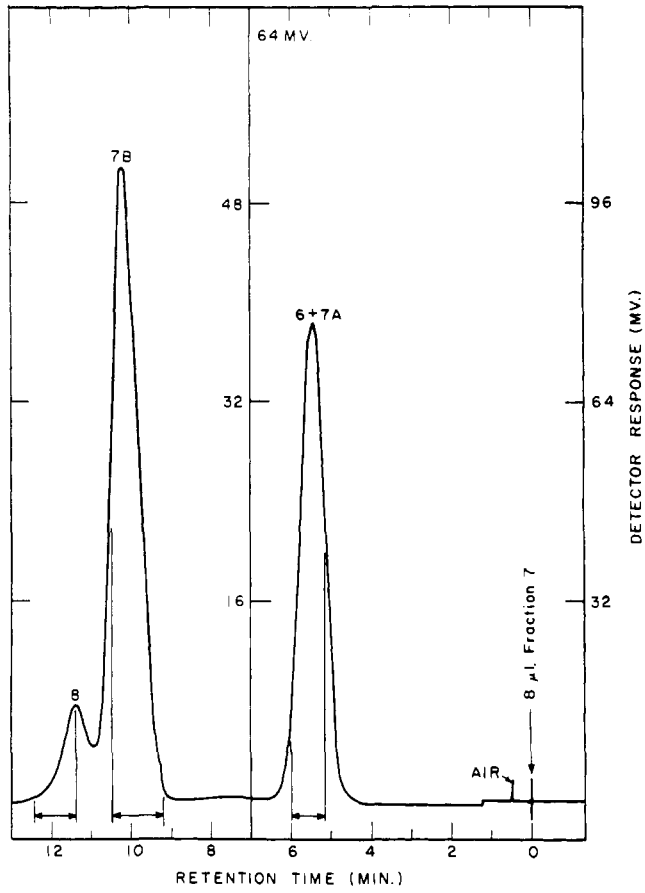

Figure 6. Fraction 7 chromatographed on 20% DEGS column

reference compounds (Table IV) suggested the presence of *n*-propanol, *n*-amyl acetate, *n*-hexyl acetate, and methyl acetate (*6*). Only the latter compound had been reported previously.

A number of fractions could be only generally characterized by their spectra as containing an acetate, an unsaturated ketone, an ester, an unsaturated alcohol, and a mixture of an alcohol and an acetate. The components of four fractions were unknown.

The compounds and fractions described are only partially responsible for the typical odor of bananas. Further investigations are necessary to elucidate their individual contributions to banana odor and their origin in the fruit.

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FOODSTUFFS ANALYSIS

Nonvolatile Acids of Blueberries

PERICLES MARKAKIS, ANDRZEJ JARCZYK,[1] and S. P. KRISHNA

Food Science Department, Michigan State University, East Lansing, Mich.

PRIOR TO THE DEVELOPMENT of chromatographic methods of analysis, qualitative and quantitative determination of acids present in small proportions in biological materials was a time-consuming and exacting operation. Minor acids were, therefore, studied only in cases of special interest. Foods were usually analyzed for two or three major acids (*9*), such as citric, malic, tartaric, or oxalic (*11*), and, of course, ascorbic acid (*17*).

[1] Present address, Zaklad Technologii, Owocow i Warzyw, Warszawa, Poland.

There is both academic interest and practical importance in the study of the individual acids of fruits. Acids are known to participate actively in the metabolism of the fruit (*16, 7*). They also definitively affect the flavor or off-flavor (*15*) of fruit and fruit products, and they may be involved in discolorations (*10, 12*).

A technique originally developed by Busch *et al.* (*4*) for the separation and determination of the Krebs cycle acids and further improved by Palmer (*14*) and Hulme and Wooltorton (*8*) appeared suitable for the study of the nonvolatile acids of blueberries, a fruit of rapidly increasing economic importance (*2*). Previously, Nelson (*13*) identified citric and malic acids in blueberries, and Kohman (*11*) determined the oxalic acid content of this fruit. Recently, Dewey and coworkers (*3, 19, 20*) studied, among other characteristics, the change of titratable acidity of blueberries during ripening.

The purpose of the present study has been to detect and quantitatively estimate as many as possible of the non-

Unripe and ripe blueberries of two cultivated varieties, Rubel and Jersey, were analyzed for nonvolatile acids. Acidified water extraction, lead precipitation, gradient elution column chromatography, paper chromatography, and titration were the chief analytical procedures used. Glutamic, aspartic, shikimic, quinic, galacturonic, glyceric, glycolic, succinic, glucuronic, citramalic, malic, citric, malonic, chlorogenic, caffeic, and phosphoric acids were tentatively identified and quantitatively estimated. Oxalic acid was determined independently. On an equivalent basis, more malic, chlorogenic, and phosphoric, and less citric and quinic acids were present in the acid mixture extracted from ripe than from unripe berries. Ripe Rubel berries contained more citric acid per 100 grams of fruit than Jersey berries of the same physiological age.

volatile acids of two varieties of blueberries at two stages of the development of the fruit.

Experimental

Blueberries, *Vaccinium corymbosum* L., of the Jersey and Rubel varieties were collected from mature bushes cultivated in southwestern Michigan. The first collection of Jersey berries was made 6 to 8 days before the appearance of red coloration, and that of Rubel berries 2 to 3 days before red coloration. The ripe fruit of both varieties was harvested 6 to 8 days after the first red color appeared on the berries. Quantities (0.5 kg.) of each of the four categories of blueberries were sealed in polyethylene bags and stored at −23° C. until analyzed.

Fifty grams of ripe, or 25 grams of unripe, berries were dropped in 100 ml. of boiling water, boiled for 5 minutes, disintegrated hot in a Waring Blendor for 3 minutes at high speed, transferred back to the original beaker with the aid of 50 ml. of water, and boiled for another 10 minutes. To the hot slurry were added 2 ml. of 1*N* HNO_3 (*6*), and the mixture was left to cool. After reaching room temperature, its volume was made to 250 ml. and filtered through cotton. Two hundred milliliters of the filtrate were concentrated to ca. 60 ml. in a vacuum flash-evaporator and diluted to 250 ml. with 95% ethanol. The precipitated pectins were filtered out by means of a milk filter, and 200 ml. of filtrate were transferred into a 250-ml. centrifuge flask containing a Teflon-coated magnet. The pH of the solution was adjusted to 7.5 with 2*N* NH_4OH, and lead subacetate dissolved in a few ml. of water was added in quantity equivalent to about twice the original titratable acidity of the sample. The mixture was stirred magnetically for 5 min., 0.2 gram of Celite was added, the flask was filled with 80% ethanol and centrifuged again. A second decantation, redispersion, and centrifugation followed. If the second or third supernatant was not clear, it was not decanted; instead, the sediment was dispersed anew, the pH of this suspension adjusted to 8.5 with NH_4OH and centrifuged again. The sediment obtained after the third decantation was finally dispersed in 150 ml. of 50% ethanol, and the suspension was saturated with H_2S. After 5 minutes of mixing with the aid of a magnetic stirrer, the flask was centrifuged, and the supernatant was checked for soluble lead by bubbling H_2S. If precipitation was complete, the solution of the free acids was concentrated to ca. 15 ml. in the flash evaporator, passed through a column of Dowex 50W × 8, 50–100 mesh, 10 cm. long by 0.7 cm. in diameter, and finally 25 to 30 ml. of acid solution were obtained. Representative aliquots were titrated to determine the acid content, and portions corresponding to a total acidity of 1 to 1.5 meq. were used for fractionation.

Dowex 1 × 8 acetate, 200–400 mesh, was used for fractionating the mixture of the acids. Commercial Dowex 1 × 8 chloride was converted to Dowex 1 × 8 acetate by first removing the fines of the chloride and then washing it, as a column, with 1*N* sodium acetate solution until free of Cl^-. Excess sodium acetate was displaced by 0.1*N* acetic acid. A resin column 33 cm. long and 0.7 cm. in diameter was formed (an Exax 10-ml. buret may be used for the purpose). After the sample was applied to the column, 15 ml. of water were passed through before the concentration gradient elution system, which consisted of a mixing flask, an eluant, reservoir, and a pressure regulator, was connected to the column. A 125-ml. suction flask, the side arm of which was connected with the column by a capillary glass tube and tygon sleeves, was used as a mixing flask. A 300-ml. separatory funnel, to the tip of which was fused a capillary glass tube reaching close to the bottom of the mixing flask, served as reservoir of the eluant. An air pressure regulator (Moore Products Co., Philadelphia 24, Pa.) was used to keep the pressure in the system at 70 inches of water. Mixing was accomplished by a magnetic stirrer. The level of the liquid in the mixing flask was kept below the rubber stopper connecting reservoir and flask. The first eluting solution consisted of 100 ml. of 3*N* acetic acid, the second, of 50 ml. of 6*N* acetic acid, and the third, of 275 ml. of 6*N* formic acid. Eighty-one fractions of 5.25 ml. each were collected in an automatic fraction cutter (Rinco Instruments, Greenville, Ill.).

The fractions were dried in a vacuum oven at 40° C. and then paper chromatographed or titrated. For paper chromatography, Whatman No. 1 sheets, 46 × 57 cm., were used, and the fractions, which had been redissolved in 50% ethanol, were spotted 2 cm. apart, 7 cm. away from the long edge of the sheet. The spotted papers were irrigated descendingly by the upper phase of a mixture of 1-butanol and 3*N* formic acid, 50:50 by volume. The lower phase of the mixture was used for vapor equilibration. After 12 hours of irrigation, the papers were dried in an air draft and sprayed with a 0.05% solution of bromphenol blue (Na salt) in 50% ethanol. For the quantitative determination, the fractions were redissolved in hot water and titrated with 0.02*N* NaOH, using phenolphthalein as indicator. Intensely colored fractions were titrated electrometrically to pH 8.1.

Oxalic acid was determined by Baker's method (*1*), independently of the ion exchange procedure. The aliquots used for the final titration were four times larger than those originally suggested by Baker. The Ca oxalate was washed on a fine sintered glass filter rather than by centrifugation, and the filter with the precipitate was transferred into a wide-mouth, conical flask for permanganate titration.

For identification, 35 known acids were passed through the column in small groups to determine their effluent volumes, and also were paper chromatographed on the same paper with the unknowns. The fluorescence of chlorogenic and caffeic acids under ultraviolet light and the molybdate test for phosphoric acid (*5*) were used as additional evidence for the identity of these acids.

Silica gel chromatography was used to confirm the identity of citric and malonic acids. The fractions of the Dowex 1 × 8 chromatography which contained these acids were combined

Table I. R_f × 100 Values of Blueberry Acids Separated by Column Chromatography and Spotted Beside Known Acids[a]

Acid	Known	Unknown
Glutamic	17	17
Aspartic	12	12
Shikimic	38	39
Quinic	23	23
Galacturonic	8	8
Glyceric	42	41
Glycolic	58	58
Succinic	71	71
Glucuronic	10	10
Citramalic	62	62
Malic	50	50
Citric	44	44
Malonic	66	65
Chlorogenic	69	69
Caffeic	78	76
Phosphoric	22–30	26

[a] Solvent: 1-butanol + 3*N* formic acid (v./v.); paper: Whatman No. 1; descending run.

after titration, passed through Dowex 50W × 8, concentrated in vacuo and chromatographed on silica gel by the method of Zbinovsky and Burris (*21*).

Results and Discussion

The following acids, in order of emergence from the column, have been tentatively identified in the blueberry varieties examined: glutamic, aspartic, shikimic, quinic, galacturonic, glyceric, glycolic, succinic, glucuronic, citramalic, malic, citric, malonic, chlorogenic, caffeic, and phosphoric. Some acids emerged from the column in pairs which were subsequently separated by paper chromatography. Such pairs were the glyceric and glycolic, succinic and glucuronic, citric and malonic, and chlorogenic and caffeic acids. Attempts to separate these pairs by reducing the fraction to size in column chromatography were not very successful. Malonic acid was detected only when the paper was heavily spotted, since this acid is present in very small quantities in comparison to citric acid. Citric and isocitric acids were not separable by the ion exchange and paper chromatographic methods used here. Silica gel chromatography, however, separates them cleanly with peaks at 66 ml. for citric acid and 88 ml. for isocitric acid. When the acid(s) to be confirmed were co-chromatographed on silica gel with citric and isocitric acids separately, it was shown that only citric acid was present. This agrees with Nelson's (*13*) findings. In the same runs, malonic acid appeared with the expected peak at 14 ml.

Table I presents data of a paper chromatographic run in which the blueberry acids, after separation by column chromatography, were spotted next to known acids.

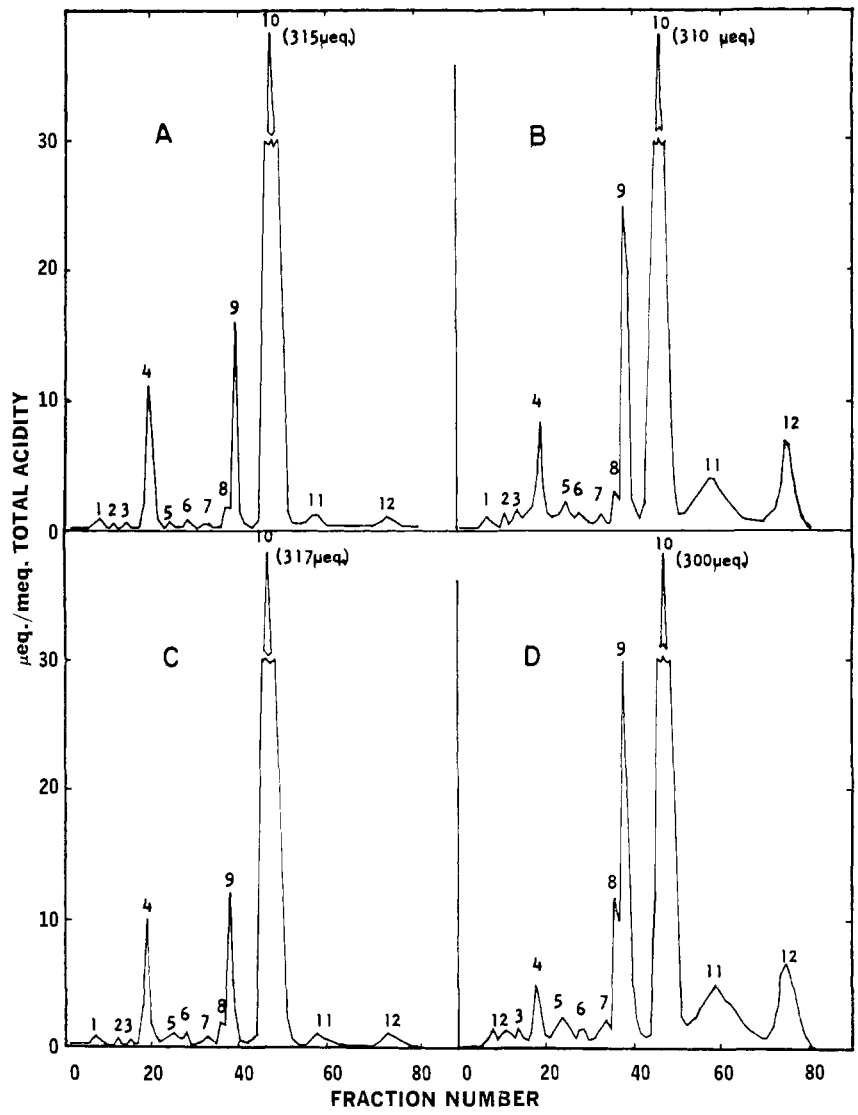

Figure 1. Titration of column chromatographic fractions of acids of Rubel and Jersey blueberries

Tentative identity of acids: 1. glutamic; 2. aspartic; 3. shikimic; 4. quinic; 5. galacturonic; 6. glyceric and glycolic; 7. succinic and glucuronic; 8. citramalic; 9. malic; 10. citric and very little malonic; 11. chlorogenic and little caffeic; 12. phosphoric

(*A*) unripe Rubel blueberries; (*B*) ripe Rubel blueberries; (*C*) unripe Jersey blueberries; (*D*) ripe Jersey blueberries

The quantitative results of four typical analyses were graphically presented in Figure 1. Except for the unseparated pairs, resolution of the column chromatographic procedure is generally satisfactory as indicated by the sharpness of the peaks. Chlorogenic and caffeic acids tend to appear in a rather large number of fractions; this, however, does not seem to affect adversely the over-all performance of the column. A large portion of the anthocyanins were removed, along with the alkaline substances, by the Dowex 50W × 8 resin treatment. The remaining anthocyanins did not interfere with the column separation of the acids, and most of the colored fractions could be titrated with phenolphthalein since the anthocyanin red disappeared as the pH approached 7.0.

Nevertheless, the column fractionation of the acids as applied here is not free from undesirable features. Reducing sugars, if not entirely removed from the sample, become oxidized while passing the column and result in the ghost acid spots of the first two or three fractions. A series of unexpected weak acid spots of R_f 0.10 also appears in fractions No. 50 to 75, and there are reasons to suspect they are an artifact.

Although the quantitative recovery of acids from ion exchange columns is generally satisfactory, a greater or smaller percentage of some acids, such as glyceric and citric, may not be finally accounted for (*4*, *8*, *14*), and oxalic acid does not seem to yield itself to such analysis (*14*). The over-all recoveries in experiments here were in higher than 90%.

Table II summarizes salient traits of the acid profile of the four blueberry categories analyzed. The figures represent the average of two determinations performed on two different extracts of the same sample o blueberries.

The citric acid value also covers the concentration of malonic acid which on the basis of the silica gel chromatography is estimated as being of the same low order with that of each of the "other acids," 0.1 to 0.2 meq. per 100 grams of fruit. Caffeic acid, however, may be responsible for up to 20 or 30% of the acidity of the chlorogenic-caffeic fractions.

In calculating the acid concentrations per 100 grams of fruit, the phosphoric acid values were multiplied by $^3/_2$, for only two thirds of this acid was titratable with phenolphthalein as an indicator.

These data indicate that in both the Jersey and Rubel blueberries the relative proportions of the acids change as the fruit matures, malic, chlorogenic, and phosphoric acids increasing with ripening, quinic and citric decreasing. The concentration of phosphoric acid per 100 grams of fruit showed essentially no change in the two stages of maturity studied, whereas the concentration of most of the other acids decreased considerably. Figure 1 shows a higher proportion of citramalic and galacturonic acids in the mature berries. The increase in the latter acid is probably connected with the metabolism of pectins. It is difficult to discuss the acids of blueberries in biochemical or physiological terms, since so little is known about the acid metabolism in fruits generally (*18*). Some authors (*7*) have even questioned the operation of a Krebs cycle as it is understood in animal tissues, and there is no satisfactory explanation for the accumulation of certain acids, such as the citric acid in blueberries, in concentrations far exceeding those of the rest of the acids.

Comparison of the two blueberry varieties analyzed for acids indicates that while qualitative differences are not apparent, ripe Rubel berries contain more citric acid per 100 grams of fruit than Jersey berries of the same physiological age. The higher titratable acidity of the Rubel berries observed by Dewey and coworkers (*3, 20*) and here must be due primarily to citric acid. In terms of acid equivalents per 100 equivalents of acidity extracted, the ripe Rubel berries were found to contain more citric acid but less malic acid, than the ripe Jersey berries.

Table II. Nonvolatile Acids of Rubel and Jersey Blueberries

	Rubel				Jersey			
	Unripe		Ripe		Unripe		Ripe	
Acid	Eq./100 eq. total acidity	Mg./100 grams fruit	Eq./100 eq. total acidity	Mg./100 grams fruit	Eq./100 eq. total acidity	Mg./100 grams fruit	Eq./100 eq. total acidity	Mg./100 grams fruit
Citric	85.9	2665	75.2	578	89.7	3215	71.6	477
Malic	2.0	65	5.8	47	1.9	71	7.2	50
Quinic	1.9	177	1.7	39	1.8	193	1.3	26
Chlorogenic[a]	0.7	121	3.0	127	0.7	140	3.1	115
Phosphoric	0.6	15	2.8	16	0.6	16	3.0	15
Oxalic	0.2	6	0.7	4	0.2	5	0.6	3
Other acids[b]	3.1	..	4.6	..	2.1	..	5.9	..

[a] Plus caffeic acid.

[b] An average figure indicating the order of concentration of each of these acids would be 0.2 meq. per 100 grams of unripe fruit and 0.1 meq. per 100 grams of ripe fruit.

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