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Note

Comparison of spectrodensitometric and colorimetric methods for quantifying starch hydrolysis products separated by thin-layer chromatography

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Indirect and direct methods have been used to quantify carbohydrates from thin-layer chromatograms[1]. Maltooligosaccharides and simple sugars have been quantified indirectly by measuring the color[2–5] or radioactivity[6] of the extracted spots. Raadsveld and Klomp[4] described the difficulties encountered with indirect quantitative determinations of complex sugar mixtures in solution. They developed elution techniques and colorimetric methods for measuring 9–10 sugars per lane on thin-layer chromatographic (TLC) plates. Elution techniques for removing sugars from various types of gels have been described[7,8]. Scott[7] reported that sugar losses after elution were 4.8% for chromatographed spots and 1.9% for unchromatographed spots. These losses were attributed partly to the spot becoming irreversibly bound to the gel as it dried during spot application. Other sources of error such as spotting, plate development, treatment of the spot, and measurement have been identified[9].

Direct quantification by densitometry has been a rapid method for the analysis of maltooligosaccharides[1,10,11]. Several factors significantly affect the precision of the densitometric method[10–13]. Single-beam instruments are less precise than dual-beam instruments[10,11]. Dallas[12] concluded that uniform layer thickness, time of development, and correct positioning of the scanning light beam over the chromatogram were the most important of all the factors considered. Welch and Martin[13] reported that the preparation of standard curves for each plate helped to decrease error.

A reliable and rapid quantitative method is needed to assess relative differences in the enzymatic susceptibility of modified and unmodified starches. This paper reports on a comparison of a colorimetric method with a densitometric method for quantifying starch hydrolysis products separated on thin-layer chromatograms.

MATERIALS AND METHODS

Preparation of hydrolyzates

Hydroxypropyl distarch phosphate from tapioca (Stein, Hall & Co., New York, N.Y., U.S.A.) was selected as the substrate for hydrolysis with hog pancreatic α-amylase (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.). The hydrolyzate was prepared by the method of Conway and Hood[14] and stored at −18° for one week, the duration of the analyses.

Thin-layer chromatography

The hydrolysis products were separated on scored, silica gel G (type 60; EM Laboratories, Elmsford, N.Y., U.S.A.) plates made up with 0.02 *M* sodium acetate. For the colorimetric method, six 20-μl aliquots of the starch hydrolyzate and 1, 2 and 3 μl of a standard solution of maltotriose and maltose (1 μg of each sugar per μl) were each applied with a Hamilton 10-μl syringe (Hamilton, Whittier, Calif., U.S.A.) in 1 cm-bands on nine separate lanes (2 cm wide) on a plate with a gel thickness of 500 μm. For the densitometric method, five 3-μl amounts of the hydrolyzates and 1, 5 and 10 μl of a standard solution of maltotriose and maltose (1 μg of each sugar per μl) were spotted on every other lane (1 cm wide) of a plate with a gel thickness of 250 μm. Both plates were run in *n*-propanol–water (7:1, v/v) up to the 14-cm mark for three successive developments.

Colorimetric quantification

The colorimetric method of Ponte *et al.*[15] for quantifying reducing sugars separated by TLC was used as an indirect measurement of the hydrolysis products. The bands of sugars were visualized in an iodine tank after the plate had been heated for 10 min in a 75° forced-air oven. Within 15 min the bands were bright yellow. The plate was removed from the tank, and the bands were circled immediately with the point of a needle. As soon as the yellow color disappeared, each band was scraped from the plate into 15-ml centrifuge tubes. Five milliliters of diphenylamine reagent (10 ml isopropanol, 40 ml glacial acetic acid, 0.50 g diphenylamine, 50 ml concentrated HCl) was added to each tube. Reagent blanks were prepared from regions of the plate that contained no sugar. The tubes were set in a boiling water bath for 30 min, followed by rapid cooling to room temperature in a cold water bath (5°). Each tube was centrifuged at 750 $\times$ *g* for 10 min. The supernatants were decanted and their absorbances measured at 630 nm with a spectrophotometer (Beckman Acta CII, Wakefield, Mass., U.S.A.) which had been zeroed with the reagent blanks. The absorbance of each band was converted to micrograms by using the maltotriose standard curve prepared from the same plate. The maltotriose standard curve could be used for all samples because equal amounts of maltose, maltotriose and maltotetraose had similar absorbance measurements with the diphenylamine reagent. Standards for higher molecular weight maltooligosaccharides were not available. The precision of the method was determined by calculating the coefficient of variability.

Densitometric quantification

Hydrolysis products were quantified directly on TLC plates after color development with diphenylamine–aniline–phosphoric acid (DAP) spray reagent[16] using the double-beam spectrodensitometer Model SD 3000 (Schoeffel, Westwood, N.J., U.S.A.) and a 10-in. strip chart recorder. The lanes on the TLC plate were scanned at 630 nm with a slit width of 0.5 mm. Peak areas on the densitometer scan were computed by multiplying the peak height by the width at half height. The areas were converted to weights of the various sugars using the maltose standard curve run on the same plate. The mean, standard deviation and coefficient of variability were calculated for each of the hydrolysis products.

In order to evaluate the precision of direct quantitative analysis by densitometry, the sources of variability inherent to the method were determined. Four major

sources of error were: (1) the instrument itself, (2) poor lane–beam alignment, (3) errors in spotting and (4) plate-to-plate variation. The error due to the densitometer was assessed by scanning the same spot five times without moving the plate from the scan plate frame. The same procedure was repeated to determine the error due to the lane–beam alignment except that the plate was taken off and put back on the frame between scans. The error in spotting was estimated by scanning five spots of equal volume on the same plate. Plate-to-plate error was calculated by scanning five spots of equal volume on five different plates. The coefficient of variability was calculated for each source of error. In addition, different sample loads were evaluated by scanning five spots each of 1, 5, and 10 μg of maltose and maltotriose and by calculating the coefficient of variability for each quantity.

RESULTS AND DISCUSSION

The results from the two methods used to quantify the hydrolysis products of the modified tapioca starch are compared in Table I. Both methods gave comparable mean values for the G_2 and G_4 oligosaccharides. The mean value for G_3 was somewhat higher by colorimetric than by densitometric measurement. The standard deviation values were somewhat higher for G_3 and G_4 by colorimetric measurement causing the coefficient of variability to be greater for the colorimetric method than for the densitometric method.

TABLE I

DENSITOMETRIC AND COLORIMETRIC QUANTIFICATION OF OLIGOSACCHARIDES IN MODIFIED STARCH HYDROLYZATES

S.D. = standard deviation. C.V. = coefficient of variability = 100 × S.D./mean.

Oligosaccharide	*Densitometry**		*Colorimetry***	
	Mean ± S.D. (mg per 100 mg starch)	*C.V. (%)*	*Mean ± S.D. (mg per 100 mg starch)*	*C.V. (%)*
G_2	17.8 ± 3.5	19.7	18.0 ± 3.0	16.7
G_3	24.7 ± 3.9	15.8	32.2 ± 4.9	22.1
G_4	12.7 ± 2.4	18.9	13.8 ± 3.7	26.8

* $n = 5$, 3 μl of hydrolyzate was spotted.
** $n = 6$, 20 μl of hydrolyzate was spotted.

Although the two methods seemed to be comparable in precision, we found that the densitometric method had three advantages over the colorimetric method. First, the densitometer was more sensitive to smaller amounts of products than was the colorimetric method. For example, the lower limit of sensitivity was 1 μg for densitometry, but 10 μg for colorimetry. This was an advantage because time was saved in spotting lesser amounts of hydrolyzate. Second, the entire amount of time to do the densitometric analysis was about 30% less than with the colorimetric method. Finally, the densitometric procedure was easier to perform on a large number of samples at one time. With a single scan of one lane, all the products in one hydrolyzate were measured, whereas in the colorimetric method, each product had to be quantified in-

dividually. In addition, the latter method had a rather unpleasant aspect to it; the diphenylamine reagent contained large amounts of acetic acid and hydrochloric acid which were irritating to the eyes and skin.

TABLE II

SOURCES OF VARIATION IN DENSITOMETRY

All measurements made on 10-μg spots of maltose.

Source of variation	*Measurements*	*Coefficient of variability (%)*
Plate-to-plate	5 spots, 5 plates	21.7
Spotting volume	5 spots, 1 plate	10.3
Lane–beam alignment	1 spot, 5 scans	0.6
Densitometer	1 spot, 5 scans	2.8

All measurements made on 10-μg spots of maltose.

In order to ascertain what caused the variability in the densitometric measurements, five contributing factors were evaluated statistically. Four of them were determined by making five replicate measurements of 10 μg spots of maltose (Table II). The two major causes of error were the variation from plate-to-plate and the inability to spot the same volume of sample in different lanes. The error due to lane–beam alignment and due to the densitometer itself were quite small in relation to the total error. The plate-to-plate error contains the other three sources of variation as well as differences between plates. Similarly, the error due to variations in spotting volume contains error from the two minor sources. If these other three sources of error are subtracted out of the value for plate-to-plate error, the plate-to-plate error is about equal to the spotting volume error. Standard error values of densitometric measurements of sugars spotted on the same plate were reported by Moczar *et al.*[17] and by Pruden *et al.*[18] to be 1.4–5.4% (5 determinations per plate) and 2.8–5.6% (10 determinations per plate), respectively. These values are significantly lower than our corresponding value (Table II) probably because their determinations were made on prepared plates with uniform gel thicknesses.

TABLE III

VARIABILITY IN DENSITOMETRIC METHOD DUE TO AMOUNT SPOTTED

Maltose spotted (μg)	*Coefficient of variability ($n = 5$) (%)*
1	29
5	12
10	14

Another cause of variation was due to the quantity of sugar spotted (Table III). There was a great deal more variation among spots of 1 μg than among spots of 5 or 10 μg. In order to minimize error in densitometric measurements, spotting volumes should be applied carefully, development procedures carried out under similar condi-

tions each time and the densitometric scans made with the same settings on the densitometer. A series of known amounts of standards must be run on each plate for a standard curve in order to avoid high plate-to-plate errors.

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