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Note

The separation of malto-oligosaccharides by high-performance, thin-layer chromatography using multiple developments

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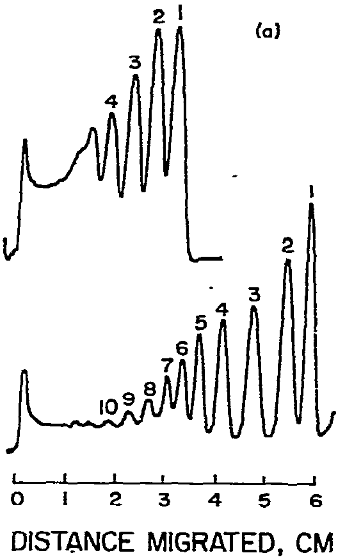
The advantages of using high performance thin-layer chromatography (h.p.t.l.c.) for the separation of oligosaccharides have already been discussed in a previous communication from this laboratory[1]. The separation of oligosaccharides with a degree of polymerization up to about 10 on h.p.t.l.c. plates is more rapid than similar separations reported in the literature[2,3].

A valuable technique in thin-layer chromatography (t.l.c.) is the use of multiple developments. After each development with a solvent system, the plate is dried and then redeveloped with the same solvent. This has the effect of decreasing the spot width, as on redevelopment the bottom of a spot starts moving before the top does, and the distance from top to bottom of the spot decreases. Obviously, no improvement in spot resolution is observed for compounds that migrate to the end of the plate, but a considerable improvement is obtained for spots that are in the middle of the plate. Thoma has demonstrated mathematically[4] that the best separation occurs at a migration distance corresponding to R_F 0.63. In the same paper, he showed that D-glucose and D-fructose were not separated by paper chromatography in a single development, but were separated after three replicate developments. Multiple developments may be performed in several ways[5]. In this note, we discuss the advantages of using unidimensional, multiple-development chromatography for the separation of oligosaccharides by h.p.t.l.c. This is the simplest form of multiple development. The plate is completely dried after each development, and the solvent path-length is the same for each development.

Figs. 1a and b show the same series of oligosaccharides separated with 2:2:1 (v/v) 1-butanol–ethanol–water as solvent. Fig. 1a illustrates a single development lasting 1 h; Fig. 1b, six replicate developments each lasting 1 h, resulting in a dramatic improvement. In Fig. 1a only 4 partially resolved peaks are shown; in Fig. 1b, 10 peaks may be seen. The same improvement was not observed when the plate was immersed continuously for 6 h, as the leading peaks ran into the solvent front, and all peaks were broader, resulting in lower detection limits. The resolution between D-glucose and maltose does not quite reach the baseline. Baseline resolution of the



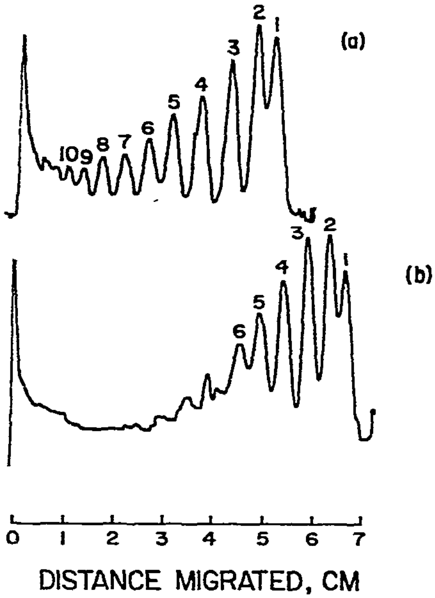


Fig. 1 (left). Chromatograms developed with 2:2:1 (v/v) 1-butanol–ethanol–water as solvent: (a) Single development of 1 h; and (b) six replicated 1-h developments. Numbers indicate the degree of polymerization. 1, D-glucose, 2 and higher, malto-oligosaccharides.

Fig. 2 (right). Chromatograms developed with 7:3 (v/v) acetone–water as solvent: (a) Single development of 30 min; and (b) two replicated 30-min developments. See legend to Fig. 1 for composition of mixture.

first four members of the series may be achieved with the same solvent system by use of two developments of 45 min, one of 60 min, and one of 75 min.

The best solvent-system for multiple developments is that in which the individual compounds to be separated have low R_F values, even though this entails a larger number of replicate developments to obtain optimum separation between a given pair of peaks. Conversely, a solvent in which the individual components to be separated have high R_F values is poorly suited for multiple developments. This is illustrated by considering the solvent system 7:3 (v/v) acetone–water, in which the oligosaccharides have high R_F values due to the high water-content. This gave, in a single development, a fair separation with 10 partially resolved peaks (see Fig. 2a). However, a second development with the same solvent system led to a very poor separation (see Fig. 2b).

A somewhat better separation was obtained with 3:1 (v/v) acetone–water, where the R_F values are lower, and the separation achieved in a single run, for oligomers with a d.p. of 4 (or higher), is inferior to that achieved with 7:3 (v/v) acetone–water. However, the separation was improved through 6 replicate developments, but the separation of D-glucose from maltose was slightly decreased during the last three developments (see Figs. 3a and 3b).

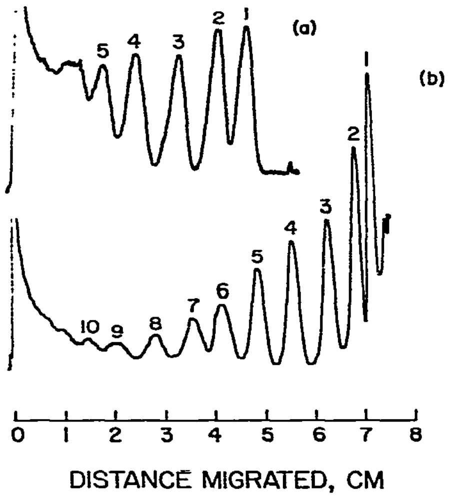


Fig. 3. Chromatograms developed with 3:1 (v/v) acetone–water as solvent: (a) Single development of 30 min; and (b) six replicated 30-min developments. See legend to Fig. 1 for composition of mixture.

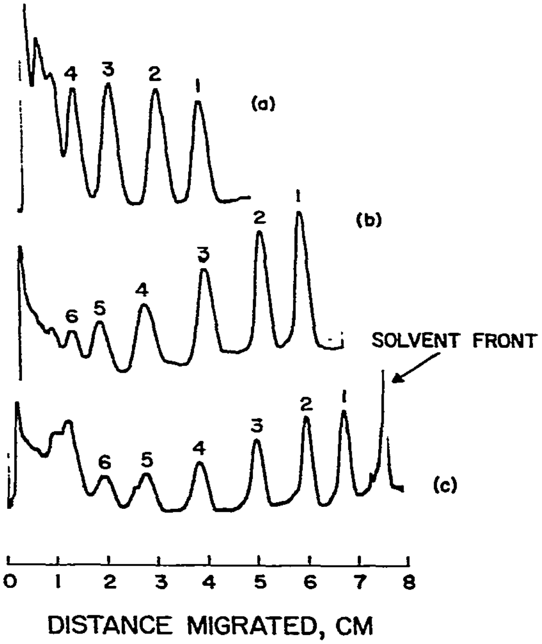


Fig. 4. Chromatograms developed with 4:1 (v/v) acetone–water as solvent: (a) Single development of 30 min; (b) three replicated 30-min developments; and (c) six replicated 30-min developments. See legend to Fig. 1 for composition of mixture.

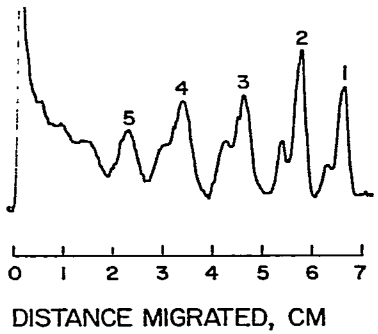


Fig. 5. Chromatogram after five replicated developments with 4:1 (v/v) acetone–water as solvent, the plate being dried in an oven at 105° between developments. See legend to Fig. 1 for composition of mixture.

Acetone–water (4:1, v/v) is a solvent system in which the oligosaccharides have relatively low R_F values but that is well suited to multiple developments, as shown in Figs. 4a–4c. Fig. 4a shows a baseline resolution from D-glucose to maltotriose after a single development, whereas Fig. 4c shows a baseline resolution from D-glucose to maltopentose after six developments. The best separation between peaks occurs at a distance ~6/10th of the solvent path-length (see Fig. 4c). It should be pointed out that generally no baseline separation is present when two spots are separated by such a minimum distance that there is just no visual overlap. Such a separation generally shows overlap when measured with the scanning spectrophotometer, because of the greater sensitivity of the instrument.

In performing these multiple developments, care must be taken not to heat the plates while removing the solvent, as this can lead to sample decomposition. Fig. 5 shows one of the earlier runs (five developments in 4:1, v/v, acetone–water) in which the solvent was removed by placing the plate in an oven for a few min at 105°. Sample decomposition is clearly shown by the presence of additional peaks. In subsequent runs, the solvent was removed by blowing a stream of air over the plate for 5 min at 30°. This did not dry the plate quite as well as heating in the oven, as evidenced by the need for slightly longer development times. Typically, the first run (plate dry) took about 25 min, whereas subsequent runs took about 30 min.

As noted earlier, better separations are achieved by choosing a solvent of low R_F value, coupled with a large number of multiple developments. The procedure is tedious and, in practice, is unlikely to be performed for much more than 10 developments, even though 36 developments for a dye mixture have been reported[6]. Thus, 4:1 (v/v) acetone–water appears to be a good solvent for resolving the lower members of an oligosaccharide series. It should, however, be noted that a commercial apparatus, available for the more sophisticated technique of programmed multiple developments, allows up to 99 consecutive developments to be performed automatically. This should give excellent separations with such a low-R_F solvent as 19:1 (v/v) acetone–water.

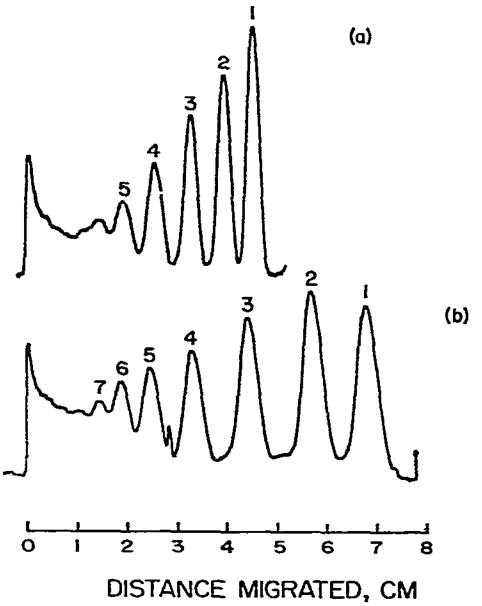


Fig. 6. Chromatograms developed with 2:2:1 (v/v) 2-propanol–acetone–water as solvent: (a) Three replicated 30-min developments at 25°; and (b) two 30-min developments at 55°. See legend to Fig. 1 for composition of mixture.

Performing t.l.c. at an elevated temperature presents the considerable advantage that migration rates are faster, and consequently separations more rapid. Similarly, performing multiple developments at elevated temperature (see Fig. 6b) results in a more rapid separation than that obtained at room temperature (see Fig. 6a); thus two developments at 55° gave a better separation than three developments at room temperature. The development time of 30 min at both temperatures was the time required for the solvent to run through the silica gel plate at room temperature. Continuous development was employed at 55° to allow for the faster rate of solvent migration at the higher temperature.

EXPERIMENTAL

Materials. — Precoated silica gel HPTLC plates (E. Merck, Darmstadt, West Germany, No. 5633) were cut into sections 2.5 × 10 cm. Each plate was scored with a line at 7.5 cm from the sample origin to ensure a reproducible path-length for multiple developments. Fisher-certified A.C.S. solvents were used. The oligosaccharides were obtained as a gift from Dr. A. W. Wight of the C.S.I.R., Pretoria, South Africa.

Sample application. — A solution (0.2 μL, corresponding to 4 μg) of 2.0% of oligosaccharide mixture in 70% aqueous ethanol, was deposited on the plate with a Pt/Ir capillary (Antech GmbH, Bad Dürkheim, West Germany) attached to an EVA Chrom-Applicator (W+W Electronic Scientific Instrument Co., Basel, Switzerland).

Recording of chromatograms. — The spots were visualized according to the method of Hansen[3]: The plate was dipped into a solution consisting of aniline (4 mL), diphenylamine (4 g), acetone (200 mL), and 85% phosphoric acid (30 mL), and then was placed in an oven for 20 min at 110°. The plate was allowed to cool, and was then scanned with a Zeiss KM3 chromatogram spectrophotometer set at 620 nm.

Chromatography. — (*a*) *At 25°*. In a glass tank (26 × 22 × 7 cm) lined with Whatman chromatography paper, the solvent mixture (100 mL) was added. The paper was allowed to wet with the solvent before chromatography was initiated. Up to six plates were placed in the tank. After the first development, all the plates were removed from the tank and dried in a stream of air for 5 min at 30°. Five of the six plates were then replaced in the chromatography tank. The procedure was repeated for the number of replicate runs required. The number of plates replaced in the tank was decreased by one after each drying period. The tank was dried after three developments, and fresh solvent and paper were used to ensure that there was no change in solvent composition during the developments.

(*b*) *At 55°*. The technique has been described previously[7].

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