GRAPE PIGMENTS

Concord Grape Pigments

Colloidal Blue Pigments of Juice

D. W. INGALSBE, A. M. NEUBERT, and G. H. CARTER

Identification of the Anthocyanins

D. W. INGALSBE, A. M. NEUBERT, and G. H. CARTER

U. S. Fruit and Vegetable Products Laboratory, Prosser, Wash.

THERE is usually no difficulty in meeting color requirements in single strength Concord grape juice, but color is more often a limiting factor in grade for frozen concentrated sweetened grape juice (9). Losses in color during processing and detartration storage contribute to lack of adequate color in such products. Reports on losses of color in grape juice have been concerned with color changes occurring in bottled juices stored at or above room temperature through action of heat, light, oxygen, or for other reasons (3, 4). Such changes are presumably due to pigment decomposition and would have little if any effect on color of concentrates that are stored in a frozen condition.

The present study was promoted by the observation that color loss during detartration is due to pigment precipitation rather than decomposition. Essentially complete recovery of the color lost from the juice can be realized by extracting the tartrate sludge with 1% aqueous HCl. Much of this recovered pigment can be reprecipitated by raising the pH of the extract to that normal for grape juice. The present study was made to isolate and identify the insoluble pigments in grape juice that are responsible for loss in color during detartration. The predominant anthocyanin pigments of Concord grapes and juice were isolated and identified, and their order of development in the grape berry was studied.

The report on the anthocyanin pigments in grapes by Ribéreau-Gayon (6) was found particularly useful in this study for the technique described and in identifying the several pigments. Reports on anthocyanin pigments in Concord grapes, *Vitis labrusca*, (1, 5, 8) were made before the publication of Ribéreau-Gayon's monograph. Handicapped by the lack of this technique and supporting data, these workers failed to resolve the complex nature of the anthocyanin pigments of Concord grapes and juice.

COLLOIDAL BLUE PIGMENTS OF JUICE

Experimental Procedures and Results

Tartrate sludges were obtained from experimentally processed Concord grape juices, commercial bulk detartration tanks of both Washington and New York state produced juices, and commercial bulk frozen concentrates. The sludges were extracted and the pigments separated into fractions by a series of treatments in chromatographic columns as outlined in Figure 1. No differences were observed between Washington and New York tartrate sludges, either in the fractions separated or in the pigments detected.

The columns, varying in diameter from 1 to 5 cm., were prepared from coarse cellulose powder and from silicic acid powder (100-mesh, 12% loss on ignition). Pigment preparations were dried on the packing material in a stream of air at room temperature. These preparations were then packed firmly into the columns over pure packing material. Elution was by gravity, using the upper, alcoholic layer of a *n*-butanol-acetic acid–water mixture (4:1:5), 1.0% aqueous HCl, 1.0% methanolic HCl, and pH 3.5 buffer (MacIlvaine's) as solvents. In all cases, pigments soluble in an eluant were removed in a highly concentrated front. Elution was continued until the color in the eluant approached a constant value.

Absorbance of juices, tartrate sludges, and various pigment fractions were measured at 515 mμ on samples suitably diluted with pH 1.0 buffer (Clark and Lubs) (7). An Evelyn colorimeter with macro tubes was used. Dried pigment preparations that were difficult to dissolve in the buffer were first dissolved in a small volume of methanolic HCl. A pH of 1.0 was selected as most suitable for measuring color in Concord juice and pigment fractions because changes in absorbance with pH were much less in the vicinity of 1.0 than in the pH range

normal for grape juice (pH 3.0 to 3.8). Also, visible turbidity that could not be removed by filtration was less at pH 1.0. A color unit was arbitrarily established as the product of absorbance and the volume in liters.

The amount of color shown for each fraction in Figure 1 was obtained in fractionating a 1-gallon jug of experimentally prepared juice. In a number of such fractionation studies, the amount of color recovered from the tartrate sludge varied from 20 to 40% of the color of the freshly prepared juice from which the sludge precipitated. Color losses were increased when decanted juices were filtered, indicating that pigment precipitation was not complete. Also, color losses by precipitation increased as detartration storage increased from 5 to 12 months. Considerable variation also occurred in the relative amounts of pigment obtained in subsequent fractions.

Two-dimensional paper chromatographs were used to determine pigments present in Concord grapes and tartrate sludge and to follow their separation into various fractions. One percent methanolic HCl solutions of the pigments were used. The first dimension was developed with the lower, aqueous phase of a *n*-butanol-acetic acid–water mixture (4:1:5) (BAW) and the upper, alcoholic layer was used for the second dimension, according to the method of Ribéreau-Gayon (6). The general pattern of spots obtained was quite reproducible, but there was some variation in R_f values in the different chromatographic runs, depending on temperature, direction of development on the paper, age and method of solvent preparation, and other factors. Figure 2 represents a typical two-dimensional chromatogram of the methanolic HCl extract, fraction VI in Figure 1. The chromatographic data presented in Figure 1 refer to spots shown in Figure 2.

From 20 to 40% of the color of Concord grape juice is lost through precipitation of pigment during detartration storage. Fourteen anthocyanin pigments found in the tartrate precipitate were also found in Concord grapes and juice. Two pigment fractions isolated from the precipitate form clear, deep blue, colloidal dispersions in aqueous solutions in the pH range of grape juice. One, minor in amount present, consists of anthocyanin pigments combined with metals. The other consists of two pigments identified as p-coumaric acid esters of delphinidin 3-monoglucoside and cyanidin 3-monoglucoside. These two anthocyanins and their p-coumaric esters predominate in Concord grapes and juice. The p-coumaric esters are probably responsible for adsorption of soluble anthocyanin pigments and their removal from juice by precipitation during detartration. They are also considered responsible, in the form of a colloidal dispersion, for most of the blue color of Concord grape juice.

Single-dimension chromatograms using the butanolic phase of BAW and other commonly used solvents were found unsatisfactory because of the many pigments present. A maximum of six poorly defined spots could be obtained, and these often showed two or more colors in visible or ultraviolet light.

The blue quality and colloidal properties of some of the pigments present in tartrate sludge were apparent in the preparation of fraction IV. The color of the aqueous extract, fraction III, changed from red to purple when the pH was adjusted to the range 3 to 4. The red color was restored to the solution and a black sludge containing pigment and potassium bitartrate was recovered by settling and centrifugation. The freshly flocculated pigment passed through filter paper. Centrifuging permitted its recovery as a thick black paste that contained in excess of 90% water. When this paste was washed by repeated suspension in distilled water, a point was reached where centrifugation no longer settled the pigment and the supernatant solution remained black. Addition of NaCl again permitted separation, showing that presence of an electrolyte was necessary to cause flocculation. Thus, it was apparent that the blue color was the result of colloidal dispersion rather than a dissolved pigment.

The presence of 14 anthocyanin pigments in fraction VI, as shown in Figure 2, suggests that most, if not all, of the anthocyanin pigments in Concord grapes must be present in the tartrate sludge. This also suggests that the color loss during detartration is the result of adsorption of soluble anthocyanin pigments by insoluble material that precipitates and that adsorption is not selective.

Isolation of Anthocyanin-Metal Fraction. The column treatment of fraction VI was used to separate pigments that remained as a brown spot at the origin from those that moved with the solvents in Figure 2. Part of the pigment present in this brown spot could be eluted from the cellulose column by means of 1% aqueous HCl. This fraction (VIII in Figure 1) changed from deep red to a clear, deep blue as the pH of the eluant was raised to 3.5 by addition of 2 to 5% NaOH. Within a few minutes, a black floc formed and settled, leaving a clear, colorless solution.

Two quantities of fraction VIII, 0.54 and 1.29 grams, were prepared from tartrate sludge and air dried. Chromatograms of these preparations usually showed traces of poorly defined red smears in addition to a heavy brown spot at the origin. One preparation contained 9.1% ash and the other 2.2%. Semiquantitative spectrographic analyses showed iron and vanadium predominant, with traces of aluminum, copper, sodium, nickel, silicon, and titanium. No evidence was found of bismuth, calcium, cadmium, cobalt, manganese, molybdenum, lead, tin, strontium, or zinc. A methanolic HCl solution of fraction VIII that had been held at room temperature for some time before rechromatographing showed spots corresponding to 1, 2, 3, and 5 in Figure 2 and a brown streak in the direction of the aqueous phase. The appearance of these pigments after prolonged hydrolysis indicates that fraction VIII consists of anthocyanin pigments combined with metals.

Isolation of Acylated Anthocyanin Pigment Fraction. One per cent aqueous HCl solutions of fraction VII in Figure 1 also changed from clear, deep red to clear, deep blue as the pH was raised to 3.5 The pH 3.5 solution was also unstable and formed a black floc which settled and left a clear, light red solution on standing. Thus, apparently the anthocyanin-metal fraction VIII is not the only material present in tartrate sludge capable of forming a blue colloidal dispersion. Fraction VII contained all of the anthocyanin pigments found in fraction VI except the spot at the origin (Figure 1). From this, it is apparent that the material responsible for the precipitation of pigments in fraction VII at pH 3.5 is capable of adsorbing and removing from solution a substantial portion of all of the anthocyanins present.

The use of a silicic acid column to separate pigments soluble in aqueous pH 3.5 buffer from those that are insoluble was resorted to when it was found impossible to make the separation from cellulose powder. Pigments soluble in pH 3.5 buffer could be removed from silicic acid in a very concentrated front, and the column changed from red to blue during elution. The silicic acid appeared to act as a scaffolding to hold the colloidal blue pigment while the soluble pigments were removed. During this elution, blue pigment often leaked in thin streams into the pure silicic acid at the column bottom. If these streamers leaked through the column, they formed a thin layer on the eluant. A small amount of blue pigment was probably also eluted with the first small volume of pH 3.5 buffer because the pH of this first eluant was lowered by HCl removed from the pigment in the column. Chromatograms of the pH 3.5 buffer-soluble pigments from the silicic acid column, fraction IX in Figure 1, were very similar to the chromatogram shown in Figure 2, but spots 6 and 7 were much lighter.

The residual pigment on the silicic acid could be removed in a very concentrated front by elution with 1% methanolic HCl. When this eluant, fraction X in Figure 1, was chromatographed, only spots 6 and 7 were obtained. When the pigments in fraction X were diluted with aqueous 1% HCl and then neutralized to pH 3.5 with 2 to 5% NaOH, the color changed from deep red to a clear, deep blue. This color change became apparent as the pH approached 2.5. The solution was unstable and a blue-black floc formed and precipitated, leaving a clear, colorless solution. On the basis of the anthocyanin pigment map developed by Ribéreau-Gayon using similar chromatographic techniques (6), the position of spots 6 and 7
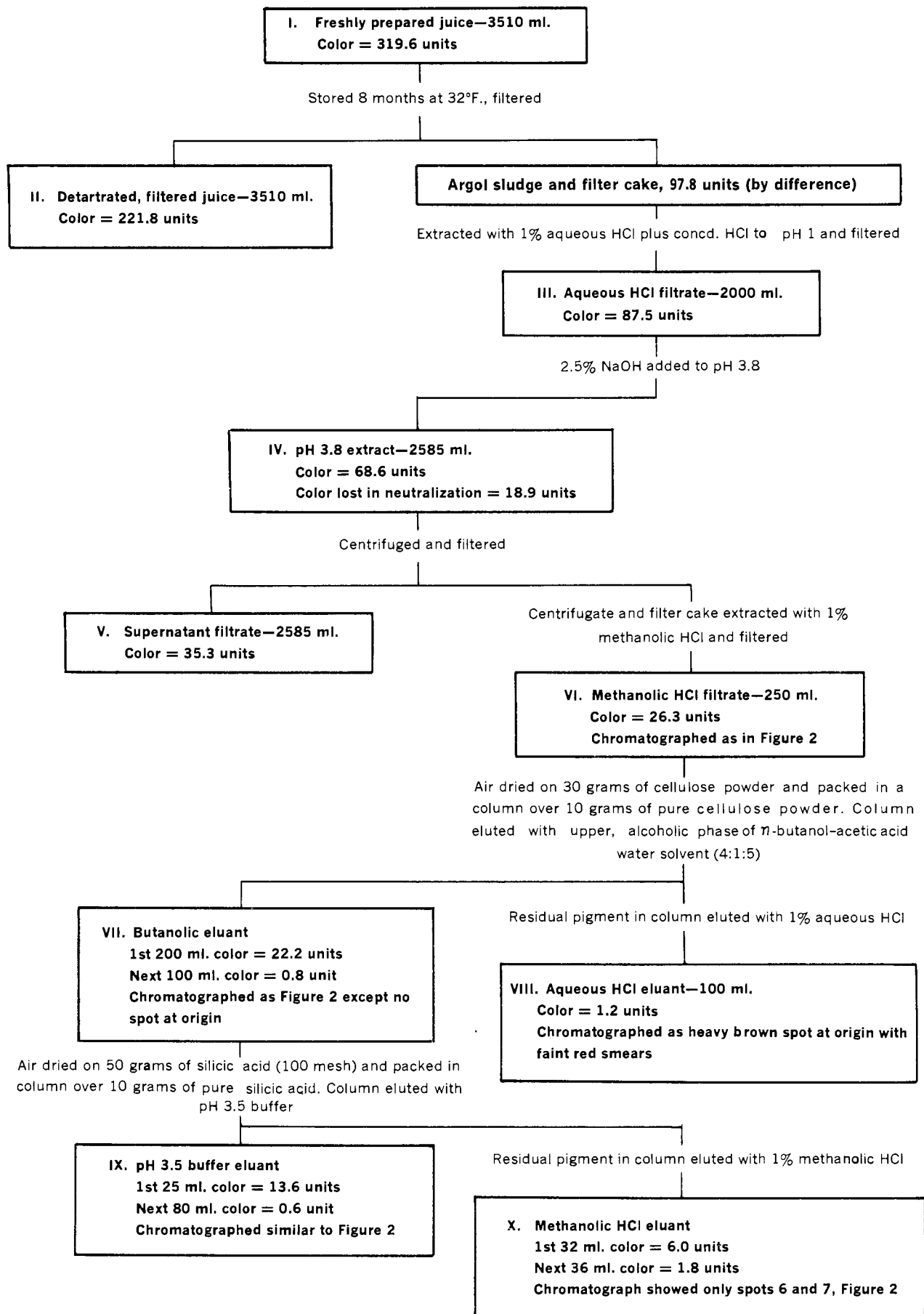
I. Freshly prepared juice—3510 ml.
   Color = 319.6 units

Stored 8 months at 32°F., filtered

II. Detartrated, filtered juice—3510 ml.
    Color = 221.8 units

Argol sludge and filter cake, 97.8 units (by difference)

Extracted with 1% aqueous HCl plus concd. HCl to   pH 1 and filtered

III. Aqueous HCl filtrate—2000 ml.
     Color = 87.5 units

2.5% NaOH added to pH 3.8

IV. pH 3.8 extract—2585 ml.
    Color = 68.6 units
    Color lost in neutralization = 18.9 units

Centrifuged and filtered

V. Supernatant filtrate—2585 ml.
   Color = 35.3 units

Centrifugate and filter cake extracted with 1%
methanolic HCl and filtered

VI. Methanolic HCl filtrate—250 ml.
    Color = 26.3 units
    Chromatographed as in Figure 2

Air dried on 30 grams of cellulose powder and packed in a
column over 10 grams of pure cellulose powder. Column
eluted with upper, alcoholic phase of n-butanol–acetic acid
water solvent (4:1:5)

VII. Butanolic eluant
     1st 200 ml. color = 22.2 units
     Next 100 ml. color = 0.8 unit
     Chromatographed as Figure 2 except no
     spot at origin

Residual pigment in column eluted with 1% aqueous HCl

VIII. Aqueous HCl eluant—100 ml.
      Color = 1.2 units
      Chromatographed as heavy brown spot at origin with
      faint red smears

Air dried on 50 grams of silicic acid (100 mesh) and packed in
column over 10 grams of pure silicic acid. Column eluted with
pH 3.5 buffer

IX. pH 3.5 buffer eluant
    1st 25 ml. color = 13.6 units
    Next 80 ml. color = 0.6 unit
    Chromatographed similar to Figure 2

Residual pigment in column eluted with 1% methanolic HCl

X. Methanolic HCl eluant
   1st 32 ml. color = 6.0 units
   Next 36 ml. color = 1.8 units
   Chromatograph showed only spots 6 and 7, Figure 2

Figure 1.   Flow diagram of grape juice pigment fractionation
Color units= absorbance X volume in liters; absorbance measured at 515 mμ and pH 1.0

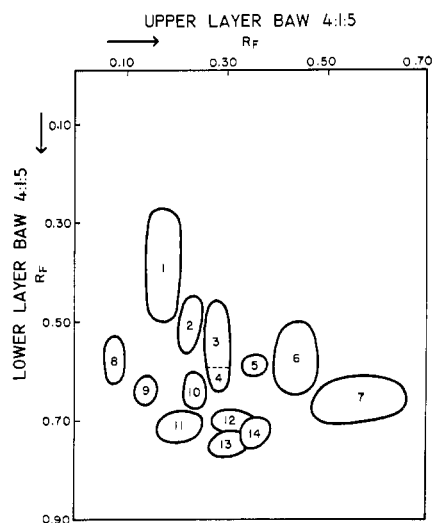**Figure 2. Paper chromatogram of Concord grape pigments**

[Butanol–acetic acid–water (4:1:5) solvent]

Table I. Data on Individual Spots in Chromatogram

Pigment No.	Approximate Quantitative Order[b]	R_f[a] Lower Layer BAW[c]	Upper Layer BAW	Color Visible	Ultraviolet[d]	Probable Pigment[e]
1	1	0.39	0.19	Purple	Purple	Delphinidin 3-monoglucoside[f]
2	5	0.51	0.23	Purple	Deep pink	Petunidin 3-monoglucoside
3	3	0.52	0.28	Mauve	Deep pink	Cyanidin 3-monoglucoside[f]
4	6	0.58	0.30	Purple	Purple	Malvidin 3-monoglucoside
5	7	0.58	0.35	Mauve	Deep pink	Peonidin 3-monoglucoside
6	2	0.58	0.43	Purple	Purple	p-Coumaric acid ester of delphinidin 3-monoglucoside[f]
7	4	0.66	0.54	Mauve	Deep pink	p-Coumaric acid ester of cyanidin 3-monoglucoside[f]
8	10	0.58	0.08	Purple	. . .	3,5-Diglucoside
9	8	0.64	0.14	Mauve	Deep pink	3,5-Diglucoside
10	7	0.64	0.24	Purple	Deep pink	3,5-Diglucoside
11	9	0.71	0.20	Mauve	Bright pink	3,5-Diglucoside
12	10	0.70	0.31	Mauve	. . .	Acylated 3-5-diglucoside
13	10	0.73	0.35	Pink	Bright pink	Acylated 3-5-diglucoside
14	10	0.75	0.31	Mauve	. . .	Acylated 3-5-diglucoside

[a] R_f values varied somewhat depending on direction of development on paper, temperature, and other factors.
[b] Estimated visually on basis of size and color intensity of spots.
[c] n-Butanol–acetic acid–water (4:1:5).
[d] Long-wave ultraviolet lamp.
[e] By comparison with Ribéreau-Gayon pigment map (6).
[f] Pigments isolated and identified by their aglycones and sugar and acid moieties.

indicate that these pigments are acylated derivatives of anthocyanins. Air-dried preparations of fraction X were ash-free but required about 110 hours under vacuum at 70° C. to attain constant weight, during which the weight loss was about 25%.

Not previously reported is the comparative insolubility of acylated derivatives of anthocyanin pigments in aqueous solution. Dried preparations of fraction X were quite insoluble in 1% aqueous HCl. After an air-dried sample in pH 1.0 buffer was shaken periodically during a 1-month period, the absorbance of the filtered solution was only 0.15, which indicates that the apparent solubility of these pigments in pH 1.0 aqueous solutions is probably due to ease of dispersion as a colloid from the hydrated form in which it precipitates.

One per cent methanolic HCl solutions of fraction X had an absorption spectrum typical of anthocyanin pigments, when measured over the range 230 to 700 mμ by means of a Beckman Model DU spectrophotometer. Maximum absorptions appeared at 285 and 535 mμ. Fraction X also had a much lower absorbance at 515 mμ and pH 1.0, per unit weight (12.6 color units per gram of oven-dried material), than did pure chrysanthemin hydrochloride (85.0 color units per gram) or a mixture of Concord grape anthocyanin pigments that had been purified by reprecipitation as the picrate by the procedure of Anderson (7) (86.6 color units per gram). A synthetic preparation of malvidin had an absorption equivalent to 121.1 color units per gram.

The hydrophylic, colloidal nature of the insoluble pigments in fraction VIII and X, and their ability to form clear,

deep blue colloidal dispersions in the pH range normal for grape juice suggests that they are responsible for the precipitation of pigments during detartration storage and for the purple color of grape juice. Mixtures of pigment fractions from grape juice that are truly soluble in aqueous solution, such as fraction V in Figure 1, and the mixed, purified anthocyanin pigment preparation were red in aqueous solutions in the pH range of grape juice.

Pigment fraction VIII was a minor constituent of the precipitated tartrate sludge. In terms of color units, the amount separated as fraction VIII was only 1.4% of the color in the tartrate sludge. Fraction X was a much more important constituent, representing almost 9% of the color in the tartrate sludge. Fraction X was even more important, on a weight basis, since its coloring capacity is much less than that

of soluble anthocyanins. The total color units recovered from the tartrate sludge in Figure 1 would be equivalent to about 1 gram of mixed, soluble anthocyanins, and the 7.8 color units isolated in fraction X represent more than 0.6 gram of this material on an oven-dry basis. The fact that the pigments in fraction X are hydrophilic and contain over 90% water in the form in which they precipitate suggests that they have a prominent place among pigments in grape juice on the basis of volume occupied. A material of this nature would be quite likely to absorb the soluble anthocyanin pigments during flocculation. The ability of the pigments in fraction X to do this is demonstrated by the fact that most of the pigments found in fraction IX could be precipitated by raising the pH of the pigments in fraction VII (from which fraction VIII had been removed) to 3.5 in aqueous solution.

IDENTIFICATION OF THE ANTHOCYANINS

Experimental Procedure and Results

Extracts of fresh or frozen grape skins, prepared according to the method outlined by Bockian et al. (2), were used in chromatographic studies on grapes. Preparations of acylated pigment, recovered from tartrate sludge as fraction X by the procedure described earlier, were used to isolate and identify several of the pigments. The two-dimensional chromatographic technique described in the first section was used to separate the anthocyanin pigments.

Figure 2 represents a typical chromatogram of pigments from mature Concord grape skins. The pigments represented by numbers 8 through 14 were present in small amounts and were not resolved sufficiently to determine their exact number. However, these pigments were resolved in enough different samples to ascertain that they are present in grape skins, juice, and precipitated tartrate sludge. Pigments 3 and 4 chromatographed as a more or less con-

tinuous oval with the end having the higher R_f in the first dimension being purple and most of the spot being mauve. Table I presents data on the individual spots and indicates the identity of these spots as they may be anticipated from the anthocyanin map developed by Ribéreau-Gayon (6), who used a similar technique. Pigments 1, 3, 6, and 7 were identified in this laboratory from their respective aglycones, sugar moiety (glucose), and, in the case of the acylated pigments 6 and 7, their acid moiety (p-coumaric acid).

Chromatograms of pigment extracts from fresh or frozen skins of Concord grapes that were just beginning to color (soluble solids of 12.7%) showed pigments 1 and 3 predominant. There were only faint spots at 6 and 7, and a few faint spots at sites of Figure 1 numbered higher than 7. As maturity increased, pigments 6 and 7 became more prominent, and the group above 7 filled out and became more intense. In mature grapes, pigments 1, 3, 6, and 7 were dominant and represented about 70% of the total. The brown area at the origin was present in samples from all maturities, but appeared to be less heavy as maturity increased. The chromatograms of mature grape skins were very similar to those obtained from tartrate sludge in the first section of this report, except that pigments 6 and 7 were somewhat heavier in the sludge extracts.

A pigment preparation containing only the two acylated pigments, 6 and 7, was separated by streaking on Whatman No. 3MM paper and developing with the upper phase of BAW solvent. The two separated bands were cut from the sheets and eluted with 1% methanolic HCl. When these eluants were chromatographed in two dimensions, one separated pigments 1 and 6 and the other pigments 3 and 7. Thus, partial deacylation of the pigments occurred in the several days required to make the elution and rechromatograph the separated pigments. Similar hydrolysis occurred when 1% methanolic HCl solutions of the mixed pigments were allowed to stand at room temperature for several days.

Pigments 6 and 7 were hydrolyzed to their aglycones by refluxing for 30 minutes in $2N$ HCl after dilution to an absorbance of about 0.8. The aglycones were extracted from the hydrolyzate with isoamyl alcohol and chromatographed in two dimensions using the upper layer of an n-butanol–$2N$ HCl mixture (1:1) as the first solvent and Forestal solvent (acetic acid–HCl–water, 30:3:10) as the second solvent. Authentic samples of delphinidin, cyanidin, malvidin, and petunidin were used as standards. Only delphinidin and cyanidin were found in the hydrolyzate from the mixed acylated pigments 6 and 7. Only delphinidin was found in the isolated preparation of pig-

ment 6, which contained some pigment 1, and only cyanidin was found in the isolated preparation of pigment 7, which contained some pigment 3.

After removal of HCl from the aqueous hydrolyzate by extraction with di-n-octylmethylamine–chloroform (1:9), the sugars were chromatographed in a single dimension using n-butanol–ethanol-water (10:1:2) as solvent and aniline hydrogen phthalate reagent as developer. Arabinose, galactose, and glucose were used as standards. Only glucose was found. The same standard sugars and unknowns were compared using ethyl acetate–pyridine–water (10:1:3) and ethyl acetate–acetic acid–water (3:1:3). Glucose and galactose were not completely separated, but only one spot was found for the unknown, and this had the same R_f as glucose. Ribéreau-Gayon (6) found the only heteroside in grapes was glucose.

Acid moieties were liberated from both mixed and separated acylated pigments 6 and 7 by saponification of a 1% aqueous HCl solution by adding 50% NaOH to a concentration of $2N$ and holding at room temperature for 5 minutes before re-acidification with concentrated HCl. The solution was extracted with ethyl ether before and after saponification, and these extracts were chromatographed in one dimension with water-saturated ethyl acetate, n-butanol–acetic acid–water (4:1:5), and n-propanol–ammonium hydroxide (7:3) as solvents. Caffeic and p-coumaric acids were used as standards. Spots were identified in ultraviolet light with and without ammonia fumes. Only p-coumaric acid was found in the saponification products of pigments 6 and 7. When the mixed preparation of these pigments was saponified, p-coumaric acid was the principal acid found, but a trace of caffeic acid was also detected. Neither caffeic nor p-coumaric acid was in the ether extracts made before saponification.

Discussion

The identification of pigment 1 as the hydrolysis product of acylated pigment 6, the aglycone of both 1 and 6 as delphinidin, and the presence of only glucose as the sugar moiety established pigment 1 as delphinidin 3-monoglucoside. This is in agreement with the pigment map of Ribéreau-Gayon. Identification of p-coumaric acid as the acid moiety in pigment 6 establishes it as a p-coumaric acid ester of delphinidin 3-monoglucoside. Similarly, identification of pigment 3 as a hydrolysis product of acylated pigment 7, the aglycone of both as cyanidin, and glucose as the only sugar moiety, establishes pigment 3 as cyanidin 3-monoglucoside and pigment 7 as its p-coumaric acid ester. This is also in agreement with the Ribéreau-Gayon

map. The identity of pigment 3 as cyanidin 3-monoglucoside was confirmed by means of two-dimensional chromatograms with a crystalline preparation of chrysanthemin hydrochloride as a standard.

On the basis of the Ribéreau-Gayon pigment map, pigment 2 may be anticipated as petunidin 3-monoglucoside, pigment 4 as malvidin 3-monoglucoside, and pigment 5 as peonidin 3-monoglucoside. These pigments were not isolated for identification. Hydrolysis of all the anthocyanin pigments in mature Concord grape skins revealed the presence of peonidin, malvidin, and petunidin as well as cyanidin and delphinidin as the only aglycones. Approximately 35% of the aglycones was cyanidin, 35% was delphinidin, 15% was petunidin, 10% was malvidin, and 5% peonidin.

Because of the minor amounts present, no attempt was made to isolate or identify the individual pigments in the group 8 through 14. On the basis of the Ribéreau-Gayon map, these would be expected to be 3,5-diglucosides of pigments 1 through 5 and acylated derivatives of these pigments. Pigments 12, 13, and 14 were confirmed as acylated 3,5-diglucosides by comparing saponified and unsaponified skin extracts of mature grapes. Pigments represented by spots 6, 7, 12, 13, and 14 were greatly diminished or disappeared upon saponification with appreciable increases in pigments 1, 4, and the 3,5-diglucosides. Ten pigments might be expected to appear in this group if all possible derivatives of p-coumaric acid of all possible diglucosides of pigments 1 through 5 were present.

On the basis of observation on the formation of these pigments in the grape skins, it would appear that the monoglucosides of delphinidin and cyanidin are the first pigments to form in the Concord variety. As the amounts of these pigments increase, other pigments are formed through addition of methyl groups in the 3' and 5' positions, the addition of a second glucose in the 5 position, and the esterification of these pigments with p-coumaric acid. No evidence was found in these studies of a second acylated p-coumaric acid derivative (bis form) as reported by Ribéreau-Gayon for *Vitis vinifera* and *labrusca* (6). Traces of caffeic acid were also detected in hydrolyzates of grape pigments by Ribéreau-Gayon, but no suggestions were made concerning possible derivatives of this acid.

These results conflict with earlier reports that suggested the predominant anthocyanin pigment in Concord grapes is malvidin 3-monoglucoside (8) or delphinidin 3-monoglucoside (5). Anderson (7) failed to identify the pigment isolated from Concord grapes but found the aglycone to have a methoxy content equivalent to petunidin or an equimolecular mixture of delphinidin and malvidin.

If the techniques of Ribéreau-Gayon are used, the authors' observations on the pigments of *Vitis labrusca* var. Concord more nearly compare with data presented by him for *Vitis labrusca*. Bearing in mind that the variety of *Vitis labrusca* used by Ribéreau-Gayon was not specified and may not have been Concord, the authors find the following differences. Fourteen anthocyanin pigments, instead of 11, are found in Concords. Cyanidin 3-monoglucoside is present in much larger amounts than found by Ribéreau-Gayon. Malvidin 3-glucoside is not the predominant pigment and is present in much smaller amounts, and a larger percentage of the pigments is acylated. Climatic differences could be responsible for the varying proportions of the pigments. Ribéreau-Gayon found wide differences between *Vitis vinifera* grown in France and California in the relative proportions of certain pigments present. A further possible reason for the differences in the amounts of acylated pigments is that Ribéreau-Gayon extracted grape skins with 1% aqueous HCl, while the authors used 1% methanolic HCl. The two major acylated pigments in Concords

have a limited solubility in aqueous solutions and may not be completely extracted by 1% aqueous HCl.

Acknowledgment

The authors acknowledge their indebtedness to C. E. Sando for supplying a crystalline preparation of chrysanthemin hydrochloride; to Earl C. Spaeth, Arlington, Va., for supplying samples of synthetic malvidin, petunidin, and delphinidin; to H. C. Lukens, Western Regional Research Laboratory, Albany, Calif., for the spectrographic analyses of pigment ash; to Leonard Jurd, Western Regional Research Laboratory, Albany, Calif., for suggestions made during the course of this study; and to personnel of Kraft Foods Division, National Dairy Products Corp., Prosser, Wash., Glenview, Ill., and Dunkirk, N. Y., for providing material for experimentation.

Literature Cited

(1) Anderson, R. J., *J. Biol. Chem.* **57**, 795 (1923).
(2) Bockian, A. H., Kepner, R. E.,

Webb, A. D., *J. Agr. Food Chem.* **3**, 695 (1955).
(3) Pederson, C. S., Beattie, H. G., Stotz, E. H., *N. Y. State Agr. Expt. Sta. Geneva, N. Y. Bull.* **728**, November 1947.
(4) Pederson, C. S., Tressler, D. K., *Ibid.*, **676**, August 1936.
(5) Pratt, D. E., Powers, J. J., Somaatmadja, D., *Food Res.* **25**, 26 (1960).
(6) Ribéreau-Gayon, P., "Recherches sur les Anthocyannes des Vegétaux Application au Genre Vitis," Librairie Generale de L'enseignement, Paris, 1959.
(7) Sastry, L. V. L., Tischer, R. G., *Food Technol.* **6**, 82 (1952).
(8) *Ibid.*, p. 264.
(9) U. S. Agricultural Marketing Service, *Federal Register* **22**, F. R. 6077, August 2, 1957.

ESSENTIAL OILS

Determination of Botanical and Geographical Origin of Spearmint Oils by Gas Chromatographic and Ultraviolet Analysis

D. MORISON SMITH,
WILLIAM SKAKUM,
and LEO LEVI

Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada

OIL OF SPEARMINT is a popular flavoring agent, used extensively in chewing gums and tooth pastes. Its output in the United States has increased steadily during the last quarter of a century. In 1960, more than a million pounds were produced in Indiana, Michigan, and Washington (*11*, *16*).

Taxonomists presently recognize two species of spearmints cultivated in the United States, namely *Mentha spicata* L. cultivar common or native American spearmint and *M. cardiaca* Gerard ex Baker cultivar Scotch or Highland spearmint. The former is not truly native to North America but was introduced from Europe (*7*) during the seventeenth century and has since been widely grown. Originally, it was the only source of oil of spearmint in the United States. Scotch or Highland spearmint was brought to America about 50 years ago. Its history, botanical characteristics, and nomenclature were discussed in detail by Hocking (*12*). Phylogenetically, it is wholly unrelated to *M. spicata* (*14*).

Criteria of identity and standards of quality have been established for spearmint oil by government agencies as well as trade organizations in various countries (*2*, *3*, *5*, *13*, *15*). Canadian Food and Drug Regulations specify at present that spearmint essence, extract, or flavor shall be prepared from spearmint or oil of spearmint, obtained from leaves and flowering tops of *M. spicata* L., and shall contain not less than 3% by volume of oil of spearmint (*5*). In view of the taxonomic classification of spearmint now generally recognized, these regulations are being revised to include preparations derived from *M. cardiaca* also.

The present study, a continuation of work on the genus *Mentha*, constitutes a detailed examination of spearmint oils of different geographical and botanical origins. It also demonstrates the application of gas chromatographic techniques to the characterization of these complex products, and relates carvone contents thus obtained to those determined by ultraviolet spectrophotometry.

Experimental

Gas Chromatographic Analyses.
APPARATUS. Gas chromatograph. Burrell Kromo-Tog K-2 equipped with thermal conductivity detector cell and separate heating baths for column and detector, respectively.

Column. Glass tubing, 0.6 cm. inside diameter.

Packing. Ucon Polar 20% on Celite, 30-60 mesh obtained from Burrell Corp. Length of packing, 230 cm.

SAIB (sucrose diacetate hexaisobutyrate) 20% on acid-washed Chromosorb W, 60–80 mesh. Length of packing 200 cm.

Carrier gas. Helium, inlet pressure 1.1 atm.; outlet pressure 1.0 atm.

Recorder. 0 to 1 mv. full scale deflection.

Chart speed. 0.5 inch per minute.

MATERIALS. Spearmint oils. Commercial and experimental samples of different geographical and botanical origins.

Spearmint oil constituents. Reference specimens obtained from various sources of supply.