Phenolic Constituents in Core Tissues and Ripe Seed of McIntosh Apples

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Two major phenolic constituents of the core tissues and seeds of McIntosh apples were identified as chlorogenic acid and phloridzin by means of paper partition chromatography. Phloretin, caffeic acid, p-coumaric acid, phloretic acid, and traces of ferulic acid were identified after acidic and alkaline hydrolysis of the core tissue extracts. Hydrolysis of an alcohol-soluble fraction of seed extracts yielded p-hydroxybenzoic acid. All of the phenolic acids identified in the core tissue, except ferulic acid, were oxidized in the presence of crude apple phenolase and may be considered as possible substrates in enzymatic core-browning reactions.

A DISCOLORATION which often occurs in the core area of McIntosh apples during cold storage at 40° F. or below is known as "core flush" or "brown core" and may be due to oxidative enzymic browning reactions. Therefore a first step in studying the causes and nature of this low temperature disorder was to identify some of the simpler phenolic constituents of the core tissues.

Certain polyphenolic substances such as catechins, flavonol glycosides, leucoanthocyanins, and chlorogenic acid occur in apple fruit (*11, 12*) and some simpler phenolic acids have been found in the leaf tissue, mainly in a bound form (*7*). Phloridzin, a dihydrochalcone glucoside, is a major phenolic constituent of the leaves, bark, roots, and seeds of apple (*6*) and is apparently restricted in the subfamily Pomoideae to the genus *Malus*. It has not previously been reported as a constituent of the fruit.

Phenolic acids are of widespread occurrence in plants and exist in the cells combined as labile esters or glycosides for the most part. Acids, containing the acrylic or propanoic side chain, have been reported to be associated with the metabolism and interactions of indole-3-acetic acid (IAA) during growth processes (*4, 13*).

Most phenolic acids are phytotoxic (*8*), but are probably harmless to the plant because in the native state they are glycosides or esters. If during storage and senescence these compounds were hydrolyzed, toxic products could accumulate in the tissue, possibly killing cells and serving as substrates for enzymatic browning reactions.

Methods and Materials

Material for core tissue extracts was obtained from apples picked 2 to 3 weeks prior to the regular harvest date, picked at the regular harvest date, and stored for 2 months at 32° F. Extracts were prepared from the seeds and seed parts removed from apples stored at 32° F. for two months.

The phenolic acids used as markers were obtained from commercial sources. After recrystallization from hot water they gave sharp melting points and were chromatographically pure when tested.

Extraction. ETHER EXTRACT. Some of the early work was accomplished using the peroxide-free diethyl ether extract described for indole acids (*3*).

ETHYL ACETATE EXTRACT. This method was devised to minimize enzymic and acid-catalyzed hydrolysis of labile compounds and yield the free phenolic compounds only. The tissues were frozen and ground at approximately −60° C. Fifty grams of fresh core tissue slices were placed in the stainless steel chamber of a Servall Omnimixer containing cold (−60° C.) ethyl acetate (200 ml.). The chamber was clamped in a Dewar flask containing an ethanol–dry ice mixture, where the tissue froze almost immediately. Fifty grams of anhydrous sodium sulfate were added to the mixture, which was blended for 3 minutes in the Omnimixer, then filtered, and the solution was evaporated to a small volume under reduced pressure. Aliquots of this solution were used for chromatography.

SEED EXTRACTS. The same procedure was used to extract seed coats and embryos of ripe apple seeds. At the same time 250 grams of ripe seeds were dried in a low temperature oven, suspended in boiling 80% ethanol, then transferred to a blender and homogenized for 3 minutes. After filtration, the extract was evaporated to approximately 25 ml. and streaked on Whatman 3 MM filter paper for chromatography. Smaller aliquots were also used for spotting.

HYDROLYSIS. Hydrolysis to yield bound phenols was effected with either 2*N* NaOH at room temperature for 5 hours or 2*N* HCl in a boiling water bath for 1 hour, after the ethyl acetate or ethanol had been removed under reduced pressure. When the solutions were cooled and acidified, as necessary, the hydrolyzates were again extracted twice with ethyl acetate and the combined extracts evaporated to a small volume for chromatography.

Chromatography. One-dimensional chromatograms of the material contained in the ether extracts were developed in the solvents previously described (*3*). The cold ethyl acetate extracts and hydrolyzates were subjected to two-dimensional chromatography on Whatman No. 1 filter paper, using the ascending technique. The components were well separated when the solvent front climbed 12 inches above the starting line. The papers were run in the first direction using benzene–propionic acid–water (2:2:1) and then in the second direction using 2% acetic acid. The air-dried papers were examined under short-wave ultraviolet light (mineralite lamp) for fluorescence and then sprayed with either diazotized sulfanilic acid or diazotized *p*-nitroaniline (*2*). Unknown substances in the extracts were identified by comparison with the behavior of authentic compounds in all cases.

APPLE PHENOLASE PREPARATION. A crude phenolase preparation (PPO), described by Siegelman (*9*) as suitable for use as a chromatographic reagent, was sprayed on both two-dimensional chromatograms of an apple core extract and chromatograms containing standard phenolic substances known to occur in apples.

Results and Discussion

Core Tissues. In preliminary studies where one-dimensional chromatograms of both core and cortex tissue extracts were used, it was observed that the concentration of phenolic acids in the core tissues far exceeded that of the cortex. This might represent total phenolic acids, since the extracts were obtained by extraction with diethyl ether and possibly enzymic hydrolysis liberated acids that occurred naturally as glycosides or esters.

There was essentially no qualitative difference in phenolic acids of preharvest and stored apples. The R_f values, color reactions, and ultraviolet fluorescence data for phenolic constituents and their distribution as free or bound forms in the core tissues and seeds are outlined in Tables I and II.

One of the major constituents of the ethyl acetate extract of the core tissues was phloretin-2-β-glucoside (phloridzin), which had never been identified in the fruit but which is a major constituent of bark, roots, seeds, and leaves of various varieties of apples, including McIntosh (*6*). Hutchinson and coworkers (*6*) found this glycoside in concentrations as high as 10% of the dry weight of roots of *Malus robusta*. Its concentration in the core tissues of McIntosh apples, however, was estimated to be in the order of 300 to 400 p.p.m. (on a fresh weight basis), as determined spectrophotometrically.

Phloridzin was identified by color reactions with diazotized sulfanilic acid, diazotized *p*-nitroaniline, and phenolase (PPO) spray (Table I) and with Ehrlich and DMCA reagents (*3*). The R_f values in four solvent systems agreed with those of standard phloridzin obtained from the Nutritional Biochemical Corp. On chromatograms of acid hydrolyzates obtained from core tissue extracts, phloridzin was detected as phloretin, the aglycone, which had other R_f values (Table I).

One other phenolic substance in the ethyl acetate extract of the core tissue was identified by its behavior on the chromatogram, blue fluorescence, weak color reaction with both diazonium salts, and R_f values in four solvents (Table I) as chlorogenic acid. Moreover, the ultraviolet absorption spectrum of the eluate of the fluorescent spot on the chromatogram was identical to that of a standard chlorogenic acid solution. Chlorogenic acid is widespread in plant tissue, is a well known constituent of apples, and is considered to be the major substrate in the phenolase reaction which takes place at the surface of cut fruit (*5*).

Other unknown spots present on two-dimensional chromatograms of ethyl acetate extracts could not be definitely identified. One of these gave a spectral curve in the ultraviolet region similar to chlorogenic acid, and reacted immediately after spraying with apple phenolase preparation. This substance also remained on the origin when benzene–propionic acid–water (2:2:1) was used as a solvent, as did chlorogenic acid. These findings suggest that this substance is an isomer of chlorogenic acid. Small amounts of isochlorogenic acid have been found in apple fruit as well as the depside, *p*-coumarylquinic acid (*10*).

After hydrolysis of the ethyl acetate extract with 2*N* hydrochloric acid, phloretic acid, caffeic acid, chlorogenic acid, and *p*-coumaric acid were identified. The caffeic acid and *p*-coumaric acid were derived either from acid-labile glycosides or from the depsides, chlorogenic and *p*-coumarylquinic acids, respectively. Hydrolysis of the extract at room temperature with 2*N* sodium hydroxide yielded large amounts of *p*-coumaric acid and phloretic acid, and traces of ferulic acid, indicating that these acids occur in the core tissues as alkali-labile esters. The phloretic acid possibly was derived through alkaline cleavage of phloridzin.

Ibrahim and Towers (*7*) found phloretic acid in apple leaves after acidic hydrolysis and considered it to be derived from acid-labile glycosides in the leaf. In the core tissues of McIntosh apples, picked 2 weeks prior to the regular harvest date, another substance thought to be *p*-hydroxyphenylacetic acid was tentatively identified by color reaction and R_f values on chromatograms of acid hydrolyzates (Table II).

Seeds. The phenolic acids in apple seeds (Table I) resemble closely those found in core tissue. Ferulic acid, *p*-coumaric acid, and phloretic acid occurred as glycosides or esters in both core tissue and seed. *p*-Hydroxybenzoic acid, which was not apparent in the core tissues, was released from seeds after acid and alkaline hydrolysis and may be present in a combined form. *p*-Hydroxybenzoic acid was found by Ibrahim and Towers (*7*) in leaf tissue, supporting their view that it is present in leaves as an acid-labile glycoside.

Phenolase Reactions. When chromatograms of the naturally occurring phenolic acids of McIntosh core tissue were sprayed with a crude apple phenolase preparation (PPO), yellow, brown, and red-brown spots were obtained

Table I. Chromatographic Behavior of Phenolic Acids

Compound	Solvents,[a] R_f A	B	C	D	Colors[b] PPO	Diaz-S	Diaz P-N	UV
Phloridzin	0.03	0.40	0.33	0.65	Yellow	Yellow	Yellow-brown	—
Phloretin	0.15	0.10	0.50	0.92	Yellow	Yellow	—	—
p-Hydroxybenzoic acid	0.60	0.65	0.19	—	—	Yellow	—	abs
p-Coumaric acid	0.70	0.45	0.30	0.90	Red	Red	Blue	abs
Phloretic acid	0.70	0.76	0.45	0.88	Red	Red	Purple	abs
Ferulic acid	0.80	0.40	0.20	0.90	—	Purple	Blue-green	fl
Caffeic acid	0.30	0.35	0.02	0.77	Red-brown	Buff	—	fl
Chlorogenic acid	0.03	0.59	0.02	0.54	Brown	Light buff	—	fl
p-Hydroxyphenylacetic acid	0.60	0.78	0.39	—	Brown	Red	Purple	abs

[a] A, benzene–propionic acid–water (2:1:1).
B, 2% acetic acid.
C, 2-propanol–ammonia–water (8:1:1).
D, butanol–acetic acid–water (4:1:1).
[b] PPO, phenolase spray (Siegelman).
Diaz-S, diazotized sulfanilic acid.
Diaz P-N, diazotized *p*-nitroaniline.
abs, absorption.
fl, fluorescence.

Table II. Distribution of Phenolic Acids in Core Tissues and Seeds

Compound	Ether Extract	Ethyl Acetate Extract: Free	NaOH	HCl	Ethanol Extract	Hydrolysis: 2N HCl	2N NaOH
		In Core Tissues				In Seeds	
Phloridzin	++	++	Trace	−	+++[a]	−	++[a]
Phloretin	−	−	−	++	−	+++	Trace
Chlorogenic acid	+	++	−	+	++	−	−
Caffeic acid	−	−	−	++	−	+	−
p-Coumaric acid	+	−	++	+	−	++	+
Ferulic acid	+	−	+	−	−	++	Trace
Phloretic acid	+	−	++	Trace	−	+	++
p-Hydroxyphenylacetic acid	−	−	−	+[b]	−	−	−
p-Hydroxybenzoic acid	−	−	−	−	−	+	+

[a] In seed coat, not in embryo.
[b] In acid hydrolyzate of extract from apples taken 2 weeks prior to regular harvest date.

Table III. Action of Polyphenolase (Apple) in Core Tissue

Compound	Degree of Reaction[a]	Color (PPO Reagent)[b]
Phloridzin	+	Yellow
p-Coumaric acid	+	Brown
Ferulic acid	−	No color
Caffeic acid	++	Brown
Chlorogenic acid	++	Brown
Phloretic acid	+	Red-brown
p-Hydroxyphenylacetic acid	+	Red-brown
Phloretin	+	Yellow

[a] + Reaction after 20 minutes in humid chamber.
++ Reaction within 1 minute.
− No reaction.
[b] Polyphenolase.

except with ferulic acid (Table III). *p*-Coumaric acid, phloretic acid, and *p*-hydroxyphenylacetic acid showed up as reddish brown spots after the chromatogram was placed in a humid chamber for 20 minutes, but the dihydric phenols, chlorogenic acid and caffeic acid, reacted immediately, yielding brownish colorations. Phloridzin and its aglycone, phloretin, were oxidized but gave yellow rather than brown colors.

Most of the phenolic acids present in core tissues are potential substrates for apple phenolase (both cresolase and catecholase). Phloridzin gave only a yellow color with apple phenolase and, although a major constituent of core tissues, is not a browning substrate. Its chemical or enzymatic degradation, however, produces phenolic acids (*1*) which brown readily.

This high concentration of phenolic compounds in the core area could account for the browning being localized there. Further research is required, however, to determine whether free phenolics are actually released during the very late stages of 32° F. storage and whether the specific enzymes capable of this release mechanism are present in stored fruit.

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LEAF PROTEINS AS FOODSTUFFS

Amino Acid Composition of Leaf Protein Concentrates

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Leaf protein concentrates, extracted from green leaves by mechanical methods, were analyzed for their amino acid composition. Samples from nine plant species harvested under different conditions of fertilization and maturity did not show large variations in amino acid content. On an amino acid compositional basis, leaf protein concentrate should be a well balanced source of dietary protein, if supplemented with synthetic methionine. Comparisons with other foodstuff proteins are presented and the future role of leaf protein concentrates in increasing the supply of high quality protein is discussed.

IT HAS been estimated that there are 1800 million hungry people in the world whose major nutritional deficiency is an inadequate supply of protein (*27*). Inasmuch as lands suitable for agriculture are already near maximum use, the protein supply could most easily be increased by more efficient utilization of existing protein. Almost all the amino acids consumed by man are initially synthesized in the leaves of green plants. There is some loss when they are concentrated into protein of seeds or tubers and a very great waste when plant protein is converted into animal products for human consumption (*18*). In many operations technological progress has replaced the animal. Perhaps efficiency could be greatly increased if mechanical power and technology were used to separate plant protein from fiber and concentrate it into consumable forms. Mechanical methods of separating and concentrating leaf proteins have been described by Pirie and associates (*3, 22–26, 28, 29*) and Chayen *et al.* (*8*). Leaf protein concentrate (LPC) can be extracted and processed, leaving a residue which might have value as a ruminant feed. This paper presents amino acid analyses of LPC extracted by mechanical processes.

Chibnall, Rees, and Lugg (*9*) reported that proteins extracted by various methods from green plants showed only slight differences in amino acid composition. Wilson (*35*) found that the amino acid composition of leaf proteins from different species was remarkably similar even at various stages of growth and noted that differences in maturity and nitrogen fertilization caused a marked change in the nonprotein nitrogen fraction, depending on the species.

While determination of amino acid provides a guide to the quality of proteins, the final answer to whether LPC could be used as a source of dietary protein lies in feeding experiments. Early feeding experiments suggested a low nutritive value (*4–7, 10–12, 17*), which now seems to have been the result of improper processing of the leaf protein. Recently Duckworth (*15*) found the gross protein value of LPC was 82 compared to 74 for soybean meal. He showed that heating LPC above a critical temperature of about 84° C. drastically reduced the gross protein value. This may explain some of the poor results obtained in earlier feeding trials. Barber (*1*) reported that LPC was nutritionally equal to white fish meal when fed to pigs. Duckworth (*14*), in similar experiments, confirmed Barber's data and concluded that inclusion of relatively small amounts of LPC in the diet improved the efficiency of feed conversion. These data indicate that the feeding value of LPC may be relatively high, if care is taken in processing. Waterlow (*34*) fed human infants recovering from