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A Gas–Liquid Chromatographic Method for Analysis of Phenolic Acids in Plants

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A GLC–MS method was developed to analyze phenolic acid extracts from plant material. Extracted acids were converted to their methyl esters by refluxing in 3 M hydrogen chloride in methanol, and the esters were reacted with bis(trimethylsilyl)trifluoracetamide plus 10% trimethylchlorosilane to silylate the phenolic groups. Derivatives of standard *p*-hydroxybenzoic, gallic, ferulic, sinapic, caffeic, *p*-coumaric, gentistic, and protocatechuic acids prepared by this procedure were analyzed by GLC on 3.75 m × 0.032 cm columns of 3% SE-30 and OV-17 on 100–120-mesh Chromosorb W. GLC–MS analysis of extracts from sphagnum moss, instant tea, and Stuart pecan kernels revealed the presence of *p*-hydroxybenzoic, coumaric, and protocatechuic acids, coumaric, gallic, and caffeic acids, and *p*-hydroxybenzoic, vanillic, coumaric, protocatechuic, syringic, gallic, gentistic, and (*p*-hydroxyphenyl)acetic acids, respectively.

Numerous methods have been reported for determining microquantities of phenolic acids in plants and have involved separations by paper chromatography, thin-layer chromatography (TLC), column chromatography (Geissman, 1962; Ribêreau-Gayon, 1972), or gas–liquid chromatography (GLC). Except when analysis is by GLC, qualitation of naturally occurring phenolics is usually based on TLC or paper chromatographic separations, followed by determination of absorption maxima in the UV region, identification of their colored complexes subjectively, and/or determination of R_f values. These procedures are not entirely satisfactory because of time requirements, resolving power, and lack of adaptability to quantitative procedures. Phenolic acids have been analyzed by GLC as their trimethylsilyl ether/esters (Chapman et al., 1970; Morita, 1972). However, investigators have reported difficulties in silylating dihydroxyphenolic acids (Karlson and Svendsen, 1965; Blakely, 1966). Phenolic acids have also been analyzed by GLC as their methyl ether/methyl esters (Erickson et al., 1973); however, several phenolic acids with similar methoxyl groups yield identical derivatives when completely methylated. Salomonsson et al. (1978) described a GLC technique for analyzing phenolic acids as their ethyl ether/ethyl esters.

By the GLC method we now report, the inadequacies of some of the preceeding methods have been eliminated by improved derivatization and analytical procedures, and analyses of phenolic acid isolates from natural products are now both rapid and quantitative.

EXPERIMENTAL SECTION

Reference Compounds. *p*-Hydroxybenzoic, vanillic, gallic, ferulic, sinapic, caffeic, *p*-coumaric, syringic, gentisic, protocatechuic, and (*p*-hydroxyphenyl)acetic acids were obtained from commercial sources. All reference acids and some of their corresponding methyl esters were analyzed by mass spectrometry for purity and spectral characteristics by means of the direct insertion probe. Standard mixtures of the trimethylsilyl ether/esters of these acids were prepared by the following procedure. One-tenth milligram of each acid was dissolved in 5 mL of 3 M hydrogen chloride in methanol; this mixture was divided into duplicate 2.5-mL aliquots, placed in screw-capped test tubes, and refluxed for 0.5, h for esterification. Hydrogen chloride and methanol were partially removed under vacuum (10 torr), and 0.2 mL of methylene chloride was added to the residue. Evaporation was repeated until the mixture was free of hydrogen chloride odor. The residue was then dissolved in 2 mL of redistilled acetonitrile, 0.1 mL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) plus 10% trimethylchlorosilane (TMCS) (Regis Chemical Co.) was added, and the mixture was allowed to stand for 15 min. After silylation was accomplished, the solution was concentrated to 0.5 mL with a stream of purified N_2 for GLC analysis.

GLC–MS Analysis. GLC analyses were made with a 3.75 m × 0.32 cm glass column packed with 3% SE-30 on 100–120-mesh Chromosorb W. The oven temperature was programmed from 120 to 225 °C at 4 °C/min, and the helium flow was 25 mL/min. Additional analyses were made with a 3.75 m × 0.32 cm glass column packed with 3% OV-17 on 100–120-mesh Chromosorb W. The OV-17 column was programmed from 130 to 225 °C at 3 °C/min, and the helium flow was 28 mL/min. The injector and detector were maintained at 250 °C. Both columns were used a Perkin-Elmer Model 900 gas chromatograph that was equipped with a flame ionization detector and was connected to a Du Pont 21-490B mass spectrometer equipped with differential pumping on the analyzer section (Senter and Horvat, 1976). Phenolic acid derivatives for

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MS analyses were separated with the 3% SE-30 column by using GLC conditions as described. MS conditions were as follows: ion source pressure, 4×10^{-6} torr; source temperature, 200 °C: ionizing voltage, 70 eV; scan rate, 10 s/decade from 15 to 500 amu.

Plant Materials. Sphagnum moss, pecan kernels, and instant tea were selected as representative materials from a low lipid, a high lipid, and a processed plant material, respectively. In the case of the high lipid product, extraction with petroleum ether was required prior to phenolic acid analyses. Phenolic acids were extracted from the samples by refluxing 5 g of plant material for 2 h in 50 mL of methanol containing 0.1% concentrated HCl. After filtration, the filtrate was taken to dryness under vacuum with a rotary evaporator; the residue was resuspended in 1 M hydrochloric acid, refluxed for 1 h, cooled, and then filtered. The hydrolyzed filtrate was centrifuged, and the supernatant was extracted with ethyl acetate (3 × 20 mL). The extract was then shaken with 5% aqueous sodium bicarbonate (3 × 20 mL) which, after acidification to pH 3.5 with 1 M hydrochloric acid, was extracted with ethyl acetate (2 × 25 mL). Residual moisture was removed from the ethyl acetate extract with anhydrous sodium sulfate. The extract was then taken to dryness under vacuum (10 torr) to yield the phenolic acid fraction.

The extracted acids were then taken up in 5 mL of 3 M hydrogen chloride in methanol and refluxed for 0.5 h for esterification. The reaction mixture was evaporated, and silylation was performed using the technique described above for Reference Compounds with the exception that the volume of acetonitrile was decreased to 1.8 mL and the volume of BSTFA plus TMCS was increased to 0.2 mL. Aliquots from this reaction were used for GLC analysis.

Standard curves relating quantities of phenolic acids to GLC peak areas of the silyl ether/esters were prepared from authentic compounds. Peak areas were determined with an Infotronics CRS 204 integrator and a linear relationship was found in all the phenolic acids investigated within a range of 0–2 μg.

RESULTS AND DISCUSSION

Quantitative extraction of phenolic compounds from plant tissue is difficult. However, Ribéreau-Gayon (1972) found that "in successive extractions of plant material the relative amounts extractable in each fraction are reasonably constant; therefore, it is possible to obtain information about the relative amounts in plant tissue even though they have not been extracted completely from the tissue". These observations were confirmed in the present study when duplicate samples of the plant materials were subjected to three successive 2-h extractions with 0.1% HCl in methanol. The extracts contained ca. 65, 24, and 11% of the extractable phenols, respectively, and repeatability of extraction for the duplicate samples was excellent. Similar results were obtained for all three types of samples.

More efficient and repeatable results were obtained from methanolic–HCl extractions of the plant materials than with other means of extraction such as 80% ethanol and direct hydrolysis of ethanol extracted plant residues (Geissman, 1962; Guenzi and McCalla, 1966; Salomonsson et al., 1978).

Acid hydrolysis of the ester and glycosidic bonds was preferred to yield the di- and trihydroxyphenolic aglycones of the indigenous acids. Alkaline hydrolysis tended to destroy these compounds with caffeic and gallic acid being reduced by 66 and 45%, respectively, in instant tea; recovery of gallic acid was reduced by 69% in pecan. Protocatechuic acid reacted in a similar fashion. Alkaline hydrolysis of methanolic–HCl extracts from sphagnum moss resulted in a 77% reduction in the quantity of this acid recovered. However, alkaline hydrolysis was preferred for analysis of *p*-coumaric acid (Table II). Basic hydrolysis of extracts from sphagnum moss and instant tea yielded ~1.5 times as much coumaric acid as acid hydrolysis.

In the present study, efficiency of partitioning the phenolic acids from the aqueous hydrolysate was enhanced by the use of ethyl acetate rather than ethyl ether. A 5% solution of sodium bicarbonate effectively removed the acids from the ethyl acetate, as evidenced by their absence in GLC–MS analyses of the residue. Derivatization of the isolated acids offered little trouble in repeatability and stability of the derivatized compounds, the main requirements of this procedure being the absence of moisture during and after derivatization and the conversion of the acids to their respective methyl esters prior to forming the trimethylsilyl ether. The trimethylsilyl ether/ester derivatives were stable, showing no signs of decomposition even when kept in closed vials for as long as 2 months.

Efficiency of esterification of the phenolic acids was determined by reacting 2 mg of *p*-hydroxybenzoic, protocatechuic, gallic, *p*-coumaric, ferulic, and caffeic acid individually with 2.5 mL of 3 M hydrogen chloride in methanol in capped vials for 1 h at 110 °C. Reactions were run in duplicate. Known volumes (0.2 mL) of the reaction mixture were removed at 15-min intervals, the methanol–hydrogen chloride was distilled off at 10–20 torr, and the sample was redissolved in methanol for analysis by high-performance liquid chromatography (LC) by using a Waters Associates Bondapak C_{18} column. The reactants were eluted from the column with 30–70% methanol in water plus 1% acetic acid. The flow rate was 1.5 mL/min and the column was maintained at room temperature. Efficiency of conversion was determined by plotting ratio of peaks heights of esters to peak heights of acids against concentration of acids. The same reaction products were further analyzed by mass spectrometry. Sufficient solution to yield 2 μg of solid was taken up in a capillary tube, the methanol was carefully evaporated, and the sample was then analyzed by direct insertion probe. MS conditions were as follows: ion source pressure, 4×10^{-6} torr; source temperature, 200 °C; ionizing voltage, 70 eV; scan rate, 100 s/decade from 15 to 500 amu; probe temperature, 250 °C. The ion current due to the methyl ester parent ion as a function of time was determined, and standard curves were prepared by plotting area under the ion current curve against percentage of ester in the standard mixtures of methyl ester and phenolic acid. Esterification of phenolic acids with 3 M hydrogen chloride in methanol at 110 °C was complete in 15 min. High-performance LC and MS analyses indicated an average conversion of 98 ± 1 and 97 ± 2%, respectively, and is in agreement with data previously reported by McFadden (1973).

Percent conversion of phenolic acid methyl esters into trimethylsilyl ethers was determined by analyzing the reaction products from preparations of various methyl esters and silylating reagents with mass spectrometry. The ether/esters were prepared by dissolving 2 mg of phenolic acid methyl ester in 1.5 mL of redistilled acetonitrile, adding 0.1 mL of BSTFA plus TMCS, and then thoroughly agitating the mixture. A 0.2-mL volume was taken, and solvent and silylating agent were distilled from the derivative at 10–20 torr and 40 °C. A small quantity (20–50 μg) of the remaining oil was drawn into a capillary tube, the tube was placed into the direct insertion probe, and the sample was volatized into the ion source at 150–175 °C. Repeated MS scans were made while the temperature was increased to 250 °C. The MS was operated at max-

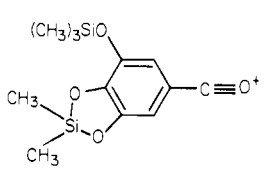
Table I. Prominent Ions in Mass Spectra of Trimethylsilyl Derivatives of Phenolic Acid Methyl Esters

acid	m/e and rel abundance of prominent ions					
p-hydroxy-benzoic	73[a]	75	125	193	209	224 (M)
	0.20[b]	0.80	0.24	0.26	1.00	0.77
vanillic	73	75	193	223	239	254 (M)
	0.35	0.16	0.43	1.00	0.45	0.38
gentisic	73	75	193	281	297	312 (M)
	0.35	0.16	0.05	0.05	1.00	0.17
proto-catechuic	73	75	193	281	297	312 (M)
	0.50	0.11	1.00	0.08	0.05	0.29
syringic	73	75	253	269	284 (M)	
	0.76	0.70	1.00	0.80	0.70	
p-coumaric	73	75	219	235	250 (M)	
	0.51	0.40	0.33	0.62	1.00	
gallic	73	75	281	369	385	400 (M)
	0.41	0.13	1.00	0.06	0.08	0.54
caffeic	73	75	219	307	323	338 (M)
	0.75	0.12	1.00	0.07	0.03	0.42
sinapic	73	75	279	295	310 (M)	
	0.55	0.25	0.40	0.25	1.00	
ferulic	73	75	249	265	280 (M)	
	0.67	0.88	1.0	0.33	0.89	

[a] m/e. [b] Relative abundance.

imum sensitivity with the electron multipler at 2800 V. Percent conversion of the phenolic acid methyl esters to trisilyl ether/esters was quantitative as determined by these analyses. The parent ion of the methylesters of p-hydroxybenzoic, gallic, caffeic, protocatechuic, p-coumaric, and ferulic acids was completely absent in the mass spectra when the spectrometer was operated at its maximum sensitivity. In all cases, the mass spectrum of the silyl ether/ester from probe analyses was consistent with that obtained by GLC–MS. Standard curves relating quantities of acids with peak areas of silyl ether/esters were linear in the weight range investigated.

The spectra from GLC–MS analysis of the trimethylsilyl ether/methyl esters of the reference phenolic acids were similar. As shown in Table I, there were prominent ions at m/e 73, 75, M, M – 15, and M – 31. All the phenolic acids investigated had fairly prominent molecular ions, M, and m/e agreed with the molecular weights calculated. The base peak was generally M – 15 for the esters containing monotrimethylsilyl ether groups. Spectra of those esters with two adjacent trimethylsilyl groups (derivatives of o-dihydroxyphenolic acids) showed base peaks at M – (31 + 88). These ions could be represented as having a cyclic moiety due to loss of the methoxide and tetramethylsilane. For the trimethylsilyl ether/methyl ester of gallic acid, the structure of the base peak could be represented as



Similar cyclic structures could be drawn for the base peaks of derivatized caffeic (m/e 219) and protocatechuic (m/e 193) acids. Morita (1972) postulated a cyclic structure for the m/e 193 base peak from the spectra of trimethylsilyl ether/trimethylsilyl ester of protocatechuic acid. These intense base peaks could be useful in identifying these compounds in complex natural mixtures. Also, protocatechuic acid and other o-dihydroxybenzoic acids could

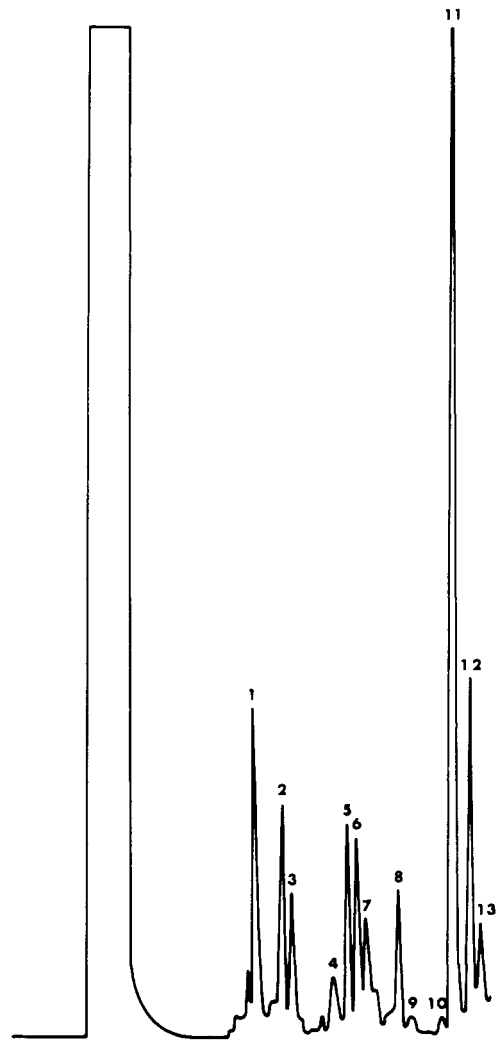


Figure 1. GLC of trimethylsilyl ether/methyl esters of phenolic acids from pecan kernels on a 3% OV-17 packed column. Peaks 1, 3, 5, 6, 8, 9, 10, and 11 are p-hydroxybenzoic, (p-hydroxyphenyl)acetic, vanillic, gentisic, protocatechuic, coumaric, syringic, and gallic acids, respectively.

be readily distinguished from other isomeric dihydroxybenzoic acids.

Phenolic acids in pecans kernels, instant tea, and sphagnum moss were determined by the analytical procedure herein described. Table II lists these acids and the amounts present in these types of plant materials. Those acids identified in instant tea have been previously reported as constituents of epicatechin gallate, theogallin, p-coumarylquinic acid, chlorogenic acid, and neochlorogenic acid (Geissman, 1962). Figure 1 shows a chromatogram of the trimethylsilyl ether/methyl esters of phenolic acids from pecan kernels on a 3% OV-17 packed column. Peaks 1, 3, 5, 6, 7, 9, 10, and 11 are due to p-hydroxybenzoic, (p-hydroxyphenyl)acetic, vanillic, gentisic, protocatechuic, coumaric, syringic, and gallic acids, respectively. Although 13 compounds are represented in Figure 1 and were repeatedly present in the derivatized samples, compounds corresponding to peaks 2, 4, 9, 12, and 13 did not give the typical mass spectral patterns of derivatized phenolic acids and were apparently solvent impurities. Compounds 12 and 13 were identified as phthalates. Our research revealed the presence of protocatechuic, p-coumaric, and p-hydroxybenzoic acids in sphagnum moss and confirms the p-hydroxybenzoyl grouping previously reported by Lindberg and Theander (1952). No attempt was made to identify the cis isomers of the substituted

Table II. Content of Phenolic Acids of Some Plant Material

acid	content, μg/g Stuart pecans	instant tea	sphagnum moss
gallic	132.8[a] (91.6)[b]	640.4 (422.6)	
gentisic	7.7		
vanillic	6.0		
protocatechuic	7.2 (5.4)		66.5 (51.2)
p-hydroxybenzoic	14.8		28
(*p*-hydroxyphenyl)-acetic	2.9		
coumaric	tr	24.5 (36.9)	31.5 (47.2)
syringic	tr		
caffeic		255 (114.7)	
Σ phenolic acids, %	0.0034	0.018	0.0025

[a] Results are given in micrograms per gram of dry plant material and are the means of triplicate analyses (pecan samples were also defatted). The sum of the identified phenolic acids are given as percent of dry weight of plant materials. [b] Values in parentheses were obtained from 4-h hydrolysis with 2 M aqueous NaOH.

cinnamic acids in the plant materials analyzed.

Due to its simplicity, reproducibility of reactions, and stability of the derivatives formed, the procedure herein reported for the GLC–MS analysis of plant phenolic acids appears to be adequate for both qualitative and quantitative analysis.

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Conversion of Parathion to Paraoxon on Soil Dusts As Related to Atmospheric Oxidants at Three California Locations

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Parathion-treated soil dusts were exposed outdoors near air-quality monitoring stations at three California locations. Results indicated that paraoxon production from parathion was controlled by atmospheric ozone concentration and type of soil dust. The ratio of paraoxon to parathion after 24 h ranged from an average of 0.117 with Mocho dust at a coastal site to 0.943 with Hanford dust at an inland site. The correlation coefficient (r) between daily paraoxon production and daily peak ozone was 0.776, 0.701, and 0.773 for San Joaquin, Hanford, and Mocho dusts. Adding the additional variables of maximum temperature and minimum relative humidity only slightly improved correlations. In areas with relatively high ozone levels, the daily average, highest hourly average, and peak ozone concentration were equally good indicators of paraoxon formation rate. Neither total radiation nor UV was correlated with paraoxon production or with atmospheric ozone. Both parathion and paraoxon volatilized rapidly when no soil dust particles were present.

Exposure of agricultural workers to paraoxon residues has been shown to be responsible for the infrequent episodes of worker illnesses following parathion application to citrus in California (Spear et al., 1977a,b). Paraoxon, the highly toxic alteration product of parathion, is carried on dust particles originating from the soil or leaf surface to the workers' clothing or skin, from which it is dermally adsorbed. Under field conditions in the Central Valley of California, parathion persists for long periods on dry soil and can be oxidized to the highly toxic paraoxon at the soil surface (Spencer et al., 1975). Dislodgeable residues on foliage are predominately associated with dust particles (Popendorf et al., 1975). Popendorf and Leffingwell (1978), who reported on natural variations in the decay and oxidation of parathion foliar residues in 17 central California orange groves, found that most of the parathion was converted to paraoxon during the first 2–4 days after application and that residue oxidation and decay were correlated strongly with dry, stable weather conditions. Adams et al. (1976, 1977) reported that dust can influence the oxidation of parathion to paraoxon and stabilize adsorbed paraoxon.

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