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Note

Continuous quantitative thin-layer chromatography of oligosaccharides

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There is often a need to determine, either on an absolute or a relative basis, the amount of each individual in a complex mixture of oligosaccharides. Such mixtures may contain many components (*cf.* Fig. 1) and typical examples are the studies of enzyme mechanisms in degradation of dextrans[1] and hemicelluloses[2].

Schaffler and Juckes[3] have previously reported the quantitative analysis of three ketose trisaccharides in molasses by continuous thin-layer chromatography (TLC), but in their method the oligosaccharides were washed from the plate for subsequent colorimetric analysis after chromatography. The problems of variable diffusion on the plate, which affect direct densitometry (see below), were thereby avoided at the cost of considerable additional time. Welch and Martin[4] have analysed mixtures of sucrose, raffinose and stachyose by direct densitometry on the plate after single ascent TLC at room temperature, but reported that "significant difference between sets was due to the differences in spot diffusions caused by uncontrollable tank and development variations". They resolved this problem by running a separate standard for each component of each sample. Shellard and Alam[5] have also observed variation of densitometer response with R_F value in TLC of alkaloids and concluded that the effect was due to diffusion during chromatography.

We needed to analyse mixtures of oligosaccharides of higher degree of polymerization (DP) than had previously been studied by quantitative TLC and were concerned that, with the prolonged continuous TLC needed to separate such compounds, diffusion effects would become more important. As models we have used mixtures of the homologous series of isomaltose oligosaccharides upto DP 9 and have investigated the major factors which influence densitometer response after visualisation of the components on the plate.

EXPERIMENTAL

Materials and methods

The isomaltodextrins will be referred to as either IM_n or B_n where IM refers

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to the linear oligosaccharide, B refers to the oligosaccharide with a single α-(1→3) branch and n in both cases refers to the degree of polymerization. IM_3–IM_9 were prepared by Streamer[6] and the IM_2 was a gift from Dr Hubert Schiweck, Süddeutschen Zucker-AG. B_5 and B_6 were prepared in this laboratory by enzymic hydrolysis of B-512 native dextran. The structures of these isomaltodextrins have been investigated by proton magnetic resonance spectroscopy and by hydrolysis with specific enzymes followed by examination of the products by TLC.

Chromatography

TLC was conducted in the continuous mode at 30 ± 2° using 20 × 20 cm pre-coated plates (Merck 5721, 0.25 mm silica gel). The continuous mode of chromatography was achieved by attaching a strip of Whatman No. 17 paper to the top of the plate with clips. The chromatography solvent was *n*-propanol–nitromethane–water (50:20:30). The plates were activated at 105° for 30 min prior to use. The carbohydrate solutions (5 μg/μl) were spotted on the plates at least 2 cm apart on a line 1.5–2.0 cm from the end of the plate using a 10-μl Hamilton syringe to give a spot in the form of a bar not more than 5 mm long and 2.5 mm wide. The compounds were detected after chromatography by spraying the plate with 50% sulphuric acid followed by heating at 110° for 30 min.

Densitometry

The densitometer used was a Kipp and Zonen DD2 Model. This is a single-beam, adjustable-slit, transmission instrument with a filter-type wavelength selector. The plates were scanned at right angles to the direction of elution with a recorder to densitometer scan rate of 1.25:1.0. The area under the curve was measured with a planimeter taking the average of 3 readings in each case and this value is termed the "densitometer response".

Precision

The precision of the method was evaluated by chromatographing eight 15-μg samples of the isomaltodextrin IM_4 for 4 h.

RESULTS AND DISCUSSION

Preliminary experiments with constant amounts of glucose and isomaltose chromatographed for varying times (2–25 h) at room temperature showed that the densitometer response increased with distance moved on the plate upto a maximum at about 12 h for each substance (12 and 9.6 cm, respectively). These experiments confirm the observations of Welch and Martin[4], who noted the increase in densitometer response with spot size and suggested that diffusion of the spot increased the efficiency of interaction with the localizing agent in terms of densitometer response. The latter workers, however, failed to observe the subsequent decrease in densitometer response with further diffusion of the spot (*i.e.* longer running of the chromatogram) and we conclude that this effect is due to a portion of the more diffuse material failing to produce a significant densitometer response.

During the above preliminary experiments the higher oligosaccharides gave diffuse spots that were inadequately resolved. It seemed likely that the use of higher

temperatures would speed up the separation and perhaps reduce diffusion. It also appeared that variations in room temperature during the prolonged chromatography may be deleterious to the resolution. Chromatography of a wide range of oligosaccharides was therefore carried out at 30 ± 2°, and Fig. 1 shows that very much improved resolution was obtained. It is possible that still higher temperatures would further improve the resolution, but would cause problems in retention of solvents. All subsequent chromatography was therefore carried out at 30°. Dallas[7] has examined the effect of distance of development on peak area and concluded that peak area varied less with the distance of development than with the time of contact of the solute with the mobile phase. Dallas recommended standardization of elution time if chromatograms were to be compared by densitometry. The results of our research show that, while control of elution time is important, control of temperature is also important and attention should be given to both.

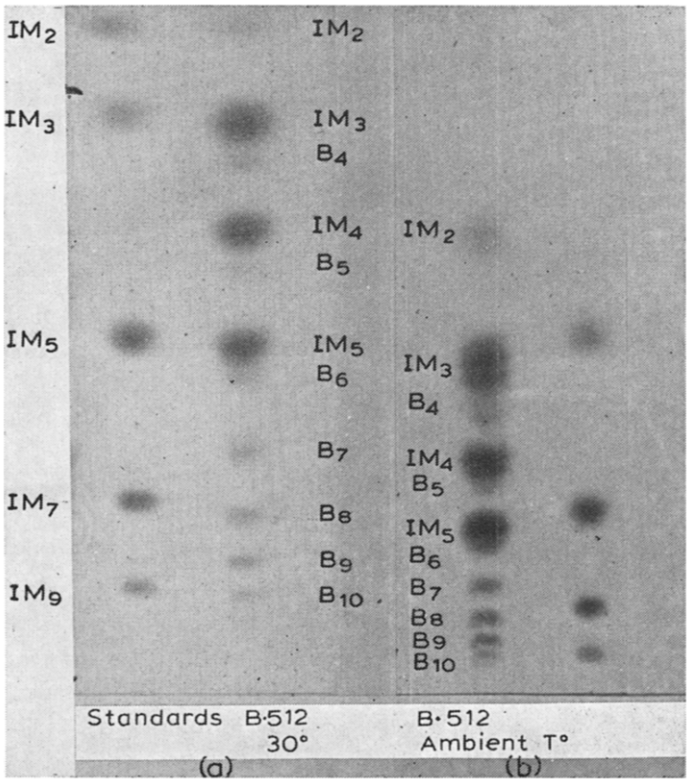


Fig. 1. Continuous TLC of isomaltodextrins from an enzyme hydrolysate of B-512 native dextran. (a) TLC at 30° and (b) at ambient temperature (23 ± >3°). Elution time 19 h.

The relationship between densitometer response and quantity of IM_2–IM_9 is shown in Fig. 2. The relationship is non-linear, in agreement with the results of Shellard and Alam[5], but a linear relationship is obtained in the usual way by plotting peak area against the square root of quantity (μg) (Fig. 3)[8]. The curves shown in Fig. 3

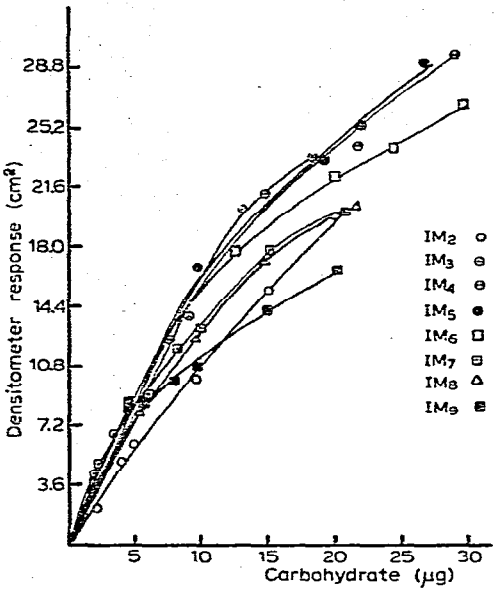


Fig. 2. Relationship between densitometer response and quantity of isomaltodextrins. TLC at 30° for 19 h.

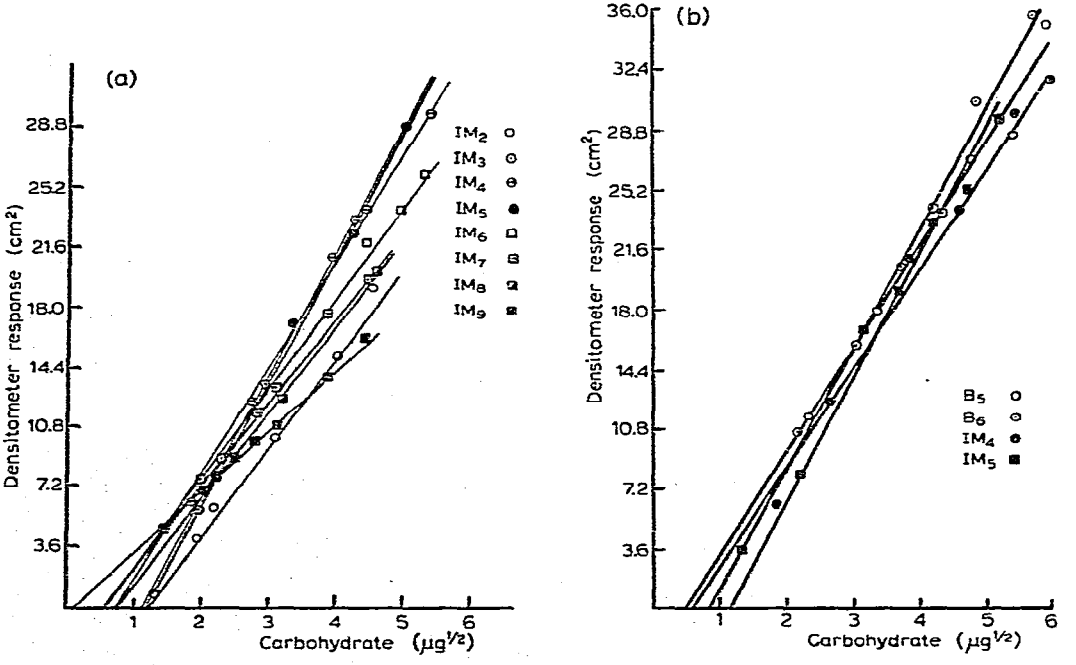


Fig. 3. Relationship between densitometer response and square root of quantity of isomaltodextrins. (Not all experimental points are shown on the graphs.)

TABLE I

REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS OF THE ISOMALTODEXTRINS IM_2–IM_9, B_5 AND B_6

See also Fig. 3.

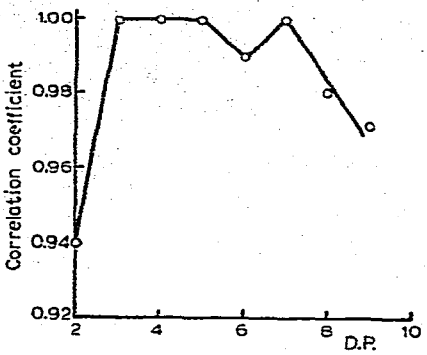
Isomaltodextrin	*Regression equation* ($y = $)	*Correlation coefficient*
IM_2	$78.48x - 100.74$	0.94
IM_3	$104.94x - 117.73$	1.00
IM_4	$80.60x - 45.00$	1.00
IM_5	$104.68x - 122.52$	1.00
IM_6	$77.04x - 50.09$	0.99
IM_7	$74.44x - 63.28$	1.00
IM_8	$71.03x - 39.51$	0.98
IM_9	$50.01x - 10.02$	0.97
B_5	$87.50x - 47.45$	0.98
B_6	$102.75x - 89.76$	0.99

were obtained from a linear regression analysis of the relationship between peak area and square root of quantity of oligosaccharide. The regression analysis yielded equations of the form

$$y = mx + C$$

where: y = peak area and $x = (\mu g \text{ oligosaccharide})^{\frac{1}{2}}$. The regression equations and correlation coefficients are listed in Table I. The relationship between peak area and square root of quantity of oligosaccharide varies significantly over the homologous isomaltodextrin series. There is a suggestion of a pattern in the relationship, however, which is revealed by plotting the correlation coefficients *versus* the degree of polymerisation (DP) (Fig. 4). Evidently, the optimum range of DP in terms of correlation of densitometer response with loading, under the conditions of the experiment, lies approximately between DP 3 and 7 (the inflexion at DP 6–7 in Fig. 4 is of doubtful significance). Welch and Martin[4] have previously shown an increase in precision with R_F value for the series fructose, glucose, sucrose, but did not detect a maximum within this limited series. The optimum in Fig. 4 probably results from an optimization in the degree of diffusion of the spot during chromatography and for different times of development of the chromatogram it is likely that the optimum DP may occur at different values. The conclusions are supported by plotting densitometer response against DP for two different loadings on the plate (Fig. 5, values derived from curves in Fig. 3a). For each loading the optimum response occurs at about DP 3–4. The subsequent decrease at higher DP (*i.e.* lower R_F) is probably associated with less than optimum diffusion and also with a slow decrease in reactivity in the higher oligosaccharides towards charring with sulphuric acid.

The precision of quantitative thin-layer chromatography using densitometry has been investigated by a number of people. Frei[8] and Dallas[7] have both concluded that it was a satisfactory method for quantitative analysis (*e.g.* coefficients of variation (C.V.) between 1.4 and 7.8%). Our results with IM_4 ($n = 8$, $\bar{x} = 12.2$, S.D. = 0.84, C.V. = 6.9%) indicate that the method is adequate for many purposes.



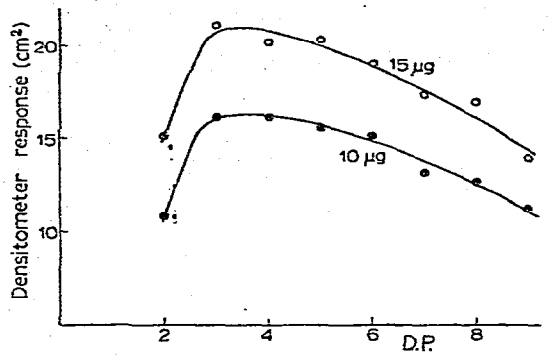


Fig. 4. Relationship between correlation coefficient and DP of isomaltodextrins.

Fig. 5. Relationship between densitometer response and DP of isomaltodextrins.

For quantitative TLC of higher oligosaccharides we therefore recommend that elution be carried out in the continuous mode at a constant temperature, which is as high as is convenient (with respect to the solvent mixture employed). A calibration curve (as Fig. 3a) must be determined for each component to be analysed, but, provided that the time and temperature of elution are kept constant, it is not necessary to run standards on each plate.

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