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Note

Quantitative estimation of malto-oligosaccharides by high-performance thin-layer chromatography

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The separation and quantification of oligosaccharides were performed long ago using adsorption techniques on a charcoal–Celite column[1], gel permeation chromatography on various supports[2] or ion-exchange resins[3]. However these methods are complicated and time consuming.

High-performance liquid chromatography allows the time of separation to be reduced with a resolution of up to 17 glucose units[4,5]. Separation of oligosaccharides of up to 35 glucose units has even been achieved but chemical derivatization is necessary prior to chromatography[6]. Paper[7,8] and thin-layer chromatography[9,10] have been successfully applied. Recently Nurok and Zlatkis[11,12] presented improvement and simplification in separating malto-oligosaccharides by using high-performance thin-layer chromatography (HPTLC).

Dextrins and glucose syrups are generally defined by their dextrose-equivalent (DE), *i.e.* the percentage of reducing sugars in a product, calculated as dextrose. These starch hydrolysates can be obtained by various means (acid, α-amylases, β-amylase or glucoamylase) which differently affect the final molecular distribution of the oligosaccharides. It is therefore useful to have a simple method of quantifying the malto oligosaccharides in starch hydrolysates. In this respect, HPTLC has been found to be the method of choice owing to its simplicity, good reproducibility and resolution. However, some difficulties can be encountered which will be discussed in this report.

EXPERIMENTAL

Materials

Chromatography was performed on HPTLC plates (10 × 10 or 20 × 10 cm) pre-coated with silica gel SI 60 (E. Merck, Darmstadt, G.F.R.). Glucose monohydrate and maltose monohydrate were obtained from E. Merck and maltotriose from Hayashibara Biochemical (Okayama, Japan).

Maltose and maltotriose were purified from any contaminant sugar by adsorption chromatography on a charcoal–Celite column eluted with water followed by increasing concentrations of aqueous ethanol as described by Whistler and Durso[13]. Maltose was eluted with water and maltotriose by 10% ethanol. The purified maltose was crystallized in 85% ethanol. Less than 0.2% maltotriose was finally detected in maltose.

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Maltohexaose was obtained by hydrolysis of amylose by α-amylase from *Bacillus subtilis* to a dextrose equivalent of 27 and fractionated on a charcoal–Celite column with increasing concentrations of ethanol in water.

The concentrations of samples were determined by phenol–sulphuric acid reagent with glucose as reference[14].

Chromatography

Pre-coated HPTLC plates were prewashed with solvent (1-propanol–acetone–water, 50:40:10) and dried at 20 and 120°C. Samples up to 4 μl were applied to the plate on 0.5–1-cm lines, 1 cm apart using a Linomat III applicator (Camag, Basle, Switzerland). The plate was developed at 20°C two to three times in a closed glass tank lined with Whatman chromatographic paper. Two compositions of the solvent system (1-propanol–acetone–water) were used, depending on the separation desired: 45:30:25 and 50:40:10.

After each development the plate was dried with air followed by drying *in vacuo* for 10 min. The spots were visualized according to the method of Hansen[9] by dipping the plate rapidly into a solution of aniline (4 ml), diphenylamine (4 g), acetone (200 ml) and 85% phosphoric acid (30 ml) and then heating at 120°C for 15 min. The spots were scanned on a TLC scanner (Camag) at 546 nm with a 0.3 mm slit and a scanning speed of 0.5 mm/sec. The signal was integrated with a Hewlett-Packard integrator connected to a 3356 computer.

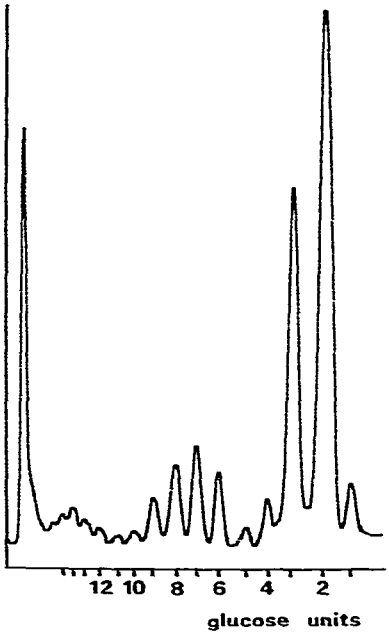
RESULTS AND DISCUSSION

Application of the sample in a 1 × 10-mm line was carried out with an automatic system which overcomes the "creep back" and "capillation" effects[15]. A minimum volume of 2 μl was necessary to ensure a satisfactorily precise volume. For higher volumes, the sample was preferably diluted with alcohol to avoid a broadening of the deposit by diffusion. Manual application by experienced workers with a 1-μl syringe has also given satisfactory results, but triplicates are recommended.

The best separation of the malto-oligosaccharides was obtained with three elutions of 40–55 min each with 1-propanol–acetone–water at volume proportions of 45:30:25 for the first and third development and of 50:40:10 for the second. Nurok and Zlatkis[12] found that a higher performance could be obtained at 55°C with a shorter elution time, but this temperature produced a diffusion of the spots and a consequently lower resolution.

The chromatogram of amylopectin digested by α-amylase from *Aspergillus oryzae* (Fig. 1) shows a complete separation of the peaks up to twelve glucose-unit chains. A complete separation of the oligosaccharides below ten glucose units was achieved with only two elutions with the same solvent mixture as shown in Fig. 2. When the second development was made with the less polar solvent, the peak separation was reduced (Fig. 3).

The method of detection is of great importance for the final precision of the quantification. It should always be made in the same way, by a rapid dipping of the plate in order to ensure a uniform wetting. The contact of the stationary phase with the acid produces bubbles which can disturb the uniformity of the wetting. Therefore it is advised to keep the plate in the reagent as long as bubbles appear, *i.e. ca.* 10 sec. A longer dipping time can result in diffusion or even dissolution of the sample.



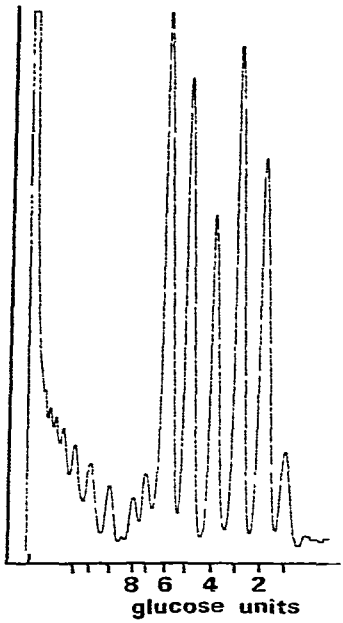


Fig. 1. Malto-oligosaccharide distribution of amylopectin hydrolysed with α-amylase from *Aspergillus oryzae* (see Table II). The first and third developments were with 1-propanol–acetone–water, (45:30:25) and the second with the same system in the proportion 50:40:10.

Fig. 2. Malto-oligosaccharide distribution of an amylopectin hydrolysed with α-amylase from *Bacillus subtilis* (see Table II). Two developments with 1-propanol–acetone–water (45:30:25).

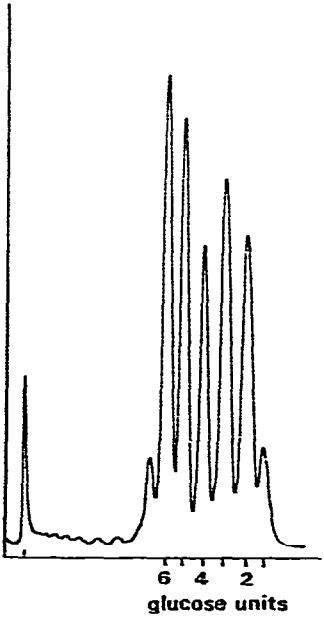


Fig. 3. Malto-oligosaccharide distribution of an amylose hydrolysed with α-amylase from *Bacillus subtilis* (see Table II). The first development was with 1-propanol–acetone–water (45:30:25) and the second with the same system in the proportion 50:40:10.

Welch and Martin[15] have noted that the densitometric response increases with the spot size in normal thin-layer chromatography, which results in different responses for each chain length of the oligosaccharides. With HPTLC plates, the increase in size of the spots was very low and the response factor of each oligosaccharide was found to be nearly equal when the amount was calculated as glucose equivalent, with the exception of glucose whose spot produced a greenish colour instead of a deep blue. Table I shows that the relative standard deviation for single measurement of eight applications of a maltohexose-maltotriose mixture on the same plate (20 × 10 cm) varies below 2.5%, which is in the range reported by Gauch *et al.*[16] and Dallas[17]. Furthermore the densitometric response for both sugars was found to be equal to one. The densitometric response for increased amounts of maltose and maltotriose are shown in Fig. 4. A good linearity of response was obtained below 1 μg of sugar applied to the plate, the response of maltose being somewhat above that of maltotriose.

TABLE I

PEAK AREA OBTAINED BY APPLYING THE SAME CARBOHYDRATE MIXTURE EIGHT TIMES ON THE SAME PLATE

	Maltotriose (0.89 μg)*		*Maltohexaose (0.90 μg*)*
	30640		30920
	30590		30771
	31766		30988
	30982		31006
	29713		31171
	29639		30456
	29991		31086
	29649		31211
Mean area	30371		30952
S.D. (%)	2.1		0.6
Response factor		0.99 ± 0.02	

* Expressed as glucose.

Variations in the peak intensity were observed between plates caused essentially by variations at the detection stage. Therefore external standards, *i.e.* pure maltose or maltotriose, have to be applied to each plate. The detection reagent is very sensitive to oxygen and light, but the plates can be stored for a few months by coating the silica gel layer with a polyethylene foil and by keeping the plates in the dark in an aluminium bag.

This simple technique has been applied to the determination of the molecular distribution of the malto oligosaccharides obtained by α-amylolysis of amylose and amylopectin by two types of α-amylases. The α-amylase from *Aspergillus oryzae* produces mostly maltose, maltotriose and α-limit dextrins from amylopectin as shown in Fig. 1. The amount of each sugar in the hydrolysate is detailed in Table II. The DE calculated from the molecular composition gives the same value as the measured one. The bacterial α-amylase from *Bacillus subtilis* produces essentially

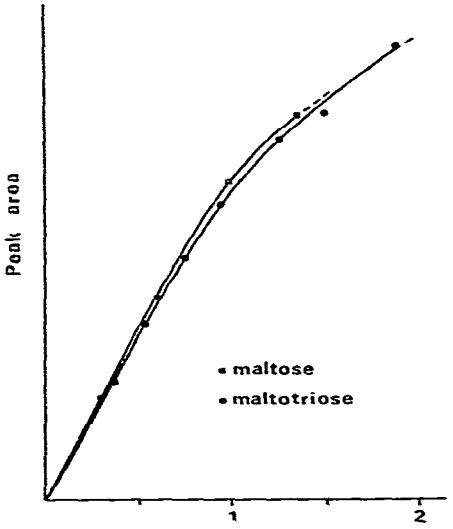


Fig. 4. Relationship between peak area and quantity of maltotriose and maltose spotted, expressed as glucose. Three developments as in Fig. 1.

TABLE II

QUANTITATIVE DISTRIBUTION OF THE OLIGOSACCHARIDES FORMED BY THE HYDROLYSIS OF AMYLOPECTIN (FIG. 2) AND AMYLOSE (FIG. 3) WITH α-AMYLASE FROM *BACILLUS SUBTILIS* AND OF AMYLOPECTIN WITH α-AMYLASE FROM *ASPERGILLUS ORYZAE* (FIG. 1)

Glucose units (n)	*Bacillus subtilis*		*Aspergillus oryzae, amylopectin (3.0 μg)*
	Amylose (3.4 μg)	*Amylopectin (3.7 μg)*	
1	4.7*	3.5*	3.9*
2	17.8	9.8	38.1
3	19.4	13.0	19.5
4	13.0	7.8	2.2
5	17.3	10.8	1.0
6	20.0	14.1	3.3
7	4.1	2.0	4.6
8		1.6	4.3
9		2.2	2.5
$17 > n > 9$	3.4 $n > 7$		6.7
$n > 17$			14.0
Dextrose calc.	33.1	20.8	34.6
equivalent meas.**	32	20	35

* Determined enzymatically.

** Reducing sugars.

oligosaccharides between 2 and 6 (ref. 18) as shown in Fig. 2 with a predominance of maltohexaose. With amylopectin the same pattern was obtained beside the branched limit-dextrins. The calculated DE was in good agreement with the colorimetric determination in each digest.

This chromatographic procedure has been successfully applied along with other chromatographic methods to the characterization of commercial maltodextrins[19].

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