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REVIEW ARTICLE

Analytical procedures for determination of phenolics active herbal ingredients in fortified functional foods: an overview

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Abstract

Fortification of foods with phenolic compounds is becoming increasingly popular due to their beneficial physiological effects. The biological activities reported include antioxidant, anticancer, antidiabetic, anti-inflammatory, or neuroprotective effects. However, the analysis of polyphenols in functional food matrices is a difficult task because of the complexity of the matrix. The main challenge is that polyphenols can interact with other food components, such as carbohydrates, proteins, or lipids. The chemical reactions that occur during the baking technologies in the bakery and biscuit industry may also affect the results of measurements. The analysis of polyphenols found in fortified foods can be done by several techniques, such as liquid chromatography (HPLC and UPLC), gas chromatography (GC), or spectrophotometry (TPC, DPPH, FRAP assay etc.). This paper aims to review the available information on analytical methods to fortified foodstuffs while as presenting the advantages and limitations of each technique.

Keywords Polyphenols · Fortified foods · Analytical methods · Medicinal plants

Introduction

In the last decades consumer demands for different food products have changed. Nowadays foods are not intended to only satisfy the hunger for humans but also to prevent nutrition-related diseases or improve the physical and mental wellbeing of consumers [1]. In recent years, functional foods have gained popularity because these products can help reduce the risk of disease. It is estimated by "Market Research" [2] that the global market of the functional food industry will reach $167 billion in 2025. The concept of functional food was first used in Japan in the 1980s. This notion eventually became widespread throughout the world. Although these foods have not yet been defined by legislation in Europe, most agree that functional foods are healthy foods or food components that have a potentially positive effect on health beyond basic nutrition [3].

Polyphenols are secondary metabolites in various plant materials that have many beneficial effects on the human body and health [4]. The phenolic compounds have primarily antioxidant and anti-inflammatory effects; in addition, the results of recent research have shown that they can have a preventive or therapeutic effect on cardiovascular diseases, neurodegenerative disorders, obesity or cancer [5]. For this reason, a number of studies have been published where various foods were fortified with polyphenol-rich plant extract [6–10]. However, the health implications of bioactive polyphenols are determined by their bioavailability to a great extent, which is influenced by many factors, including phenolic structure, chemical interactions, food processing, and matrix. The development of fortified food is valueless if the active ingredients are not stable in the food matrix or if they are not absorbed throughout the digestive system. In this context, encapsulation processes play an instrumental role in protecting the bioactive components in the food matrix as well as favor their absorption in the gastrointestinal tract [11, 12]. Consumers expect that the products they use in everyday life are safe and high quality. Therefore, the development and application of analytical methods in the field of plant-fortified functional food analysis are crucial. However, studies in this direction are considerably complex because herbal preparations contain numerous active ingredients. Moreover, polyphenols may interact with the food matrix components [13, 14]. Spectrophotometric methodologies such as 2,2-diphenyl-1-picrylhydrazyl radical scavenging- (DPPH),

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Folin-Ciocalteu-, or ferric reducing antioxidant power (FRAP) assay are widely used for fortified food analysis. [15–17]. In comparison with chromatography-based technologies, these methods are less sensitive and specific. Currently, high-pressure liquid chromatography (HPLC) with diode array (DAD) or mass spectrometry (MS) detection is the most widely used analytical approach for polyphenol analysis in fortified food matrices [18, 19].

This report includes a discussion of how plant extracts appear in fortified functional foods, the effects of the extracts on different foods, the effect of these products on human health, and finally, it describes the analytical methods used for the quantification and determination of phenolic compounds from fortified foodstuffs.

Application of plant extracts in food

Over the last decade, foods fortified with herbal extracts rich in phenolic substances have become widespread (Table 1).

As shown in Table 1, plant extracts are typically used in food fortification in the form of freeze-dried. However, phenolic compounds uses are substantially limited due to their instability during food processing and storage or in the gastrointestinal tract. The behavior of phenolic compounds in the human body can be affected by the structure and composition of the food matrix and the class of polyphenol. In liquid matrices, the polyphenols are more readily bioaccessible whereas, if the matrix is solid, the polyphenols contained must first be extracted to be bioaccessible and potentially bioavailable. In the case of the human body, the extraction is carried out by the gastrointestinal tract where both the mechanical action during mastication and the chemical action during the digestive phase contribute to the extraction of phenolics from solid matrices. The extraction of polyphenols from solid matrices is influenced by various factors such as temperature, pH, the type of solvent used, and so on. These variables can also affect the extraction efficiency of different polyphenols in the gastrointestinal tract [33]. For example, studying the bioaccessibility of olive polyphenols (verbascoside, hydroxytyrosol, and oleuropein) in fortified taralli, Cedola et al. [26] realized that the combined effect of enzymatic activity and pH changes helps degrade part of the bioactive compounds in the gastric and intestinal phase. Polyphenols have been shown that can reduce the digestive rate of starch, thus modulating the glycemic response to carbohydrates [34]. Consequently, extracts from plants have recently been incorporated in cereal-based products to help alleviate type 2 diabetes mellitus [35]. However, some studies suggest that polyphenols have lower effects on starch digestion in the case of fortified food. For example, Kan et al. [36] observed lower starch digestion inhibition when bread was fortified with berry extracts when compared to the co-ingestion of berry polyphenols with bread during in vitro assay. Coe and Ryan [37] applied an in vitro dose–response analysis to determine the optimal dose of a baobab fruit extract and green tea extract for reducing rapidly digestible starch in white bread. Although bread fortified with tea extract (0.4%) and baobab fruit extract (1.9%) did not reduce the satiety or glycemic response, white bread with added baobab fruit extract increased insulin economy by reducing the amount of insulin needed for given blood glucose. In some cases, lipids can also have a positive effect on the bioavailability of polyphenols, as they are able to "capture" and protect them from degradation in the gastrointestinal tract or the formation of insoluble complexes [38]. In a previous study, Ortega et al. [39] reported that higher fat content might have a positive effect on the stability of cocoa polyphenols, possibly due to the improved micellarization during digestion. Nowadays, dairy products are one of the most ideal carrier matrices for the delivery of bioactive plant ingredients to the human body. For example, yogurt is an excellent delivery vehicle for phenolic compounds of plant extracts because the low pH (~4.1–4.5) of yogurt contributes to the stability of phenolic compounds during storage [40], while the presence of proteins maintains the integrity of phenolic compounds during digestion, increasing their bioaccessibility [41]. Other dairy products, such as cheese or milk can also serve as a suitable matrix for the controlled release of phenolic compounds. Lamothe et al. [42] showed that the green tea extract addition to cheese and milk promoted polyphenol-protein complex formation, which significantly improved polyphenol stability in a simulated gastrointestinal environment and enhanced the antioxidant activity.

Polyphenols in free form may also have negative effects on the taste of different food due to their strong astringency effect [6]. Moreover, freeze-dried plant extracts with larger particles (> 25 micron) often cause a sandy mouthfeel [43]. To avoid these drawbacks, delivery systems have been developed, and among them, encapsulation plays a predominant role. Encapsulation is a process to entrap phenolic active ingredients within a wall material. The wall materials used for encapsulates must be food-grade, biodegradable, and stable in the food system during processing, storage, and consumption. Among the used wall materials, polysaccharide- and protein-based polymers are widely used for encapsulation. According to the obtained particle size, capsules are called micro- or nanocapsule. Microparticles are generally in the 1–123 µm size range, while the size of nanoparticles ranges approximately from 1 to 200 nm. Commonly applied encapsulation techniques for the purpose of encapsulating phenolic extracts are as follows: anti-solvent precipitation, electrospraying, spray-drying, and atomization/coagulation (Table 2).

Table 1 Applications of plant extracts in various foodstuffs

Plant extract	Applied form	Concentration of plant extract used	Food product tested	Main results	References
Dairy products					
Roselle calyces (*Hibiscus sabdariffa* L.)	Freeze-dried	1%	Goat-milk yogurt	Increased α-glucosidase inhibitory activity and decreased pH. The fortified yogurt stored under refrigerated conditions (4 °C) had acceptable shelf life during the study period (15 days)	[20]
Safflower (*Carthamus tinctorius* L.)	Freeze-dried	0.1, 0.5, and 1.0%	Yogurt	Increased protein, fat, lactose, TPC, TFC, and antioxidant activity. Lower moisture content and pH value. Reduced ROS production in LPS-induced HT-29-cells. Safflower extract downregulated α-glucosidase and lipase activities	[21]
Argel (*Solenostemma argel* Hayne)	Freeze-dried	0.1 and 0.2 g/100 mL	Set-type yogurt	Increased acidity, water-holding capacity, viscosity, TPC, and antioxidant activity. Reduced pH, syneresis, texture, and TBARS values. Also reduced L* value and increased a* and b* values	[22]
British yellowhead (*Inula britannica* L.)	Freeze-dried	0.25, 0.5, 0.75, and 1%	Cheddar type cheese	Higher extract concentrations resulted in higher protein and ash contents, with a concomitant decrease in pH, total solid, and fat content. Higher TPC and antioxidant activities compared to the control. Fortification gave a more desirable texture without change of taste and odor of the cheeses	[23]
Cereal-based products					
Blue pea (*Clitoria ternatea* L.)	–	5, 10, 15, and 20%	Sponga cakes	Higher TPC and antioxidant activity. Reduced TBARS[f] values. Lower L*, a*, and b* values were observed with increasing concentration of blue pea	[24]
Green tea (*Camellia sinensis* L.)	Freeze-dried	0.5, 0.8, and 1.1%	Rye breads	Catechins in rye bread samples significantly increased with the increasing level of green tea extract. Catechin stability was in the order: epigallatocatechin < epigallatocatechin gallate < epicatechin gallate. The antioxidant effectiveness decreased in the following order: 1.1% > 0.8% > 0.5% > control	[25]
Olive (*Olea europaea* L.)	Liquid	75 g	Taralli	Higher TPC, TFC, and antioxidant activity compared to the control. Decreased abundant polyphenol present in taralli in the gastric and intestinal phase. Polyphenol stability during in vitro digestion was in the order: verbascoside > hydroxytyrosol > oleuropein	[26]

Table 1 (continued)

Plant extract	Applied form	Concentration of plant extract used	Food product tested	Main results	References
Glasswort (*Salicornia europaea* L.)	vacuum oven-Dried	20 mL/50 g	Durum wheat fresh pasta	Increased antioxidant activity compared to the control. Higher TPC and TFC contents after digestion	[27]
Spearmint (*Mentha spicata* L.)	Liquid	2.5, 5, and 7.5%	Bread	Higher TPC, DDPH radical scavenging activity, and FIC ability compared to control. Spearmint extract at 2.5% showed the highest organoleptic properties	[28]
Meat and meat products					
Turmeric (*Curcuma longa* L.)	–	250, 500, and 750 mg/kg	Fresh lamb sausages	Reduced lipid oxidation and increased antioxidant capacity	[9]
Perilla (*Perilla frutescens* L.)	Freeze-dried	0.03%	Surimi fish balls	Retarded lipid and protein oxidation. Reduced TBARS values and protein carbonyl contents	[29]
Drumstick-tree (*Moringa oleiferia* L.)	Powdered	0.1%	Goat meat patties	Higher TPC compared to control. Retarded lipid peroxidation	[30]
Confectionery products					
Cinnamon (*Cinnamomum zeylanicum*)	Powdered	0.5 and 1%	Dark chocolate	Higher TPC and decreased texture properties (hardness) as compared to the control. A whitening of chocolate surface was observed during storage (60 days)	[31]
Roselle calyces (*Hibiscus sabdariffa* L.)	Suspension	20.0%	Cupcakes	Fortification increased the, ash, fiber, ascorbic acid, and total anthocyanins content in the cupcakes. Also increased a* value and decreased b* and L* values	[32]
Nettle (*Urtica dioica* L.)	Freeze-dried or syrup	2.0%	Milk, semisweet, and dark chocolates	Higher TPC, chlorogenic acid and flavonoid derivate compared to control. Nettle extract increased the astringency and bitterness. Dark chocolates showed better sensory properties than semisweet and milk chocolates	[6]

TPC total phenolic content, *TFC* total flavonoid content, *ROS* reactive oxygen species, *LPS* lipopolysaccharide, *HT-29* the human colorectal cell line, *TBARS* thiobarbituric acid reactive substances, *DPPH* 2,2-Diphenyl-2-picrylhydrazyl, *FIC* ferrous-ion chelating

Table 2 Studies on encapsulation of polyphenols for food fortification

Food product tested	Plant extract	Wall material	Encapsulation method	Capsule properties	Main results	References
White and milk chocolate	Cinnamon (*Cinnamomum burmannii* Blume)	Shellac and xanthan gum	Anti-Solvent precipitation	The average size of the colloidal nanoparticles was 191 nm, while the size of the lyophilized nanoparticles ranges from 9 to 104 µm	Higher TPC and antioxidant activity of chocolates. The presence of the particles up to 2% does not alter the fineness of the chocolate. Slower polyphenol release	[44]
Biscuits	Green tea (*Camellia sinensis* L.)	Gelatin and zein	Electrospraying	Gelatin particles were quasi-spherical. Zein particles were smaller, had a rougher surface and a greater content of small fibrils. In both cases, particles had a size in the submicron range	Encapsulation efficiency of catechins was close to 90 g/100 g (gelatin) and 85 g/100 g (zein). Decreased total catechin recovery from biscuits enriched with microcapsule	[45]
Cottage cheese	Rosemary (*Rosmarinus officinalis* L.)	Alginate	Atomization/coagulation	Round or pear-like form. Small brown droplets were also noticed, which was related to the presence of the extract. Particle size ranges between 51.1 and 122.6 µm	Encapsulation efficiency of around 100%. Unchanged nutritional values compare to the control. Microencapsulated extracts showed better antioxidant properties throughout storage	[12]
Yogurt	Doum tree (*Hyphaene thebaica*)	Lecithin and chitosan	Liposomes	The sizes of liposomes were ranges between 261 and 483 nm depending on the concentration of doum extract	Encapsulation efficiency was higher than 70%. 5% liposome addition was recommended to increase the antioxidant activity of yogurt	[46]
Milk	Turmeric (*Curcuma longa* L.)	Dextrin	Spray-drying	The mean diameter of turmeric extract nanoemulsions powder was approximately 200 nm	Nanoemulsions effectively protected the encapsulated turmeric extract from degradation during storage. Milk showed noticeable yellowing, particularly for higher concentrations (>10 mg/mL). Encapsulation was able to protect curcuminoids during digestion	[11]

TPC total phenolic content

Sampling, clean-up, and extraction techniques

Correct sample preparation is an important step for the success of analytical processes. Although the interactions between polyphenols and macronutrient of foods generally have a positive effect on the bioaccessibility of phenolics [38, 42], they can cause a multitude of problems in the analysis, including the generation of emulsions, sample turbidity, ion suppression in MS detection, blockage, or irreversible damage/adsorption onto stationary phases of HPLC, etc. [47, 48].

Functional foods fortified with plant extracts can be classified into three broad categories according to their rheological properties: liquid (milk), semi-solid (yogurt, mayonnaise), and solid (chocolate, cake, cheese, etc.) samples. In general, solid samples require more complex and time-consuming treatments than liquids or semi-solid samples. These steps mainly depend on the food matrix, the plant extracts, most specifically the chemical properties of active ingredients, and the type of used analytical techniques. The main challenge for analysts is to maximize recovery of the analytes and minimize the interferences by use of appropriate extraction and clean-up treatments [49]. The general scheme of pretreatment and extraction methods of polyphenols from fortified foods is shown in Fig. 1.

Solid samples are usually subjected to particle size reduction either by grating [50], crushing [51], or grinding [52]. It is well known that surface area increases with the decrease in particle size. The main advantage of this step is increasing the interaction with solvent and mass transfer of phenolics. For dairy products, while some authors measure liquid and semi-liquid samples immediately after centrifugation and filtration [28], others use lyophilization as a sample preparation step and then use the powdered sample for further clean-up stage [53] or extraction [12]. To eliminate the water content, fortified cereal-based and meat products, such as pasta [27], bread [25], cookies [54], or sausage [9], are also often lyophilized. This step is mainly necessary for high-fat content samples before lipid removal because many organic solvents cannot easily penetrate food containing much water, and therefore degreasing would be inefficient. Solvent extraction (SE) using a vortex mixer is one of the most commonly used and easiest methods for removing the lipid phase from food matrixes. This method generally includes initial extraction with *n*-hexane (usually three times), centrifugation and/or filtration, discarding of fat-containing supernatants, and removal of residual organic solvent from the defatted sample by air-drying for 24 h [55]. Its main drawbacks are the time and effort required to carry out the extraction and the large volumes of applied organic solvents [56]. Furthermore, hexane as a lipophilic solvent could extract lipophilic phenolic compounds alongside

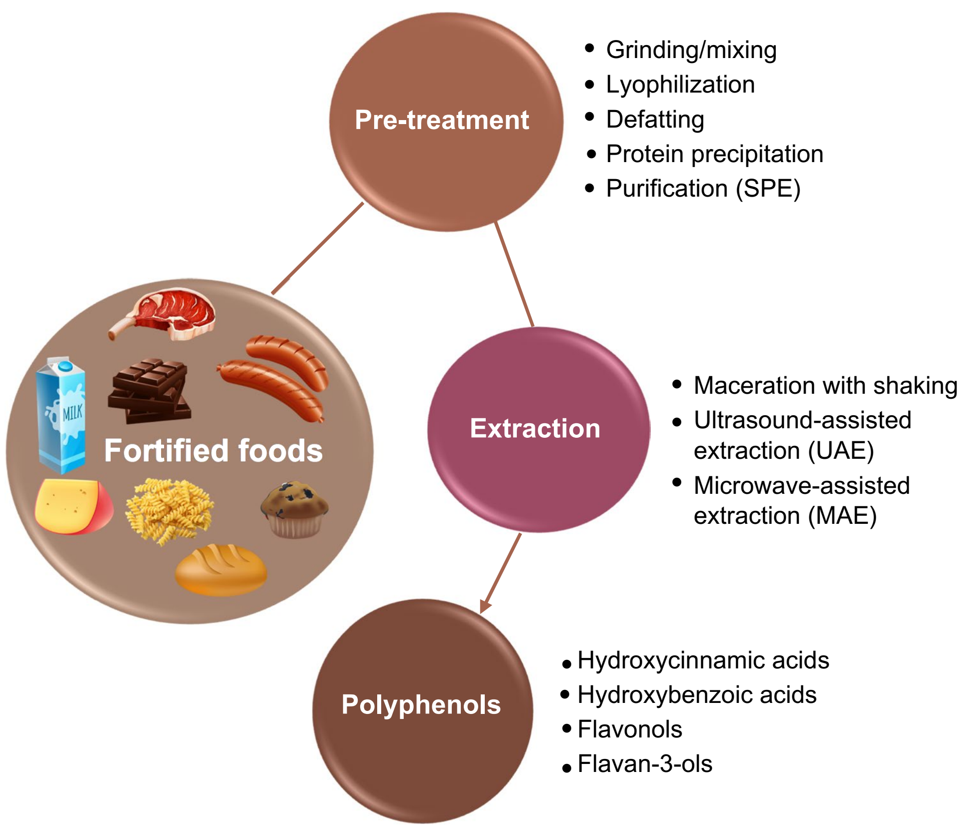

Fig. 1 General scheme of polyphenol extraction experiments from fortified foods

targeted lipids [57]. Martini et al. [58] recently reported a non-solvent method. The process is based on centrifugation and allows the cocoa butter to precipitate in 10 min from the chocolate extract.

In most cases, especially for dairy products, another main step in sample preparation is the precipitation of contaminant protein because the interaction between polyphenols and proteins can affects the release of phenolics from the matrix during the extraction step [49]. Proteins in foods are usually removed using isoelectric precipitation by a mineral acid such as hydrochloric acid and sulfuric acid at its isoelectric point during the pretreatment of samples. Despite this, Zhang et al. [59] in their study indicated that a saturated solution of lead acetate and 5% potassium oxalate is more suitable for protein removal from fortified milk than isoelectric precipitation. Acid precipitation combined with alkaline treatment was used for purslane-fortified Greek-style yogurt by El-Sayed et al. [17]. For this, yogurt samples were homogenized with distilled water, and the pH was readjusted by the addition of 2.5 mL HCl to 4. After centrifugation, the pH of the supernatants was adjusted to pH 7.0 using NaOH and re-centrifuged for the further precipitation of proteins.

For fortified foodstuffs, there can be no specific extraction rules. The main considerations that the analyst must be considered for the extraction, are the nature of the food and the nature of the ingredients used for fortification [60]. The techniques range from conventional solvent extraction (maceration with shaking/stirring) [7, 25, 40] to modern methods like ultrasound-assisted extraction [19, 61, 62] and microwave-assisted extraction [63]. In recent years, these methods have been discussed by many authors in several excellent review articles [64–67]. Therefore, this part of the review focuses on a brief presentation of the extraction conditions used to extract phenolic compounds from various fortified food matrices (Table 3).

In some cases, solid-phase extraction (SPE) has also been reported as a method for purifying phenolic compounds from fortified liquid samples before instrumental detection. For instance, SPE on a column of modified silica (C18) allowed the recovery of phenolics by elution with methanol [69] or *N, N*-Dimethylformamide [59] from fortified milk beverage.

Analytical methods

Spectrophotometric methodologies

The indirect measurement of phytochemicals has been applied for decades. The used spectrophotometric techniques are based on electron/proton transfer reactions and usually are linked to the antioxidant capacity. Depending on what kind of reaction involved, the assays can be classified into two groups: electron transfer (e.g. DPPH, ABTS, FRAP, TPC) and hydrogen atom transfer-based methods (e.g. ORAC). It is important to note that no single method is enough to determine the antioxidant property since different assays can give widely different results. Differences between methods can be ascribed to reaction media. Certain phenolic compounds may not be soluble in reaction media cannot express radical scavenging activities. For example, some assays measure only the hydrophilic antioxidants (e.g., Folin, FRAP, or ABTS), while others (e.g., DPPH) detect only those soluble in alcoholic solvents [70]. In addition, it should be noted that non-phenolic components found in the food matrix may also cause interference during antioxidant analysis. The Folin-Ciocalteu assay is the most widely used procedure for quantification of total phenolics in plant extract supplemented food. This assay is a colorimetric method based on electron transfer reactions between the Folin-Ciocalteu reagent and phenolic compounds. However, the method is not specific for total phenolic content determinations, as the Folin-Ciocalteu reagent can react with food compounds (especially if they are present in large amounts), such as vitamins, amino acids, proteins, carbohydrates, organic acids, and so on, thereby skewing the results. For example, sugars in high-sugar foods (e.g., chocolate) can cause interference if present in high concentrations. Barišić et al. [71], in their study, compared the classical and the modified Folin-Ciocalteu assay (acidic

Table 3 A brief summary of the experimental conditions for conventional and nonconventional extraction techniques for fortified food material

Extraction methods	Maceration with shaking	Ultrasound-assisted extraction	Microwave-assisted extraction
Matrix	Yogurt, cheese, cupcake, bread	White chocolate, soy milk, durum wheat spaghetti, biscuit	Dark chocolate
Common solvents used	Methanol, water, aqueous ethanol/methanol, or acidified methanol	Methanol, aqueous methanol, or acidified acetone/ethanol	Aqueous methanol
Temperature (°C)	Ambient or can be heated	Ambient or can be heated	60
Time required (min)	45–300	15–60	5
Sample: solvent ratio (g/mL)	from 1:1 to 1:40	from 1:4 to 1:20	1:20
Reference	[7, 28, 40, 68]	[10, 19, 52, 61]	[63]

conditions without the adding of Na_2CO_3) for total phenolic analysis in dark- and milk chocolates. The authors pointed out that sugars cannot interact with the Folin-Ciocalteu reagent under acidic conditions. They also highlighted that up to 40% higher total polyphenol concentrations can be measured using the standard method. Belščak-Cvitanović et al. [6] reported in their study a total phenolic content of approximately 2.5–3.0 mg GAE/g and 8.0–13 mg GAE/g for milk- and dark chocolate, respectively. In another study, Jahangir et al. [31] measured 9.86 mg GAE/g of total phenolic value for dark chocolate. In both studies, the standard Folin-Ciocalteu assay was used under non-acidic conditions and the results obtained were consistent with the values measured by Barišić et al. [71], which ranged from 1.70 to 3.63 mg GAE/g for milk and 7.54–12.71 mg GAE/g for dark chocolate, confirming the fact that these results are often overestimated due to sugar interference. Similar observations were also made for yogurts. However, the Folin-Ciocalteu reactivity of yogurts is derived from the degradation of milk protein, which may result in the release of phenolic amino acids and non-phenolic compounds such as sugars and proteins, which can interfere with the analysis [28]. For example, Kim et al. [40] observed an increasing tendency in total phenol content during storage (7 days) of yogurt in both the presence and absence of lotus leaf, which was explained by proteolysis of milk protein, which released amino acids with phenolic side chains. Besides, the authors noted that the metabolism of microbes could also have produced new phenolic acids, which could contribute to the increased total polyphenol values. In contrast, Cho et al. [72] reported a temporary decrease in the total phenolic value of plain and olive leaf supplemented yogurt due to the decomposition of polymeric phenolic compounds in the presence of lactic acid bacteria during refrigerated storage (15 days).

In the case of bread, it has been shown that the chemical reactions that occur during the baking process may also affect the results of measurements. Baiano et al. [73], in their study showed a different phenolic distribution between the crust and crumb of bread enriched with vegetable waste extracts, which was explained by the baking process and the type of phenolic compounds. On one hand, it is well known that the stability of phenolic compounds differs [74], and on the other hand, that higher temperatures (> 110 °C) enhance the Maillard reaction [75]. Since the temperature of the crumb never exceeds 100 °C while the crust can reach also higher temperatures than 205 °C, it can be assumed that in the work of Baiano et al. [73], the heating altered the phenolics to different extents and in different ways in the inner and outer part of the loaf.

Another most frequently employed spectrophotometry method is the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. It is an accepted method for screening the antioxidant activity of plant extracts and based on the electron donation of antioxidants to neutralize DPPH radical. However, the application of this assay is limited by certain drawbacks, similar to the Folin-Ciocalteu assay. Feng et al. [76], in their study, mentioned that yogurt itself has certain antioxidant effects as a result of a lot of amino acids and small molecular peptides with antioxidant activity produced during its fermentation. Fadavi and Beglaryan [14] reported that the whey proteins containing active groups can reduce the antioxidant activity of peppermint-enriched UF-Feta cheese. Besides, it was also shown that with an increase in rennet values, antioxidant activity decreases due to the interaction of intermediate size peptides of cheese with polyphenols of peppermint. A similar observation was reported in the ferric reducing antioxidant power (FRAP) assay. For example, El-Sayed et al. [17] described a reduced antioxidant activity of purslane-fortified Greek-Style yogurt during cold storage due to milk protein–polyphenol interactions. Another major limitation of the DPPH assay is the overlapped spectra of compounds that absorb in the same wavelength range as DPPH. For example, Raikos et al. [77] attributed the decreased antioxidant activities of salal berry-fortified yogurt beverages to the loss of fruit anthocyanin content. In another study, Shori et al. [28] found a weak correlation between total phenolic content and DPPH radical scavenging activity of spearmint-fortified bread, thereby pointed out that other constituents, such as proteins and amino acids, may also have affected the scavenging activity. Furthermore, Bhat et al [78] observed that DPPH scavenging activities of bakery products are also influenced by the Maillard reaction products (e.g. mellanoidins).

High performance liquid chromatography

In comparison to spectrophotometric methodology, high-performance liquid chromatography (HPLC) allows more accurate determination for polyphenols. HPLC (or, more recently ultra-high-performance liquid chromatography; UHPLC) with UV–Vis spectroscopy and/or mass spectrometry (MS) is one of the most common approaches for quantitative analysis of phenolic compounds in fortified food samples. Generally, the MS system is coupled with low-resolution mass analyzers (LRMS), especially triple quadrupole (TQ) mass spectrometers. For example, 8 polyphenol compounds were quantified by UHPLC-TQ MS/MS in sausages fortified with grape seed extract [13]. Oh et al. [79] quantified 10 polyphenols in yogurt fortified with *Morus alba* leaf extract using TQ. In another study [80], 13 polyphenols were quantified by LC-TQ MS/MS in milk fortified with pomegranate peel extract. High-resolution mass spectrometry (HRMS) has become increasingly prominent in recent years for the determination of polyphenols in food. In comparison to LRMS techniques, the HRMS becomes highly advantageous when working with complex matrices, which main contain many isobaric interferences. The main advantage of HRMS in polyphenol analysis is that the highly accurate measurement (< 5 ppm) allows the possibility of unambiguously determining the elemental composition of known and new constituents. Due to the highly diverse structure of phenolic compounds and the complexity of food matrices, this sensitivity is almost indispensable when targeting polyphenols in fortified matrices [81–83]. Q-TOF instruments coupled with electrospray ionization are the most commonly employed for the analysis of polyphenols in foods. The Q-TOF is a hybrid quadrupole flight mass spectrometer in which the third quadrupole is replaced by a time-of-flight tube [84]. Quite recently, Muhammad et al. [19] quantified 8 phenolic compounds in cinnamon-fortified white chocolate using the Q-TOF–MS system. In another recent study [58], this approach allowed tentative identification of 153 and 125 individual phenolic compounds in green tea and turmeric fortified dark chocolate respectively. There is no doubt that with the advances in chromatography technologies in the past decade, HPLC or UHPLC coupled with low or high-resolution mass analyzers have enabled rapid and more accurate separation of phenolics with significantly reduced time and cost. Despite this, diode-array detectors (DAD) coupled with HPLC are the most frequently used systems for quantitative and qualitative analysis. Although HPLC–DAD is cheap and robust, it has several disadvantages, such as i) compound identification is only feasible by retention time and UV-spectra, ii) low detection and quantification limits in complex matrices iii) complicated to choose the correct standard. A summary of the researches reported over the last five years (2017–2021) using HPLC techniques for the analysis of phenolic compounds in various fortified foods is presented in Table 4.

As shown in Table 4, in almost all cases, reverse-phase (RP) C18 columns are used for the separation of phenolic compounds in fortified food samples. Aside from this, examples using other stationary phases such as C8 or even high strength silica (HSS) T3 can also be found in the literature. Typical columns in most of the reported HPLC analysis are 150–250 mm in length, internal diameters of 4.6 mm, and are usually filled with 3–5 µm porous silica particles. The UPLC systems use shorter (100–150 mm) and narrower (1–2.1 mm) columns packed with small size particles (≤ 1.8 µm), which can allow faster analysis. Generally, during the separation of phenolics by RP-HPLC acidified water (with low concentrations of formic acid or acetic acid) and acetonitrile or occasionally methanol as organic solvents (in some cases also acidified with formic acid or acetic acid) are employed as mobile phases. The wavelength selected for determining phenolic compounds is an important criterion and generally ranges between 254 and 520 nm.

Complex food matrices may cause interferences that can lead to incorrect determinations and even false positive or negative results. Thus, every HPLC methodology should be validated to evaluate the ability of the method to provide reliable quantitative results. Zhang et al. [59] validated a method for the determination of four flavone C-glucosides (homoorientin, orientin, vitexin, and isovitexin) in bamboo leaves-fortified milk by RP-HPLC–DAD wherein several performance characteristics such as linearity, repeatability, recovery, LOD, and LOQ were evaluated. A linear calibration curve was obtained with $r^2 > 0.9995$. The stability of the method was examined by estimating the precision in terms of repeatability and was found to be acceptable with values of ≤ 3.2% for all compounds. Recoveries were in the range 81–92%. The LOD and LOQ values were less than 0.03 and 0.09 µg/mL, respectively. Moreover, there were no impurities or co-elution observed (match factor ≥ 98%). A method was developed and validated for the simultaneous quantification of flavan-3-ols, glycosylated flavonols, and benzoic acid derivatives in sausages fortified with grape seed extracts by Ribas-Agustí et al. [13]. The method was performed with a LOD ranging from 1 to 60 mg/100 g depending on the phenolic compounds. In this study, the recovery of procyanidins and epigallocatechin gallate ranged from 61 to 69%, which can be explained by the fact that these compounds may interact with the proteins of sausages. With other phenolic compounds, the recoveries changed between 75 and 96%. Wang and Zhou [89] described an HPLC–PDA method to determine the catechins in bread fortified with green tea extracts. The calibration curves for epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, catechin gallate, and gallocatechin gallate were linear between 1 and 50 ppm, respectively. The determination coefficients were ≥ 0.999; the recovery rate varied from 92 to 94%. The authors also showed that the retention levels of green tea catechins in freshly baked bread were ca. 83% and 91%. Overall, one piece of bread (53 g) contained 150 mg of green tea extract/100 g of flour provided 28 mg of tea catechins, which was 35% of those infused from one green tea bag (2 g). The losses were be explained by the isomerization/epimerization and degradation of tea catechins during the various bread-making stages including mixing, thawing, proofing, and baking.

Gas chromatography

Few studies have used gas chromatography for the analysis of polyphenols in fortified foods, because these compounds are not volatile. Therefore, before the injection onto GC, a derivatization step is required to ensure good vaporization of the sample and obtain volatile and thermostable derivatives [90]. Derivatization of phenolic compounds in fortified foods can be performed at 70 °C in 20–30 min. Trialkylsilylation is the preferred derivatization method to increase the volatility of phenolic compounds. Although there exists

Table 4 Summary of high-performance-liquid chromatography analysis of phenolics in fortified foods

Plant extract	Analyte	Food product analyzed	Measurement method	Stationary phase	Mobile phase composition and elution program	References
Lemon balm (*Melissa officinalis* L.)	Rosmarinic acid	Cupcake	UPLC-DAD-ESI(−)-MS, $\lambda = 280$ and 370 nm	Spherisorb S3 ODS-2 C18 (4.6×150 mm; 3 µm) 0.5 mL/min, 35 °C	0.1% formic acid in H_2O (A), 100% ACN (B), 5 min: 15%B, 5–10 min: 15–20%B, 10–20 min: 20–25%B, 20–30 min: 25–35%B, 30–40 min: 35–50%B	[7]
Olive (*Olea europaea* L.)	Verbascoside, hydroxytyrosol, oleuropein	Taralli	HPLC-DAD, $\lambda = 240$, 280, and 330 nm	Zorbax C18 (150×4.6 mm; 5 µm) 1 mL/min, n.r	2.5% acetic acid in H_2O (A), 100% ACN (B), 0–10 min: 10–20%B, 10–35 min: 20–40%B, 35–45 min:40–100%B, 45–50 min: 100–10%B	[26]
Chinese cinnamon (*Cinnamomum cassia*)	Coumaric acid, syringic acid, ferulic acid, quercetin, quercetin- 3-rhamnoside, kaempferol, cinnamaldehyde	Yogurt	HPLC–UV, $\lambda = 270$ and 360 nm	Ascentis C18 (250×4.6 mm; 5 µm) 1 mL/min, n.r	0.1%formic acid in H_2O (A), 100% ACN (B), 0–0.5 min: 0–3%B, 0.5-10 min: 3–10%B, 10-34 min: 10–40%B, 34-40 min: 40–100%B	[41]
Lemon balm (*Melissa officinalis* L.), peppermint (*Mentha piperita* L.), lavender (*Lavandula angustifolia* L.) rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.)	Rosmarinic acid, chlorogenic acid, caffeic acid, luteolin	Goat's milk	HPLC-DAD, $\lambda = 278$ nm	Zorbax C18 (250×4.6 mm; 5 µm) 1 mL/min, n.r	2% formic acid in H_2O (A), 2% formic acid in ACN (B), 0-25 min: 90–60%A, 25–50 min: 60–30%A	[18]
Korintje cinnamon (*Cinnamomum burmannii* Blume)	Catechin, epicatechin, procyanidin B1 and B2, 3,4- dihydroxybenzaldehyde, *p*- hydroxybenzaldehyde, protocatechuic acid, apigenin	White chocolate	UPLC-ESI(+)-Q-TOF-MS	Acquity HSS T3 (100×2.1 mm; 1.8 µm) 0.4 mL/min, 40 °C	0.1% formic acid in H_2O (A), 0.1% formic acid in ACN (B), 0–1 min: 3%B, 7 min: 15%B, 14 min: 22%B, 17 min: 30%B, 22–24 min: 100%B, 26–30 min: 3%B	[19]
Sakura green tea (*Camellia sinensis* L.), turmeric (*Curcuma longa* L.)	Hydroxycinnamic acids, hydroxybenzoic acids, flavan- 3- ols, ellagitannins, flavonols, flavones	Dark chocolate	LC-ESI(−)-Q-TOF-MS/MS	HxSil C18 (250×4.6 mm, 5 µm) 1.0 mL/min, n.r	1% formic acid in H_2O (A), 1% formic acid in ACN (B), 0–0.5 min: 4%B, 0.5–60 min:4–30%B, 60–66 min:100%B	[58]

Table 4 (continued)

Plant extract	Analyte	Food product analyzed	Measurement method	Stationary phase	Mobile phase composition and elution program	References
Olive (*Olea europaea* L.)	Oleuropein	Yogurt	HPLC-DAD, $\lambda = 280$ nm	Supelcosil LC-ABZ (250 × 4.6 mm, 5 µm) 0.9 mL/min, ambient	1%formic acid in H_2O (A), 100% MeOH (B), 0-3 min: 5–15%B, 3-13 min:15–25%B, 13-25 min: 25–35%B, 25-35 min:35–45%B, 35–40 min:45–50%B, 40-45 min: 50–100%B, 45–50 min: 100–5%B	[72]
Nettle (*Urtica dioica* L.)	Hydroxycinnamic acids, hydroxybenzoic acids, flavan- 3- ols, glycosylated flavonols	Bread	UHPLC-DAD-ESI(−)-MS/MS, $\lambda = 254$ and 280 nm	Syncronis C18 column (100 × 2.1 mm, 1.7 µm) 0.3 mL/min,40 °C	0.01% acetic acid in H_2O (A), 100% ACN (B), 2 min: 5%B, 2–12 min: 5–95%B, 12–20 min: 95–5%B	[51]
Burnet rose (*Rosa Spinosissima* L.)	Flavonols, flavan-3-ols, anthocyanins, ellagitannins	Yogurt	HPLC-PDA^{g}, $\lambda = 254$, 280, 360, and 520 nm	Cadenza Imtakt CD-C18 (75 × 4.6 mm; 5 µm) 1.0 mL/min, 30 °C	4.5% formic acid in H_2O (A), 100% ACN (B), 0–1 min: 5%B in A, 1–20 min: 25%B in A, 20-26 min: 100%B, 26–27 min: 5%B in A	[85]
Cistus (*Cistus incanus* L.)	*p*-coumaric acid, ferulic acid, rutin, ellagic acid, phloridzin, 3- hydroxybenzoic acid, (+)- catechin, protic acid, gallic acid	Wheat bread	HPLC-DAD, $\lambda = 320$ and 280 nm	Cosmosil 5C18-MS-II (250 × 4.6 mm; 5 µm) 1 mL/min, n.r	2% acetic acid in H_2O (A), 100% MeOH (B), 0 min: 95%A, 10 min:70%A, 25 min: 50%A, 35 min: 30%A,40 min: 95%A	[86]
Rosemary (*Rosmarinus officinalis* L.)	Rosmarinic acid	Jelly candy	HPLC-DAD, $\lambda = 280$ and 330 nm	Zorbax SB-C18 (250 × 4.6 mm; 0.25 µm) 1 mL/min, n.r	0.05% formic acid in H_2O (A), 100% ACN (B), 0 min: 5%B, 10 min: 15%B, 30 min: 25%BA, 35 min: 30%BA, 50 min: 55%BA, 55 min: 90%B, 70 min: 100% B	[87]
Green tea (*Camellia sinensis* L.)	(−)-epigallocatechin gallate, (−)- epicatechin gallate, (−)- epigallocatechin, (−)- epicatechin, (−)- gallocatechin gallate, (−)- gallocatechin, (+)- catechin	Biscuits	HPLC-DAD, $\lambda = 280$ nm	Zorbax SBC8 (150 × 4.6 mm; 3.5 µm) 0.9 mL/min, 30 °C	100% ACN (A), 0.1% H_3PO_4 in H_2O, started with 100 mL/100 mL of eluent B, decreasing to 90 mL/100 mL B in 20 min and to 85 mL/100 mL in 60 min	[45]

Table 4 (continued)

Plant extract	Analyte	Food product analyzed	Measurement method	Stationary phase	Mobile phase composition and elution program	References
Grape seed (*Vitis vinifera* L.)	Gallic acid, epicatechin, procyanidin B1 and B2	Yogurt	HPLC-DAD, $\lambda = 277$ nm	Dionex C-18, 120 A° (250 × 4.6 mm; 5 µm) 0.3 mL/min, 30 °C	1% acetic acid in H_2O (A), 100% ACN (B), 0–20 min: 95– 87%A, 20–30 min: 87%A; 30–46 min: 87–78%A, 46– 55 min, 78–10%A, 55– 65 min: 10%A	[88]

DAD diode-array detection, *ESI* electrospray ionization, *MS* mass spectrometry, *ACN* acetonitrile, *Q-TOF* quadrupole time-of-flight, *MeOH* methanol, *PDA* photo diode-array detection, *n.r* not reported

a great variety of commercially available silylating reagent, the most common in the literature surveyed regarding phenolic compounds has been bis(trimethylsilyl)trifluoroacetamide (BSTFA). Using an appropriate polar solvent such as pyridine can also favor the dissolution of the analyzed material in the derivatizing reagent. The reported works (only two studies) were using FID [91] and MS [92] detection for the analysis of fortified yogurts and edible oils, respectively. Fused silica capillary columns with lengths of 30 m and inner dimensions of 0.25 mm were used in both reported studies. The used column coating material was 5% phenyl-95% dimethyl-polysiloxane (HP-5 and ZB-5). The temperature program is generally based on gradients, using initial temperatures ranging from 70 °C to 80 °C, and final temperatures between 300 °C and 320 °C, achieved in different steps and with rate increases ranging from 4 to 20 °C/min. The authors have injected 1 µl derivatized sample volume into the columns at a split (1:20 ratio) or splitless mode. Helium is used as the carrier gas at a flow-rate of between 0.6 and 2.4 ml/min.

Karaaslan et al. [91] have identified nine individual bioactive phenolic compounds in callus yogurt by GC-FID. According to the data, approximately 72% of the polyphenols present in the yogurt were identified, and the total amount of characterized and quantified phenolic compounds from callus extract-fortified yogurt was 55.8 mg/L. Although these data indicate that the used method was acceptable for the phenolic analysis in fortified yogurt samples, the authors suggest a stronger separation system such as GC–MS/MS or LC–MS for better detection of phenolic compounds found in foods. In another study, Salta et al. [92] quantified 17 phenolic compounds in enriched vegetable oils (olive oil, sunflower oil, palm oil, and a vegetable shortening) by GC–MS analysis. After the olive leaf extract enrichment, oleuropein was predominated in all cases. Moreover, supplementation of olive oil with the extract resulted in a concentration increase of tyrosol, hydroxytyrosol, maslinic acid, caffeic acid, quercetin, protocatechuic acid, and vanillethanediol. Besides, the authors reported good linearity in the range of quantitation limit and up to 20-fold concentration for each phenolic compound.

Conclusion and future perspectives

As food fortification with plant extracts is becoming more popular around the world, there will be an increasing need to monitor the quality and safety of these products. However, the analysis of polyphenols is relatively difficult because of the complexity of food matrices. In this study, we pointed out that, besides the interactions between polyphenols and food ingredients, new compounds that formed during the technological process may affect the results of measurements. From the literature, it is clear that the utilization of encapsulated polyphenols could effectively improve the performance of functional foods.

Although most studies on the analytical characterization of plant-fortified foods use classical pretreatment and extraction techniques for sample preparation, recent sample preparation trends follow an even more straightforward and economic analysis. During the past decade, several new analytical approaches have been appeared and used for the polyphenol analysis from different non-fortified food. An example is the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) technique, which was successful adopted for the determination of phenolic compounds from non-fortified solid [93] and semi-solid [94] as well as liquid [95] samples.

Based on the available literature, it could be stated that the use of spectroscopic techniques is limited by the lack of their specificity. All in all, rapid, accurate, and sensitive chromatographic techniques are becoming increasingly important for polyphenol analysis. Taking into account the complexity of fortified foodstuffs, the use of LRMS and HRMS techniques will certainly increase in the future. At the same time, it should be considered that these instruments are very expensive and not available in many laboratories.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Compliance with Ethics requirements This article does not contain any studies with human or animal subjects.

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