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## Review Article

# Advanced Analytical Methods for Phenolics in Fruits

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Phenolic compounds are a group of secondary plant metabolites, many with health-promoting properties that are present in all parts of plants. They have an aromatic structure, including either one or more hydroxyl groups giving them the ability to stabilize free radicals and protect biological tissues against damage related to reactive oxygen species. Phenolic compounds are concentrated in the fruit of plants, and therefore, the fruit can be an important dietary source of these phytochemicals, which exist as monomers, or bound to one another. Polyphenolic compounds are classified into different subclasses based upon the number of phenol ring systems that they contain, saturation, and length of the carbon chain that bind the rings to one another. The phenolic acids present in fruit tissues protect the plant against disease, infections, UV radiation, and insect damage. For this reason, the beneficiary effects of phenolic compounds are continually being investigated for their health-promoting properties and for meeting increased consumer demand for healthy nutritious food. Due to the functional properties of polyphenolic compounds, there is increased interest on improving extraction, separation, and quantification techniques of these valuable bioactive compounds, so they can be used as value-added ingredients in foods, pharmaceuticals, and cosmetics. This review provides information on the most advanced methods available for the analysis of phenolics in fruits.

## 1. Introduction

Fruits are a good source of compounds with phenolic functionality including phenols, lignins, lignans, coumarins, tannins, phenolic acids, and flavonoids which are important in the human diet [1]. These phenolic secondary metabolites comprise of an aromatic benzene ring with one or more hydroxyl groups (e.g., polyphenolics) that can exist as a simple monomeric phenolics (simple phenolics) or a complex polymerized polyphenolic molecules. These molecules generally play a critical role in plant defense mechanisms including pathogen or insect attack, ultraviolet light, and mechanical damage in plants [2]. They also play an important role in human health, protecting against damage induced by reactive oxygen or nitrogen species. Phenolics are classified based upon their carbon skeleton and in accordance with the number of phenol units present. Phenolics arise from either phenylalanine or tyrosine amino acids that are deaminated into cinnamic acids (C6-C3)

and are primarily derived from the phenylpropanoids [3, 4]. Simple phenolic acids (C6C1) are divided into two groups: hydroxybenzoic acids (e.g., benzoic acid and gallic acid) and hydroxycinnamic acids (e.g., caffeic, ferulic, p-coumaric, sinapic, and cinnamic acids) [5]. These molecules play a vital function to protect the organism against to biotic or abiotic stress factors [6–10]. Most phenolic compounds are highly reactive, are therefore not stable, and can degrade into a wide variety of products. In the last decade, many epidemiological studies indicate a direct relationship between consumption of fruits and the prevention of diseases such as cancer and those related to inflammation and oxidative stress [11]. Monomeric phenolic compounds can polymerize to form more complex phenolic compounds. These include flavonoids, tannins, and lignin. Complex phenolics have a range of biological activities (e.g., antioxidants) and functional characteristics (e.g., natural colorants, astringency, etc.) and are of great interest to the food industries, cosmetic industry, and allied health fields.

Flavonoids ( $C_6C_3C_6$ ) are complex phenolics that can be classified into six subclasses that include flavonols (wines, teas, and red grapes), flavanones (citrus), flavones (green leafy species), isoflavones (soybeans), anthocyanins (blue, red, and purple berries), and flavanols (tea and cocoa) and are present principally as glycosylated, esterified, and polymerized derivative forms in fruits [12, 13]. Flavonols play an important role as antioxidants; for example, they protect ascorbic acid from autoxidation in juices and which can lead to juice discoloration. Although flavonoids are abundant in fruit, and fruits or beverages can be a significant source of dietary flavonoids, levels will vary depending on the varieties, environmental conditions, soil, and climatic factors [14]. Berries are a good source of quercetin and its derivatives (e.g., quercetin-4'-O-glucoside, diglucoside, and rutinoid), whereas the most abundant dietary flavanone glycoside is hesperetin-7-O-rutinoside present in citrus fruits [15]. Peterson et al. [16] reported that the most prevalent dietary flavanone aglycones are naringenin, hesperetin, isosakuranetin, and eriodictyol. The same authors demonstrate that a citrus fruit is also a primary source of narirutin, eriocitrin, didymin, neohesperidin, naringin, hesperidin, neoeriocitrin, and poncirin. The ratio of these compounds to each other can vary. For example, narirutin and naringin were detected in grapefruit in high ratios, while the levels of hesperidin and narirutin in oranges and eriocitrin in lemons were even higher. In addition, some flavanone glycosides such as 7-rutinoside are tasteless, in contrast to neohesperidin (7-O-neohesperidoside), naringin, and hesperetin (naringenin-7,4-O-neohesperidoside) which have an intense bitter taste isolated from bitter oranges and grapefruit [17]. Apigenin (4',5,7-trihydroxyflavone) is another key flavone found in fruits, vegetables, spices, and herbs and is abundant in grapefruit, beverages, some vegetables, and herbal plants such as chamomile (*Matricaria chamomilla*) [18]. Isoflavones are present in plants in the glycosylated forms but are converted to aglycone forms through the action of intestinal microflora [19]. Isoflavones are detected commonly in legumes such as green beans, fava, and soybeans, and among them, genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) and daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) are the two major forms of dietary isoflavones and are consumed in soy products. Fermented soy products also contain an additional seven isoflavone aglycones in significant levels [20]. Due to the structural similarities to human hormone estrogen, isoflavones have potent estrogenic properties [21].

Anthocyanins are another important class of flavonoids that are colorful water-soluble glycosides and acylglycosides of anthocyanidins. 3-O-glycosides or 3,5-di-O-glycosides of malvidin, delphinidin, pelargonidin, cyanidin, petunidin, and peonidin are known as the most common natural anthocyanins and are classified based on the number and position of hydroxyl and methoxy groups.

Anthocyanins are responsible for the brilliant colors of various plant parts including flowers and leaves and especially fruits having red, blue, purple colors, particularly strawberries, blueberries, black currants, cherries, raspberries, and

red and purple grapes. Anthocyanidins are also responsible for the color of red wines. Their color based upon the degree of methylation and with pH is discrete from other phenolics by the range of colors each forms [22–24]. Color differences of anthocyanins depend on the substitutions of the B ring, the pattern of glycosylation, and the degree and nature of esterification of the sugars with aliphatic or aromatic acids, and also on the pH, temperature, type of solvent, and the presence of copigments. Berries are a good source of anthocyanins, and 100 g of berries can provide up to 500 mg of anthocyanins [25–31].

Flavan-3-ols (e.g., catechin and epicatechin) are the core structure of condensed tannins (i.e., oligomeric and polymeric proanthocyanidins) and are the most complex subclass of flavonoids. Flavan-3-ols have been previously reported as an antioxidant, chemopreventive, and immunoregulation agents [32]. Procyanidins exist in a wide range of foods (especially tea and cocoa) and often exist in foods in a range of galloylated forms.

Most widely used techniques for phenolics are HPLC (high-performance liquid chromatography), LC/MS, GC (gas chromatography), GC/MS, UV-Vis spectrophotometry, mass spectroscopy, electrochemical, and fluorometric methods [33]. Liquid chromatography mass spectrometry (LC/MS) is used to determine phenolics in both APCI and ESI [34] techniques, ABTS+(2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl radical) [35].

## 2. Sample Preparation and Extraction Methods

Sample preparation and extraction methods varied widely based on the nature of the sample matrix of the fruit or vegetable and based on the chemical structures of the phenolic compounds being extracted [12]. As most samples contain a mixture of simple and complex polyphenolic compounds, such as phenolic acids, flavonoids, anthocyanins, and proanthocyanins, it is critical to choose a suitable method for sample preparation and extraction. Proteins, carbohydrates, lipids, or other elements may play negative effect to extraction of phenolics. In addition, it is not always possible to extract fresh samples, and special preparation techniques such as lyophilization, nitrogen pulverization, or drying (oven, air, shade, etc.) may be needed [36–38]. Particle size of extracted material and solvent-to-solute ratios need to be considered. As seen in Figure 1, there are many reliable qualitative and quantitative methods available for the measurement and characterization of the phenolic content in different natural products [1]. Moreover, the success of these techniques will depend on the most effective sample preparation and extraction methods [12]. Extraction efficiency is greatly influenced by solvent choice and composition (organic or inorganic) and plays a critical role in the extraction yield of phenolics from fruits and vegetables. Generally, for the extraction of phenolics, water, acetone, ethyl acetate, alcohols (i.e., methanol, ethanol, and propanol), and their various percentages of mixtures are used [27, 39]. In addition to the solvent type extraction conditions, parameters such as temperature and duration also influence the yield of phenolics.

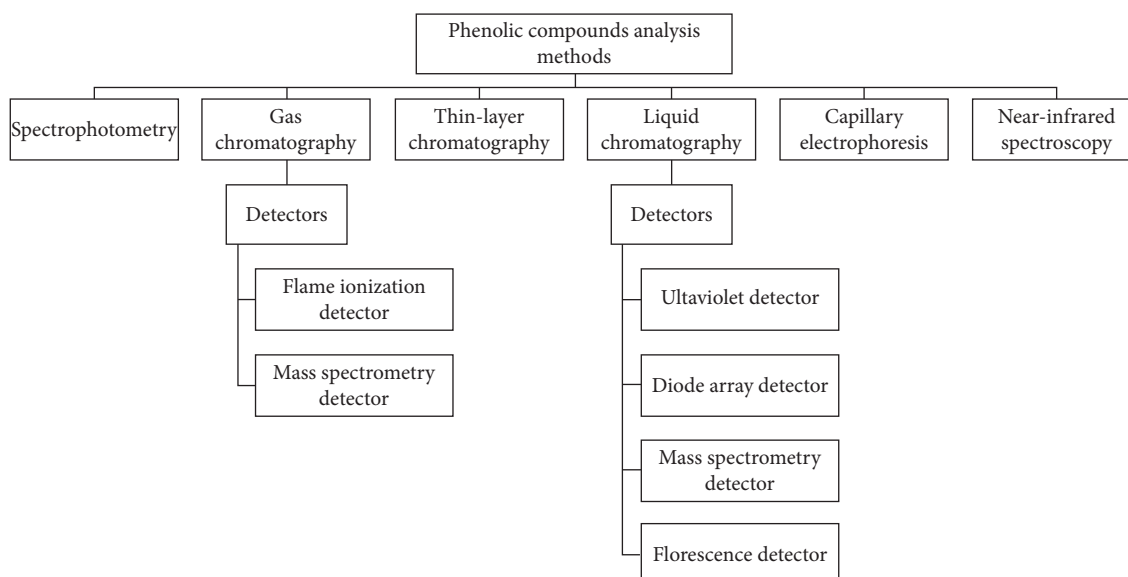


FIGURE 1: Methods used for both qualitative and quantitative analysis of phenolics [1].

Khoddami et al. [2] previously reported that recovery of phenolics varied from one sample to another sample. It is also reported that acid- or base-catalyzed hydrolysis is also an important consideration for the stability of the phenolics in extracts [40, 41]. Davidov-Pardo and Marn-Arroyo [42] reported that the extraction pH plays an important role in the extraction efficiency of phenolic compounds, and the same authors implied that catechins and their isomers are detected more efficiently in alkaline conditions as compared with acidic ones. Extraction of phenolic compounds are commonly done using either liquid-liquid or solid-liquid extraction technique. However, liquid-liquid extraction has some disadvantages because of using costly and potentially toxic solvents. For this reason, improved extraction methods such as solid-phase microextraction (SPME) and solid-phase extraction (SPE) techniques are used to extract phenolics from liquid samples. In general, inexpensive and simple methods such as soxhlet, reflux, and maceration processes are the more conventional procedures used to recover phenolics from solid samples [43]. In addition, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), ultrasound-microwave-assisted extraction (UMAE), supercritical fluid extraction (SFE), subcritical water extraction (SCWE), and high hydrostatic pressure processing (HHPP) are the methods that help us to shorten extraction times and decrease the release of toxic pollutants through reducing organic solvent consumption and are relatively simple to perform. Pulsed electric field (PEF) is also another extraction technique that can be applied at room temperature conditions and performed in a matter of seconds requiring low energy to increase cell membrane breakdown in mass transfer [44] which were applied previously in several fruits such as strawberry and grapes [45].

**2.1. Quantification of Phenolics.** The analysis of phenolic acids and flavonoids by liquid and/or gas chromatography

techniques is the most widely and commonly applied methods for the quantification of phenolics in fruits and vegetables. In addition, spectrophotometric assays are used as nonspecific methods used for evaluating the levels of phenolics in many fruits and vegetables [46, 47].

**2.2. Spectrophotometric Assays.** Although, fruits differ in the quantity and types of phenolic antioxidants, degree of conjugation, and composition of sugar, total phenolic compounds can be estimated in fruits using the reagent proposed by Otto Folin and Vintila Ciocalteu [48] and recently modified by Li et al. [49]. This Folin–Ciocalteu method is robust, highly reproducible (as long as sugars are corrected for), convenient, and fast, requiring only a UV spectrophotometer. The method is typically standardized with either gallic acid, rutin, or a combination of pinocembrin/galangin (2:1) [50, 51]. The Method is based on a reaction of the chemical reagent (i.e., tungsten and molybdenum) with phenolic electron transfer. The phenolic compounds are oxidized to phenolates by the reagent at alkaline pH in a saturated solution of sodium carbonate resulting in a blue molybdenum-tungsten complex and can be measured at 765 nm [48]. The absorbance of each sample can be compared with those obtained from the standard curve, and the obtained data are expressed as  $\mu\text{mol}$  gallic acid equivalents per gram of fresh or dry matter. Because the reaction is quantitative and presumable, the analysis of a mixture of phenols can be recalculated based on any other standard. The assay comprises of monophenols and provides predictable reactions based on the phenols and provides measuring of all compounds readily oxidizable under the reaction conditions.

**2.3. Chromatographic Determination of the Phenolic Profile.** Recently, chromatographic techniques such as high-performance liquid chromatography (HPLC), HPLC electrospray ionization mass spectrometry (HPLC-ESI/MS), gas

chromatography-mass spectrometry (GC/MS), capillary electrophoresis (CE), and near-infrared (NIR) spectroscopy techniques are developed for identification, separation, and quantification of phenolics [1]. Phenolic content of plant materials can be measured and identified using HPLC employing different stationary phase-solvent combinations and various detectors [52]. HPLC relies on comparisons of unknown compounds with standard reference compounds to make both qualitative and quantitative analytical measurements. Columns can be selected to impart specific separations based on the stationary phase type and the size and structure of the packing materials to which the stationary phase is bound to [52]. Detector choice can also be manipulated to enhance detection and especially quantification. Phenolic compounds can easily be measured using UV-Vis, photodiode array detection (DAD), fluorometric detection (FLD), and electrochemical detection (ECD). Each stationary phase-detector combination will provide specific information on the phenolic composition of a sample. For example, UV detection can be used to measure benzoic acid at 246–262 nm, gallic acid at 271 nm, and 275 nm for syringic acid. Two different wavelengths 225–235 nm and 290–330 nm can be used to measure cinnamic acids, but the common wavelength of 280 nm is issued for the general analysis of phenolics [52]. However, many factors such as sample purification, column and detector types, solvents used as mobile phase and solvent purity, and their pH affect HPLC analysis of phenolics. It is previously reported that mixtures of water, methanol, acetonitrile, formic and acetic acids, and trifluoroacetic acid are used for mobile phase for phenolic compounds in reversed phase (RP) chromatography using octadecyl silica columns [52]. Generally, among the HPLC detectors, UV-Vis and DAD detectors are more common compared to the fluorometric detection (FLD). Common stationary phases include  $C_{18}$  RP columns employing an acidified mobile phase (e.g., acetic acid, formic acid, phosphoric acid or citrate) and ammonium acetate buffers of organic solvents (acetonitrile, methanol, ethanol, tetrahydrofuran, 2-propanol etc.). Detection efficiency can be improved by using SPE cartridges composed of styrene-divinylbenzene to purify phenolic compounds from crude extracts prior to HPLC analysis. The wavelength selected for monitoring phenolics is an important criterion [51] and generally ranges between 190 and 380 nm. Gradient elution is generally preferred rather than isocratic elution. Some of the authors previously reported that phenolics such as flavonones, flavonoids, and flavan-3-ols of plum, blueberry, raspberry, strawberry, orange, apple, and tea are possible to be measured by common HPLC techniques [27, 53, 54]. In general, for identification and quantification of phenolics, individual stock solutions of each standard are prepared in methanol and stored at  $-20^{\circ}\text{C}$  until analysis. The working standard mixture solutions are made by diluting the appropriate amount of each stock standard solution to obtain at least 5 calibration levels (final concentrations of 5, 10, 20, 200, and 1000  $\mu\text{g}/\text{mL}$ ) [51]. Measurements of flavanols, hydroxycinnamates, flavonols, and anthocyanins of fruits can be detected at 280, 320, 360, and 520 nm by using HPLC. External standards are used to quantify the phenolic

compounds [55]. Stable isotopes can also be used to quantify phenolic compounds when HPLC-ESI/MS is being used as described below.

**2.4. Mass Spectroscopic (MS) Measurements.** HPLC-ESI/MS is used to increase the range of phenolic compounds detected in a sample and to improve sensitivity as compared with standard chromatographic methods. HPLC-ESI/MS is a robust and selective quantification method that is effective at measuring the complex array of phenolics typically found in fruits and vegetables [52]. Mass spectrometry methods can be performed on a variety of instruments including electrospray ionization ion trap instruments (HPLC-ESI-ITMS) [56], triple quadrupole instruments (HPLC-ESI-QQQ-MS/MS), and time-of flight instruments (HPLC-ESI-TOF/MS). The mass spectrometer (MS) is an analytical detector that gives both qualitative and quantitative measurements based on separation of ions by their  $m/z$  ratio and 0.01% correction. Mass spectrometry involved three stages: ionization, mass analysis, and detection of ions [52]. Separation of phenolic compounds is best achieved in aqueous-organic extracts of foods with HPLC prior to MS analysis although GC can also be used. The most common solvent reduction and ionization technique is electrospray ionization (ESI). This can be performed using different voltages to create negative or positive pseudomolecular ions ( $[\text{M}+\text{H}]^{+}$  or  $[\text{M}-\text{H}]^{-}$ ) that can be accelerated into the mass analyzer [52]. The mass analyzer separates ions based on the flight path as with a magnetic ( $B$ )/electric ( $E$ ) field separation, time-of-flight (TOF) in a field free region, or by altering ion trajectories using quadrupole and ion trap mass analyzers. Detection is usually achieved with an ion multiplier tube. Triple quadrupole analyzers and ion trap analyzers are often used when higher sensitivity and specificity, or structural information is required for identification. Fidelity of MS measurements can be increased using MS/MS techniques. For example, a common technique is to create product ions through collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer ( $Q_2$ ), analyzing the fragment ions in the second analyzer of the instrument ( $Q_3$ ). Paper chromatography (PC), thin layer chromatography (TLC) techniques, high-speed counter current chromatography (HSCCC), capillary electrophoresis (CE), and supercritical fluid chromatographic techniques are also used to identify the phenolic compounds [2, 11, 57–59].

### 3. Conclusion

Phenolic compounds are one of the most important classes of phytochemicals with both functional and health promoting properties. Fruits are the excellent sources of these compounds, and improved methodology for the extraction, isolation, separation, identification, and quantification of the full range of the phenolic content of fruits is critical for understanding the health potential of various fruits, as well as good sources for these compounds.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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