Table III. Influence of Copper on Phenoloxidase Activity[a]

molar ratio (Cu to PO)	% rel act.	molar ratio (Cu to PO)	% rel act.
0:1	100.0[a]	1:2	124.3[c]
1:4	117.4[b]	1:1.25	136.8[d]

[a] Data presented in Table III are average values of duplicate results for two experiments. Numbers followed by the same letter are not significantly different (α = 0.05) by Duncan's multiple-comparison test.

as an inactive precursor, it became activated before the purification process.

Influence of Copper on Phenolase Activity. Addition of copper stimulated the phenolase–DOPA reaction (Table III), and the data presented indicate that the enzyme activity increased with copper concentration. Addition of copper acetate at a molar ratio of 1:1.25 (Cu to PO) increased PO activity by approximately 37% while the same salt at a molar ratio of 1:4 (Cu to PO) only stimulated the reaction by approximately 17%. The values reported in Table III are corrected for catalysis of the reaction by copper. Activation by copper was also observed with the various fractions recovered in the purification process. Various workers such as Bailey et al. (1959) and Mathew and Parpia (1971) have also demonstrated a copper dependency of phenoloxidases.

Conclusions. In conclusion, the 30 000 molecular weight band is phenoloxidase (EC 1.10.3.1) made up of a single polypeptide chain. We also observed that the various fractions from the purification of PO were activated by copper, which eliminates the possibility that activation of the purified enzyme by copper was a simple case of stripping copper from the enzyme with subsequent recovery of activity on adding the ion. Further studies in our laboratory will be designed to determine the kinetic properties of white shrimp PO in relation to properties of homologous enzymes derived from other crustacean species as well as investigate the efficacy of various treatments to prevent melanosis as alternatives to the use of sulfites in crustacean species.

Registry No. PO, 9002-10-2.

LITERATURE CITED

Affinity Chromatography. Principles and Methods; Pharmacia Fine Chemicals: Uppsala, Sweden, 1979; p 15.
Aylward, F.; Haisman, D. R. *Adv. Food Res.* **1969**, *17*, 1.
Bailey, M. E.; Fieger, E. A.; Novak, A. F. *Food Res.* **1959**, *25*, 557.
Cobb, B. F., III In Proceedings of the Conference on the Handling, Processing and Marketing of Tropical Fish, Tropical Products Institute, 1977; pp 405–411.
Davis, B. J. *Ann. N.Y. Acad. Sci.* **1964**, *121*, 404.
Gomori, C. *Methods Enzymol.* **1955**, *1*, 138–146.
Gornall, A. G.; Bardawill, C. J.; David, M. M. *J. Biol. Chem.* **1949**, *177*, 751.
Horowitz, N. H.; Fling, M.; Macleod, H. A. Sheoka, N. *J. Mol. Biol.* **1960**, *2*, 96.
Katoh, S.; Kambayashi, T.; Deguchi, R.; Yoshida, F. *Biotechnol. Bioeng.* **1978**, *XX*, 267.
Laemmli, U. K. *Nature (London)* **1970**, *227*, 660.
Madero, C. F. Ph.D. Dissertation, Texas A & M University, 1982.
Mathew, A. G.; Parpia, H. A. B. *Adv. Food Res.* **1971**, *19*, 75.
Neville, A. C. In *Biology of the Arthropod Cuticle*; Springer-Verlag: New York, 1975; pp 116–260.
Ott, L. *An Introduction to Statistical Methods and Data Analysis*, 2nd ed.; Duxbury: Boston, 1984; pp 339–347.
Park, E. Y.; Luh, B. S. *J. Food Sci.* **1985**, *50*, 678.
Pinhey, K. G. *J. Exp. Biol.* **1930**, *7*, 19.
SAS User's Guide: Statistics, 5th ed.; SAS Institute: Cary, NC, 1985.
Savagaon, K. A.; Sreenivasan, A. *Fish. Technol.* **1978**, *15*, 49.
Thomas, P.; Janave, M. T. *J. Food. Sci.* **1973**, *38*, 1149.
Tolbert, N. E. *Plant Physiol.* **1973**, *51*, 234.
Vamos-Vigyazo, L. *CAC Crit. Rev. Food Sci. Nutr.* **1981**, *15*, 49.

Received for review September 30, 1986. Accepted April 1, 1987. Florida Agricultural Experiment Station Journal Series No. 7558. This work was supported by a grant from the Gulf and South Atlantic Fisheries Development Foundation, Inc., Foundation Project No. 27-01-49373/10000.

Total Phenolics and High-Performance Liquid Chromatography of Phenolic Acids of Avocado

Andrew M. Torres,* Terry Mau-Lastovicka, and Ruhi Rezaaiyan

Total phenolic concentrations as gallic acid equivalents were determined for leaves, fruits (mesocarp), and seeds (cotyledons) of several cultivated varieties of avocado (*Persea americana* Mill.). Concentrations were generally highest in cotyledons and lowest in the mesocarp of fruits. Phenolic acids were extracted by alkaline hydrolysis and identified with high-performance liquid chromatography, spectrophotometry, and thin-layer chromatography. Sixteen phenolic acids were identified, and in general, every tissue of every variety had the same array. The commonly occurring gentisic acid was not detected.

Plant phenolics include a great diversity of compounds such as simple phenols, phenolic acids, coumarins, flavonoids, tannins, and lignins (Robinson, 1963; Ribereau-Gayon, 1972; Harborne, 1964, 1984). In the strict sense used here, phenolic acids have a benzene ring, a carboxylic acid, and one or more phenyl hydroxyl groups that may become methylated to produce methoxy groups. Phenolic acids commonly occur as esters and/or ethers in combination with various sugars and aliphatic or aromatic acids and hydroxy acids. Most plant phenolic acids are derivatives of either benzoic (benzenecarboxylic; C_6–C_1) or cinnamic (phenylpropanoid or phenylacrylic; C_6–C_3) acids (Roston and Kissinger, 1982). Cinnamic acid is in turn derived by the deamination of the amino acid L-phenyl-

Department of Botany, University of Kansas, Lawrence, Kansas 66045.

0021-8561/87/1435-0921$01.50/0

alanine, and benzoic acid is metabolized from shikimic acid or by the removal of an acetate from cinnamic acid (Gross, 1981).

Phenolic acids are generally sequestered in the cell vacuole or in special tissues and are normal precursors of many other compounds. However, when cells are ruptured, the phenols are released and become oxidized, atmospherically and enzymatically, to quinones, which in turn polymerize and produce a brown or rust color. This is the well-known browning reaction seen on cut surfaces of apples, potatoes, and avocados (*Persea americana* Mill.). The polymers also bind to and inactivate enzymes so that special measures must be taken to protect plant enzymes during extraction (Loomis, 1974; Torres, 1984).

Avocado phenolics have been studied mainly because of the economic significance of the browning reaction (Kahn, 1975, 1977; Golan et al., 1977; van Lelyveld et al., 1984) and because of the possible association between phenols and root rot resistance (Brune and van Lelyveld, 1982). These studies have employed paper or thin-layer chromatography (TLC), but none have used the more sensitive technique high-performance liquid chromatography (HPLC).

Because there are very many possible phenolic compounds, the present study was limited to the phenolic acids, that is, the moieties remaining following the removal of the sugar or acid esters by alkaline hydrolysis. Even with this limitation, there can be many phenyl hydroxy and phenyl methoxy derivatives of benzoic and cinnamic acids. For example, considering only three of five possible substitutions on the benzene ring (carbon 1 is occupied by the acid group), there could be three monohydroxy (*p*-, *m*-, and *o*-; or 4, 3, 2); six dihydroxy; six trihydroxy; 10 monohydroxy, monomethoxy; 16 monohydroxy, dimethoxy; and 16 dihydroxy, monomethoxy molecules. In total, 57 benzoic and 57 cinnamic acid derivatives are possible, all of which have at least one phenyl hydroxy. Many of these, of course, would not be expected to occur in plants.

Since phenolic acids and their derivatives are significant components of taste and odor, are involved in the browning reaction, and are thought to be involved in growth regulation and in disease and herbivore resistance, it is important to identify those that occur in crops of economic significance. The purpose of this study was (1) to determine and compare the total phenolic content of different avocado tissues—young and mature leaves, mesocarp (the edible portion of the fruit), and seed cotyledons—in different cultivated varieties; (2) to identify with HPLC, spectrometry, and TLC the array of benzoic and cinnamic phenolic acids found in avocados; and (3) to determine whether or not this array varied between tissues or between varieties.

MATERIALS AND METHODS

Fresh material of the cultivars Hass, Duke, Fuerte, and Gwen was collected at the same time from mature trees in plantings of the University of California, Riverside, CA, and shipped by air express to Lawrence, KS. Entire young and mature leaves and thinly sliced cotyledons and ripe mesocarps were weighed and lyophilized for 2 days. Freeze-dried tissues were quickly weighed for fresh to dry weight ratios, hermetically sealed in plastic bags, and stored at −20 °C until needed. Fruits of Duke were not available for this study.

Total phenols were extracted by grinding freeze-dried tissue with a chilled mortar and pestle in liquid nitrogen. A 100-mg portion of powder was weighed into a 50-mL beaker with 20 mL of 100% methanol. The beaker was covered with plastic wrap and stirred for 2 h at room temperature. The sample was filtered, transferred to a separatory funnel, and shaken with 20 mL of hexane. The lower methanol layer with phenols was washed five times more with fresh hexane. The methanol solution was filtered and adjusted to a volume of 10 mL for measurement of total phenol content using a modification of the Folin–Ciocalteau method of Amerine and Ough (1974): A 0.1-mL sample was delivered to a 15-mL test tube and mixed with 6 mL water, 0.5 mL of Folin reagent was added, mixed, and allowed to stand between 1 and 8 min when 1.5 mL of 20% sodium carbonate was added, and the total volume was brought to 10 mL with water. The mixtures incubated for 2 h at 50 °C. Absorbance was read on a spectrophotometer at 765 nm. Five replicates of three samples of each tissue were analyzed. The standard curve was prepared with known concentrations of gallic acid. Comparisons were based on nested analyses of variance and the Student–Newman–Keuls for multiple comparisons among means (Sokal and Rohlf, 1969).

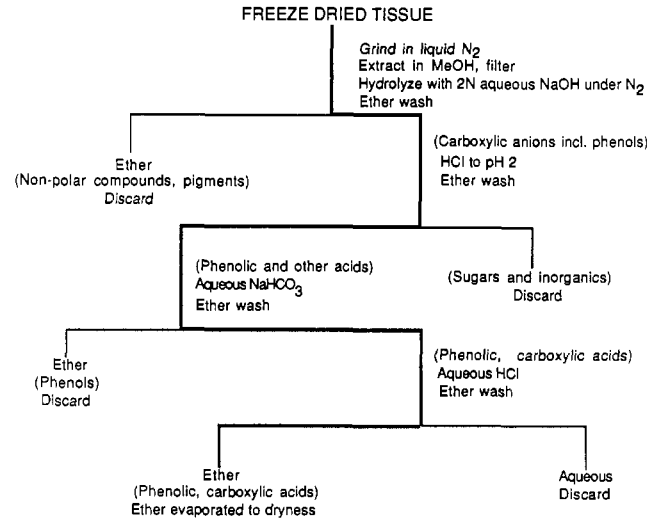

Figure 1. Flow chart for extraction of phenolic acids from avocado tissues. Additional details in text.

Phenolic acids were extracted with a modification of the method of van Lelyveld et al. (1981) as shown in Figure 1. The freeze-dried tissue in a chilled mortar was covered with liquid nitrogen and ground to powder. Methanol was added to the powder (10 mL/g) in a filtering flask. Nitrogen gas was immediately bubbled through the mixture, which was kept anaerobic with a nitrogen-filled balloon. The mixture was stirred for 30 min and filtered through No. 1 Whatman paper into a filtering flask. The methanol was evaporated with a stream of nitrogen gas. NaOH (2 N, 4 mL/g of powder) was added to the dried extract, and nitrogen was bubbled through the mixture through the arm of a filtering flask. A balloon was attached to the mouth of the flask and inflated with nitrogen to ensure an anaerobic atmosphere. The mixture was allowed to stand overnight at room temperature for hydrolysis. The extract was filtered through a Millipore microfilter (0.45 μm), delivered to a separatory funnel, and washed 3× with ether (8 mL/g of powder) to remove the nonpolar substances. The ether was discarded, and the aqueous extract was acidified to pH 2 with concentrated HCl. The extract was washed 3× in a separatory funnel with ether. The aqueous layer containing sugars and inorganics was discarded, and 5% sodium bicarbonate (48 mL/g of powder) was added to the ether layer (with acids and phenols) and shaken for 2 min. The lower, aqueous layer containing the phenolic acids in anionic form was removed and acidified to pH 2 with concentrated HCl; the ether layer with phenols was

discarded. The acid layer containing the free phenolic acids in protonated form was returned to the separatory funnel and shaken 3× with ether. The ether layer with the phenolic acids was evaporated to dryness in a stream of nitrogen gas. The residue was taken up in about 1 mL of methanol and filtered with a Millipore microfilter. Extracts were tested with TLC for chlorogenic acid (the quinic acid ester of caffeic acid), which should not have been present in hydrolyzed preparations. If necessary, the samples were concentrated by evaporating some of the methanol with nitrogen. Samples were analyzed while fresh or stored at 2–3 °C in stoppered tubes in the dark.

A programmable Spectra-Physics HPLC, Model 8700, was fitted with an Alltech Associates 4.6 mm × 25 cm column and a 4.6 mm × 10 cm precolumn packed with 8-μm reversed-phase ODS Adsorbosphere. The variable-wavelength UV detector was a Kratos Spectraflow 757 set at 0.02 AUFS, and the strip chart recorder was a Fisher Recordall 5000 set at a speed of 0.5 cm/min. Two solvents were used: 5% aqueous acetic acid (A) and acetonitrile 100% (B). The gradient was 100–90% A and 0–10% B over 30 min, then 90–40% A and 10–60% B over 10 min, 40–25% A and 60–75% B over the next 10 min, and finally 25–0% A and 75–100% B over the final 10 min to purge the column. The same gradient was used between analyses to ensure no compounds were left in the column. Phenolic acid standards were from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical Co. (Milwaukee, WI). They were prepared as 1 mM solutions in methanol. All solvents were HPLC grade and were filtered and degassed prior to use. Samples were filtered prior to use. Samples of 20 μL were injected. The 1 mL/min flow rate generated a pressure between 1200 and 600 psi. Retention times and the solvent composition when the standards eluted were recorded. Retention times of the peaks of the avocado extracts relative to that of *p*-coumaric acid as the internal standard were calculated. Each extract was run at 254, 275, and 300 nm.

Absorbances of standards were determined with a Shimadzu UV-160 programmable UV–vis recording spectrophotometer. The standards were made up in solvents of the same composition as that of the assumed phenolic acid at the time of HPLC elution. Scanning was between 200 and 400 nm.

TLC was carried out on precoated 3.5 × 10 cm silica gel plates with a fluorescent indicator. Plates were spotted with standards alongside avocado extracts and were cospotted with extracts.

RESULTS AND DISCUSSION

Total phenolic content can vary in different species of the same genus (Gartlan et al., 1980), in the same species at different times of year, and in the same tissue at different stages of growth (Lowman and Box, 1983; Janzen and Waterman, 1984).

Golan et al. (1977) found that Fuerte mesocarp had significantly higher total phenolics than Lehrman (28.7 vs. 2.8 mg/100 g fresh weight (FW) chlorogenic acid equivalents). They also found a difference in phenol content between the proximal and distal ends of the mesocarp of the same fruit. Prabha and Patwardhan (1980) measured total phenolics in several avocado fruit tissues (peel, mesocarp, seed, seed coat) and found they varied widely between the tissues. Total phenolics in the mesocarp were 1 mg/g FW for unripe fruit and 8 mg for ripe fruit (gallic acid equivalents). The cultivar used was not identified. Van Lelyveld et al. (1984) reported 0.096 mg/g FW total phenols as catechol equivalents for Fuerte mesocarp. The differences in the reported values may reflect variation in the phenols used for the calibration curves, the extraction methods, or possibly the degree of ripeness of the fruits.

Table I. Total Phenols (mg/g FW ± SE) in Avocado Tissues as Gallic Acid Equivalents[a]

cultivar	mesocarp	cotyledon	young leaf	mature leaf
Hass	1.8 ± 0.0[a]	24.6 ± 0.0[b]	19.3 ± 0.2[c]	17.5 ± 0.1[d]
Gwen	1.8 ± 0.0[a]	16.5 ± 0.1[d]	17.0 ± 0.1[d]	16.5 ± 0.1[d]
Fuerte	1.1 ± 0.0[a]	29.8 ± 0.1	24.2 ± 0.1[b]	15.0 ± 0.1
Duke			22.0 ± 0.0	18.5 ± 0.0[c]

[a] Values with different letters are different from each other at the 0.05 significance level. Those with the same letter are not significantly different from each other. Unmarked values are significantly different from each other and from all others.

The total phenolic concentrations of tissues examined in this study are given as gallic acid equivalents in Table I. In Gwen the concentrations in cotyledons and leaves were about the same. In Hass and Fuerte the cotyledons had significantly higher phenolic concentrations than any other tissue. The high cotyledon concentration is in accord with our earlier experiences with the preparation of extracts from various tissues for isozyme analysis in that seeds require the most protection to maintain enzyme activity (Torres, 1984). In contrast, mesocarp requires no protective measures whatever.

Several authors have emphasized the importance of the extraction method in determining the types of compounds that will be obtained (Julkunen-Tiitto, 1985; Steele et al., 1969; van Lelyveld et al., 1981). This factor may account for the relatively few phenolic acids reported for avocados to date. Ramirez-Martinez and Luh (1973) reported two phenolic acids (caffeic and *p*-coumaric) from the mesocarp of Fuerte. Golan et al. (1977) identified four phenolic acids in the mescarp of Fuerte and Lehrman (caffeic, *p*-coumaric, protocatechuic, ferulic). Prabha and Patwardhan (1980) reported two (caffeic, *p*-coumaric) in an unnamed variety, and five phenolic acids (caffeic, *p*-coumaric, ferulic, *p*-hydroxybenzoic, and 3,4-dihydroxybenzoic) were reported by Brune and van Lelyveld (1982) from the mesocarp of Fuerte and four other varieties.

With the extraction method used here, several nonphenolic carboxylic acids would be present and those with conjugated double bonds would also absorb in the ultraviolet and be detected by HPLC. Therefore, attention was focused on those peaks of the chromatogram between two standards: gallic acid, the most polar, and cinnamic acid, the least polar (see Figure 2).

Tentative identifications were made by comparing relative retention times of the standards with those of the peaks of the avocado extracts. Standards for the tentatively identified peaks were then coinjected with the avocado extract to determine whether a single amplified peak, a peak with a shoulder, or two peaks resulted. If a single, symmetrical peak resulted, a further test was the comparison of the maximum absorbance of a compound in the extract with that of its assumed corresponding standard as determined with the recording spectrophotometer (see Figure 3). An identification was considered confirmed if the absorbances matched. To eliminate the variability of absorbance due to solvent, the standards were all dissolved in a solvent identical with that of the peak at the time of its elution.

The 16 phenolic acids detected by HPLC in avocados are listed in Table II. Retention times relative to *p*-coumaric acid, a prominent component in all extracts, are also given along with an indication of each peak's relative height among the compounds in the scan of its highest absorbance. Figure 3 is a typical scan. The order of elution of the standards was as reported by others (Wulf and

Table II. Avocado Phenolic Acids Identified with HPLC, Retention Times Relative to 4-Hydroxycinnamic Acid (RRT), Maximum Absorbance among 254-, 275-, 300-nm Chromatograms, Spectrophotometer Absorbance Maxima, and Peak Height Relative to the Entire Scan of Its Maximum Absorbance Wavelength

acid	common name	RRT × 100	max abs	spec abs max	rel pk ht[a]
4-hydroxybenzoic	*p*-hydroxybenzoic	46	254	255	M
2,3-dihydroxybenzoic	*o*-pyrocatechuic	64	254	245, 315	L
2,4-dihydroxybenzoic	β-resorcylic	58	254	256, 294	L
2,6-dihydroxybenzoic	γ-resorcylic	33	254	246, 306	L
3,4-dihydroxybenzoic	protocatechuic	29	254	256, 296	L
3,5-dihydroxybenzoic	α-resorcylic	27	254	249, 307	M
3,4,5-trihydroxybenzoic	gallic	17	275	271	L
3-hydroxy-4-methoxybenzoic	isovanillic	76	254	262, 294	L
4-hydroxy-3-methoxybenzoic	vanillic	66	254	260, 290	L
4-hydroxy-3,5-dimethoxybenzoic	syringic	80	275	275	L
2-hydroxycinnamic	*o*-coumaric	125	275	275, 325	M
3-hydroxycinnamic	*m*-coumaric	118	275	235, 277	M
4-hydroxycinnamic	*p*-coumaric	100	300	235, 310	H
3,4-dihydroxycinnamic	caffeic	68	300	246, 322	L
4-hydroxy-3-methoxycinnamic	ferulic	120	300	240, 328	H
4-hydroxy-3,5-dimethoxycinnamic	sinapic	123	300	246, 336	M

[a] Key: L, low; M, medium; H, high.

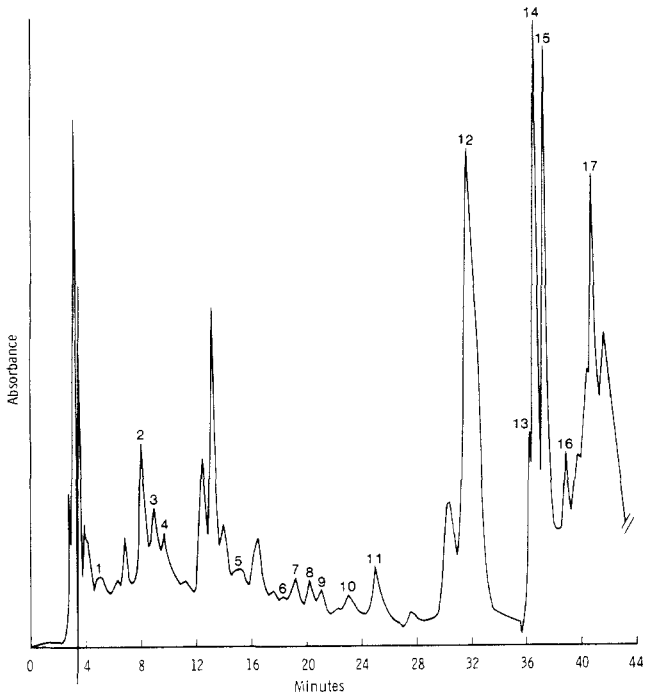

Figure 2. HPLC chromatogram of avocado extract. Peak numbers: 1, 3,4,5-trihydroxybenzoic; 2, 3,5-dihydroxybenzoic; 3, 3,4-dihydroxybenzoic; 4, 2,6-dihydroxybenzoic; 5, 4-hydroxybenzoic; 6, 2,4-dihydroxybenzoic; 7, 2,3-dihydroxybenzoic; 8, 4-hydroxy-3-methoxybenzoic; 9, 3,4-dihydroxycinnamic; 10, 3-hydroxy-4-methoxybenzoic; 11, 4-hydroxy-3,5-dimethoxybenzoic; 12, 4-hydroxycinnamic; 13, 3-hydroxycinnamic; 14, 4-hydroxy-3-methoxycinnamic; 15, 4-hydroxy-3,5-dimethoxycinnamic; 16, 2-hydroxycinnamic. Peak 17 is cinnamic acid.

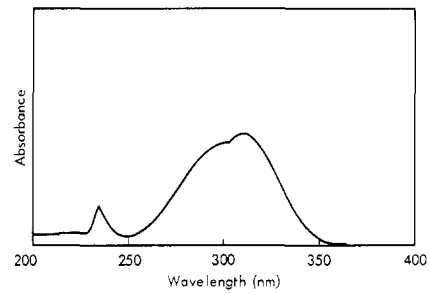

Figure 3. Spectrophotometric spectrum of 4-hydroxycinnamic acid dissolved in 82% aqueous–5% acetic acid–18% acetonitrile, the HPLC solvent composition at the time of its elution. The maximum absorbance among the three HPLC wavelengths used was 300 nm.

Nagel, 1976; Hardin and Stutte, 1980; Banwart et al., 1985), and the same general observations were made—polarity is increased most by hydroxys at the 4-position, followed by those at the 3- and 2-positions. Methoxys and the acrylic groups reduce polarity and increase retention times. Ratios of phenolic acid pairs with the same substituents, one a benzoic and the other a cinnamic acid derivative, can be calculated from data of Table II. For example, the ratio for *p*-hydroxybenzoic and *p*-hydroxycinnamic acid is 2.17. In each instance, of course, the cinnamic acid derivative pair member was retained longer since it was less polar.

The four benzoic acid derivatives said to be universal (Harborne, 1984)—*p*-hydroxy, protocatechuic, vanillic, and syringic—were present in all tissues of all varieties. Six other benzoic acid derivatives were identified, and most of these occurred in low amounts. The four commonly occurring cinnamic acid derivatives—ferulic, sinapic, caffeic, and *p*-coumaric—were also present in all tissues and varieties. Two others were detected, and both occurred in medium amounts (Table II). The very widespread gentisic acid, 2,5-dihydroxybenzoic acid (Tomaszewski, 1960), was not detected in avocados.

In general, all tissues of all varieties had the same array of phenolic acids, and we cannot be certain that the few apparent exceptions were not due to low concentrations. However, examples in which the absence seems genuine include sinapic acid from Fuerte mesocarp and cotyledon, but not from its leaves, and *o*-coumaric acid from Duke leaves.

Some peaks in the HPLC chromatograms remained unidentified even after attempted matches against 11 other phenolic acid standards (see Figure 2). The unidentified peaks may be any of several types of compounds. For example, they could be cis–trans isomers of identified cinnamic acid derivatives, coumarins from the cyclization of the C3 group, other acids with conjugated double bonds such as abscissic acid and unsaturated fatty acids, or various phenylacetic acids that also absorb in the ultraviolet. One possible unidentified compound that is more polar than gallic acid and with the same parent chromophore is digallic acid, which has five hydroxys. Others may be unknown naturally occurring phenolic acids.

The only attempt to quantify the phenolic acids was to score the heights of peaks as low, medium, or high in the chromatograms of each peak's greatest absorbance (see Table II). Absolute values probably vary widely with the

Table III. Avocado Leaf Tissue Phenolic Acids Identified by TLC, Solvent Systems, and R_f's (×100)

phenolic acid	solvent system[a]				
	BMA	BMA1	EMT	TCA	TEF
4-hydroxybenzoic		28	41		60
2,3-dihydroxybenzoic	56	36			59
2,4-dihydroxybenzoic	53	35			
2,6-dihydroxybenzoic		6			54
3,4-dihydroxybenzoic		24			51
3,5-dihydroxybenzoic	25	14			
3,4,5-trihydroxybenzoic		10			
3-hydroxy-4-methoxybenzoic	57	38			
4-hydroxy-3-methoxybenzoic		37	67		
4-hydroxy-3,5-dimethoxybenzoic		49			
2-hydroxycinnamic				36	
3-hydroxycinnamic		36			
4-hydroxycinnamic		42			
3,4-dihydroxycinnamic	41	24			
4-hydroxy-3-methoxycinnamic		55			
4-hydroxy-3,5-dimethoxycinnamic	61	47			

[a] Solvent systems: BMA = benzene–methanol–acetic acid, 90:16:8; BMA1 = the same components, 35:4:2; EMT = ether–methanol, 18:1, used with plates first treated by dipping in saturated sodium phosphate; TCA = toluene–chloroform–acetone, 8:5:7; TEF = toluene–ethyl formate–formic acid, 50:40:10.

season and physiological state of the tissue.

Detection of phenolic acids in extracts by paper chromatography and TLC requires, in comparison with HPLC, considerably greater concentrations of extracts. Perhaps this is another reason that so few phenolic acids had heretofore been reported. Identification of a phenolic acid was considered confirmed when the standard and an extract spot migrated to the same position on a plate and had identical UV fluorescent characteristics. In addition, cospotting of extract and standard had to result in a single symmetrical spot with no hint of a dumbell shape. All 16 phenolic acids identified by HPLC and spectrometry were also identified by TLC (Table III), and most were identified in more than one solvent system.

HPLC is a very useful and efficient method for the study of plant phenolics and can provide considerable information about the phenolic acid composition of suitably prepared extracts. In addition, preliminary studies indicate that HPLC data can lead to further analyses using gas chromatography, with or without mass spectroscopy, to quantify the phenolic acids and to identify additional compounds in derivatized extracts.

ACKNOWLEDGMENT

We thank B. O. Bergh of the University of California, Riverside, CA, for providing all the plant materials, S. R. Gollapudi, Lester Mitscher, Robert Hanzlik, John Landgrebe, and Albert Burgstahler for advice on various technical aspects of this study, and Rose Wessling for excellent technical assistance. This work was supported by a U.S.–Israeli Binational Agricultural Research and Development grant.

Registry No. *p*-Hydroxybenzoic acid, 99-96-7; *o*-pyrocatechuic acid, 303-38-8; β-resorcylic acid, 89-86-1; γ-resorcylic acid, 303-07-1; protocatechuic acid, 99-50-3; α-resorcylic acid, 99-10-5; gallic acid, 149-91-7; isovanillic acid, 645-08-9; vanillic acid, 121-34-6; syringic acid, 530-57-4; *o*-coumaric acid, 583-17-5; *m*-coumaric acid, 588-30-7; *p*-coumaric acid, 7400-08-0; caffeic acid, 331-39-5; ferulic acid, 1135-24-6; sinapic acid, 530-59-6.

LITERATURE CITED

Amerine, M. A.; Ough, C. S. *Wine and Must Analysis*; Wiley: New York, 1974.
Banwart, W. L.; Porter, P. M.; Granato, T. S.; Hassett, J. J. *J. Chem. Ecol.* **1985**, *11*, 383–395.
Brune, W.; van Lelyveld, L. J. *Phytopath. Z.* **1982**, *104*, 243–254.
Gartlan, J. S.; McKey, D. B.; Waterman, P. G.; Mbi, C. N.; Struhsaker, T. T. *Biochem. Syst. Ecol.* **1980**, *8*, 401–422.
Golan, A.; Kahn, V.; Sadovski, A. Y. *J. Agric. Food Chem.* **1977**, *25*, 1253–1260.
Gross, G. G. "Phenolic Acids". In *The Biochemistry of Plants. Secondary Plant Products*; Conn, E. E., Ed.; Academic: New York, 1981; Vol. 7, pp 301–315.
Harborne, J. B. *Biochemistry of Phenolic Compounds*; Academic: New York, 1964.
Harborne, J. B. *Phytochemical Methods*; Champman and Hall: New York, 1984.
Hardin, J. M.; Stutte, C. A. *Anal. Biochem.* **1980**, *102*, 171–175.
Janzen, D. H.; Waterman, P. G. *Biol. J. Linn. Soc.* **1984**, *21*, 439–454.
Julkunen-Tiitto, R. *J. Agric. Food Chem.* **1985**, *33*, 213–217.
Kahn, V. *J. Sci. Food Agric.* **1975**, *26*, 1319–1324.
Kahn, V. *J. Sci. Food Agric.* **1977**, *28*, 233–239.
Loomis, W. D. In *Methods Enzymol.* **1974**, *31*, 528–545.
Lowman, M. D.; Box, J. D. *Aust. J. Ecol.* **1983**, *8*, 17–25.
Prabha, T. N.; Patwardhan, M. V. *J. Food Sci. Technol.* **1980**, *17*, 215–217.
Ramirez-Martinez, J. R.; Luh, B. S. *J. Food Sci. Agric.* **1973**, *24*, 219–225.
Ribereau-Gayon, P. *Plant Phenolics*; Hafner: New York, 1972.
Robinson, T. "Aromatic Compounds". In *The Organic Constituents of Higher Plants*; Burgess: Minneapolis, MN, 1963; pp 45–69.
Roston, D. A.; Kissinger, P. T. *J. Liq. Chromatogr.* **1982**, 5 (Suppl. 1), 75–103.
Sokal, R. R.; Rohlf, F. J. *Biometry*; Freeman: San Francisco, CA, 1969.
Steele, J. W.; Bolan, M.; Audette, R. C. S. *J. Chromatogr.* **1969**, *40*, 370–376.
Tomaszewski, M. *Bul. Acad. Polon. Sci.* **1960**, *8*, 61–65.
Torres, A. M. *J. Hered.* **1984**, *75*, 300–302.
Van Lelyveld, L. J.; Alcock, C. M.; Nel, E. *J. Hortic. Sci.* **1981**, *56*, 285–288.
Van Lelyveld, L. J.; Gerrish, C.; Dixon, R. A. *Phytochemistry* **1984**, *23*, 1531–1534.
Wulf, L. W.; Nagel, C. W. *J. Chromatogr.* **1976**, *116*, 271–279.

Received for review November 24, 1986. Accepted June 4, 1987.