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DETERMINATION OF GLUCOSE, FRUCTOSE AND SUCROSE IN MOLASSES BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A method is described for the simultaneous determination of sucrose, fructose and glucose in molasses by high-performance thin-layer chromatography. The relative standard deviations obtained by this method are 1.1% for sucrose, 2.2% for fructose and 4.3% for glucose.

INTRODUCTION

The determination of sucrose, fructose and glucose is of importance to the sugar industry but is also of interest to many other fields as evidenced by the large number of papers including the determination or separation of these compounds. Chromatographic methods include thin-layer chromatography (TLC)[1–7], paper chromatography[8], gas chromatography[9,10] and high-performance liquid chromatography[11–15]. TLC has the advantage of simplicity and allows the simultaneous determination of several samples. With the recent advent of high-performance TLC (HPTLC) a higher precision is attainable than with classical TLC–densitometry and analysis times are considerably shortened[14]. Hsu *et al.*[15] recently reported the determination of sucrose in molasses with a relative standard deviation of 2.2%. This is less than half the value reported by classical TLC–densitometry for glucose, fructose or sucrose. We report here on the simultaneous determination of these sugars by HPTLC.

EXPERIMENTAL

Materials and reference standards

Molasses samples were provided by Dr. J. Bruijn (Sugar Milling Research Institute, Durban, South Africa). D-Fructose (J. T. Baker, Phillipsburg, N.J., U.S.A.), glucose (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and sucrose (Matheson, Coleman and Bell, Houston, Texas, U.S.A.) were dried in a vacuum desiccator for 72 h at room

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temperature and were all found to be chromatographically pure. Certified ACS reagent grade ethyl acetate and pyridine were obtained from Fisher Scientific.

All the standards and molasses solutions were prepared immediately before analysis. The molasses solutions contained 1.5 g of molasses in 1 l of 70% ethanol (*i.e.* 300 ng/200 nl). For quantitative analysis a calibration curve was included in each plate and consisted of three points spotted from three solutions: a concentrated solution containing 0.15 g fructose, 0.1 g glucose and 0.5 g sucrose in 1 l of 70% ethanol (respectively 30, 20 and 100 ng in 200 nl). The other solutions were prepared from the concentrated solution. The latter was diluted by a factor of 0.6 and 0.8 respectively.

Chromatography

Chromatography was performed on 10 × 10 cm HPTLC plates coated with silica gel 60 (E. Merck, Darmstadt, G.F.R.). These plates were dipped in a 0.2 *M* aqueous solution of monobasic potassium phosphate. The wet plates were dried at 85° for 45 min. After cooling for *ca.* 2–3 h at room temperature, the plates were stored in a desiccator.

A volume of 200 nl of molasses solution and reference standard solutions was applied to the HPTLC plate using a 200-nl Pt–Ir capillary (Antech, Bad Dürkheim, G.F.R.) attached to an EVA Chrom-Applicator (W + W Electronic Scientific Instrument Co., Basle Switzerland). Using the data pair technique[16], 12 spots (3 standards and 3 samples) were applied 0.7 cm apart in the sequence: standard 1, sample 1, standard 2, sample 2, standard 3, and sample 3.

The spot diameter was held to less than 1.5 mm by spotting *ca.* 30–40 nl at a time with a 200-nl Pt–Ir capillary. A vacuum line was held close to the surface of the plate to hasten evaporation of solvent. The developing chamber was a glass crystallization dish, 5 cm deep and 15 cm in diameter, fitted with two glass covers each 10 × 20 cm. The cover glasses were arranged such that there was a slit 0.3 × 16 cm between them. This chamber was lined with filter paper (Whatman chromatography paper, medium flow-rate), a volume of 55 ml developing solvent system, ethyl acetate–pyridine–water (8:2:1), was added and the chamber allowed to stand for 10 min to permit vapor equilibration.

Chromatography was initiated by placing an HPTLC plate in the chamber with the silica gel surface touching one side of the slit. The plate was developed continuously for 30 min during which time the solvent front moves 6 cm. The plate was then dried at room temperature in a stream of air. The procedure was repeated twice to give a total of three developments. This yields baseline separation between fructose, glucose and sucrose.

The sugars were visualized by dipping the plate in a solution containing 4 g diphenylamine, 4 ml aniline, 30 ml of 85% H_3PO_4, in 200 ml of acetone for about 3 sec. This reagent has been previously described by Hansen[7]. The plate was then allowed to stand in the laboratory atmosphere for 15 min to effect initial drying. Excess visualizing reagent was wiped from the back of the plate and the plate was placed in an oven at 110° for 20 min.

The plates were allowed to cool to room temperature and then scanned from the back with a KM-3 spectrophotometer (Carl Zeiss, New York, N.Y., U.S.A.) in the reflectance mode for visible absorption. The spectrophotometer parameters were: λ = 390 nm, slit length 6 mm, slit width 0.4 mm, scanning speed 50 mm/min. Three-

gration of spot areas was performed using a Spectra-Physics minigrator (Spectra-Physics, Santa Clara, Calif., U.S.A.). Calculation of the concentration in each sample was made from a calibration graph constructed for each plate.

RESULTS AND DISCUSSION

The best separations of glucose and fructose by TLC have been reported on plates impregnated with a suitable salt[1,5]. Our attempts to separate glucose, fructose and sucrose with a variety of solvent systems on non-impregnated plates failed. A good separation between these sugars has been reported by Welch and Martin[4] on TLC plates impregnated with monobasic potassium phosphate by dipping in a 0.1 *M* aqueous solution of the salt and then drying. We find that the optimum concentration of monobasic potassium phosphate is 0.2 *M* for HPTLC plates using the development conditions described below.

The solvent system ethyl acetate–pyridine–water (8:2:1) was the same as that used by Welch and Martin[4]. Continuous development in a non-saturated tank was found to give the best results. However the actual configuration of the tank and how the plates were placed in the tank was found to be critical, as it affects the saturation of the atmosphere. Thus, when the front of the plate was directly exposed to the open slit of the development chamber, three 15-min developments were required for baseline separation between glucose, fructose and sucrose. Unfortunately this configuration gives an uneven solvent front and cannot be used for quantitative analysis. A perfectly even solvent front is obtained by allowing the silica gel surface to rest against the slit, *i.e.* the front of the plate is directly exposed to a somewhat more concentrated solvent atmosphere. This configuration requires three 30 min developments for baseline separation between fructose, glucose and sucrose. Fig. 1A shows the separation achieved for a synthetic mixture and Fig. 1B shows the separation achieved for

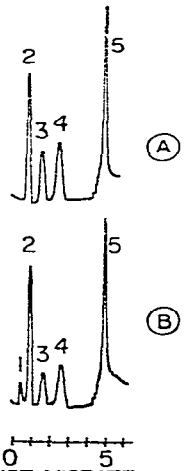

Fig. 1. A, HPTLC chromatogram of sugar mixture (50 ng each). 2 = Sucrose; 3 = glucose; 4 = fructose; 5 = solvent front. B, HPTLC chromatogram of molasses sample (300 ng); 1 = Kestose and higher sugars; 2 = sucrose; 3 = glucose; 4 = fructose; 5 = solvent front.

a molasses sample. The small peak before sucrose in the molasses sample corresponds to material that remains at the origin and consists of kestose and possible traces of higher sugars.

The spots were visualized by dipping in the reagent described by Hansen and then drying in an oven at 110°. It was found necessary to lower the plates into the dipping reagent and to remove the plates from the dipping solution with a rapid r ovement. If this is not done the solution streaks and spikes are seen when the plates are scanned.

After preliminary drying the plates were placed in an oven at 110°. When the spots first appear sucrose is blue, glucose is light brown and fructose is pink. On further heating all the spots become a similar blue color. The spots were scanned using the Zeiss KM-3 spectrophotometer in the reflectance mode. Plates were scanned from the back as this resulted in a larger response than when they were scanned from the front. The absorption spectra for the three sugars are shown in Fig. 2. The absorption maxima are at about 390 nm which was the wavelength chosen for scanning the plates. Other authors have reported scanning plates at about 620 nm for sucrose or glucose. With prolonged heating of the plate the absorption does increase in this region. However we found that this resulted in a somewhat lower precision due to slight darkening of the background. The effect of the heating period on precision is shown in Table I, which lists results obtained by spotting the same molasses sample eight times. The best values for relative standard deviation are found at heating periods of 20 min and 30 min. Slightly lower values of relative standard deviation are found when a synthetic mixture of sugars is used.

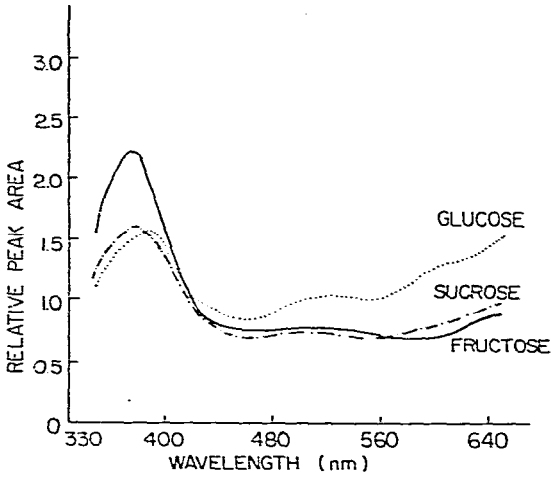

Fig. 2. Absorption spectra of sucrose, glucose and fructose (50 ng each).

A 20-min heating period was chosen for all developments discussed below. Under these conditions typical values of relative standard deviation are 1.0% for sucrose, 1.8% for fructose and 2.5% for glucose. For all heating periods sucrose has the lowest relative standard deviation and glucose the highest value. This may be related to the chemical stabilities of the compounds which would be expected to decrease in the order sucrose, fructose, glucose. It has been shown by Scott[6] that sugars

TABLE I

EFFECT OF HEATING PERIOD ON RELATIVE STANDARD DEVIATION* FOR FRUCTOSE, GLUCOSE AND SUCROSE IN A MOLASSES SAMPLE

Heating period (min)	*Relative standard deviation (%)*		
	Fructose	*Glucose*	*Sucrose*
10	3.1	6.5	2.1
20	1.8	2.5	1.0
30	1.8	2.7	0.8
40	4.3	4.8	1.4
60	4.5	5.1	1.6

* Relative standard deviation determined by scanning eight individual spots. Each spot scanned twice and mean used for calculation. Results combined by data pair technique to give four values for calculation of relative standard deviation.

do decompose on drying on silica gel. Fig. 3 shows calibration curves for the three sugars. The plots are near linear at low concentration but are more curved at higher concentration. All the curves can be described by the expression, $y = ax^b$ where y is the integrated area, x is the concentration in ng and a and b are constants. Table II lists the constants a and b after different periods of heating together with a correlation coefficient. In all cases the fit as determined by the correlation coefficient is good. An inspection of the coefficient b shows that the plot for sucrose is the least linear but that the deviation from linearity remains constant with the heating time for all compounds. After an initial increase in intergrated area there is a fall off with heating time as shown by coefficient a.

The intensity of spot color is a function of some factors that are difficult to control accurately such as the time that the plate is in the dipping solution and the temperature of the oven. However if a calibration curve is included on each plate, satisfactory results are still obtained as shown in Table III which shows the reproducibility obtained when the same sample is spotted on each of five plates. The sample and the three standards for the calibration curve were all spotted in duplicate

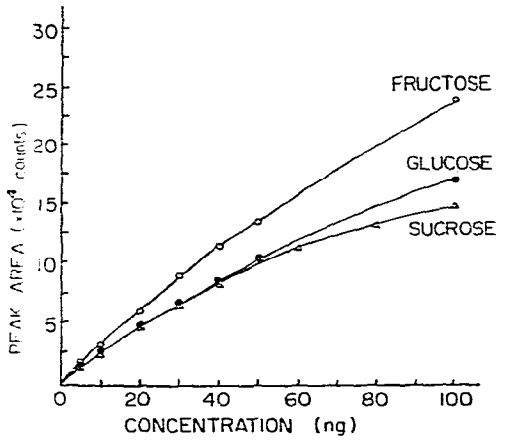

Fig. 3. Calibration curve of sugars separated by HPTLC.

TABLE II

NON-LINEAR REGRESSION ANALYSIS OF STANDARD CALIBRATION CURVE OF FRUCTOSE, GLUCOSE AND SUCROSE

Model $y = ax^b$ where x = concentration (ng) and y = integrated area. R^2 = Correlation coefficient.

Heating period	*Fructose*	*Glucose*	*Sucrose*
20	$a = 0.443$	$a = 0.3713$	$a = 0.6648$
	$b = 0.8717$	$b = 0.8347$	$b = 0.6812$
	$R^2 = 0.9973$	$R^2 = 0.9987$	$R^2 = 0.9968$
60	$a = 0.3491$	$a = 0.3522$	$a = 0.5891$
	$b = 0.8786$	$b = 0.8361$	$b = 0.6816$
	$R^2 = 0.9968$	$R^2 = 0.9992$	$R^2 = 0.9960$

TABLE III

REPRODUCIBILITIES OF SUGAR DETERMINATIONS IN A MOLASSES SAMPLES

Compounds	*Mean**	*Standard deviation for 5 determinations*	*Relative standard deviation** for 5 determinations (%)*
Fructose	26.23	0.59	2.2
Glucose	17.66	0.75	4.3
Sucrose	77.67	0.83	1.1

* ng/300 ng molasses.

** The molasses sample was spotted twice on each of five plates using the data pair technique. The mean from each duplicate was used for the calculation of relative standard deviation.

and the results combined using the data pair technique. The relative standard deviation for the five determinations for sucrose is 1.1%, for fructose 2.2% and 4.3% for glucose.

The accuracy of the method was tested by performing a recovery test. The results of the recovery test are listed in Table IV. Recoveries appear independent of concentration and vary between about 80% for fructose and 91% for sucrose. The incomplete recoveries may be due either to some decomposition of the sugars on the plate or due to the matrix effect[17] where the spot shape effects the quantitation of a compound.

The precision reported here for sucrose (relative standard deviation 1.1%)

TABLE IV

RECOVERY TEST OF THE SPIKED MOLASSES SAMPLE WITH STANDARD FRUCTOSE, GLUCOSE AND SUCROSE

Compounds	*Originally present (ng)*	*Added ng*	*Recovered ng*	*Recovery (%)*
Fructose	26.70	30	24.52	81.7
		15	11.82	78.8
Glucose	18.10	20	17.43	87.2
		10	8.72	87.2
Sucrose	77.30	80	72.77	91.0
		40	36.33	90.8

and fructose (relative standard deviation 2.2%) is better than has previously been reported for these sugars in the TLC literature. This precision may be considered as being due to a combination of the improved plates, spotting apparatus and scanning spectrophotometer that were used.

The method should also be applicable to the determination of trisaccharides in molasses even though we have not investigated this aspect. However the integrated area for the trisaccharide peak (typically about 1.3%) is of the same order as that reported by Kort *et al.*[9].

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