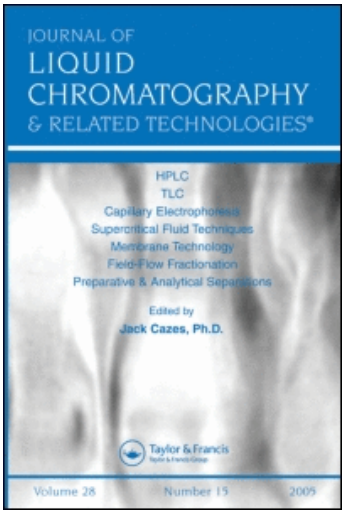


Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Liquid Chromatographic Determination of Phenolic Acids of Vegetable Origin

Daryl A. Roston[a]; Peter T. Kissinger[a]
[a] Department of Chemistry, Purdue University West Lafayette, Indiana

To cite this Article Roston, Daryl A. and Kissinger, Peter T.(1982) 'Liquid Chromatographic Determination of Phenolic Acids of Vegetable Origin', Journal of Liquid Chromatography & Related Technologies, 5: 12, 75 — 103
To link to this Article: DOI: 10.1080/01483918208082216
URL: http://dx.doi.org/10.1080/01483918208082216

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(Suppl. 1), 75-103 (1982)

LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENOLIC ACIDS OF VEGETABLE ORIGIN

Daryl A. Roston and Peter T. Kissinger
Department of Chemistry
Purdue University
West Lafayette, Indiana 47907

ABSTRACT

This brief review describes recent findings on the liquid chromatographic determination of monocyclic phenolic acids that are endogenous to plant materials. A major portion of the review deals with the separation of phenolic acid mixtures by bonded phase chromatography. Sample preparation techniques, ultraviolet absorption detection, and electrochemical detection are also considered.

INTRODUCTION

Phenolic acids have been shown to be ubiquitous in plant materials (1). Their widespread presence in samples of vegetable origin has generated interest in diverse branches of science. Because they have been implicated as important intermediates in secondary plant metabolism, phenolic acids are of interest to investigators engaged in fundamental biochemical research (1-3). Phenolic acids have also been demonstrated to be important factors for the taste, aroma, color, and stability of many food materials (4). Hence, they are the subject of extensive investigation in the area of food science. Although

not the subject of this review, phenolic acids are also encountered in the metabolism of aromatic amino acids in animals.

Numerous analytical methods have been employed for the isolation and identification of phenolic acids. Traditional approaches have involved thin-layer chromatography and paper chromatography (5-6). More recently, gas chromatography with pre-column derivatization has been utilized (4,7-9). As with many other areas in need of improved analytical methodology, the 1970's witnessed the advent and increasing popularity of liquid chromatography for the separation and identification of phenolic acids in plant materials and related matrices. This review emphasizes three major areas: liquid chromatography conditions, detection schemes, and sample handling prior to chromatography. The reviewed literature has been limited to recent studies concerned with compounds of C_6-C_1, C_6-C_2, and C_6-C_3 carbon skeletons. The bulk of the literature deals with the separation and identification of hydroxy and hydroxy-methoxy derivatives of benzoic and cinnamic acid. Structures for these compounds are detailed in Table 1.

LIQUID CHROMATOGRAPHY

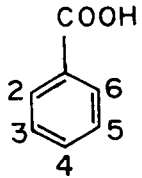
An abbreviated summary of the liquid chromatographic methods and conditions employed for the separation of phenolic acid mixtures is given in Table 2. Several types of liquid chromatography have been employed, including liquid-liquid, classical anion-exchange, and various forms of bonded-phase chromatography. Bonded-phase chromatography (especially reverse-phase) has enjoyed the most extensive use.

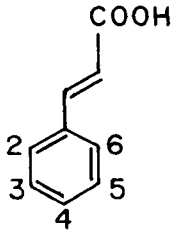
Liquid-Liquid and Anion-Exchange Chromatography

All of the recent applications of liquid chromatography for the separation of phenolic acid mixtures have employed bonded

TABLE 1

Benzoic and Cinnamic Acid Derivatives





Substitution	Trivial Name	Substitution	Trivial Name
2-OH	Salicylic acid	2-OH	o-Coumaric acid
3-OH	m-Hydroxybenzoic acid	3-OH	m-Coumaric acid
4-OH	p-Hydroxybenzoic acid	4-OH	p-Coumaric acid
2,4-di-OH	β-Resorcyclic acid	3,4-di-OH	Caffeic acid
2,5-di-OH	Gentisic acid	3-OCH_3,4-OH	Ferulic acid
2,6-di-OH	γ-Resorcyclic acid	3,5-di-OCH_3,4-OH	Sinapic acid
3,4-di-OH	Protocatechuic acid		
3,5-di-OH	α-Resorcyclic acid		
3,4,5-tri-OH	Gallic acid		
3-OCH_3,4-OH	Vanillic acid		
3,5-di-OCH_3,4-OH	Syringic acid		

TABLE 2

Liquid Chromatographic Studies

Stationary Phase	Chromatographic Conditions: Isocratic	Chromatographic Conditions: Gradient	Compounds Studied: Phenolic Acids	Compounds Studied: Flavonoids	Compounds Studied: Natural Esters	Reference
silica gel		x	x		x	10
silica gel	x		x			11
alkyl-amine	x		x			12
pellicular anion exchange resin	x		x			13
pellicular anion exchange resin	x		x	x		14
pellicular anion exchange resin	x		x	x		15
pellicular anion exchange resin		x	x	x		16
polyamide	x		x		x	17
diol		x	x		x	18
alkyl phenyl	x		x			19
alkyl phenyl		x	x	x		20
C_2	x		x			21

C_8		x			x	18
C_{18}	x	x	x			19
C_{18}	x		x	x	x	22
C_{18}		x	x	x	x	23
C_{18}		x	x		x	24
C_{18}		x	x			25
C_{18}	x		x			26
C_{18}	x				x	27
C_{18}	x				x	28
C_{18}	x		x			29
C_{18}		x	x	x	x	30
C_{18}	x		x			31
C_{18}	x		x	x		32
C_{18}		x	x	x		33
C_{18}	x		x			34
C_{18}	x		x			35
C_{18}		x		x	x	36
C_{18}	x		x			37

phase systems (17-37). Prior to the dominance of bonded-phase chromatography in the late 1970's, liquid-liquid, ion-pairing, and anion-exchange chromatography were used (10-16). In general, these reports described the use of a single chromatographic system for a specific application (10,11,13-16) without information on the effect of changes in the two phases on the separation of interest. Consequently, they are of limited utility in terms of the selection of chromatographic conditions for separations of other mixtures of related compounds.

Two of the earliest applications of liquid chromatography for the separation of phenolic acids utilized normal phase LC with silica gel as stationary phase (10,11). Nagels and Parmentierreported the use of a gradient mobile phase consisting of chloroform, hexane, and tert-butanol for the separation of cinnamic acids and their corresponding quinic acid esters (10). Hovermann and co-workers employed a mobile phase consisting of methylene chloride, ethanol and water for the separation of cis and trans isomers of ferulic and p-coumaric acid. Several communications have employed anion-exchange chromatography (13-16). In each case the stationary phase was a pellicular anion exchange resin. Three of the communications reported the use of isocratic conditions with the aqueous mobile phase buffered in the pH range 3.7 to 4.7 (13-15). A gradient mobile phase was used in the fourth study (16). Terweij-Groen and Kraak investigated the separation of mixtures of cinnamic, benzoic, and sulfonic acids with an ion-pair system consisting of aqueous acids solutions as mobile phases and long-chain aliphatic amines as stationary phases (12). Important parametersfor the separation werethe liquid-liquid distribution and ion-pair formation. Systematic studies of the effect of the type of amine, pH, and methanol content were reported.

Bonded-Phase Chromatography

The use of bonded-phase chromatography characterizes much of the literature concerned with the liquid chromatographic separation of phenolic acids. Four aspects of bonded-phase chromatography important to the resolution of phenolic acid mixtures have been addressed by the current literature: mobile phase composition, bonded-phase material, structure-retention effects, and the chromatography of naturally occurring esters. A summary of the reported findings pertaining to each area is given below.

Mobile Phase Composition. Generally, mobile phases for the bonded phase separation of phenolic acids have been mixtures of distilled water, an organic solvent, and a weak organic acid. The use of an electrolyte as mobile phase modifier has also been frequently reported. Organic solvents employed as mobile phase modifiers included methanol (19,22,23,25-27,29,30,33,34,35), ethanol (20,36), propanol (37), butanol (19,25,31,33), and acetonitrile (21,24,32). Generally, the quantity of organic solvent modifier employed was tailored to the separation addressed in each study. Weak organic acids used as mobile phase modifiers included acetic acid (19-22,25,26,30-35,37), phosphoric acid (23,24,27,36), and citric acid (29). Modification of mobile phase composition with organic acids lowers the pH of the mobile phase and supresses ionization of the acidic functional groups. Ionization suppression aided separations based on the hydrophobicities of the phenolic acids. Several studies reported the use of electrolytes as mobile phase modifiers. Ammonium acetate (19,25,33,37), sodium chloride (29), and tetrabutyl ammonium phosphate (30) were all employed. In each case the electrolyte was used in conjunction with organic solvent and organic acid mobile phase modifiers. The role of

electrolyte in the separation has not been well-defined. However, it has been suggested that the increased ionic strength enhances the separation by decreasing intra-molecular hydrogen bonding (25).

Systematic studies of the effect of parameters such as organic modifier content and pH on bonded phase separations have been reported in several communications (21,22,29). Using a C_2 alkyl bonded phase, Grodzinska-Zachweija and co-workers found linear log k´ - % acetonitrile relationships for seven cinnamic acid derivatives (21). The linear decrease in log k´ values was observed when the acetonitrile content of the aqueous mobile phase was increased from 5% to 25% (V/V). Working with a mixture of ten compounds, including nine benzoic and cinnamic acid derivatives, Wulf and Nagel examined changes in retention induced when the percentage of methanol in the aqueous mobile phase was increased from 0% to 20% (22). A C_{18} alkyl bonded phase was employed. They observed linear log k´ - % methanol relationships for all of the studied compounds. In addition, it was shown that the log k´ - % methanol plots for each class of phenolic acid (benzoic and cinnamic) resulted in a set of nearly parallel lines. Hence, for a mixture of phenolic acids of a given class, relative retention did not change as the percentage of methanol increased. However, the slope of the log k´ - % methanol plots was steeper for cinnamic acids. Therefore, the selectivity for a given benzoic acid-cinnamic acid pair decreased as methanol content increased.

A study of the effect of pH on the retention of several benzoic and cinnamic acid derivatives was conducted by Price and co-workers (29). Employing a constant ionic strength buffer, they varied the pH of the aqueous mobile phase from 3 to 6 and monitored the retention change for the various acids. The stationary phase was C_{18} material. A sigmoidal retention time - pH relationship was observed for each acid. The increase

in retention with decreasing pH appeared to level-off at pH 3. In addition, the pH-retention relationships indicated that the phenolic acids had pK_a values between 4 and 5. Hence, retention is markedly dependent on pH in this region.

An important decision concerning mobile phase composition regards the choice of an isocratic or gradient system. Information tabulated in Table 2 indicates that thirteen of the studies employing bonded-phase systems employed isocratic conditions while nine used gradients. The isocratic-gradient choice was apparently predicated on the range of hydrophobicities exhibited by the compounds present in the studied mixture. Flavonoid compounds ($C_6 - C_3 - C_6$ carbon skeleton) are often found in the same matrices as single-ring phenolic acids. Consequently many of the communications that have dealt with the bonded-phase separation of mixtures of phenolic acids have also considered flavonoid compounds (20,22,23,30,32,33,36). It is evident that the increased hydrophobicity of the larger flavonoid molecules necessitates the use of gradients if they are to be determined simultaneously with single-ring phenolic acids. All of the studies that reported practical separations of mixtures of phenolic acids and flavonoid compounds employed gradient mobile phases (23,30,33,36). Three communications reported the use of different conditions to optimize separations of phenolic acids and flavonoid compounds in separate experiments (20,22,32).

Bonded-Phase Materials. Six bonded phase materials have been utilized for the separation of phenolic acid mixtures: diol (18), polyamide (17), alkyl phenyl (19,20), C_2 (21), C_8 (18), and C_{18} (19,22-37). C_{18} has been the most popular material, however, substantial evidence in support of its widespread use has not been reported. The literature does not contain adequate comparisons of the performance of various materials. Only one such study was reported. Scrivin, Day, and Willis compared the separation of eight benzoic acid

derivatives on alkyl-phenyl and C_{18} bonded phase columns (19). A 5% acetic acid aqueous mobile phase was employed. The results shown in Table 3 indicate that better separation was observed with the alkyl phenyl column. The popularity of C_{18} materials can be attributed to their widespread commercial availibility and use in other applications of liquid chromatography. Representative retention data from studies employing C_{18} materials is tabulated in Table 4.

Structure-Retention Effects. A limited amount of information is available concerning the effect of phenolic acid structure on retention. Because it is considered to be the determinant factor in reverse-phase chromatography, all of the retention-structure relationships have been interpreted in terms of hydrophobicities. Retention-structure effects for phenolic acids have been attributed to three features of the phenolic acid structure, the acidic side chain, the nature of the ring substituents, and the position(s) of the ring substituent(s) on the aromatic moiety. For the cinnamic acids, an additional factor is cis-trans isomerization.

Findings illustrating the effect of the acidic side chain on retention were reported in two communications (22,25). Using an isocratic - C_{18} system, Wulf and Nagel calculated the relative retention ($\alpha = k_2/k_1$) for pairs of cinnamic and benzoic acid derivatives that had the same ring substituents and ring substituent positions (22). Reported α values were as follows: caffeic acid - protocatechuic acid, 4.45; p-coumaric acid-p-hydroxy benzoic acid, 4.24; and o-coumaric acid-salicylic acid, 1.98. As expected, the cinnamic acid derivatives were more strongly retained. Murphy and Stutte observed similar results with a C_{18}-gradient system (25).

Ring substituent-retention relationships for bonded phase chromatography are illustrated by the retention data tabulated

TABLE 3

Comparison of the Performance of Alkyl Phenyl and C_{18} Columns (19)

Compound	t_r (min) phenyl	C_{18}
3,4,5-trihydroxybenzoic acid	3.0	2.4
3,5-dihydroxybenzoic acid	5.5	3.4
2,5-dihydroxybenzoic acid	6.2	4.7
2,4-dihydroxybenzoic acid	8.1	6.1
4-hydroxybenzoic acid	6.7	5.0
3-hydroxybenzoic acid	8.1	5.7
2-hydroxybenzoic acid	8.7	5.2
4-hydroxy-3-methoxybenzoic acid	7.2	5.8

TABLE 4

Representative Retention Data From Studies Using C_{18} Stationary Phases

	Compound		t_r (min)	k´
A (22)	Gallic acid	3,4,5-trihydroxybenzoic acid	2:02	0.49
	Protocatechuic acid	3,4-dihydroxybenzoic acid	2:52	1.10
	p-Hydroxybenzoic acid	4-hydroxybenzoic acid	4:12	2.07
	d-Catechin	3-3',4',5,7-pentahydroxyflavanone	6:12	3.54
	Caffeic acid	3,4-dihydroxycinnamic acid	8:03	4.89
	Salicylic acid	2-hydroxybenzoic acid	9:20	5.83
	Chlorogenic acid	3-caffesylquinate	11:38	7.51
	p-Coumaric acid	4-hydroxycinnamic acid	13:22	8.78
	o-Coumaric acid	2-hydroxycinnamic acid	17:10	11.56
	Ferulic acid	4-hydroxy-3-methoxycinnamic acid	24:14	16.73
	Cinnamic acid		33:03	23.20

B (25)	Gallic acid	3,4,5-trihydroxybenzoic acid	5:31	0.46
	Gentisic acid	2,5-dihydroxybenzoic acid	6:48	0.81
	Protocatechuic acid	3,4-dihydroxybenzoic acid	7:45	1.06
	p-Hydroxybenzoic acid	4-hydroxybenzoic acid	11:41	2.10
	Salicylic acid	2-hydroxybenzoic acid	13:59	2.71
	Vanillic acid	4-hydroxy-3-methoxybenzoic acid	15:35	3.14
	Caffeic acid	3,4-dihydroxycinnamic acid	19:08	4.08
	Syringic acid	4-hydroxy,3,5-dimethoxybenzoic acid	20:54	4.55
	Benzoic acid		23:08	5.14
	p-Coumaric acid	4-hydroxycinnamic acid	28:31	6.57
	Ferulic acid	4-hydroxy-3-methoxycinnamic acid	33:53	8.00
	Sinapic acid	4-hydroxy-3,5-dimethoxycinnamic acid	37:17	8.90
	Cinnamic acid		50:09	12.31

mobile phase: A - water: acetic acid (95:5)

B - butanol: methanol: acetic acid: water (1:5:2:92), 0.018 M ammonium acetate for 10 minutes, then a 20 minute linear gradient to butanol: methanol: acetic acid: water: (2.5:12.5:2:83), 0.018 M ammonium acetate

in Table 4B. Vanillic acid (4-hydroxy-3-methoxybenzoic acid) has a significantly longer retention time than protocatechuic acid (3,4-dihydroxybenzoic acid). The methoxy group of vanillic acid increased the compound's retention relative to protocatechuic acid. Gallic acid (3,4,5-trihydroxybenzoic acid) is retained less than gentisic acid (2,5-dihydroxybenzoic acid). An additional hydroxy group on the gallic acid moiety decreased its retention relative to gentisic acid. In general, hydroxy groups render a molecule more polar and decrease retention while methoxy groups substantially increase hydrophobicity and increase retention.

The substituent position-retention relationship has been elaborated by studies reporting the separation of various mixtures of ortho, meta, and para isomers of hydroxy-benzoic acid (22,25), hydroxy-cinnamic acid (coumaric acid) (22,26), and hydroxyphenyl-acetic acid (35). C_{18} stationary phases were employed in each study. A para-meta-ortho elution sequence was observed, with the ortho isomers eluting last. The elution order suggests that the proximity of the hydroxy substituent and the carboxylic acid group in the ortho isomer decreases the solvation of the molecule and increases retention.

A more subtle structural feature that affects retention is the cis-trans isomerization of the cinnamic acid derivatives. Figure 1 depicts the cis and trans isomers of cinnamic acid. Two communications have reported the separation of cis and trans isomers with reverse-phase chromatography systems (26,31). Caccamese and co-workers indicated that the cis isomers of ferulic and sinapic acids were retained longer than the trans isomers (26). On the other hand, Hartley and Buchan reported that the trans isomers of p-coumaric, ferulic, caffeic, and sinapic acid eluted after the cis isomers (31). C_{18} stationary phases were used in each case. The former study utilized a water-acetic acid-methanol mixture as mobile phase,

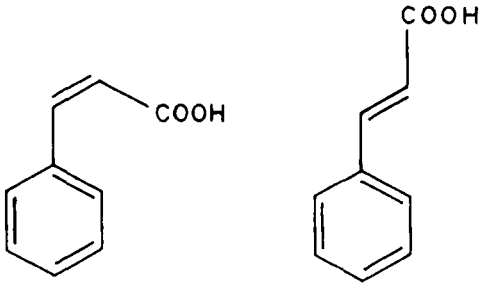


FIGURE 1. cis and trans Isomers of Cinnamic Acid

while the latter employed a mixture of water, acetic acid, and butanol.

Naturally Occurring Esters. Findings concerning the chromatography of naturally occurring phenolic acid esters are important because they extend the analytical capabilities of the liquid chromatographic approach. Most of the work has centered on the esters of benzoic or cinnamic acid derivatives with quinic acid, tartaric acid, or glucose. Structures of the latter three compounds are shown in Figure 2. The ester linkages are considered to consist of the phenolic carboxylic acid group bonded to one of the hydroxy oxygens of glucose, quinic acid, or tartaric acid. Esters of quinic acid and caffeic acid, termed the chlorogenic acids, have been the most thoroughly studied naturally occurring esters. Chlorogenic acid is depicted in Figure 3. The term chlorogenic acid usually refers to the compound with the ester linkage at the 3-hydroxy group of quinic acid. Isomers of chlorogenic acid with ester linkages at the 1,4, and 5-hydroxy groups of quinic acid have also been investigated. The 5-hydroxy quinic acid-caffeic acid ester is frequently termed neochlorogenic acid (1). Esters with more than one caffeic acid molecule per quinic acid moiety are usually referred to as isochlorogenic acid (27).

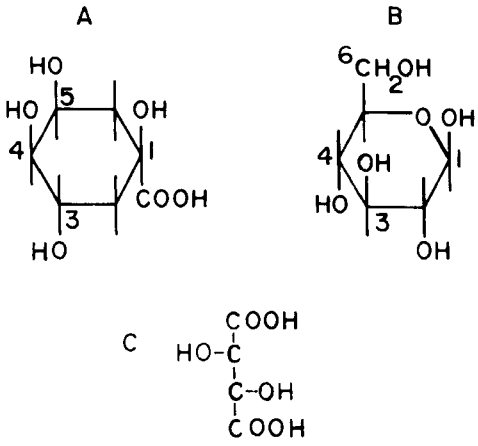


FIGURE 2. Compounds Found as Naturally Occurring Esters with Phenolic Acids: (A) Quinic acid, (B) Glucose, (C) Tartaric acid

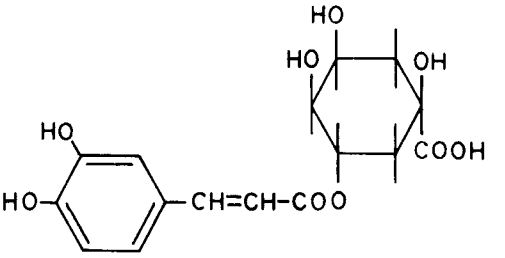


FIGURE 3. Chlorogenic Acid

The most extensive work on the liquid chromatographic separation of natural phenolic acid esters has been completed by Nagels and co-workers (18). Using synthesized esters, they studied mixtures of phenolic acids, glucose-phenolic acid esters, and quinic acid-phenolic acid esters with C_8 and diol bonded-phase systems. Employing a C_8-gradient system, they separated an eleven component mixture containing the quinic acid and glucose esters of caffeic acid, p-coumaric acid, and ferulic acid. Glucose ester linkages were at the 1-hydroxy group. For the

quinic acid esters, the linkages were at the 5-hydroxy group. When the phenolic acid was present in both forms, the glucose ester eluted prior to the quinic acid ester. Unesterified acids were not present in the mixture. The separation of a mixture containing 1,3,4, and 5-hydroxy esters of quinic acid and caffeic acid (chlorogenic acid isomers) with similar C_8-gradient conditions were also reported. A normal phase system comprised of a diol bonded phase and a gradient mobile phase was shown to resolve a fifteen component mixture which included cinnamic acids, benzoic acids, and four phenolic acid-glucose esters. The mixture contained two unesterified acid-glucose ester pairs. In each case, the glucose derivative was retained longer than the unesterified acid. The same system was used to resolve five phenolic acid-quinic acid esters. The elution order of the quinic acid esters observed with the diol bonded phase was inverted relative to the elution order observed with the C_8 stationary phase. A mixture of 5,4, and 3-hydroxy quinic acid-caffeic acid esters was also separated with the diol-gradient system. Again, elution order was inverted relative to the sequence observed with the C_8 stationary phase (classical "normal" vs "reverse" phase behavior).

Additional findings concerning the liquid chromatographic separation of naturally occurring esters were reported by Ong and Nagel (24). Using a C_{18}-gradient system they separated five compounds that had been extracted from grape juice and were subsequently identified as esters of cinnamic acid derivatives. Three of the isolated compounds were phenolic acid-tartaric acid esters. The other two, termed "tri-esters" were found to be esters of tartaric acid, a phenolic acid, and glucose. The "tri-ester" of each cinnamic acid derivative eluted just prior to its tartaric acid ester. Unesterified caffeic acid had longer retention than any of the identified natural esters. The nature of the "tri-ester" linkages were

not elucidated, however, it was found that their retention was markedly dependent on a mobile phase pH change from 2.3 to 3.4. An identical pH-retention dependence was observed for the phenolic acid-tartaric acid esters. It was suggested that the pH dependence in the 2.3 to 3.4 range was probably due to the ionization of the tartaric acid carboxylic acid groups,suggesting that at least one of the carboxylic acid groups of tartaric acid was not bound in the "tri-ester."

An additional communication reported the separation of a mixture of chlorogenic acids isomers that had been isolated from sweet potatoes. Using a C_{18}-isocratic system Walter and co-workers observed that the isochlorogenic acid isomers were retained longer than 3 and 4-hydroxy quinic acid-caffeic acid esters (27).

DETECTION

Ultraviolet absorption has been the most popular detection mode for the liquid chromatographic determination of phenolic acids. Wavelengths utilized include (nm's): 235 (12), 254 (12, 16,19,20,22,25,29,30,32,33,35,36), 270 (11), 275 (31), 280 (10, 16,18,22,30), 290 (34), 303 (13), 313 (27), 320 (21,24), 350 (23), and 360 (36). Minimal information pertaining to optimum wavelength selection has been reported, however, the ultraviolet spectral properties of benzoic and cinnamic acid derivatives have been described (1). A limited number of studies have employed electrochemical detection (14,15,17,37), however, liquid chromatography with electrochemical detection (LCEC) possesses several characteristics which render it ideal for phenolic acid determinations. Among the most important factors are good selectivity, favoring easily oxidized phenols, and superior detection limits (often a hundredfold lower than for UV absorption).

Electrochemical detection can be understood by considering the hydrodynamic voltammogram (detector potential-current plot) shown in Figure 4. As the detector potential is increased in the positive direction the oxidation of electroactive eluant A commences. At a sufficiently positive potential the current response becomes independent of the detector potential, indicating that the current is limited by the mass transport of the electroactive species to the electrode surface. The potential where the current response is one-half of its diffusion limited value is termed the $E_{1/2}$. For the electrochemical detection of compound A, the detector potential is poised at or near the potential where the current response is diffusion limited. A detector response results when the chromatographic zone of compound A passes through the detector, producing an instantaneous exchange of electrons due to the oxidation of A molecules. The magnitude of the recorded amperometric response is proportional to the number of molecules

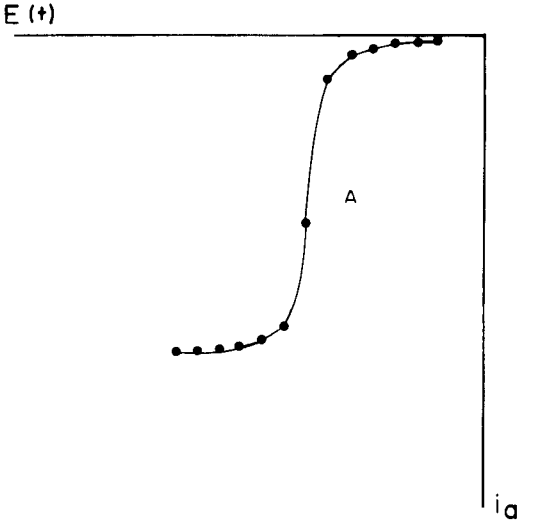


FIGURE 4. Hydrodynamic Voltammogram for Oxidizable Analyte (A).

eluting over the detector-electrode. A more extensive description of the principles of electrochemical detection are given elsewhere (38).

An important advantage of LCEC is the low detection limits achieved for many determinations. Typical detection limits are in the picomole range (38). The marked sensitivity of LCEC experiments is achieved with detectors of the design shown in Figure 5. The detector housing consists of a thin teflon gasket sandwiched between two blocks of Kel-F. The working electrode is positioned in the lower block. Typical electrode materials are glassy carbon and carbon paste (38). Reference and auxiliary electrodes necessary for implementing potential control are also

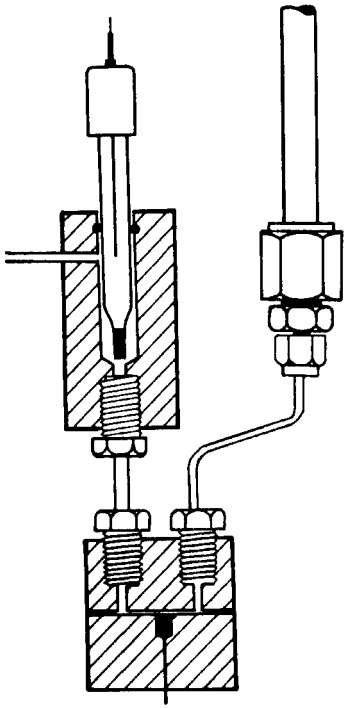


FIGURE 5. Thin-Layer Amperometric Detector Cell.

housed in the detector assembly. A detailed description of the detector cell and instrumentation requirements has been reported elsewhere (39). An important consideration for electrochemical detection is that it is not compatible with all types of chromatography. Amperometric detection requires the presence of electrolyte in the mobile phase. Most LCEC experiments are performed with buffer salt concentrations of ~0.05 M. Therefore, electrochemical detection is most compatible with reverse-phase chromatography (including ion-pairing and ion-exchange). Direct electrochemical detection is not practical with normal phase chromatography because of the low dielectric constants of the mobile phases employed.

Most phenolic acids are electroactive and can be determined by oxidative LCEC. Electrochemical oxidations of phenols can be quite complex. The oxidation mechanism is dependent on several factors including solvent composition, pH, electrode potential, and phenol structure. A general, complete mechanism for the electrochemical oxidation of phenols has not been put fourth, however, evidence points to the existence of two primary oxidation products: a phenoxy cation and a phenoxy radical. Either product can react to form secondary products that are also electroactive.

For LCEC determinations, the most important consideration is the detector potential required for the oxidation of the phenolic acid(s) of interest. A wide range of $E_{1/2}$ values has been reported (40). The ease of oxidation of the phenolic acid will affect the selectivity and detection limits of the assay. When more extreme detector potentials are employed, more compounds are oxidized, resulting in more complex chromatograms. In addition, as the detector potential is increased, the background current and noise are increased, raising the detection limits. In general, the ease of oxidation of a phenolic acid

can be predicted from the ring substitution. Groups capable of donating electron density increase the ease of oxidation while electron withdrawing groups have the opposite effect. Hence, di- and tri-hydroxy substituted phenols are easier to oxidize than mono phenols. Figure 6 depicts the relative oxidation potentials for hydroxy and methoxy substituted phenolic acids.

APPLICATIONS AND SAMPLE PREPARATION

Table 5 provides an overview of the use of liquid chromatography for the determination of phenolic acids in a variety of samples. Listed in Table 5 are the investigated materials and identified phenolic acid constituents reported in several studies. Plant materials, food, and beverages of vegetable origin dominate the list of studied samples. A critical aspect of the analysis of "real" samples is the allied preparation procedure. A diversity of procedures have been reported as sample preparation prior to the liquid chromatographic separation of extracted sample components. In each case, the preparation procedure was tailored to the investigation at hand. Sample preparation for the determination of phenolic acids usually consists of several steps which can be classified into one of three categories: homogenization, preliminary clean-up, and hydrolysis. Important aspects of sample

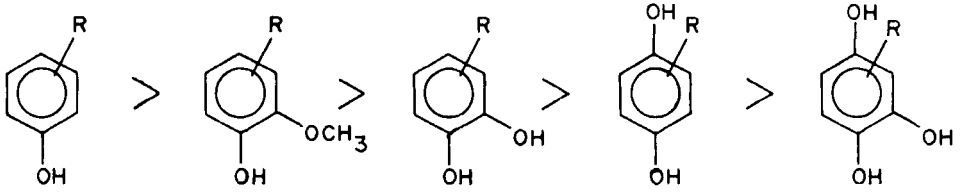


FIGURE 6. Effect of Ring Substitution on the Oxidation Potentials of Phenolic Acids.

TABLE 5

LC Applications for the Determination of Phenolic Acids

Sample	Compounds Identified	Reference
cacao beans	Vanillic acid	15
	Protocatechuic acid	
	Ferulic acid	
	2-Hydroxyphenylacetic acid	
	p-Coumaric acid	
	Caffeic acid	
beer, wine	Vanillic acid	16
	Ferulic acid	
	p-Coumaric acid	
	Caffeic acid	
	Gallic acid	
sunflower meal	Chlorogenic acid	17
tobacco leaf tissue	Neochlorogenic acid	23
	Chlorogenic acid	
	4-O-Caffeoylquinic acid	
	Caffeic acid	
soybean leaf tissue	Gallic acid	25
	Protocatechuic acid	
	p-Hydroxybenzoic acid	
	Salicylic acid	
	Vanillic acid	
	Caffeic acid	
	p-Coumaric acid	
	Ferulic acid	

preparation for the liquid chromatographic determination of phenolic acids are discussed below.

Preliminary Clean-up. Preliminary clean-up consisted of a series of extractions. Two extraction schemes are generally employed. In several studies, the homogenized sample was subjected to an extensive extraction with a relatively polar organic solvent such as chloroform (15) or petroleum ether (17,23) to remove lipid materials and other neutral compounds prior to the analytical extraction. After the clean-up extraction, the analytical extraction was performed on the "de-fatted" sample with organic solvents such as ethanol (17), ethylacetate (15), and 80% aqueous methanol (23). The second scheme involves the performance of a preliminary liquid-solid extraction on the sample followed by additional liquid-liquid extractions on the original extract. Solvents employed for initial liquid-solid extractions were 2% acetic acid in boiling water (25), ethanol (27,34), dilute sodium hydroxide (31), and 2M HCl (32). A related approach consisted of a preliminary liquid-solid extraction with subsequent clean-up by preparative liquid chromatography (10,24). Silica gel (10), and a polyamide material (24) were utilized as preparative stationary phases.

Hydrolysis. An important preparative step for the determination of phenolic acids with liquid chromatography is acid or base hydrolysis. Hydrolysis serves to cleave the ester linkages of phenolic acids bound in naturally occurring esters. Therefore, concentrations determined after the use of hydrolysis conditions are representative of bound and unbound phenolic acid content. Hydrolysis is usually achieved by subjecting the extracted materials to elevated temperatures at high or low pH. More explicit descriptions of hydrolysis conditions for the determination of phenolic acids have been published elsewhere

(41). Four of the reviewed communications reported the use of hydrolysis conditions for phenolic acid determinations (24,25, 31,32). In two of the studies, hydrolysis conditions were employed to affect preliminary extractions of phenolic acids from plant materials (31,32). In the other two investigations, hydrolysis was performed on previously extracted materials (24,25). An important factor (which has too often been ignored) is that many phenolic compounds are destroyed in a base hydrolysis step, especially those with low oxidation potentials, due to oxidation by dissolved oxygen. This fact can seriously reduce recovery and also contribute new products to the sample mixture.

REFERENCES

1. Ribereau-Gayon, P., Plant Phenolics, Hafner Publishing Co., New York, 1972, Chpt 4.

2. Geissman, T.A. and Crout, D.H.G., Organic Chemistry of Secondary Plant Metabolism, Freeman, Cooper and Co., San Francisco, 1969, Chpt. 5.

3. Towers, G.H.N., Biochemistry of Phenolic Compounds, Harborne, J.B., eds., Academic Press, New York, 1964, Chpt. 7.

4. Maga, J.A., Simple Phenols and Phenolic Compounds in Food Flavor, CRC Critical Reviews in Food Science and Nutrition, 10, 323, 1978.

5. Harborne, J.B., Phytochemical Methods, Chapman and Hall, London, 1973, Chpt. 2.

6. Seikel, M.K., Biochemistry of Phenolic Compounds, Harborne, J.B., eds., Academic Press, New York, 1964, Chpt. 2.

7. Steele, J.W. and Bolan, M., Phytochemistry of the Salicaceae, III. A Gas-Liquid Chromatographic Procedure for the Identification of Plant Phenols, J. Chromatogr., 71, 427, 1972.

8. Vande Casteele, K., DePooter, H., and Van Sumere, C.F., Gas Chromatographic Separation and Analysis of Trimethylsilyl Derivatives of Some Naturally Occurring Non-Volatile Phenolic Compounds and Related Substances, J. Chromatogr., 121, 49, 1976.

9. Schulz, J.M. and Herrmann, K., Analysis of Hydroxybenzoic and Hydroxycinnamic Acids in Plant Material, II. Determination by Gas-Liquid Chromatography, J. Chromatogr., 195, 95, 1980.

10. Nagels, L. and Parmentier, F., Kinetic Study of Possible Intermediates in the Biosynthesis of Chlorogenic Acid in Cestrum Poeppigii, Phytochem. 15, 703, 1976.

11. Hovermann, W., Rapp, A., and Ziegler, A., A Method for Separating Phenolic Acids by High Pressure Liquid Chromatography, Chromatographia, 6, 317, 1973.

12. Terweij-Groen, C.P. and Kraak, J.C., Ion-Pair Phase Systems for the Separation of Carboxylic Acids, Sulphonic Acids, and Phenols by High-Pressure Liquid Chromatography, J. Chromatogr., 138, 245, 1977.

13. Kramer, U. and Kishan, B., Determination of Trace Phenol and Isomeric Hydroxybenzoic Acids in Aqueous Benzoic Acid Solutions by Aqueous Liquid Chromatography, J. Chromatogr., 89, 348, 1974.

14. Kenyhercz, T.M. and Kissinger, P.T., A New Approach to the Phenolic Components in Beer. Application to the Determination of Sinapic, Ferulic, and p-Coumaric Acids, J. Agric. Food Chem., 25, 959, 1977.

15. Kenyhercz, T.M. and Kissinger, P.T., Determination of Selected Acidic, Neutral, and Basic Natural Products in Cacao Beans and Processed Cocoa. Liquid Chromatography with Electrochemical Detection, Lloydia, 41, 130, 1978.

16. Charalambous, G., Bruckner, K.J., Hardwick, W.A., and Linnebach, A., Determination of Beer Flavor Compounds by High Pressure Liquid Chromatography, Tech. Quart., Master Brew. Ass. Amer., 10, 74, 1973.

17. Felice, L.J., King, W.P., and Kissinger, P.T., A New Liquid Chromatography Approach to Plant Phenolics. Application to the Determination of Chlorogenic Acid in Sunflower Meal, J. Agric. Food Chem., 24, 380, 1976.

18. Nagels, L., Van Dongen, W., DeBrucker, J., and DePooter, H., High-Performance Liquid Chromatographic Separation of Naturally Occurring Esters of Phenolic Acids, J. Chromatogr., 187, 181, 1980.

19. Scriven, F.M., Day, W.R., and Wills, R.B.H., Analysis of Hydroxybenzoic Acids by High Pressure Liquid Chromatography, J. Liq. Chromatogr., 2, 125, 1978.

20. Vanhaelen, M. and Vanhaelen-Fastre, R., High-Performance Liquid, Gas-Liquid, and Thin-Layer Chromatography of Naturally Occurring Flavonoids, Phenolic, and Related Compounds, J. Chromatogr., 187, 225, 1980.

21. Grodzinska-Zachweija, Z., Bieganowska, M., and Dizdo, T., Effect of Mobile Phase Composition on the Partition of Phenolic Acids in Reversed-Phase TLC and HPLC, Chromatographia, 12, 555, 1979.

22. Wulf, L.W. and Nagel, C.W., Analysis of Phenolic Acids and Flavonoids by High-Pressure Liquid Chromatography, J. Chromatogr., 116, 271, 1976.

23. Court, W.A., High-Performance Reversed-Phase Liquid Chromatography of Naturally Occurring Phenolic Compounds, J. Chromatogr., 130, 287, 1977.

24. Ong, B.Y. and Nagel, C.W., High-Pressure Liquid Chromatographic Analysis of Hydroxycinnamic Acid-Tartaric Acid Esters and Their Glucose Esters in Vitris Vinifera, J. Chromatogr., 157, 345, 1978.

25. Murphy, J.B. and Stutte, C.A., Analysis for Substituted Benzoic and Cinnamic Acids Using High-Pressure Liquid Chromatography, Anal. Biochem., 86, 220, 1978.

26. Caccamese, S., Azzolina, R., and Davino, M., Separation of cis and trans Isomers of Naturally Occurring Hydroxycinnamic Acids by High-Pressure Liquid Chromatography, Chromatographia, 12, 545, 1979.

27. Walter, Jr., W.M., Purcell, A.E., and McCollum, G.K., Use of High-Pressure Liquid Chromatography for Analysis of Sweet Potato Phenolics, J. Agric. Food Chem., 27, 938, 1979.

28. Walter, Jr., W.M. and Purcell, A.E., Evaluation of Several Methods for Analysis of Sweet Potato Phenolics, J. Agric. Food Chem., 27, 942, 1979.

29. Price, Jr., W.P., Edens, R., Hendrix, D.L., and Deming, S.N., Optimized Reverse-Phase High-Performance Liquid Chromatographic Separation of Cinnamic Acids and Related Compounds, Anal. Biochem., 93, 233, 1979.

30. Qureshi, A.A., Prentice, N., and Burger, W.C., Separation of Potential Flavoring Compounds by High-Performance Liquid Chromatography, 170, 343, 1979.

31. Hartley, R.D. and Buchan, H., High-Performance Liquid Chromatography of Phenolic Acids and Aldehydes Derived From Plants or From the Decomposition of Organic Matter in Soil, J. Chromatogr., 180, 139, 1979.

32. Charpentier, B.A. and Cowles, J.R., Rapid Method of Analyzing Phenolic Compounds in Pinus elliotti Using High-Performance Liquid Chromatography, J. Chromatogr., 208, 132, 1981.

33. Hardin, J.M. and Stutte, C.A., Analyses of Phenolic and Flavonoid Compounds by High-Pressure Liquid Chromatography, Anal. Biochem., 102, 171, 1980.

34. Davino, M., Catara, A., Caccamese, S., and Perrotta, G., Analysis of Post Infectional Phenolic Acids in Lemons Inoculated with Phoma Tracheiphila by High-Pressure Liquid Chromatography, Rivista di Patologia Vegetale, S. IV, 15, 35, 1979.

35. Warthen, Jr., J.D. and Mandava, N., Separation of Isomeric (Hydroxyphenyl) Acetic Acids by Reversed-Phase High-Performance Liquid Chromatography, J. Chromatogr., 144, 263, 1977.

36. Niemann, G.J., Flavonoids and Related Compounds in Leaves of Pinaceae, III* Pinus jeffreyi, Z. Naturforsch, 33c, 777, 1978.

37. Roston, D.A. and Kissinger, P.T., Identification of Phenolic Constituents in Commercial Beverages Using Liquid Chromatography with Electrochemical Detection, Anal. Chem, in press.

38. Kissinger, P.T., Amperometric and Coulometric Detectors for High-Performance Liquid-Chromatography, Anal. Chem., 49, 447A, 1977.

39. Kissinger, P.T., Bruntlett, C.S., Bratin, K., and Rice, J.R., Trace Organic Analysis, National Bureau of Standards Special Publication No. 519.

40. Felice, L.J. and Kissinger, P.T., Determination of Homovanillic Acid in Urine by Liquid Chromatography with Electrochemical Detection, Anal. Chem., 48, 794, (1976).

41. Ribereau-Gayon, P., Plant Phenolics, Hafner Publishing Co., New York, 1972, Chpt. 3.