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Profiling methods for the determination of phenolic compounds in foods and dietary supplements

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Abstract

Profiling methods are needed that separate and detect all the phenolic compounds in a single extract of a food material. These methods must be comprehensive, rapid, and rich in spectral information. Fourteen methods that meet, or have the potential to meet, these criteria have been selected from the recent literature for review. In general, the methods employ a single aqueous methanol extraction, separation on a reversed-phase C column, and detection by UV/vis spectroscopy and mass spectrometry. The variations in extraction, separation, and detection are discussed. An increasingly important aspect of these methods is the archiving of data to permit cross-comparison of samples and standards and retrospective analysis. This review shows that the necessary technology is available to achieve the desired analytical goals.

Keywords

Chromatographic profiling; Phenolic compounds; Flavonoids

Introduction

This review will examine selected methods that have been used, or have the potential to be used, as profiling methods for phenolic compounds in food materials. Phenolic compounds are ubiquitous in the plant kingdom. More than 8,000 phenolic compounds have been isolated in a wide variety of forms [1]. Characterization of phenolic compounds in the food supply requires methods that are comprehensive, rapid, and rich in spectral information. These comprehensive methods can be best described as “profiling” methods. In general, a profiling method should separate and detect as many of the components as possible in a single extract of a food material. The profiling method may be the first step in an elaborate analysis procedure, allowing identification of known and unknown compounds.

The ideal profiling method should be as simple as possible, should detect all the compounds present, should provide as much information as possible for each peak in the chromatogram for the purpose of identification, structural evaluation, and quantification, and should accomplish all this in a single chromatographic run. In addition, the method should be applied in a standardized manner that allows comparison of compounds in new materials with existing standards and literature data and provides the opportunity for retrospective analysis, i.e., reexamination of archived data when new standards or compounds of interest appear.

Profiling methods are the logical convergence of two philosophical approaches: classical analytical chemistry and systems biology. Analytically, there has always been the demand to expand from highly optimized, single-component analyses to multicomponent analyses; to extract, separate, detect, identify, and quantify as many components as possible in a single procedure. This has been particularly true for food composition where methods have increasingly focused on the development of nutrient profiles for classes of foods [2, 3]. Biologically, recent research has focused on understanding the organism as a whole using genomics, transcriptomics, proteomics, and, finally, metabolomics [4]. Since the metabolome is defined as an inventory of small molecules, the plant metabolome represents potential human nutrients and health-promoting compounds. Thus, a plant (or animal) metabolome and a nutrient profile are synonymous. Lacking a universal solvent, one must determine an unbiased profile using a series of extractions, each aimed at a different range of polarities. This review will focus on extraction, separation, and detection conditions suitable for phenolic compounds.

Phenolic compounds

Phenolic compounds constitute the largest class of nonnutrients found in the plant kingdom, with more than a dozen subclasses (Fig. 1) and hundreds of compounds in many of the subclasses, e.g., more than 450 flavonols, 400 flavones, 350 flavanones, 300 isoflavones, 19 anthocyanidins, and 350 chalcones. This large variety arises from extensive glycosylation of the aglycone backbones and acylation of the glycosides [5]. The most recognized compounds are the phenolic acids (Fig. 1, A, B), flavonoids (Fig. 1, C–K), and the flavonoid polymers (Fig. 1, L). More than 5,000 different glycosylated flavonoids have been reported and phenolic acids are equally numerous, existing in many derivatized forms and/or bound to insoluble components [1]. Polymerized flavonoids (proanthocyanidins) can be found in many plant materials linked with a single bond or double bonds and may have lengths in excess of ten units [6].

Phenolic compounds are secondary metabolites. They are not involved in growth and energy metabolism and are usually generated in response to environmental stress (e.g., predation, attack by microorganisms, UV light levels). They have also been shown to have a role in the production of root nodules and the plant's immune response. Phenolic compounds are derived from universally present precursors (acetylcoenzyme A, amino acids, and shikimate) and have arisen from the divergence of genes originally associated with metabolism [7]. Consequently, the phenolic compounds found in plants tend to follow taxonomic lines and can be used for botanical identification. Although there are thousands of phenolic compounds in the plant kingdom, there are relatively few in any particular taxonomic group.

Nutritional interest has focused on phenolic compounds since the early 1980s when epidemiological studies showed that diets rich in fruits and vegetables were correlated with a reduced risk of chronic diseases [8]. The time frame necessary to prove the essentiality of phenolic compounds to human health is considerably longer than that for traditional nutrients. Nutritional epidemiological studies cover many years and are dependent on

accurate intake surveys and food databases. Despite continued clinical and laboratory research that has shown that phenolics, in general, and many specific flavonoids have a wide variety of health-promoting effects [8], the essentiality of phenolic compounds is still being debated and none have recommended dietary reference intakes. A careful evaluation of the impact of phenolic compounds on human health requires a food composition database for these compounds. This database, in turn, requires profiling methods that are capable of identification and quantification of phenolic compounds in large numbers and wide varieties of foods.

Profiling methods

The analysis of phenolic compounds is particularly challenging because they are found in all plant materials and in a wide variety of forms. For the thousands of phenolic compounds, there are hundreds of published analytical methods and numerous reviews [9–16]. The USDA Database for the Flavonoid Content of Selected Foods [17] cites 199 papers that provided analytical results. Citations for another 200 papers that provided no data, only reporting method design, optimization, or compound identification, are available upon request.

The vast majority of the methods in the papers cited above were developed for a specific plant material (e.g., oranges) or a family of materials (e.g., all varieties of oranges or citrus in general). Very few were developed specifically as profiling methods. While the general chemical similarities of the phenolic compounds require that these methods have a great many common features, specific chemical features of individual compounds have resulted in a wide variety of optimized parameters for extraction or separation. For many methods, the breadth of applicability can be expanded by altering these parameters. Greater breadth, however, is accompanied by greater compromise. The specific chemical characteristics of the many phenolics will dictate that a single method cannot be optimized for every compound. Compromises in extraction and separation will affect both identification and quantification.

Tables 1, 2, 3, 4 present 14 profiling methods for phenolic compounds that have been selected from the literature of the last 15 years [18–31]. This is not meant to be a comprehensive list. The selected methods provide a historical perspective and serve as examples of methods that can meet, or can potentially meet, the criteria set forth in the “Introduction.” In some cases, the methods were directed at a limited number of food materials, but could be readily modified for application to a greater breadth of materials than originally described [19, 20, 22, 25, 27]. In recent years, however, methods have appeared that were designed to cover a wide range of compounds in any type of food material [21, 23, 24, 26, 28, 29] or as metabolite profiling methods [30, 31]. For these methods, liquid chromatography (LC) is the method of choice for separation and diode array detection) and mass spectrometry (MS) detection are the detection methods of choice.

In general, the methods in Table 1 employ a single extraction step, a single separation step, and tandem DAD and MS detection. These characteristics appear best suited to a profiling method. There are some exceptions, however. Methods are included that use multiple extractions [22], multiple chromatographic runs [28], and tandem MS (MS/MS) [30] or ion trap MS (IT/MS) [27, 31] detection. Both of the latter methods are used in conjunction with collision-induced dissociation (CID). In recent years, LC-DAD-MS, usually with electrospray ionization (ESI), has been used increasingly for characterization of compounds in complex foods and botanical materials [5, 9–13]. It provides a wealth of data; retention times and UV/vis and mass spectra. These data can provide either positive identification, by matching the characteristics of the analytical peaks to those of standards or well-

characterized plant materials reported in the literature, or provisional identification, based on structural information for the compound subunits. For example, the aglycone, the glycosides, and acylation can be identified for glycosylated flavonoids. The advantages/disadvantages of the methods in Table 1 will be discussed in the following sections.

Hertog et al. [18] described what may be considered the first profiling method for flavonoids in 1992. They employed aqueous MeOH extraction and acid hydrolysis for the identification of five flavone and flavonol aglycones in six different fruits and vegetables using LC-DAD. Conversion of the glycosides to aglycones served several purposes: it reduced the number of compounds to be identified, simplified the separation process, provided compounds for which standards were available, and yielded values for the flavonoids in the forms which were believed to be absorbed. With use of this method, flavonoids could be identified and quantified on the basis of UV/vis absorption (200–600 nm) and available aglycone standards.

Numerous researchers have followed the lead of Hertog et al. and used acid hydrolysis and LC-DAD as the basis for profiling methods [26, 32–36]. Merken and Beecher [3] developed an LC-DAD method for the determination of 26 flavonoid aglycones in five phenolic subclasses based on a combination of acid hydrolysis (flavones, flavonols, and anthocyanidins) and direct extraction (flavan-3-ols and flavanones) with quantification using aglycones and some glycosidic flavanones. Harnly et al. [28] used this method for the determination of 20 flavonoids in 59 fresh vegetables, fruits, and nuts sampled across the USA. A similar approach was used by Franke et al. [36] for the determination of 12 polyphenols (in four phenolic subclasses) in Hawaiian fruits and vegetables.

Other researchers used LC-DAD for characterization of phenolic compounds without hydrolysis [19–21, 24]. However, positive identification and quantification required the availability of appropriate glycosylated standards or a second chromatogram after hydrolysis of the sample. Sakakibara et al. [24] developed an LC-DAD method for the identification of “all” polyphenols (over 100 compounds from 11 phenolic subclasses) in vegetables, fruits, and teas, based on retention times and UV/vis spectra. Quantification was based on acid hydrolysis and external aglycone standards.

LC-DAD-MS has been commonly used for profiling methods since the turn of the century [22, 23, 26, 27, 30, 31]. Cuyckens and Claeys [23] used time-of-flight (TOF) MS (LC-DAD-ESI/TOF/MS) for the fast characterization of flavonoid glycosides and identified 12 flavonoids (three subclasses) in a hawthorn extract. Schieber et al. [22] developed an analytical system for the separation and identification of phenolic acids and flavonoids using LC-DAD-ESI/MS and identified 27 phenolic compounds (seven subclasses) in apples and pears. Papagiannopoulos et al. [27] also used LC-DAD-ESI/CID/MSⁿ for the identification and quantification of 41 extractable phenols (five subclasses) in carob fruit and fruit products. Lin and Harnly [29] developed an LC-DAD-ESI/MS method for the identification of glycosylated flavonoids and other phenolic compounds and demonstrated its versatility by identifying 78 compounds (eight subclasses) in fruit, beans, and herbs. Stobiecki et al. [30] developed an LC-DAD-ESI/CID/MS/MS method for profiling phenolic glycoside conjugates in *Arabidopsis thaliana* and identified 16 phenolic compounds from three subclasses. Farag et al. [31] used both LC-DAD-ESI/MSⁿ and quadrupole TOF (QTOF) MS (LC-DAD-ESI/QTOF/MS) for profiling *Medicago truncatula*.

The methods just listed have many common features because they are all aimed at extracting, separating, identifying, and/or quantifying the same subclasses of compounds. The chemical characteristics, more specifically the polarity, of the phenolic compounds dictate the extraction and chromatographic conditions that provide optimum recovery and

separation. In general, the methods in Table 1 used extraction with aqueous alcohol, acetone, or dimethyl sulfoxide (DMSO), chromatographic separation with a reversed-phase column and either an aqueous MeOH or an acetonitrile (ACN) gradient, and detection by UV/vis spectroscopy and MS (of some form). These instrumental approaches have been combined with a number of chemical pretreatments to provide further information and to facilitate quantification. Acid hydrolysis is used to strip off the sugars and produce the aglycones. Base hydrolysis of the sample is used to free covalently bound phenolic acids from the sample matrix and base hydrolysis of the extract is used to deacylate the sugars. A more detailed discussion of each aspect of the analytical methods follows.

Extraction

The first step of the method is the removal of the analytes from the sample matrix. This step is far more critical for quantification than for identification, providing a detectable fraction of each compound is extracted. For a profiling method, as envisioned here, extraction conditions should be as mild as possible to maintain the integrity of the components, i.e., to accurately characterize the endogenous phenolic compounds. This assumes that the compounds are not covalently bound to the sample matrix. This assumption is generally valid for the flavonoids and the flavonoid polymers, but not for phenolic acids. Tura and Robards [16] define the fidelity between the phenolic profiles of the starting material and the extract as a basis for judging the success of an extraction. In most cases, a successful extraction will not remove interferences. In addition, the extraction must also avoid chemical artifacts arising from hydrolysis, oxidation, and isomerization [16]. This usually entails the addition of an antioxidant, such as *tert*-butyl-4-hydroxyanisole, di-*tert*-butyl-4-methylphenol, or *tert*-butylhydroquinone, and/or a polyphenol oxidase inhibitor, such as sodium fluoride.

Phenolic acids commonly form covalent bonds with the sample matrix. In forage materials, cross-linked phenolic acids reinforce the lignin support structure. Acid and alkaline hydrolysis, prior to extraction, increase the quantitative yield. This pre-extraction chemical treatment, however, will significantly alter the forms and yields of the other phenolic components. Since the purpose of the profiling step is to characterize the endogenous phenolic compounds, preextraction treatment is not useful. Instead, a method using pre-extraction chemical treatment would be useful as the second step (following profiling) in the analytical process.

There are reviews [9, 10, 12, 14, 16] covering all aspects of the extraction of flavonoids from plant materials and the difficulties of postextraction acid hydrolysis [10, 16, 37, 38]. In general, phenolic compounds are weak organic acids ($pK_a=8-12$), can range from hydrophilic to hydrophobic, and are usually readily extracted into aqueous alcohol [16]. Glycosylation tends to render flavonoids less reactive and more water-soluble. Aqueous MeOH, EtOH, acetone, and DMSO are the most commonly reported solvents for extraction, with a wide range of water-to-solvent ratios. The range of ratios reflects the variation in the polarity of the phenolics, the specific compounds targeted by the research [39], and the range of end points used to characterize the extraction efficiency, e.g., chromatographic peak area of a single compound, the sum of peak areas for multiple compounds, or total phenolic assays, such as the Folin-Ciocalteu assay.

Aqueous MeOH is probably the most frequently used solvent, although extraction of anthocyanins is enhanced using acidified MeOH or acetone [37, 40] and some flavanones are known to be more soluble in DMSO [41, 42]. In Table 2, eight of the 12 methods that extracted phenolic compounds from solid samples used MeOH concentrations that varied from 60 to 100%. Three of the methods used 50–70% acetone and one method used diethyl

ether. To cover the variation of solubilities, multiple extractions with large solvent-to-solid ratios are employed [21, 41]. This generally provides quantitative extraction, but it produces a large dilution effect that is usually countered by evaporation of the solvent and redissolution of the solid. This last step makes DMSO, with its low volatility, less attractive as a solvent. Several researchers have reported enhanced extraction efficiencies for MeOH or EtOH when using 5–10% DMSO. Most of the extraction methods used an antioxidant or simply failed to list it in the methods section.

Numerous studies have reported the use of fractionation methods, primarily based on solid-phase extraction for liquid samples or following an initial extraction step. Giusti et al. [43] routinely removed anthocyanins from the other phenolic compounds using ethyl acetate. Parejo et al. [44] used seven fractions collected from thin-layer chromatography. Schieber et al. [22, 45] and Hakkinen et al. [32] separated ionic and neutral phenolic fractions using solid-phase extraction and ethyl acetate at a pH of 1.5 and 7.0. These approaches provided more concentrated and purer fractions that were then subjected to reversed-phase chromatography. Fractionation is most successful when specific compounds are targeted. For a profiling method, the added time of preparation and the potential loss of components is a major concern. Fractionation is not an attractive procedure for rapid profiling of phenolic compounds.

Postextraction acid hydrolysis has been used to strip sugars from the flavonoid backbones to reduce the complexity of subsequent chromatograms and permit calibration with more readily available aglycone standards. Most researchers have used variations of the method of Hertog et al. [18]; extracting with 62.5% MeOH, adding a sufficient quantity of 6 M HCl to bring the final concentration to 1.2 M HCl in 50% aqueous MeOH, and refluxing at 90 °C for 2 h. Numerous reports have questioned the appropriateness of this method for all flavonoid glycosides [32, 37–39]. Merken and Beecher [46] reported that aglycone concentrations were time-dependent; increasing with hydrolysis of the glycosides and decreasing as a result of acid-induced degradation. They proposed a kinetic method that involved sampling of the refluxing sample every half hour for 5 h. Peaks from the ten chromatograms were then used to determine the initial glycoside concentration. This method was not suitable for flavan-3-ols or flavanones, which degraded very rapidly. A second, direct extraction was used to analyze these two subclasses [28, 36].

Separation

The quality of any chromatographic separation is dependent on the characteristics of the compounds to be separated and their interactions with the column and the solvent. An examination of the separation methods used in the 199 reports used for the USDA flavonoid database shows almost 199 different separation schemes. This is not surprising considering the many different columns and solvents that are available. In addition, these studies targeted many different phenolic compounds in many different foods. A profiling method for phenolic compounds requires uniform separation across the range of polarities; from the most polar (hydroxybenzoic and hydroxycinnamic acids) to the least polar (aglycones and polymethoxylated flavonoids) compounds. This target of a fairly wide polarity range does serve to produce some uniformity in the chromatographic conditions, but, as shown in Table 3, there are still a wide variety of separation schemes.

The 14 selected profiling methods listed in Table 3 employed 13 different C18 reversed-phase columns. Two columns were used in more than one study [18, 19, 22, 27] and one study used two columns [23]. It should be noted that the studies listed in Table 3 covered a period of 15 years and that the column technology has advanced considerably during this interval. Only reversed-phase columns were used since phenolic compounds are weak acids

that can be separated as neutral, relatively hydrophobic compounds in a weak acid matrix. Normal-phase columns have been used for proanthocyanidins to provide separation of the monomers through the decamers and a broad peak for the higher polymers [6]. A wide variety of reversed-phase columns are available and the characteristics of each column are slightly different. Several comparative studies have been reported for the separation of flavonoids [23, 29, 47, 48] and each selected different columns. In practice, no column is optimum for all regions of the chromatogram. Best results for the entire range of polarities are achieved with multiple columns. However, a profiling method provides an initial characterization of a sample obtained under a set of standard conditions. Thus, a column must be selected, despite the compromises, and used routinely before proceeding to more targeted separations.

The two primary solvents used for the mobile phase are MeOH and ACN. In general, the solvents are used in a binary system with a weak acid. The elution gradients for MeOH usually started at 5–10% (v/v) and ended at 40–100% (Table 3). The gradients for ACN started at 0–10% and finished at 30–90%. Separation of the more polar phenolic acids is highly dependent on the pH of the mobile phase [47] because they are weak acids. A weakly acidic mobile phase will suppress ionization and enhance the separation on a reversed-phase column. Several studies have presented tables for the retention times for phenolic compounds with a wide range of polarities [24, 29]. The large range of final solvent concentrations reflects the fact that one of the largest differences between methods is their separation of nonpolar compounds.

With DAD, the initial acid concentration ranged from 2 to 10% phosphoric or acetic acid, adjusted to a pH of 2–4. The final acid concentration was usually lower. The less polar aglycones and polymethoxy flavonoids are less prone to ionize and can be separated in mobile phases with a pH around 7. MS detection requires lower (0.1–2%) but isocratic acid concentrations. Cuyckens and Claeys [23] concluded that, for MS detection, formic acid was preferable to acetic or trifluoroacetic acid for an ACN/water mobile phase. Optimum concentrations were 0.1% for negative ionization and 0.5% for positive ionization. When an MeOH/water mobile phase was used, 1% acetic acid offered the best sensitivity [23].

In Table 3, nine of the studies used an ACN/water mobile phase and five used MeOH/water. One study used a mixture of the two, which is not uncommon. The polarity range of the gradients used for MeOH and ACN in Table 1 averaged out to be roughly the same. Using the Snyder polarity index, the MeOH gradients started at 8.7 (the value for water is 9.0) and ended at 7.4 (MeOH is 6.6). The ACN gradients started at 8.9 and ended at 7.5 (ACN is 6.2). Thus, both solvents have been used to separate compounds over the same range of polarities. Most of the chromatographic separations took 60–70 min, although a few took approximately 30 min. Ironically, one might anticipate that the shorter separation times were used with MS detection which could resolve overlapping peaks using selective ion monitoring, but that was not the case.

Figure 2 presents chromatograms for the elution of 76 phenolic compounds in four plant materials [29]. Labels for 11 hydroxycinnamates in navel orange (between 7- and 15-min elution time) are not given. There is considerable overlap between the different subclasses of phenolics, which makes it difficult to provide a simple elution order; however, some generalizations can be made. The hydroxybenzoic and hydroxycinnamic acids are generally eluted first and the aglycones and polymethoxylated flavonoids are generally eluted last. The glycosidic linkage site affects retention times; for the same aglycone and glycoside, the elution order is 7-*O*-glycoside, 3-*O*-glycoside, and 4'-*O*-glycoside [5]. The glycoside will affect the elution order; for the same aglycone and linkage site, the elution order is galactoside, glucoside, pentoside (xyloside, arabinopyranoside, and arabinofuranoside),

rhamnoside, and glucurono-side [5]. With the same glycoside at the same position, the elution order of the aglycones is flavanones, flavonols, and flavones [5, 22]. The retention time for individual flavonoids is, in general, inversely related to increasing glycosylation [5].

Detection

UV/vis spectroscopy and MS are the primary methods of detection employed with LC separation of phenolic compounds. The multiple conjugate bonds make phenolic compounds strong chromophores with strong UV and, for the anthocyanidins and some flavonols, visible absorption bands (Fig. 3). In addition, the molecular structure of the phenolic compounds makes them fairly easy to ionize by ESI and atmospheric-pressure chemical ionization. In general, sensitivity is greater with negative ionization [5, 24], but this is highly compound specific. Electrochemical detection has been used with a series of voltammetric or coulometric detectors but has had limited use. Fluorescence detection has also had limited application since few phenolic compounds fluoresce.

The robustness of UV/vis detection offers the best means of quantifying phenolic compounds and can also be used for distinguishing flavonoid subclasses (Fig. 3). UV/vis spectroscopy and MS, in the total ion count mode, have comparable detection limits for flavonoids, approximately 10 ng in an LC peak. MS has better detection limits in the single ion monitoring (SIM) mode, <1 ng [5]. However, the information-rich fragmentation patterns of MS are critical to the identification of the many different compounds and are lost in the SIM mode. Table 3 shows the impact of the availability of smaller, less expensive, and more user friendly quadrupole mass spectrometers as LC detectors in the late 1990s. Today, LC-DAD-MS and, more specifically, LC-DAD-ESI/MS are recognized as necessary tools for the identification and quantification of phenolic compounds in foods [5, 9, 11–13].

The attractiveness of MS for the identification of organic compounds has been enhanced in recent years by the development of triple-stage quadrupole MS and IT/MS for detection of selected fragments (MS/MS). These spectrometers used improve specificity by using CID to produce characteristic daughter ions. This capability allows deconvolution of overlapping peaks and reduces the demand for high-resolution chromatography. Many mass spectrometers can be operated in a multiscan mode which allows polarity switching and in-source CID. This makes it possible to simultaneously monitor negative and positive ions and to obtain spectra with high and low fragmentation energies (Fig. 4), thus providing a limited imitation of MS/MS detection. The fragmentation patterns from a high voltage potential provide more information about the structure and conjugates of a compound than the low potential necessary to preserve and identify the parent ion. Unfortunately, the multiscan mode also results in a poorer signal-to-noise ratio since less time is spent at each mass. Stobiecki [13] and Cuyckens and Claeys [5, 23] have written excellent reviews on the utility of CID and fragmentation patterns for identifying phenolic compounds.

The combination of UV/vis and MS detection has many features that recommend it for phenolic compounds. From a single chromatographic peak, LC-DAD-MS with multi-scan or MS/MS detection can determine the molecular mass of the parent phenolic compound, identify the aglycone, determine the number and type (hexoside, deoxyhexoside, or pentoside) of the glycoside, identify acylation of the glycosides, and, from some fragmentation patterns, determine the location of the glycosidic bonds [13, 29]. More specific information can be acquired using MS/MS, but this requires a targeted approach. Specific daughter masses must be selected in advance. LC-DAD-MS with in-source CID can be operated in the discovery mode and allows automatic acquisition of fragmentation patterns. This eliminates the possibility of missing data as a result of a priori decisions. Overall, LC-DAD-MS is the current technique of choice for a profiling method.

Identification/quantification

The wealth of data collected by multisignal MS or MS/MS allows two levels of identification of compounds; positive and provisional. Positive identification can be achieved if reference standards are available and a detailed comparison of the retention time and the spectra shows a match. This requires running the appropriate standard in conjunction with the sample or employing a standardized approach that allows the sample to be compared with archived data. Provisional identification is achieved when the subunits are positively identified but the positions of the glycosidic and acyl linkages have not been confirmed. In many cases, taxonomic data provide a good basis for postulating the correct structure, but positive identification requires analysis by NMR. Identification of specific compounds based solely on the UV/vis spectra is problematic. When chromatograms display multiple, partially overlapping peaks, confirmation of the peak identification by MS is preferred.

The ability to simultaneously acquire data in both the positive and the negative ionization mode presents an important advantage to the identification process as some compounds are detectable in only one mode. Fragmentation patterns provided by CID, with either the positive or the negative ionization, are much more informative with respect to structure [5, 13, 23]. As stated earlier, fragmentation data can be obtained by in-source CID or MS/MS. MS/MS targets a single mass for collision and therefore has fewer interferences. In-source CID allows operation in the discovery mode but is less specific since all the masses are subjected to CID. In combination with LC, however, the use of high fragmentation energies can be used to provide information-rich spectra on a routine basis.

Figure 5 presents the high-energy and low-energy spectra of didymin, an O-linked flavanone rutinoside, and illustrates the advantage of using high and low fragmentation energies. The low-energy spectra (100 V) clearly show the parent ion for didymin and weak intensities for the monosaccharide (Y_1^+) and aglycone (Y_0^+). At the high fragmentation energy (250 V), the parent ion cannot be seen and the aglycone ion is much more prominent. Neither spectrum offers information that allows identification of the 7-position as the site for the glycosidic linkage; however, this is the most common site for flavanones. In this case, the structure was previously confirmed with reference standards. The high fragmentation energy spectra for C-linked glycosides can be even more informative, with respect to linkage sites, since the high-energy spectra show the sequential destruction of the glycosides through cross-ring cleavage [5, 9, 14].

The level of identification necessary for nutrition and food analysis is a question worth considering. Provisional identification of phenolic compounds can be achieved by profiling methods using in-source CID or MS/MS. Achieving positive identification can be time-consuming and expensive, possibly requiring use of different columns, solvents, MSⁿ, and NMR. Spectroscopic fingerprints and chromatographic profiles have been used for many years for authentication of plant-derived materials, such as wines, juices, and oils [16], without positive identification of all the individual compounds. Profiling methods with archiving can readily categorize peaks as positively identified, provisionally identified, and unknown. This status can be accepted or positive identification of provisional and unknown peaks can be pursued at a later time.

What level of identification is necessary for determining bioavailability and bioefficacy? Gentle extraction processes can provide fidelity of the phenolic compounds with respect to their form in the original sample matrix, but the chemical forms are usually modified in the digestive tract through hydrolysis, oxidation, fermentation, enzymatic reactions, and/or interactions with other food components. Thus, in most cases, the microenvironment of the

plant material has no relevance to the absorption process. This observation led to the use of acid hydrolysis for analysis of the flavonoid aglycones since the data showed that all flavonoids were absorbed as aglycones. This is still true for isoflavones and many flavonoids. Recent research, however, has shown that some monoglucosylated flavonoids are absorbed through a glucose transport mechanism. Other studies have suggested that the sugar and position are critical to the absorption process. This suggests that positive identification is necessary to assess bioavailability. It is probably safest to say that more research on the chemical interactions of phenolic compounds during the digestive process and more knowledge regarding absorption mechanisms are necessary before the necessary level of identification can be specified.

Table 4 shows that a wide variety of phenolic compounds have been identified in a wide variety of food materials. This abbreviated table does not show that the number of identified compounds in a given category usually exceeded the number of compounds for which there were standards. In other words, 20 flavonols may have been identified even though there were only three standards available. Thus, in most cases, the identification was provisional, based on UV/vis and mass spectra, not on comparison with authentic standards.

Quantification of phenolic compounds is a challenging task. Historically, the main obstacle has been the lack of standards. With thousands of glycosylated flavonoids, there have not been and never will be standards for most compounds. The lack of standards was another driving force for the development of acid hydrolysis methods for the analysis of flavonoids. Removal of the glycosides produces a smaller number of aglycones and a relatively greater number of available standards. Standards for nonflavonoid phenolics are even more difficult to find. In addition, the cost of the standards that are available can be quite prohibitive.

The primary means of quantifying phenolic compounds is UV/vis absorption and, as stated earlier, this can be used to distinguish the different phenolic subclasses. In many cases, the absorption coefficients of the compounds and the wavelengths of maximum absorption do not change significantly with glycosylation because the glycosidic and alkyl residues are poor chromophores. It is common for researchers to use the aglycone as a standard for many of the glycosylated forms, especially for O-linked compounds. The same cannot be said for C-linked glycosides, which can establish a new series of conjugated bonds and significantly alter the absorption spectra; however, C-linked glycosides are not very prevalent.

Quantification using MS is generally less accurate and has poorer precision. Although specificity is enhanced through the use of in-source CID or MS/MS, accuracy of quantification is relatively unaffected. Accuracy is primarily affected by ionization efficiency and sample bias due to ion suppression interferences is common. The poorer precision of MS (10–15%) is a result of the variability of the ionization process. As a rule of thumb, MS is used for identification of compounds and UV/vis absorption is used for quantification. Both measurement systems require standards for accurate quantification.

Table 4 shows that quantification of phenolic compounds lags behind identification. There is a need for a systematic evaluation of the absorption coefficients and wavelength maxima of phenolic compounds with a variety of carbohydrate and acylated conjugates. General rules for the similarity of spectra would be extremely useful for the quantification process. A systematic study of absorption coefficients for more than 150 flavonoids was published more than 35 years ago [49]. An update is desperately needed.

Quantification places much more emphasis on the efficiency of the extraction process [16]. Compromises associated with the extraction of a large number of phenolics for a profiling method may result in inaccuracies (low bias) for some compounds. The degree of inaccuracy will vary depending on the phenolics present and the extraction conditions.

However, the degree of inaccuracy must be considered in light of the natural variability of phenolic compounds in plants. Because many of the phenolics are influenced by plant stress, concentration variations with respect to location, growing conditions, and processing conditions can be large, even for the same cultivar. A recent study showed that the standard error for flavonoids in foods purchased across the country, with no discrimination with respect to cultivar, was approximately $\pm 180\%$ [28]. The relative precision of the method used in the study was greater than desired ($\pm 12\%$), but was still small compared with the sample variation.

The high natural variability of the phenolic concentrations raises the question as to what level of quantification is necessary for databases, especially if there is no discrimination with respect to cultivar. In many cases, compound identification may be sufficient for the vast majority of phenolic compounds which are found at low levels in relatively few plants. Identification implies the presence of the compound above a threshold level, but provides no further quantitative information. For epidemiological studies, quantitative values are necessary to establish the impact of phenolic compounds on human health. For these studies, a quantitative database with cultivar data would be preferred.

Archiving

Application of profiling methods to plant materials will generate a tremendous amount of data. To obtain maximum usefulness from this data, a profiling method must be applied in a standardized manner to all samples and standards. This means that each analysis no longer provides standalone data, but instead contributes to a database that can be used for cross-referencing compounds in a wide variety of samples and standards. For example, Lin et al. [50] recently used the spectra for hydroxycinnamic acid derivatives in navel orange peel to identify the same compounds in dry beans on the basis of archived data. A database for a profiling method should include chromatograms and UV/vis and mass spectra of every compound and the positive or provisional identification.

Proper archiving of analytical results can also provide the capability of retrospective analysis. As new standards become available or as new phenolic compounds draw scientific interest, data collected from archived analyses can be mined for identification and possible quantification of these compounds.

Every method listed in Tables 1, 2, 3, 4 can be used to establish a database if applied in a systematic, standard manner. These databases would be even more useful if the mass spectra obtained from ESI were more reproducible between instruments. However, the need to organize the data from profiling methods is imperative.

Summary

The technology exists for the development of profiling methods that are comprehensive, rapid, and data-rich. These methods are needed for the characterization of food materials and the establishment of food composition databases. This review has considered those methods applied to the analysis of aqueous alcohol extractions of phenolic compounds. Extraction with different solvents is necessary to cover the full range of small metabolic compounds found in food materials. Profiling methods are an answer to the demand for timely information on the nutrients and health-promoting compounds in an ever-changing food supply.

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Fig. 1. Structural skeletons of common phenolic compounds found in food materials

Fig. 2.

Liquid chromatography (LC) chromatograms with UV absorption: navel orange peel (350 nm), soybean seeds (270 nm), Fuji apple peel (270 nm), cranberry (270 nm), Fuji apple peel (520 nm), and cranberry (520 nm). Compounds for navel orange peel are glycosylated flavones (1, 2, 5, 6, 7, 9, 10), glycosylated flavanones (3, 4, 8, 11, 12), and polymethoxyflavones (13–18); for soybean seed they are glycosylated isoflavones (1–7) and isoflavone aglycones (8–10); for Fuji apple peel they are anthocyanins (1, 2), hydroxycinnamic acid (3), flavan-3-ols (4, 6), proanthocyanidins (5, 7–9), glycosylated flavonols (10–17), and glycosylated dihydrochalcones (18, 19); and for cranberry they are anthocyanins (1–4), flavan-3-ols (5), glycosylated flavonols (6–16), and flavonol aglycones (17, 18). Full identification of these peaks can be found in [29]

Fig. 3. UV/vis absorption spectra: **a** 1 quercetin 3-*O*-galactoside (flavonol), 2 sinengetin (flavone), 3 cyanidin 3-*O*-galactoside (anthocyanin), 4 chlorogenic acid; **b** 1 hesperidin (flavanone), 2 epicatechin (flavanol), 3 genistin (isoflavone), 4 phloridzin (dihydrochalcone) [29]

Fig. 4.

LC chromatograms of elder flower extract: **a** UV absorption at 350 nm, **b** total ion count (TIC) for positive ionization at 70 V, **c** TIC for positive ionization at 250 V, **d** TIC for negative ionization at 70 V, **e** TIC for negative ionization at 250 V, and **f** UV absorption at 350 nm of acid-hydrolyzed elder flower extract. Compounds are hydroxycinnamic acids and derivatives (*E1*, *E2*, *E3*, *E4*, *E5*, *E13B*, *E14B*, *E15*), flavonol glycosides (*E6*, *E7*, *E8*, *E9*, *E10*, *E11*, *E12*, *E13A*, *E14A*), and flavonol aglycones (*E16*, *E17*, *E18*). Full identification of these peaks can be found in [29]

Fig. 5. Structure of didymin (a flavanone-*O*-rutinoside) and mass spectra obtained with positive ionization and *A* a low fragmentation voltage and *B* a high fragmentation voltage [29]

No.	Matrix	Pre-Extraction Treatment	Extraction	Pre-Analysis Treatment	Ref
1	5 vegetables & 1 fruit	freeze dried, powdered	62.5% MeOH, 2 g/l TBHQ	acid hydrolysis (40 mL extract + 10 mL 6 M HCl)	18
2	2 wines	none	none	filtered	19
3	8 citrus juices	filtered	25 ml sample diluted with 20 ml DMF and heated at 90 C for 10 min	centrifuged	20
4	5 pear varieties 4 apple varieties red wine pomace 2 green bean varieties lentils	homogenized	99% MeOH, 1% BHT 80% MeOH, 1% BHT	filtered	21
5	2 apple varieties 3 pear varieties	mashed, centrifuged, & pasteurized	70% acetone	evaporate acetone and extract with 100% EA at pH 1.5 and pH 7.0	22
6	hawthorn extract (commercial)	none	none	none	23
7	63 fruits, vegs, & teas	freeze dried, powdered	identification 90% MeOH, 0.5% AA acid hydrolysis see Hertog, method #1	Added 50 nM flavone in DMSO as IS	24
8	clovers	finely ground	80% MeOH	none & acid hydrolysis	25
9	Hypericum perforatum Rhodolia rosea 2 green teas 2 grape red wines 2 orange juices	freeze dried, powdered filtered	100% MeOH with IS of dextromethorphan dilute with 100% MeOH	none	26
10	carob fruits & products	air dried	pressurized liquid extraction with 50% MeOH at 60 C	none	27
11	59 fresh fruits, vegs, & nuts	freeze dried, powdered powdered	acid hydrolysis see Hertog, method #1 90% MeOH, 0.4 g/l TBHQ	none	28
12	3 fruit, 1 bean, 1 herb	air dried, powdered	60% MeOH	none, heating at 80 C, and acid hydrolysis	29
13	<i>Arabidopsis thaliana</i>	freeze dried, powdered	80% MeOH	none	30
14	<i>Medicago truncatula</i>	finely ground	80% MeOH	none, heating at 80 C, and acid hydrolysis	31

Table 2. Selected methods—sample preparation

AA - acetic acid
 EA - ethyl acetate
 MeOH - methanol
 DMSO - dimethylsulfoxide
 MBS - metabisulfite
 IS - internal standard
 ACN - acetonitrile
 DMF - dimethyl formamide
 BHT - 2,6-di-*tert*-butyl-4-methylphenol
 SPE - solid phase extraction
 IS - internal standard

Table 1

Selected methods—overview

No.	Authors	Purpose	No Sub-Classes	No of Compounds	Ref
1	Hertog et al.	Rapid method for quantitative determination of 5 major flavonoid aglycones in vegetables and fruits	2	5	18
2	Lamuela-Raventos & Waterhouse	A direct HPLC separation of wine phenolics	6	21	19
3	Mouly et al.	Simultaneous global method for separation and quantification of flavanones and polymethoxylated flavones in citrus juices	2	12	20
4	Escarpa & Gonzalez	Optimization and validation of a single chromatographic method as approach to determine the phenolics compounds from different sources	8	46	21
5	Schieber et al.	Establish analytical system for separation of phenolic acids and flavonoids of apple and pear	7	26	22
6	Cuyckens & Claeys	Optimization of an LC method for the fast characterization of flavonoids, even if reference compounds are not available	3	12	23
7	Sakakibara et al.	Simultaneous determination of all polyphenols in vegetables, fruits, and teas	11	104	24
8	Wu et al.	Robust inclusive method to study the isoflavonoid profile of red clover	1	31	25
9	Tolonen & Uusitalo	Fast screening method for the analysis of total flavonoids in plants and foodstuffs	6	79	26
10	Papagiannopoulos et al.	Identify and quantify the main extractable polyphenols in carob pods and products	5	41	27
11	Harnly et al.	Flavonoid content of US fruits, vegetables, and nuts	5	20	28
12	Lin & Harnly	A screening method for the identification of flavonoids and other phenolic compounds using standard analytical approach for plant materials	8	78	29
13	Stobiecki et al.	Profiling phenolic glycoside conjugates in leaves of <i>Arabidopsis thaliana</i>	3	16	30
14	Farag et al.	Large scale and systematic identification of polyphenols in <i>Medicago truncatula</i>	5	35	31

Table 3
Selected methods—columns, solvents, gradients, and detectors

No.	column	solvent	gradient A/B start A/B end	Time	Detection	Ref
1	Novapak C18	25% ACN, 0.025 M KH_2PO_4	iso	25	DAD	18
	150 × 3.9, 4 μm , Waters	45% MeOH, 0.025 M KH_2PO_4	iso			
2	Novapak C18	50 mM $\text{NH}_4\text{H}_2\text{PO}_4$, pH 2.6	100/0/0	60	DAD	19
	150 × 3.9, 4 μm , Waters	20% a, 80% ACN	0/80/20			
		0.2 M H_3PO_4 , pH 1.5				
3	Alltima C18	ACN	100/0	49	DAD	20
	250 × 4.6, 5 μm , Alltech	4% AA	70/30			
4	Nucleosil 120 C18	0.01 M H_3PO_4	95/5	25	DAD	21
	250 × 4.6, 5 μm , MetaChem Tech.	MeOH	0/100			
5	Aqua C18	2% AA	90/10	65	DAD	22
	250 × 4.6, 5 μm , Phenomenex	50% ACN, 0.5% AA	0/100		ESI/MS	
					neg	
6	Xterra RP-18	0.5% FA	87/13	15	DAD	23
	250 × 3, 5 μm , Waters	0.5% FA in ACN	77/23		ESI/TOF/MS	
7	Capcell pak C18	10% MeOH, 50 mM NaH_2PO_4	100/0	95	DAD	24
	100 × 4, 5 μm , Shiseido	70% MeOH	0/100			
	Prodigy ODS	0.1% FA				
8	150 × 3.2, 5 μm , Phenomenex	0.1% FA in ACN				25
9	SymmetryShield RP-8	0.1% FA	88/12	23	DAD	26
	50 × 2.1, 3.5 μm , Waters	100% MeOH	10/90		ESI/MS	
					pos & neg	
10	Aqua C18	1% AA	95/5	77	DAD	27
	150 × 2, 5 μm , Phenomenex	1% AA in ACN	40/60		ESI/CID/MS ⁿ	
					neg	
11	Zorbax Eclipse XDB-C18	0.5% TFA	90/6/4	60	DAD	28

No.	column	solvent	gradient A/B start A/B end	Time	Detection	Ref
	250 × 4.6, 5 μmm, Agilent	0.5% TFA in MeOH 0.5% TFA in ACN	0/85/15			
12	Symmetry C18	0.1% FA	90/10	70	DAD	29
	250 × 4.6, 5 μmm, Waters	0.1% FA in ACN	35/65		ESI/MS	
					pos & neg	
					hi & low FV	
13	SuperSpher 100 RP-18	5% ACN, 0.5% FA	9%	60	DAD	30
	250 × 2, 5 μmm, Merck	95% ACN, 0.5% FA	0/100		ESI/CID/MS/MS	
					pos & neg	
14	Reversed-phase, C18	0.1% AA	95/5	70	DAD	31
	250 × 4.6, 5 μmm, Baker	ACN	Oct-90		ESI/MS ^a	
					pos & neg	
					ESI/QTOF/MS	
					pos	

AA - acetic acid

DAD - diode array detector

QTOF - quadrupole time-of-flight

FA - formic acid

ESI - electrospray ionization

CID - collision induced dissociation

FV - fragmentation voltage

MS - mass spectrometry

Table 4

Selected methods—compounds identified/quantified

No.	Compounds Identified	Compounds Quantified	Standards	Ref
1	5 vegetables, 1 fruit	L, O	L, O	18
2	2 wines	none	Ag(nc), B, C, Lg, P(nc)	19
3	8 citrus juices	Ng, Np	Ng, Np	20
4	5 pear varieties	none	B, Bd, C, Dg, I, L, Lg, O, P(nc)	21
	4 apple varieties			
	red wine pomace			
	2 green bean varieties lentils			
5	2 apple varieties	B, Bd, C, D,	B, Bd, C, D, Dg, L, Lg, P	22
	3 pear varieties	Dg, L, Lg, P		
6	hawthorn extract (commercial)	none	Lg, Og,	23
7	63 fruits, vgs, & teas	A, B, Bd, C, I, L,	A, B, Bd, D, I, Ig, L,	24
		Lg, N, O, Og, T	Lg, N, Ng, O, Og, T	
8	red, white, & alsike clover	I, Ig, Iga	I, Ig, Iga	25
9	Hypericum perforatum	A, C	A, Ag, C, L, Lg, N, O, Op	26
	Rhodolia rosea			
	2 green teas			
	2 grape red wines			
	2 orange juices			
10	carob fruits & products	B, Bd, C, Lg, P	B, C, L	27
11	59 fresh fruits, vgs, & nuts	A, C, L, O, N, Ng	A, C, L, O, N, Ng	28
12	3 fruit, 1 bean, 1 herb	none	Ag, B, Bd, C, Dg, L, Lg, Ng, Og, Op	29
13	<i>Arabidopsis thaliana</i>	none	none	30
14	<i>Medicago truncatula</i>	none	I, Ig, N, Ng	31

A-anthocyanidins, B-hydroxybenzoic & hydroxycinnamic acids, C-catechins, D-chalcones & dihydrochalcone I-isoflavones, L-flavonols, N-flavanones, O-flavones, P-proanthocyanidins, T-theaflavins, Q-anthraquinones

a-acylated, d-derivative, g-glycoside, p-polymethoxy

(nc)-non commercial standards, all others purchased from vendors