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THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF SUGAR MIXTURES

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SUMMARY

Some general remarks are made concerning the difficulties experienced in the quantitative determination of the composition of complex sugar mixtures in solution. The application of thin-layer chromatography to this analytical problem is discussed. A technique for obtaining a satisfactory separation of nine to ten sugars has been worked out. Methods of determining reducing sugars and keto-sugars in amounts of 0 to 100 μg are mentioned, as well as a technique for removing the sugar-containing area from the thin-layer plate. Figures illustrating the quantitative aspects of the method are given.

INTRODUCTION

The determination of the composition of complex sugar mixtures, such as occur in foodstuffs or in vegetable matter, has always been an intricate chemical problem. Only in a limited number of cases, where usually no more than two specified sugars were known to be present — *e.g.* sweetened condensed milk, confectionery or jam — is it possible to estimate their concentrations quantitatively by classical methods of reductometry and polarimetry or by modern enzymatic methods. For the analysis of more complicated mixtures of sugars, various ingenious techniques have been developed in the course of time, in which use is made of specific reactions such as oxidation or reduction, fermentation and enzymic conversion. In applying these techniques, however, several serious difficulties may be experienced:

(a) They can be used only if the type and number of the sugars in the product are known in advance, and only if the sugar combination fits the chosen procedure.

(b) It is necessary to verify whether the conditions for applying specific chemical or biochemical reactions can be fulfilled in a satisfactory manner.

(c) The analytical techniques are mostly very time-consuming.

(d) The results obtained are often interconnected, *i.e.* an error in the determination of one component may affect the results of the estimation of the other components.

For these reasons it would be interesting to know the possibilities of various forms of chromatography for the separation of sugar mixtures, as after separation it would only be necessary to determine the amounts of the various individual sugars. Such techniques would be independent — within certain limits — of the number of sugars in the sample, while specific reagents would seldom be required. The method in question must be adaptable to the quantities of the sugars to be determined. Thus,

analytical data of each sugar would be obtained which are not interconnected with the results of the other sugar determinations. For each sugar the analytical results can be tested for reproducibility and percentage recovery.

An example of such an analytical method is the analysis of honey, as given in the Official Methods of Analysis issued by the A.O.A.C. Mono-, di- and polysaccharides are separated on a column of charcoal (and filter aid), and subsequently the amounts of the individual sugars or of the simple sugar mixtures are determined[1].

It has been reported by several authors that fairly complex sugar mixtures could easily be separated by paper chromatography. Similar results could be obtained in an even shorter time by thin-layer chromatography, a method which offers even greater possibilities because materials other than cellulose, *e.g.* silica gel, can be used. Most of the work concerned with paper or thin-layer chromatography deals with the qualitative aspects of sugar analysis, however. Only a few workers have reported on the quantitative determination of sugars after the thin-layer chromatographic separation of a sugar mixture.

In some cases the amounts of sugar were estimated by densitometric evaluation of the sugar spots on the chromatograms. MOCZAR *et al.*[2] reported standard errors of the mean, varying from ± 1.4 to ± 5.4 % for different sugars, and in experiments conducted by WOLFROM *et al.*[3] a coefficient of variation of ± 3 % was mentioned. A few other papers mention extraction from the carrier material and the measurement of the sugar concentration in the extract by colorimetry. According to BANCHER *et al.*[4–6] an accuracy varying from 3 to 5 % was found. SAMUILENKO[7] does not refer to the reproducibility of his determination. A standard deviation of ± 10 % could be calculated from results in a paper by IGLÓY AND MIZSEI[8] dealing with the determination of sorbose.

Some years ago we made a study of the fermentation of lactose and the transitory occurrence of glucose and galactose in fresh cheese. By means of paper chromatography a good separation between the three sugars was obtained, although developing times of 40 h were normal[9]. We now have modified this method to such an extent that fairly complex sugar mixtures can be separated by thin-layer chromatography in a reasonable length of time. As a micromethod for the estimation of reducing sugars[10] was also available, it was thought worth trying to combine the two methods into one for the analysis of complex sugar mixtures.

EXPERIMENTAL

Chromatographic procedure

Cellulose powder MN 300 from Macherey, Nagel & Co (516 Düren, G.F.R.) was used to prepare the plates for thin-layer chromatography. A mixture of 15 g of this powder with 90 ml distilled water, was applied to five plates, 20 × 20 cm, resulting in a layer approximately 0.3 mm thick. In some cases a layer thickness of 0.5 mm may be used.

To check the effectiveness of the separations and to estimate the recovery during quantitative analysis, aqueous solutions containing 1 or 2 g of one or more sugars per 100 ml were prepared. Volumes of 0.005 to 0.010 ml were applied to a base line, at 2 cm from the edge of the plate, with the aid of an Agla micrometer syringe, from which

the solution could be ejected in a stream of small droplets. At a later stage a Hamilton semi-automatic micro-pipette was also used for the purpose.

After the plates had been allowed to dry, they were developed by ascending chromatography in closed glass jars, using a mixture of distilled water with freshly distilled ethyl acetate and pyridine in a ratio of 25:100:35. This mixture has the advantage of being homogeneous over a large temperature range around 20°. To achieve effective separation, the chromatographic run was preferably made three times, the second and third treatment being carried out after the solvent front in the preceding run had reached the top of the cellulose layer and the plate had been dried. After the third run the sugar spots were made visible by spraying the plate with a visualising reagent* and drying for 15 min at a temperature of 100°.

Fig. 1 is a reproduction of a chromatogram showing the separation of a mixture of nine sugars, *viz.* lactose, maltose, sucrose, glucose, galactose, mannose, arabinose, xylose and rhamnose. A series of average R_F values calculated from a number of chromatograms is recorded in Table I.

In order to make quantitative sugar determinations, a reference mixture was applied to the base line at the left- and right-hand side of the plate. The central section of this base line (of 8 to 15 cm length) was used for the mixtures to be analysed. After the chromatogram had been developed, a clean glass plate was placed over this central area, in order to prevent it from being contaminated by the spray liquid which is used to make the reference spots visible. Subsequently a network of lines was traced with a

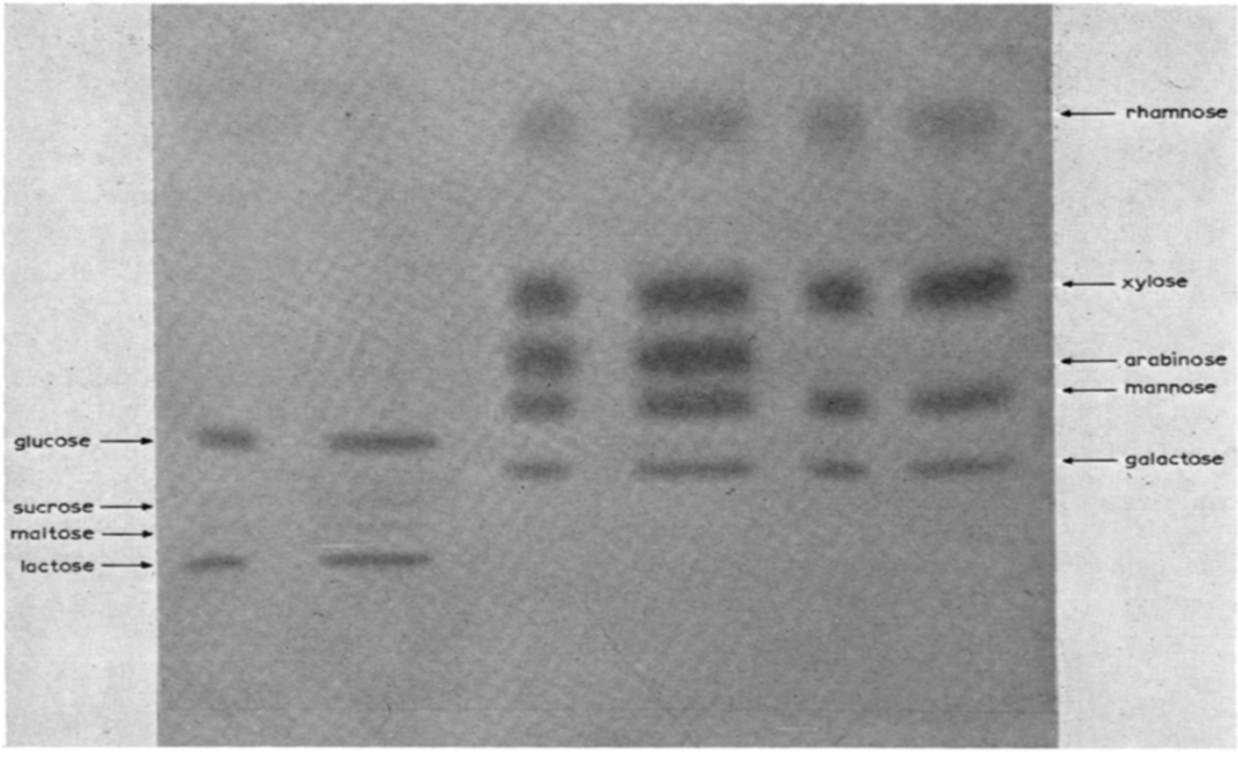


Fig. 1. Thin-layer chromatogram showing the separation of nine sugars.

* Prepared by dissolving 1.3 ml orthophosphoric acid (85%) and 0.93 ml freshly distilled aniline in 100 ml 70% ethanol. This reagent can be kept for several weeks if it is stored in the dark at a temperature of 5°.

soft lead pencil to mark the areas of the individual sugars, as indicated in Fig. 2. Each area was scraped off carefully by means of a small sharpened stainless-steel spatula; the scrapings were collected on a clean piece of parchment paper and transferred to a small centrifuge tube. As a control, an equal area of cellulose layer without sugar was scraped off. The scrapings in the tubes were mixed with exactly 1 ml of water and, after centrifuging for 10 min at 3,000 r.p.m., exactly 0.5 ml was taken for analysis.

TABLE I

AVERAGE R_G VALUES FOR VARIOUS TYPES OF SUGAR

Conditions: layer thickness, 0.3 mm; material, cellulose powder MN 300 (Macherey, Nagel & Co); developing mixture, water–ethyl acetate–pyridine (25:100:35). Three consecutive runs.

Type of sugar	R_G *value* (*glucose* = 100)	*Type of sugar*	R_G *value* (*glucose* = 100)
Lactose	36	glucose	100
Maltose	53	mannose/fructose	115
Sucrose	66	arabinose	131
Galactose	83	xylose	155
Glucose	100	rhamnose	210

Determination of reducing sugars

Some methods based on the reducing properties of these sugars have been adapted to the determination of microgram quantities. A few years ago, such a method was published by GUINN[11], who made use of the reduction of potassium cyanoferrate(III). In our laboratory KOOPS'[10] method is frequently used; he adapted the procedure of CHERONIS AND ZYMARIS[12] for the estimation of small amounts of lactose in cheese. Unfortunately this method does not seem to have found much application in practice: we only found MOCZAR *et al.*[2] mentioning a similar procedure as a useful method in their experiments. These methods are based on the reduction of tetrazolium compounds in an alkaline medium, which leads to the formation of intensely coloured formazans. In the original method of KOOPS *p*-anisyltetrazolium chloride (Fluka) was used as the specific reagent. Some time after publication this reagent was withdrawn from the market by the producer and replaced by another.

Reagents

Stock solution. 1 g Tetrazolium Blue Chloride [Standard-Fluka] for bacteriology (3,3'-dianisole-4,4'-bis-2,5 diphenyl-tetrazolium chloride) is dissolved in 40 to 50 ml lukewarm methanol or ethanol. The mixture is transferred to a 100 ml volumetric flask and diluted to the mark with distilled water.

When kept in the refrigerator, the stock solution can be used for over one year.

Reagent. The tetrazolium reagent is prepared by mixing one part of stock solution with three parts of a 0.3 *M* NaOH solution.

Solvent mixture. Chloroform and ethanol (96 %), freshly distilled, are mixed in a ratio of 5:4.

Determination

Exactly 0.5 ml of a sugar solution (containing 0 to 100 μg sugar) is transferred to a test tube with a Cornwall pipette, and mixed with exactly 0.5 ml of the reagent.

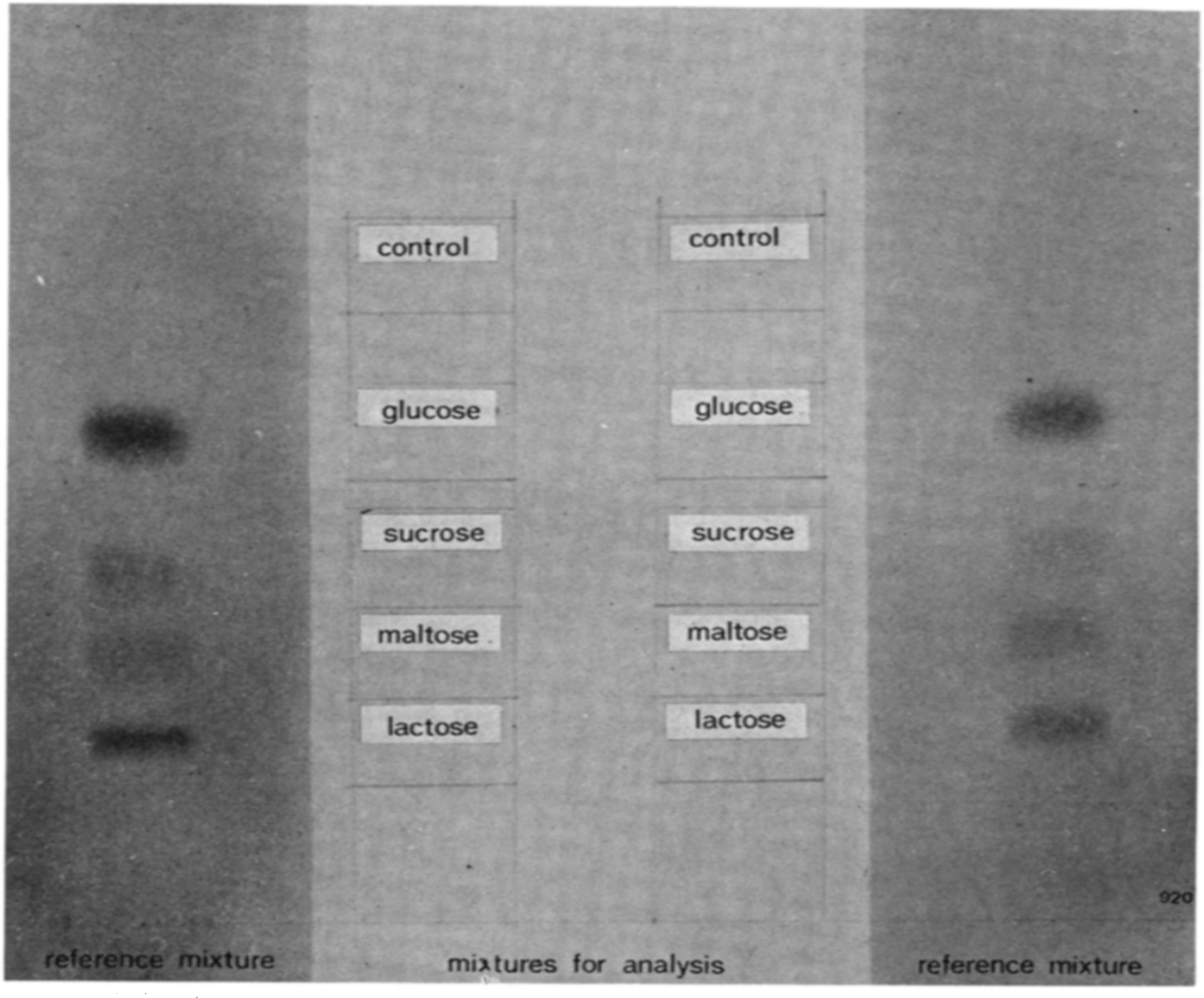


Fig. 2. Thin-layer chromatogram showing the separation of four sugars and the preparation of the plate for quantitative determination.

A glass bead serves as a boiling aid. The contents of the tube are heated to their boiling point on a small flame covered by a copper gauze in approximately 20 sec and kept boiling for 45 sec. Immediately afterwards the tube and its contents are cooled in tap water. The insoluble formazan is dissolved by adding a small amount of the solvent mixture while stirring with a glass rod, and transferred to a 50 ml volumetric flask which is then filled to the mark with the solvent mixture.

The extinction is measured with a spectrophotometer at 615 nm or with a suitable photoelectric colorimeter (*e.g.* Kipp-Engel, filter S 61) in a 1 cm cuvette.

Standard curves for the various sugars

The procedure as described in the preceding section is carried out with 1% aqueous solutions of the various sugars. The relation between the extinction, determined with a Kipp-Engel colorimeter using filter S 61, and the amount of sugar (glucose, mannose and maltose) is presented in Fig. 3.

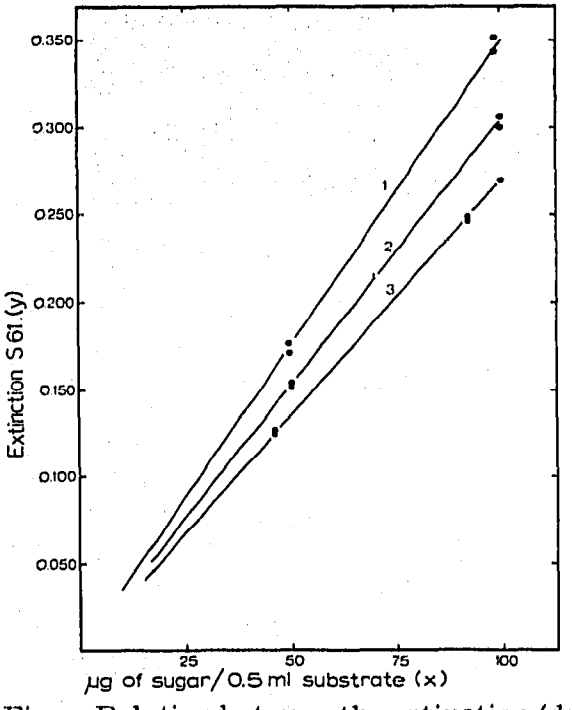
From our estimations the following equations, expressing the relation between extinction and amounts of sugar, could be derived:

$y = 0.00268\ x$ (maltose)
$y = 0.00273\ x$ (lactose)
$y = 0.00284\ x$ (galactose)
$y = 0.00292\ x$ (rhamnose)
$y = 0.00303\ x$ (mannose)
$y = 0.00306\ x$ (xylose)
$y = 0.00312\ x$ (arabinose)
$y = 0.00350\ x$ (glucose)
y = extinction (1 cm cuvette, Kipp-Engel colorimeter, filter S 61).
x = μg of sugar in 0.5 ml, as used for the determination.

It stands to reason that these equations refer only to the conditions in our laboratory. It is advisable for every analyst to determine his own reference lines or equations and to check these data regularly, to allow for any possible changes in equipment or reagents.

Determination of sucrose and other ketoses

To determine the amount of sucrose on a microgram scale, KULKA's[13] method was used, which is based on the reaction of keto-sugars with resorcinol in an acid medium.



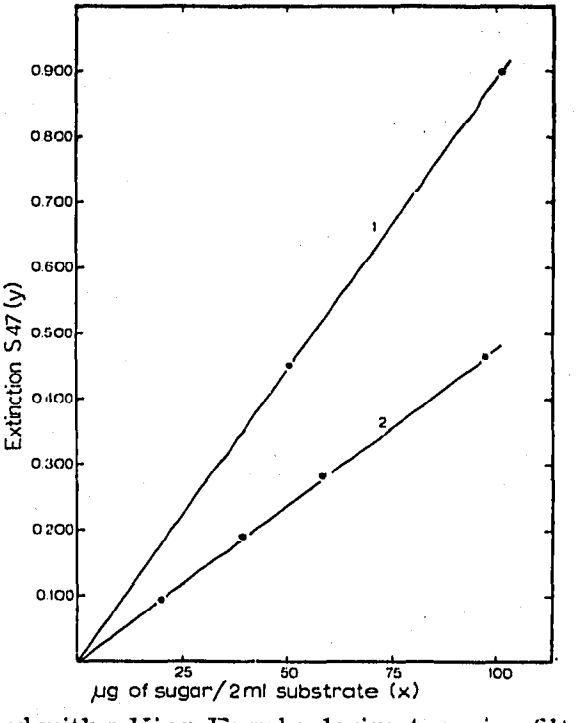


Fig. 3. Relation between the extinction (determined with a Kipp-Engel colorimeter using filter S 61) and the amount of sugar. 1 = Glucose ($y = 0.00350\ x$); 2 = mannose ($y = 0.00303\ x$); 3 = maltose ($y = 0.00268\ x$).

Fig. 4. Relation between the extinction (determined with a Kipp-Engel colorimeter using filter S 47) and the amount of sucrose (2) and fructose (1).

Reagents

Solution A. 50 mg resorcinol dissolved in 100 ml absolute ethanol.

Solution B. 216 mg ferric ammonium sulphate dissolved in 1 l hydrochloric acid (d = 1.18).

Determination

2 ml of a solution containing between 0 and 100 μg sucrose or fructose is mixed in a test tube with exactly 3 ml of both solution A and solution B. The tube is heated for 40 min in a water bath at a temperature of 80°, then cooled in ice water for 2 min and finally brought up to room temperature by immersion in water at 20° for 5 min. The solution is transferred to a 1 cm cuvette and the extinction is measured in a photoelectric colorimeter (Kipp-Engel) using filter S 47, or in a spectrophotometer at a wavelength of 480 nm.

Standard curves for various sugars

Fig. 4 shows the reference curves that express the relation between the extinction and the amounts of sucrose or fructose in 2 ml of substrate.

Quantitative determination of sugars by thin-layer chromatography

Measurement of the sugar solutions

In order to find out the exact amount of sugar deposited on the base line of the thin-layer plate, the volume of the liquid ejected by the Agla micrometer syringe, starting from the 0.005 ml mark, was determined. From the average weight of 16 of these volumes (S_D 1.1 %) and the density of water, an average volume of 0.00490 ml was calculated.

The purity of the sugars is another important factor to be reckoned with in establishing the amount of sugar. However, we think we have found a reasonable approximation by using the reductometric method of Luff-Schoorl. Our preparations of lactose, glucose and sucrose were shown to contain 90.5 %, 98.8 % and 97.5 % of anhydrous sugar, respectively. This means that, by using 0.005 ml drops of a solution containing 2 % of each of these sugars from the Agla micrometer syringe, 89 μg lactose, 97 μg glucose and 96 μg sucrose were deposited on the thin-layer plate.

Estimation of the sugars

Nine portions of 0.005 ml of the mixture of lactose, glucose and sucrose were applied to the base line of a thin-layer plate. At both ends of the base line two reference spots were applied, as described above.

TABLE II

YIELD AND REPRODUCIBILITY OF SUGAR DETERMINATIONS

Amount of sugar deposited on thin-layer plate (μg)	*Amount of sugar in each individual area, according to determination* (μg)									*Recovery*		S_D ±	
	1	2	3	4	5	6	7	8	9	μg	%	μg	%
Lactose 89	89	90	89	90	90	89	96	98	89	91.1	102.3	3.3	3.6
Glucose 97	92	97	94	99	97	99	100	100	97	97.2	100.2	2.7	2.7
Sucrose 96	90	91	94	94	93	93	95	94	90	92.7	96.5	1.9	2.05

After treatment of the plate, lactose and glucose were determined by the Tetrazolium Blue method, and sucrose by the method of KULKA as described above. Table II contains the results of the analyses. The average values and the S_D were calculated from these figures.

DISCUSSION

From the figures in Tables I and II an impression can be gained regarding the possibilities of the method. Its prime merit is that it allows the quantitative analysis of complex sugar mixtures to be made with fair accuracy. Table I shows that the method does not result in a chromatographic separation between mannose and fructose. Nevertheless the amount of either sugar can be established if both are present simultaneously. The total quantity of the two sugars can be determined by the Tetrazolium Blue method, whereas the estimation of fructose by the resorcinol method is not affected by the presence of mannose.

It is important that the ease with which the different manipulations can be carried out permits the volume of liquid delivered by each pipette to be checked regularly, and the yield of each individual sugar to be estimated. Every analyst can easily ascertain the possibilities for each particular case, and test the accuracy and reproducibility of his results.

Finally, the qualitative analysis of sugar mixtures of unknown composition by the method described may facilitate the investigation of such mixtures in a classical way. If only one or two sugars appear to be present, the determinations can often be conducted along conventional lines.

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