A RAPID QUANTITATIVE METHOD FOR THE ROUTINE DETERMINATION OF OLIGOSACCHARIDES IN CANE MOLASSES BY THIN-LAYER CHROMATOGRAPHY

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Abstract

A rapid method for the separation of fructosyl oligosaccharides in cane molasses is described. The procedure is based on the use of pre-coated silica gel plates with a novel solvent system and continuous development. The ferricyanide method for reducing sugars has been adapted for the colorimetric estimation of fructosyl oligosaccharides, by inversion prior to colour development. Calibration curves for individual t.l.c. determinations were linear in the 0-100 μg range.

Introduction

An accurate estimation of the sucrose content of molasses is an important factor in process control. The standard methods for determining sucrose in molasses are known to be influenced by non-sucrose constituents, particularly oligosaccharides. A great deal of work has been carried out on the raffinose content of beet molasses. Paper chromatography followed by visual or in some cases colorimetric estimation is the method most commonly used[1,2,3,4,5,6]. Schneider, *et al*[7], compare several of these methods. Bichsel *et al*[8] determine raffinose and 6-kestose densitometrically on paper. Saglietto and Mantovani[9] separate raffinose and 6-kestose from sucrose by t.l.c. and estimate them spectrofluorimetrically.

However, very little work on the oligosaccharide content of cane molasses has been done. Kamoda *et al*[10], concerned about the quality of raw sugar, separated the oligosaccharides from sucrose on cellulose and determined them with anthrone. Staker[11] measured the overall levels of oligosaccharides in cane-derived materials by an automated carbon column-anthrone method.

Preliminary experiments showed that Natal cane molasses contained substantial amounts of kestose (Du Boil *et al*[12]).

At the 15th meeting of the International Commission for Uniform Methods of Sugar Analysis[18], the following correction factor was recommended for cane molasses when the invertase method was used:

$$\% \text{ sucrose} = \frac{19}{20} [(\% \text{ total invert}) - (\% \text{initial invert} - 1{,}07\% \text{ kestose}]$$

The kestoses were separated from sucrose by t.l.c. and colorimetrically estimated.

Experimental

Sample preparation

Molasses (26 g) was transferred to a 100 ml Kohlrausch flask and dissolved in about 50 ml of water. A 54° brix lead subacetate solution was added and the flask agitated for 30 minutes. After making up to volume the mixture was filtered through a S. & S. No. 613 filter paper using a filter aid. The first few millilitres of filtrate were discarded. The clarified leaded filtrate was applied to the chromatogram.

A sucrose standard, concentration 5 μg/μl was used throughout the determination.

Thin-layer chromatography

Pre-coated silica gel plates (Merck, 20 × 20 cm²) were used. The plate was prepared for sample application as indicated in Figure 1.

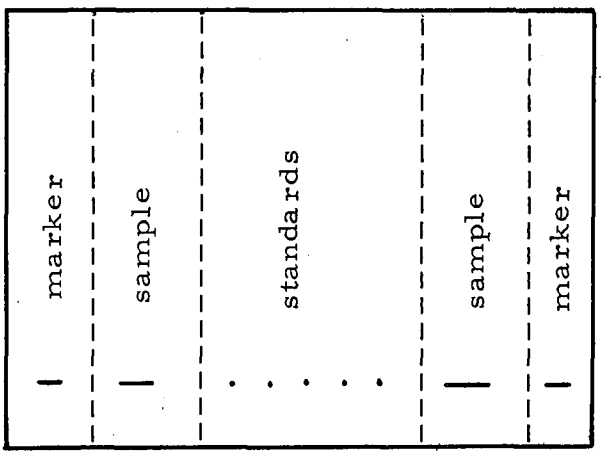


FIGURE 1: Preparation of t.l.c. plate for spotting.

Sucrose standards were applied to the plates in 3 to 4 mm spots with a Hamilton micro-syringe. Molasses samples were streaked, normally 10 μl/1,5 cm was applied. Multiple spotting was necessary. The spot was dried in a cold air stream before respotting. Chromatograms were developed in a Shandon continuous tank for 23 hours, at 25°C without equilibration in butanol (water saturated): ethanol (100 : 85). The chromatograms were dried at room temperature. After covering up the appropriate sections of the plate with aluminium foil and cellulose tape, the marker strips were sprayed with diphenylamine (De Stefanis and Ponte[17]). The positions of the oligosaccharides and the various sucrose standards on the unsprayed sections were located by reference to the guide strips with approximately 0,5 cm allowance on either side of the sugar spots, Figure 2.

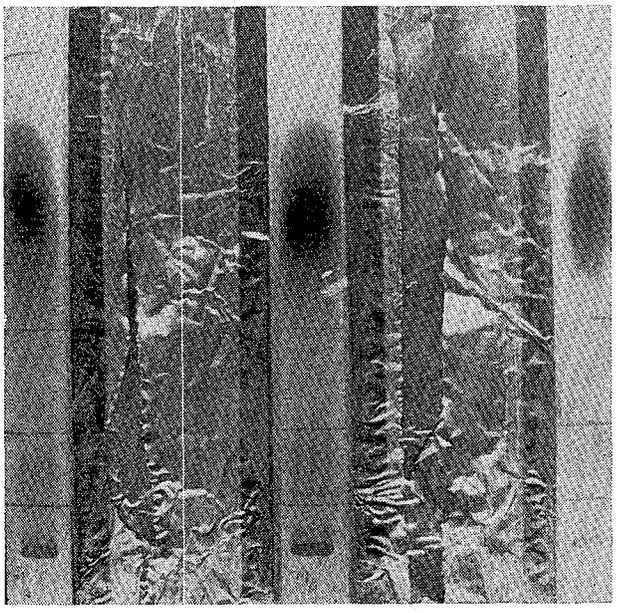


FIGURE 2: Sprayed t.l.c. plate guide strips showing the bands ready for elution.

Elution and determination

After removing the sprayed sections the rest of the plate is sprayed with a 1% invertase (in 0,5% KH_2PO_4) solution. The plate was left for 45 minutes at 40°C. The silica containing the sugars together with a blank silica square were scraped off the plate and eluted, with distilled water (1 ml) in a test tube for 10 minutes. To this 0,05% potassium ferricyanide (2 ml) and 0,52% sodium carbonate (2 ml) were added and the solution was boiled for exactly 15 minutes. After cooling in ice, the samples were transferred to micro-centrifuge tubes and spun at 2 500 rpm for five minutes. The clear supernatant liquid was read at 420 nm in a Beckman DBG spectrophotometer.

Results

The development of a suitable routine procedure has proved to be difficult. Most of the published methods attempted failed to separate higher oligosaccharides when they occurred simultaneously with high invert or sucrose concentrations. Silica gel layers were finally chosen because of their speed and high adsorptive capacity.

The marker strip technique was used for the determination, necessitating reproducible layers. For this reason pre-coated silica gel plates were chosen. A single ascent does not separate the higher sugars from sucrose. Multiple development is very tedious and gives rise to artefacts. A Shandon continuous tank gave a very good separation. Plates were spotted or streaked and run overnight. The solvent system butanol saturated with water and ethanol (100 : 85) produced the best separation (Schäffler and Du Boil[14]).

A study of the ferricyanide method for the routine analysis of reducing sugars was recently carried out by G. Comrie[15]. Sensitivity, reproduci-

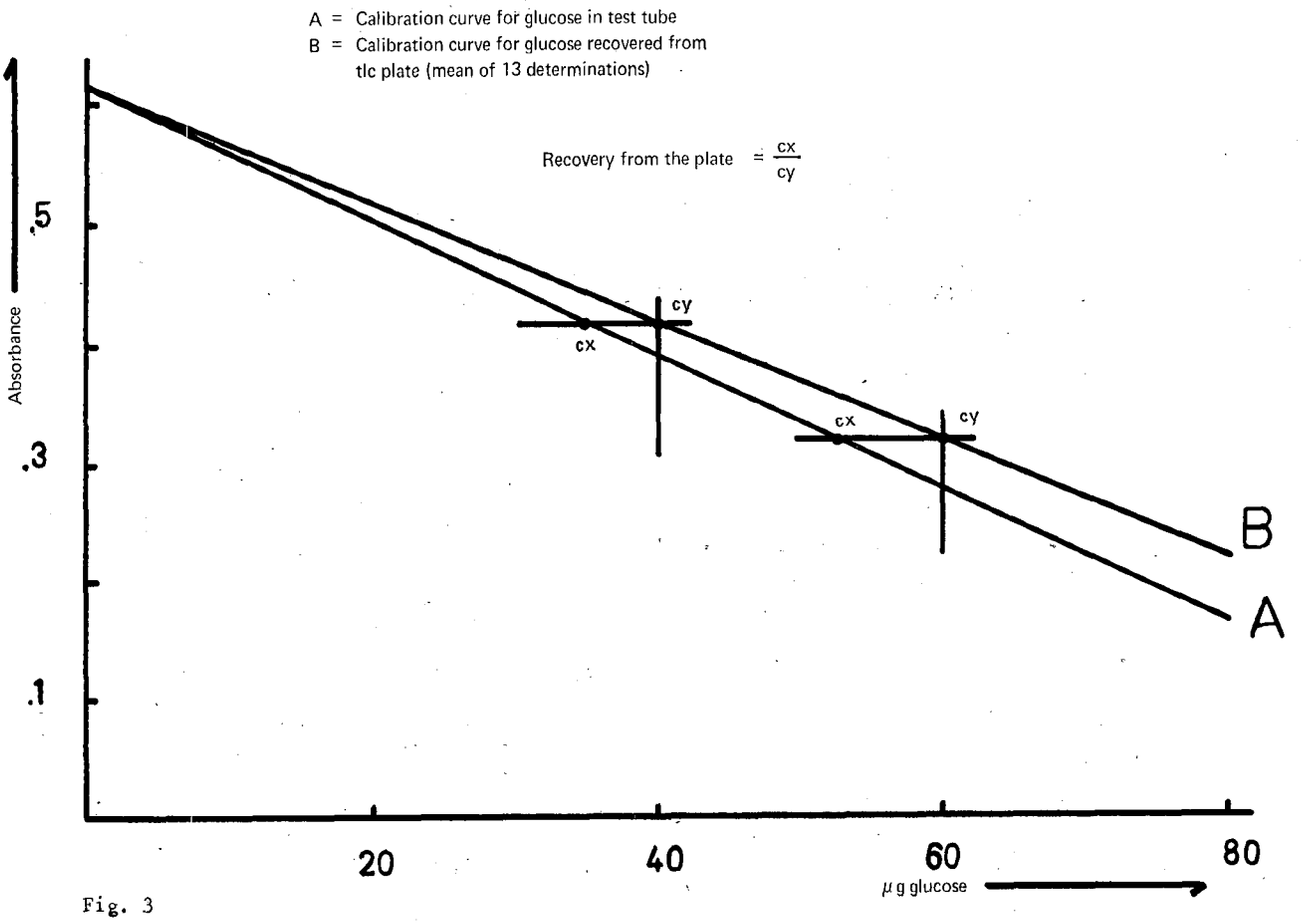


Fig. 3

bility and accuracy were good. The method was adapted to the determination of fructosyl oligosaccharides by spraying the t.l.c. plate with invertase prior to elution and colour development. Removal of silica gel by centrifuging before colorimetry was found to be adequate.

Calibration curves for glucose were drawn up to test recoveries from t.l.c. plates, as shown in Figure 3.

Recovery of the sugars from t.l.c. plates was measured by removing and eluting about 7,5 cm^2 of silica including the sample. A similar amount was taken for the blank. (See Table I.)

Determination of Oligosaccharides by Thin-Layer Chromatography.

TABLE I

Recovery of glucose from t.l.c. plates.

Amount spotted	30μg	60μg	80μg
Amount recovered	25,6	52,8	69,8
	26,8	52,8	69,2
	24,4	55,6	68,6
Mean recovery	25,6	53,7	69,2
Mean % recovery	85,3	89,5	86,5
Standard deviation	0,98	1,28	0,49

Sugar recovery was not complete (total mean % recovered — 87,1%, overall standard deviation of 0,92), a certain amount of sugar was irreversibly adsorbed by the plate (Scott[16]). It was decided to use a separate calibration curve for each plate. Reproducibility was good. The mean recovery for a series of glucose standards was 99,7%.

Although the method worked well for reducing sugars its application was ultimately aimed at oligosaccharides. Therefore sucrose-glucose mixtures together with standard glucose solutions were streaked on a t.l.c. plate, and run for 4½ hours in ethyl acetate-acetic acid-water (6 : 3 : 2). A good separation was obtained. The recoveries were calculated from the glucose standards. All plates were sprayed with invertase before commencing with the determination. (Table II.)

Determination of Oligosaccharides by Thin Layer Chromatography

TABLE II

Sugar Recoveries.

Glucose spotted	Recovered	% Recovered
30 μg	30,5	101,7
50 μg	48,35	96,8
Sucrose spotted		
30 μg	29,74	99,1
50 μg	48,54	97,1

Mean sucrose recovery 98,1%

The oligosaccharides were hydrolysed with a commercial preparation of invertase (Convertit). The time of inversion used by Fric and Kubaniova[19] for sucrose was 40 minutes for an 0,2% invertase solution. Throughout the work mentioned in this paper a 1% solution of invertase was used. To test for complete inversion a molasses sample (27/6) was analysed as usual by t.l.c. Three different inversion times were used. Analysis was carried out in duplicate. Results are shown in Table III.

Determination of Oligosaccharides by Thin Layer Chromatography

TABLE III

Inversion Time	μg kestose		% kestose
25 minutes	34,6	34,9	1,67
45 minutes	34,4	36,8	1,70
115 minutes	34,2	—	1,63

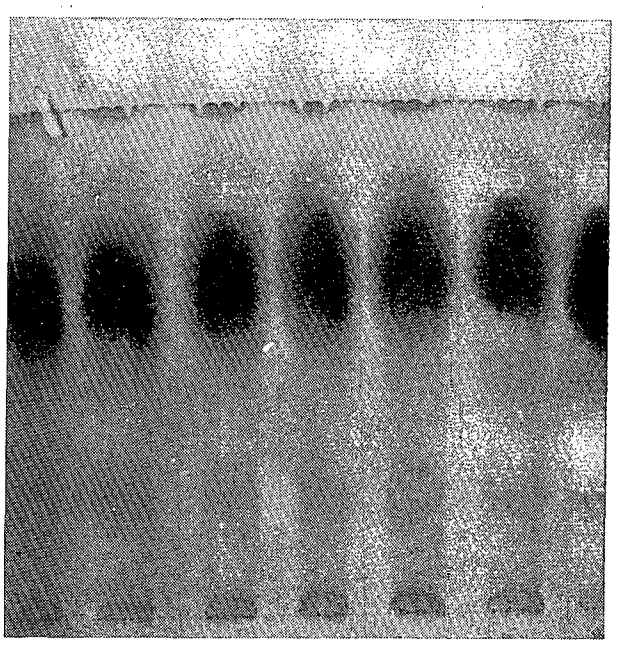
There is no significant difference between the three inversion times. An inversion time of 45 minutes was therefore chosen.

Despite the fact that a blank was used with every determination the invertase solution used was tested for reducing activity. The difference between the blanks, sprayed and not sprayed with invertase is smaller than the variation between blanks from individual determinations.

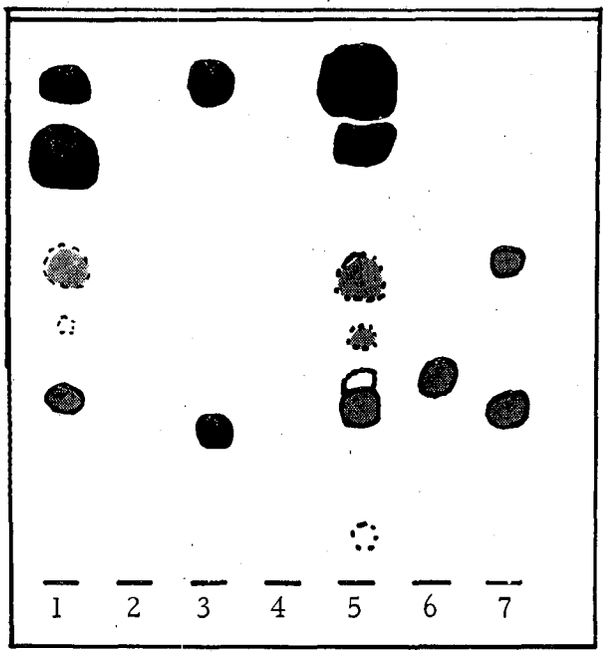
Each analysis was accompanied by a separate calibration curve for that particular t.l.c. plate. After 28 plates the degree of variation looked small. Therefore an overall calibration curve was derived from the 28 individual curves and an analysis of variance carried out.

$F = 1,46$ $\qquad F_{27/112/0,05} = 1,63.$

Therefore there is no significant difference between the overall calibration curve and the individual curves. The series of sucrose standards which are tedious to apply to each plate could be left out. This would allow more time and plate area for molasses analysis.



CHROMATOGRAM 1: Varying amounts of molasses, oligosaccharides can be seen below large sucrose spots.



CHROMATOGRAM 2: Mill molasses. 3: Glucose and raffinose. 5: High-test molasses. 6: 1-kestose. 7: Neo- and 6-kestose.

The above method was applied to Empangeni final molasses. A series of weekly composites were analysed for oligosaccharide content. (See chromatogram No. 1). Chromatogram No. 2 shows that kestoses are the main oligosaccharides present besides sucrose. The areas on the t.l.c. plate corresponding to neo-kestose, 1- and 6-kestose were removed and the concentration estimated from the sucrose standard curve. The sucrose % was corrected by subtracting the % kestose from the results obtained by the Lane and Eynon method. (Tables IV and V.)

Similar analyses were carried out on seasonal composites from Felixton, Amatikulu and Darnall. (Tables VI and VII.)

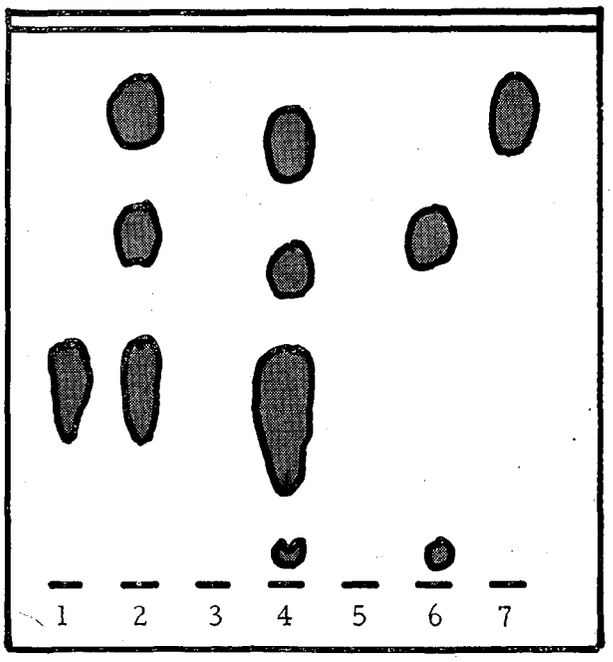
Conclusions

At the time of writing this paper no absolute method was available for the analysis of sucrose, so it is impossible to assess the accuracy of the correction factor recommended for kestoses by I.C.U.M.S.A. However, from mere visual comparison of chromatograms it is obvious that in some cases the oligosaccharide content is considerable. Although other sugars are present in the molasses the kestoses seem to constitute the major portion Their measurement is therefore important. The oligosaccharide content of final molasses does not seem to show any seasonal variation and the amounts do not differ much from mill to mill.

The above method was also tested for reproducibility on a single molasses sample (27/6). A coefficient variation of 6,0% was obtained for nine determinations.

As can be seen from the tables the correction factor is considerable and has a profound influence on the sucrose % values reported previously. From the recent I.C.U.M.S.A. conference kestoses percentages of up to 1,5% were reported.

Initial experiments using pre-coated cellulose plates have yielded a good separation of glucose, fructose, and sucrose in final molasses. (See chro-



CHROMATOGRAM 3: Separation on cellulose. 1: Sucrose. 2: Fructose, glucose and sucrose. 4: Mill molasses. 6: Glucose and raffinose. 7: Fructose.

Determination of Oligosaccharides by Thin-Layer Chromatography

TABLE IV

Empangeni Molasses
Kestose % determined as sucrose by T.L.C. ferricyanide.

Sample Date	% Oligosaccharide	Mean
13.6.70	1,84 1,85	1,85
20.6.70	1,67 1,90	1,79
27.6.70	1,79 1,65	1,72
5.7.70	2,01 2,10	2,06
11.7.70	2,07 1,96	2,02
1.8.70	1,45 1,59	1,52
1.8.70	1,60 1,55	1,58
30.8.70	1,86 1,80	1,83
13.9.70	1,90 1,84	1,87
4.10.70	1,68 1,82	1,75
11.10.70	2,25 2,34	2,30
18.10.70	2,23 2,27	2,25
1.11.70	1,74 1,89	1,82
8.11.70	2,08 2,09	2,09
15.11.70	2,07 2,34	2,21
22.11.70	1,60 1,52	1,56
28.11.70	1,49 1,54	1,53

matogram No. 3.) Application of the above mentioned method for their quantitation will not be difficult. This will give a more accurate estimate of the amount of sucrose present in molasses. The kestose correction factors can then be more accurately gauged.

Determination of Oligosaccharides by Thin-Layer Chromatography

TABLE V

Empangeni Molasses
Sucrose per cent corrected for oligosaccharide content

Sample (1970)	Brix	Invert %	Kestose % (as sucrose)	Sucrose %	Sucrose % corrected
13,6	88,4	11,1	1,85	38,37	36,52
20,6	88,7	11,1	1,79	38,17	36,38
27,6	88,8	11,5	1,72	38,21	36,49
5,7	88,4	11,5	2,06	38,65	36,59
11,7	89,1	13,2	2,02	37,43	35,41
1,8	88,3	13,0	1,55	35,80	34,25
30,8	89,1	15,7	1,83	34,83	33,00
13,9	90,2	14,4	1,87	37,53	35,66
4,10	89,2	14,6	1,75	37,30	35,55
11,10	89,6	13,4	2,30	37,66	35,36
18,10	88,7	12,8	2,25	38,21	35,96
1,11	87,4	11,8	1,82	38,70	36,88
8,11	87,6	11,5	2,09	38,38	36,29
15,11	87,2	11,4	2,21	37,65	35,44
22,11	86,9	11,4	1,56	36,87	35,31
28,11	88,4	11,5	1,53	39,06	37,53

Determination of Oligosaccharides by Thin-Layer Chromatography

TABLE VI

Per cent oligosaccharides in final molasses (seasonal composite)

Sample	Per cent Oligosaccharide	Mean
Felixton	1,84 1,85	1,85
Amatikulu	2,08 2,33	2,15
Darnall	1,96 2,08	2,02
Empangeni	—	1,89

Determination of Oligosaccharides by Thin-Layer Chromatography

TABLE VII

Sucrose per cent corrected for oligosaccharides in factory composites

Sample	Brix	Reducing Sugar %	Kestose %	Sucrose %	Sucrose % corrected
Felixton	87,6	15,20	1,85	34,48	32,62
Amatikulu	88,0	14,60	2,15	35,87	33,72
Darnall	86,0	14,60	2,02	33,45	31,43

Summary

A new t.l.c. spectrophotometric method has been developed for the determination of oligosaccharides in molasses. Although the method is not specific for kestoses, they constitute a large percentage of oligosaccharides in cane and the kestose correction factor for sucrose will give a better idea of the actual amount of sucrose in molasses.

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Discussion

Mr. Bruijn: I see that there was a certain amount of irreversible absorption. This is contrary to paper chromatography where basically you get 100% recovery. Is the irreversible absorption on the plate related to the distance the spot has travelled? If not, what is the mechanism.

Mr. Schaffler: It may have some relation to the distance the sugar has travelled on the plate.

However work by Fairbairn showed that the loss of sugar from the point of application to the distance it travelled was minimal. Probably some of the sugar is lost at the point of application due to the heat of the dryer. Recovery tests on pure sugars have shown that there is a certain amount of irreversible absorption by the silica gel and this absorption depends on the heat treatment the sugars have experienced.

Mr. Bruijn: We are at present comparing molasses from diffusion factories and milling factories. We have not found many differences.

A theory has been advanced that owing to the enzymes in the cane polymerisation might occur in a diffuser and different oligosaccharides may form.

In Table VI I am amazed at the closeness of the figures for FX and EM for per cent oligosaccharides in final molasses—1,85 and 1,89. Is my conclusion correct—that this paper reports the total amount of oligosaccharides present in molasses?

Mr. Schaffler: The figure of 1,89 is the mean of the figures in Table IV whereas the figure of 1,85 for FX is a composite figure.

Mr. Bruijn: But if it was a comparable sample then the conclusion is that there is no difference.

Mr. Schaffler: As can be seen from the slides two samples were taken from each mill and all the mills show similar concentrations.

More work still has to be done on the various sugars and how they are formed in the cane, either by enzymes in the plant, bacteria or by degradation in the factory.

Mr. Bruijn: Referring to the figures in Table V, could not the isotope dilution method have been used?

Mr. Schaffler: Yes, but it is rather lengthy and this method is fairly quick. In all cases we have been rather pessimistic about the amount of kestose. In other words, when raffinose is present we determine it as sucrose and its reducing activity is less than that of sucrose.

There is a possibility, with the isotope dilution method, that when sucrose is precipitated as barium saccharate, trisaccharides are occluded.

We have now received a gas chromatograph and from reports overseas we can expect good results from it. The determination of invert sugar together with sucrose only takes twenty-five minutes and the error is about two per cent.

Mr. Jennings: It seems good to have a low sucrose in molasses figure but then the sucrose must be somewhere else which means a higher undetermined loss.

We will have to investigate the presence of oligosaccharides in the incoming juice.

If this is an undetermined loss then it is one that is directly affected by the non-sucrose content of the molasses.

Dr. Matic (in the chair): If it can be determined it will no longer be undetermined.

Some slides were shown of oligosaccharides in high test molasses and also ordinary molasses from the refinery. It is rather worrying to see the amount of oligosaccharides in HTM and in refinery molasses that have not been inverted, particularly if it is assumed that the oligosaccharides in HTM are formed during inversion.

The possible effect of refinery molasses on raw sugar quality is still not clear.

Mr. Lenferna: This paper shows that there are still undetermined substances in cane molasses.

Very often in a factory there are inexplicable changes in the process.

The paper will help us in the factory to analyse the product we are working with and will help us determine what is causing trouble at any specific time.

Dr. Matic: Unfortunately it will not give us an answer to sudden undetermined losses because the oligosaccharides are unexpectedly constant throughout the year.