terpene fraction of oil distilled from the gum oleoresin of *Bosuellia serrata* Roxb. and of oil of the leaves of the Formosa Hinoki tree. Those oils were not available to us. Ikeda reported cedar leaf oil (thuja oil) to contain 13.0% α-thujene and oil of cubeb to contain 13.2% α-thujene (*6*). α-Pinene was reported to occur in these oils in quantities of 15.6 and 12.1%. These oils were examined as possible sources of α-thujene, but we failed to resolve α-thujene from α-pinene on the preparative gas chromatograph. [Klouwen and Ter Heide recently reported that α-thujene could be separated from α-pinene on Apiezon L (*7*). This has since been confirmed by the authors.] A sample of α-thujene isolated by Ikeda from *Eucalyptus dives* was obtained from Teranishi. The infrared spectrum (Figure 6) is in agreement with that published in the Sadtler tables; the absorption observed in the ultraviolet is consistent with this structure.

Acknowledgment

The authors are grateful to Roy Teranishi, Western Regional Research Laboratory, Albany, Calif., for access to his library of infrared spectra. They thank R. A. Bernhard, of this department, for samples of orange and lemon oil.

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Received for review February 28, 1964. Accepted May 29, 1964.

ASTRINGENCY PRINCIPLES

Changes in Phenolic Content in Persimmons during Ripening and Processing

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Changes in concentration of phenolic substances extractable with methanol and aqueous methanolic solution were compared with histochemical observations and organoleptically observed changes in astringency. Decrease in extractability of phenolics by methanol accompanied loss in fluidity of cellular tannins and decrease in astringency. Oxidation was found to be responsible for loss in astringency on air drying of sliced persimmons or high speed blending. Pureeing and freezing of astringent tissue resulted in loss of astringency and decrease in phenolics even in presence of added ascorbic acid or sulfite. The leucoanthocyanin isolated from methanolic extracts of astringent tissue contained both leucodelphinidin and leucocyanidin, apparently present together in the molecule.

THE disappearance of astringency with ripening in persimmon fruit is one of the most pronounced ripening changes known. The nature of the changes occurring during loss in astringency has been investigated by botanists, plant physiologists, and chemists, but our knowledge of the composition and structure of the phenolic compound involved and of changes in its molecular structure during ripening is still incomplete.

Until very recently but few quantitative chemical data were available on changes in tannin content during ripening of astringent and nonastringent varieties. The earliest studies reported were those of Bigelow *et al.* (*2*) in the United States. Only a few of the papers published in Japan give data on "tannin" content (*33*); and even in the more recent work tannins are determined by the nonspecific acid permanganate titration procedure (*18, 19*). While it is now fairly well established that the organic tannin-like astringents of food are related to leucoanthocyanins (*1, 30*), their molecular structure is still unknown. Ito and Oshima (*14*) reported the isolation of a leucoanthocyanin identified as leucodelphinidin-3-glucoside as the main component of persimmon tannin. Their preparation was astringent at a concentration of 20 mg. per 100 ml., but apparently was of high molecular weight, because it was not chromatographically mobile in the usual phenolic solvents. Siegelman and Craft (*26*), who also reported isolation of a partially purified persimmon leucoanthocyanin, estimated its molecular weight to be 50,000.

Lloyd (*16*) first observed that the tannin present in specialized tissue cells is fluid in unripe astringent persimmons and easily spreads over cut surfaces, but in ripe fruit the tannin cells no longer

rupture readily, they resist plasmolysis, and their tannin masses shrink and become ovular. He suggested that the insolubility of the tannin in the ripe fruit was due to combination with cellulose-like polysaccharide present in the tannin cell (*17*). Siegelman and Craft (*26*), on the basis of their observation that astringent persimmon tissue lost astringency when blended at high speeds in water or its own juice but not when blended at low speeds in isotonic solution, proposed that disappearance of astringency on ripening involved changes in macromolecular structure. Swain (*29*) found that monomeric leucocyanidin was only slightly astringent but its oligomer was markedly so. Goldstein and Swain (*10*) for this reason suggested that loss in astringency occurred on ripening as a result of polymerization reactions in which the astringent oligomers were converted into insoluble and unreactive polymers.

Hillis and Swain (*11*) and subsequently Goldstein and Swain (*10*) used the difference in extractability of phenolics in 100 and 50% methanol as a measure of changes in molecular size. They assumed that the monomers and oligomers of leucoanthocyanins were soluble in 100% methanol but that the more highly polymerized products were not. In view of the present recognition of leucoanthocyanins as astringent phenolics, they used procedures for measuring total phenolics, vanillin-reactive phenolics, and leucoanthocyanin.

These procedures for estimating changes in total leucoanthocyanin content and in their molecular size were only recently applied to persimmons.

After our data obtained by these methods on changes occurring during ripening of both astringent and non-astringent varieties as well as during processing (dehydration, freezing, and freeze-drying) were presented, two recent papers (*10*, *20*) included some quantitative data on persimmons. Since our observations are more detailed, and include varieties not previously examined and data on effect of processing as well as observations on the astringent phenolic we have isolated, these data are presented here.

Experimental

Varieties and Treatments Investigated. Several varieties of astringent persimmons were examined, including Hachiya (seedless), Niu Nai (seeded), and Chein Tsein (seeded), and the non-astringent Fuyu (seeded). The fruit were either obtained from the experimental orchard at Davis or purchased locally as shown in Table I. The Chein Tsein were selected in the hard astringent state harvested near Davis on December 14, 1962, and dehydrated with and without sulfuring at Davis. At the time of dehydration, purees were prepared and frozen with added sulfite or ascorbic acid.

Table I. Variety, Source, and Ripeness of Persimmons Analyzed

Variety	Ripeness	Source	Date Examined
Fuyu	Hard	Davis	Oct. 25, 1962
	Soft	Davis	Nov. 20, 1962, after storage in carbon dioxide
Hachiya	Firm–1	Market	Nov. 20, 1962
	Firm–2	Market	Nov. 25, 1962, firm ripe slightly astringent fruit
	Soft–1	Market	As above, but after storage until Dec. 4, 1962
	Soft–2	Market	Soft fruit obtained Dec. 4, 1962
Niu Nai	Hard	Davis	Oct. 25, 1962
	Soft	Davis	Nov. 20, 1962, after storage in carbon dioxide

More unripe and astringent Hachiya persimmons were obtained from Davis on September 26, 1963, and were used in alcohol-ripening and freeze-drying experiments. The fruit from Davis was examined on arrival and after ripening at room temperature in an atmosphere of carbon dioxide. Fruit also was ripened by sealing in aluminum foil and storing at room temperature. The fruit of the Niu Nai variety on receipt was yellow-green in color, and averaged 206 grams per fruit, 7.7 cm. in diameter, and 6.9 cm. high; its soluble solids content, determined refractometrically, was 24% and the fruit tissue had a pH of 4.7. The fruit of the Fuyu variety were more immature, greenish yellow, and smaller and harder than the Niu Nai. Their average weight was 150 grams, average diameter 8.3 cm., and average height 6.2 cm.; their soluble solids content was 13.2% and their pH 5.2. After ripening in an atmosphere of carbon dioxide, the soluble solids content of the Niu Nai fruit tested about 22% and that of the Fuyu 17%.

Hachiya fruit were ripened also by treatment with alcohol. Hachiya fruit harvested in the fall of 1963, of fairly uniform size and color, weighing from 165 to 265 grams, with an average of 215 grams, were sealed by wrapping with aluminum foil. One milliliter of 40% ethanol was added to the stem end per 100 grams of fruit before sealing. The sealed fruit was stored in a covered jar for 9 days, most of the astringency being lost in 3 days at room temperature.

The Chein Tsein fruit were harvested near Davis on December 14, 1962. For dehydration 33.2 kg. of firm, fully ripened, but astringent fruit were used, the stem tissue and seeds were removed, and the remaining tissue was sliced to yield 29.2 kg. of unpeeled prepared fruit. When 14.1 kg. of sliced fruit were dehydrated in an experimental countercurrent peach dehydrator at a dry bulb temperature varying from an initial level of 48.9° C. (120° F.) to a final level of 64.4° C. (148° F.) over 17.5 hours, the yield was 4.27 kg. of dried unsulfured fruit; 15.2 kg. of sliced fruit were sulfured for 6 hours and then dehydrated as above to yield 4.21 kg. of sulfured fruit. Two lots of fruit from the same sample were stemmed, seeded, and pureed; to one lot of puree (4.55 kg.) 4.5 grams of ascorbic acid and to another lot (4.55 kg.) 8.3 grams of sodium bisulfite were added. The purees were frozen and stored at −28.9° C. (−20° F.) and the dehydrated fruit at 0° C. (32° F.). The sulfur dioxide content of the sulfited Chein Tsein puree was determined by the Ponting and Johnson (*21*) volumetric procedure and found to be 62.9 mg. of SO_2 per 100 grams as free and 96 mg. as total SO_2; that of the dried fruit was 440 mg. as free and 817 mg. as total on February 27, 1963, when these samples were investigated for distribution of extractable phenolics.

Davis-grown Hachiya fruit of the 1963 season was also freeze-dried after dipping in solutions containing 0 to 1000 mg. of SO_2 per liter. The persimmon fruit was peeled, cut into slices about 4 mm. thick, immersed in sodium metabisulfite solutions, and then freeze-dried. From 165.5 grams fresh weight there were obtained from 30.0 to 35 grams of freeze-dried tissue.

Histological Examination. The distribution of phenolics in the persimmon fruit tissue was determined qualitatively by testing cut sections with the alcoholic vanillin and concentrated hydrochloric acid reagent introduced by Lindt (*15*) and used by Bradfield and Penney (*3*) and Swain and Hillis (*31*) and by the Reeve reagent (*22*).

The tissues for microscopic examination were prepared by pressing small sections between the cover slip and slide and examined before and after staining with the Reeve reagent.

Extraction of Phenolic Substances. The tissue extracts for the quantitative analysis of phenolic substances were prepared by hot methanol extraction (*10*, *11*). The fresh fruit was halved and portions of tissue from the mesocarp region adjacent to seed cavity were cut out with a cork borer. Tissue from four segments was obtained and 2.5-gram portions of composite sample were extracted in triplicate. The cut tissue was weighed out into 25 ml. of methanol and heated to boiling for 10 minutes, the tissue being pressed out with a glass rod to facilitate penetration of solvent and extraction of phenolics. The methanol extract was then decanted from the residue and filtered, and the residue returned to the flask and further extracted with absolute methanol. The tissue was extracted five successive times, in the same manner and the filtrates were combined and made up to 100 ml. The residual tissue from the 100% methanol extraction was re-extracted three times with 50% of methanol and the filtrates obtained were made up to 50 ml. with 50% methanol.

The final residue was of an opaque white color and when tested with Reeve

reagent gave a very faint brown color, indicating that most, but not all, phenolics were removed. When this stained residue was examined microscopically, it was found that the phenolic stain was confined to large tannin cells. To compare the effect of order of extraction, a sample of fresh Hachiya fruit was extracted first with 50% methanol and then with 100% methanol. The 100% methanol extracts, particularly in the initial extraction, filtered slowly and yielded a viscous cloudy filtrate. These had to be centrifuged in a Servall ultracentrifuge (1000 × G) before analysis.

The phenolics extractable from the dehydrated sliced Chein Tsein persimmons were determined by grinding the sliced fruit in a food chopper, through first coarse and then fine cutter, mixing between these operations. An aqueous extract was prepared by boiling 25 grams of the dehydrated ground tissue of sulfured and unsulfured fruit with 400 ml. of water for one-half hour. The extract was then filtered through cotton and diluted to 500 ml. The water extract of unsulfited persimmons was diluted 1 to 25 for the Folin-Denis and vanillin assay, while that from sulfited was diluted 1 to 100. A methanol extract was prepared by heating 50-gram aliquots of ground tissue with 800 ml. of methanol under a reflux condenser for 1 hour. The methanol extract was decanted off and the residue was then re-extracted four times with successive 800-ml. aliquots of methanol. The methanolic extracts were combined to yield 3011 ml. of methanol solution from the unsulfured and 3046 ml. from sulfured fruit. A 50-ml. aliquot was removed and part was diluted 1 to 10 for assay of the Folin-Denis and vanillin-reactive phenolics and aliquots of undiluted methanol extract were used in leucoanthocyanin assay.

In addition to the above water and methanol extraction, 1.5-gram aliquots of the ground dehydrated persimmon tissue were weighed out into 25 ml. of absolute methanol and heated for 10 minutes, and after decanting off the supernatant liquid the residue was re-extracted for four successive times. The combined methanol extract was made up to 100 ml. with methanol. The residue was re-extracted three times with 50% methanol, and this extract also was made up to 100 ml. The methanol extracts from sulfited tissue were diluted 1 to 10 for vanillin and Folin-Denis assay and nonsulfited tissue extracts were diluted 1 to 5.

Similar procedures were used in extracting samples of freeze-dried Hachiya persimmons. These were extracted successively with 100% methanol, 50% methanol, and water. For comparison one lot was extracted only with water at room temperature.

Extracts also were prepared by the Siegelman and Craft procedure (*26*). Fifty- to 250-gram portions of diced fruit were blended with 100 to 500 ml. of 0.3*M* glycerol containing 0.01*M* (ethylenedinitrilo)tetraacetate. When unpeeled hard fruit was used, it was impossible to obtain satisfactory blending at low speeds, but with peeled fruit this

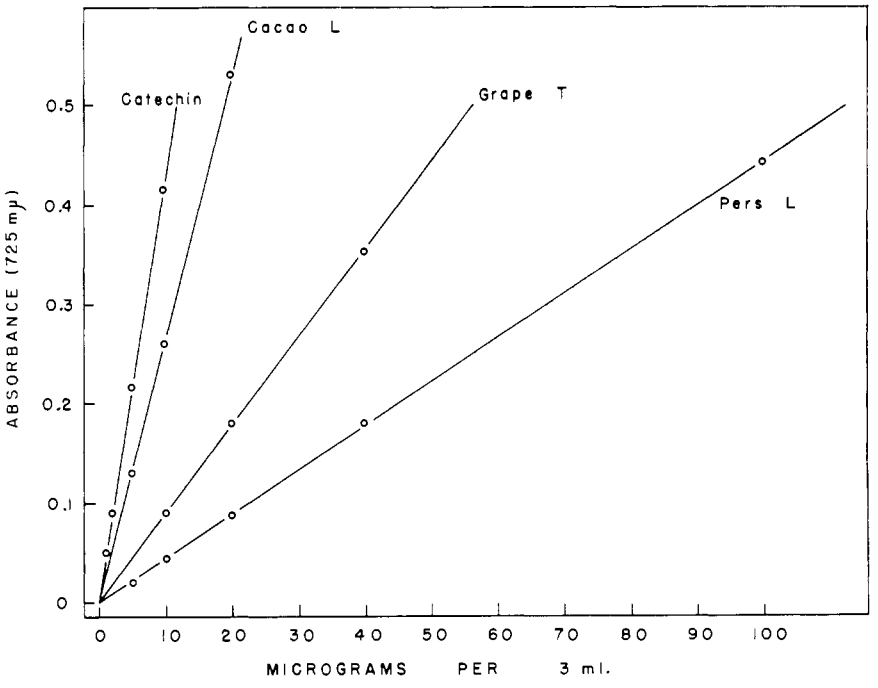

Figure 1. Folin-Denis pigment formation by catechin, cacao leucoanthocyanin, grape phlobatannin, and persimmon leucoanthocyanin

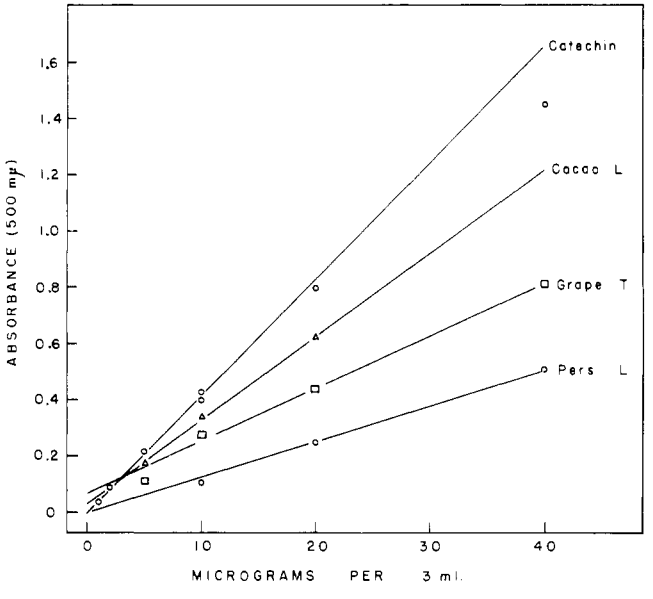

Figure 2. Pigment formation by vanillin reagent with catechin, cacao leucoanthocyanin, grape phlobatannin, and persimmon leucoanthocyanin

was possible. The mixtures were blended at 40 and 100 volts, using a Powerstat voltage controller for 5 minutes, with and without the addition of sodium sulfite at a level of 200 mg. (as SO_2) per kg. of fruit tissue. The blended extracts were stored at 32° F. and later pressed through cheesecloth and centrifuged in a Servall ultracentrifuge for 15 minutes.

Determination of Total Phenolic Substances. The total phenolic content of the extracts was determined by a modification (*10*) of the Folin-Denis (*7*) phosphomolybdate-phosphotungstate procedure. The absorbance of the blue pigment formed was determined in a Beckman Model DU spectrophotometer whose circuit was modified (*4*), against a blank prepared by using 1 ml. of water instead of the test solution. The total phenolic content was expressed as milligrams of catechin, usually per 10 grams of fresh tissue. A comparison of Folin-Denis pigment formation by several leucoanthocyanin preparations with that by catechin is shown in Figure 1. The absorbance increased linearly with concentration but more rapidly with catechin and least so with persimmon

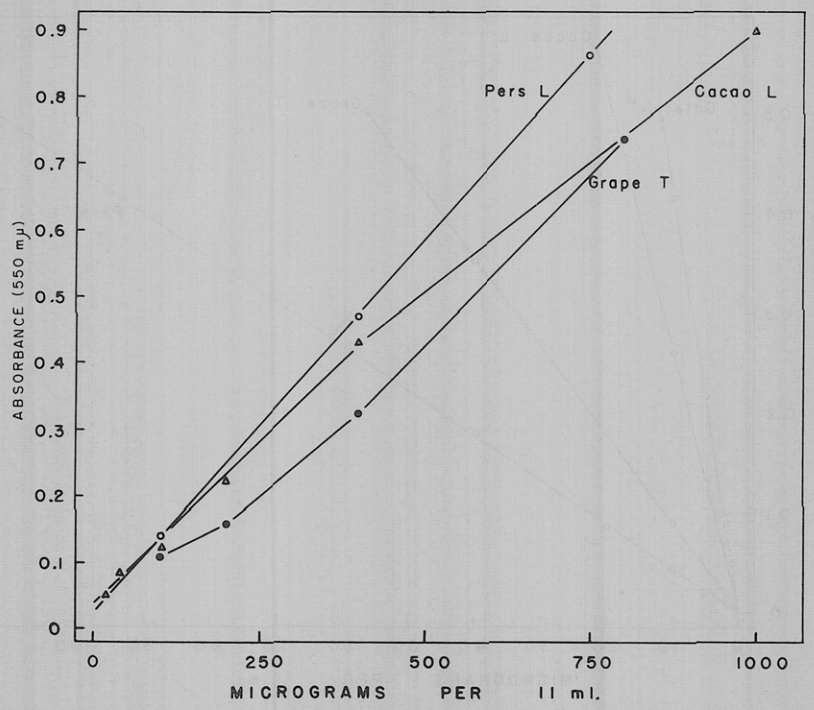

Figure 3. Pigment formation on heating acid butanol solutions of several purified leucoanthocyanin preparations

leucoanthocyanin. Apparently at a higher degree of polymerization less molybdate–tungstate is reduced under the test conditions.

Determination of Flavonols. The vanillin-reactive phenolics were determined by the use of vanillin dissolved in 70% sulfuric acid (*10*, *31*) and reported as catechin by the absorbance at 500 mμ. As shown in Figure 2, the concentration of the red carbonium ions formed from the phloroglucinol residues was greatest with catechin and least with persimmon leucoanthocyanin.

Determination of Leucoanthocyanin. The leucoanthocyanin content was determined from the concentration of anthocyanidin produced by heating in butanol–HCl (*10*, *31*). The concentration of the anthocyanidin formed (delphinidin) was determined by measuring absorbance at 550 mμ against a reagent blank using 1 ml. of water or methanol instead of the phenolic solution. The results obtained with persimmon leucoanthocyanin, shown in Figure 3, indicate a greater conversion into delphinidin than of cacao or grape leucoanthocyanin into cyanidin. Our conversion, however, was less than that previously found (*10*, *31*).

Determination of Molybdate-Reducing Vicinal Hydroxyl Groups. The vicinal di- and trihydroxyl groups were determined by the Goldstein and Swain (*10*) molybdate procedure. As the data in Figure 4 show, the yellow pigment was formed in largest amounts by catechin and in least amounts by persimmon leucoanthocyanin.

Absorption spectra were determined either in a Cary Model 11 recording spectrophotometer or in a Bausch and Lomb Model 505 recording spectrophotometer. Paper partition chromatography and column chromatography were carried out by the usual procedures.

The cacao leucoanthocyanin used was supplied by T. Swain from a preparation obtained by the procedure of Forsyth and Roberts (*8*). The persimmon leucoanthocyanin preparation was supplied by S. Ito, and the grape phlobatannin preparation was supplied by F. De Eds of the Western Utilization Research and Development Unit of the Agricultural Research Service, U. S. Department of Agriculture. It was isolated originally by C. E. Sando and E. D. Walter in 1952 from White Colombard grape pomace, air-dried at 49° C., ground and extracted first with benzene, then with 60% acetone, concentrated under reduced pressure, filtered, extracted with 1-butanol, and precipitated from butanol by petroleum ether. The vanillin was an Eastman Organic Chemicals preparation, purified by recrystallization from water. The D-catechin was an anhydrous preparation obtained from Aldrich Chemical Co. All other chemicals used were of analytical reagent grade.

Results and Discussion

Histochemical Observations. In the tissues of the fresh fruit, both in the hard unripe condition and after ripening, the tannins were confined to large tannin sacs or cells scattered throughout the mesocarp region but absent from the endocarp region. With the vanillin reagent, a cherry-pink pigment was observed in the Niu Nai, and a pink pig-

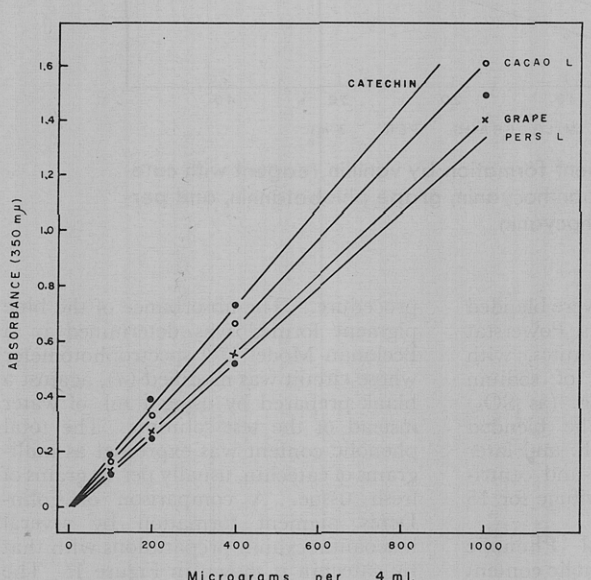

Figure 4. Determination of vicinal di- and trihydroxyl groups by molybdate

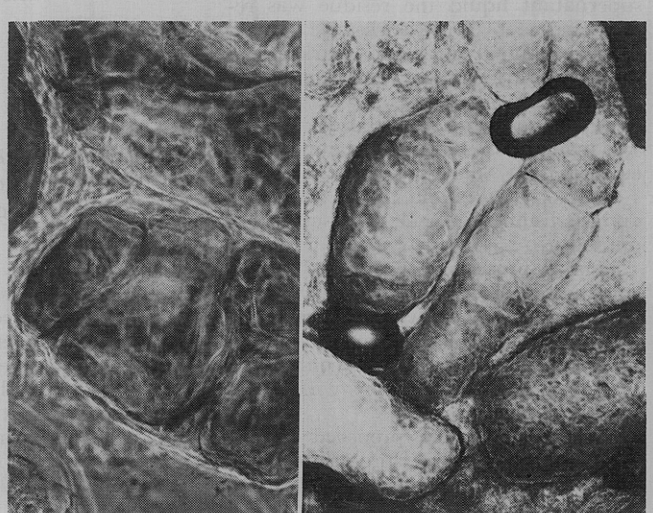

Figure 5. Tannin cells in ripe and unripe persimmon stained with Reeve reagent

Left. Ripe
Right. Unripe

ment in the Fuyu and Hachiya persimmon fruit. While some diffusion of tannins occurred during cutting of the firm fruit, as reported by Lloyd (*16*), pigmentation was localized in the large tannin cells in the mesocarp tissue between the peel and seed cavity. With the Reeve reagent (*22*) a more intense brown pigmentation occurred, indicating gallotannin rather than catechol tannin-type reaction. After storage at room temperature for 3 weeks the vanillin test was less intense but still cherry pink and the Reeve test still gave rather intense localized brown pigmentation. With seeded varieties the distribution of phenolics was essentially that reported by Lloyd (*16*). On ripening, the persimmon fruit changed from green to deep yellow to orange in color, and the mesocarp tissue became soft and gelatinous. The tannin cells still remained intact but appeared shriveled in size.

Typical photomicrographs of tannin cells in hard and ripened Hachiya persimmons are shown in Figure 5. The tannin cells on ripening were, on an average, shortened 22% along the longitudinal axis and 39% radially. In the nonastringent Fuyu persimmon the tannin cells were more thickened even in the green fruit and contained a red pigment, presumably an anthocyanin pigment, which gave the flesh a red speckled appearance. The tannin cells contained both polyphenol oxidase and peroxidase, as was found by Ito (*12*). On exposure to air the cut cells browned. This oxidative browning was not found in hard astringent fruit but began to occur during ripening, reached a maximum just prior to loss in astringency in Hachiya fruit, and again decreased in the fully ripe gelatinous fruit. Typical localized browning is shown in Figure 6. The peroxidase enzyme activity was greater with catechol than with guaiacol as substrate. Catalase activity was present at all stages of ripening but most intense in soft ripe fruit.

Effect of Ripening Dehydration or Freezing. The firm fruit of the Niu Nai, Hachiya, and Chein Tsein varieties were astringent to taste. Ripening of the Niu Nai fruit even when stored at room temperature in a loosely covered container with carbon dioxide was slow and the fruit did not lose all astringency or become typically soft in texture. Softening of fruit tissue has been reported to occur during treatment with carbon dioxide (*24*) but not when the fruit is ripened with alcohol or frozen and stored at freezing temperatures to reduce astringency (*13*, *19*, *28*). The Chein Tsein fruit when obtained was astringent but on dehydration without sulfuring lost its astringency, as reported for other astringent varieties by Cruess (*5*), Cruess and Joslyn (*6*), and Ryerson (*23*). When sulfured and then dehydrated, the fruit still retained astringency. When persimmons are freeze-dried, however, astringency is retained whether the fruit is or is not sulfited. In Japan it is the custom to sulfur whole persimmons lightly before drying (*13*). The usual procedure is to sulfur the fruit to an SO_2 content of about 30 p.p.m. before drying, which is reduced to 8 p.p.m. after drying. Matsui and Murata (*18*) reported that sulfuring up to 389 p.p.m. before drying had no effect on astringency but bleached the color. Astringency, however, is retained when the fruit is heavily sulfured. The tannin cells in dehydrated fruit could not be demonstrated in the sulfured preparation, although they were found intact in the nonsulfured material. Photomicrographs of tannin cells in rehydrated unsulfured and sulfured persimmon are shown in Figure 7. The frozen purees prepared from astringent fruits, however, were completely devoid of astringency, even though oxidative changes were inhibited by addition of sulfite or ascorbic acid. It is not known whether this loss in astringency occurred during pureeing before addition of reducing substances or during freezing.

Distribution of Phenolics in Methanol Extracts. The phenolic content of the Fuyu, Niu Nai, and Hachiya fruit as determined in methanol extracts is shown in Table II. The data summarized indicate that the nonastringent Fuyu contained considerably fewer phenolics than the astringent varieties. Interestingly enough, the phenolic content of the astringent varieties was considerably higher even after ripening, when their astringency had been largely lost. This was particularly true of the second lot of soft Hachiya which, although completely nonastringent, still contained almost ten times as much total phenolics, 8.5 times as much leucoanthocyanins, and 8.5 times as much vanillin-reactive phenolics as the hard Fuyu. While in the Fuyu variety extractability of all phenolic constituents increased appre-

Figure 6. Localized browning in cut persimmons

Left. Hard, unripe
Center. Just prior to ripening
Right. Fully ripe nonastringent Hachiya

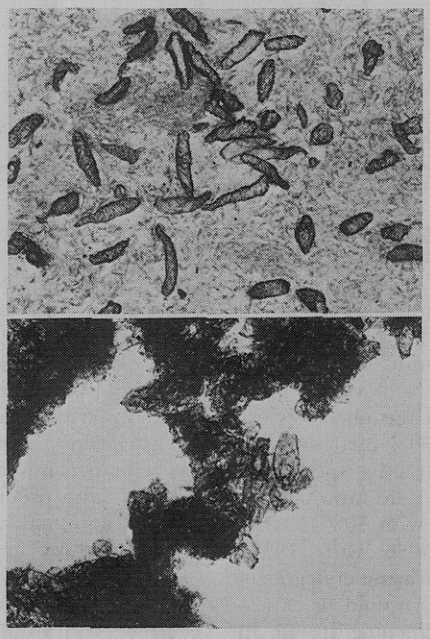

Figure 7. Tannin cells in rehydrated dried Chein Tsein persimmons

Upper. Unsulfured
Lower. Sulfured unstained

Table II. Phenolic Content in Fresh Persimmon Tissue

(As milligrams of catechin per 10 grams of tissue)

Variety	Stage of Ripeness	Methanol Concn., %	Folin-Denis	Leucoanthocyanin[a]	Vanillin	Molybdate
Fuyu	Hard	100	4.7	2.5	1.5	11.4
		50	6.6	10.3	4.2	10.8
		Total	11.3	12.8	5.7	22.2
	Soft	100	5.5	2.7	2.9	
		50	10.6	20.0	6.4	13.4
		Total	16.1	22.7	9.3	
Hachiya	Firm-1	100	170.7	196.0	53.4	
		50	20.5	29.5	11.5	...
		Total	191.2	225.5	64.9	
	Soft-1	100	161.1	248.0	57.1	
		50	20.7	25.4	9.9	...
		Total	181.8	273.4	67.0	
	Soft-2	100	90.3	67.1	36.2	
		50	19.3	30.1	11.7	...
		Total	109.6	97.2	47.9	
Niu Nai	Hard	100	107.8	143.0	32.6	243.0
		50	5.1	5.5	3.0	10.2
		Total	112.9	148.5	35.6	253.2
	Soft	100	53.6	41.8	17.5	118.0
		50	57.1	154.0	27.5	110.3
		Total	110.7	195.8	45.0	228.3

[a] As milligrams of cacao leucoanthocyanin.

Table III. Ratios of Phenolic Contents in Fresh Persimmon Tissue[a]

Variety	Stage of Ripeness	Methanol Concn., %	V/FD	LA/V	FD/MO	Ratio 50:100 FD	Ratio 50:100 V	Ratio 50:100 LA	Ratio 50:100 MO
Fuyu	Hard	100	0.328	1.64		1.41	2.70	4.04	
	Soft	100	0.520	0.96					
	Hard	50	0.627	2.48					
	Soft	50	0.603	3.13		1.93	2.35	7.40	7
Hachiya	Hard-1	100	0.312	3.67		0.146	0.208	0.151	
	Soft-1	100	0.355	4.34					
	Hard-1	50	0.474	2.57					
	Soft-1	50	0.478	2.56		0.128	0.173	0.103	
	Soft-2	100	0.380	1.85					
		50	0.604	2.56		0.207	0.322	0.450	
Niu Nai	Hard	100	0.320	4.40	0.444	0.0474	0.092	0.0386	0.043
	Soft	100	0.336	2.40	0.454				
	Hard	50	0.590	0.50	1.84				
	Soft	50	0.482	0.554	5.61	1.065	1.54	3.83	0.945

[a] V. Vanillin. FD. Folin-Denis. LA. Leucoanthocyanin. MO. Molybdate.

ciably during ripening, there was a slight decrease in total phenolics extracted in Hachiya and Niu Nai varieties on ripening, but this was accompanied by an appreciable increase in leucoanthocyanins and a smaller increase in vanillin-reactive phenolics.

The ratios between the phenolics extracted with 50 and 100% methanol and between the various reactive species are shown in Table III. The ratio of total phenolics extractable with 50% methanol to that extractable with 100% methanol, used as a measure of degree of polymerization (*9*, *10*), would be expected to increase on ripening if loss in astringency was accompanied by polymerization. This definitely occurs in Niu, Nai, but not in Hachiya persimmons; with the nonastringent Fuyu persimmon the ratio increased for all phenolics except the vanillin-reactive ones. With the Niu Nai persimmons a 22-fold increase in this ratio occurred with Folin-Denis values, a 97-fold increase with leucoanthocyanin values, and a 17-fold increase with vanillin values. With bananas, where the change in astringency on ripening is as dramatic as with persimmons, an 8-fold increase in this ratio occurred with Folin-Denis values, a 46-fold increase in leucoanthocyanin values, and 14-fold increase in vanillin values (*10*). With fruit such as peaches, little change in this ratio occurred with Folin-Denis or vanillin values and less than a threefold increase occurred in leucoanthocyanin values. An increase in extractability of phenolics in 50% methanol on ripening would be expected also if the initial treatment with 100% methanol resulted in cleavage of a secondary valence bonding of phenolics by a cellular protein or polysaccharide constituent. This is known to occur when water-insoluble gelatin-tannates are treated with ethanol (*32*).

Table IV. Effect of Order of Extracting with 100% or 50% Methanol on Phenolic Content Extracted from Hachiya Persimmons (Firm-2)

(Results as mg. of catechin per 10 grams of tissue)

Methanol Concn., %	FD	V	LA[a]
100	99.0	39.4	125
50	13.5	5.5	10.7
Total	112.5	44.9	135.7
50	111.0	43.8	101.0
100	1.0	0.8	2.1
Total	112.1	44.6	103.1

[a] As milligrams of cacao leucoanthocyanin per 10 grams of fresh tissue.

To test the latter, the phenolics extracted from Hachiya persimmon tissue by treatment first with 100% methanol and then with 50% methanol were compared with those obtained when the order of extraction was changed. The results obtained (Table IV) indicate that the total quantity of phenolics obtained and their distribution are essentially unchanged. The Hachiya persimmons used here, however, were firm ripe and not particularly astringent. If the tannins had been bound by a cellular constituent(s) by bonds similar to those by which tannic acid is bound by gelatin, and treatment with absolute methanol had cleaved these bonds as first reported for tannin-gelatin by treatment with ethanol by Trunkel (*32*), considerably more phenolic material would be expected to be extractable by 50% methanol after rather than before extraction with 100% methanol. In the persimmons tested, however, 88% of the total phenolics were extracted with 100% methanol and over 99% with 50% methanol.

With the Niu Nai persimmons (Table II), 95.5% of the total phenolics were extracted with 100% methanol from the hard fruit, and only 48.4% from the ripened but still somewhat astringent fruit. Under the same conditions 96.3% of the leucoanthocyanins in hard fruit and only 21.4% in the ripened fruit were extracted with 100% methanol. If this decrease in extractability of leucoanthocyanins were due to polymerization, one would expect a change in either the hydroxylation or substitution pattern in the extractable phenolics. The change in substitution pattern in the A ring is shown by the decrease in percentage of vanillin-reactive phenolics extractable with 100% methanol which occurred during ripening, even though

the total vanillin-reactive phenolics increased. A change in hydroxylation pattern is shown in a decrease in vicinyl hydroxyl groups extractable with 100% methanol as measured by molybdate which occurred during ripening, but total molybdate reactive phenolics decreased. While qualitatively these changes are in the proper direction if condensation involved the B-ring hydroxyls, quantitatively they do not agree with the marked decrease in extractability of leucoanthocyanins.

The data on the phenolic content of dehydrated persimmons are shown in Table IV, calculated on an equivalent fresh weight basis and expressed as milligrams of catechin per 10 grams fresh weight. As shown in Table V, the total phenolics obtained by serial extraction with methanol were considerably higher than those obtained by a single prolonged extraction; about a 15% higher yield was obtained with unsulfured fruit and a 300% higher yield with sulfured fruit.

The unsulfured and nonastringent persimmon tissues contained fewer total phenolics, vanillin-reactive phenolics, and leucoanthocyanins than did the sulfured astringent persimmon. The aqueous extract did not yield the expected delphinidin on heating with butanol–HCl but instead a brown pigment indicating appreciable oxidation. The total water-extractable phenolics, calculated from the Folin-Denis values, expressed on a fresh weight basis, in the water extract of unsulfured fruit amounted to 7.7 mg. in comparison with 110 mg. in astringent Niu Nai, 161 mg. in astringent Hachiya, and 11 mg. in nonastringent Fuyu. The total 100% methanol–extractable phenolics in unsulfured fruit amounted to 10.7 mg. per 10 grams of fresh fruit (weight basis).

The considerably higher values for phenolic content of the sulfured dehydrated persimmons were not due to interference by either residual sulfur dioxide or ascorbic acid. Sulfur dioxide is known to reduce the Folin-Denis reagent (*27*) and so is ascorbic acid (*25*). As shown in Figure 8, under our test conditions, sulfur dioxide reduces Folin-Denis reagent less than catechin. In the aliquot of the diluted extract used for Folin-Denis assay, however, the sulfur dioxide present would be 0.004 mg., considerably below the range at which interference would be expected. Interference from ascorbic acid is about 55% of that due to phenolics present. Persimmons, however, on the average contain 10 mg. of ascorbic acid per 100 grams of fruit tissue, and the aliquot used for analysis would contain too little ascorbic acid to be detectable. The interference of sulfur dioxide in the vanillin and leucoanthocyanin tests is less than in the Folin-Denis assay. One milliliter of sodium bisulfite solution

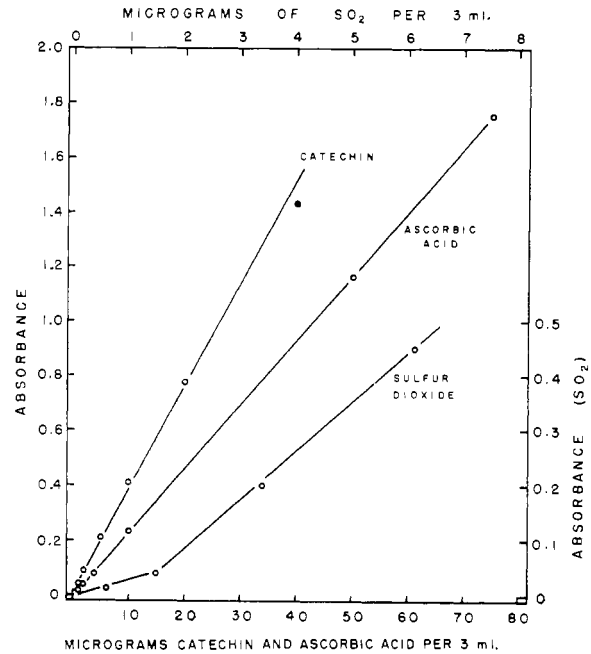

Figure 8. Interference of ascorbic acid and sulfur dioxide in Folin-Denis test

equivalent to 125 mg. of SO_2 per liter added to aliquots containing 4 μg. of resorcinol caused only a 10% increase in vanillin value; and when added to 500 μg. of grape phlobatannin caused a 17% decrease in leucocyanidin value.

The phenolic content of the sulfured dehydrated persimmons, expressed on a fresh weight basis, is appreciably lower than that found for astringent fresh persimmons (Table II). The phenolics present in the frozen purees from the same lot of fruit, which were completely nonastringent to taste, are shown in Table VI. In comparison with the data shown in Table V, the sulfited puree was lower in all phenolics but relatively lower in vanillin-reactive phenolics, and higher in leucoanthocyanin content. The relative decrease in total Folin-Denis value was 55%, in vanillin-reactive phenolics 63%, and in leucoanthocyanin 35%. The extracts of the frozen persimmons were prepared from 2.73 to 4.85 grams of tissue, the methanolic extracts were made up to 100 ml., and aliquots of this extract were diluted 1 to 5 for the analysis. The levels of sulfur dioxide in the test mixture were 3.2 to 6.2 μg., at which interference with the Folin-Denis values would be appreciable. The values reported for the persimmon puree frozen with added ascorbic acid were about 70% too high and the corrected values for the Folin-Denis values of 100% methanol extract would be 3.0 mg. per 10 grams.

Acetone, ethanol, and methanol are compared in Table VII as solvents for phenolics present in dehydrated sulfured persimmons. While more total phenolics were extracted with 100% methanol than with other undiluted solvents, more were extracted with 50% acetone or ethanol than with 50% methanol. Methanol was a better solvent for vanillin-reactive phenolics than ethanol, and also for leucoanthocyanins. Acetone interfered with vanillin-reactive phenolics.

Aqueous extracts of freeze-dried Hachiya persimmons prepared by blending 10-gram samples of unripe fruit with 240 ml. of water and centrifuging clear contained from 11 to 30 mg. of total phenol per gram of dry tissue, from 3 to 10 mg. of vanillin-reactive phenolics, and from 11 to 26 mg. of leucoanthocyanin at SO_2 levels varying from 0 to 100 p.p.m. The samples at all levels of residual sulfur dioxide were astringent. This was confirmed by freeze-drying another somewhat riper lot of fruit. Apparently the loss in astringency during air dehydration is largely due to oxidation.

Ripening in Alcohol. The fruit stored with 40% alcohol at room temperature was examined periodically. Initially it was astringent and remained astringent after 1 and 2 days' storage. On the third day most of the astringency was lost and on the fourth day the fruit was completely nonastringent but still firm. Thereafter it gradually softened. The phenolic content of the fruit tissues after storage for 0 to 8 days is shown in Table VIII. This indicates close agreement between loss in astringency and reduction in extractability of phenolics in 100% methanol. In comparison with these values the Folin-Denis phenolic content of water extracts decreased only from 3.41 mg. per gram initially to 2.2 mg. per gram after 8 days; the vanillin values decreased from 0.17 to 0.14, and the leucoanthocyanin content increased from 0.7 to 1.5 mg. per gram.

Table V. Phenolic Content of Extracts of Dehydrated Chein Tsein Persimmons

(As milligrams of catechin per 10 grams of FW tissue)

Extraction Method		FD	V	LA[a]
Hot 100% methanol	Unsulfited	10.7	2.1	...
	Sulfited	32.2	11.2	50.0
Hot water	Unsulfited	7.7	4.2	32.2
	Sulfited	50.3	28.2	88.0
100% methanol followed by 50% methanol	Unsulfited	8.4	3.0	4.0
	Sulfited	54.4	18.6	18.0
	Unsulfited	3.9	6.9	...
	Sulfited	37.1	16.7	24.6
Total 100% + 50%	Unsulfited	12.3	9.9	4.0
	Sulfited	91.5	35.3	42.6

[a] As milligrams of cacao leucoanthocyanin.

Table VI. Phenolic Content of Extracts of Frozen Chein Tsein Persimmons

(Milligrams of catechin per 10 grams)

Solvent	Antioxidant	FD	V	LA[a]
100% methanol	Ascorbic acid	10.1	2.0	...
	Sulfite	16.2	2.0	...
50% methanol	Ascorbic acid	13.6	9.5	24.1
	Sulfite	25.0	11.1	27.6
Total	Ascorbic acid	23.7	11.5	24.1
	Sulfite	41.2	13.1	27.6

[a] As milligrams of cacao leucoanthocyanin per 10 grams.

Table VII. Phenolic Content of Extracts of Sulfured Dehydrated Persimmons

(As milligrams per 10 grams of fresh fruit)

Solvent		Concn.	FD	V	LA
Acetone		100	1.4	5.8	...
		50	65	44.7	125
		Total	66.4	50.5	125
	Ratio	50:100	46.5	7.7	...
Ethanol		100	9.6	2.1	...
		50	65.5	24.5	103
		Total	75.1	26.6	103
	Ratio	50:100	6.8	11.5	...
Methanol		100	27.7	9.6	18.6
		50	31.7	17.5	103
		Total	59.4	27.1	121.6
		50:100	1.15	1.82	5.5

Table VIII. Changes in Phenolic Content during Ripening in Alcohol

(As milligrams per grams of dry tissue)

Days Stored	Methanol Concentration, %								
	100	50	0	100	50	0	100	50	0
	Folin-Denis Values			Vanillin Values			Leucoanthocyanin		
0	44.3	2.8	4.1	15.3	1.4	1.4	32.1	2.3	3.2
1	23.2	2.9	...	9.5	1.5	...	15.5	2.5	...
2	9.8	9.3	2.0	0.8	5.4	0.7	7.2	9.8	1.7
3	2.4	20.7	2.2	2.2	8.0	1.2	1.5	19.0	2.9
4	4.1	30.0	2.2	1.7	9.3	0.9	0.0	24.7	1.5
6	4.2	29.5	3.9	1.5	7.3	1.7	0.0	23.9	5.5
8	3.6	11.8	9.2	16.7	6.1	2.2	...	11.5	2.1

Distribution of Phenolics in Blended Extracts. Siegelman and Craft (*26*) had observed that extractability of leucoanthocyanins and astringency decreased on blending fruit tissue at high speeds or for a longer time in isotonic solution. To obtain further data on this, extracts of Niu Nai, Hachiya, and Fuyu persimmons were prepared by blending at low and high speeds, with and without added sulfite. Sulfite was added to reduce oxidation suspected to be involved. As the data summarized in Table IX indicate, the extractable phenolics obtained by this procedure were much lower than those obtained by methanol extraction.

The extracts of unripe Niu Nai fruit were blended on October 25, 1962, and stored at 0° C. until November 10, when they were filtered and analyzed. Those of ripe fruit were blended on November 19 and analyzed on November 28. The extracts of unripe Hachiya fruit were blended on November 19 and analyzed on November 28; those of ripe Hachiya were blended on December 4 and analyzed on December 7. The extracts of firm Fuyu were blended on October 25 and analyzed on November 10; those of ripened Fuyu were blended November 20 and analyzed November 28.

With firm Niu Nai there was little difference between phenolics extracted by low or high speed blending in absence of added SO_2, but addition of SO_2 resulted in marked increase in extractable phenolics at high speed. On ripening, neither addition of SO_2 nor speed of blending had an appreciable effect. The firm Hachiya fruit behaved differently, in that at low speed more phenolics were extracted than at high speed in both the absence and presence of SO_2. In the presence of SO_2 the quantity of total phenolics and vanillin-reactive phenolics increased but that of leucoanthocyanins decreased. With Fuyu, SO_2 had little effect on speed of blending. The values reported in Tables VII, VIII, and IX (Niu Nai and Hachiya) are not corrected for SO_2 content, but under the conditions of analysis, error due to this would be low.

The blended fruit extracts were stored at 0°C. for 3 to 16 days before filtration and analysis and it is likely that on standing the tissue particles adsorbed phenolics. Preliminary investigations made later indicated that while the blended extracts, at either low or high speed, were astringent when tasted immediately after blending, on storage for a few days they lost astringency. The fruit tissue particles remaining after separation by filtration and centrifugation were deeply stained with Reeve reagent and the intensity of this staining increased on storage after blending. It is likely that adsorption of soluble phenolics from the broken tannin cells by cellular material was more responsible for changes in astringency during blending and storage than depolymerization or oxidation.

While it is not possible to confirm this with fresh astringent fruit because of lateness of season, data on possible changes in phenolic content during storage of blended extracts were obtained using frozen whole Hachiya and frozen Chein Tsein puree. The Hachiya fruit were obtained December 11 and were slightly astringent fresh; when tasted after storage for about 4 months they were completely nonastringent. The residue after blending with isotonic glycerol solution was slightly astringent. The blended extracts after preparation were stored for 11 days at 0°C. before filtration but, as shown in Table X, little if any change in phenolic content occurred during this period of storage. Representative aliquots of 125 grams of tissue were blended with 250 ml. of isotonic glycerol solution and the phenolic content of the filtrate was determined as

Table IX. Phenolic Constituents in Persimmons Extracted by Blending

(As milligrams per 10 grams of fresh weight)

Blending Voltage	FD	V	LA	V/LA
Niu Nai Persimmons, Fruit Hard[a]				
40	12.4	2.8	25.4	0.11
100	13.5	2.8	29.1	0.10
Ratio 40:100	0.92	1.0	0.87	
+ 200 P.P.M. SO_2				
40	38.3	9.7	50.3	0.19
100	10.7	1.2	6.8	0.18
Ratio 40:100	3.5	7.8	7.5	
Niu Nai Persimmons, Fruit Soft				
40	5.0	1.2	2.7	0.46
100	4.88	1.4	3.1	0.45
Ratio 40:100	1.02	0.9	0.9	
+ 200 P.P.M., SO_2				
40	9.80	1.6	3.4	0.46
100	10.2	1.6	4.2	0.37
Ratio 40:100	0.96	1.0	0.8	
Hachiya, Fruit Hard				
40	29.6	10.0	69.0	0.15
100	7.15	2.3	6.9	0.34
Ratio 40:100	4.2	4.3	10.0	
+200 P.P.M. SO_2				
40	44.40	13.7	50.1	0.27
100	15.75	4.1	10.6	0.39
Ratio 40:100	2.32	3.3	4.86	
Fuyu Persimmons, Fruit Hard[b]				
40	5.8	1.8	3.9	0.460
100	5.6	1.1	6.0	0.185
Ratio 40:100	1.03	1.63	0.66	
+200 P.P.M. SO_2				
40	8.5	1.8	5.7	0.316
100	9.8	2.3	8.5	0.276
Ratio 40:100	0.9	0.8	0.7	
Fuyu Persimmons, Fruit Soft				
40	3.5	1.4	4.3	0.330
100	2.8	1.2	6.4	0.193
Ratio 40:100	1.20	1.14	0.68	
+200 P.P.M. SO_2				
40	5.9	1.7	6.0	0.280
100	6.1	2.1	9.9	0.224
Ratio 40:100	0.93	0.81	0.66	

[a] LA as milligrams catechin per 10 grams of fresh weight. [b] LA as milligrams cacao leucoanthocyanin per 10 grams of fresh weight.

Table X. Effect of Storage after Blending on Vanillin-Reactive Phenolics

Date Filtered	Blending Speed	Absorbance	Vanillin-Reactive Phenolics as Catechin per 10 Gram Fresh-Weight
Hachiya, Frozen Whole Fruit			
3-1-63	40	0.46	0.317
	100	0.46	0.318
3-4-63	40	0.50	0.342
	100	0.50	0.341
3-11-63	40	0.50	0.344
	100	0.50	0.346
Chein Tsein, Frozen with Ascorbic Acid			
3-1-63	40	1.21	0.833
	100	1.26	0.874
3-4-63	40	1.23	0.847
	100	1.22	0.839
3-11-63	40	1.27	0.881
	100	1.24	0.854
Chein Tsein, Frozen with SO_2			
3-1-63	40	1.47	0.011
	100	1.45	0.998
3-4-63	40	1.50	1.032
	100	1.46	0.998
3-11-63	40	1.47	1.011
	100	1 46	0.998

vanillin-reactive phenolics. The absorbance values reported are for the pigment formed in undiluted filtrates and the phenolics are expressed as catechin, in milligrams per 10 grams fresh weight.

Absorption Spectra of Persimmon Phenolics. The absorption spectra of methanol extracts from astringent persimmons differed markedly from those of nonastringent. The spectrum of Niu Nai persimmon tissue extracts had a sharp characteristic absorption band at 280 mμ, decreased to a minimum at 252 mμ, and then rapidly increased as wave length decreased. That of Fuyu, on the other hand, had a maximum at 258 mμ and decreased to a minimum at 236 mμ. In the visible region there was considerable absorption in the region of 400 to 480 mμ but little absorption at higher wave lengths. The absorption spectra of the anthocyanidins produced by heating methanolic extracts in butanol-HCl showed the characteristic maximum at 555 mμ without a noticeable increase in absorbance at 450 mμ, characteristic of phlobaphene production (*11*).

Persimmon Leucoanthocyanin. We were not able to obtain a purified preparation from Niu Nai persimmons by the procedure used by Ito and Oshima (*14*), because of the rapid denaturation by acetic acid. We were able to prepare a purified leucoanthocyanin by precipitation of phenolics from a 70% methanol extract with neutral lead acetate, deleading with sulfuric acid in methanol, precipitating with ethyl ether, and repeating solution in methanol and precipitation with ether. From an aliquot of methanol extract representing 300 grams of persimmon flesh we obtained 2.38 grams of light cream-colored astringent preparation which behaved chromatographically like Ito and Oshima's preparation. When applied in moderate amounts to paper it yielded delphinidin on acid hydrolysis. On applying larger volumes of acid hydrolyzate it was found to contain both delphinidin and cyanidin in the ratio of about 5 to 1, thus confirming Siegelman and Craft's observation (*26*). On heating with dilute acid it did not yield any detectable anthocyanidins but yielded several mobile phenolics (*26*).

Acknowledgment

The cooperation of C. O. Hesse, Department of Pomology, at Davis, in arranging for the supply of persimmon fruit, and of M. W. Miller, Department of Food Science and Technology, in arranging for the dehydration and freezing of persimmons at Davis, is gratefully acknowledged. The technical assistance of Mary Lou Gotthold in analysis and of T. Chen and M. Morris in fruit preparation is acknowledged.

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Received for review September 3, 1963. Accepted May 18, 1964. Division of Agricultural and Food Chemistry, 145th Meeting, ACS, New York, N. Y., September, 1963. Presented in part at 129th annual meeting, AAAS, Philadelphia, December 26, 1962, and at Pacific Coast Section Meeting, AAAS, Stanford, July 19, 1963. Investigations supported in part by Public Health Service research grant EF-00801-1 from the Division of Environmental Engineering and Food Protection.

SUGAR ESTIMATION

Refractometric Dry Solids as an Indicator of the Sugar Content of Papaya Fruit

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Where the range in sugar contents among examined fruits was substantial, near perfect correlations, $r = 0.987$ and 0.991, between the refractometric dry solids (RDS) of the fruit juice and the sugar content of papaya (*Carica papaya* L.) fruit were obtained. The correlation was less impressive, $r = 0.610$, where sugar concentrations differed only slightly from one lot of fruits to another, but was adequate enough not to preclude the use of RDS as an indicator of sugar content.

OFTEN an estimate of the sugar content in plant tissue can be quickly obtained from the refractometric dry solids (RDS) of the cell sap. It is not precise, but where sugar is the predominant water-soluble constituent, the method has been used routinely and advantageously—e.g., in grape, melons, etc. (*1, 2, 4, 7*).

In breeding trials and in some cultural experiments with papaya, a fruit crop commercially important in Hawaii and in other tropical-subtropical areas, the juice RDS has been accepted as a measure of sugar content. Unfortunately, the relationship between the two characteristics, or the reliability of the former as indicator of the latter, has escaped any previous establishment. The refractometric dry solids denote content of any solute, and do not selectively disclose the level of sugar as sometimes inferred. In this study the degree of correlation has been determined.

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Materials and Methods

Samples of papaya (*Carica papaya* L.) fruit were provided by R. A. Hamilton of the station. The plants were grown at the Manoa, Poamoho, and Waimanalo farms. Three evaluations were performed. In the first, fruits of six strains with a substantial range in sugar contents were employed; another test considered Solo papaya fruits harvested during the two extremes in season, summer and winter. In both trials, samples were harvested at a stage of maturity when at least 50% of the fruits' external surface was yellow, and were analyzed when they attained complete yellowness after storage in the laboratory. The third evaluation explored Solo fruits which were in varying stages of maturity or ripeness when harvested. The stages were fully-yellow, half-yellow, a trace-of-yellow, mature-green, and immature-green. The first four were recognized by their external coloration; the mature-green fruit was distinguished from the immature-green by its larger size and content of fully-developed, dark seeds.

Samples for analyses consisted of three replicate fruits. The RDS was obtained with a Zeiss F_3 hand refractometer. The freshly expressed, unfiltered sap of the pulp was examined. Expectedly, the RDS's vary slightly with the morphologic position within a given fruit wherein the sample is obtained. Thus, the pulp from an entire fruit was first blended mildly and then examined.

Extracts for sugar determination were prepared from 50 grams of pulp by these steps: mixed with 1 gram of $CaCO_3$ and 100 ml. of distilled water, homogenized 5 minutes in a Waring Blendor, boiled 5 minutes and filtered hot with 5 grams of washed Filter Cel, rinsed with two 50-ml. portions of boiling distilled water, combined filtrates, cooled, and diluted to 250 ml. The absence of anthrone-reactive substances in further extracts of the residue was considered as evidence of complete extraction. Aliquots of the extracts were clarified by Loomis's method (*3*) and hydrolyzed by adding $^1/_{10}$ their volumes in concentrated HCl and allowing to stand at room temperature overnight. After neutralization, the sugar content was