oxide values for both dosage treatments decreased and the free fatty acids increased during storage of the samples in cans at 24° C.

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FRUIT JUICE CONSTITUENTS

Chromatographic Comparison of Nonvolatile Acids of Fresh and Stored Apple Juice Concentrate

M. L. BUCH, E. C. DRYDEN, and CLAUDE H. HILLS

Eastern Regional Research Laboratory, Philadelphia 18, Pa.

The nonvolatile acids of fresh and of storage-darkened apple juice concentrate were separated by the use of paper chromatography following silicic acid partition chromatography. No difference could be detected in the acids between the fresh and the darkened concentrate. The use of three different solvent mixtures and six spray reagents for paper chromatography, together with 41 reference acids, allowed tentative identification of galacturonic, quinic, phosphoric, citric, malic, chlorogenic, citramalic, caffeic, succinic, and lactic acids by their R_f values and colors produced with the spray reagents. Seven acids present in minor amounts were not identified.

APPLE JUICE can be concentrated at a low temperature to 70° Brix. The resulting product is much more stable to decomposition by microorganisms, and the reduced water content saves in shipping, packaging, and storage costs. Although the concentrate is more stable than fresh juice, it does darken upon storage. The rate of darkening is influenced by the varieties of apples from which the concentrate was produced, as well as by other factors.

There is evidence that this deterioration during storage is due to the Maillard or nonenzymatic browning reaction. In his recent review of browning reactions, Hodge (*10*) has pointed out that organic acids have been shown to be involved both directly in a reducing sugar–organic acid reaction and synergistically in a reducing sugar–amino acid–organic acid type of reaction. This study was undertaken to find out if there were any changes in the identity and amount of acids when apple juice was concentrated and allowed to darken. Qualitative and quantitative changes could be due either to participation of the acids in the browning reaction or to normal degradation of sugars and other compounds.

Materials and Methods

Preparation of Concentrate The apple juice was prepared from a blend of two parts of Jonathan and one part each of McIntosh, Northern Spy, and Stayman Winesap apples. Immediately after pressing, the juice was passed through an apparatus for essence recovery (*8*), depectinized, and filtered. The treatment of the juice is that used in the preparation of full-flavor apple juice concentrate. The treated juice (18.6° Brix) was frozen and stored for experimental work (*7*).

A sample of this juice was concentrated in a laboratory vacuum still to 69° Brix and stored in an incubator at 100° F. After several months the darkened sample was removed and diluted to 18.6° Brix.

Ion Exchange Treatment A column containing Zeo Rex cation exchange resin (Permutit Co.) was regenerated with 2*N* hydrochloric acid and washed until the effluent produced a negative chloride test as described by Porter, Buch, and Willits (*18*). A De Acidite (Permutit Co.) anion exchange column was regenerated with 1*N* sodium hydroxide and washed until the effluent was neutral to phenolphthalein (*18*). The juice or diluted darkened concentrate was mixed with an equal volume of water, run through the Zeo Rex resin, and then rinsed with four bed-volumes of water. The effluent (sugars plus acids) and rinse were then run through the De Acidite column, followed by a four-bed-volume rinse with water. De Acidite was chosen because it is a weakly basic resin and does not cause degradation of sugars, as was found to be the case with strongly basic resins (*13, 17*). The acids were eluted from the column with an excess of 0.1*N* sodium hydroxide. This eluate, containing the sodium salts of the acids plus sodium hydroxide, was run through a column of regenerated Zeo Rex. This normality of alkali was found to be sufficient for complete recovery and was much better than the stronger alkali usually employed (1*N* or 2*N*), because one pass through the cation column would free the acids. The stronger alkali required several passes with regenerations after each pass, or another larger column had to be set up. The effluent, containing only the free acids, was concentrated in vacuo to a definite volume and used for chromatographic analysis. In later work Dowex

50 (Dow Chemical Co.) was substituted for Zeo Rex, as the Zeo Rex seemed to undergo some deterioration upon prolonged contact with sodium hydroxide.

Paper Chromatography The descending technique was employed with $18^1/_4 \times 22^1/_2$-inch sheets of Whatman No. 1 filter paper. The solvents used were *n*-butyl alcohol–acetic acid–water (4:1:5), *n*-amyl alcohol–5*M* aqueous formic acid (1:1) (*5*), and ethyl alcohol–ammonium hydroxide–water (20:1:4) (*14*).

The developed papers were sprayed with slightly alkaline bromophenol blue when the first two solvents were used, and with Universal Indicator at pH 9 to 10 when the third solvent was used. In addition, spray reagents were used which differentiate some of the acids by means of the colors produced. Naphthoresorcinol (0.1%) in a 95% ethyl alcohol solution of (1*N*) phosphoric acid (*4*), when sprayed on the chromatogram heated at 80° C. for 5 minutes and observed after heating and again after 24 hours, was useful with a few other acids, as well as for the typical uronic acid reaction. When 1% aqueous ferric chloride (*1*) was used, observations were made immediately and after 24 hours. The other four spray reagents (*5*) were 10% acetic anhydride in pyridine, heated at 100° C. for 5 minutes, saturated aqueous ammonium vanadate, 2% ceric ammonium nitrate in 1*N* nitric acid, and ammoniacal silver nitrate.

The amount of acid solution of fresh or darkened concentrate which was to be chromatographed varied, because the concentration of the component acids varied. It was generally adjusted so that the amount of a particular acid to be compared with a standard acid was in the range of 20 to 100 γ. Therefore, when the concentration was optimum for the acid present in lowest quantity, the amount of malic acid might be as high as 15 mg.

Column Chromatography A silicic acid partition column was used, and the procedure was essentially that of Marvel and Rands (*15*). A column 19 mm. in diameter was packed to a height of approximately 160 mm. with 18 grams of silicic acid which had been ground with 10.8 ml. of water and then slurried with chloroform. The sample was added to the column by the procedure described by Wise (*19*). Two grams of silicic acid was mixed with 1.2 ml. of an aqueous solution of the acids (the same proportion of water to silicic acid as was used in the rest of the column) and transferred to the top of the column.

The *n*-butyl alcohol–chloroform solvents used were those of Marvel and Rands (*15*), except that 50 ml. of 40–60 and 50 ml. of 20–80 chloroform–butyl alcohol were substituted for 100 ml. of the 30–70 mixture. Also, after 100 ml. of the saturated 100% butyl alcohol solvent, a mixture of 3 volumes of water-saturated 50–50 chloroform–butyl alcohol plus 1 volume of absolute ethyl alcohol was used to remove the last of the acids.

By use of a volumetric fraction cutter (Gilson Medical Electronics, Madison, Wis.) 140 10-ml. fractions were collected. Each fraction was titrated with 0.02*N* sodium hydroxide after the addition of phenol red indicator and enough ethyl alcohol to produce a single phase. The ethyl alcohol homogenization of the titration solution was developed by Buch, Porter, and Willits during investigations of the acids of maple products.

The silicic acid used was Mallinckrodt, 100-mesh, especially prepared for chromatographic analysis. The chloroform was U.S.P. grade and the *n*-butyl alcohol was Eastman Kodak, boiling point 116–118° C. Neither solvent required redistilling.

A large scale column, containing 200 grams of silicic acid, was used to eliminate part of the malic acid. A proportionally larger sample of the acid solution was put on the column, and the fractions which contained the major part of the malic acid were removed and discarded. The rest of the fractions were combined and shaken in a separatory funnel with excess aqueous sodium hydroxide and the water layer was separated. This solution, containing sodium salts of the acids plus sodium hydroxide, was run through a cation exchange column, acid form. The effluent, which now contained the free acids, was concentrated in vacuo to dryness, redissolved in water, and run on the smaller, 20-gram silicic acid column. The fractions from this column were titrated, and those fractions contributing to a particular peak combined. Each set of combined fractions was then extracted with alkali and run through a cation exchange column as before. After concentration, the solution was chromatographed on paper. Phenol red indicator was also extracted and was therefore present in each fraction. However, as its R_f was near that of malic acid, it did not interfere with most of the other acids. This procedure was followed with the fresh juice and with a sample of concentrate which had been stored for 8 months at 100° F. and had become rather dark.

Results and Discussions

Table I shows R_f values and colors produced by spray reagents with standard acids and with the acids of fresh and darkened concentrate. Only those concentrated acids for which the peak effluent volume is given were recovered from a silicic acid column and rechromatographed on paper. The others could not be recovered because of their extremely low concentration.

Apple Concentrate Acid Acid A appears to be galacturonic acid, which probably arises from pectin degradation during depectinization of the juice. Paper chromatography indicated no difference in concentration of this acid fraction between fresh juice and darkened concentrate. Recovery from the silicic acid column was poor and no quantitative estimate could be made by this method.

Acid B was stronger in the darkened concentrate than in the juice, although present in both. The concentration was too low for reliable quantitative analysis using a silicic acid column. The pink or brown color produced with naphthoresorcinol suggests that it may contain a keto group. It gave a negative test with ninhydrin, as did all the other acids.

Acid C is hard to detect, both because of its low concentration and because of interference from acids of similar R_f values. It comprised less than 0.1% of the total acidity.

Acid D appears to be quinic acid (*12*, *17*) and acid E phosphoric, both of which have been reported in apple tissue or juice. They have the same peak effluent volume on a silicic acid column; however, when these acids were run on a large column, extracted, evaporated to dryness, and rerun on a small column, acid E disappeared. Assuming that the acid left is only D and that all of E is present in the aqueous sample and none in the dried sample, the amount of each is shown in Table II.

Acid F appears to be citric acid, which has been reported in apple fruit (*6*). It occurs too close to malic acid on the column for resolution to be good, but paper chromatographic evidence indicates that there is no difference in concentration between the juice and the darkened concentrate. It accounted for approximately 0.8% of the total acid.

Acid G is probably malic acid, which has long been recognized as the principal acid in apples.

There was not enough acid H to be detected on two-dimensional paper chromatograms or on a silicic acid column. When run one-dimensionally with *n*-amyl alcohol–formic acid, it formed a crescent-shaped spot just below the leading edge of malic acid. This peculiar shape may have been caused by the extremely large amount of malic acid, which pushed this unknown acid ahead of it and distorted the shape of the spot, so that the concentration per unit area was increased enough to be just barely detectable. Chlorogenic acid, which has been found in apples (*2*, *16*), fluoresces under ultraviolet illumination and turns brown immediately with ammoniacal silver nitrate, as does acid H.

Table I. Chromatographic Constants of

Reference Acid	Rf Values: n-Amyl alcohol–formic acid	Rf Values: n-Butyl alcohol–acetic acid	Rf Values: Ethyl alcohol–ammonia	Spray Reagents: Acetic Anhydride in Pyridine: Daylight	Acetic Anhydride in Pyridine: UV, 24 hr.	Ammonium Vanadate: Daylight	Ammonium Vanadate: Daylight 24 hr.	Ceric Ammonium Nitrate: Daylight or UV	Ceric Ammonium Nitrate: UV, 24 hr.
Sulfuric	0.04	0.14	0.14	...	...	Yellow	W. gray	...	...
Galacturonic	0.02	0.19	0.21	...	w	W. yellow	Gray	+	w
2-Ketogluconic	0.04	0.24	0.28	...	...	...	Gray	+	+
Hydroxypyruvic	0.06	0.27	0.12	...	...	Yellow	...	...	...
Quinic	0.09	0.31	0.37	...	...	...	Gray	+	w
Meconic[a]	0.08	0.16	0.07	...	...	W. yellow	...	+	+
Mesotartaric	0.11	0.30	0.13	...	...	...	Gray	+	w
Ascorbic[a]	0.12	0.44	0.34	Yellow	+	Gray	Gray	+	+
Isoascorbic	0.12	0.37	0.39	...	+	...	Gray	+	+
d-Tartaric	0.15	0.37	0.16	...	w	Red	Red	+	w
Phosphoric	0.19	0.33	0.04	...	...	Yellow	Yellow-gray	+	...
Dihydroxymaleic	0.22	0.31	0.02	...	...	...	W. Gray	w	...
Mesoxalic	0.24	0.31	0.12	...	...	...	Gray	+	+
Chelidonic	0.24	0.35	0.37	...	...	Yellow	...	+	...
Citric	0.23	0.43	0.05	...	+	Yellow	Gray	+	+
Isocitric	0.24	0.51	0.06	...	...	W. yellow	Gray	+	+
Dihydroxytartaric	0.29	0.36	0.13	...	...	Blue	Gray	+	+
Malic	0.33	0.55	0.19	...	...	Yellow	Gray	+	+
Chlorogenic[a]	0.39	0.67	0.43	Yellow	+	Yellow	Brown	+	+
Pyroglutamic	0.40	0.61	0.43	...	...	Yellow	...	...	...
Gallic	0.46	0.67	Streak	...	...	Brown	Brown	+	+
Maleic	0.55	0.46	0.26	...	...	Yellow	...	...	...
Citramalic	0.51	0.67	0.25	...	...	...	Gray	+	+
Kojic[a]	0.52	0.70	0.45	...	+	...	Gray	+	+
Malonic	0.55	0.60	0.15	...	...	Yellow	...	+	...
Succinic	0.61	0.78	0.25	...	...	Yellow	...	...	...
Lactic	0.61	0.74	0.50	...	...	W. yellow	Gray	+	+
Caffeic[a]	0.73	0.85	0.48	Brown	+	Yellow	Brown	+	+
Glutaric	0.76	0.81	0.28	...	...	Yellow	...	...	...
Adipic	0.81	0.84	0.35	...	...	Yellow	...	...	...
Phthalic	0.82	0.77	0.31	...	...	Yellow	...	...	...
Monochloroacetic	0.83	0.79	0.55	...	...	Yellow	...	...	...
Fumaric	0.83	0.83	0.30	...	...	Yellow	...	...	...
Trichloroacetic	0.85	0.70	0.72	...	w	Yellow	...	...	...
Mandelic	0.85	0.87	0.60	...	...	Yellow	Gray	+	w
Gentisic[a]	0.86	0.88	0.65	...	+	...	...	+	...
Furoic	0.87	0.89	0.56	...	...	Yellow	...	+	w
Benzoic	0.92	0.88	0.66	...	...	...	...	...	...
Salicylic[a]	0.93	0.87	0.71	...	+	...	...	Brown	+
Tropic	0.94	0.93	0.50	...	...	Yellow	...	...	...
Stearic	0.97	0.90	0.51(streak)	...	+	...	...	w	...
Apple Concentrate Acid									
A	0.03	0.20	0.21	...	...	?	Gray	+	...
B	0.05	0.25	...	...	+	...	Gray	+	+
C	0.07	0.18	0.47	...	...	Yellow	...	...	...
D	0.08	0.31	0.38	...	...	...	Gray	+	+
E	0.18	0.34	0.04	...	...	Yellow	Gray	+	...
F	0.22	0.42	0.04	...	+	W. yellow	Gray	+	+
G	0.34	0.56	0.18	...	...	Yellow	Gray	+	+
H[a]	0.39	...	...	...	+	...	...	...	...
I	0.43	0.59 or 0.64	0.47?	...	+	...	Gray	+	+
J	0.51	0.68	0.24	...	...	...	Gray	+	+
K	0.54	0.50 or 0.24	...	...	...	W. yellow	...	...	...
L	0.73	0.83	...	...	+	...	...	...	...
M	0.60	0.76	0.26	...	...	Yellow	...	...	...
N	0.60	0.72	0.51	...	...	...	Gray	+	...
O	0.77	...	...	...	?	Yellow	...	?	?
P	0.81	0.50 or 0.82	0.51?	...	?	Yellow	...	...	...
Q	0.95	0.90	...	...	...	Yellow	...	+	+

w = Weak.
+ = Positive.
[a] Fluorescent under ultraviolet illumination before spraying.

Standard Acids and Apple Concentrate Acids

Reference Acid	Ferric Chloride		Naphthoresorcinol		Ammoniacal Silver Nitrate	Peak Effluent Volume on Silicic Acid Column	Possible Identity
	Daylight	Daylight, 24 hr.	Daylight	Daylight, 24 hr.	Daylight		
Sulfuric	...	...	W. brown	W. brown	Brown		
Galacturonic	...	White	...	Blue	Yellow	1220	
2-Ketogluconic	Yellow	Pink	...	...	Yellow		
Hydroxypyruvic	...	...	...	...	White		
Quinic	Yellow	Pink	...	...	Yellow	1070	
Meconic[a]	Pink	Pink	...	...	Red-brown		
Mesotartaric	...	White	...	...	White		
Ascorbic[a]	Yellow	...	W. brown	W. brown	Immed. brown		
Isoascorbic	Yellow	White	W. brown	W. brown	Immed. brown		
d-Tartaric	Yellow	White	...	...	White		
Phosphoric	...	White	...	...	Gray	1060	
Dihydroxymaleic	...	...	...	...	Brown		
Mesoxalic	Yellow	White	W. yellow	Green	Tan		
Chelidonic	...	Brown	W. Gray	W. Gray	Pink		
Citric	...	White	...	...	White	790	
Isocitric	...	W. pink	...	...	White		
Dihydroxytartaric	Yellow	White	...	W. brown	White		
Malic	W. yellow	White	...	...	White	750	
Chlorogenic[a]	Gray-green	Gray	...	...	Immed. brown		
Pyroglutamic	...	Pink	...	...	Pink	530	
Gallic	Gray	Brown	...	...	Immed. brown		
Maleic	...	Pink	...	...	White		
Citramalic	W. pink	W. pink	...	...	White	590	
Kojic[a]	Pink	Pink	...	...	Red-brown		
Malonic	...	...	...	...	Yellow		
Succinic	...	Pink	...	...	White	440	
Lactic	W. yellow	White	...	...	Yellow	430	
Caffeic[a]	Gray-green	Gray	...	...	Immed. brown		
Glutaric	...	W. white	...	...	White	340	
Adipic	...	...	...	...	White	260	
Phthalic	...	...	...	...	Gray		
Monochloracetic	...	...	...	...	White		
Fumaric	W. Gray	W. white	...	...	White	340	
Trichloracetic	...	...	W. Pink	W. Pink	White		
Mandelic	Yellow	...	...	...	Yellow		
Gentisic[a]	Yellow	Gray	...	...	Brown		
Furoic	...	...	...	...	White		
Benzoic	...	...	...	...	White		
Salicylic[a]	...	...	...	...	White		
Tropic	...	...	...	...	Yellow		
Stearic	Yellow	...	...	...	White		
Apple Concentrate Acid							
A	...	w	...	Blue	Yellow	1230	Galacturonic
B	Yellow	...	Pink	Brown	White	1110	
C	...	...	...	...	White	1350	
D	Yellow	Pink	...	...	Yellow	1070	Quinic
E	...	White	...	...	Gray		Phosphoric
F	...	White	...	...	White	790	Citric
G	...	White	...	...	White	750	Malic
H[a]	...	...	...	...	Immed. brown		Chlorogenic
I	...	...	...	...	Yellow	590	
J	...	W. pink	...	...	White	590	Citralmalic
K	...	...	...	...	Yellow	530	
L	...	...	...	...	Immed. brown		Caffeic
M	...	Pink	...	...	White	430	Succinic
N	...	White	...	...	Yellow	430	Lactic
O	...	...	...	...	...	330	
P	...	...	...	...	White	250	
Q	...	...	...	...	White	140	

w = weak.
+ = Positive.

[a] Fluorescent under ultraviolet illumination before spraying.

Acid I was weak and did not show up very well on paper chromatograms. It was easier to see when rechromatographed on paper after being separated from most of the other acids on a silicic acid column.

Acid J is probably citramalic, which has been reported in apple peel (*11*).

Table II. Quantitative Analysis of Fresh and Darkened Concentrate

	Meq. in 100 Ml. of 18.6° Brix Juice	
Acid	Fresh juice	Darkened concentrate (diluted)
Total acids	13.4	13.6
D	0.16	0.16
E	0.69	0.74
G	11.4	11.6
I, J	0.15	0.15
M, N	0.27	0.26

Acid K was detected on paper chromatograms only after recovery from a silicic acid column.

Acid L, like H, shows up only under the most favorable conditions. As chlorogenic acid is made up of quinic acid and caffeic acid, it is reasonable to suppose that a trace of caffeic acid could be present.

Acids M and N appear to be succinic and lactic, respectively. Both have been found in apple fruit (*3, 9*). They were adequately resolved only in the ethyl alcohol–ammonia solvent and there seemed to be more of N than of M.

Acids O and P are weak and might be glutaric and adipic, respectively.

Acid Q is also weak and forms a rather diffuse spot.

All these identifications are tentative and will have to be confirmed by actual isolation of the acid and formation of derivatives. All acids present in the fresh juice remained in the darkened concentrate and no diminution in their concentration could be detected, but a larger amount of each acid will have to be available in order to get a true quantitative picture. This can probably be done by running several samples through a large silicic acid column, combining all fractions except malic, and rechromatographing on a column of the usual size. These studies will be the subject of future research.

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FOOD ANALYSIS

Determination of Benzoic and Salicylic Acids in Food Products

DUANE T. ENGLIS, BRUCE B. BURNETT[1], ROBERT A. SCHREIBER, and JAMES W. MILES[2]

Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Ill.

Benzoic and salicylic acids have long been used for preservation of food. As benzoic acid in quantities not exceeding 0.1% is permitted under the Federal Food, Drug, and Cosmetic Act, it has been employed widely. Both the processor and regulatory authority therefore must make certain that the approved quantity has not been exceeded. Salicylic acid cannot legally be added, but as it is ordinarily more effective than benzoic acid, it occasionally finds its way into foods.

[1] Present address, Carothers Research Laboratory, Experimental Station, E. I. du Pont de Nemours & Co., Wilmington, Del.
[2] Present address, College of Pharmacy, University of Kentucky, Louisville, Ky.

The acidity of the medium in which these acids are used is of major influence upon their preservative action (*20, 22*). Among the products in which one or both of the acids may be expected are ketchup, fruit concentrates, jams, preserves, and margarines. In general, methods for the determination of the two acids are similar, in that they are commonly extracted from an aqueous solution acidified with hydrochloric acid with the use of some volatile organic solvent, which later may be evaporated off. Among the solvents used are ether (*12, 13*), chloroform (*1, 15, 16*), and benzene (*4*). Sometimes separation is effected by steam distillation (*24*). Final estimation has been made gravimetrically (*9*), volumetrically by titration of the acids in alcohol solution with a standard base, and in the case of the salicylic acid by formation of a colored compound with ferric ion (*18, 23*).

Methods for analyzing mixtures of benzoic and salicylic acids have received limited attention. A tentative method of the Association of Official Agriculture Chemists (*3*) for their estimation in a compound ointment involves extraction of the acids and determination of total acidity by titration with a standard base. The salicylic acid is determined bromometrically and the benzoic acid is calculated by difference from the total acidity.

A review of the literature indicates