[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE OHIO STATE UNIVERSITY]

Chromatography of Cuban Blackstrap Molasses on Clay; Some Constituents of an Odor and Pigment Fraction[1]

BY W. W. BINKLEY[2] AND M. L. WOLFROM

In continuation of our work[3-5] on the chromatographic isolation of constituents of cane juice and cane blackstrap molasses, we have isolated from the latter a fraction which is only weakly held by the adsorbents employed. Concentrated in this fraction are the odoriferous constituents to which the molasses, and the rums derived therefrom, owe in the main their characteristic taste and odor. The concentration of such a fraction had been noted previously.[5] Further amounts have now been prepared and subjected to some detailed study. The fraction least adsorbed on a fuller's earth type of clay was isolated from 1 kg. of a typical Cuban cane blackstrap molasses by ethanol–acetone elution in the presence of some water. The aqueous solution obtained after organic solvent removal was extracted with petroleum ether and the extract on solvent removal yielded a viscous, green liquid with a strong odor of cane molasses. The yield was 0.4% of the original molasses. The odor of this material was highly volatile and disappeared completely on standing in the laboratory for several weeks. It could be trapped by condensation at low temperatures. The condensate (Fraction A) reacted with 2,4-dinitrophenylhydrazine. It is under further investigation.

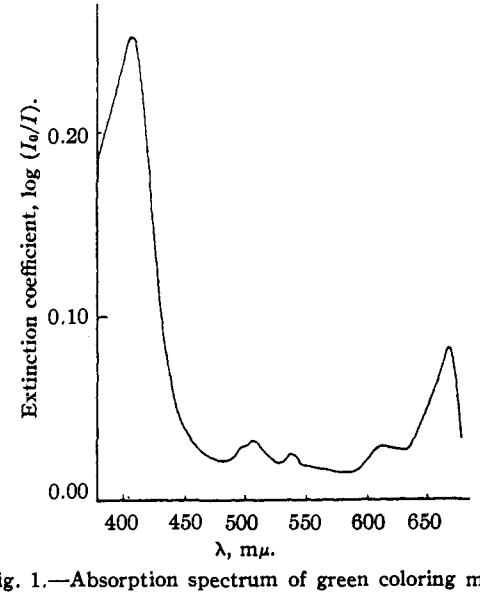

Fig. 1.—Absorption spectrum of green coloring matter (in ether) from Cuban Blackstrap molasses: Beckman spectrophotometer (Model DU); 1.00-cm. cell; measured by Mr. Bernard Wildi of this Laboratory.

The fraction (Fraction B) residual after Fraction A removal was further chromatographed on Silene-EF[6] and was divided into six fractions (Table I). The green color of this hydrocarbon-soluble mixture was identified as chlorophyll *a* by its absorption spectrum (Fig. 1). This spectrum shows that the substance is nearly pure chlorophyll *a* containing no more than a few per cent. of what is probably chlorophyll *b* as indicated by the slight absorption in the region 560–570 mμ. The presence of such unaltered chlorophyll *a* in blackstrap molasses seems remarkable.

TABLE I

EFFLUENT FRACTIONS FROM THE CHROMATOGRAM FROM 2.0 g. OF FRACTION B FROM CANE BLACKSTRAP MOLASSES[a]

Fraction	Eluate volume, ml.	Color	Yield, mg.	Substances present
1	250	Golden	511	Fats, "melissyl" alcohol, sterols
2	100	Colorless	39	"Melissyl" alcohol, trace of sterols
3	250	Green	146	Sterols, chlorophyll[a]
4	250	Green	149	Sterols, chlorophyll[a]
5	150	Light green	8	Chlorophyll[a]
6[b]			882	

Total 1735 (86.7%)

[a] See experimental portion for details. [b] By acetone extraction of column.

From the various Silene fractions there was isolated "melissyl" (synonym "myricyl") alcohol, a sterol fraction and a fat fraction. "Melissyl" alcohol was found free in the molasses, which had however been subjected to some alkaline treatment in its manufacture. Melissyl alcohol has been reported as a major component of the cane wax[7-9] isolable from the clarification muds formed in the manufacture of raw sugar. The melting point of natural "melissyl" alcohol will vary with the source. Its formula is generally considered to be $CH_3(CH_2)_{28}CH_2OH$ but actually it is very probably a mixture of close homologs, as has been

(1) Presented before the Division of Sugar Chemistry and Technology at the 112th Meeting of the American Chemical Society, New York, N. Y., September, 1947.

(2) Sugar Research Foundation Fellow of The Ohio State University Research Foundation (Project 190).

(3) W. W. Binkley, Mary G. Blair and M. L. Wolfrom, THIS JOURNAL, **67**, 1789 (1945).

(4) W. W. Binkley and M. L. Wolfrom, *ibid.*, **68**, 1720 (1946).

(5) W. W. Binkley and M. L. Wolfrom, *ibid.*, **69**, 664 (1947).

(6) L. W. Georges, R. S. Bower and M. L. Wolfrom, *ibid.*, **68**, 2169 (1946).

(7) J. E. Q. Bosz, *Arch. Suikerind. Nederland-Indië*, **28**, 969 (1920); *Chem. Centr.*, **91**, III, 637 (1920).

(8) N. L. Vidyarthi and N. Narasingarao, *J. Indian Chem. Soc.*, **16**, 135 (1939); *C. A.*, **33**, 9700 (1939).

(9) R. T. Balch, U. S. Patent 2,381,420 (1945); R. T. Balch, "Wax and Fatty Byproducts from Sugarcane," No. 3, Technological Report Series, Sugar Research Foundation, Inc., 1947.

demonstrated by Koonce and Brown[10] for the "melissyl" alcohol fraction of carnauba wax.

The sterol fraction ((m. p. 142–143°, cor.), $[\alpha]^{26}D$ −38° in chloroform) isolated was a typical phytosterol such as has been reported in studies on crude cane wax.[9] It is undoubtedly a mixture of the sitosterol type but was obtained in this work in too small amount to allow of further fractionation.

Saponification of the main fat fraction yielded further quantities of "melissyl" alcohol, glycerol (identification by a color test) and an unsaturated acid that on catalytic hydrogenation yielded stearic acid and on hydroxylation yielded a tetrahydroxystearic acid which was shown by its melting point (156°, cor.) and analysis to be essentially α-sativic acid.[11] The parent unsaturated acid is thus in all probability linoleic acid. It is of interest to note that this acid is the major acid present in cane wax.[9]

Finally, we wish to call attention to the demonstration that the chromatographic techniques established for separation of carbohydrate mixtures are also applicable to the separation of non-carbohydrate systems.

Experimental

Chromatographic Isolation of an Odor and Pigment Fraction.—The Cuban cane blackstrap molasses employed in this work was the same sample described previously.[3,5] One kilogram of this molasses was diluted with 250 ml. of water and made into a smooth paste by the addition of 400 g. of clay.[12] This paste was added with good agitation to a mixture of 3.6 liters of absolute ethanol and 2.4 liters of acetone; the agitation was continued for one hour after the addition of the paste. The supernatant liquor was removed with the aid of a siphon. The precipitate was transferred to a percolator and washed with 3 liters of acetone. The supernatant liquor and the percolator effluent were combined and concentrated to a volume of approximately 1 liter under reduced pressure at a bath temperature of 50°. This concentrate was poured into a 2-liter liquid-liquid extractor,[13] diluted with 1 liter of water and extracted for twenty-four hours with petroleum ether (b. p. 30–60°). A turbid, green extract was obtained. Removal of solvent under reduced pressure yielded a viscous, green liquid with a strong odor of cane blackstrap molasses; yield 4.1 g. (0.41% of the original molasses).

Separation of the Molasses Odor Fraction (Fraction A).—The concentrate (4.1 g.) was heated for two hours at 100–110° under a pressure of 0.03 mm. and the distillate (Fraction A) that formed was collected in traps surrounded with a mixture of solid carbon dioxide and acetone. The traps were washed with ethanol (95%). The collected washings had a strong odor of molasses and gave a precipitate with 2,4-dinitrophenylhydrazine reagent. The undistilled concentrate (Fraction B, 4.0 g.) had a fatty odor. The nature of Fraction A is under further investigation. It is highly volatile.

Chromatography of the Residual Fraction (Fraction B).—An amount of 2.0 g. of the residual concentrate dissolved in 40 ml. of benzene, was added at the top of a 150 × 80 mm. (diam.) column[14] of a mixture (150 g.) of 5 parts (by wt.) of "Silene-EF"[15] and 1 part of "Celite."[16] The chromatogram was developed with 1250 ml. of 500/1 benzene[17]/ethanol[18] (volume ratio). The effluent was collected in five fractions and pertinent data relative to these fractions are listed in Table I. The column was extruded and extracted for twenty-four hours with acetone in a Soxhlet extractor. The substances eluted from the adsorbent (Fraction B-6) were waxy solids with a strong fatty acid odor. They were completely soluble in 0.1 *N* sodium hydroxide.

Anal. Saponification equiv., 694 (1.44 ml. of 0.1 *N* sodium hydroxide per 100 mg.).

Isolation of "Melissyl" Alcohol, a Sterol Fraction and a C_{18} Unsaturated Fatty Acid Fraction from Fraction B-1.—Fraction B-1 (Table I) was a viscous, light amber liquid with a fatty odor.

Anal. Unsaponifiable matter, 65%; saponification equiv., 741 (1.35 ml. of 0.1 *N* sodium hydroxide per 100 mg.); free fatty acid, absent; iodine no., 96 (7.57 ml. of 0.1 *N* I_2 per 100 mg.).

An amount of 426 mg. of Fraction B-1, 5 ml. of ethanol (95%) and 0.5 ml. of aqueous 50% potassium hydroxide were heated under reflux for two hours on a steam-bath. The reaction mixture was cooled to room temperature and extracted with ether. The ether-soluble fraction (Fraction B-1-a) was washed with water until free of alkali; yield 267 mg.

An amount of 242 mg. of Fraction B-1-a, dissolved in 5 ml. of benzene was added at the top of a 175 × 35 mm. (diam.) column of a mixture (45 g.) of 5 parts (by wt.) of "Silene-EF" and 1 part of "Celite." The chromatogram was developed with 90 ml. of 500/1 benzene/ethanol. The column was extruded and two well-defined zones, a narrow upper zone 70 to 75 mm. from the top of the column and a wide zone at the bottom of the column, were detected with the aid of ultraviolet light. The zones were isolated and eluted with acetone. Zone yields for the upper and lower zones were 124 and 110 mg., respectively after solvent removal. The material from the upper zone crystallized from ethanol (95%) as needles; yield 40 mg., m. p. 139–141° (cor.); Liebermann–Burchard sterol test[19] positive. Plates were obtained from a benzene solution of the lower zone; yield 30 mg., m. p. 83–84° (cor.). These crystalline substances were identical with those of a similar nature (phytosterol fraction and "melissyl" alcohol, respectively) described and further characterized below.

The potassium hydroxide solution (Fraction B-1-b), containing fatty acids and glycerol, from the saponification of Fraction B-1 was acidified with hydrochloric acid and extracted with three 25-ml. portions of chloroform. The water layer was set aside for glycerol recovery (see below). The residue, 137 mg., from the chloroform extracts was a soft paste with a fatty odor. This paste reacted with alkaline permanganate and bromine in carbon tetrachloride; its ethanolic solution was acid to litmus; its lead salt was soluble in ether.

Anal. Neutral equiv., 302 or 3.31 ml. of 0.1 *N* sodium hydroxide per 100 mg., iodine no., 118 or 9.28 ml. of 0.1 *N* I_2 per 100 mg.

Following the general procedure of Brown and coworkers[20] for the separation of unsaturated from saturated fatty acids by low temperature crystallization, an amount of 150 mg. of the above fatty acid fraction was dissolved

(10) S. D. Koonce and J. B. Brown, *Oil & Soap*, **21**, 167, 231 (1944).

(11) B. H. Nicolet and H. L. Cox, THIS JOURNAL, **44**, 144 (1922).

(12) Florex XXX, a Florida fuller's earth, supplied by the Floridin Co. of Warren, Pa.

(13) S. E. Q. Ashley and W. M. Murray, Jr., *Ind. Eng. Chem., Anal. Ed.*, **10**, 367 (1938).

(14) Column dimensions refer to the adsorbent.

(15) A hydrated calcium acid silicate produced by the Columbia Chemical Co., Barberton, Ohio.

(16) No. 535, a siliceous filter-aid manufactured by Johns-Manville Co., New York, N. Y.

(17) All benzene employed was free of thiophene.

(18) Absolute ethanol was employed in all chromatographic operations.

(19) C. Liebermann, *Ber.*, **18**, 1803 (1885); H. Burchard, Diss. Rostock (1890), *Chem. Centr.*, **61**, II, 25 (1890).

(20) J. B. Brown, *Chem. Revs.*, **29**, 333 (1941).

in 1 ml. of acetone and cooled to −50 to −60° by a bath of solid carbon dioxide and trichloroethylene. The frozen mass was allowed to thaw slowly until an amorphous solid separated. This solid was collected rapidly on a filter and washed with 0.5 ml. of cold acetone; yield 30 mg. An ethanolic solution of this solid yielded a non-crystalline, highly solvated, amorphous solid. A viscous liquid (Fraction F) was obtained from the filtrate of the above precipitation after removal of the acetone; yield 120 mg. This liquid (120 mg.) was dissolved in 10 ml. of absolute ethanol and hydrogenated in the presence of 50 mg. of platinic oxide. After six alternate evacuations and additions of hydrogen (to 1 atm. pressure), the reaction mixture was warmed to 50° and shaken for several hours. The catalyst was removed by filtration. The filtrate was allowed to reflux for two hours with 150 mg. of potassium hydroxide to saponify any ethyl esters formed during the hydrogenation. This solution was diluted with 50 ml. of water and acidified with hydrochloric acid. The precipitated fatty acid was collected on a filter; yield 116 mg. An ethanolic (95%) solution of the precipitate gave crystals; m. p. 68–68.5°. Recrystallization from absolute ethanol gave plates, m. p. 69–69.5° (cor.) undepressed on admixture with an authentic specimen of stearic acid (m. p. 70–71°, cor.).

In another experiment, an amount of 100 mg. of the above unsaturated fatty acid fraction (Fraction F) was dissolved in 30 ml. of 0.03 *N* potassium hydroxide and to the cooled (5°) solution was added dropwise under good agitation a solution of 20 mg. of potassium permanganate in 5 ml. of water. Five minutes after the completion of the addition, the reaction mixture was decolorized with sulfur dioxide. A white, flocculent precipitate formed which was crystallized from ethanol; yield 30 mg. (0.008% original molasses), m. p. 156° (cor.).

Anal. Calcd. for $C_{17}H_{31}(OH)_4CO_2H$: C, 62.04; H, 10.42. Found: C, 61.96; H, 10.28.

The aqueous layer from the fatty acid isolation was adjusted to *p*H 7–8, evaporated to dryness on a steam-bath and extracted with ethyl acetate. A few droplets of a viscous liquid was obtained after solvent removal and this residue gave a positive pyrogallol–sulfuric acid test for glycerol.[21]

Isolation of "Melissyl" Alcohol from Fraction B-2.—This fraction was crystalline. Plates were obtained on recrystallization from benzene; yield 20 mg. (total yield of "melissyl" alcohol, 0.01% original molasses), m. p. 83–84° (cor.) undepressed on admixture with a specimen of "melissyl" alcohol (m. p. 87–87.5°, cor.) separated from carnauba wax by fractional crystallization. The mother liquor showed a positive Liebermann–Burchard[19] sterol test.

(21) S. P. Mulliken, "Identification of Pure Organic Compounds," Vol. I, John Wiley and Sons, New York, N. Y., 1904, p. 169.

Anal. Calcd. for $C_{30}H_{52}O$: C, 82.11; H, 14.24. Found: C, 81.74; H, 14.18.

Conversion to the acetate with sodium acetate and acetic anhydride gave crystals; m. p. 66–67° (cor.).

Characterization and Further Isolation of the Phytosterol Fraction and Identification of Chlorophyll *a*.—Fractions B-3 and B-4 were crystalline. When an ethanolic solution of these fractions was allowed to evaporate at room temperature, nearly colorless, elongated plates formed near and on the bottom of the container. The green color and some crystals collected higher up on the walls of the container. Careful washing with cold ether removed nearly all of the green color. The washings were used for the determination of absorption spectra (see below). A total of 250 mg. of crystals was obtained from Fractions B-3 and B-4. These were combined and yielded colorless crystals from ethanol (95%); m. p. 142–143° (cor.), $[\alpha]^{26}D$ −38° (*c* 4.9, 2-dm. tube, chloroform), Liebermann–Burchard sterol test[19] positive. A benzene solution of these crystals fluoresced in ultraviolet light.

Anal. Calcd. for $C_{27}H_{46}O$: C, 83.88; H, 11.99. Found: C, 83.64; H, 11.90.

The material of Fraction B-5 was amorphous. The green colored substance from Fractions B-3, B-4 and B-5 was combined and its absorption spectrum in ether solution is shown in Fig. 1. The spectrum is that of chlorophyll *a* with a probable slight admixture of chlorophyll *b* or pheophytin *a*; yield 0.00004% original molasses (calcd. from absorption spectrum).

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Summary

By chromatographic methods the following trace constituents were isolated from a sample of Cuban cane blackstrap molasses: "melissyl" alcohol, chlorophyll *a* (identification by absorption spectrum), a phytosterol fraction (m. p. 142–143°, $[\alpha]^{26}D$ −38° in chloroform) and a fat fraction in which the presence of glycerol and linoleic acid was indicated. The highly volatile odor fraction was concentrated and shown probably to consist in part of carbonyl compounds.

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