase (RP) or hydrophilic interaction (HILIC) mode, and multidimensional chromatographic systems, as they are described in the following sections.

### 2.1.1 High Pressure Liquid Chromatography

The basic principle of HPLC is that it relies on the different polarities of the constituents of a sample in order to separate them. It is different from standard column chromatography since it requires pressure in order to force the solution pass through the column. HPLC is widely used for the separation of phenolic compounds employing RP-C18 analytical columns. The mobile phase consist of an aqueous solution and an organic solvent, followed by detection with UV, diode array (DAD) or tandem mass spectrometry (MS/MS) [16]. The developments and applications of RP-HPLC methodologies in the separation of phenolic compounds in foods and beverages have been reviewed up to 2014 [16]. Ouchemoukh et al. [24] developed an RP-HPLC-analytical methodology coupled to electrospray time-of-flight mass spectrometry (ESI-TOF-MS) and achieved successful separation for 35 phenolic compounds in honeys, that belonged to different chemical classes, such as phenolic acids, flavonoids and phenyl alcohols (Figure 2) using a Rapid Resolution Liquid Chromatography system (RRLC) equipped with a vacuum degasser, autosampler, binary pump and thermostated column department. They employed an RP-C18 analytical column (4.6 x 150 mm, 1.8 \( \mu m \) particle size, ZORBAX Eclipse plus) and the eluting mobile phase, consisted of A: 0.5 mL/100 mL of acetic acid in deionised water and B: methanol (MeOH), pumped at 1.2 mL/min, applying a multi-step linear gradient, as it is described in Table 1 and the column temperature was maintained at 35°C [24]. Despite the effective separation of the target analytes, the method is time consuming with each analysis run lasting for 40 min. Can et al. [25] developed an HPLC methodology coupled with a UV-Vis detector in 280 and 315 nm, for the determination of 18 phenolic compounds in different type Turkish honeys using an RP Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 \( \mu m \)), thermostated at 30°C and a gradient program with two solvents (A: 80% acetonitrile (ACN) in MeOH, B: 2% acetic acid in distilled water). All targeted compounds were separated with this methodology, but analysis is time consuming. Thus, the major short-comings of RP-HPLC methodologies are that it requires long elution times and presents reduced selectivity for the separation of co-eluting compounds. Moreover, because of its speed and its reliance on the polarities of the phenolic acids (Figure 2a), flavonoids and their glycosides (Figure 2b), compounds with structural similarity and polarities can possibly exit the chromatographic system nearly at the same or exactly at the same time. Another weakness is adsorption. Since HPLC typically uses glass columns packed with beads of different materials that may bind with the chemicals of the sample mixtures that pass through the column [26]. The increasing need for better chromatographic performance and high throughput resulted in the development of advanced chromatographic techniques, such as UHPLC and HILIC. In addition, multidimensional chromatography shows excellent analytical performance, for the analysis of complex mixtures, increasing peak capacity.

### 2.1.2 Ultrahigh Pressure Liquid Chromatography

UHPLC is the advanced version of HPLC, using narrow-bore columns that are packed with very small particles (< 2 \( \mu m \)), operating in high pressures. UHPLC systems present high sensitivity, improved resolution, and short time analysis compared to conventional HPLC [6]. Decreasing the size of particles results in gaining higher peak efficiency [21]. Consequently, the sensitivity is higher with narrow peaks and low detection limits (LODs), decreasing the volumes of organic solvents. For the analysis of phenolic constituents in functional foods, RP-C18 columns are frequently used. Vasic et al. [26] performed separation on a Syncronis C18 column (100 x 2.1 mm, 1.7 \( \mu m \) particle size). The mobile phase consisted of (A) water with 0.1% acetic acid and (B) 100% ACN. [27]. An RP-C18 Poroshell120 column (3.0 mm x 100 mm, 2.7 \( \mu m \)) was used for the separation of 11 phenolic acids and flavonoids in barley samples in a UHPLC-PDA and UHPLC-QTOF-MS chromatographic system [28]. UHPLC are applied
<table>
<thead>
<tr>
<th>Functional Food</th>
<th>Compounds</th>
<th>Chromatographic Separation Technique</th>
<th>Analytical Column</th>
<th>Column Temperature</th>
<th>Mobile Phases</th>
<th>Detection</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honey</td>
<td>35 phenolic acids and flavonoids</td>
<td>HPLC</td>
<td>RP-C18 ZORB-BAX Eclipse (4.6 x 150 mm, 1.8 µm)</td>
<td>35°C</td>
<td>A: 0.5 mL /100 mL of acetic acid in deionised water B: MeOH (flow rate: 1.2 mL/min, gradient: 0 min, 5 % B; 30 min, 76.1 % B; 33 min, 100 % B; 34 min, 5 % B)</td>
<td>ESI-TOF-MS/MS</td>
<td>[4]</td>
</tr>
<tr>
<td>Honey</td>
<td>18 phenolic acids and flavonoids</td>
<td>HPLC</td>
<td>RP-C18 Zorbax Eclipse XDB column (4.6 x 150 mm, 5 µm)</td>
<td>30°C</td>
<td>A: 80% ACN in MeOH, B: 2% acetic acid in distilled water (flow rate: 1 mL/min, gradient: 0–2 min, 95% B; 2–8 min, 95–90% B; 8–11 min, 90–85% B; 11–13 min, 85–75% B; 13–17 min, 75–70% B; 17–30 min, 70–65% B; 30–33 min, 65–0% B; 33–38 min, 0–0% B; 38–40 min 95% B; 40–48 min, 95% B)</td>
<td>PDA</td>
<td>[25]</td>
</tr>
<tr>
<td>Honey</td>
<td>52 phenolic acids and flavonoids</td>
<td>UHPLC</td>
<td>RP-C18 Synchromis column (100 x 2.1 mm, 1.7 µm)</td>
<td>30°C</td>
<td>(A) water with 0.1% acetic acid and (B) 100% ACN (flow rate: 0.3 mL/min, gradient: 0.0–1.0 min 5% B, 1.0–16.0 min from 5% to 95% (B), 16.0–16.1 min from 95% to 5% (B) and then 5% (B) for 4 min)</td>
<td>Orbitrap-MS/MS</td>
<td>[27]</td>
</tr>
<tr>
<td>Seed (Barley)</td>
<td>11 phenolic acids and flavonoids</td>
<td>UHPLC</td>
<td>RP-C18 Poroshell 120 column (3.0 x 100 mm, 2.7 µm)</td>
<td>30°C</td>
<td>(A) deionized water and 0.1% acetic acid (B) ACN with 0.1% acetic acid (flow rate: 0.38 mL/min, gradient: 0-1 min, 0-10%; B; 1-5 min, 10-26%; B; 5-7.5 min, 26-35%; B; and 7.5-13 min, 35-100%)</td>
<td>PDA</td>
<td>[28]</td>
</tr>
<tr>
<td>Wine</td>
<td>251 phenolic acids, flavonoids, lignans and polyphenols</td>
<td>UHPLC</td>
<td>RP-C18 Knauer Blue-Orchid column (2 mm x 100 mm, 1.8 µm)</td>
<td>30°C</td>
<td>(A) deionized water with 0.1% acetic acid and 5mM ammonium formate (B) MeOH with 0.1% acetic acid and 5mM ammonium formate (flow rate: 0.22 mL/min, gradient: started from 5% MeOH to 90% MeOH within 35 min)</td>
<td>QTOF-MS/MS</td>
<td>[29]</td>
</tr>
<tr>
<td>Table olives</td>
<td>11 secoiridoids, phenylethanois and their glucosides</td>
<td>UHPLC</td>
<td>RP-C18 Poroshell 120 column (3.0 x 50 mm, 2.7 µm)</td>
<td>20°C</td>
<td>(A) deionized water with 0.01% acetic acid (B) ACN with 0.01% acetic acid (flow rate: 0.7 mL/min, gradient 10% B, 0-2 min; 10 30% B, 2-3 min; 30 65% B, 3-5 min)</td>
<td>LC-MS/MS</td>
<td>[30]</td>
</tr>
<tr>
<td>Functional Food</td>
<td>Compounds</td>
<td>Chromatographic Separation Technique</td>
<td>Analytical Column</td>
<td>Column Temperature</td>
<td>Mobile Phases</td>
<td>Detection</td>
<td>Ref.</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------</td>
<td>-------------------------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>--------------</td>
<td>-----------</td>
<td>-----</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>36 phenolic compounds (phenolic acids, lignans, flavonoids, seco-roidoids) and free fatty acids</td>
<td>UHPLC</td>
<td>RP-C18 Acclaim RSLC column (2.1 x 100 mm, 2.2 µm) with a pre-column of ACQUITY UPLC BEH C18 (1.7 µm), Van Guard Pre-Column.</td>
<td>30°C</td>
<td>(A) 90% water, 10% MeOH and 5 mM ammonium formate, (B) 100% MeOH and 5 mM ammonium formate. (flow rate 0.2 mL/min, gradient: 1% of (B) for 1 min, increasing to 39 % for the next 2 min, and increasing to 99.9 % (flow rate 0.4 mL/min) in the following 11 min, at a flow rate 0.48 mL/min</td>
<td>ESI-QTOF-MS/MS</td>
<td>[32]</td>
</tr>
<tr>
<td>Goji berry</td>
<td>29 phenolic acids, flavonoids, hydroxycinnam-ics, alkylphenols, stilbenes, lignans, phenylethanoids</td>
<td>UHPLC</td>
<td>RP-C18 Agilent Zorbax eclipse plus (50 x 2.1 mm, 1.8 µm)</td>
<td>30°C</td>
<td>(A) water (B) MeOH</td>
<td>ESI-QTOF-MS/MS</td>
<td>[31]</td>
</tr>
<tr>
<td>Tea</td>
<td>42 phenolic acids, flavonoids and phenylethanoids</td>
<td>UHPLC</td>
<td>RP-C18 column BEH SHIELD (3.0 x 150 mm, 1.7 µm)</td>
<td>40°C</td>
<td>(A) water with 0.1% formic acid and (B) ACN with 0.1% formic acid. (gradient (min/%A): 0.0/100, 9.91/74, 18.51/35, 18.76/0, 23.76/0, 23.88/100, 26.00/100)</td>
<td>PDA MS/MS</td>
<td>[34]</td>
</tr>
<tr>
<td>Pistachios</td>
<td>58 phenolic acids, gallocateins and flavonoids</td>
<td>HPLC</td>
<td>- Kinetex RP-C18 core-shell (250 mm) with a SecurityGuard Ultra C18 guard column (4.6 x 2 mm)</td>
<td>35°C</td>
<td>(A) water with 1% (v/v) formic acid (B) MeOH with 1% (v/v) formic acid (gradient: isocratic at 2% B for 10 min, from 2 to 37% B in 27 min, isocratic at 37% B for 5 min, from 37 to 40% B in 18 min, from 40 to 60% B in 10 min, from 60 to 100% B in 20 min, isocratic at 100% for 14 min, from 100 to 2% B in 1 min, and isocratic at 2% B for 7 min) -UHPLC gradient: isocratic at 2% B for 1 min, from 2 to 37% B in 7 min, from 37 to 40% B in 5.2 min, from 40 to 100% B in 4.8 min, isocratic at 100% for 6 min, from 100 to 2% B in 1 min, and isocratic at 2% B for 5 min. Total run time was 30 min at a flow rate of 0.3 mL/min</td>
<td>DAD-ESI-MS/MS DAD-ELSD</td>
<td>[35]</td>
</tr>
<tr>
<td>Functional Food</td>
<td>Compounds</td>
<td>Chromatographic Separation Technique</td>
<td>Analytical Column</td>
<td>Column Temperature</td>
<td>Mobile Phases</td>
<td>Detection</td>
<td>Ref.</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------</td>
<td>-------------------------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>--------------</td>
<td>-----------</td>
<td>------</td>
</tr>
<tr>
<td>Seeds (poppy, sunflower, pumpkin, sesame and flaxseed)</td>
<td>463 flavonoids, phenolic acids, lignans and stilbenes</td>
<td>UHPLC</td>
<td>RP-C18 Knauer BlueOrchid column (100 2 mm, 1.8 µm particle size).</td>
<td>30°C</td>
<td>(A) water with formic acid and ammonium formate and ammonium formate (gradient: 5% B to 95% B within 34 min; flow rate: 220 µL/min)</td>
<td>ESI-QTOF-MS/MS</td>
<td>[36]</td>
</tr>
<tr>
<td>Potatoes (peels)</td>
<td>55 polyphenols</td>
<td>UHPLC</td>
<td>RP-C18 (100 x 2.1 mm, 1.8 µm)</td>
<td>30°C</td>
<td>(A) water with 0.1% formic acid and (B) ACN with 0.1% formic acid (gradient: 0–1 min, 2% B; 1–2.5 min, 10% B; 2.5–6 min, 15% B; 6–7.5 min, 50% B; 7.5–9.5 min, 98% B and 9.5–10 min, 2% B at a rate of 0.5 mL/min)</td>
<td>ESI-MS/MS</td>
<td>[40]</td>
</tr>
<tr>
<td>Saffron</td>
<td>5 phenolic acids and flavonoids</td>
<td>UHPLC</td>
<td>RP-C18 Zorbax (150 x 4.6 mm, 5 µM)</td>
<td>30°C</td>
<td>(A) water with 0.1% formic acid and 5 mM ammonium formate and 5 mM ammonium formate, (gradient: 10% B to 90% B from 0.01 min to 8 min, hold for 3 min and back to 10% B in 0.1 min and re-equilibrated for 5 min)</td>
<td>ESI-MS/MS</td>
<td>[41]</td>
</tr>
<tr>
<td>Beverage from strawberries</td>
<td>64 phenolic acids, flavonoids and tannins</td>
<td>UHPLC</td>
<td>RP-C18 Zorbax SB (2.1 x100 mm 1.8 µm)</td>
<td>30°C</td>
<td>(A) water with 0.1% formic acid and (B) MeOH with 0.1% formic acid (flow rate: 0.4 mL/min, gradient: 0.0–1.0 min 5% B, 123 1.0–12.0 min from 5% to 95% (B), 12.0–12.1 min from 95% to 5% (B), then 5% (B) for 3 min)</td>
<td>MS/MS</td>
<td>[38]</td>
</tr>
<tr>
<td>Berries (skin, pulp and seeds)</td>
<td>22 phenolic acids, flavonoids and coumarins</td>
<td>UHPLC</td>
<td>RP-C18 Hypersil Gold (100 x 2.1 mm, 1.9 µm)</td>
<td>40°C</td>
<td>(A) water with 0.2% formic acid (B) ACN (flow rate: 0.4 mL/min, gradient: 0.0–2.0 min 5% B, 2.0–12.0 min from 5% to 95% (B), 12.0–12.1 min from 95% to 5% (B), then 5% (B) for 3 min)</td>
<td>DAD-MS Orbitrap-MS/MS</td>
<td>[37]</td>
</tr>
<tr>
<td>Functional Food</td>
<td>Compounds</td>
<td>Chromatographic Separation Technique</td>
<td>Analytical Column</td>
<td>Column Temperature</td>
<td>Mobile Phases</td>
<td>Detection</td>
<td>Ref.</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------</td>
<td>-------------------------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>--------------</td>
<td>----------</td>
<td>-----</td>
</tr>
<tr>
<td>Berries</td>
<td>60 phenolic acids and flavonoids</td>
<td>UHPLC</td>
<td>RP-C18 Zorbax Eclipse Plus column (2.1 mm × 100 mm, 1.8 µm)</td>
<td>35°C</td>
<td>(A) water with 0.025% acetic acid (B) ACN with 0.025% acetic acid (flow rate: 0.4 mL/min, gradient: 0.3 min, 2 15% B; 0.3 4.9 min, 15 50% B; 4.9 5.4 min, 50 100% B; 5.4 6.1 min, 100 B; 6.1 8.0 min, 100 2% B)</td>
<td>MS/MS</td>
<td>[39]</td>
</tr>
<tr>
<td>Supplements (36 different vegetables, fruits and berries)</td>
<td>119 phenolic acids, flavonoids and anthocyanins</td>
<td>UHPLC</td>
<td>RP-C18 Blue-Orchid (50 x 2 mm, 1.8 µm)</td>
<td>30°C</td>
<td>(A) 0.1% formic acid ACN (B) 0.1% aqueous formic acid. (anthocyanins: flow-rate of 0.3 mL/min, 2 min of 5% solvent A in B, the proportion of A was increased linearly to 30% over a period of 10 min, followed by 3 min of 80% solvent A and then 5 min at the start conditions to re-equilibrate the column, phenolics: flow rate of 0.3 mL/min, 5% A for 3 min, changing linearly from 5 to 40% A in B over 9 min. After 4 min of 80% solvent A, the column was re-equilibrated for 5 min)</td>
<td>MS/MS</td>
<td>[42]</td>
</tr>
<tr>
<td>Berries</td>
<td>Anthocyanins and total phenolics</td>
<td>UHPLC</td>
<td>RP-C18 (Acquity UPLC BEH C18, (2.1 x 100 mm and 1.7 µm particle size)</td>
<td>30°C</td>
<td>(A) water with 2% formic acid (B) MeOH (flow rate: 0.4 mL/min, gradient: 0 min, 15% B; 3.30 min, 20% B; 3.86 min, 30% B; 5.05 min, 40% B; 5.35 min, 55% B; 5.64 min, 60% B; 5.94 min, 95% B; 7.50 min, 95% B)</td>
<td>QTOF-MS</td>
<td>[43]</td>
</tr>
</tbody>
</table>
not only in targeted screening methodologies, but in untargeted metabolomics studies, as well. Rocchetti et al. [28] introduced a metabolomics approach with the development of a UHPLC-ESI-QTOF-MS method for the phenolic profiling of Chardonnay wines from different origins, achieving successful separation and identification for 251 phenolic acids, flavonoids, phenylethanoids, lignans and polyphenols [29]. Johnson et al. [30] used a UHPLC-MS/MS system equipped with an RP-C18 Poroshell 120 column (3.0 x 50 mm, 2.7 µm) for the separation and detection of phenolic alcohols (Figure 2c) and secoiridoids (Figure 2d) in Spanish-style green, Californian-style black ripe, and Greek-style table olives. Hydroxycinnamic acids, flavonols, flavanols, glycoside forms of anthocyanins and flavonols were identified in Italian and Chinese Coji berries with a UHPLC-QTOF-MS with an RP-C18 Agilent Zorbax eclipse plus column (50 x 2.1 mm, 1.8 µm), under a water methanol gradient elution [31]. An RP-UHPLC-ESI-QTOF-MS/MS methodology was developed for the target and non-target determination of phenolic compounds in extra-virgin olive oil samples using an Acclaim RSLC C18 column (2.1 x 100 mm, 2.2 µm) with a pre-column of ACQUITY UPLC BEH C18 (1.7 µm, VanGuard Pre-Column) within 11 min. The separation was operated at 30°C. The solvents used consisted of: (A) 90% H₂O, 10% MeOH and 5 mM ammonium formate, (B) 100% MeOH and 5 mM ammonium formate [32]. Thirty six phenolic compounds were determined through target, suspect and non-target screening, including phenolic acids, flavonoids, lignans and phenylethanoids, while the method also enabled the determination of free fatty acids with retrospective analysis. The extraction procedure was also optimized, suggesting the mixture of MeOH:water (80:20, v/v) as the most appropriate extraction solvent in olive oil samples [33]. Guerreiro Pereira et al. [34] developed a UHPLC-PDA-MS methodology for the identification of antioxidants in medicinal plants which are used as additives in functional beverages, employing a BEH C18, with ethylene bridged hybrid technology, providing extraordinary levels of efficiency, peak asymmetry and chemical stability. Forty two phenolic acids, flavonoids and phenylethanoids were determined in different organs of *H. italicum subsp. Picardii*, including roots, vegetative aerial-organs and flowers that are used for tea making with UHPLC coupled to photodiode array (PDA) and MS [34]. Ersan et al. [34] optimized the extraction procedure, developed a novel UHPLC-DAD coupled to evaporative light scattering detector (ELSD) method for the determination of phenolics in pistachios, and compared it to an HPLC method in terms of separation efficiency and quantitation. According to the results, analyses with UHPLC were 3.7 times faster and the core-shell particle HPLC column allowed superior peak resolution [35]. The detected antioxidants exhibited a wide range of polarity, ranging from high water-soluble gallotannins and flavonoid glycosides to less polar anacardic acids. The potential health benefits of nuts consumption were investigated after the development of a UHPLC-ESI-QTOF-MS methodology that was applied in poppy, sunflower, pumpkin, sesame and flaxseed matrices for the determination of lignans. Based on the results, all matrices proved to be excellent sources of phenolics, displaying high antioxidant capacity and worth receiving increasing attention as functional foods, since in addition to their nutritional benefits (vitamins, amino acids, trace elements, fiber), they also contain lignans and flavonoids [36]. Panteli et al. [37] evaluated the antioxidant capacity of 13 varieties of berries in Serbia with a UHPLC-ESI-MS/MS methodology. A novel functional beverage made from strawberry by fermentation was proposed by Álvarez-Fernández et al. [38]. This interesting approach describes the transformation of strawberries into a beverage by gluconic and acetic fermentation and the subsequent monitoring of the nonanthocyanin content within 90 days of storage in two different temperatures (27-30°C and 4°C). Dudonné et al. [39] reported for the first time the complete characterization of proanthocyanidins in native Canadian berries, identifying over 60 phenolic compounds in less than 10 min with UHPLC-MS/MS and verifying the contribution of their antioxidant compounds in beneficial health effects. UHPLC coupled to ESI-MS/MS has also been applied for the investigation of the antioxidant profile of potatoes [40], saffron [41] and in the polyphenolic characterization of food supplements containing 36 different vegetables, fruits and berries [42]. Machado et al. [43] optimized the phenolic extraction from residues of blackberries by evaluating the influence of temperature (60, 80 and 100°C) and solvent type (water, acidified water pH=2.5, ethanol and ethanol with water 50% v/v) using pressurized liquid extraction (PLE) and selected ethanol with water (50% v/v) as the ideal solvent at 100°C. All recent applications of RP-HPLC and RP-UHPLC methodologies for the determination of antioxidants in functional foods are summarized in Table 1.

### 2.1.3 HILIC Chromatography

HILIC is an alternative to RP-HPLC separations of polar, basic or weak acidic samples. HILIC is characterized as normal phase chromatography, using polar columns in aqueous-organic mobile phases for the separation of the analytes.
The separation mechanism of HILIC is opposite to RP systems. It uses hydrophilic stationary phases with RP type eluents. Any polar chromatographic surface, such as simple unbonded silica silanol or diol bonded phases, amide bonded phases, amino or anionic bonded phases, zwitterionic bonded phases and cationic bonded phases, can be used for HILIC separations [45]. As for the mobile phase, typically HILIC uses ACN with a small amount of water [38]. Aprotic solvents miscible with water such as THF or dioxane can be used, as well. In order to control the pH and ion strength of the mobile phase, ammonium acetate and ammonium formate are usually added. Other salts like sodium perchlorate can be used in order to increase the mobile phase polarity and effect elution. HILIC has been used for the separation of small phenolic compounds in functional foods, counting fewer applications compared to UHPLC [45]. The selectivity and the retention of phenols is affected by the pH, the fraction of the organic solvent and the concentration of the used buffer. pH affects the retention by shifting the ionization of the column material and the analytes. The ionized analytes exhibit stronger strong retention on HILIC columns, since they are more hydrophilic in their ionized form compared to neutral. Recently, the use of amide-embedded stationary phases for the LC separations of small phenolic compounds has become very popular. Aral et al. [46] developed an amide-embedded silica based stationary phase and studied the retention time behaviors of six.

Figure 3. Relation of a (a) 1D LC system to a (b) 2D LC system
different groups of phenolic compounds, including anilines, sudan dyes. The chromatographic behavior of the developed stationary phase was compared to commercially available RP column ACE C-18 under HILIC conditions. According to the experimental data, the functional groups on the stationary phases influence the selectivity probably through secondary interactions with the model compounds. The retentions on the stationary phase of the moderately polar compounds are higher than those of the highly polar compounds, because of the hydrophobic and hydrophilic interactions between the analytes and the stationary phase.

2.1.4 Multidimensional Chromatography
Taking into consideration that food matrices are complex, in some cases a single column chromatographic methodology may not achieve the separation of the antioxidants of interest, especially if the target compounds are isomers or have structural similarity. Comprehensive two-dimensional liquid chromatography (LC x LC) is a promising technique for the analytical separation of phenolics from food matrices in offline, on-line or stop-flow modes [38]. Two dimensional LC includes one-dimensional (1-D) isocratic and gradient elution LC. Multidimensional systems offer great improvements in the resolving power over 1D-LC. Figure 3 shows the relation between 1D-LC and 2D-LC systems. Especially, the combination of HILIC and RP is challenging because of the orthogonal group-type separations that are achieved [47]. Muller et al. [49] developed a generic HILIC x RP-LC-DAD-MS methodology, utilizing dilution of the first dimension flow and employing large volume injection in the second dimension by kinetic optimization of experimental parameters in an attempt to provide maximum performance [49]. With this methodology, 149 phenolics were tentatively identified in rooibos tea, wine and grapes. In first dimension, separations were performed on an Xbridge Amide column (150 x 1.0 mm, 1.7 µm, Waters) and the mobile phase consisted of (A) 0.1% formic acid in ACN and (B) 0.1% formic acid in water at 11 µL/min flow rate. In second dimension, the first dimension eluent was diluted with 0.1% formic acid in water at 99 µL/min flow rate and a Kinetex C18 column (50 x 3.0 mm, 1.7 µm, Phenomenex, Torrance, USA) was used for the analysis of grape seeds, and a Zorbax Eclipse Plus C18 column (50 x 3.0 mm, 1.8 µm, Agilent) was used for tea, wine and grape analysis. The columns were thermostated at 50°C [49]. Lu et al. [50] developed an online HPLC methodology with silica capillary tube with polyamide coating, coupled to ion mobility spectrometry (IMS) for the determination of nine phenolic acids in seedling roots. The separation was performed by gradient elution: (A) MeOH with 0.1% acetic acid (v/v) on a narrow diameter RP-C18 column with ammonia as the sheath fluid. IMS was employed to assist in the determination of the co-eluted compounds that could not be separated by HPLC, in order to achieve a highly selective and versatile two-dimensional chromatographic analysis. The proposed method enabled effective separation of coumaric acid, chlorogenic acid, protocatechuic acid, caffeic acid, vanillic acid, sinapic acid, ferulic acid, benzoic acid and salicylic acid. The limit of detection (LOD) ranged from 0.05 to 5 ng and the recovery of the spiked samples ranged from 85.10% to 100.51% [50]. In another work, a novel (LC x LC) method was developed and introduced as a powerful alternative to conventional LC, coupled to DAD and MS for the determination of the polyphenolic fraction of pistachio kernels from different geographical origins. Pistachios are a source of different groups of valuable phytochemicals such as flavan-3-ols, anthocyanins, proanthocyanidins, flavonoids, phenolic acids and stilbenes, presenting excellent biological activities. A 150-mm micro-bore cyano column (2.7 µm) and 50-mm superficially porous C18 silica column (2.7 µm) were employed in the first and second dimension, respectively, to overcome conventional LC limitations, especially in terms of resolving power [51]. Lu et al. developed an LC LC-ESI-MS/MS method with shifted gradients in the second dimension for the phenolic characterization of pomegranates, providing simultaneous separation and identification of 37 phenolic bioactive constituents [52]. Toro-Uribe et al. [53] evaluated the phenolic content of cocoa with the determination of 30 main compounds, including 3 xanthines, 2 flavan-3-ols and 24 oligomeric procyanidins, using a novel on-line LC x LC-MS/MS methodology, after studying different modulation strategies to enhance the method performance. High resolving power and peak capacity were achieved with focusing modulation set-up. The use of active modulation with the addition of a make-up flow increased the solvent strength mismatch produced between dimensions. Recently, an innovative 3-dimensional LC x LC-IMS methodology was introduced by Venter et al. [54] for the analysis of phenolic compounds in various natural products. The combination of HILIC, RP-LC and IMS provided excellent separation of the phenolic compounds in three dimensions. The incorporation of IMS improved MS sensitivity and mass spectral data quality. IMS also enabled the separation of trimeric procyanidin
isomeric species, which could not be differentiated by High Resolution Mass Spectrometry (HR-MS) or HILIC x RP-LC. A comprehensive LC x LC method was developed coupled to DAD and MS to obtain the phenolic profiles of grapevine canes from several varieties [55]. The method was optimized and the combination of diol and C18 columns produced the best results, enabling the determination of 81 phenolic components in around 80 min, showing good separation capability, effective peak capacity and orthogonality. Table 2 presents all recent applications of LC x LC methodologies for the determination of antioxidants in functional foods.

### 2.2 Gas Chromatography

The application of GC is vital for the analysis of non-polar and semi-polar phenolic antioxidants. Despite the fact that GC and LC are complementary techniques, the evolution of HPLC led both analysts and instrument manufacturers to use primarily HPLC [56]. GC is mainly applied for the analysis of the lipid fraction of functional foods. There are only a few works using GC-MS for the identification of phenolic antioxidants in functional foods. An interesting study by da Silva et al. describes the application of GC in the determination of phenolic compounds in native fruits from South America [57]. Karabagias et al. [58] proposed the application of a non-target headspace solid-phase microextraction (HS-SPME) methodology in combination with GC-MS for the determination of volatiles in greek honeys and the exploitation of natural organic compounds. In another interesting study, a GC-MS method was developed complimentary to an LC-MS methodology for the simultaneous determination of more than 40 minor compounds that belonged to different chemical classes, just in a single run, in order to evaluate the profiles

<table>
<thead>
<tr>
<th>Functional Food</th>
<th>Compounds</th>
<th>1st dimension Chromatographic Column</th>
<th>2nd dimension Chromatographic Column</th>
<th>Detection</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plants (seedling roots)</td>
<td>9 phenolic acids</td>
<td>InertSustain C18 analytical column (100 x 3.0 mm, 2.1 µm)</td>
<td>InertSustain C18 analytical column (100 x 3.0 mm, 2.1 µm)</td>
<td>ESI-IMS UV</td>
<td>[50]</td>
</tr>
<tr>
<td>Pistachios</td>
<td>51 polyphenolic compounds</td>
<td>Ascentis Express Cyano (ESCN) column (150 x 1.0 mm, 2.7 µm)</td>
<td>Ascentis Express C18 column (50 x 2.1 mm, 2.7 µm)</td>
<td>PDA-ESI-MS/MS</td>
<td>[51]</td>
</tr>
<tr>
<td>Pomegranate</td>
<td>37 polyphenolic compounds</td>
<td>Cyano column (1.0 mm)</td>
<td>C18 superically-porous particle column</td>
<td>PDA/ESI-MS</td>
<td>[52]</td>
</tr>
<tr>
<td>Cocoa</td>
<td>30 phenolic compounds (xanthines, flavanols and oligomeric procyanidins)</td>
<td>Lichrospher Diol-5 column</td>
<td>C18 partially porous column (30 x 4.6 mm, 2.7 µm)</td>
<td>ESI-MS/MS</td>
<td>[53]</td>
</tr>
<tr>
<td>Chestnut, red wine, grape seed and rooibos tea</td>
<td>Ellagitannins and gallotannins, procyanidins, flavonoid and non-flavonoid phenolics</td>
<td>HILIC separations were performed using an Acquity UPLC BEH Amide column (150 x 1.0 mm, 1.7 µm)</td>
<td>Kinetex C18 core-shell column (50 x 3.0 mm, 1.7 µm)</td>
<td>UV x IMS-MS</td>
<td>[54]</td>
</tr>
<tr>
<td>Grapevine</td>
<td>51 phenolic compounds (procyanidins, stilbenes, flavonoids)</td>
<td>Lichrospher diol-5 (150 x 1.0 mm, 5 µm)</td>
<td>Ascentis Express C18 column (50 x 4.6 mm, 2.7 µm)</td>
<td>ESI-MS/MS DAD</td>
<td>[55]</td>
</tr>
<tr>
<td>Functional Food</td>
<td>Compounds</td>
<td>Capillary Column</td>
<td>Analysis Temperature</td>
<td>Carrier gas</td>
<td>Detection</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------</td>
<td>------------------------------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Honey</td>
<td>10 Benzene derivatives and phenolic volatiles</td>
<td>DB-5MS (cross linked 5% PH ME siloxane) (60 m x 320 µm i.d., 1 µm film thickness)</td>
<td>40-260°C</td>
<td>Helium, at a flow rate of 1.5 mL/min</td>
<td>MS</td>
</tr>
<tr>
<td>Virgin olive oil</td>
<td>Simple phenols and polyphenols (phenolic acids, secoiridoids, flavonoids)</td>
<td>5%-phenyl-methyl polysiloxane (HP-5MS) capillary column (30 m x 0.25 mm i.d., 0.25 µm)</td>
<td>140-310°C</td>
<td>Helium, at a flow rate of 1 mL/min</td>
<td>MS</td>
</tr>
<tr>
<td>Honey from strawberry trees</td>
<td>Homogentisic acid</td>
<td>TraceGOLD TG-5MS capillary column (5% diphenyl, 95% dimethylpolysiloxane; 30 m length, 0.25 mm id, 0.25 µm film thickness) HP-5ms Ultra Inert capillary column</td>
<td>60°C for 1 min, raised at 10°C/min to 210°C and held for 1 min, raised at 40°C/min to 280°C</td>
<td>Helium, at a flow rate of 1 mL/min</td>
<td>MS</td>
</tr>
<tr>
<td>Wood vinegar</td>
<td>9 phenolic compounds</td>
<td>HP-5ms Ultra Inert Capillary Column</td>
<td>60°C held for 10 min, then increased by 2°C/min to 80°C and held for 5 min, raised by 2°C/min to 110°C and held 6 min, raised by 2°C/min to 120°C and held for 5 min, finally raised by 2°C/min to 180°C and held at 180°C for 2 min</td>
<td>Helium, at a flow rate of 0.8 mL/min</td>
<td>MS</td>
</tr>
<tr>
<td>Brazilian native fruits</td>
<td>Flavonoids, phenolic acids and their derivaties</td>
<td>RTX-5MS 30 m x 0.25 mm 0.25 µm</td>
<td>80°C (1 min), raising at 20°C/min to 250°C, remaining at 250°C for 1 min (9 min 30 s), raising at 6°C/min to 300°C, remaining at 300°C for 5 min (13 min 20 s), raising at 15°C/min to 310°C, remaining at 310°C for 5 min (5 min 40 s), raising at 20°C/min to 320°C, for 10 min</td>
<td>Helium</td>
<td>MS</td>
</tr>
</tbody>
</table>
Antioxidants in functional foods

of different virgin olive oils that were produced in laboratory scale [59]. Because of the chemical complexity of the compounds, the chromatographic conditions were optimized to cover a wide range of volatilities. Thus, the temperature ramp was tested from 120 °C to 320 °C at 3 °C/min. Finally, 4 °C/min ramp from 140 °C to 310 °C produced chromatograms with the best resolution/analysis time ratio. The injection volume was also optimized, testing injections of 0.1, 0.5 and 1 µL of sample in splitless and split modes (1:10, 1:25 1:50 and 1:75 as split ratios) and 1 µL was selected as the optimum injection volume with a split of 1:25 [59]. A GC-MS method was developed and validated for the determination of homogentisic acid (HGA) in strawberry tree honey [60]. The method was optimized and ethyl acetate was selected as the most effective solvent for the extraction, among dichloromethane and ethyl ether. HGA recovery improved with the addition of NaCl and a decrease in pH, since the addition of salt influenced the ionic strength of the solution and decreased the solubility of HGA in the aqueous phase, enhancing its partitioning in the organic [60]. In another study, the volatile antioxidant profile of wood vinegar was evaluated using GC-MS. A total of 17 chemical compounds were determined and 9 of them were phenolic compounds (2,6-dimethoxyphenol, 3,5-dimethoxy-4-hydroxytoluene, and 1-(4-hydroxy-3,5-dimethoxyphenyl)-ethanone, 2-methoxyphenol, 4-ethyl-2-methoxyphenyl, and 1-(4-hydroxy-3-methoxyphenyl)-2-propanone, catechol, 3-methoxy-1,2-benzenedi-ol, and 4-methyl-1,2-benzenediol) [61]. In this study, more than 70% of the chemical components were identified as phenolic compounds and the antioxidant activity assay showed great antioxidant activity [61]. GC-MS was employed for the determination of cinnamic acid, protocatechuic acid, p-coumaric acid, m-coumaric acid, gallic acid, sinapic acid, 6,7-dihydroxycoumarin, 3-D-glycopyranoside, epicatechin, kaempferol, quercetin and myricetin in the ethanolic extracts of leaves, seeds and pulp of 4 Brazilian native fruit species [62]. Prior to GC-MS analysis, derivatization was carried out with MSTFA in order to reduce the polarity of the functional groups and facilitate separation. Gallic acid and epicatechin were the most abundant antioxidants in these Brazilian fruits [62]. All applications of GC-MS in the determination of antioxidants in functional foods are summarized in Table 3.

2.3 Future Perspectives

The major goals in analytical separation techniques will always be the same: increase accuracy and selectivity, decrease detection and quantification limits, solvents volume and costs. Increasing analysis speed will always be vital, as well. The automation of the sample preparation process and the use of microextraction techniques [62] in combination with novel hybrid on-line systems [63] could make analytical operations more robust and precise, opening new areas of research. LC and GC conditions should be developed and optimized to provide short time of analysis, while providing the adequate selectivity and accuracy. The automation of the extraction process and the use of two-dimensional chromatographic systems, coupled to high resolution detection, despite the instrumentation cost, will play a major role in the future investigation of complex food matrices.

3.0 Conclusions

The aim of this review was to gather all the recent advances in the development and optimization of LC methodologies for the separation of phenolic compounds in functional foods. The use of HPLC provides increased throughput. The application of UHPLC offers several benefits, compared to conventional HPLC, such as better separation efficiency and sensitivity, decreasing solvents volumes and waste and shortening the time of analysis. In addition, the development of comprehensive multidimensional LC systems proves promising, offering high resolution, and has already found several applications in the investigation of the phenolic profile, especially in complex matrices, like foodstuff, and is expected to play a significant role in the field of analytical separations in the future.

4.0 Summary Points list

- Functional foods gain considerable attention because of their high nutritional value and antioxidant activity.
- UHPLC in reversed phase RP is widely used for the separation of phenolic compounds in food matrices.
- The development of multidimensional methods is advantageous compared to conventional HPLC methodologies for phenolic analysis of complex matrices.

5. References

29. Rochetti G, Gatti M, Bavaresco L, Lucini L. Untarget-
Antioxidants in functional foods

Reviews in Separation Sciences

Kalogiouri N and Samanidou VF.

Antioxidants in functional foods


49. Muller M, Tredoux AGJ, de Villiers A. Application of Kinetically Optimised Online HILIC RP-LC Methods Hyphenated to High Resolution MS for the Analysis of Natural Phenolics. Chromatographia 71(6), 181-196 (2010).


