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# Phenolic Acids in Foods: An Overview of Analytical Methodology

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Phenolic acids are aromatic secondary plant metabolites, widely spread throughout the plant kingdom. Existing analytical methods for phenolic acids originated from interest in their biological roles as secondary metabolites and from their roles in food quality and their organoleptic properties. Recent interest in phenolic acids stems from their potential protective role, through ingestion of fruits and vegetables, against oxidative damage diseases (coronary heart disease, stroke, and cancers). High performance liquid chromatography (HPLC) as well as gas chromatography (GC) are the two separation techniques reviewed. Extraction from plant matrixes and cleavage reactions through hydrolysis (acidic, basic, and enzymatic) are discussed as are the derivatization reagents used in sample preparation for GC. Detection systems discussed include UV−Vis spectroscopy, mass spectrometry, electrochemical, and fluorometric detection. The most common tandem techniques are HPLC/UV and GC/MS, yet LC/MS is becoming more common. The masses and MS fragmentation patterns of phenolic acids are discussed and tabulated as are the UV absorption maxima.

**KEYWORDS: Phenolic acids; analytical methodology; HPLC; gas chromatography; mass spectrometry; antioxidant, foods**

## INTRODUCTION

Vascular plants synthesize a diverse array of organic molecules, referred to as secondary metabolites. These partake in a variety of roles in plant life ranging from structural to protection. Phenolic acids are one such group of aromatic secondary plant metabolites widely spread throughout the plant kingdom (*1*).

Phenolic acids are a subclass of a larger category of metabolites commonly referred to as "phenolics". The term phenolics encompasses approximately 8000 naturally occurring compounds, all of which possess one common structural feature, a phenol (an aromatic ring bearing at least one hydroxyl substituent) (*2*). Current classification divides the broad category of phenolics into polyphenols and simple phenols, based solely on the number of phenol subunits present (*3*). Polyphenols possessing at least two phenol subunits include the flavonoids, and those compounds possessing three or more phenol subunits are referred to as the tannins (hydrolyzable and non-hydrolyzable).

The name "phenolic acids", in general, describes phenols that possess one carboxylic acid functionality. However, when describing plant metabolites, it refers to a distinct group of organic acids (**Table 1**). These naturally occurring phenolic acids contain two distinguishing constitutive carbon frameworks: the hydroxycinnamic (**Xa**) and hydroxybenzoic (**Xb**) structures. Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring create the variety. In many cases, aldehyde analogues (**Xc**) are also grouped in with, and referred to as, phenolic acids (e.g., vanillin). Caffeic, *p*-coumaric, vanillic, ferulic, and protocatechuic are acids present in nearly all plants (*4*). Other acids are found in selected foods or plants (e.g., gentisic, syringic).

**Aim and Scope.** Isolating, identifying, purifying, and observing patterns present in foods has been a rich, albeit disjointed, area of study, owing to the diversified functions of phenolic acids in both plant life and in human sensory qualities. The methodology in measurement systems for polyphenols, including flavonoids and tannins, have received a great deal of attention and have been extensively reviewed and assessed (*5*−*9*). Few reviews focusing exclusively on the analytical methodology for the analysis on phenolic acids have been written (*10, 11*).

This review is a compilation of the different methodologies and techniques used in the analysis of phenolic acids in foods and some nonfood sources, generated in the diversified research arenas from 1982 to 2002. The literature articles have been grouped according to two major chromatographic (separations) techniques (high performance liquid chromatography and gas chromatography (HPLC, GC)) and presented mainly in the form of tables. Relative to sample preparation, only investigations that have attempted to release or "free" the hydroxycinnamic and hydroxybenzoic acid derivatives from their ester attachments have been considered. This choice was made for two reasons: it obliges one to carefully examine these steps in the sample preparation process, namely extraction and bond cleaving steps;

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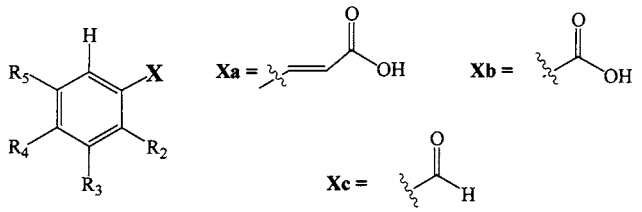
**Table 1.** Structures of the Prominent Naturally Occurring Phenolic Acids




| $R_2$ | $R_3$ | $R_4$ | $R_5$ | X | code | common name |
|---|---|---|---|---|---|---|
| H | H | H | H | a | **1** | cinnamic acid |
| –OH | H | H | H | a | **2** | *o*–coumaric acid |
| H | H | –OH | H | a | **3** | *p*–coumaric acid |
| H | –OH | H | H | a | **4** | *m*–coumaric acid |
| H | $–OCH_3$ | –OH | H | a | **5** | ferulic acid |
| H | $–OCH_3$ | –OH | $–OCH_3$ | a | **6** | sinapic acid |
| H | –OH | –OH | H | a | **7** | caffeic acid |
| H | H | H | H | b | **8** | benzoic acid |
| –OH | H | H | H | b | **9** | salicylic acid |
| H | H | –OH | H | b | **10** | *p*–hydroxybenzoic acid |
| H | $–OCH_3$ | –OH | H | b | **11** | vanillic acid |
| H | $–OCH_3$ | –OH | $–OCH_3$ | b | **12** | syringic acid |
| H | –OH | –OH | H | b | **13** | protocatechuic acid |
| –OH | H | H | –OH | b | **14** | gentisic acid |
| –OH | –OH | –OH | –OH | b | **15** | gallic acid |
| H | $–OCH_3$ | $–OCH_3$ | H | b | **16** | veratric acid |
| H | $–OCH_3$ | –OH | $–OCH_3$ | c | **17** | syringealdehyde |
| H | $–OCH_3$ | –OH | H | c | **18** | vanillin |

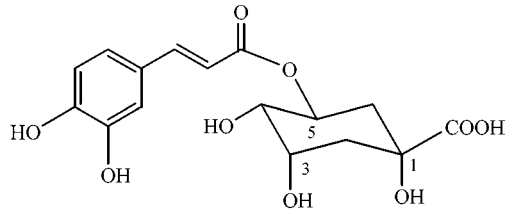



**Figure 1.** Structures of 5-caffeoylquinic acid (chlorogenic acid)

and despite the renewed interest and potential evidence concerning health benefits, little is known about the absorption (i.e., which derivative) and metabolism of phenolic acids.

**Source and Roles of Phenolic Acids in Plants.** *Biosynthetic Origin.* Both benzoic and cinnamic acid derivatives have their biosynthetic origin from the aromatic amino acid L-phenylalanine, itself synthesized from chorismate, the final product in the shikimate pathway (**Figure 1**) (*12, 13*). Subsequent conversion of L-phenylalanine to the various hydroxycinnamic acids involves a three-step sequence referred to as the "general phenylpropanoid metabolism", owing to its almost universal appearance in synthesis of aromatic secondary metabolites. The first step in the phenylpropanoid metabolic path is the stereospecific deamination (anti) of phenylalanine, generating the *trans*-double bond in the cinnamic backbone. Hydroxylation of the aromatic ring at position 4 generates *p*-coumarate. Subsequent formation of the CoA ester (an activated form of the ester) occurs via enzymatic conversion (*13*). Other steps, which intervene after the formation of *p*-coumarate and prior to the CoA ligase reaction, involve additional hydroxylation of the aromatic ring and methylation reactions, giving other derivatives (caffeate, ferulate, and sinapate). The benzoic acid derivatives have two proposed origins: the main pathway is the side chain degradation (loss of an acetate) of the corresponding hydroxycinnamic acids derivatives; the other source is an alternate path stemming from an intermediate in the shikimate pathway, and involves a series of enzymatic reactions converting 3-dehydroshikimate to various benzoic acid derivatives (*12*).

**Location and Roles in Plants.** Although a great deal is still unknown regarding the roles of phenolic acids in plants, they have been connected with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy (*14*−*16*). Cinnamic and benzoic acid derivatives exist in virtually all plant foods (e.g., fruits, vegetables, and grains) and are physically dispersed throughout the plant in seeds, leaves, roots, and stems (*17, 18*). Only a minor fraction exists as "free acids". Instead, the majority are linked through ester, ether, or acetal bonds either to structural components of the plant (cellulose, proteins, lignin) (*19*−*23*), or to larger polyphenols (flavonoids), or smaller organic molecules (e.g., glucose, quinic, maleic, or tartaric acids) or other natural products (e.g., terpenes) (*24, 25*). These linkages give rise to a vast array of derivatives. It is this diversity that is one of the major factors in the complexity of the analysis of phenolic acids.

The diversity of these components is not exclusive to their roles or their structural characteristics. Phenolic acids are not homogeneously distributed throughout plant tissues (*27*). In addition, there exists a large variability found during various stages of maturation (*28*). Growing conditions, such as temperature, are known to affect the phenolic acid content as well (*29*).

**Phenolic Acids in Foods.** Elucidating their roles in plant life is only one aspect of the many investigations concerning phenolic acids. One vast area of interest has been in food quality (*1, 30*). Phenolic acids have been associated with color, sensory qualities, and nutritional and antioxidant properties of foods (*31*). One impetus for analytical investigations has been the role these phenolics have in the organoleptic properties (flavor, astringency, and hardness) of foods (*3, 32*−*35*). Additionally, the food industry has investigated the content and profile of phenolic acids, their effect on fruit maturation, prevention of enzymatic browning, and their roles as food preservatives (*18, 30, 36*). Their role in the oxidative processes in foods further complicates their analysis (*37*).

Phenolics behave as antioxidants, due to the reactivity of the phenol moiety (hydroxyl substituent on the aromatic ring). Although there are several mechanisms, the predominant mode of antioxidant activity is believed to be *radical scavenging* via hydrogen atom donation. Other established antioxidant, radical-quenching mechanisms are through electron donation and singlet oxygen quenching (*4*). Substituents on the aromatic ring affect the stabilization and therefore affect the radical-quenching ability of these phenolic acids. Different acids therefore have different antioxidant activity (*38, 39*). The antioxidant behavior of the free, esterified, glycosylated, and nonglycosylated phenolics has been reported (*40*).

**Roles of Phenolic Acids in Human Health.** The literature provides a wealth of information that correlates a diet high in fruits and vegetables with the maintenance of health and disease prevention. Current thought links the high antioxidant content of fruits and vegetables with the inhibition of oxidative damage diseases such as coronary heart disease, stroke, and cancers (*41*−*45*). Certain foods are even classified as functional foods, owing to their established healthful protective effects (*46, 47*).

Typically, when discussing phenolics in plants foods, flavonoids are the predominant class described (*48*), because they account for approximately two-thirds of the dietary phenols (*49*). However, phenolic acids account for almost all of the remaining third, and there is an increasing awareness and interest in the

antioxidant behavior and potential health benefits associated with these simple phenolic acids. It is their role as dietary antioxidants that has received the most attention in recent literature (*39*, *50*−*52*). Because of their ubiquitous presence in plant-based foods, humans consume phenolic acids on a daily basis. The estimated range of consumption is 25 mg−1 g a day depending on diet (fruit, vegetables, grains, teas, coffees, spices) (*53*).

Although the exact nature of the antioxidant or protective effects in vivo is not well established, there exist some preliminary investigations (*54*−*56*) . A great deal more has been written on the in vitro and chemical antioxidant behavior of phenolics (*39*, *52*, *57*, *58*). Only the metabolic fates of caffeic, ferulic, chlorogenic, and sinapic acid have been explored. Chlorogenic acid is most likely metabolized by the colonic microflora (*59*, *60*). Bourne et al. (*61*) report that only 11−25% of ferulic acid ingested is excreted in urine as free ferulic acid or as glucuronide conjugate. Certain common and reccurring excreted metabolites reported are hippuric acid (*62*) and ferulic and isoferulic acid (*63*), as well as glucuronides and sulfate conjugates of the phenolic acids (*60*).

Beyond the protective antioxidant behavior, other biological activities of phenolic acids have been reported. Caffeic acid, one of the most prominent naturally occurring cinnamic acids, is known to selectively block the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma, and allergic reactions (*64*). Other studies have reported that caffeic acid and some of its esters might possess antitumor activity against colon carcinogenesis (*65*, *66*). Recent investigations by Maggi-Capyeron et al. (*67*) have linked a series of phenolic acids (**3**, **5**−**7**, **13**, **15**) with the inhibition of AP-1 transcriptional activity. AP-1 is an activator protein implicated in the processes that control inflammation, cell differentiation, and proliferation. Caffeic acid derivatives (e.g., dicaffeoylquinic and dicaffeoyltartaric acids) have been shown to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase. This enzyme catalyzes the integration of viral DNA into the host chromatin. Therefore, these hydroxycinnamate derivatives are currently being investigated for their potential antiviral therapy (*68*).

These recent investigations describing the potential healthful dietary impact of phenolic acids and their metabolism have reenergized the interest in developing analytical methodologies for their detection and measurement from not only plant sources but also human fluids. Therefore, it is important for future reference to compile the previously developed methods, as well as the sample handling and preparation.

## ANALYTICAL METHODS

**Food Sample Manipulation.** *Sample Preparation.* Although sample preparation is considered "a means to an end" in most investigations, it is nonetheless of central importance to accurate analysis (*8*). This step varies a great deal, owing to the diversity in food and plant matrixes. Wines, spirits, and clear juice samples require minimal manipulation, whereas whole fruits require a sequence of steps. In the case of wines, the alcohol is most often removed from the sample via rotary evaporation, and the residue is taken up in a small volume of the solvent used in the chromatographic separations (*69*). In an attempt to standardize and simplify sample preparations for wine analysis and to prevent loss or decomposition of components, some investigators directly injected the wine sample after filtration through a 0.45 $\mu$m membrane (*70*, *71*). Thompson seedless grape juice has been prepared in the same manner (*72*). In other cases, wines were acidified (∼pH 2) and saturated with NaCl prior to extraction with ethyl acetate. The organic extracts, combined and reduced, were taken up in a small aliquot of the mobile phase (*73*, *74*). An alternate method of treating juice reported by Naim et al. (*75*) was to mix orange juice with silica gel prior to freezing, freeze-drying the sample, and then generating a powder used in further manipulations.

Soft fruits (berries, grapes) are either crushed, pressed, or freeze-dried, followed by grinding to generate a powder of a defined particle size (*76*). In some investigations, it is the must (raw juice) that is treated, in others it is the mash, in yet others it is the peel, depending upon focus of the research. More solid fruits (e.g., apples, avocadoes, olives) and vegetables are either homogenized (using a blender) in an alcoholic solvent or freeze-dried and ground (*25*, *77*). Beveridge et al. (*78*) immersed broccoli and carrot pieces in liquid $N_2$ prior to breaking the frozen tissue via a freezer/mill centripep. Finally, analysis of solid grains and beans (wheat, rice, lentils) most often involves grinding the grains into fine flour (*79*).

Techniques employed for component stabilization are, for the most part, not discussed. However, a few preparations do address this issue. Heating the freshly pressed juice or must denatures polyphenol oxidase (PPO), an enzyme responsible for the degradation and browning of fruits and subsequent alteration of phenolic acid content, which is triggered upon cell damage. This method, referred to as HTST (high-temperature short-time), is most often used in the processing and storage of fruit juice (*80*). Oxidation of phenolics to their respective quinones is also hindered via addition of ascorbic acid, a water-soluble antioxidant (*76*).

*Extraction.* Extraction techniques need to take into account the location of phenolic acids in the plant. Most phenolic acid derivatives present in the plant matrix are stored in vacuoles and are commonly extracted in alcoholic or organic solvents. The exceptions are those bound to insoluble carbohydrates and proteins within the plant matrix. Often, saponification prior to extraction is employed to cleave the ester linkage to the cell walls. As an example, Kroon et al. incubated bran cell walls in 1M NaOH for 24 h at 37 °C to remove the ester-linked ferulic acid (*78*, *81*, *82*).

Considering the variety in food matrixes involved, there is surprisingly a great deal of coherency in the choice of solvents for extracting the phenolic acids and their conjugates. The common solvents for extractions are hot water, methanol, ethanol, acetone, and ethyl acetate, the latter being the most common. In one reference, the authors describe an extraction from olive oil with *N*,*N*-dimethylformamide (DMF) (*83*). Although the extraction methods for hydroxycinnamic acids are based mainly on polarity, acidity, and hydrogen-bonding capabilities of the hydroxyl groups of the aromatic ring, often choice of extraction solvent depends on the food. Escarpa et al. (*77*) report using 100% methanol for apples and pears and 80% aqueous methanol for green beans, lentil and pomace. Extraction was carried out in the absence of light and in the presence of 1% BHT (2,6-di-*tert*-butyl-4-methylphenol). Montedoro et al. (*84*) report that for olive oil, 80% aqueous methanol yields the highest extraction rates. Smolarz et al. (*85*) report the successful extraction of phenolic acids and their methyl esters with chloroform. Reported extraction times vary from 1 min to 6 h using Soxhlet extraction (*86*). In some cases, mechanical means to enhance molecular interaction are employed: vortex followed by centrifuge (*84*), sonication, mechanical stirring, and continuous rotary extraction (*87*, *88*). More recently, microwave irradiation has been used to enhance extraction (*89*). Extractions are, almost always, repeated 2−3

times, and extracts are combined. However, Sosulski et al. (*79*) extracted ground wheat, oats, corn, brown rice, and potatoes six times, using a methanol−acetone−water (7:7:6) mix at room temperature. In one investigation on extraction conditions, Palma et al. (*90*) compared the extraction of various phenolic compounds in boiling (65 °C) methanol under atmospheric conditions with pressurized liquid extraction (PLE) under inert and dark conditions at various temperatures ranging from 40 to 150 °C. All of the simple phenolics were stable under PLE conditions, whereas in boiling methanol, the recoveries were lower.

Often, food samples contain both polyphenolics and simple phenolics. The most commonly reported technique to fractionate is based on acidity (*91*). The p$K_a$ of the phenolic hydrogen is around 10, whereas that of the phenolic carboxylic acid proton is between 4 and 5. Removal of neutral compounds is performed after treatment with NaOH. A sequence of acidification, treatment with $NaHCO_3$, and extraction steps then isolates the phenolic acids (*92*−*94*). Delage et al. (*95*) adjusted the pH of crude apple juice to 7, extracted with ethyl acetate to remove the flavonoids from the juice, and then adjusted the pH to 2 to extract the phenolic acids. The extraction solvent was then evaporated and the sample taken up in the chosen chromatographic solvents.

Solid-phase extraction (SPE) is a commonly used technique to fractionate as well as to remove unwanted components from the sample. By eluting with solvents of varying pH, larger phenolics and sugars are separated from the smaller phenolic components. Recovery rates are not often discussed, however, those that have been reported are conflicting. Glowniak et al. (*96*) report recovery rates of 98.5%, using columns with quaternary amine sorbent, whereas Wrolstad et al. (*97*) claim that their recovery rates are 10% and variable from $C_{18}$ cartridges for the phenolic acids but near 100% for the larger polyphenols (flavanol aglycones and flavanol-3-ols and ellagic acid). Beveridge et al. (*78*) report that recoveries, from C18 Sep-Pak, for commercially available standards range from 101 to 104%. Benassi et al. (*98*) report that with or without a $C_{18}$ Sep-Pak or ion exchange columns as part of their sample preparation, their chromatographic results were the same.

*Hydrolysis.* Many of the initial investigations focused on separating and attempting to identify the ester content of plant foods. This proved to be a formidable task, due to the variety of these derivatives (*25, 99, 100*). These derivatives often have nearly identical UV spectra. The alcohol moiety to which the phenolic acid is bound often does not contain a chromophore (e.g., quinic acid in chlorogenic acid). The retention behavior changes due to the difference in polarity, yet the UV spectra are nearly identical. Minor bathochromic shifts are observed when an ester does contain chromophores such as the carbon−oxygen double bond caffeoyl tartrate in wines (*101*). Hydrolysis of the ester to a carboxylic acid has been one strategy employed to simplify the analysis and gain a more specific picture of the phenolic acid profile in foods. There are two main procedures to cleave the ester bond reported in the literature, acidic hydrolysis and saponification. A third, less prevalent technique is cleavage through the use of enzymes (esterases).

Although reaction times and temperatures for the acidic hydrolysis conditions vary a great deal, this general method involves treating the plant extract or food sample itself with inorganic acid (HCl) at reflux or above reflux temperatures in aqueous or alcoholic solvents (methanol being the most common). Acid ranged from 1 to 2 N aqueous HCl, and the reaction times ranged from 30 min (*77*) to 1 h. Gao et al. (*102*) examined a series of acid hydrolysis media in an attempt to find a system for maximum preservation of phenolic acids. A mixture of 2 N HCl and methanol (1:1, v/v) at 100 °C for 1 h yielded the highest recovery (30−65%). Both methyl esters and carboxylic acids were monitored. Solvents such as ethanol, *tert*-butyl alcohol, and 2-propanol gave lower results, and aqueous HCl is reported to have destroyed the hydroxycinnamic acids. Krygier et al. (*103*) report that loss under acidic conditions varies with the phenolic acid, ranging from 15 to 95% loss for *o*-coumaric and sinapic acids, respectively.

Saponification entails treating the sample with a solution of NaOH with reported concentrations ranging from from 1 to 4 M. Most of the reactions are allowed to stand at room temperature for time ranging from 15 min (*104*) to overnight (*92*), although Shahrzad et al. (*105*) report hydrolyzing grape juice with 1.5 M NaOH for 62 h. Some investigations mention that the reactions are carried out in the dark, as well as under an inert atmosphere such as argon or nitrogen gases (*97*). Minor modifications to this typical method include saponification with constantly agitation for 24 h at 37 °C degrees (*81*). Lam et al. (*23*) carried out saponification under harsh conditions for the purpose of comparison and conducted the saponification with 4M NaOH, for 2 h at 170 °C. Torres et al. (*92*) carried out the saponification under dark, anaerobic conditions, by purging the reaction with $N_2$. Zgorka et al. (*27*) performed basic hydrolysis using $NaBH_4$ and 1% $Ba(OH)_2$ at 100 °C for 15 min.

Enzymatic reactions have been reported to release phenolic acids (mainly ferulic and *p*-coumaric acids). These enzymes (pectinases, cellulases, amylases) are employed for the degradation of the carbohydrate linkages. The mode of action by which these acids are released is the cleavage of an acetal or hemiacetal bond found between carbohydrate moieties and the hydroxyl groups off the aromatic ring, and not by ester cleavage reactions. Meyer et al. (*106, 107*) discusses the release of phenolics using Grindamyl pectinase, a commercial enzyme having pectinolytic, cellulitic (cellulose) and hemicellulitic but no esterase activity. Andreasen et al. (*108*) discuss and compare several different enzyme preparations for the release of phenolic acids from the cell wall of rye grains. Smith et al. (*109*) used the enzyme Driselase (a commercial cellulase) to determine the location of the ester linkage of ferulic acid. Driselase contains glycanases but no esterase activity. By isolating the feruloyl oligosaccharides released after digestion, they established to which sugar ferulic acid was esterified. Again, some acid derivatives are extracted from the cell walls, but it is not the ester bond that is cleaved. Cinnamoyl esterases are known, but little has been discussed with respect to the analytical use of these enzymes for the release of phenolic acids (*110, 111*). However, Yu et al. (*112*) reported that a sequential acid, alpha-amylase and cellulase hydrolysis might be applicable to the release of phenolic acids from barley.

Wrolstad et al. (*113*) reported a reduction in the amount of chlorogenic acid and an increase caffeic acid in their investigation on Thompson seedless grape juice. The commercial enzyme preparation used for clarification of the juice demonstrated this hydrolytic activity, but no mechanism is proposed. Baranowski et al. (*101*) reported using an enzyme (Pectinol 10 M) to hydrolyze the tartrate esters of ferulic, caffeic, and *p*-coumaric acids in white wines. Only the trans form is recognized by this enzyme.

Some of the linkages between the hydroxyl groups of the aromatic ring and the sugars or other phenolics are ether linkages. Again, this occurs mainly in the structural components of the plants. Sun et al. (*86*) have reported a method to determine

**Table 2.** UV Absorbance Maxima ($\lambda_{max}$) of Selected Phenolic Acids[a]

| phenolic acid | UV $\lambda_{max}$ (nm) |
|---|---|
| gallic | 217, 272 |
| protocatechuic | 218, 260, 295 |
| gentisic | 213, 239 (s), 332 (m) → 370 |
| caffeic | 220, 240 (br) 294 (ps), 326 |
| vanillic | 219, 261, 294, → 320 |
| syringic | 218, 276 → 328 |
| *p*-coumaric | 226, 312 → 361 |
| feruilic | 218, 236 (br), 295 |
| veratric | 218, 262, 295 |
| sinapic | 238, 326 |

[a] Legend: → = extends out to; (s) = shoulder; (m) = moderate abs.; (br) = broad; (ps) = pre-shoulder.

the hydroxycinnamic acids participating in either ester or ether linkages in the cell wall polymers. These authors refer to mild hydrolysis (1M NaOH at room temperature) cleaving the ester linkages and hot alkaline hydrolysis (4 M NaOH at 170 °C for 2 h) cleaving both ether and ester linkages. Ether-linked derivatives were estimated by the difference between total and ester-linked acid derivatives. Lam et al. (*23*) employed 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to selectively cleave benzyl ethers, thereby elucidating the position of the ether linkage of ferulic and p-coumaric acid to lignin in grass cell walls.

No one definitive method for hydrolysis has yet been developed. Although the additional hydrolysis step creates new questions with respect to sample preparation, analyte stability and recoverability, cleaving the ester bond greatly simplifies the analysis by reducing the number of derivatives. Much research is required to develop a system that quantitatively releases the acids from food matrixes and at the same time prepares them for chromatographic analysis. Development of a robust sample preparation methodology would be of great assistance.

**Chromatographic Separations.** *Early Chromatographic Investigations.* Early investigations used either paper chromatography (PC) or thin-layer chromatography (TLC). Paper chromatography consisted of Whatman filter paper as the stationary phase. For TLC, the stationary phase varied, including silica gel, cellulose, and polyamide layers (*114*, *115*). TLC was considered a convenient separation method. It is fast, inexpensive, and several samples can be examined at the same time, side by side. Although less used in the analysis of foods, TLC is still commonly used for the determination of phenolic acids in plant material in natural product analysis (*116*−*118*). The main disadvantages of TLC are limited quantitation aspects. Estimations of recovery with preparatory TLC have, however, been reported, including that, of the total loss of ferulic acid (45%) during sample preparation and analysis, 30% was lost in the preparative TLC step (*75*).

Historically, the most common colorimetric method employed for quantification of phenolic acids was the Folin−Ciocalteu reagent (FCR). FCR involves the reduction of a phosphomolybdic−phosphotungstic acid to a blue colored complex in alkaline solution. The phosphomolybdic−phosphotungstic−phenol complex generated in solution gives an easily detected absorbance at 760 nm (*119*). However, quantification is problematic, in that other components in the food extracts behaved as reducing agents. Identification of individual phenolic compounds is not possible with this method.

*HPLC.* In the last twenty years, the analytical technique that has dominated the separation and characterization of phenolic compounds is HPLC with reverse phase (RP) column technology. Many of the reviews concentrating on phenolics have focused on the flavonoids with a section dedicated to phenolic acids (*7*, *37*, *120*, *121*). Only a few have focused exclusively on the separation techniques for phenolic acids (*11*, *122*).

Table 4 in this review lists representative examples of the methods devised and columns employed in RP-HPLC separations of phenolic acids. The table also contains a summary of the sample preparation steps and lists detection methods. These are organized and tabulated according to food sample, i.e., wine and beer (W), fruit and juices (J), grain (G), leaves and plant parts (L) and olives and olive oil (O). Phenolic acids detected and separated are also indicated.

There is a great deal of uniformity in column choice for HPLC. Considering the diversity of stationary phases available, columns chosen for the determination of phenolic acids are almost exclusively composed of a $C_{18}$ stationary phase with an internal diameter ranging from 2.1 to 5 mm (the most common being 4.6 mm). Investigations using mass spectrometry coupled to liquid chromatography report using columns with smaller i.d. (2.1 mm to 1.1 mm) (*123*). The particle size in these columns is, in most cases, either 3 or 5 $\mu$m. Two exceptions are Li et al.

**Table 3.** Fragmentation Patterns of the TMS Derivatives of Phenolic Acids[a,b]

| phenolic acid | $M^{\bullet+}$ | $[M-15]^+$ | $[M-89]^+$ | $[M-177]^+$ | other common ions: $[M-30]^+$, $[M-59]^+$ + others |
|---|---|---|---|---|---|
| cinnamic | 220 (70) | 205 (100) | 131 (78) | *c* | 161 (84) |
| *o*-coumaric | 308 (54) | 293 (53) | 219 (18) | 131 (3) | 147 (100) |
| *p*-coumaric | 308 (100) | 293 (98) | 219 (87) | 131 (3) | 249 (70) |
| ferulic | 338 (100) | 323 (54) | 249 (32) | 161 (3) | 308 (46)[4] |
| sinapic | 368 (64) | 353 (57) | 279 (30) | 191 (6) | 338 (100)[d] 161 (39) |
| caffeic | 396 (66) | 381 (20) | 307 (8) | 219 (100) | |
| *p*-hydroxybenzoic | 282 (28) | 267 (100) | 193 (39) | 105 (2) | 223 (72) |
| vanillic | 312 (86) | 297 (100) | 223 (55) | 135 (4) | 267 (81), 282 (47)[d] |
| syringic | 342 (86) | 327 (100) | 253 (33) | 165 (4) | 297 (61), 312 (84)[d] |
| protocatechuic | 370 (52) | 355 (23) | 281 (4) | 193 (100) | |
| gentisic | 370 (10) | 355 (100) | 281 (4) | 193 (2) | 223 (10) |
| gallic | 458 (78) | 443 (46) | 369 (4) | 281 (100) | |
| 2,3,4 THB | 458 (3) | 443 (100) | 369 (2) | 281 (27)) | |

[a] GC/MS analysis was carried out on a Varian 3400 gas chromatograph interfaced to a TSQ 700 mass spectrometer. Electron impact ionization mode was used for MS (ionization energy was set to 70 eV). Samples were injected (1$\mu$L) with the splitless mode on to a 30 m × 0.25 mm i.d., 0.5$\mu$m DB5–5MS capillary column The carrier gas was helium, with a linear velocity of 30–35 cm/s. Derivatization was achieved by heating the phenolic acid in the presence of BSTFA in pyridine (excess of a 1:1 by volume mixture) for 50 min at 50 °C. [b] Numbers in parentheses are relative abundance. [c] Scans were recorded from *m*/*z* 60–500. [d] Loss of 30 u (a molecule of formaldehyde) through rearrangement of the methoxy substituent.

**Table 4.** Representative Examples of High-Performance Liquid Chromatography with Reversed-Phase Columns for the Analysis of Phenolic Acids. Sample Preparation and Detection Methods Are Included

| | sample source and phenolic acids detected | sample preparation and hydrolysis conditions | stationary phase and guard column | mobile phase and method | detector | ref |
|---|---|---|---|---|---|---|
| J | grape and berry extracts **15**, **13**, **10**, **11**, **7**, **12**, **3**, **5**, **6** | hydrolysis: $CH_3OH$-2N HCl 100 °C, 1h; the hydrosylate was injected | $C_{18}$, SuperPac Pep-S (250 × 4 mm, 5 $\mu$m) guard column (4 × 10 mm) | A: formic acid; water (1:99, v/v); B: $CH_3OH$; 17–22% B, flow rate, 0.9 mL/min inj. volume 30 $\mu$L. | PDA | 102 |
| J | orange juice Cou[a], **5**, **6**, **7** | 2 N NaOH, 4h, RT; adjusted pH to 4.5 with $H_3PO_4$; sample was centrifuged, supernatant extracted 2x with ethyl acetate | adsorbosphere HS $C_{18}$, (250 × 4.6 mm, 5$\mu$m) | tested seven methods: 1: 30% $CH_3OH$ 70% $H_2O$ 2: 20% $CH_3CN$ 80% $H_2O$ 3: 21% THF 79% $H_2O$ 4: 15% $CH_3OH$ 10.5% THF 74.5% $H_2O$ 5: 10% $CH_3CN$ 10.5% THF 79.5% $H_2O$ 6: 15% $CH_3OH$ 10% $CH_3CN$ 75% $H_2O$ 7: 10% $CH_3OH$ 6.7% $CH_3CN$ 7% THF 76.3% $H_2O$ flow rate: 1.0 mL/min inj volume 20 $\mu$L; best resolution was with solvent system 3. | PDA | 145 |
| J | grape juice **3**, **7**, Chl[b], **15**, **13** | juices: filtered and injected | Supelcosil LC-18 (250 × 4.6 mm, 5$\mu$m) guard column ODS-10 40 × 4.6 mm) | A: 0.07M $KH_2PO_4$ adjusted to pH 2.5 with phosphoric acid B: $CH_3OH$ flow rate: 1.0 mL/min. inj volume was not indicated. | PDA | 113 Q[c] |
| J | kiwi fruit juice **13**, HBa[d], **7**, Chl, **3** | kiwi fruit crushed, mash was enzymatically treated (pectolytic), and filtered through Celite; adjusted pH to 7 with NaOH to remove weakly acidic phenolics (passed through C-18 cartridge), then adjusted eluent to pH 2 | Spherisorb RP C-18 (250 × 4.6 mm, 5$\mu$m) | A: $H_2O$ B: $CH_3CN$ flow rate: 1.0 mL/min inj volume 20 $\mu$L | PDA | 91 Q |
| J | fruit juices **10**, **13**, **15**, **11**, **12**, **5**, **7**, **6**, Chl[e] | juices (orange, apple, pineapple, peach, apricot, pear and grape juice) were extracted with diethyl ether and ethyl acetate | $C_{18}$ Nova-pak (300 × 3.9 mm, 5$\mu$m) | A: 2% acetic acid B: $CH_3OH$/acetic acid/ $H_2O$ (30:2:68) flow rate: 0.7 mL/min inj volume not indicated | 280 and 340 nm | 146 Q |
| J | grape must, apple, and peach **7**, **5** **15**, **13**, hBa, **11**[f] | juice samples were extracted with diethyl ether followed by ethyl acetate; after evaporation of organic solvents, sample was taken up in $CH_3OH/H_2O$ (1:1,v/v) | Nova-pak $C_{18}$ (300 × 3.9 mm, 4 $\mu$m) | A: $H_2O$ /acetic acid (98:2, v/v) B: $H_2O/CH_3CN$/acetic acid (78:20:2, v/v/v) flow rate: 0.7 mL/min inj volume not indicated | PDA (210–360 nm) | 147 |
| J | oranges and grapefruits **6**, **5**, Cou, **7** | juice samples were hydrolyzed (2M NaOH, under $N_2$, 4 h, RT then acidified; clear supernatant was extracted with EtOAc | LiChrosopher 100 RP-18 (250 × 4.6 mm, 5 $\mu$m) | acetic acid in water/ $CH_3OH$ (77:23, v) flow rate: 1 mL/min inj volume 10 $\mu$L | monitored at 300 nm | 33 Q |
| J | apple juice **15**, **13**, **3**, Chl | apples were crushed and pulp was pressed; running juices were used for this investigation; adjusted to pH 2, then extracted with ethyl acetate to isolate phenolic acids | Spherisorb ODS 2 $C_{18}$ (250 mm × 4.6 mm, 5 $\mu$m) | A: $H_2O$ –HCl (99.8:0.02:v/v) B: $CH_3OH$–HCl (99.8:0.02:v/v flow rate: 1.8 mL/min temp held at 40 °C | PDA benzoates (255 nm) cinnamic acids (290, 320 nm) | 95 |

**Table 4 (Continued)**

| | sample source and phenolic acids detected | sample preparation and hydrolysis conditions | stationary phase and guard column | mobile phase and method | detector | ref |
|---|---|---|---|---|---|---|
| J | grape pomace **3**, **16**, **14**, **7**, **17,** aldehyde of **13** | sample was milled in coffee grinder and dried at 40 °C for 24 h; stored at –20 °C; extracted using pressurized liquid extraction (PLE) with $CH_3OH$ at various temperatures (inert and dark conditions.) | LiChrospher column | A: $CH_3OH$-acetic Acid-$H_2O$ (10:2:88) B: $CH_3OH$-acetic Acid-$H_2O$ (90:2:8) flow rate: 1 mL/min | PDA and fluorescence detection –351 nm excitation and 453 nm emission. | 90 |
| J | berries (11 different) **7**, **3**, **5**, **10**, **15**, Ell | freeze-dried berry samples were extracted with aq. $CH_3OH$ (50%), hydrolyzed overnight with 1.2 M HCl at 35 °C. | ODS–Hypersil $C_{18}$ (100 × 4.0 mm, 3$\mu$m) | A: 50mM $NH_4H_2PO_4$ adjusted to pH 2.6 B: 20% A with 80% $CH_3CN$ C: 0.2 M orthophosphoric acid adjusted to pH 1.5 with $NH_3$ flow rate: 0.5 mL/min inj volume not indicated. | PDA Ell and **10**–260 nm **15**–280 nm Others at 320 nm. | 148, 149 |
| J | fruit juices **15**, **7**, **5**, chl | juices were pretreated (0.1 M HCl) then run through a Sep-Pak $C_{18}$ cartridge and a Bond Elute PSA cartridge | L-column ODS (250 mm × 4.6 mm, 5$\mu$m) | A: 5 mmol/L $KH_2PO_4$ (pH 2.5) B: $CH_3CN$ isocratic run (A–B, 41:9, v/v) flow rate: 1.0 mL/min column temp was 40 °C | PDA | 150 |
| J | apple pomace, apple juice, and pear fruits **15**, **13**, **10**, chl, **7**, **12**, **3**, **5**, **6**, **1**. | pH fractionation, liquid–liquid extraction and SPE are employed. | Aqua 5u $C_{18}$ (250 mm × 4.6 mm) guard column $C_{18}$ ODS (4 × 3.0 mm) | A: $CH_3OH$ + 0.045% formic B: $H_2O$ + 0.045% formic flow rate: 1 mL/min column temp was 25 °C. | PDA monitored at 280, 320 and 370 nm) spectra from 200 to 600 nm. MS–ESI (negative ion mode) | 151 |
| J | black and green grape and cherry juice **7**, Chl, **5**, **15**, | juice sampes were hydrolyzed (2M NaOH, pH 12.5 under argon at RT) for either 48 (cherry) or 62 (grape) h; samples were acidified to pH 3.4, then extracted with ethyl acetate. | LiChrospher 100-RP18 (120 mm × 4 mm, 5$\mu$m) Precolumn RP-18 (4 mm × 4 mm) | isocratic $H_2O$–ethyl acetate–acetic acid (95.6:4.1:0.3, v/v) flow rate: 0.5 mL/min Inj volume: 20 $\mu$L run time not indicated | UV detection 280 and 320 nm | 105 Q |
| J L W | apples, white wine, turnip, pear, cauliflower, cabbage **15**, **13**, Chl, **14**, **7**, **12**, **6**, **3**. | fruit and vegetable samples were chopped into small pieces and blended with 80% $CH_3OH$; after filtration, evaporation and adjustment to pH 2 (with HCl) the samples were extracted with ethyl acetate (3x). $C_{18}$ sep-Pak cartridge was used after samples were evaporated and redissolved in small amount of $CH_3OH$. | Zorbax ODS (250 mm × 4.6 mm, 10 $\mu$) | isocratic: $CH_3OH$/4% acetic acid (27:73) column temp was 40 °C flow rate: 0.8 mL/min inj volume: 10 $\mu$L | UV detector set at 313 nm | 124 Q |

**Table 4 (Continued)**

| | sample source and phenolic acids detected | sample preparation and hydrolysis conditions | stationary phase and guard column | mobile phase and method | detector | ref |
|---|---|---|---|---|---|---|
| J<br>L<br>G | potato, apple, tomato, carrot, crisp bread, red raspberry, strawberry, apple juice, rose wine **7**, **3**, **5**, **10**, **11**, **12**, **13**, **15** | both acidic and basic hydrolyses were performed<br>basic: sample was treated with 10 M NaOH, covered with $N_2$, stirred overnight (16h) at RT;<br>concentrated acidic: HCl was then added and to 85°C for 30 min<br>results from acid and base hydrolyzilates were added to give total phenolic acids. | Intersil ODS-3 (150 mm × 4 mm, 3 $\mu$m) C-18 guard column. | A: 50 mM $H_3PO_4$, pH 2.5<br>B: $CH_3CN$ Column temp was 35 °C<br>flow rate: 0.7 mL/min<br>inj volume 10 $\mu$L. | PDA focus on 254, 270, 280 and 329 nm) | 152 Q |
| W<br>J | white wines and juices **15**, **13**, **14**, **11**, **12**, Cou, **5**, **7** [g] | juices: filtered and injected<br>wines: samples concentrated to remove ethanol. | Nucleosil 120 $C_{18}$ (250 × 4 mm, 5 $\mu$m) | A: Glacial acetic acid in $H_2O$ at pH 2.65<br>B: 20% A 80% $CH_3CN$<br>flow rate: 1.5 mL/min<br>inj volume 100 $\mu$L<br>column temp at 40°C | PDA | 69 Q |
| W<br>J | fresh wine pomace, apples, pears, fresh green beans, dried lentils. **15**, Chl, **7**, Cou | apples and pears peeled, green beans homogenized, lentils ground; extraction in the absence of light and in the presence of 1% BHT[12] used<br>ultrasound: apples and pears (100% $CH_3OH$); red wine pomace, green beans and lentils (80% aq $CH_3OH$)<br>filtration: SPE (Sephadex LH-20)<br>hydrolysis: 2 N HCl at 100 °C for 30 min, constant shaking conditions | Nucleosil 120 $C_{18}$ (250 × 4.6 mm, 5 $\mu$m) | A: 0.01 M aqueous phosphoric acid<br>B: 100 % $CH_3OH$ | PDA | 77 Q |
| W | white wines **15**, **13**, **10**, **11**, **7**, **12**, Cou, **5** | wine samples were filtered through 0.45 $\mu$m filter, adjusted to pH 2.5 and diluted with the mobile phase | SpherisorbODS-2 (250 mm × 4.6 mm, 5 $\mu$m) | A: aqueous $H_2SO_4$ (pH 2.5)<br>B: $CH_3OH$<br>flow rate: 0.5 mL/min for the first 10 min (monitored at 210 nm) then to 0.7 mL/min and monitored at 278 nm<br>inj volume 20 $\mu$L. | PDA monitored at 278 nm for phenolic acids | 98 |
| W | sherry wines **15**, **13**, **14**, **10**, m-HB, **11**, **7**, **12**, **5**, **6**, **2**, 2,6–DMB[i], 3,4,5–TMC[j] | samples filtered 0.45$\mu$m membranes; two stages (1) SPE (LiChrolut $C_{18}$) and (2) SAX anion exchanger separating neutral and acidic species | LiChrospher $C_{18}$ (250 × 4 mm, 5 $\mu$m) | A: $CH_3OH$-acetic acid–$H_2O$ (10:2:88)<br>B: $CH_3OH$–acetic acid–$H_2O$ (90:2:8)<br>flow rate: 1 mL/min<br>inj volume 20 $\mu$L. | PDA | 88, 153 Q |
| W | sherry wines **15**, **13**, **14**, **10**, **11**, **7**, 2,6-DMB, **12**, **5**, **16**, **6**, **2**, 3,4,5-TMC | diethyl ether extract (0.8 rpm /3h) of 100 mL sherry wine dissolved in 100 mL of sat′d NaCl solution; dried organic solvent with sodium sulfate, evaporated to a volume of 0.5 mL then 5 mL with $CH_3OH$ | $C_{18}$, (250 × 4 mm, 5 $\mu$m) gave best results; five columns tested | A: 10% $CH_3OH$–2% aq acetic acid<br>B: 90% $CH_3OH$–2% aq acetic acid<br>flow rate: 1 mL/min<br>inj volume 20 $\mu$L | PDA (210–390 nm) | 87 |

**Table 4 (Continued)**

| | sample source and phenolic acids detected | sample preparation and hydrolysis conditions | stationary phase and guard column | mobile phase and method | detector | ref |
|---|---|---|---|---|---|---|
| W | wine **15**, **13**, **10**, **11 7**, **12**, **3**, **5** | extracted wine with ethyl acetate at two different pH's; first at pH 7 (removal of alcohols) and the pH 2 (adjust with 2M HCl to remove phenolic acids) | MCH 10 $C_{18}$ (300 × 4 mm) | A: $CH_3OH$<br>B: $H_2O$ (adjusted to pH 2.5 with $HClO_4$) | UV–Vis (280 nm) | 94 Q |
| W | red wine **15** [k] | wine was filtered (0.45$\mu$m membrane) and injected directly | ODS hypersil (250 × 4 mm, 5 $\mu$m) guard column: LiChrospher 100 RP-18 | A: ethyl acetate<br>B: $CH_3OH$<br>C: $H_2O$<br>flow rate: 0.4 mL/min for 5 min, then 0.5 mL/min<br>inj volume 20 $\mu$L. | UV–Vis | 70 Q |
| W | wine **15**, **13**, **11**, **7**, **3**, **12**, **5**, **10** [l] | direct injection | Merck Superspher RP18 (250 × 4 mm) | A: 1% acetic acid in $H_2O$<br>B: 5% acetic acid in $H_2O$<br>C: $CH_3CN$–acetic acid–$H_2O$ (30:5:65, v/v/v)<br>flow rate: 0.5 mL/min<br>column temp was 22.5 °C<br>authors emphasize thermostated column due to long analysis time | PDA | 71, 154 |
| W | white wines **15**, **14**, **11** or MHB, **7**, **9**, **3**, **5** | wine samples were acidified (pH 2 with 1M HCl) and saturated with NaCl; extraction with ethyl acetate | ULTRA-SPHERE ODS (250 × 4.6 mm) Spherisorbe ODS2 (250 × 4.6 mm) | isocratic: mobile phase 2% (v/v) 2-propanol, 2% (v/v) acetic acid, 8.7% (v/v) HPLC grade $CH_3OH$, 0.018 M ammonium acetate, and 87.3% (v/v) HPLC grade $H_2O$<br>inj volume 20 $\mu$L | electrochemical detection Ag/AgCl ref, carbon polyethylene working electrode | 73 Q, 74 |
| W | red and white wines **15**, **13**, **11**, **7**, **12**, **3**, **5** [m] | wine samples: both direct injection and sample preparation as follows: (pH adjusted to 2 with 0.1 M HCl)<br>extraction with diethyl ether<br>organic samples evaporated, taken up in $CH_3OH$, filtered and injected | Nova-Pak $C_{18}$ (150 × 3.9 mm, 4 $\mu$m) Nova-Pak $C_{18}$ precolumn | A: $CH_3OH$–acetic acid–$H_2O$ (10:2:88, v/v/v)<br>B: $CH_3OH$–acetic acid–$H_2O$ (90:2:8, v/v/v)<br>gradient program: flow rate: 1 mL/min<br>inj volume not indicated | UV and fluorescence detectors in series ($\lambda_{ex}$ 278 $\lambda_{em}$ 360) (15 min) then switched to $\lambda_{ex}$ 330, $\lambda_{em}$ 374 min | 127 Q |
| W | beer **7**, **3**, **14**, **11** | beer sample was acidified with acetic acid then run through aC-18 Sep-Pak cartridge<br>phenolic acids were eluted with 0.5 ammonia soln then sample was reacidified with acetic acid | Brownlee RP-18 ODS (100 mm × 4.6 mm, 5 $\mu$m) | isocratic method 15% v/v methanol soln containing 0.1 M ammonium acetate (pH 4)<br>sample loop 200 $\mu$L<br>flow rate: 1.0 mL/min | electrochemical detection scan rate 2.0V/s–1scan/s over a 1.2 V range. | 155 |
| O | olives **11**, **7**, **3** | initial treatment of olives with NaOH (2.5%w/v)<br>extracted with 80% (v/v) ethanol containing 1% sodium metabisulfite<br>pH adjustment to 3.0 with 6N HCl | Spherisorb ODS-2, 5 $\mu$m | initial: 95% water (pH adjusted to 2 with phosphoric acid) and 5% $CH_3CN$<br>20 min 20% $CH_3CN$<br>20 min 50% $CH_3CN$<br>final 10-min 75% to purge column. | PDA (200–380 nm) | 34 Q |

**Table 4 (Continued)**

| | sample source and phenolic acids detected | sample preparation and hydrolysis conditions | stationary phase and guard column | mobile phase and method | detector | ref |
|---|---|---|---|---|---|---|
| O | virgin olive oil **13**, **10**, **11**, **7**, **12**, **3**, **5**, **2**, 1 | extraction: variety of solvents tested and compared | $C_{18}$ Erbasil Column (150 × 4.6 mm, 5 $\mu$m) | A: 2% acetic acid in $H_2O$<br>B: $CH_3OH$ | PDA (190–367 nm) | 84 |
| O | table olives; **11**, **3**, **6**, **5**, **2** | olives were boiled, salted, dried used official extraction method: EEC commission Regulation No 2568/91 Official J, L 248 05.09.1991 | Baker-Bound $C_{18}$ (250 mm × 4.6 mm, 5 $\mu$m) | A: acetic acid-$H_2O$ (98:2:v/v)<br>B: $CH_3OH$<br>flow rate: 1 mL/min | monitored at 278 nm | 156 |
| O | olives and oils **15**, **7**, **11**, **10**, **12**, **6**, **3**, **5**, 1 + other phenols in oil | extraction procedure was taken from Cartoni et al. (*123*) | Nucleosil ODS (250 mm × 2.1 mm, 5 $\mu$m) precolumn filter A.318 was used | A: $CH_3OH$ +0.045% formic<br>B: $H_2O$ + 0.045% formic Flow rate: 150 $\mu$L/min<br>at then end of column, a 1.1 M $NH_3$ added at a rate of 1/7 $\mu$L/ min – resulting in a solution with pH 7. | API–MS/MS triple quadropole MS) | 129 |
| O | virgin olive oils **11**, **3**, **5** | oil: extracted with 0.1 M sodium bicarbonate buffer (pH 8.3)<br>samples were then acidified with 1:1 HCl (pH 2) and again passed through phenyl cartridges eluted with $CH_3OH$ | for oil: $C_{18}$ Nucleosil (250 mm × 2.1 mm, 5 $\mu$m)<br>for standards: $C_{18}$ Nucleosil (250 mm × 1.1 mm, 5 $\mu$m) | $CH_3CN$–$H_2O$ (17:83) – 0.08% acetic acid<br>flow rate: 250 $\mu$L/min–<br>$CH_3CN$–$H_2O$ (18:82) – 0.02% TFA<br>flow rate: 50 $\mu$L/min | fluorescence ($\lambda_{ex}$ 264 $\lambda_{em}$ 354) switched to $\lambda_{ex}$ 310, $\lambda_{em}$ 430 nm at 14 min; UV and MS (APCI) negative-ion mode | 123 Q |
| L | aerial parts: *Echinacea* **13**, Chl, **10**, **11**, **7**, **12**, **3**, **5** | soxhlet extraction with $CH_3OH$ for 6 h. Initial SPE (octadecyl), pH adjustment to 7.0–7.2, followed by SPE (quaternary amine)<br>final elution with 0.2 M $H_3PO_4$–$CH_3OH$ (1:1, v/v) | ODS Hypersil (200 × 4.6 mm, 5 $\mu$m) | isocratic conditions: $CH_3OH$/water/ acetic acid (25:75:1, v/v) | UV (254 nm) | 96 Q |
| L | avocado (leaves, fruit and seeds) **10**, **13**, **15**, **11**, **12**, **2**, **3**, **7**, **5**, **6** | tissue was frozen with liquid $N_2$ and ground<br>extracted with $CH_3OH$<br>saponified overnight under anaerobic $N_2$ with 2N NaOH<br>series of acid/base extractions to fractionate. | ODS Adsorbosphere (250 mm × 4.6 mm, 8 $\mu$m) guard column | A: 5% aq acetic acid<br>B: 100% $CH_3CN$<br>flow rate: 1 mL/min | monitored at 254, 275 and 300 nm | 92 |
| L | plants from *Lamiacea* family **13**, **10**, **14**, Chl, **12**, **7**, **11**, **5**, **3**, rosmarinic acid | dry, pulverized samples were refluxed with $CH_3OH$<br>filtered through SPE micro-columns (octadecyl Baker Bond) after adjustment to pH 7.0–7.2 with $NaHCO_3$ samples were passed through quaternary amine SPE micro columns. | Hypersil (200 × 4.6 mm, 5 $\mu$m) | isocratic: $CH_3OH$–acetic acid–$H_2O$ (25:1:75)<br>flow rate: 1 mL/min | variable $\lambda$ detector (254 nm) | 157 Q |
| L | mistletoe plants **1**, **15**, **10**, **13**, Chl, **14**, **3**, **5**, **9**, **4** | dried and finely powdered plant material was extracted with $CH_3OH$<br>fractions were divided equally: half were treated with 1N HCl and the other 1N KOH in a boiling water bath for 15 min. | LiChrospher RP-18 (250 × 4.6 mm, 5 $\mu$m) | isocratic mobile phase $H_2O$ –$CH_3CN$–phosphoric acid (85:13.8:1.2, v/v/v) | 320 nm | 158 Q |

**Table 4 (Continued)**

| | sample source and phenolic acids detected | sample preparation and hydrolysis conditions | stationary phase and guard column | mobile phase and method | detector | ref |
|---|---|---|---|---|---|---|
| L | *Asclepias Syriaca* L. (Milkweed) **10**, **3**, **13**, **7**, **15**, **5** | dried leaves were extracted with boiling $CH_3CH_2OH$ (95%)<br>extracts were divided and both acidic (36% HCl, 100 °C, 1h) and basic ($NaBH_4$, 1% $Ba(OH)_2$, 100 °C 15 min) hydrolysis were performed | ODS–Hypersil $C_{18}$ (200 × 4.6 mm, 5 $\mu$m) | isocratic eluent $CH_3OH$–acetic acid–$H_2O$ (25:1:75, v/v/v)<br>flow rate: 1 mL/ min<br>column temp was 25 °C | UV–Vis detector 254 nm | 27 Q |
| L | plant material (*Eleuthero-coccus senticosus*) **13**, Chl, **10**, **11 7**, **12**, **3**, **5** | dried, pulverized plant material was refluxed in $CH_3OH$ for 1h<br>samples were first passed through octadecyl SPE cartridges<br>eluent was brought to pH 7.0–7.2 with 5% $NaHCO_3$ and then pass through quaternary amine SPE cartridges<br>analytes were desorbed with 0.2 M phosphoric acid and methanol | (1) ODS–Hypersil $C_{18}$ (200 × 4.6 mm, 5 $\mu$m) and (2) Symmetry $C_{18}$ (250 × 4.6 mm, 5 $\mu$m) | (1) $CH_3OH$–acetic acid–$H_2O$ (23:1:77, v/v/v)<br>flow rate: 1 mL/ min<br>(2) $CH_3OH$-0.001 M $H_3PO_4$ (23:77, v/v)<br>flow rate: 1 mL/ min | UV 254 nm PDA 254 nm and 280 nm fluorescence: ex: 230 nm em: 350 nm | 159 Q |
| G | barley and malt **11**, **3**, **5** (trans and cis) | extracted with $CH_3OH$<br>hydrolyzed with 2 N NaOH at RT for 4 h<br>acidified with 2N HCl (pH 1) extracted three times with ethyl acetate | adsorbosphere $C_{18}$ (150 mm × 4.6 mm) | A: water- pH 2.6 with orthophosphoric acid)<br>B: $CH_3CN$<br>flow rate: 0.8 mL/min<br>column temp 35°C<br>inj volume 20 $\mu$L | PDA | 82 Q |
| G | wheat bran 5 | in insoluble residues<br>hydrolysis with 1M NaOH constant agitation at 37°C | Spherisorb ODS–II (240 × 10 mm, 5 $\mu$m) | column was equilibrated with 28% $CH_3OH$ in 0.01% acetic acid for 7 mL<br>flow rate: 1 mL/min. | monitored at 310 nm | 81 |
| G | wheat, rice, rye, barley straws **5**, **3**, **12**, **6**, **11**, **10** | Samples were ground to pass a 1.2 mm screen<br>saponified under (a) 1 M NaOH for 18 h under $N_2$ or (b) 4M NaOH for 2 h at 170 °C. | Hichrom H5ODS (250 mm × 4.6 mm) | A: $H_2O$–$CH_3OH$–acetic acid (89:10:1)<br>B: $H_2O$–$CH_3OH$–acetic acid (90:9:1)<br>flow rate: 1 mL/min at RT | UV at 320 nm | 86 Q |
| G | barley **7**, **5**, **3**, (cis- and *trans*-isomers) | samples were extracted with 95% ethanol and hexane in ultrasonic bath<br>treatment with 2M NaOH at 20 °C for 16 h under $N_2$<br>after centrifugation, samples were acidified (6M HCl) and extracted with ethyl acetate | Nova-Pack $C_{18}$ (300 × 3.9 mm) | A: $H_2O$–acetic acid (89:2, v/v)<br>B: $H_2O$ –$CH_3CN$–acetic acid (78:20:2, v/v/v) | PDA 210–400 nm | 125 Q |
| G | wheat bran as dietary supplement, cornmeal and oat bran **15**, **10**, **11**, **12**, **5**, **18**, **17** | sequential extractions:<br>overnight shaking with $CH_3OH$ at RT (extraction of phenolic acids)<br>–0.1 M NaOH at 110°C for 10 min (extraction of alkali-labile lignin)<br>acidified with 37% HCl<br>samples were filtered with 0.22 $\mu$m nylon filter | Nova-Pak phenyl column (150 mm × 3.9 mm, 3 $\mu$m) | $CH_3OH$–$H_2O$ (30:70, v/v) with 0.01% acetic acid and 0.2 mM $Et_4NI$, pH of 5.67±0.1<br>flow rate: 0.3 mL/min<br>column temp 10 ±0.1 °C<br>eluate is split-fraction is diverted to provide flow-rate of 11$\mu$L/min into ES interface | ES-MS selected ion monitoring (SIM) monitored negative ion mode ($[M–H]^-$ | 128 |

[a] Authors do not state whether it is *o*- or *p*- coumaric acid. [b] Chlorogenic acid. [c] The letter Q designates that the investigation was quantitative. [d] Described by the authors as a hydroxybenzoic acid derivative; position of substitution not defined. [e] In addition to the simple phenolics, the authors separated several tartaric esters. [f] Also includes the separation of derivatives of phenolic acids: tartrates, quinates, and glucosylates. [g] Authors list both cis and trans isomers for **5**, Cou and **7** acids. [h] 2,6-Di-*tert*-butyl-4-methylphenol. [i] 2,6-Dimethoxybenzoic acid (internal standard). [j] 3,4,5-Trimethoxycinnamic acid. [k] Other phenolics in the analysis were resveratrol, quercetin, and rutin. [l] Caftaric and coutaric acids (tartaric esters of caffeic and *p*-coumarate respectively). [m] Flavonoids were also part of this analysis.

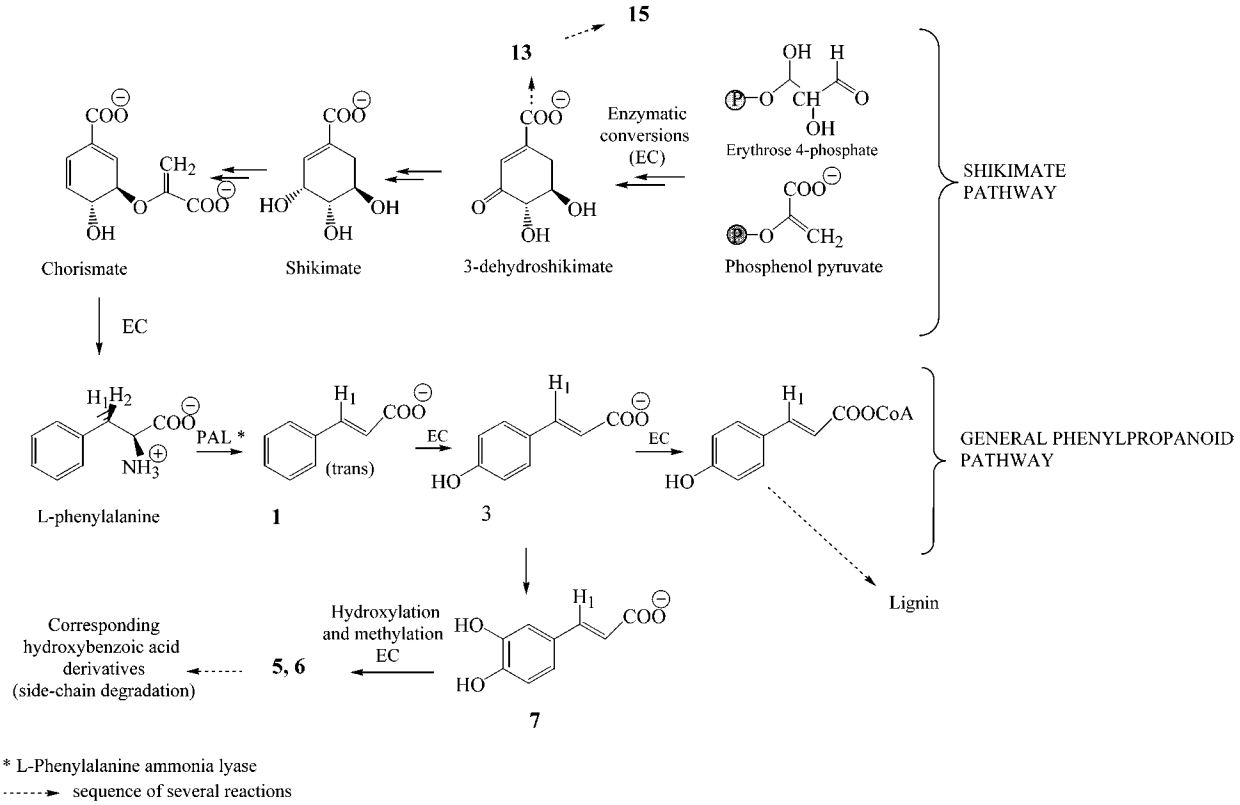



**Figure 2.** Biosynthetic pathways to hydroxybenzoate and hydroxycinnamate derivatives.

(*92*) and Torres et al. (*124*), who report working with columns having 8 and 10 $\mu$m particles, respectively. Column lengths range from 100 to 300 mm. Most investigations do not explain their choice of column. A few exceptions are Häkkinen et al. (*76*), who report the results from testing three different $C_{18}$ columns, and Guillen et al. (*87*), who chose from five different $C_{18}$ columns. Many authors do not report using a guard column.

Although there is a large variety in percent compositions of solvent systems, the types of solvents remain fairly consistent. Gradient elution systems are more frequently employed than their isocratic counterparts. Only one study mentions a convex gradient (*90*). Linear gradients involving an aqueous (often labeled A) phase and an organic phase (B) predominate.

Typically, an acid is added to the aqueous phase, although some report adding a small amount of acid to the alcoholic solvents as well (*95*). However, Lopez et al. (*70*) do not add an acid, but instead describe a three solvent system (ethyl acetate, methanol, and water). Acetic acid is most often chosen as the acidic additive, but sulfuric, perchloric, phosphoric (*113*), trifluoroacetic (*78*), hydrochloric (*95*), and formic acids are also reported (*94, 98, 113*).

The organic solvents with the acidic aqueous solvent are predominantly methanol and acetonitrile, but propanol, butanol, tetrahydrofuran, and ethyl acetate are employed as well. In certain investigations, the mobile phases (A and B) consist of mixtures of the organic and aqueous solvents. Borzillo et al. (*156*) report solvent A as methanol−acetic acid−water (10:2:88, v/v/v) and solvent B as containing methanol−acetic acid−water (90:2:8, v/v/v). Hernanz et al. (*125*) describe their organic phase as consisting of water, acetonitrile, and acetic acid (78:20:2, v/v/v). In a few methods, the mobile phases contain buffers such as $H_3PO_4$/ $KH_2PO_4$ or acetic acid/ammonium acetate and sodium citrate buffer (pH 5.4) (*112*). In one early investigation, the authors claim that separation could not be achieved without the use of ammonium acetate in their solvent (*126*).

Run times for the methods vary a great deal, ranging from 30 to 150 min. Some methods have been developed to include larger polyphenols (flavonoids) as well as phenolic acids, thereby increasing the analysis time. In addition, some reports include equilibration time within the programmed run, while others have it as a separate step. Flow rates range from 0.15 to 1.8 mL/minute, the most common being 1 mL/min. In many cases, injection volumes are not indicated; however, those that were reported have ranged from 10 to 20 $\mu$L. Again, not all authors mention column temperature; however, the reported temperatures range from 20 to 45 °C. Roggero et al., (*71*) examining phenolic acids and polyphenols in one run, kept the column at 22.5 °C. They emphasized that the long analysis time (150 min) required constant temperature for reproducibility. Gioacchini et al. (*128*) report that, in order to achieve highly reproducible retention times, their column was thermostated at 10 ± 0.1 °C.

*Detection.* Detection techniques for HPLC investigations have been overwhelmingly by UV−Vis with photodiode array (PDA) detection, a common method of monitoring at wavelengths from 190 to 380 nm. Phenolic acids with the benzoic acid carbon framework have their $\lambda_{max}$ in the 200 to 290 nm range (**Table 2**). The only exception is gentisic acid, which has an absorbance that extends to 355 nm (**Figure 3**). The cinnamate derivatives, due to the additional conjugation, show an additional broad absorbance band from 270 to 360 nm. The single most common wavelength used for monitoring occurred at 254 nm. This is perhaps due to convention based on the strong energy line from earlier lamps, because it is not the overall $\lambda_{max}$. Because many of the absorption spectra are very similar (**Figure 3B**), peaks assignments are made with both the retention times as well as by comparing the UV−Vis spectra of analytes to purchased standards.

Other less common means of detection, coupled to LC, have been through electrochemical detection (EC), fluorescence (F),

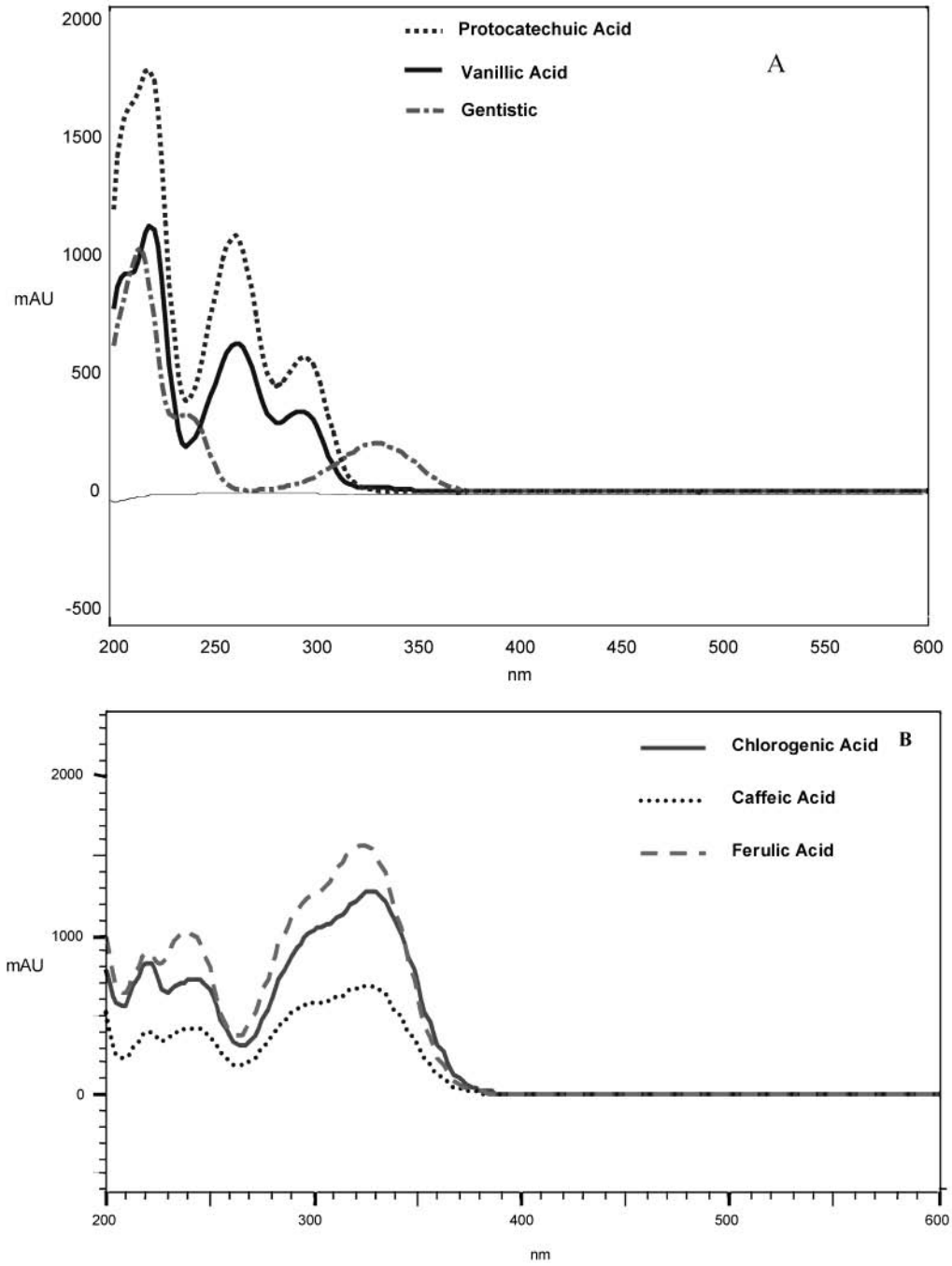



**Figure 3.** (A) UV–Vis spectroscopic overlay of protocatechuic, vanillic, and gentisic acids. (B) UV–Vis spectroscopic overlay of ferulic, caffeic, and chlorogenic acids.

and MS. Using a carbon polyethylene electrode and an Ag/AgCl reference electrode, Malher et al. (*74*) employed EC for the analysis of Vidal Blanc wines. Electrochemical behavior provided the additional information for the identification of phenolic acids not previously identified with LC/UV.

Rodriguez-Delgado et al. (*127*) used fluorescence and absorbance detectors in series. The excitation wavelength was 278 nm, and the emission was 360 nm for the first 17.5 min of their 35 min chromatographic run. For the remaining 16.5 min, the excitation was 330 nm, and the emission wavelength was 374 nm. The automatic switching of detection parameters was based on elution characteristics previously established and was used to distinguish certain polyphenols. The wavelength employed for the UV absorbance was 280 nm. Both selectivity as well as increased sensitivity (for certain of the phenolic acids) was achieved using this serial detection method. Monitoring with both UV and fluorescence assisted in distinguishing between nonfluorescent and fluorescent overlapping peaks.

Analytical methods for phenolic acids using LC coupled to MS detection is an emerging field. Gioacchini et al. (*128*) developed a method for the determination of phenolic acids in wheat, oat bran, and cornmeal, using HPLC-MS with an electrospray interface (ESI). The LC separation consisted of a methanol−water mix with 0.01 acetic acid and 0.2 mM tetraethylammonium iodide as the ion-pairing reagent. Diverting a portion of the solvent (eluent splitting) allows for a small flow rate (11 $\mu$L/min) into the ESI and MS for ion generation and detection, respectively. The mass detection portion was carried out in the negative ion mode (i.e., $[M-H]^-$). The authors report very high sensitivity (detection limits ranging from 1 to 6 ng/

injection) monitoring with this HPLC-MS-ESI technique. Retention time and mass $[M-H]^-$ confirmed the identification.

Identification and quantification was achieved for several phenolics in olives and oil via HPLC-MS/MS (*129*). The interface between the liquid chromatography and the MS detector in this case was carried out with atmospheric pressure ionization (API). After determining the precursor ion in full scan, negative-ion mode, the product ions were determined using MS/MS. To achieve high specificity in addition to high sensitivity, the authors monitored the HPLC-MS/MS analysis in multiple reaction mode (MRM). Cremin et al. (*130*) report an LC/ES-MS method to analyze hydroxycinnamates (**5**, **7**, chlorogenic acid) in human urine and plasma.

Although not yet prevalent in the literature, these LC-MS techniques are proving to be very powerful for the analyses of complex matrixes, because complete chromatographic separation is not always necessary, in part due to the mass selectivity of detection.

**Gas Chromatography.** Gas chromatography (GC) is another major chromatographic technique employed for the analysis of phenolic acids in plants. Earlier work was typically performed with flame ionization detection (FID) (*131*−*133*). One of the major concerns with this technique is the low volatility of phenolic compounds. Nevertheless, there are still many examples of GC for the separation of phenolic acids.

*Derivatization: Reagents and Process.* One chemical characteristic of the hydroxyl group, the hydrogen bonding capability, increases the melting point. Analysis in the gas phase requires a chemical modification step, often referred to as derivatization, in addition to sample isolation, clean up, and preparation. The hydroxyl groups are converted to ethers or esters. Although GC performed on underivatized phenols and acids is rare, there exist some methodological investigations. Christov et al. (*134*) describe flame ionization and electron capture as detection methods in the analysis of underivatized acids.

Derivatization can be a challenge for analytes in complex food matrixes. The glycosides present in the food matrix interfere with the chemical modification of the analytes of interest (*135*). Methods developed for the analysis of phenolic acids from varied sources are listed in **Table 5**. Sample preparation and derivatization reagents are also summarized. Coupling of the two gas-phase techniques, GC and MS, is a major analytical tool. The more definitive information offered by MS detection lends itself well to the identification and quantification of phenolic acids in certain foods (*136*).

There are a variety of reagents used to modify and generate volatile derivatives. Diazomethane is often used for the generation of methyl esters. Although solutions of diazomethane react efficiently with carboxylic acids, it must be generated in the laboratory, and is explosive (*137*). Husek et al. (*138*) employed ethyl and methyl chloroformate for the formation of ethyl and methyl esters, respectively. Dimethyl sulfoxide with methyl iodide in an alkaline medium is another a procedure for methylation. However, methyl esters can lead to some confusion, as they are naturally occurring in some plant-based material. Smolarz et al. (*85*) reported that, in their analysis of organic extracts in certain species of *Polygonum*, they were unable to differentiate between the naturally occurring methyl esters and the free acids. By synthesizing the silylated derivatives, they could distinguish between the free versus bound acids.

The most common derivative is, by far, the trialkylsilyl group. It is generated via covalently linking the alkyl substituted silicon atom to the oxygen of the hydroxyl groups. The most common alkyl substituent has been a methyl group, hence the trimethylsilyl (TMS) derivative. Although there exists a great variety of commercially available silylating reagents, the most common in the literature surveyed regarding phenolic acids have been *N*,*O*-bis-(trimethylsilyl)acetamide (BSA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and *N*,*O*-bis-(trimethylsilyl)-trifluoroacematide (BSTFA).

There are many advantages to generating the silylated derivatives instead of other derivatizating agents. The reaction is simple. A typical derivatization procedure most often takes place in a screw-capped vial and can be, for some reagents, an instantaneous process. In addition, both functional groups are derivatized (acids and phenols) in the same step. The reaction is mostly free of unwanted side products potentially caused by TMS delivery reagents reacting with silylated derivatives (*135*). Moreover, many of the minor products or artifacts have been well described and documented (*139*). In addition, the byproducts of these reactions are extremely volatile, elute very early, and do not interfere with the analysis (*140*). The reaction involves dissolving the dried sample in a base (e.g., pyridine or ethylamine) and the TMS delivery reagent then heating the reaction vial for 20−60 min.

Although there have been some investigations that have sought to speed up this procedure, most of the literature indicates that heating is still the predominant technique. Chu et al., (*141*) reasoning that the heat transfer was a slow process, devised a microwave derivatization procedure cutting the time to 30 s. Phenols and carboxylic acids are relatively reactive and are easy functional groups to silylate. However, in some instances, the derivatives can be unstable once removed from the silylating medium and exposed to the moisture in the air. A common step taken to prevent decomposition is to cover the silylated derivatives with an organic solvent (e.g., hexane, isooctane) after removal of the solvent from the derivatization reaction step.

*Chromatographic Methods.* Although there are fewer examples of gas chromatography to survey, there exists a great deal of variety in the methodology developed. In the more recent literature, fused silica capillary columns are the most common employed. Lengths are 25−30 m, and the inner dimensions range from 0.25 to 0.5 mm. The stationary phase thickness is typically 0.25 $\mu$m, although there is one exception where Husek et al. (*138*) used a column with an 11$\mu$m film thickness. The most common column coating material is DB 5, which contains 5% phenyl silicone and 95% methysilicone.

Leung et al. (*104*) are one of the few groups who reported isothermal methods for separating phenolic acids. Otherwise, most of the investigations report a variety of temperature gradients, steps, and rate increases, ranging from 2 to 40 °C/min. Initial column temperatures range from 40 to 140 °C, and 75−80 °C seems to be commonly chosen.

*Detection.* In the older studies, FID is common, but MS has become widespread. Most of the GC/MS work is performed in the electron ionization (EI) mode, with the ionization voltage set to a standard 70 eV. The spectra are collected from *m*/*z* 39−650 in continuous scanning mode. Although many of these silylated phenolic acids are isomers, i.e., have the same masses, their dissimilar cleavage patterns can be used as an additional means for identification, an important concern for the analysis of complex food matrixes where few commercially available standards exist.

The benzene ring is a stable functional group and does not typically show much fragmentation. However, silylation of the hydroxyl groups generates additional distinct fragmentation patterns. In most cases, the molecular ion $[M^{+\bullet}]$ for the TMS

**Table 5.** Examples of GC and GC/MS Application for the Analysis of Phenolic Acids with Preparation and Derivatization Methodology Included

| sample source and analyte | sample prep and derivatization reagent | column | temp method | ionization technique and detector | ref |
|---|---|---|---|---|---|
| propolis (bee glue) **7** + several other phenolics[a] | propolis extraction with pet ether and diethyl ether; both derivatized and underivatized preparations were investigated; BSTFA[b] was used for derivatization | 9 m × 0.25 mm, 0.25 $\mu$m film thickness SE–54 fused-silica capillary column<br>smaller columns after split: 10 cm × 0.25 SE–54 fused-silica | 80–280at a rate of 20 °C min $^{-1}$, 280–300 °C rate 2 °C min $^{-1}$, 10 min hold 300 °C<br>gas flow split 1:1 to 2 detectors<br>inj temp 320 °C<br>det temp 350 °C | comparison of electron-capture and FID | 134 |
| sunflower seeds 1, mHBA, **10**, **11**, Cou, **12**, **5**, **7** | seeds dehulled and defatted; extracted with 70% aq acetone<br>hydrolysis: 1N NaOH in boiling bath for 15 min; 2N HCl heated at 100°C for 30 min<br>TMS derivatives prepared from BSA | two columns: 6% OV-1 on 80–100 mesh Chromosorb W. 3% OV-17 on Chromosorb W (3 m × 3 mm o.d) | several methods reported:<br>isotherm: 215 °C<br>programmed: 130–220 °C<br>170–215 °C<br>170–260 °C<br>at 4 or 5 °C/min | FID | 104 |
| standards: **8**, **9**, **11**,**13**, **14**, **15** | methyl chloroformate and ethyl chloroformate were used for the formation of alkyl esters; reaction medium consisted of hexane-chloroform-pyridine (40:10:1) medium | CP–SIL 5 CB fused silica capillary column (25 m × 0.32 mm, 0.11 $\mu$m) | injection via split mode (1:20) at 240 °C<br>linear temp program at 10 °C /min ranges up 0–270 °C (18 min run). | FID | 138 |
| olive leaves and roots and soil | plant material boiled in 2M HCl for 45 min then extracted with ethyl acetate; extracts were dried and Tri-sil/BSA added heated at 70 °C for 30 min | SPB-1 fused silica capillary column (30 m × 0.32 mm, 0.25 $\mu$m) | 138 °C held for 38 min to 150 °C at 1 °C/min, hold for 12 min<br>injection via split mode (1:5) at 260 °C<br>carrier gas: He<br>flow rate: 2.8 mL/min<br>detector at 300 °C | FID | 160 |
| cereal (wheat, oats, corn, brown rice potatoes) **12**, **6**, **3**, **5**, **11** | series of extractions were performed, highest recovery was with methanol–acetone–water (7:7:6, v/v/v) silylated with Tri-Sil/BSA[c] | GC: 2.0 m × 2 mm o.d. glass column packed with 3% OV-1 on 80–100 mesh Chromosorb W<br>GC/MS: WCOT capillary column of fused silica coated with OV-101 (24 m × 0.2 m) | CG: 120 °C –300 °C at a rate of 4 °C /min<br>GC/MS: 40–150 °C at 40 °C /min, 150–300 °C at 4 °C/min, held at 300 °C for 10 min. | GC: FID<br>GC/MS: ionizing voltage 70 eV; scan time 2.5 s *m*/*z* 40 –800 | 79 Q, 103 |
| standards: **15**, **14**, **11**, **7**, **5**, **3** | the acid was dissolved in 0.5 mL of pyridine and 0.3 mL of (BSA) was added;<br>accelerated derivatization: microwave oven at high power –30 s; comparison between BSTFA/thermal heating and BSA/ microwave oven | HP-5MS capillary column (30 m × 0.25 mm, 0.25 $\mu$m) | initial oven was set to 80 °C; then raised to 240 °C at 15 °C min –$^{1}$; then held for 3 min<br>run time 14.7 min<br>carrier gas: He (99.995% purity)<br>flow rate: 1 mL/min | MS | 141 |
| taxons of *Polygonum* L. genus **8**, **1**, **10**, **13**, **3**, **11**, **7**, **5** | refluxed pulverized plant material in $CHCl_3$ for 30 min filtered with SPE– RP $C_{18}$ silica gel<br>derivatization, methylation: to dry KOH in DMSO[d], $CH_3I$ was added as well as silylation with BSTFA 20 min at 90 °C | XTI-5 fused-silica capillary column (30 m × 0.25 mm, 0.25 $\mu$m) | initial column temperature was 140 °C, held for 2 min, then raised to 300 °C at a rate of 5 °C min–$^{1}$<br>carrier gas: He (99.995% purity)<br>flow rate: 1 mL/min<br>inj temp: 300 °C | EI-MS spectra were collected at *m*/*z* 50–650. | 85 |

**Table 5.** Continued

| sample source and analyte | sample prep and derivatization reagent | column | temp method | ionization technique and detector | ref |
|---|---|---|---|---|---|
| american cranberry fruit **8**, **11**, **3**, **5**, **7**, **6**, **2** | cranberry fruit was ground in distilled water; aliquot was acidified with 1N HCl (pH 2) and extracted with ether; organic phase was extracted with 5% $NaHCO_3$; derivatization involved dissolution in pyridine and treatment with BSTFA + TMCS[e] (heated for 30 min at 60 °C) | DB-5 fused-silica capillary column (30 m × 0.35 mm, 0.25 $\mu$m film) | initial oven was set to 80 °C, then raised to 120 °C at a rate of 5 °C $min^{-1}$; 120 °C to 240 °C at a rate of 10 °C $min^{-1}$; 240 °C to 280 °C at a rate of 20 °C $min^{-1}$<br>carrier gas: He (99.995% purity)<br>flow rate: 1 mL/min<br>inj temp: 280 °C<br>inj vol: splitless 1 mL | MS spectra were scanned from *m*/*z* 50 to 550 at a rate of 1.5 scans /sec | 136 Q |
| distilled alcohol beverages **11**, **15**, + other smaller acids and aldehydes.[f] | used SAX purification of samples; silylation was performed by heating MSTFA in $CH_3CN$ at 80 °C for 20 min | DB-5 ms fused-silica capillary column (30 m × 0.35 mm,0.25 $\mu$m film) | initial oven was set to75 for 2 min, then raised to 100 °C at a rate of 25 °C $min^{-1}$; 100 °C–300 °C at a rate of 10 °C $min^{-1}$; 300 °C for 5 min<br>carrier gas: He<br>flow rate: 1.1 mL/min<br>inj temp was set to 250 °C; interface to 290 °C<br>splitless injection 1 min after injection | MS-electron impact mode (EI) spectra were scanned from 39 to 450 u at a rate of 1.66 scans/s. | 140 Q |
| **13**, **11**, **15**, hcin[g] samples taken from an Egyptian mummy | methanolysis: formation of methyl esters and Sylon-HTP[h] (pyridine/HMDS/TMSC, 9:3:1, v/v/v) | 30 m × 0.25 mm i.d. fused-silica DB-5 (poly 5% phenyl, 95%methylsiloxane) | 40–130 °C rate 9 °C $min^{-1}$, 130–290 °C rate 2 °C $min^{-1}$, 290 °C for 10 min | EI-MS spectra were collected in total ion monitoring mode: scan range *m*/*z* 20–650) | 161 |
| wheat **10**, **11**, **3**, **12,** and **5** | sample was derivatized with excess MSTFA (xs)[i] | 30 m × 0.25 mm i.d. (0.25 $\mu$m stationary phase thickness) DB-5 MS | 80°C hold 1 min, to 160 °C at 10 °C/min, 160–235 °C at 5 °C/min hold for 5 min | EI with GC/MS CID for GC/MS MS helium gas (99.999% purity) | 14, 15 |

[a] Pinocembrin and galangin and phenylethylcaffeate. [b] N,O-*bis*(trimethylsilyl)trifluoroacetamide. [c] *N*,*O*-bis(trimethylsilyl)acetamide in either pyridine or dimethyl formamide (DMF). [d] DMSO = dimethyl sulfoxide. [e] TMCS = trimethylchlorosilane. [f] Vanillin, syringealdehyde. [g] Unspecified regiochemistry, authors simply state a hydroxycinnamic acid; Hcin is the chosen abbreviation. [h] Pyridine/hexamethyldisilazane/trimethylchlorosilane. [i] MSTFA: *N*-methyl-*N*-trimethylsilyltrifluoroacetamide.

derivatives of phenolic acids is a prominent peak in the mass spectrum (**Table 3**). Gentisic and 2,3,4,-trihydroxy benzoic acid are the exceptions. Both these acids possess a substituent at the 2-position on the benzene ring. The "ortho effect" is an established phenomenon. The adjacent relationship permits a molecular interaction between substituents facilitating decomposition or fragmentation. Generation of the [M-15] fragment, loss of a methyl group, via alpha-cleavage, is a well-established cleavage pattern for both TMS ethers and esters and provides the base peak for several of these analytes. TMS esters can, however, undergo a subsequent fragmentation generating the [M-59] fragment, as is the case with cinnamic (*m*/*z* 161), *p*-coumaric (*m*/*z* 249), and *p*-hydroxybenzoic acid (*m*/*z* 223). After the loss of a methyl group from the TMS group, $CO_2$ is expelled after rearrangement, giving [M-59] (**Figure 4**A). Loss of *O*TMS, [M-89], is also a fragmentation pathway common for derivatized carboxylic acids, because the acylium cation is a stable species. (**Figure 4B**) The predominant [M-30] fragment involves losing a molecule of formaldehyde and represents the cleavage of the methoxyl substituent of the phenyl ring (**Figure 4C**). For sinapic acid (also referred to as sinapinic acid), this pattern produces the base peak. Ferulic acid also shows a substantial *m*/*z* 308 fragment.

For gallic, caffeic and protocatechuic acids, the major fragmentation route generates a [M-177] peak. While Diekman et al. (*142*) and McClosky et al. (*143*) propose a structure (**Figure 4D**) and mechanism for loss of a fragment with this mass that involves adjacent TMS groups, there are no reports that describe the origin of this fragment for phenolic acids. Although gallic, caffeic, and protocatechuic have *O*TMS groups that are vicinal, the cleavage pattern proposed by McClosky et al. would involve breaking the benzene ring, which seems unlikely.

## DISCUSSION

The impetus for developing analytical separation methods for phenolic acids has been multifaceted. Much of the existing analytical methodologies originated from the interest in these analytes for their biological roles as secondary metabolites, that is, as markers for taxonomic studies, for their ecological effects (e.g., allelopathic behaviors), and even as a description of maturation stages. Another portion of the analytical work was

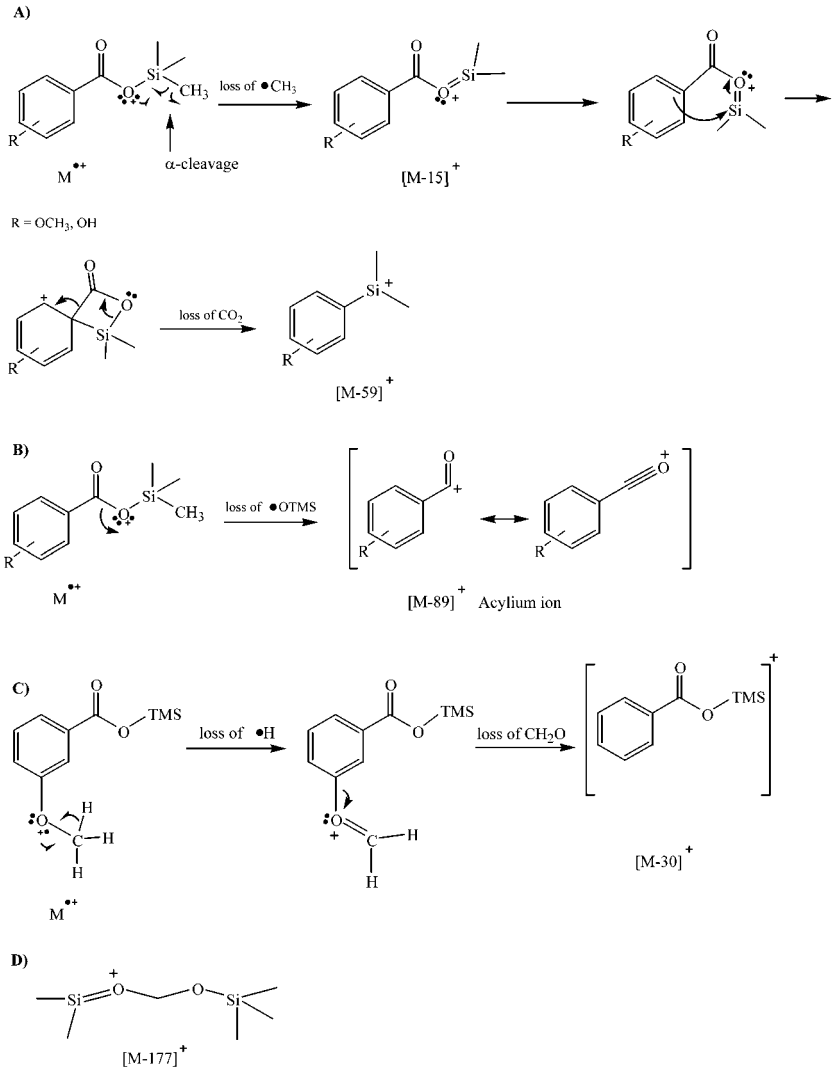



**Figure 4.** (A) Mechanism for the loss of [M-15] and [M-59]. (B) Formation of the acylium ion [M-89]. (C) Loss of a molecule of formaldehyde [M-30]. (D) Fragment with M of 177 u.

developed to understand the impact of these acids on organoleptic properties or food flavors and preservation (oxidative damage control) and prevention of adulteration. A more recent interest stems from the purported role phenolic acids play in the protection, through a diet high in fruits in vegetables, against diseases caused by oxidative damage. As yet, no analytical methods have been established or devised from a nutritional perspective. That is, no measurements of phenolic acids content in commonly consumed foods, as a means to assess dietary intake, are underway. Despite the fact that these phenolic acids are purported to have health benefits, the exact mechanism of absorption, of disease prevention, or which species (free or conjugated) is bioavailable is not well understood (*144*). To assist in the epidemiological investigations, databases and analytical methods need to be established.

HPLC with reverse phase column is the most widespread chromatographic technique in the investigations of phenolic acids. Photodiode array allowing for real time UV−Vis spectra are the most prevalent detection method. Retention times and UV−Vis absorption spectra have typically been the identification criteria for most of the reported investigations. Considering the multitude of components and complexity of plant matrices, identification of peaks is a difficult issue. Other detection methods have been investigated, namely electrochemical and fluorometric systems with the aim of increasing specificity. LC/MS seems to be the next wave to address this complexity.

With GC coupled to MS, mass, as well as fragmentation patterns, has become a more definitive identification tool, although isomeric compounds still need retention time data for confirmation. Due to the similarities in structures and the resiliency of the aromatic ring, the cleavage patterns under MS do not necessarily generate distinctive fragments. To overcome the low volatility of these compounds, silylation has become the major derivatization technique. With the increasing use of LC/MS, the additional chemical modification steps might become unnecessary. However, among other issues, the isomeric masses of these phenolic acids generate identification difficulties.

Alcoholic−aqueous solvents are the predominant means of extraction. Hydrolysis or saponification is still the most common means of "freeing" the acids, even though it is still unclear how much, if any, of the acids decompose under these conditions. Enzymatic release perhaps is the next area of scrutiny.

Many of the separation methods have been developed from a qualitative perspective. For qualitative analyses, sample preparation is not crucial; however, it is crucial for quantitative investigation of components in foods. Despite a great number of analytical investigations that have been performed for phenolic acids, as well as their larger analogues, their separation

and quantitation still proves difficult due to, in part, the problems and uncertainties in sample preparation. No systematic investigations on the effects of the various sample preparation methods have been reported. It appears, in fact, that the field is in need of a robust sample preparation technique.

## ABBREVIATIONS

BHT, 2,6-di-*tert*-butyl-4-methylphenol; BSA, N,O-bis(trimethylsilyl)acetamide; BSTFA, *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide; FCR, Folin-Ciocalteu Reagent; FID, flame ionization detection; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; PLE, pressurized liquid extraction; PD, photodiode array; PPO, polyphenol oxidase; SPE, solid-phase extraction; TMCS, trimethylchlorosilane; TMS, trimethylsilyl; TMSO, trimethylsilyloxy.

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