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QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS OF SUCROSE IN THE PRESENCE OF SUGAR OXIMES USING A BUFFERED OXIMATION REAGENT AND GLASS CAPILLARY COLUMNS

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SUMMARY

Aqueous sugar solutions containing fructose, glucose and sucrose can be derivatised rapidly with a novel oximating reagent, incorporating an organic buffer. The buffered reagent produces rapid and reproducible oximation of the monosaccharides without hydrolysing sucrose or affecting its silylation.

Reasons for the use of a SP-2250 glass capillary for the separation of sugars in cane molasses are also given.

INTRODUCTION

Sucrose, fructose and glucose are the main carbohydrates present in sugar cane juice and subsequent factory processing streams. Their estimation is important in assessing both sugar cane quality and factory performance[1]. The approximate ranges of the three sugars during processing are listed in Table I.

TABLE I
RANGE OF SUGAR LEVELS IN CANE SUGAR FACTORY STREAMS
Concentrations are expressed as % sugar in sample.

Stream	*Fructose (F)*	*Glucose (G)*	*F/G*	*Sucrose*
Mixed juice	0.2–0.6	0.2–0.6	1.0	9–13
Syrup	1–2	1–2	0.9–1.1	50–60
Final molasses	6–11	3–9	1.1–2.4	26–33

Gas–liquid chromatography (GLC) provides a means of measuring these three sugars more specifically than traditional titration and polarisation techniques which are readily influenced by impurities[2,3].

The volatilisation of sugars prior to GLC is normally achieved by converting the sugars into their trimethylsilyl (TMS) derivatives. The TMS ethers of monosaccharides possess the following disadvantages:

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(1) the proportions of each anomer will depend on solvent composition, temperature and the length of time the sugar has been dissolved;

(2) the overlap between fructose and glucose leads to inaccurate results;

(3) the overlap of the two major monosaccharides with other minor constituents in cane molasses will also give inaccurate results;

(4) the signal-to-noise ratio for a monosaccharide producing multiple peaks is obviously lower than a sugar producing a single peak. This is extremely important when the sugar is present in low concentration (see Table I).

Various chemical procedures have been proposed to inhibit anomerisation. Sweeley *et al.*[4] suggested oximation prior to silylation. Brobst[5] developed an *in situ* oximation–silylation procedure for sugars in aqueous solution. The reagents for this procedure are available commercially from a single supplier[6].

Aqueous sugar solutions covering a wide concentration range (0.5–35%) were derivatised in this laboratory, and although excellent qualitative results were obtained we noticed that sucrose (a non-reducing disaccharide and the sugar of prime importance to the sugar technologist) was hydrolysed during the oximation of fructose and glucose. This was due to the acidity of the oximation reagent.

This paper describes an oximation reagent incorporating an organic buffer. The buffered reagent produced rapid and reproducible oximation of monosaccharides without hydrolysing sucrose. Xylose and trehalose were added as internal standards for the monosaccharides and sucrose respectively. Reasons for using an SP-2250 glass capillary column are also given.

EXPERIMENTAL

Materials

Fructose (low in glucose), glucose (AnalaR), sucrose (Aristar), xylose (Biochemical) and trehalose dihydrate (Biochemical) were obtained from BDH (Poole, Great Britain). All reference sugars were dried *in vacuo* over phosphorus pentoxide and stored in a desiccator. The following reagents were commercially available: pyridine (for analysis; E. Merck, Darmstadt, G.F.R.); hydroxylamine hydrochloride (M & B reagent; May & Baker, Dagenham, Great Britain); hexamethyldisilazane (HMDS) (Ohio Valley, Manetta, OH, U.S.A.), stored under nitrogen and refrigerated; trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.), refrigerated; dimethylaminoethanol (BDH laboratory reagent).

Preparation of derivatives

Hydrolysis of sucrose during oximation. A calibration solution was prepared by dissolving sucrose (600 mg) and trehalose (660 mg) in 2.4 ml of distilled water (solution A). Aliquots were silylated directly or oximated prior to silylation.

The aliquot (5 μl) in a screw cap vial (3 ml) fitted with a PTFE-lined silicon disc was silylated by adding pyridine (0.5 ml), HMDS (0.45 ml) and TFA (0.05 ml) in rapid succession. The vial was hand shaken, capped and placed in an ultrasonic bath at 80°C for 10 min. The sample was degassed prior to injection.

An aliquot (5 μl) in a 3-ml vial was treated with 0.5 ml of oximation reagent (2.5 g of hydroxylamine hydrochloride in 100 ml of pyridine). The sample was placed in an ultrasonic bath at 80°C for 30 min. After cooling for 10 min, HMDS (0.45 ml) and TFA (0.05 ml) were added. The silylation was carried out at 80°C for 10 min.

pH of oximation reagent. Aliquots (5 μl) of solution A were treated with various amounts of dimethylaminoethanol (0, 9, 18 and 27 μl), followed by the oximating reagent in pyridine (0.5 ml) (2.5%, w/v). Oximation and silylation were carried out as above.

Effectiveness of buffered oximation reagent. A calibration standard containing the following sugars was prepared: fructose, 150 mg; glucose, 150 mg; xylose, 150 mg and water, 2.4 ml. Aliquots (in triplicate) were treated with various amounts of dimethylaminoethanol (0, 18 and 27 μl), followed by oximation and silylation as above.

Effect of new buffered oximation reagent on sucrose hydrolysis. Three calibration standards bracketing the sucrose concentration range for cane molasses were prepared:

	S_1	S_2	S_3
Trehalose (mg)	660	660	660
Sucrose (mg)	500	600	700
Water (ml)	2.4	2.4	2.4

Aliquots were silylated in triplicate above. The buffered oximation reagent was prepared by adding 2.5 g of hydroxylamine hydrochloride to pyridine (100 ml). Dimethylaminoethanol (270 μl) was added to 5 ml of this hydroxylamine solution just before it was needed. The solution was mixed thoroughly (solution B). Aliquots for oximation were prepared in triplicate. Oximation with solution B was identical to the procedure described above.

Gas chromatography. A Hewlett-Packard 5840 gas chromatograph equipped with an autosampler was employed. Experimental details are listed in Table II.

TABLE II

EXPERIMENTAL DETAILS FOR GLC SEPARATION OF OX–TMS SUGAR DERIVATIVES

Column	15 m × 0.25 mm I.D. glass capillary, coated with SP-2250 (obtained from SGE, North Melbourne, Australia)
Inlet pressure	25 kPa
Pre-column flow-rate	10.6 cm^3/min
Column flow-rate	0.4 cm^3/min (nitrogen)
Split ratio	25:1
Injection volume	4 μl (0.15 μl onto column)
Injector/flame ionization detector temperature	250/250°C
Oven program	150°C for 2 min, 150–240°C at 8°C/min
Make-up gas	60 cm^3/min (nitrogen)
Hydrogen	40 cm^3/min
Air	330 cm^3/min

RESULTS AND DISCUSSION

Sucrose hydrolysis

Use of Brobst's procedure to oximate an aqueous sucrose–trehalose mixture prior to silylation produced a lower sucrose response factor (1.075) than that obtained

for direct silylation (1.097). Fig. 1A clearly indicates significant sucrose hydrolysis, as fructose and glucose can be detected easily. The pH of the hydroxylamine reagent was found to be 5.4, Stadler's table[15] indicates about 0.15% sucrose inversion per hour at 80°C at this pH.

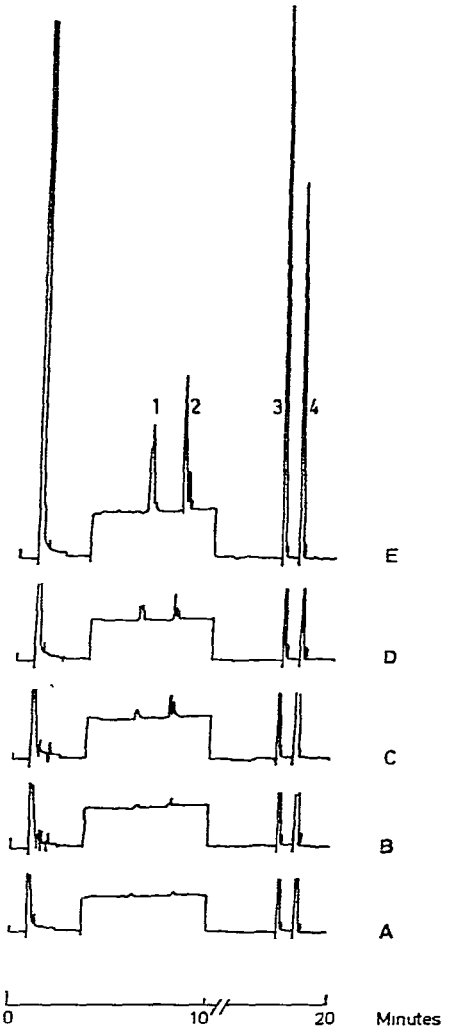

Fig. 1. Sucrose hydrolysis during oximation: E = unbuffered oximation reagent, pH = 5.4; D = buffered oximation reagent, pH = 6.5; C = buffered oximation reagent, pH = 7.1; B = buffered oximation reagent, pH = 7.4; A = direct silylation (no oximation). Peaks: 1 = OX–TMS-fructose; 2 = OX–TMS-glucose; 3 = TMS–sucrose; 4 = TMS-trehalose. Attenuation: for monosaccharides, 2 ↑ 3; for disaccharides, 2 ↑ 7.

To ascertain whether 80°C was necessary for oximation, lower temperatures were investigated. At 50°C and a reaction time of 30 min, oximation was incomplete. At this temperature sucrose hydrolysis was still apparent. No reference to such

hydrolysis has been noted by us. Adam and Jennings[7], using methyl oximes, noticed fructose and glucose peaks in all their chromatograms; they attributed this to sucrose hydrolysis during their drying step (using phosphorus pentoxide).

Buffer

Although oximation is acid-catalysed and hydroxylamine is unstable in basic solution, Fritz *et al.*[8] developed a quantitative titrimetric procedure for carbonyl compounds employing semi-neutralised hydroxylamine hydrochloride solutions. The choice of buffer was limited by the anhydrous conditions and the fact that the hydrochloride of the base used should be soluble in the solvent. These workers found either 2-dimethylaminoethanol or 2-diethylaminoethanol to be suitable.

Effect of pH of the oximation reagent on sucrose hydrolysis

Dimethylaminoethanol was used to raise the pH of the oximation (OX) reagent without causing any undesirable precipitation reactions. The effect of neutralising the OX reagent before oximation can be seen in Fig. 1 and Table III.

TABLE III

EFFECT OF pH OF OXIMATION REAGENT ON SUCROSE RESPONSE FACTOR

Base = Dimethylaminoethanol. B:A = Equivalents base relative to equivalents HCl. Response factors, *K*, are the means of three sample preparations. R.S.D. = Relative standard deviation.

Base (μl)	*B:A*	*pH*	*Expected hydrolysis (%/h)*	*K*	*R.S.D.*
0	0 :1	5.4	≈0.15	1.077	0.9
9	0.5:1	6.5	0.01	1.093	0.7
18	1 :1	7.1	0.003	1.095	0.1
27	1.5:1	7.4	<0.001	1.097	0.3
—	Direct silylation	—	—	1.097	0.3

The response factor for sucrose using an OX reagent at a pH of 7.4 was virtually identical to that obtained for direct silylation. Fig. 1 also indicates that with the buffered reagent sucrose hydrolysis was not significant. Any detectable quantities of fructose and glucose are probably minute impurities in Aristar sucrose. No significant hydrolysis of other disaccharides such as trehalose, maltose or cellobiose was observed when using Brobst's procedure.

Effect of pH on monosaccharide oximation

Buffering the oximation reagent eliminated sucrose hydrolysis. The effect of increasing the pH of the oximation reagent on the actual oximation of fructose and glucose is noted in Table IV. It is obvious that effective oximation of fructose and glucose can be obtained even in slightly alkaline solution, as excellent agreement over the range pH 5.4–7.4 was obtained.

TABLE IV

EFFECT OF pH OF OXIMATION REAGENT ON FRUCTOSE AND GLUCOSE RESPONSE FACTORS

Number of samples in each case: 12. Oximation time = 30 min. Internal standard: xylose. These response factors are the means of three sample preparations.

pH	K_F	*R.S.D.*	K_G	*R.S.D.*
5.4	1.072	0.2	1.058	0.1
6.5	1.076	0.3	1.058	0.1
7.1	1.075	0	1.058	0.1
7.4	1.076	0.3	1.058	0.2
Mean	1.075	0.3	1.058	0.1

Decreasing oximation reaction time

We have shown that excellent quantitation of fructose and glucose can be obtained by using a slightly alkaline oximation reaction prior to silylation. For all these studies an OX reaction time of 30 min at 80°C was adopted. To reduce sucrose hydrolysis even further, an OX reaction time of 10 min at 80°C was investigated. It can be seen from Table V that statistically there was no difference between the two reaction times. This reduction in sample preparation time is obviously advantageous for routine high throughput analysis.

TABLE V

EFFECT OF OX REACTION TIME ON MONOSACCHARIDE RESPONSE FACTORS

Numbers of samples: 12. Mean difference between response factors at different OX reaction times, $D = 0.001$. t_{exp} = Calculated Student's t value for paired observations. Critical t value, $t_{1-\alpha}$, ($\alpha = 0.05$) = 2.776.

	K_F	K_G
Time		
30 min	1.075	1.058
10 min	1.076	1.058
t_{exp}	−0.06	−0.11

Comparison of sucrose response factors: direct silylation vs. OX/silylation

Accurate sucrose analysis should be independent of monosaccharide oxime formation. The effect of the new buffered OX reagent was investigated by preparing three aqueous sucrose–trehalose standards, bracketting our normal cane molasses sample range (see Experimental). The comparison is presented in Table VI. There was

TABLE VI

SUCROSE CALIBRATION STANDARDS FOR CANE MOLASSES

Number of samples: 12. $t_{exp} = -1.91$; $t_{1-\alpha}$ ($\alpha = 0.05$) = 2.31.

	K_S
Direct silylation*	1.106
OX–silylation**	1.109

* Silylation conditions: 10 min at 80°C.

** OX time: 10 min at 80°C followed by silylation for 10 min at 80°C.

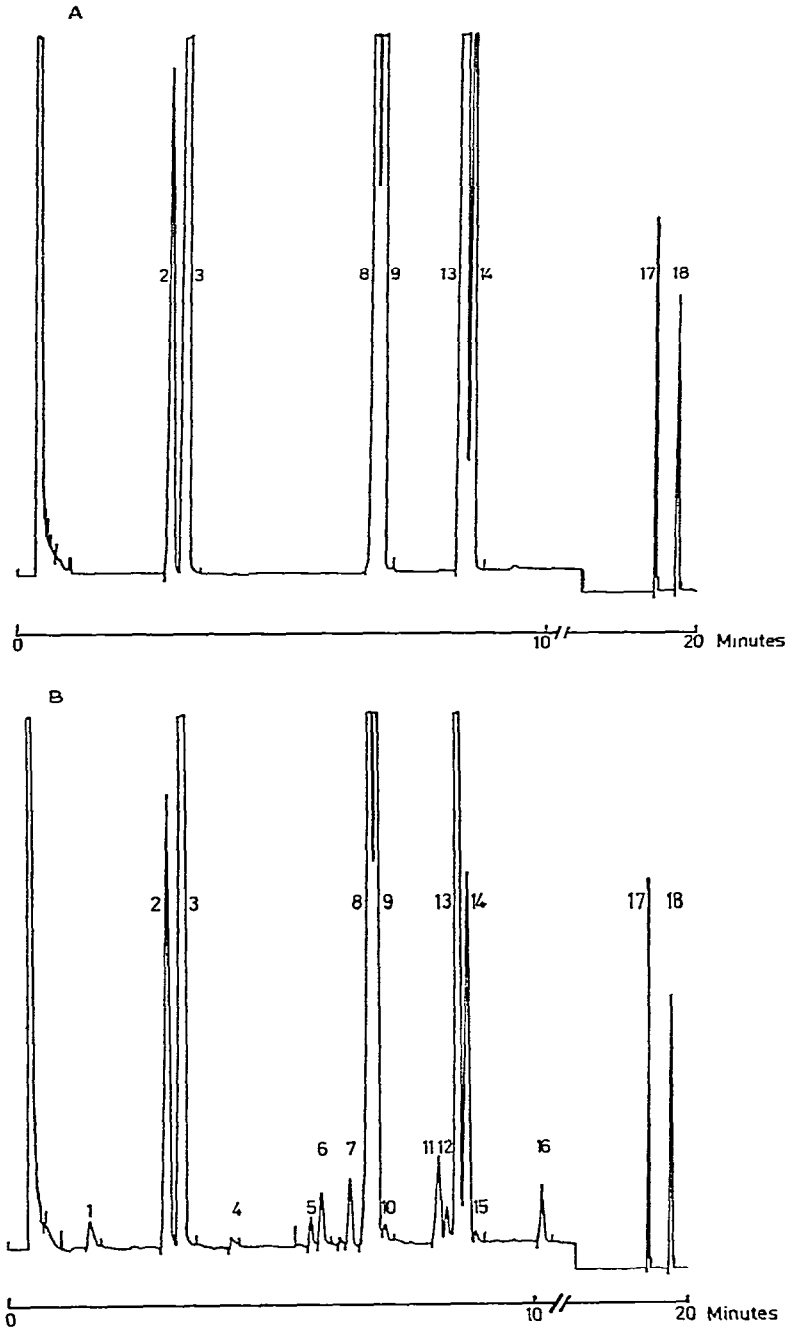

Fig. 2. Separation on SP-2250 (OV-17) wall-coated glass capillary columns: A = Calibration standard; B = cane molasses sample. OX–TMS derivatives: 2,3 = xylose; 8,9 = fructose; 13,14 = glucose; 17 = sucrose; 18 = trehalose. Other peaks: 6 = mannitol; 11 = mannose; 16 = inositol; 1, 4, 5, 7, 10, 12, 15 = unknowns. Chart speed: for monosaccharides, 2.5 cm/min; for disaccharides, 0.25 cm/min. Attenuation for mono- and disaccharides was 2 ↑ 5 and 2 ↑ 7, respectively.

no statistical difference and in our opinion the new buffered reagent effectively produces monosaccharide oximes without hydrolysing sucrose.

Epimerisation of monosaccharides at pH 7.5

In dilute alkaline solutions the monosaccharides can undergo profound changes[9]. Glucose can produce its epimers mannose and fructose. To investigate the effect of the higher pH (7.4) of the oximation reagent on monosaccharide epimerisation, dilute aqueous solutions containing either fructose or glucose were subjected to the OX–TMS procedure at pH 7.4. No evidence of epimerisation was observed.

Use of glass capillary columns

Packed columns were used initially during the development of the GC procedure for sugars[3,10]. We recently utilised stainless-steel capillaries coated with OV-17 for an extensive study of carbohydrate changes during sugar boiling[11]. However, glass capillaries offer certain advantages due to better peak separation and sharper peaks: greater column efficiencies; better column deactivation; often better resolution in less time; more information and more accurate results.

We therefore switched to glass capillaries. SP-2250 (OV-17) wall-coated columns produced an excellent separation of OX–TMS-xylose (internal standard), fructose, glucose, TMS-sucrose and trehalose (internal standard), Fig. 2A. Carrier gas flow was optimised (average flow velocity, 16 cm/sec), thus all OX–TMS sugars produced acyclic *syn* and *anti* isomers. Despite the separation of each monosaccharide into its respective doublets, each sugar was well separated.

An aqueous molasses sample was subjected to the OX–TMS procedure, Fig. 2B. Besides fructose and glucose, approximately ten minor constituents could also be observed in the molasses sample. We have been monitoring sugar products for over 3 years and the pattern depicted in Fig. 2B is completely characteristic of South African cane molasses. Some of these peaks, mannitol, mannose and inositol, have been identified. The use of columns with lower efficiency and selectivity can result in the overestimation of both fructose and glucose by about 6–11% in South African cane molasses.

A further advantage of the use of capillary columns is their ability to separate the geometric isomers of each monosaccharide OX–TMS derivative. The peak area ratio under controlled conditions is characteristic for the isomers of each sugar. A change in this area ratio can often indicate an impurity co-eluting with one or the other isomer. This is of practical importance for low concentrations of fructose and glucose.

Several authors have indicated that OX/TMS derivatives are either unstable[12] or produce inconsistent quantitative results[2]. The method described in this paper has been in constant use for the past 3 years and thousands of aqueous commercial solutions have been chromatographed.

The acceptance of this method by the South African sugar industry has been realised by ensuring careful attention to the analytical detail. A paper describing the routine procedures and method evaluation techniques should be published shortly[14].

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