SEPARATION OF CHITIN OLIGOSACCHARIDES BY THIN-LAYER CHROMATOGRAPHY

R. F. POWNING AND H. IRZYKIEWICZ
Division of Entomology, CSIRO, Canberra, A.C.T. (Australia)
(Received December 27th, 1966)

INTRODUCTION

Chromatography of chitin oligosaccharides on paper has already been described[1,2]. Thin-layer chromatography is generally regarded as giving better results than paper chromatography and the following investigations were carried out to determine the best conditions for the thin-layer technique. The separation of acetylglucosamine and oligosaccharides of glucosamine on thin layers was described by TAKEDA *et al.*[3] but the N-acetylated oligosaccharides were not examined.

MATERIALS AND METHODS

Chitin oligosaccharides were prepared substantially according to BARKER *et al.*[1] and further purified by passage through Sephadex G-25.

Layer materials

Kieselgel G, Kieselgel HR, Kieselguhr G were all Merck products. Plates, 20 × 5 cm, were spread at 0.25 mm thickness according to standard procedures with "Shandon" apparatus. After air-drying overnight, about 2 mm of the layer was removed from the edges and then the plates were stored in a sealed cabinet without further activation. Samples were applied in 0.5 μl spots along a line 3 cm from the lower end of the plate. A line was scraped in the layer material 10 cm from the origin and the plates were removed from the developing solvent immediately the solvent reached this line.

Chromatography

The solvents used in this work after preliminary trial runs with microslides were as shown in Table I. Development was carried out at room temperature (24–25°)in glass cylinders 22 cm high by 6.5 cm diameter, fitted with gas-tight glass lids. Solvent (40 ml) was added to the jar and the plate positioned so that the layer was on the inner side of the plate. No attempt was made to saturate the atmosphere in the jars by paper wetted with solvent. The solvent was removed from the plates by heating in an oven at 70° for ½–1 h.

Spray reagents

The chlorination starch–iodide technique as described for paper chromatograms[2] was modified slightly. The plates were humidified over water at 40° for 15 min and then

TABLE I

CHROMATOGRAPHY SOLVENTS

Solvent No.	*Solvent composition**								*Time (h) on*	
	n-Pro	*Isopro*	*tert.-Bu*	*n-Bu*	*Isoam*	*Eth*	*Water*	*Amn*	*Silica Gel HR*	*Silica Gel G*
1	70	—	—	—	—	—	30	—	1.6	2.8
1A	70	—	—	—	—	—	30	1	1.5	2.8
2	—	72	—	—	—	—	27	—	2.7	3.5
2A	—	72	—	—	—	—	27	1	2.8	3.5
3	—	—	70	—	—	—	35	—	3.6	5.8
3A	—	—	70	—	—	—	35	1	3.7	6.7
4	—	—	—	50	—	70	40	—	1.7	2.5
4A	—	—	—	50	—	70	40	1	1.2	2.5
5	—	—	—	—	50	60	30	—	1.6	2.8
5A	—	—	—	—	50	60	30	1	1.8	2.8
6	—	—	50	—	—	70	40	—	2.3	3.4
6A	—	—	50	—	—	70	40	1	2.3	3.3

* The above components were *n*-propanol, isopropanol and *tert.*-butanol (BDH laboratory grade) and *n*-butanol, isoamyl alcohol, ethanol and ammonia (sp.gr. 0.91) (BDH analytical reagent).

chlorinated over a solution of chlorine in carbon tetrachloride for 30 min. These procedures were carried out in covered glass troughs in which the plates were supported face up above the water or the chlorine solution. After aeration for 1 h the plates were sprayed with starch–iodide reagent. With this technique 0.5 μg of oligosaccharide could easily be detected. A similar sensitivity could be obtained by spraying the plate with a solution of 0.2 *N* potassium permanganate in 4 *N* sulphuric acid and warming slightly for about 15 min. The originally pink plate changes to a brown background with white spots. Although the chromatograms can be examined at this stage, a much greater contrast can be obtained by neutralising the acid on the plate over ammonia and then spraying with saturated benzidine hydrochloride (or *o*-tolidine) in 2 % acetic acid. The spots then appear white on a dark blue background.

RESULTS

Preliminary experiments showed that results with thin-layers of microcrystalline cellulose were similar to those found for paper chromatography[2], and that alumina thin layers were inferior to those made with silica gel. On Kieselguhr G with the solvents listed in Table I, no separation was observed between the oligosaccharides, and all migrated close to the solvent front. Modification of solvents by decreasing water content had the effect of leaving an increasing amount of oligosaccharide at the origin with the remainder going to the solvent front. Silica Gel G and HR were selected for further study and the R_F values obtained are listed in Table II. All the results show a linear relationship between R_M value, log $(1/R_F-1)$, and degree of polymerisation of the oligosaccharide. A selection of these relationships is given in Fig. 1. Addition of ammonia to solvents for silica gel plates invariably reduced the R_F values and it also appeared to increase resolution slightly and to reduce tailing. The type of chromatogram obtained can be seen in Fig. 2. When two developments on Silica Gel G were used, excellent resolution of oligosaccharides was obtained (Fig. 2). Due to the re-

tarding effect of ammonia addition, solvent 1 A is thought to be most satisfactory for multiple runs. R_{F_2} values for chitohexaose were quite high, and the data closely approximated the equation, $R_{F_2} = 2R_{F_1} - R_{F_1}^2$, of STARKA AND HAMPL[4].

TABLE II

R_F VALUES FOR CHITIN OLIGOSACCHARIDES ON SILICA GEL

Silica	*Saccharide**	*Solvent*											
		1	*1A*	*2*	*2A*	*3*	*3A*	*4*	*4A*	*5*	*5A*	*6*	*6A*
HR	Nag	0.72	0.48	**	0.54	0.77	0.50	0.76	0.59	0.63	0.46	**	0.67
	biose	0.63	0.37		0.41	0.70	0.38	0.70	0.47	0.51	0.31		0.56
	triose	0.53	0.27		0.31	0.62	0.27	0.63	0.37	0.40	0.20		0.45
	tetraose	0.45	0.19		0.22	0.55	0.19	0.56	0.27	0.31	0.12		0.36
	pentaose	0.37	0.13		0.16	0.47	0.13	0.48	0.21	0.23	0.07		0.27
	hexaose	0.30	0.09		0.11	0.38	0.09	0.41	0.14	0.15	0.05		0.19
G	Nag	0.63	0.45	0.74	0.54	0.68	0.51	0.66	0.53	0.54	0.40	0.73	0.61
	biose	0.52	0.34	0.66	0.42	0.58	0.39	0.57	0.41	0.42	0.27	0.68	0.49
	triose	0.42	0.24	0.58	0.31	0.48	0.29	0.48	0.32	0.30	0.16	0.61	0.38
	tetraose	0.33	0.17	0.50	0.22	0.39	0.21	0.40	0.23	0.22	0.10	0.53	0.28
	pentaose	0.25	0.12	0.42	0.16	0.31	0.15	0.32	0.17	0.14	0.06	0.45	0.21
	hexaose	0.19	0.08	0.33	0.10	0.23	0.11	0.25	0.11	0.09	0.03	0.37	0.14

* Nag→hexaose represents the oligosaccharide series from acetylglucosamine.
** Little differentiation and bad tailing.

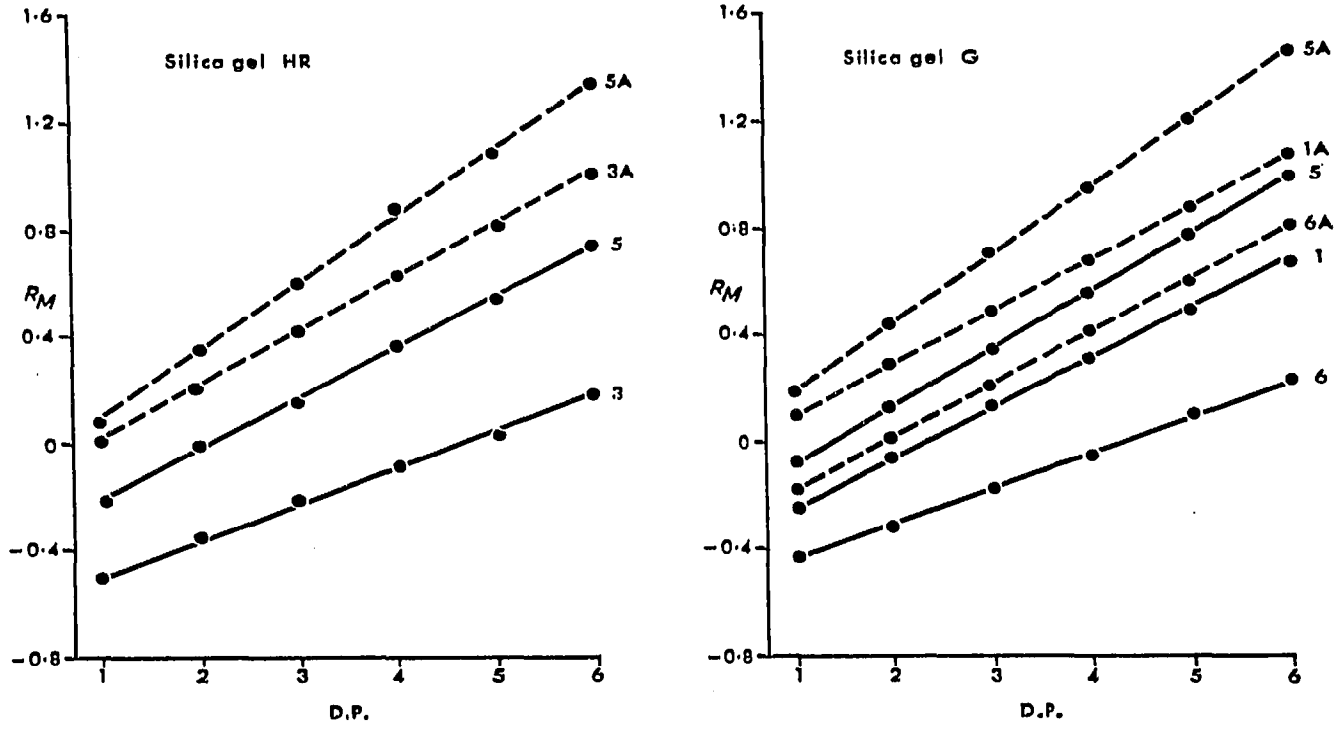

Fig. 1. Relationship between R_M value and degree of polymerisation (D.P.) of chitin oligosaccharides on silica gel thin-layer plates. Numbers on graphs refer to solvents listed in Table I. Broken lines represent solvents with added ammonia.

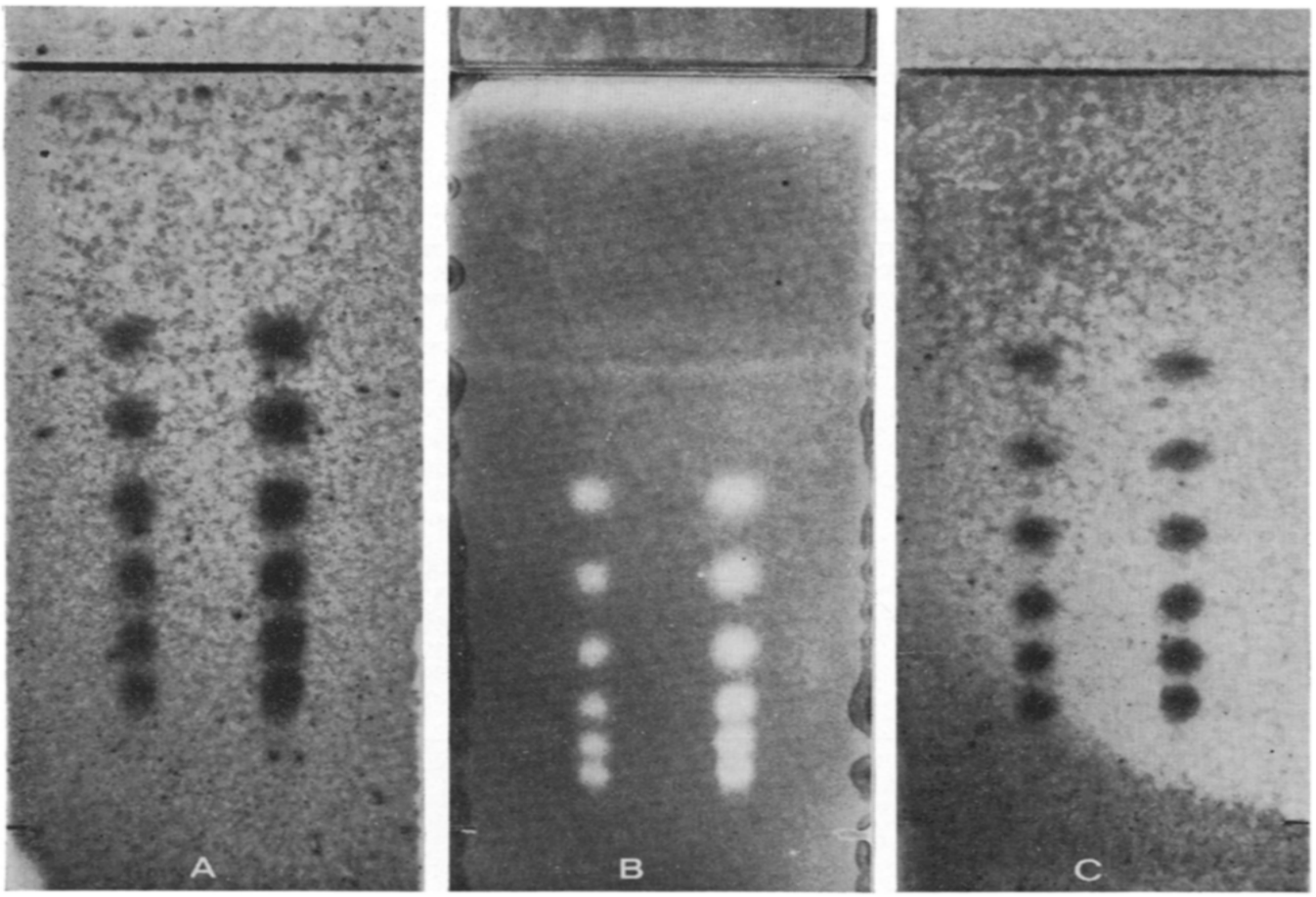

Fig. 2. Chromatograms of chitin oligosaccharides (1 and 2 μg) on Silica Gel G. (A) Single development in solvent 1; chlorine, starch–iodide treatment. (B) Single development in solvent 1A; permanganate, sulphuric acid, benzidine treatment. (C) Double development in solvent 1A; chlorine, starch–iodide treatment.

DISCUSSION

All solvents (Table I) used with Silica Gel G plates permit satisfactory separations of chitin oligosaccharides up to the hexaose. Despite slower development rates, Silica Gel G is the preferred layer material as results with plates made with Silica Gel HR were slightly inferior due to tailing. Solvents containing a lower alcohol such as *n*-propanol travelled faster than those containing higher alcohols, in particular *tert.*-butanol, however, the spots on plates developed in the latter appeared to be more compact. It is probable that the higher viscosity of *tert.*-butanol was responsible for both the longer development time and the lower diffusion of solute.

Since none of the solvents gave any resolution on Kieselguhr G plates, it appears that in this material the chromatographic process is very different from that in silica gel. Kieselguhr is a less active adsorbent than silica gel and is generally recommended for partition chromatography[5]. If the mechanism with chitin oligosaccharides on Kieselguhr G is mainly partition, the failure on this layer is probably due to the rapid decrease of solubility with increased size of the oligosaccharides. Despite the fact that the solvents used with silica gel have high percentages of water, the mechanism in this

layer is at least partly adsorption and this is supported by the effects of ammonia.

Oligosaccharides higher than chitohexaose were not available to test their behaviour in thin-layer chromatography. However, the results of double development on Silica Gel G plates show that the resolution obtained and the movement of the hexasaccharide are satisfactory for resolution at least up to the octasaccharide.

In view of the linear relationship established between size of oligosaccharide and R_M value, and the improved resolution of spots, it may be concluded that thin-layer chromatography is preferable to paper chromatography for many purposes in oligosaccharide analysis.

SUMMARY

A variety of solvents for use in thin-layer chromatography have been examined, and from these can be selected solvents appropriate for single run separation of chitin oligosaccharides at least up to the hexasaccharide, or for multiple run chromatograms with higher oligosaccharides. Best results were obtained on Silica Gel G plates. A linear relationship has been established between R_M value and degree of polymerisation of oligosaccharide for all solvents used. Ammonia addition to solvents decreased R_F values and increased resolution.

REFERENCES

1 S. A. BARKER, A. B. FOSTER, M. STACEY AND J. M. WEBBER, *J. Chem. Soc.*, (1958) 2218.
2 R. F. POWNING AND H. IRZYKIEWICZ, *J. Chromatog.*, 17 (1965) 621.
3 M. TAKEDA, T. TOMIDA, T. OMURA AND H. KATSUURA, *J. Shimonoseki Coll. Fisheries*, 14 (1965) 1.
4 L. STARKA AND R. HAMPL, *J. Chromatog.*, 12 (1963) 347.
5 D. WALDI, in E. STAHL (Editor), *Thin-layer Chromatography*, Academic Press, New York, 1965, p. 33.