ENZYMATIC QUANTITATIVE DETERMINATION OF HEXOSES, SINGLY AND IN MIXTURES WITH THEIR OLIGOSACCHARIDES

BY ROBERT ERNST AND JOSEPH ARDITTI

Department of Developmental and Cell Biology, University of California, Irvine, California, 92664, U.S.A.

(Received 16 August 1971)

SUMMARY

Quantitative determinations of a number of naturally occurring D-hexoses and their oligosaccharides can be accomplished by combinations of enzymatic methods without prior separation of the sugars. These spectrophotometric methods employ readily-available enzymes and commercial reagents. The procedures are particularly useful for the quantitative analysis of fructofuranosides, such as raffinose or stachyose, in the presence of sucrose, glucose and/or fructose; maltose or lactose mixed with galactose and/or glucose; melibiose in mixtures with raffinose, and other combinations.

INTRODUCTION

Many chemical and enzymatic methods have been described for qualitative and quantitative determination of naturally-occurring sugars (Aminoff et al., 1970; Holligan, 1971; Holligan and Drew, 1971; Lewis and Smith, 1967). The latest employ gas-liquid chromatography (GLC). Since GLC is not yet widely available to botanists, this paper presents modified enzymatic methods for the rapid quantitative determination of mixtures of oligosaccharides and their component monosaccharides. These methods, which employ readily-available enzymes and commercial reagents, have been used in studies of carbohydrate physiology of orchid seedlings (Ernst, 1967; Ernst, Arditti and Healey, 1972).

MATERIALS AND METHODS

Sugars. Melibiose and stachyose were obtained from Calbiochem, Los Angeles, California and other sugars from Pfannstiehl Laboratories, Inc., Waukegan, Illinois. Only D-sugars were employed. They were dissolved either in distilled water and kept frozen until used or in methanol—water (7:3, v/v) and stored at room temperature.

Enzymes. Fructofuranosidase (invertase) melibiase free, 116 U/mg, derived from bakers' yeast, Grade IV was obtained from Sigma Chemical Company, St Louis, Mo. An alternative grade, purchased from Calbiochem Wallenstein invertase activity 873 (Meister, 1965), gave comparable results.

Yeast α-glucosidase (maltase) suspension, 2 mg protein/ml (20 U/ml) and galactose dehydrogenase (Galactose UV-test, TC-GA 15921 TGAN) were obtained from Boehringer Mannheim Corporation (BMC), San Francisco, California.

β-Galactosidase (lactase), derived from Escherichia coli, 5.5 mg protein/ml (398 U/mg)
in saturated ammonium sulphate and a prepared galactose oxidase reagent, Galactostat, were purchased from Worthington Biochemical Corporation, Freehold, New Jersey.

Phosphoglucone isomerase (PGI from yeast) in 2.4 M ammonium sulphate, 4 mg protein/ml, 1820 U/ml at 36° C was obtained from Calbiochem.

Thin layer chromatography (TLC). Method A. Borated Silica Gel G plates, developed with methyl ethyl ketone : acetic acid : methanol (6 : 2 : 2, v/v/v), were used for separation and identification (Jeffrey, Arditti and Ernst, 1969). Maltose and the products of its hydrolysis were detected with aniline-diphenyl amine (Bailey and Bourne, 1960) followed by naphthoresorcinol-H₂SO₄ (Lato et al., 1968). This system detected sugars which could not be detected with aniline-diphenyl amine alone. Method B employs identical plates and visualizing techniques, but uses chloroform : acetic acid : water (5 : 4.3 : 0.7, v/v/v) as the developer.

Spectrophotometry. Spectrophotometric measurements were conducted in a Beckman DB-G instrument at 340 nm and 1 cm-light path. Mixing was by several gentle inversions. The cuvette chamber was maintained at 25° C to prevent bubble formation. Calculations were based on the micromolar extinction coefficient of 6.22 cm² for NADH₂ and NADPH₂ (Horecker and Kornberg, 1948).

Replication. All hydrolysates and determinations were replicated four times. The results reported are averages of these.

ASSAY OF INDIVIDUAL SUGARS AND MIXTURES

Glucose

Glucose was determined by the hexokinase/glucose-6-phosphate dehydrogenase method (Cori and Larner, 1951; Keller, 1965; Peterson and Young, 1968; Stein, 1965) with a commercial reagent, Glucose Stat-Pack (Calbiochem), referred to as 'glucose reagent'. The reaction sequence is as follows:

(hexokinase)

\[
\text{glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate (G-6-P)} + \text{ADP}
\]

(G-6-P dehydrogenase)

\[
\text{G-6-P} + \text{NADP} \rightarrow \text{NADPH}_2 + \text{6-phosphogluconate.}
\]

Fructose

Fructose was determined by converting it into glucose-6-phosphate with PGI (Schmidt, 1961) added to the glucose reagent. This enzyme mixture is referred to as the 'fructose reagent'. Thus, following glucose determination, 5 µl PGI (about 9 U) were added to each 3 ml of the glucose assay reagent containing the sugar sample. Stable absorbance values and quantitative recoveries of known amounts of sample were obtained after 15 minutes at 25° C (Fig. 1) when fructose was alone or in mixture with glucose, sucrose or raffinose.

Galactose

Measurements of galactose were by the galactose dehydrogenase method (Finch et al., 1969; Rommel et al., 1968; Wallenfels and Kurz, 1962) using Galactose UV-test.

Raffinose

Hydrolysis of raffinose was carried out under conditions recommended for inversion of sucrose (Bergmeyer and Klotzsch, 1965). Aqueous solutions of the trisaccharide,
Enzymatic determination of sugars

EXPLANATION OF FIGURES

Fig. 1–5. Solid lines show theoretical values (lower abscissa); dots represent µ moles sugar recovered. Broken line is reaction rate in minutes (upper abscissa).

Fig. 1. Fructose. Points representing recovery were obtained after 15-minutes incubation. Reaction rates were determined with 0.01 M solutions.

Fig. 2. Raffinose. Points representing recovery were obtained following 1-hour incubation with invertase. Reaction rates were determined with 0.1 M solutions.

Fig. 3. Stachyose. Points representing recovery were obtained following 1-hour incubation with invertase. Reaction rates were determined with 0.1 M solutions.

Fig. 4. Maltose. Points representing recovery were obtained following 1-hour incubation with α-glucosidase. Reaction rates were determined with 0.01 M solutions.

Fig. 5. Lactose. Points representing recovery were obtained following 75-minutes incubation with β-galactosidase in glucose reagent. Reaction rates were determined with 10 µl of 0.025 M solutions in 3 ml glucose reagent.

Fig. 6. Melibiose (▲) and raffinose (●). Points representing recovery were obtained following 1-hour incubation with Galactostat reagent.
ranging in molarity from 0.025 to 0.1, were incubated with 5.2 times their volumes of 0.1 M sodium acetate buffer (pH 4.6) containing 0.77 mg/ml invertase, at 37° C, for 1 hour. Although melibiose samples (0.1 M) were included to check for possible melibiase activity, the hydrolysed samples yielded only melibiose and fructose by TLC and glucose was also shown to be absent by enzymatic analysis. Hydrolysis was complete in 20 minutes and recovery corresponded to 96.4-98.7% (Fig. 2).

The quantity of raffinose (measured as fructose) contained in 20 µl of hydrolysed sample added to the fructose reagent is derived as follows, where O.D. Fru is observed change in optical density, total volume of reagents in cuvette = 3 ml and micromolar extinction coefficient for NADPH₂ = 6.22:

\[ \text{micromoles NADPH}_2 \text{ formed} = \frac{3 \times \text{O.D. Fru}}{6.22} = \frac{\text{O.D. Fru}}{2.073}; \]

\[ \therefore \text{micromoles NADPH}_2 \text{ per ml} = \frac{\text{O.D. Fru}}{2.073} \times \frac{1}{0.02}. \]

By correcting for the dilution factor, 6.2, occurring during the hydrolysis step and allowing for the relationship between the molecular weights of raffinose and fructose, raffinose content (%) in the sample prior to hydrolysis can be calculated. Based on a 20-µl sample in 3 ml, raffinose content (%) = \[ \frac{0.4345 \times 6.2 \times \text{O.D.} \times 504.43}{180.16} \]

where 0.4345 is a factor converting O.D. into g/100 ml fructose as follows:

\[ \frac{180.16}{2.073} \times \frac{1}{0.02} = 4345 \ \mu g/ml = 0.4345 \ g/100 \ ml (\%). \]

**Sucrose/raffinose mixtures**

Hydrolysis of sucrose and raffinose mixtures under the conditions outlined above will result in a hydrolysate containing glucose (from sucrose) and fructose (from sucrose and raffinose). Fructose values in excess of double the optical density obtained for glucose represent hydrolysed raffinose. Therefore with O.D. Glc = observed change in optical density with glucose reagent, and other factors as above, the following calculations apply:

\[ \text{micromoles sucrose} = \frac{\text{O.D. Glc}}{2.073}; \]

\[ \therefore \text{per cent sucrose in sample prior to hydrolysis} = \frac{0.4345 \times 6.2 \times \text{O.D. Glc} \times 342.3}{180.16}, \]

and, since subtraction of O.D. Glc from O.D. Fru gives fructose derived from raffinose only, as the glucose and fructose content of sucrose are equivalent,

\[ \text{micromoles raffinose} = \frac{\text{O.D. Fru} - \text{O.D. Glc}}{2.073}; \]

\[ \therefore \text{per cent raffinose in sample prior to hydrolysis} = \frac{0.4345 \times 6.2 \times (\text{O.D. Fru} - \text{O.D. Glc}) \times 504.43}{180.16}. \]
Enzymatic determination of sugars

Aqueous and methanol-water solutions of 0.025–0.075 M sucrose and 0.075–0.025 M raffinose, as well as 0.1 M sucrose only and 0.1 M raffinose only, were hydrolysed as for raffinose. TLC of samples taken after 1 hour showed absence of sucrose and raffinose and presence of melibiose, glucose and fructose. The recovery of sucrose was 97.2–101.7% and that for raffinose 95.7–98.7% when analysed with the glucose and fructose reagents. Presence of methanol had no adverse effect on this method (but see maltase assay below).

Stachyose

Hydrolysis of stachyose was carried out as for raffinose. Hydrolysis was slower than for raffinose and the optical density obtained with the fructose reagent levelled off after about 40 minutes (Fig. 3). Recoveries ranged from 93.5–96.7% (Fig. 3).

Sucrose/stachyose mixtures

Hydrolysis of mixed aqueous sucrose and stachyose solutions was carried out as for sucrose/raffinose mixtures. TLC of samples taken after hydrolysis showed absence of sucrose and stachyose, but the presence of manninotriose, glucose and fructose. In assays of mixtures in various proportions as for sucrose and raffinose, recovery ranged from 98.7 to 104.6% for sucrose and 94.2 to 95.7% for stachyose. Calculations for sucrose are those given for sucrose/raffinose mixtures, whereas stachyose recovery was calculated as follows:

$$\text{micromoles stachyose} = \frac{\text{O.D. Fru} - \text{O.D. Glc}}{2.073}$$

and, per cent stachyose in sample prior to hydrolysis

$$= \frac{0.4345 \times 6.2 \times (\text{O.D. Fru} - \text{O.D. Glc}) \times 666.6}{180.16}$$.

Maltose

Maltose was measured as glucose following hydrolysis. Aqueous maltose solutions (0.025–0.1 M) and maltose dissolved in methanol–water were hydrolysed with maltase as follows: 50 µl maltose solution, 400 µl 0.1 M sodium acetate buffer (pH 6.0), and 250 µl maltase suspension (20 U) were incubated at 37°C for 1 hour. Samples of glucose only (0.1 M) dissolved in water or methanol–water were included as controls. Unreacted maltose samples showed the presence of 0.09% glucose (by enzymatic procedure) as impurity, and minor amounts of a higher oligosaccharide (probably maltotriose) by TLC (Table 1). On completion of hydrolysis, TLC of aqueous solutions showed the presence of glucose only. With methanol–water solutions, a second spot appeared with a higher $R_F$ value than glucose. Since α-methyl-D-glucoside (meGlc) can be synthesized by action of maltase from yeast where methanol is present (Bourquelot, Hérissé and Bridel, 1913) we tested for its presence chromatographically. The data show that meGlc was present in the hydrolysate (Table 1). Hydrolysis of aqueous maltose solutions resulted in stable optical density values after 40 minutes (Fig. 4). Methanol–water solutions gave a recovery of only 67.5% with maltose and of 69.2% with glucose.

Lactose

Hydrolysis and estimation of lactose was by an adaptation of a previous method
312 ROBERT ERNST AND JOSEPH ARDITTI

(Reithel and Venkataraman, 1956). To each 3 ml of glucose reagent were added 10 µl of 0.0125–0.25 M aqueous lactose solutions, free of glucose. Following the addition of 44 units of b-galactosidase (lactase) in 20 µl, lactose was hydrolysed and measured as glucose over a period of 75 minutes. Recoveries ranged between 96.5 and 100.3% (Fig. 5). Lactose content (%) in the sample prior to hydrolysis

\[
\frac{0.869 \times \text{O.D. Glc} \times 342.3}{180.16}
\]

In this as well as all previous examples involving oligosaccharides, the presence of glucose and/or fructose (free or as phosphate) must be determined prior to hydrolysis. Calculations for oligosaccharides should be corrected accordingly.

Table 1. Thin layer chromatography of maltose, glucose and derivatives

| Carbohydrate          | Chromatography Method A | Chromatography Method B |
|-----------------------|-------------------------|-------------------------|
|                       | RF                      | Colour                   | RF                      | Colour                   |
| Glucose               | 0.57                    | blue                     | 0.25                    | blue                     |
| Maltose               | 0.43                    | blue                     | 0.14                    | blue                     |
| Maltotriose (?)       | 0.19                    | blue                     | 0.07                    | blue                     |
| a-Methyl-D-glucoside  | 0.68                    | reddish-blue             | 0.41                    | reddish-blue             |
| Unknown               | 0.68                    | reddish-blue             | 0.41                    | reddish-blue             |

**Melibiose**

Galactose oxidase oxidizes terminal galactosyl units in oligosaccharides (Avigad et al., 1962). Employing Galactostat, we obtained standard curves with melibiose, lactose, raffinose and stachyose. These were of different slopes at equivalent concentrations. Hence melibiose can be measured directly with the Galactostat reagent if other galactosyl containing sugars or galactose are absent. The same is true for raffinose stachyose and lactose.

**Melibiose/raffinose mixtures**

Melibiose present in mixtures with raffinose was determined as follows. Raffinose was hydrolysed with invertase, yielding equimolar amounts of fructose and melibiose. Total melibiose was then determined with the Galactostat reagent. Correction was made for melibiose derived from raffinose on the basis of fructose present in the hydrolysate (see raffinose determination). Thus, 0.1 M raffinose treated with invertase for 1 hour as above, was diluted with water to a concentration of 2.5 mM. Aliquots of 25–150 µl from this solution were each added to 1.5 ml Galactostat reagent and diluted to 3 ml with water. After incubation for 1 hour at 37° C, the reaction was stopped with 0.2 ml 0.5 M EDTA-Na4 (Sempere, Gancedo and Asensio, 1965) and the optical density read at 425 nm. Melibiose treated in like manner was used as control (Fig. 6).

**DISCUSSION**

Various techniques involving the enzymes glucose oxidase, invertase and melibiase in conjunction with determination of carbohydrate by reducing power, anthrone, manometry or optical rotation have been previously developed for assay of glucose, fructose, sucrose, melibiose and raffinose in various combinations (Eddy and Mapson, 1951; Potter and Williams, 1958; Böttger and Steinmetzer, 1959; Johnson et al., 1964 and De Whalley, 1965). Problems include: (i) the necessity to remove interfering substances,
Enzymatic determination of sugars

e.g. ascorbic acid with ascorbic acid oxidase; (2) colour development by the anthrone
method, which requires the inclusion of standard solutions and depends on temperature
and duration of reaction; (3) the determination of optical rotation which requires special
apparatus, and measurements may be low where large amounts of optically active species
are present (Bergmeyer and Klotzsch, 1965); and (4) melibiase is not commercially
available.

The methods described here for these five sugars have important advantages over these
techniques. They are simpler, accurate and, because of the enzymes involved, entirely
specific. Except for melibiase, they do not require a calibration curve for each analysis
and utilize equipment generally found in biological laboratories. Raffinose can also be
measured with Galactostat but this reagent will also react with galactose and other
galactosyl-glycosides. The fructose reagent can also be used to determine stachyose
after treatment with invertase (Fig. 3).

The method of quantitative analysis of sucrose/raffinose or sucrose/stachyose mixtures
should also be suitable for the determination of other galactosyl sucroses with a fructo-
furanosyl group detachable by invertase, such as verbascose (Bourquelot and Bridel,
1910) and ajugose. A difficulty may be the decreasing rate of hydrolysis with increasing
molecular weight (Adams, Richtmyer and Hudson, 1943; Courtois, 1958). Although
mixtures of glucose, fructose, sucrose and raffinose occur commonly in plant materials,
stachyose or verbascose are less widespread (Bailey, 1965) but such mixtures could be
measured quantitatively without separation by our methods.

Pan, Nicholson and Kolachov (1953) reported the determination of mixtures of
glucose, maltose and other oligosaccharides fermentable by yeast, but substantial
differences in the degree of fermentation, based upon type of yeast employed, have been
shown by Kempf and Lindemann (1954) who could not confirm the quantitative
fermentation of glucose in 90 minutes and that of maltose in 150 minutes with commercial
baker’s yeast. Quantitative analysis of glucose and maltose in mixture is readily
achieved by our method. Although maltotriose would not be distinguished from maltose,
this is not an important shortcoming because maltotriose does not occur naturally in
plant tissues (Pazur, 1970).

Lactose has been detected in such plant tissues as the fruit of Achras sapota (Ven-
kataraman and Reithel, 1958) and pollen of Forsythia (Kuhn and Löw, 1949). Chroma-
tographically purified α-galactosidase was employed by us to avoid the interferences
encountered with less pure preparations. The Galactostat reagent can be used for lactose
determination only if galactose or other sugars with a terminal galactosyl group are
absent. The Galactose UV-test reagent permits the measurement of this sugar in the
presence of other galactosides. However, analogues of D-galactose such as L-arabinose
and D-fucose will also react (Finch et al., 1969; Hu and Grant, 1968).

In addition to the mixtures we have analysed, these methods may be employed for a
variety of other combinations.

ACKNOWLEDGMENTS

This research was supported in part by grants from the American Orchid Society
Fund for Research and Education, the National Science Foundation (GB 13417), the
Orchid Digest Corporation, the Textilana Corporation, and the Office of Naval Research,
Contract NR-108-796. We thank Drs E. J. Miller and M. H. Fisch for reading and
commenting on the manuscript and Brigitta H. Flick for technical assistance.
REFERENCES

ADAMS, M., RICHTMYER, N. K. & HUDSON, C. S. (1943). Some enzymes present in highly purified invertase preparations: a contribution to the study of fructofuranosidas, galactosidas, glucosidas and mannosidas. J. Am. chem. Soc., 65, 1369.

AMNÖFF, D., BINKLEY, W. W., SCHAFER, R. & MOWRY, R. W. (1970). Analytical methods for carbohydrates. In: The Carbohydrates. Chemistry and Biochemistry, 2nd edn, Vol. II B (Ed. by W. Pigman and D. Horton), p. 739. Academic Press, New York.

AVIGAD, G., AMARAL, D. ASENSIO, C. & HORTECKER, B. L. (1962). The D-galactose oxidase of Polyporus circinatus. J. biol. Chem., 237, 2736.

BAILEY, R. W. (1965). Oligosaccharides, p. 111. Pergamon Press, Oxford.

BAILEY, R. W. & BOURNE, E. J. (1960). Colour reactions given by sugars and diphenylamine-aniline spray reagents on paper chromatograms. J. Chromatog., 4, 206.

BERGMeyer, H. U. & KLOTZSCHE, H. (1965). Sucrose. In: Methods of Enzymatic Analysis (Ed. by H. U. Bergmeyer), p. 99. Academic Press, New York.

BÖTTGER, S. & STEINMETZ, W. (1959). Die selektive enzymatische Bestimmung von Zuckern in Zuckerfabrikprodukten. Z. Zuckerind. Cal. Repub. Böhm. Mähr., 9, 16.

BOURJUQELT, E. & BRIEDE, M. (1910). Sur un sucres nouveau, le verbascose, retiré de la racine de Molène. C.r. hebld. Stanc. Acad. Sci., Paris, 151, 766.

BOURJUQELT, E., HÉRISSEY, H. & BRIEDE, M. (1913). Synthèse biochimique de glucosides d’alcools (glucosides a l’aide de la glucosidase a méthylglucoside a. Destruction de glucosidase a en milieu fortement alcoolique. C.r. hebld. Stanc. Acad. Sci., Paris, 156, 501.

COTTI, G. T. & LARNER, J. (1951). Action of amylo-1,6-glucosidase and phosphorylase on glycogen and amylopectin. J. biol. Chem., 188, 17.

COUTO, J. E. (1958). Les oligosaccharides non réducteurs contenant du galactose. In: Proceedings of the Fourth International Congress of Biochemistry, Vienna. Symposium 1. Carbohydrate chemistry of substances of biological interest (Ed. by M. L. Wolfe), p. 151. Pergamon Press, London.

DE WHALLEY, H. C. S. (1965). Raffinose. In: Methods of Enzymatic Analysis. (Ed. by H. U. Bergmeyer), p. 93. Academic Press, New York.

EDDY, P. & MAPSON, L. W. (1951). Some factors affecting anthocyanin synthesis in cross seedlings. Biochem. J., 49, 604.

ERNST, R. (1967). Effect of carbohydrate selection on the growth rate of freshly germinated Phalaenopsis and Dendrobium seed. Bull. Am. Orchid Soc., 36, 1688.

ERNST, R., ARDITTI, J. & HEALEY P. L. (1972). Carbohydrate physiology of orchid seedlings. II. Hydrolysis and effects of oligosaccharides. Amer. J. Bot., 58, 827.

FINCH, P. R., YUEN, R., SCHACHTER, H. & MOSCARIELLO, M. A. (1969). Enzymic methods for the micro- or submicro-assay of D-mannose, D-glucose, D-galactose, and L-fucose from acid hydrolysates of glycoproteins. Anayt. Biochem., 31, 269.

HOLLIGAN, P. M. (1971). Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. I. A review of techniques used for the separation, identification and estimation of carbohydrates by gas-liquid chromatography. New Phytol., 70, 279.

HOLLIGAN, P. M. & DREW, E. A. (1971). Routine analysis by gas-liquid chromatography of solublc carbohydrates in extracts of plant tissues. II. Qualitative analysis of standard carbohydrates, and the separation and estimation of soluble sugars and polyols from a variety of plant tissues. New Phytol., 70, 271.

HORTECKER, B. L. & KORNBERG, A. (1948). The extinction coefficients of the reduced band of pyridine nucleotides. J. biol. Chem., 175, 385.

HU, A. S. L. & GRANT, S. (1968). Enzymic determination of D-galactose, D-arabinose, and their homologs. Anal. Biochem., 25, 221.

JEFFREY, D. C., ARDITTI, J. & ERSNT, R. (1969). Determination of di-, tri-, and tetrasaccharides in mixtures with their component monosaccharides by thin layer chromatography. J. Chromatog., 31, 475.

JOHNSON, G., LAMBER, C., JOHNSON, D. K. & SUNDERWIRTH, S. G. (1964). Plant tissue analysis. Colorimetric determination of glucose, fructose, and sucrose in plant material using a combination of enzymatic and chemical methods. J. agric. Fd Chem., 12, 210.

Keller, D. M. (1965). An enzymatic method for glucose quantitation in normal urine. Clin. Chem., 11, 472.

KEMPPE, W. & LANDEMANN, E. (1954). Erfahrungen mit einer neuer verbesserten Hefe-Abba u m ethode zur Analyse einer Mischung von Glukose, Maltose und vergärbaren Oligosacchariden nach S. C. PAN, L. W. Nicholson und Paul Kolachov. Stärke, 6, 217.

KoNN, R. & LÖW, I. (1949) Über ein Vorkommen von Milchzucker im Pflanzenreich. Chem. Ber., 82, 479.

LIND, M., BRUNELL, B., CRUFTIN, G. & MEZZETT, T. (1968). Analysis of carbohydrates in biological fluids by means of thin layer chromatography. J. Chromatog., 36, 101.

LEWIS, D. H. & SMITH, D. C. (1967). Sugar alcohols (polyols) in fungi and green plants. II. Methods of detection and quantitative estimation in plant extracts. New Phytol., 66, 185.

MEYER, H. (1966). Yeast invertase—an elusive, but useful enzyme. Wallerstein Labs Commun., 28, 7.

PAN, S. C., NICHOLSON, L. W. & KOLACHOV, P. (1953). Analysis of a mixture of glucose, maltose, and fermentable oligosaccharides. Improved yeast fermentation method. Anal. Chem., 25, 231.

PARK, J. H. (1970). Oligosaccharides. VII. Important oligosaccharides of biological origin. B. Trisaccharides. In: The Carbohydrates. Chemistry and Biochemistry, Volume II A (Ed. by W. Pigman and D. Horton), p. 125. Academic Press, New York.

PETerson, I. J. & YOUNG, D. S. (1968). Evaluation of the hexokinase/glucose-6-phosphate dehydrogenase method of determination of glucose in urine. Anal. Biochem., 33, 207.
Enzymatic determination of sugars

Potter, E. F. & Williams, K. T. (1958). A comparison of a glucose oxidase method with an official method for the determination of fructose. J. Ass. off. agric. Chem., 41, 681.

Reithel, F. J. & Venkataraman, R. (1956). Lactose in Sapotaceae. Science, N. Y., 123, 1083.

Rommel, K., Bernt, E., Schmitz, F. & Grimmel, K. (1968). Enzymatische Galaktosebestimmung im Blut und oraler Galaktose-Toleranztest. Klin. Wschr., 46, 536.

Schmidt, F. H. (1961). Die enzymatische Bestimmung von Glucose und Fructose nebeneinander. Klin. Wschr., 39, 1244.

Sempere, J. M., Gancedo, C. & Asensio, C. (1965). Determination of galactosamine and N-acetylgalactosamine in the presence of other hexosamines with galactose oxidase. Analyt. Biochem., 112, 509.

Slein, M. W. (1965). D-glucose determination with hexokinase and glucose-6-phosphate dehydrogenase. In: Methods of Enzymatic Analysis (Ed. by H. U. Bergmeyer), p. 117. Academic Press, New York.

Wallenfels, K. & Kure, G. (1964). Über die Spezifität der Galaktosedehydrogenase aus Pseudomonas saccharophila und deren Anwendung als analytisches Hilfsmittel. Biochem. Z., 335, 559.

Venkataraman, R. & Reithel, F. L. (1958). Carbohydrates of the Sapotaceae. I. The origin of lactose in A. sapota. Archs. Biochem., 75, 443.