Phenolic and Sugar Components of Armavirec Variety Sunflower (*Helianthus annuus*) Seed Meal

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Caffeic (3,4-dihydroxycinnamic) acid, chlorogenic (3-caffeoylquinic) acid, and 3,5-dicaffeoylquinic acid have been identified in the aqueous methanol extract of defatted seed meal of sunflowers (Armavirec variety). Another disubstituted cinnamic acid has been isolated in the free state and also as a monoester of quinic acid. This aromatic acid appears to have a hydroxyl and a methoxyl substituent at either the 2,4- or 2,5-positions. Several other minor phenolic constituents have been detected but not identified. The aqueous methanol extract also yielded three disaccharides and a trisaccharide (1.9%) identified as raffinose. The disaccharide isolated in the largest amount (4.4% of the defatted meal) was sucrose. Another, which yielded only glucose on complete acid hydrolysis, is probably α,α′-D-trehalose. The third disaccharide was cleaved to glucose and fructose by the same treatment. Total phenolic content of the defatted meal was about 2.4%, and the total amount of sugars isolated, 9.7%.

Increasing interest in sunflower (*Helianthus annuus*) as a North American oilseed crop has resulted in expanded research activities on the nutritional and chemical aspects of the seed oil and meal (see Earle *et al.*, 1968, for a historical summary). This trend applies especially to the promising high-oil Russian sunflower varieties because most of the work thus far has been published in journals that are somewhat inaccessible to American investigators.

A number of workers (Smith and Johnsen, 1948; Joubert, 1955; Sechet-Sirat *et al.*, 1959; Milić *et al.*, 1968) have reported the presence of chlorogenic acid (3-caffeoylquinic acid) in sunflower seeds. This acid has been implicated in the enzymic browning of various fresh fruits and vegetables (Hulme, 1953; Weurman and Swain, 1953; Sakamura and Obata, 1963). It also causes a marked darkening of sunflower seed meals and protein concentrates derived from them by alkali extraction (Osborne and Campbell, 1897; Smith and Johnsen, 1948). Smith and Johnsen (1948) and Joubert (1955) devised schemes for removing chlorogenic acid in attempts to produce meals and protein concentrates that are not prone to this darkening effect.

Processing methods for sunflower seed meal and its quality for use in products for animal or human consumption could be affected by minor constituents not yet investigated. A project was therefore initiated to detect, isolate, and identify, if possible, any such minor constituents in seeds of one of the high-oil Russian sunflower varieties, Armavirec, currently being grown in the United States and Canada. This report describes the isolation and identification of some phenolic and sugar constituents of this seed meal.

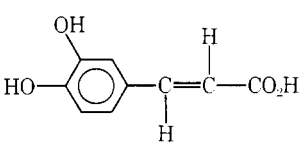
EXPERIMENTAL

Spectral Analyses. Ultraviolet spectra were determined on methanol solutions with a Beckman DK-2A recording spectrophotometer, and infrared spectra, on KBr pellets with a Perkin-Elmer 137 Infracord spectrometer. A Varian HA-100 nuclear magnetic resonance (NMR) spectrometer was used to determine 100 MHz NMR spectra of the phenolics in d_5-pyridine or d_6-acetone solutions. Chemical shifts were measured from internal tetramethylsilane = τ 10.0.

Countercurrent Distribution. Countercurrent distribution (CCD) of the phenolic mixture was done with an automatic 200-tube Craig-Post apparatus. The ethyl acetate-10% NaCl (pH 2.0) solvent pair of Corse *et al.* (1966) was modified to give a lower phase (40 ml. per tube) of 3% sodium chloride at pH 2.5. A total of 600 transfers were made with 35 ml. of upper phase per transfer. After the 200 fundamental transfers were completed, the remaining 400 were collected in a fraction collector.

Gas-Liquid Chromatography. Silylated sugars were analyzed as hexane solutions by gas-liquid chromatography (GLC) with a dual-column F&M Model 5750 chromatograph equipped with hydrogen flame detectors. A 61 × 0.3-cm. column packed with 3% OV-1 on Gas-Chrom Q (Applied Science Laboratories) was used, and the column temperature was programmed at 4° C. per minute starting either at 100° or 200° C., depending on whether monosaccharide or oligosaccharide derivatives were being analyzed. Helium was the carrier gas.

Isolation of Phenolic and Sugar Concentrates. Armavirec variety sunflower seeds were furnished by Cargill, Inc., from the 1966 U. S. crop. They were dehulled, ground, and defatted by repeated Soxhlet extraction—first with petroleum ether (b.p. 30° to 60° C.) and then with diethyl ether. Defatted meal (82 grams) was Soxhlet extracted with 1 liter of aqueous methanol for 4 hours. The meal was dried, replaced in the thimble, and reextracted with fresh solvent. This sequence was repeated once more. Removal of solvent from the combined extracts on a rotary evaporator yielded a tan gum, which solidified during overnight drying in a vacuum desiccator; yield, 15.51 grams (18.9% of meal). Since a portion of this extracted material could not be redissolved in 30 ml. of 80% aqueous methanol, this residue was filtered off as white crystals and dried (0.15 gram). This crystalline substance was later shown to be caffeic acid.



Caffeic acid

The filtration liquor was evaporated and the residue was dissolved in 100 ml. of aqueous HCl at pH 2. Repeated extraction (12 times) of the acidic solution with 100-ml. portions of ethyl acetate yielded 2.68 grams of extractable material. The pH of the aqueous phase was immediately adjusted to 5.5.

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Table I. Phenolics of Armavirec Sunflower Seeds

Phenolic Fraction	From CCD Fractions	Content in Defatted Meal, %	Paper Chromatography R_f, BAW[a]	Paper Chromatography R_f, SFFW[b]	TLC R_f, MAA[c]	Acid Hydrolysis Products[d]
A	0 to 24	0.03	0.83	0.37	0.73	...
B	0 to 24	0.03	0.76	0.21	0.44	Caffeic, quinic acids
C	35 to 54	0.08	0.73	0.57, 0.63	0.36, 0.50	Caffeic, quinic acids
D	65 to 89	<0.02	0.74	0.57	0.70	Phenolic A, quinic acid
Chlorogenic acid (E)	165 to 350	2.0	0.63	0.64	0.31	Caffeic, quinic acids
Caffeic acid[e]	...	0.2	0.79	Streaked	0.64	...

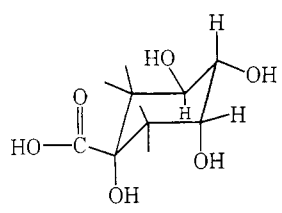
[a] Butanol:acetic acid:water (5:1:4).
[b] Sodium formate:formic acid:water (10:1.2:200).
[c] Methanol:acetone:acetic acid (12:5:3). Used with boric acid-impregnated Silica Gel G.
[d] By paper chromatography.
[e] Recovered from total extract before CCD.

Cursory examination of the two fractions by a number of techniques revealed that the ethyl acetate fraction contained the phenolic constituents and that components remaining in the aqueous phase were primarily carbohydrate in nature.

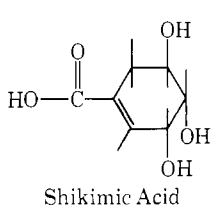
Thin-Layer Chromatography. Phenolic compounds were analyzed on thin-layer plates of boric acid-impregnated Silica Gel G (250 microns) developed in methanol:acetone:acetic acid (MAA) (12:5:3). These plates were prepared in the usual manner except the slurry of Silica Gel G was made with saturated aqueous boric acid solution instead of with water. They were dried at 110° C. for 1 hour. After development the plates were viewed under ultraviolet light and the fluorescent spots were marked. Nonphenolic components were then detected by spraying the chromatogram with a saturated solution of chromium trioxide in 50% aqueous sulfuric acid and heating for 30 to 60 minutes at 120° C.

The isolated sugars were analyzed on boric acid-impregnated Silica Gel G plates developed in chloroform:methanol:isopropanol:acetic acid (CMIA) (2:2:1:1) or benzene:methanol:acetic acid (BMA) (2:2:1). All sugars were detected with a solution of naphthoresorcinol in acidic alcohol (Lato *et al.*, 1968).

Paper Chromatography. Phenolic constituents were analyzed by the ascending technique on Whatman No. 1 or No. 4 paper. The upper phase of a butanol:acetic acid:water (BAW) (5:1:4) mixture was one of the solvents used. Another quite useful solvent system was sodium formate:formic acid:water (SFFW) (10:1.2:200) (Smith, 1958; Ibrahim and Towers, 1960). The phenolic spots were visualized by ultraviolet light and by spraying the chromatograms first with a solution of diazotized *p*-nitroaniline (Bray *et al.*, 1950) and then with a 20% aqueous sodium carbonate solution. The colors that appear are varied and depend on the particular compound (Ibrahim and Towers, 1960). Quinic acid and shikimic acid were also analyzed by paper chromatography in both the BAW and SFFW solvent systems.



Quinic Acid



Shikimic Acid

A detection method utilizing sodium periodate, sodium nitroprusside, and piperazine (Cartwright and Roberts, 1955) was used.

Sugars were analyzed by ascending paper chromatography with Whatman No. 1 paper and BAW solvent system, and also with Whatman No. 4 paper and an ethyl acetate:pyridine:water (4:3:2) system. Spots were detected with the naphthoresorcinol spray mentioned above, the aniline phthalate reagent of Partridge (1949), or the aniline-diphenylamine reagent in acetone as described by Harris and MacWilliam (1954).

Preparative Paper Chromatography. Phenolic CCD fractions were further fractionated with Whatman 3 MM paper by the descending technique with either SFFW or BAW solvent systems. Bands on the developed chromatogram were visualized under ultraviolet light, cut out, and extracted with aqueous methanol. The methanol extracts of bands from chromatograms that had been developed with the SFFW solvent system contained a considerable amount of sodium formate. Evaporation of the methanolic solvent, dissolution of the residue in water (pH 2.0), and extraction of this acidic solution with ethyl acetate were necessary to recover the phenolic components from this mixture.

Descending preparative paper chromatography on Whatman 3 MM filter paper with the BAW solvent system was applied to the neutralized (pH 7) sugar concentrate. Four bands were located by spraying strips cut from the edges of each developed chromatogram with aniline-diphenylamine reagent. The individual sugars (I, II, III, and IV) were recovered by extracting the filter paper bands with water and also with methanol.

Acid Hydrolysis of Sugars. Sugars I, II, and IV were hydrolyzed by refluxing with 0.75*N* HCl for 5 hours. Hydrolysis of sugar III was done with 0.4*N* HCl under reflux for 2 hours. All hydrolysis mixtures were neutralized with 1*N* NaOH, evaporated nearly to dryness, and dried overnight in a vacuum desiccator.

Trimethylsilylation. Samples (about 2 to 10 mg.) of unknown sugars I, II, III, and IV; authentic sugars; and the sugar hydrolysis products described above were each treated with 1 ml. of Tri-Sil reagent (Pierce Chemical Co.). After being shaken thoroughly, the mixtures were allowed to stand overnight at room temperature. Pyridine was removed in a jet of nitrogen, the *O*-trimethylsilyl (*O*-TMS) ether derivatives (Sweeley *et al.*, 1963) were dissolved in hexane, and inorganic salts were removed by centrifugation. After they were concentrated to about 0.2 ml., the hexane solutions were analyzed by GLC.

RESULTS AND DISCUSSION

Isolation and Purification of Phenolic Fractions. The CCD weight curve, together with thin-layer chromatography (TLC) of the combined fractions (Figure 1), revealed that the

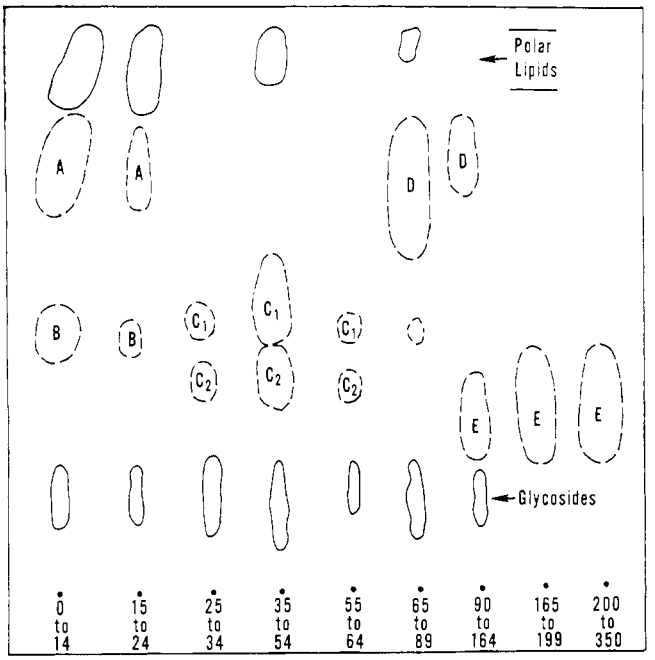


Figure 1. Thin-layer chromatogram of combined fractions from countercurrent distribution of phenolics from sunflower seed meal

Boric acid-impregnated Silica Gel G. Methanol:acetone:acetic acid (12:5:3). Broken boundaries = ultraviolet fluorescence. Solid boundaries = charred with chromium trioxide–sulfuric acid reagent

major constituent (fractions 165 to 350, later identified as chlorogenic acid) was isolated in nearly pure form and that the remainder of the complex phenolic concentrate had been resolved into simpler mixtures. Contaminants designated as polar lipids and glycosides were found by TLC to be spread throughout the first 150 CCD fractions. Detection methods used with paper chromatography did not visualize lipid materials; however, the components labeled "glycosides" in Figure 1 had characteristics consistent with those of phenolic glycosides (Goldschmid and Hergert, 1961).

CCD fractions 0 to 24, 35 to 54, and 65 to 89 were combined and subjected to preparative paper chromatography. The SFFW solvent system was used for fractions 0 to 24 and 35 to 54, and the BAW system for fraction 65 to 89. Purified phenolic fractions A, B, C, and D resulting from this separation and some of their characteristics are listed in Table I, along with data for chlorogenic and caffeic acids isolated earlier. The spots designated A, B, and D in Figure 1 are the main components of the correspondingly labeled fractions in Table I. Fraction C in Table I contains both C_1 and C_2 (Figure 1) because these two components were not sufficiently resolved on the preparative chromatograms. All four fractions were contaminated by lipid material, which apparently streaked on the preparative paper chromatograms. Fraction A, which was badly contaminated, was purified further by dissolving the phenolic material in warm (40° C.) water and the contaminant remained behind as an insoluble tar. This technique was unsuccessful when applied to fractions B, C, and D.

Chlorogenic and Caffeic Acids. The major phenolic constituent (2.0%) of defatted sunflower meal is chlorogenic acid (fraction E). Two recrystallizations of CCD fractions 165 to 350 from warm water gave a material that had infrared, ultraviolet, and NMR characteristics (Figure 2) identical with those of an authentic sample. The recrystallized material was also indistinguishable from chlorogenic acid by paper chromatography, TLC, m.p. (201° to 209° C.) and mixed m.p. (202° to 209° C.). Sondheimer (1958) reported a m.p. of 201° to 205° C.

Similarly, the solid material, described earlier as that part of the aqueous methanol extract not soluble in 30 ml. of this solvent, was identified as caffeic acid. The isolated material was identical with authentic caffeic acid by all the criteria listed above for chlorogenic acid. Three recrystallizations from warm water gave crystals with a m.p. of 215° to 218° C., and a mixture melting point with pure caffeic acid of 214° to 218° C. Sakamura and Obata (1963) reported a m.p. of 219° to 220° C. for caffeic acid.

Quinic and Shikimic Acids. Milić *et al.* (1968) reported the occurrence of free quinic acid in sunflower meal. However,

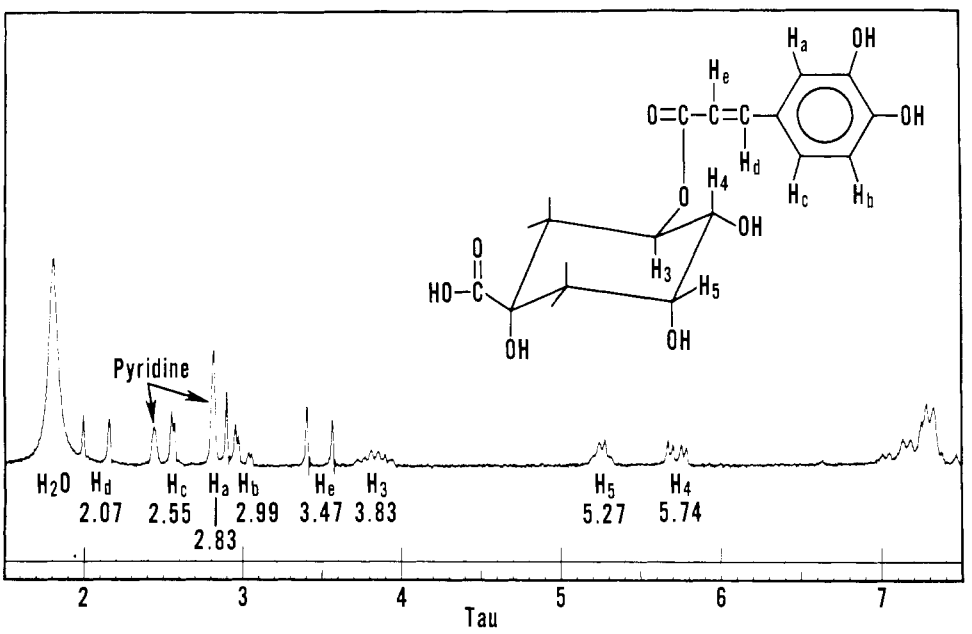


Figure 2. 100 MHz. NMR spectrum of chlorogenic acid from sunflower seed meal in d_5-pyridine

Chemical shifts measured from internal tetramethylsilane = τ 10.0

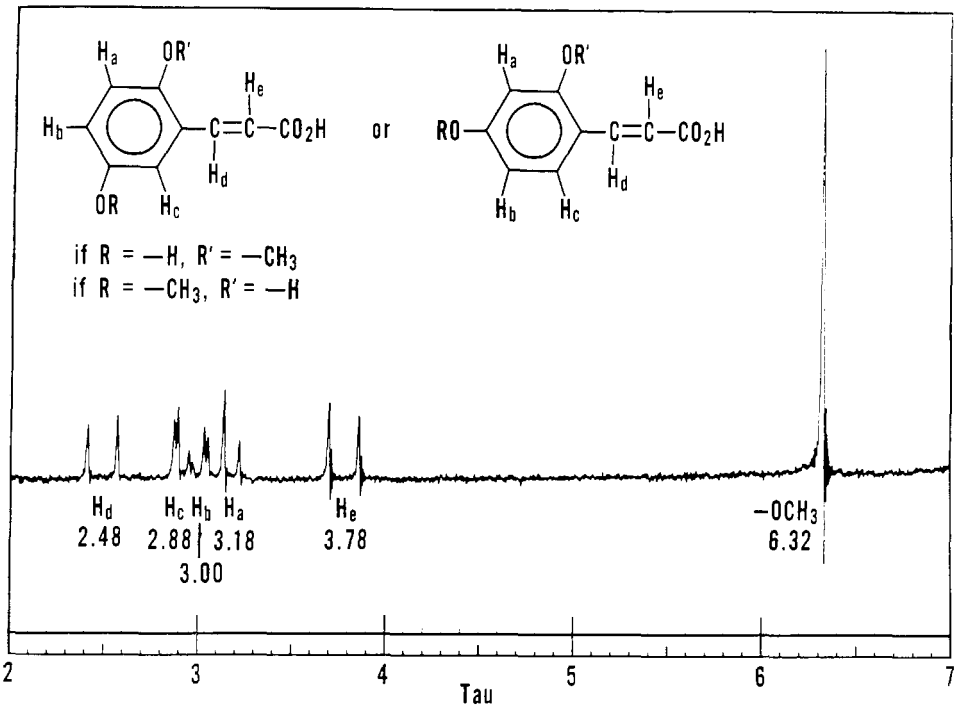
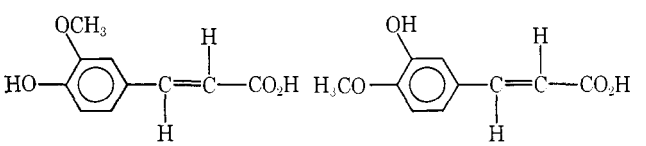


Figure 3. 100 MHz. NMR spectrum of phenolic fraction A in d_6-acetone

Chemical shifts measured from internal tetramethylsilane = τ 10.0

paper chromatographic analyses of the total aqueous methanol extract of Armavirec sunflower seed meal and detection by the periodate-nitroprusside-piperazine method failed to detect any quinic or shikimic acids. The R_f and rate of color formation for these two acids differ enough that each can be detected in the presence of the other (Goldschmid and Hergert, 1961). Authentic quinic and shikimic acid samples were analyzed for comparison.

Phenolic Fraction A. Ultraviolet analysis of fraction A gave a spectrum similar to that of caffeic acid (Schroeder, 1967) but with diminished intensity. The NMR spectrum of A in d_6-acetone is shown in Figure 3. The absence of complex signals in the τ 3.8 to 6.0 region (Corse *et al.*, 1966) clearly demonstrates that A is not an ester of quinic acid. A sharp singlet at τ 6.32 equivalent to three protons indicates that the molecule has one methoxyl group. (Methanol in d_6-acetone under these conditions gave a singlet at τ 6.43.) A strongly coupled pair of doublets, J = 16 Hz, centered at τ 2.48 and τ 3.78, are ascribed to the *trans*-olefinic protons of a cinnamic acid moiety because the corresponding protons of pure caffeic acid give signals with chemical shifts near these values. The chemical shifts of the three aromatic proton signals, τ 2.88, 3.00, and 3.18, differ slightly from those of the corresponding protons in caffeic, ferulic (3-methoxy-4-hydroxycinnamic), and isoferulic (3-hydroxy-4-methoxycinnamic) acids. The splitting pattern, however, is the same for all four compounds.



Ferulic acid Isoferulic acid

Therefore A must have three aromatic protons arranged in the same manner as caffeic, ferulic, and isoferulic acids so that two of them are *ortho* and *para*, respectively, to the third proton. Any other arrangement would give a splitting pattern (Cardillo *et al.*, 1968) quite different from that observed in Figure 3. These considerations reduce the possible structures for fraction A to the 2,4- and 2,5-disubstituted cinnamic acids with a hydroxyl group at one position and a methoxyl group at the other.

The melting point of fraction A was 168° to 170° C. after many recrystallizations. Literature values for some related synthetic compounds are: 2-hydroxy-5-methoxycinnamic acid, 191° to 193° C. (Böhme and Severin, 1957); 2-hydroxy-4-methoxycinnamic acid, 201° to 202° C. (Böhme and Severin, 1957); and 2-methoxy-5-hydroxycinnamic acid, 179° to 180° C. (Schnell, 1884). So far as is known, none of these isomers are naturally occurring and the 2-methoxy-4-hydroxy isomer has never been reported. However, Brown (1963) isolated from *Lavandula officinalis* a material reported to be 2-glucosyloxy-4-methoxycinnamic acid. From these data, it seems probable that A is a 2-methoxycinnamic acid with a hydroxyl group at position 4 or 5 since the 2-hydroxy isomer is reported to have a much higher melting point.

Phenolic Fraction B. The R_f values (Table I) for fraction B on paper chromatography and TLC agreed with those for authentic 3,5-dicaffeoylquinic acid. Infrared and ultraviolet spectra of B were also in accord with this structure, but it was apparent from the NMR spectrum (in d_5-pyridine, 75° C.) that B was a mixture of phenolics along with some contaminant. Complex signals centered at τ 3.88 and 5.30 were probably due to the protons on carbons 3, 4, and 5 of the quinic acid moiety of 3,5-dicaffeoylquinic acid (Corse *et al.*, 1966). Some of the other weak, complex bands observed at τ 3.97, 4.05, 4.51, and 5.60 are likely due to other phenolics, but the presence of nonphenolic material makes unequivocal assignment of these bands impossible.

Acid hydrolysis of fraction B (2*N* HCl at 85° C. for 2 hours) yielded only quinic and caffeic acids as determined by paper chromatographic analysis (BAW) of the product. The intensity of the caffeic acid spot (relative to the quinic acid spot) was considerably greater in the hydrolysis products of B

Table II. Sugars of Armavirec Sunflower Seeds

Sugar	Type	Content in Defatted Meal, %	Paper Chromatography R_f, BAW[a]	TLC R_f, CMIA[b]	Acid Hydrolysis Products
I	Disaccharide	1.3	0.32	0.19	Glucose, fructose
II	Disaccharide	2.1	0.26	0.20	Glucose
III	Disaccharide	4.4	0.16	0.41	Glucose, fructose
IV	Trisaccharide	1.9	0.07	0.25	Glucose, fructose, galactose

[a] Butanol:acetic acid:water (5:1:4). [b] Chloroform:methanol:isopropanol:acetic acid (2:2:1:1). Boric acid-impregnated Silica Gel G.

than in those of chlorogenic acid. These data lead to the conclusion that phenolic fraction B is primarily 3,5-dicaffeoylquinic acid, but that other unidentified phenolic(s) are present in small amounts.

Phenolic Fraction C. Fraction C showed two fluorescent phenolic components, C_1 and C_2, on TLC (Figure 1) but the preparative paper chromatographic separation did not resolve them. Some contaminant was also present. The ultraviolet and infrared spectra of C provided no specific structural clues other than confirming the phenolic nature of the constituents. Acid hydrolysis of fraction C yielded quinic and apparently caffeic acids, but the intensity of the caffeic acid spot (by paper chromatography) in this hydrolyzate was comparable to the intensity of the caffeic acid spot observed on the chromatogram of a chlorogenic acid hydrolyzate. The NMR spectrum of C (d_5-pyridine solution at room temperature) shows a pair of doublets (τ 3.52 and 3.62, J = 16 Hz) due to *trans*-olefinic protons on the carbon α to a cinnamic acid carboxyl group (such as H_a in Figure 2), whereas the spectra of monocaffeoylquinic acids show only one doublet (as in Figure 2) in this region. The signals (near τ 2.3) of the other *trans*-olefinic protons (on the carbon β to the carboxyl group) were partially obscured by a large water signal. In addition to appearing as a pair of doublets, the combined signals of the *trans*-olefinic protons (τ 3.52 and 3.62) were about as intense (compared to the quinic acid proton signals) as the signal of the corresponding proton in the spectra of monocaffeoylquinic acids (Figure 2).

These data suggest that fraction C is either a mixture of two different monocaffeoylquinic acid esters or a mixture of one caffeoylquinic acid ester and another monoester of quinic acid with an unidentified cinnamic acid derivative. If phenolic fraction A esterified with quinic acid were present in C, it would have to be in the dihydroxy form rather than the hydroxy methoxy form that was isolated since no methoxyl proton signals are observed in the NMR spectrum of C. A paper chromatographic spot due to phenolic A or to its dihydroxy form could be obscured by the caffeic acid spot. Complex signals presumably due to the protons on carbons 3, 4, and 5 of the quinic acid moiety in fraction C had chemical shifts of τ 4.02, 4.48, and 5.48. This sequence is not in accord with any of the patterns reported by Corse *et al.* (1966) for the monoesters of quinic acid. Thus the exact nature of the constituents of fraction C remains unclear.

Phenolic Fraction D. The amount of phenolic material available was very small since fraction D was badly contaminated. Doublets centered at τ 2.11 and 3.53 (J = 16 Hz) in the NMR spectrum of D (in d_5-pyridine) indicate that it contains *trans*-olefinic protons of the cinnamic acid type. A singlet due to methoxyl protons was also observed at τ 6.33, but the portion of the spectrum between τ 3.8 and 5.0 was completely obscured by a broad water signal. Acid hydrolysis of D yielded a product that contained quinic acid but apparently no caffeic acid. When subjected to acid treatment (as in the hydrolysis) and then analyzed by paper chromatography (BAW system), caffeic acid gives a blue-violet spot with diazotized *p*-nitroaniline (before the sodium carbonate spray). This colored spot was observed during analysis of the hydrolyzates of fractions B and C but not on the chromatogram of the hydrolyzate of fraction D. However after the chromatogram was sprayed with sodium carbonate solution, a brown spot with the same R_f as that of fraction A appeared. Acid-treated fraction A does not give a blue-violet spot with diazotized *p*-nitroaniline. The phenolic component of fraction D probably is a monoester of quinic acid with the compound isolated as phenolic fraction A.

Identification of Sugars. Some characteristics of the four sugars, which were isolated as noncrystalline solids without definite melting points, are listed in Table II. In addition, all four were shown to be nonreducing by their failure to react with aniline phthalate. Analysis of sugars III and IV by TLC and by GLC of their *O*-TMS ethers demonstrated that they were probably sucrose and raffinose, respectively. The acid hydrolysis products of III and IV, which were identified by GLC analysis of their *O*-TMS ethers, support this conclusion. Small amounts (less than 2% total) of two apparent tetrasaccharides were also observed on the GLC chromatogram of sugar IV *O*-TMS ether. Results of these same analyses applied to sugar II indicate that it probably is α,α'-trehalose. The identity of sugar I remains unknown. To be certain that any two *O*-TMS ether samples that were being compared had the same retention time on GLC, it was necessary to analyze them as a mixture because retention characteristics are difficult to reproduce exactly on consecutive temperature programmed runs.

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