Paper Chromatographic Determination of Raffinose in Sugar Beet Molasses

FERN JANTZEF AND A. L. POTTER[1]

Accurate knowledge of the raffinose content of molasses is important to sugar beet growers and processors. Carruthers and co-workers (4)[2] have reviewed methods commonly used for the determination of raffinose and described their limitations.

Polarimetric methods are most widely used in the sugar beet industry. Optically active substances other than the common sugars reduce the reliability of these methods.

Paper chromatographic methods are more specific and more reliable. Albon and Gross (1) determined raffinose directly. de Whalley (5) hydrolyzed raffinose to melibiose with invertase before chromatography to eliminate interference of kestoses and slow-moving colored compounds. These methods involve visual comparison of the color developed from spots of raffinose or melibiose with that from a series of standards. Thus, they are limited by subjective errors. Bevenue and Williams' method (3) is similar to that of de Whalley except that they measure the developed chromatographic spots of melibiose by reflected light. Reproducibility of these methods is approximately 10%.

Weidenhagen and Schiweck (9) obtained better chromatograms by clarifying the molasses with lead acetate and then desalting with ion-exchange resins. They reported incomplete recovery of sugars from the resins and used a correction factor.

Several methods (6, 7, 8) involve photometric measurements after elution of the raffinose- or melibiose-containing zones of paper chromatograms. These methods can give very good precision. However, elution may not recover all the sugar. .

We report a paper chromatographic method for determining raffinose directly. It avoids ion-exchange resins, invertase hydrolysis, or elution. The diluted molasses, clarified by lead acetate, is spotted on a chromatographic sheet. The sugars are separated by descending chromatography, and the raffinose spots are developed with *p*-anisidine. The resulting yellow color is measured with a color difference meter or a transmission densitometer.

This report describes a second clarification procedure, which removes more colored materials. After clarification with lead

acetate, the molasses solution is washed through a cellulose column with 80% ethanol. Both procedures gave good chromatograms with the samples analyzed, that is, raffinose appeared as a compact spot separated from other color-producing compounds. If chromatography of the lead-treated molasses does not separate raffinose from colored materials, the second procedure may be preferred.

Experimental

Apparatus.—Gardner Automatic Color Difference Meter, Model AC-2 (Gardner Laboratory, Inc., Bethesda, Md.). This instrument measures colors in terms of percent reflectance R_d and chromaticity coordinates, a (red, plus; green, minus) and b (yellow, plus; blue, minus) (2).

Photovolt Transmission Densitometer (Electronic Photometer Model 501-A with Transmission Density Unit Model 52-C).

Reagents.—Neutral Lead Acetate. Saturated aqueous solution.

Raffinose Standard (30 μg/μliter anhydrous raffinose). Dilute 3.54 g raffinose pentahydrate to 100 ml with water. Prepare working standards from this stock solution. The stock and standard solutions may be preserved by adding a few crystals of thymol and storing in a refrigerator.

Chromatographic Solvent. n-Propanol-ethyl acetate-water (7:1:2 by volume) (1).

p-Anisidine. Suspend 20 g p-anisidine in about 400 ml absolute ethanol. Dissolve by adding 100 ml concentrated hydrochloric acid. Dilute to 1000 ml with absolute ethanol.

Chromatographic Paper. Whatman No. 1, chromatographic grade paper (18¼ × 22½ inches).

Whatman Standard Grade Cellulose Powder.

Sample Treatment.—Heat the molasses with occasional stirring in a water bath at 100° C or in an oven at 110° C until it is homogeneous. Clarify the sample by one of the methods A or B below.

A. Clarification with Lead Acetate. Transfer a 5-g sample to a 25 ml volumetric flask with water. Add 1 ml lead acetate solution. This quantity precipitates interfering substances of most samples. Mix and dilute to volume. If foam hinders dilution to volume, add one drop of amyl alcohol. Let stand about 30 minutes, then filter using Eaton-Dikeman No. 509 folded filter paper.

B. Clarification with Lead Acetate and Cellulose Column. Dilute a 5-g sample of molasses and 1 ml lead acetate solution to 10.0 ml with water. Let stand about 30 minutes, filter.

Prepare the column, 30 mm in diameter, with a fritted glass disc, by pouring in a slurry of cellulose powder in absolute ethanol. Stir and let settle as the liquid flows. The final volume of settled cellulose is 120 ml.

Run 4.0 ml of the filtrate into the column. Rinse the sides of the column above the cellulose with small portions of 80% ethanol. Then add 80% ethanol continuously to the top of the column. Discard the first 75 ml of effluent. Collect the next 250 ml of effluent. Evaporate it on a steam bath to a few ml and then dilute to 10.0 ml. Qualitative tests showed that all the p-anisidine-positive sugars were in this fraction.

Paper Chromatography.—Place 10.0 μliter-spots of the solutions on a chromatographic sheet at 1.5 inch intervals. Apply on each sheet four spots of molasses solution, three spots of a raffinose standard of lower concentration, four spots of intermediate concentration, and three spots of higher concentration. Arrange the spots in groups so that the spots of any one solution are about equally distributed across the sheet. Use raffinose standards varying from 1.5 to 4.5 μg/μliter. Most samples fell in a smaller range, 1.5 to 3.0 μg/μliter. Dilute the molasses solutions if necessary so that the raffinose concentration is near that of the intermediate standard.

Separate the sugars by descending chromatography. Spot test paper strips with a molasses solution. Chromatograph the strips with the sheets and develop the strips at intervals to determine whether raffinose is well separated from other sugars. About 64 hours is usually required to separate raffinose from sugars (probably kestoses) that interfere with its measurement. When chromatography is finished, air dry the sheets.

Dip each sheet by drawing it through a trough containing p-anisidine reagent. Let the sheet dry 15 minutes in a fume hood. Heat in a forced air oven at 70° C for 2 minutes. After allowing it to stand for a few minutes at room temperature, measure the color of the developed raffinose spots with the color difference meter or the transmission densitometer.

Measurement with Color Difference Meter.—Operate the instrument according to the manufacturer's instructions. Standardize the color difference meter with a "yellow" calibrated enameled standard ($R_d = 60.1$, $a = -1.7$, $b = +22.6$) obtained from the manufacturer. Position the sheet so that a raffinose spot is centered over the aperture (1.5 inch diameter). Place the enameled standard on the paper to hold it flat. Obtain readings of R_d and b. Move the paper slightly while balancing the b chromaticity coordinate to assure the maximum reading. The yellow color fades when exposed to the light of the instrument, so the

color of each spot must be measured after an equal time of exposure. Record the b reading of each spot at 25 seconds of exposure.

For each sheet, plot the average b readings of each standard solution against the raffinose concentration. The relation between b reading and raffinose concentration is shown in Figure 1. Average the b readings for the sample on the same sheet and determine the amount of raffinose from this curve.
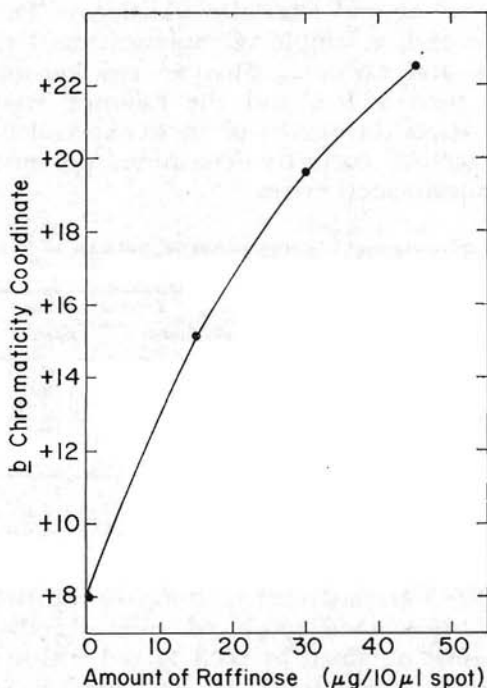

Figure 1.—Relation between b chromaticity coordinate and raffinose concentration.

Measurement with Transmission Densitometer.—Place the sheet over the aperture ($1\frac{1}{8} \times 9/16$ inches) of the transmission densitometer. Using a 440 mμ filter, adjust the instrument so that the optical density reads zero (100% transmission) on a blank area of the chromatographic sheet in the region of the raffinose spots and infinity (zero percent transmission) on the most dense area of a developed sucrose spot. Measure the maximum optical density of each raffinose spot. Determine the amount of raffinose of the sample from the standard curve for the same sheet.

Results and Discussion

Replicate determinations of about 1% raffinose in molasses by the methods presented in this report were within the following limits: If the color of the p-anisidine-raffinose spots was measured with the color difference meter, the results were within about five percent of the mean. If the transmission densitometer was used, the results were within about ten percent.

Two experiments were carried out to evaluate the accuracy of the paper chromatographic method. First, molasses samples were analyzed before and after the addition of known amounts of raffinose. Second, a sample of molasses was fermented until free of sucrose and raffinose. Sucrose and known amounts of raffinose were then added, and the raffinose was determined. Table 1 summarizes the results of these experiments. The data show that the method correctly determined the amounts of raffinose within experimental error.

Table 1.—Paper chromatographic determination of raffinose with known amounts of raffinose added.

Sample	Percent raffinose		
	Initially found[1]	Added	Found[1]
Molasses 1	1.13	0.17	1.26
2	1.08	0.36	1.46
Fermented molasses[2]	0.00	1.00	0.99
	0.00	1.30	1.34

[1]Paper chromatographic method.
[2]Free of raffinose and sucrose by treatment with Bakers' yeast. Sucrose was added after treatment to imitate original molasses (60% sucrose).

Some samples were analyzed to compare the two clarification procedures. Each method produced good chromatograms, and the raffinose values obtained by both agreed within experimental error. The simple treatment with lead acetate (A) was· satisfactory for the samples encountered. The cellulose column procedure (B) removed more colored materials from the molasses solutions. If chromatography of the lead-treated molasses does not separate raffinose from colored compounds, the cellulose column procedure may be required.

Solutions of raffinose alone and of raffinose containing sucrose in amounts equivalent to those in molasses, were chromatographed. The raffinose spots gave the same values whether sucrose was present or not. We concluded that it is not necessary to add sucrose to the raffinose standards.

Several reagents, including p-anisidine, orcinol, napthoresorcinol, and α-napthol were tested as developers of the raffinose

spots. p-Anisidine is sensitive, produced the most stable color, and gave consistent results.

Faint spots of p-anisidine-positive sugars, probably kestoses, usually appeared between raffinose and sucrose on chromatograms of the samples analyzed. Chromatography for about 64 hours removed them far enough from the raffinose that they did not interfere. Carruthers *et al.* (4) reported that 1-kestose and *neo*kestose can be chromatographically separated from raffinose, but that the mobility of 6-kestose is very similar to that of raffinose. In addition, they reported that only traces of 6-kestose are present in beet molasses. Therefore, we assume that, if 6-kestose was in the raffinose area, the color developed from it contributed only negligibly.

Table 2 lists raffinose contents determined from the same samples by polarimetric methods and by the paper chromatographic method. These data show a large variation in raffinose content of the sugar beet molasses as determined by polarimetric methods. If it is assumed that the paper chromatographic method gives the true amount of raffinose, polarimetric methods may err by a factor of three or four.

Table 2.—Comparison of raffinose content of suger beet molasses determined by polarimetric methods and by paper chromatography.

Sample	Percent raffinose	
	Polarimetric[1]	Paper chromatographic
1	1.44	1.20
2	1.19	1.18
3	1.08	1.21
4	0.25	1.01
5	0.54	1.13

[1]The values by polarimetric methods were determined at the two sugar beet plants where the samples were produced.

Summary

A paper chromatographic determination of raffinose in sugar beet molasses was developed. The treatment of the sample before chromatography is simple. Two clarification procedures are reported: A. treatment with lead acetate; B. treatment with lead acetate followed by washing the sugars through a cellulose powder column with 80% ethanol. Each procedure gave the same raffinose values. The sugars were separated by descending chromatography. p-Anisidine was chosen to develop the color of the raffinose spots because it is as sensitive as other reagents tested and produces more stable color. The intensity of the color of the spots was measured with a color difference meter or a transmission densitometer.

Replicate results were within about 5% of the mean when the color difference meter was used, and within about 10% when the transmission densitometer was used.

Acknowledgment

The authors are indebted to R. M. McCready for samples of sugar beet molasses and yeast-treated molasses, and to H. C. Lukens for assistance in using the Gardner Color Difference Meter.

Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Literature Cited

(1) ALBON, N., and D. GROSS. 1952. The chromatographic determination of raffinose in raw sugars. Analyst. 77: 410-412.

(2) AMERICAN SOCIETY FOR TESTING MATERIALS. 1955. Tentative method of test for color difference using the Hunter Color Difference Meter. Book of ASTM Standards, Part 8, pp. 1043-1046.

(3) BEVENUE, ARTHUR, and KENNETH T. WILLIAMS. 1958. Quantitative determination of raffinose or melibiose by paper chromatography. Arch. Biochem. Biophys. 73: 291-295.

(4) CARRUTHERS, A., J. V. DUTTON, J. F. T. OLDFIELD, C. W. ELLIOTT, R. K. HEANEY and H. J. TEAGUE. 15th May, 1963. Estimation of Sugars in Beet Molasses. Sixteenth Annual Technical Conference of the British Sugar Corporation Limited.

(5) DE WHALLEY, H. C. S. 1952. Raffinose in molasses and low beet products. Int. Sugar J. 54: 158.

(6) GOL'DFARB, R. I., and P. L. DANILENKO. 1960. Quantitative determination of the raffinose content of molasses by paper chromatography. Sakharnaya promyshlennost. 34 (5): 21-23. (Abstr.) Int. Sugar J. 1960, 62: 262.

(7) SCHNEIDER, F., A. EMMERICH, C. REICHEL and H. ROTHER. 1959. Uber die papierchromatographische Bestimmung von Raffinose. Zucker-Beihefte 3 (4): 95-102. (Abstr.) Int. Sugar J. 1959, 61: 317.

(8) TAUFEL, K., H. RUTTLOFF and A. TAUFEL. 1961. Zur Mikroanalytik der Raffinose. Nahrung. 5: 353-361.

(9) WEIDENHAGEN, R., and H. SCHIWECK. 1959. Papierchromatographische Raffinosebestimmung in Melasse. Zeit. Zuckerind. 9: 443-445.