Journal of Chromatography, 151 (1978) 39–49

CHROM. 10,467

PREPARATION AND THIN-LAYER CHROMATOGRAPHY OF OLIGOGALACTURONIC ACIDS

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(Received August 2nd, 1977)

SUMMARY

The preparation of oligogalacturonic acids on a relatively large scale has been achieved by chromatography on a DEAE-Sephadex A-50 column (80 × 10 cm). The yield of oligogalacturonic acids (degree of polymerization = 1–9) recovered from the hydrolysate of polygalacturonic acid (PGA) was 54.8%. Trigalacturonic acid was obtained in highest yield when 1 l of 1% PGA solution was hydrolysed by 200 mg of pectinase at 35° for 15 min. An improved thin-layer chromatographic (TLC) method was used for the detection and identification of the oligogalacturonic acids. The effects of thin-layer plates, solvent systems and spray reagents on the TLC behavior of the oligogalacturonic acids have been studied. The solvent ethyl acetate–acetic acid–water (4:2:3, v/v/v) gave the best separation on an Eastman E-13255 cellulose plate when developed twice in an ascending direction at 25°. A double spraying technique, spraying first with alkaline solution then with the chromogenic reagent, was developed with successful results. The quantitative determination of the oligogalacturonic acids by TLC is also reported.

INTRODUCTION

Oligogalacturonic acids are known to be the end products of the hydrolysis of pectic acid by endo-polygalacturonase (EC 3.2.1.15). The separation of these oligomers has been studied by many investigators. Luh and Phaff[1] and Demain and Phaff[2] isolated oligouronic acids from the yeast polygalacturonase hydrolysate by precipitating them in alcoholic solution with addition of strontium(II) chloride. After desalting with cation exchangers, the oligomers were separated by paper chromatography. Similar methods have been applied by McCready and Seegmiller[3], Wright[4] and Pressey and Allen[5]. The separation of oligouronide by Dowex resin column chromatography has been reported by Derungs and Deuel[6], Nagel and Anderson[7], Hasegawa and Nagel[8] and Nagel and Wilson[9]. Hatanaka and Ozawa[10] and Pressey and Avants[11] applied a DEAE-Sephadex column to separate the oligouronides.

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Recently, Davé *et al.*[12] reported that both saturated and unsaturated oligogalacturonic acids could be separated on an AG-1-X8 (formate) column.

For the preparation and identification of the oligogalacturonic acids, paper chromatography was used by most of the early investigators. However, very little information is available on the thin-layer chromatography (TLC) of oligogalacturonic acids, probably due to the difficulty of finding a suitable chromogenic reagent for the visual examination of the spots. An effort has been made to solve this problem by developing a double spraying technique. This paper reports the preparation of oligogalacturonic acids, which were separated by chromatography on a DEAE-Sephadex A-50-120 column. The results of the TLC of oligogalacturonic acids are also presented.

EXPERIMENTAL

Polygalacturonic acid (PGA)

A commercial PGA (No. 3491) purchased from Sunkist Growers (Ontario, Calif., U.S.A.) was purified by washing three times with ethanol, two volumes of 95% ethanol being added to an aliquot of 1% aqueous PGA solution at pH 4.0. The precipitate was collected and squeezed in a nylon cloth to apparent dryness, then re-dissolved in water to its original volume. The ethanol precipitation process was repeated twice more. Subsequently, the PGA was dried by blending with 95% ethanol. After filtration, the PGA was washed with acetone and then dried in a vacuum oven at 50° for 2 h.

Pectic enzymes

Commercial pectinase P-4625 was purchased from Sigma (St. Louis, Mo., U.S.A.). Endopolygalacturonase from *Rhizopus arrhizus* (endo-RAPG) was isolated and purified 274-fold as described previously[13].

Anhydrogalacturonic acid (AGA) assay

The carbazole method described by Rouse and Atkins[14] was used.

Determination of reducing value

The reducing value of oligogalacturonic acids was determined by the arseno-molybdate method[15].

Column chromatography of oligogalacturonic acids

The improved procedure used in this study was based on the method reported by Hatanaka and Ozawa[10] and Pressey and Avants[11]. One liter of 1% PGA solution was adjusted to pH 5.0 with 1 *M* sodium hydroxide solution and warmed to 30°, then 200 mg of pectinase were dissolved in 20 ml of 0.15 *M* sodium chloride solution and this solution was added to the PGA solution. The mixture was heated to boiling, controlling the heating time to within 15 min with constant stirring. The mixture was cooled to 25° in a water-bath and centrifuged, then the supernatant was treated with 5 g of strontium(II) chloride and two volumes of 95% ethanol. The precipitate was collected by centrifugation, then dissolved in 200 ml of water and deionized by adding 25 g of Dowex-50 (H^+) cation-exchange resin to reduce the pH to 2.0 or lower. The

resin was removed by filtration, the pH of the filtrate was adjusted to 5.0 with 1 *M* sodium hydroxide solution, then it was poured on to an 80 × 10 cm DEAE-Sephadex A-50-120 column that had previously been equilibrated with distilled water at pH 6.0. The column was eluted first with 4 l of water at pH 6.0, and then stepwise with 4-l volumes of 0.05, 0.10, 0.125, 0.150, 0.175, 0.200 and 0.225 *M* sodium chloride solution. Subsequently, 6 l each of 0.250 and 0.275 *M* sodium chloride solution were introduced. The total volume of the eluate was 44 l.

Fractions of 20 ml each were collected and analysed for their anhydrogalacturonic acid contents by the carbazole method[14]. A total of 2200 fractions were collected. Fractions corresponding to each peak were combined and concentrated in a flash vacuum evaporator at 45°. The concentrated sample (about 200 ml) was deionized by passing it through a Dowex-50 (H^+) column (30 × 2.2 cm). The deionized concentrates were frozen and lyophilized for 72 h at 0.1 mmHg pressure in a Stokes freeze-drier.

Thin-layer chromatography of oligogalacturonic acids

Both qualitative and quantitative TLC of oligogalacturonic acids was studied. Exploratory experiments on solvent systems, types of plates, spraying reagents and multiple development techniques were investigated. For the identification of the oligogalacturonic acids, Eastman E-13255 cellulose plates were used. Each spot contained 40 μg of freeze-dried sample in water. An authentic standard of α-D-galacturonic acid (Sigma) was used as a reference. The plate was developed in an ascending manner at 24° in a glass tank containing ethyl acetate–acetic acid–water (4:2:3, v/v). To develop the spots, the plate was first sprayed with 10% ammonia solution and then with bromophenol blue (50 mg per 100 ml, 95% ethanol at pH 7.0) or with mixed indicators[5]. The spots sprayed with bromophenol blue gave a yellow color on a bluish background, and those sprayed with mixed indicators yielded red color spots on a greenish background.

RESULTS

Preparation of oligogalacturonic acids

Column chromatography on DEAE-Sephadex A-50-120. Fig. 1 shows the elution profile of oligogalacturonic acids separated on a DEAE-Sephadex A-50-120 column, in which 11 peaks can be seen. Two small peaks were eluted first, then followed by peaks identified consecutively as the monomer up to the nonamer [degree of polymerization (DP) = 9]. The trimer was found in the highest yield in the end product of hydrolysis.

Table I gives the recoveries of the oligomers. The total recovery of oligogalacturonic acid (DP = 1–9) was 54.8% of the amount of PGA. The trimer was the main product of the enzymic hydrolysis, comprising 38.1% of the total amount recovered, followed by the tetramer (20.8%), pentamer (13.1%), hexamer (9.2%), heptamer (7.3%), dimer (5.9%), octamer (2.0%) and nonamer (1.2%).

Identification of the oligogalacturonic acids. Oligogalacturonic acids eluted from the DEAE-Sephadex column were detected on the basis of their R_F values when separated on the thin-layer chromatogram. Fig. 2 shows a typical thin-layer chromatogram developed twice with ethyl acetate–acetic acid–water (4:2:3, v/v). Peak

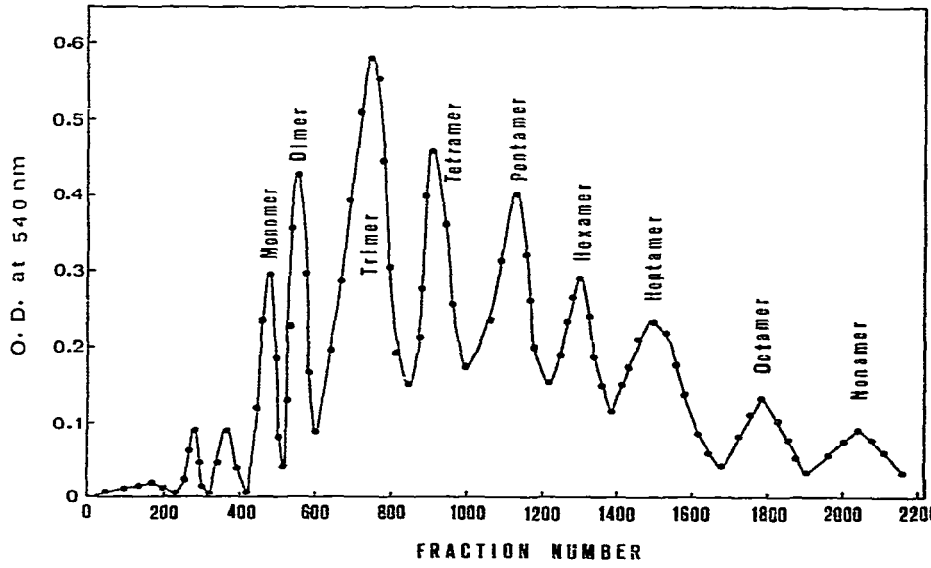

Fig. 1. Elution profile of oligogalacturonic acids separated by chromatography on a DEAE-Sephadex A-50-120 column.

TABLE I

RECOVERY OF OLIGOGALACTURONIC ACIDS ON DEAE-SEPHADEX A-50-120 COLUMN CHROMATOGRAPHY

9.52 g of polygalacturonic acid (DP = 70) were hydrolysed by pectinase (Sigma, P-4625)

Peak No.	*Oligogalacturonic acid*	*Amount (g)*	*mmole*	*Recovery (%)*
3	Monomer	0.1244	0.64	1.30
4	Dimer	0.3081	0.83	3.23
5	Trimer	1.9898	3.64	20.90
6	Tetramer	1.0824	1.50	11.37
7	Pentamer	0.6864	0.76	7.20
8	Hexamer	0.4784	0.45	5.02
9	Heptamer	0.3815	0.31	4.00
10	Octamer	0.1038	0.07	1.09
11	Nonamer	0.0650	0.04	0.68
	Total oligomers	5.2198	8.24	54.82

3 (P-3) had a mobility similar to that of authentic α-D-galacturonic acid (D-GA), and was identified as a monomer. In order of decreasing mobility, the dimer to the heptamer appeared consecutively on the chromatogram.

The oligogalacturonic acids were further identified by determining the ratio of AGA to the reducing group content (AGA/CHO) in each oligomer. It is shown in Table II that the molar ratio (AGA/CHO) was consistent with the degree of polymerization of each oligomer. The R_F and R_{ga} values of the oligomers are also presented in Table II (the R_{ga} value is the ratio of the distance travelled by the spot from the origin to that of α-D-galacturonic acid). The R_F value was obtained by developing with the solvent only once and the R_{ga} value by developing twice.

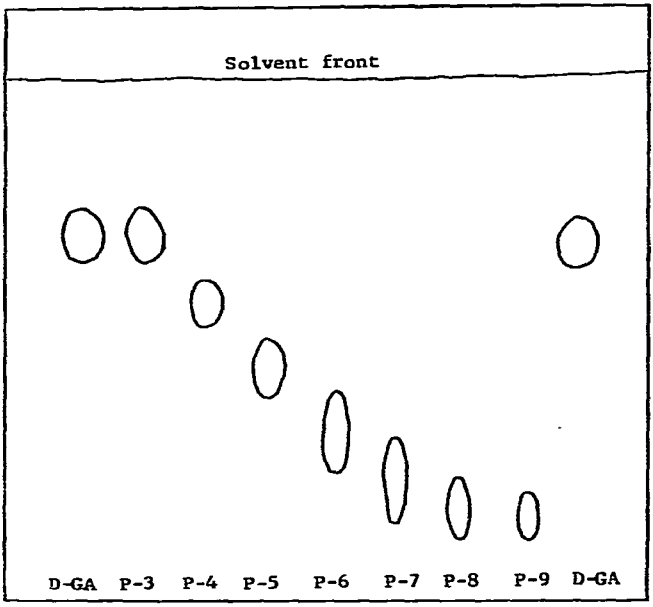

Fig. 2. Typical thin-layer chromatogram of oligogalacturonic acids. D-GA = α-D-galacturonic acid (standard); P-3 = monogalacturonic acid; P-4 = digalacturonic acid; P-5 = trigalacturonic acid, P-6 = tetragalacturonic acid; P-7 = pentagalacturonic acid; P-8 = hexagalacturonic acid; P-9 = heptagalacturonic acid. Plate: Eastman E-13255 cellulose sheet. Solvent system: ethyl acetate–acetic acid–water (4:2:3, v/v/v). Development: ascending elution, twice. Spray reagent: mixed indicators thymol blue + bromothymol blue + methyl red).

TABLE II

CHROMATOGRAPHIC MOBILITIES AND REDUCING PROPERTIES OF OLIGOGALACTURONIC ACIDS

Oligogalacturonic acid	*DP*	*Ratio (AGA/CHO)**		*R_F value (MN-300, E–A–W, 2:1:2)***	*R_{ga} value*	
		Expt. I	*Expt. II*		*E-13255, E–A–W, 2:1:2, 2×***	*E-13255, E–A–W, 4:2:3, 2×***
Digalacturonic acid	2	2.046	2.015	0.58	0.88	0.76
Trigalacturonic acid	3	2.928	3.050	0.52	0.80	0.58
Tetragalacturonic acid	4	3.842	4.002	0.44	0.60	0.37
Pentagalacturonic acid	5	5.073	5.235	0.31	0.47	0.22
Hexagalacturonic acid	6	6.095	6.139	0.19	0.27	0.12
Heptagalacturonic acid	7	7.140	6.980	0.13	0.17	0.06
Octagalacturonic acid	8	7.987	8.080	0.08	0.13	0.03
Nonagalacturonic acid	9	9.050	9.040	0.04	0.08	0.02
Decagalacturonic acid	10	9.847	10.190	0.01	0.04	0.01

* Ratio (AGA/CHO) = μmoles anhydrogalacturonic acid/μmoles reducing groups.

** MN-300 = Polygram Cel-300 TLC sheet; E 13255 = Eastman cellulose TLC sheet; E–A–W = ethyl acetate–acetic acid–water; 2× = developed twice.

Thin-layer chromatography of oligogalacturonic acids

Effects of solvents and plates. Efforts were made to improve the separation of the oligouronides. Two different solvent systems and two different cellulose TLC plates were tried. Table III shows the variation of the R_F values with the type of solvents, type of plate and the number of developments. It was found that increasing the polarity of the solvent resulted in higher R_F values of the oligogalacturonic acids. The characteristics of the oligomers are shown in Fig. 3, in which solvent I (ethyl acetate–acetic acid–water, 2:1:2, v/v/v) with a Polygram Cel-300 plate gave the highest R_F values and solvent II (ethyl acetate–acetic acid–water, 4:2:3, v/v/v) with an Eastman E-13255 plate the lowest.

TABLE III

CHROMATOGRAPHIC CHARACTERISTICS OF OLIGOGALACTURONIC ACIDS SEPARATED ON THIN-LAYER CHROMATOGRAM SHEETS UNDER DIFFERENT CONDITIONS AT 24°

Oligomer DP	*R_F or R_{ga} values, plates, solvent system and elution time (min)* **						$\log\left(\frac{R_F\,(100)}{1-R_F}\right)$	$\log\left(\frac{R_{ga}\,(100)}{1-R_{ga}}\right)$
	R_F, E-13255, E–A–W, 2:1:2, 150 min	*R_F, E-13255, E–A–W, 4:2:3, 150 min*	*R_F, MN-300, E–A–W, 2:1:2, 95 min*	*R_F, MN-300, E–A–W, 4:2:3, 95 min*	*R_{ga}, E-13255, E–A–W, 2:1:2, 2 A.D.*	*R_{ga}, E-13255, E–A–W, 4:2:3, 2 A.D.*	*(E-13255, E–A–W, 4:2:3, 1 A.D.)*	*(E-13255, E–A–W, 4:2:3, 2 A.D.)*
1	0.570	0.460	0.650	0.590	—	—	1.93	—
2	0.450	0.340	0.580	0.480	0.880	0.760	1.71	2.50
3	0.380	0.240	0.520	0.380	0.790	0.580	1.50	2.14
4	0.270	0.160	0.480	0.250	0.600	0.380	1.28	1.79
5	0.180	0.100	0.310	0.130	0.470	0.220	1.05	1.45
6	0.100	0.060	0.190	0.070	0.270	0.120	0.81	1.11
7	0.058	0.035	0.130	0.048	0.170	0.060	0.56	0.78
8	0.026	0.020	0.080	0.018	0.130	0.028	0.31	0.46
9	0.010	0	0.040	0.010	0.080	0.014	0	0.15
10	0	0	0.010	0	0.040	0.010	0	0

* Abbreviations as in Table II; 1 A.D. and 2 A.D. = developed once or twice, respectively, in ascending direction.

Multiple development of the chromatograms was also investigated. The oligogalacturonic acids were separated better on the thin-layer chromatograms when they were developed twice than when developed once. When the plates were developed twice, a solvent of higher polarity gave a better separation of large oligomers (DP $>$ 4) than small oligomers (DP $<$ 4). Fig. 4 shows the difference between the two solvent systems when the oligomers were chromatographed on an Eastman E-13255 cellulose plate. Further, a linear relationship was observed when the logarithm of the partition function was plotted against the degree of polymerization of the oligogalacturonic acids (Fig. 5). However, this linear behavior occurred only when solvent II (ethyl acetate–acetic acid–water, 4:2:3, v/v/v) was used and not with solvent I (ethyl acetate–acetic acid–water, 2:1:2, v/v/v). Fig. 5 also shows that chromatograms developed twice gave a better separation than those developed once. With the double development method, nonagalacturonic acid (DP = 9) was separated successfully from the others.

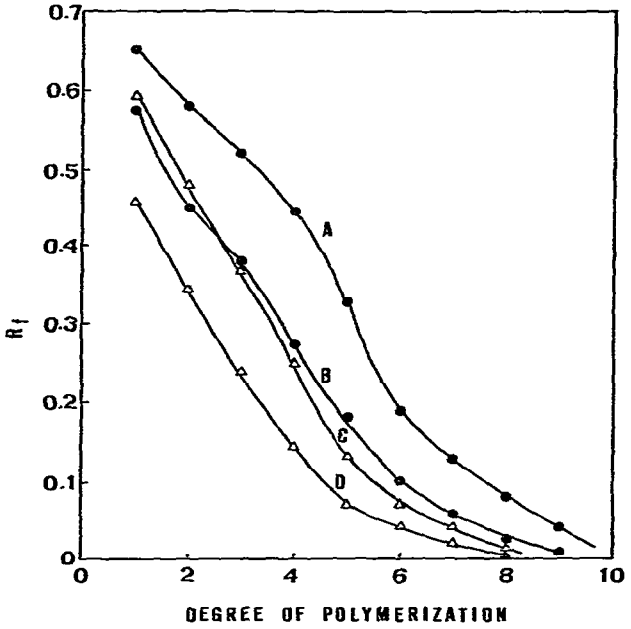

Fig. 3. Relationship between R_F values and molecular size of oligogalacturonic acids when separated by TLC with different plates and proportions of components in solvent system (ethyl acetate–acetic acid–water). A, Polygram Cel-300, solvent 2:1:2; B, Eastman 13255, 2:1:2; C, Polygram Cel-300 4:2:3; D, Eastman 13255, 4:2:3.

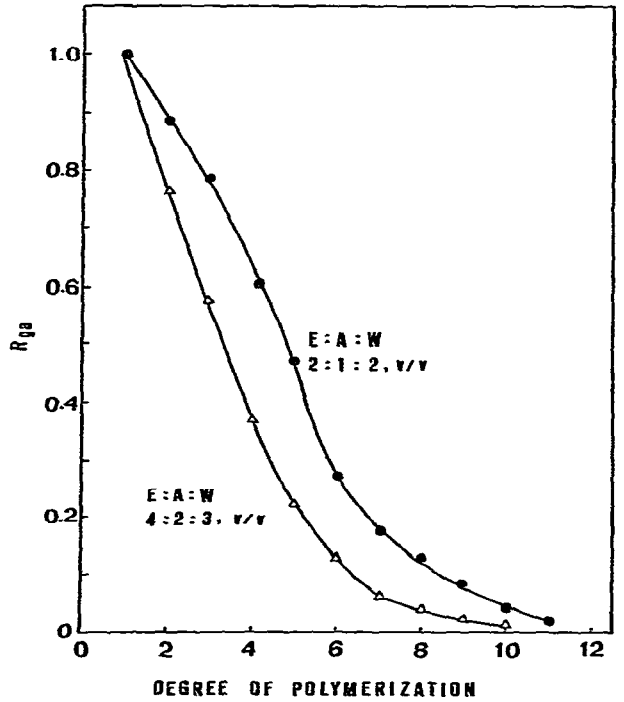

Fig. 4. Relationship between degree of polymerization and migration of oligogalacturonic acids on Eastman 13255 cellulose TLC chromatogram sheet developed twice with solvent systems of different polarity. E:A:W = ethyl acetate–acetic acid–water.

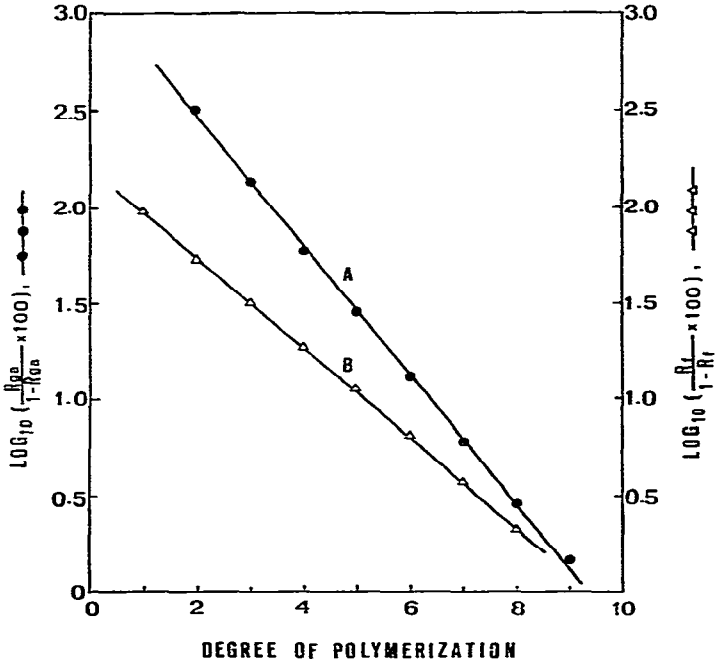

Fig. 5. Relationship between logarithm of partition function and molecular size of oligogalacturonic acid separated on an Eastman 13255 cellulose TLC plate with the solvent system ethyl acetate–acetic acid–water (4:2:3, v/v) at 25°. Ascending development: A, twice; B, once.

Detection of compounds in the spots. Several chromogenic reagents used in paper chromatography were applied to the thin-layer chromatograms but were not successful because the background of the plate had the same color as the spots of the oligogalacturonic acids. This difficulty may be attributed to the acidic residues present in the cellulose layer of the chromatogram. The acidic residues may be derived either from the acetic acid of the solvent or from the sulfate residues that served as a binder for the cellulose layer. Attempts to remove the acidic residues from the TLC plate by heating it in a vacuum oven were unsuccessful. The difficulty was overcome, however, by spraying with a dilute alkaline solution to neutralize the acidic residues.

Two carboxyl group-specific chromogenic agents have been used routinely for detection. The developed plates were first sprayed with 0.1 *M* ammonia or sodium hydroxide solution, then with either bromophenol blue or mixed indicators (thymol blue (50 mg), bromothymol blue (600 mg) and methyl red (250 mg) in 100 ml of 95% ethanol).

Two other spray reagents specific to aldehyde groups were also used, namely aniline (0.93 ml) plus orthophosphoric acid (1.3 ml, 85%) in 70% ethanol, and a 0.2% solution of *m*-phenylenediammonium dichloride plus 0.2% oxalic acid in 95% ethanol. Both reagents gave flesh-colored spots with the oligogalacturonic acids after heating at 100° for 15 min. However, as they are aldehyde group (CHO)-specific reagents, they gave a less distinct color with the oligogalacturonic acids of higher DP than with those of lower DP.

Quantitation of oligouronic acids by TLC. Quantitative analysis of the oligogalacturonic acids was effected by scraping off the acidic spots from the thin-layer chromatogram. Fig. 6 shows the technique, in which the chromatogram is divided into small squares with a pencil by referring to the guide spots on each side. Each square corresponds to an individual separated oligogalacturonic acid. Table IV gives the results of the spot (square) assays. The assay was carried out by determining the content by the carbazole method[14]. The relative amounts of the oligogalacturonic acids shown in Table IV are based on the end products when applied to the TLC plate. A 0.055-ml sample containing 216 μg of end products was applied for each hydrolysate. The results indicate that as the hydrolysis time increased, the total oligogalacturonic acids (DP $\leqslant$ 7) increased in the hydrolysate. After reaction for 20 h catalysed by endo-RAPG, 94.6% of the PGA (DP = 134) had been converted into oligomers (DP $\leqslant$ 6), in which 51% was the trimer.

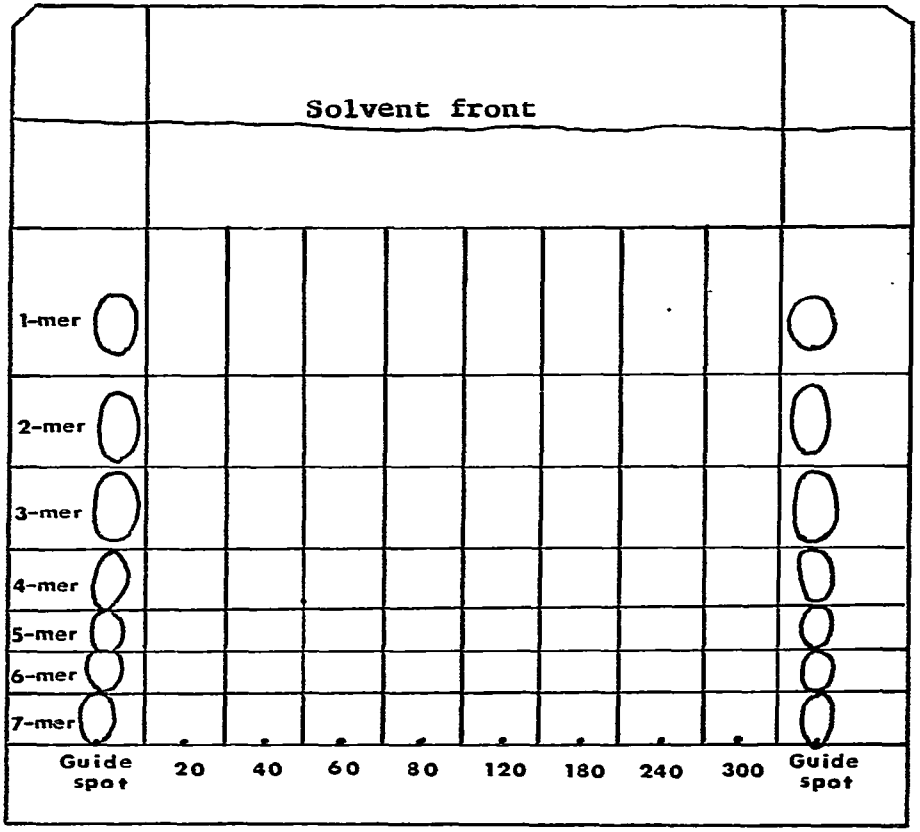

Fig. 6. Guide spot technique for TLC quantitative assay of oligogalacturonic acids when PGA-S (DP = 134) was hydrolysed by RAPG for different times at 30°. TLC plate: Eastman E-13255 cellulose sheet. Solvent system: ethyl acetate–acetic acid–water (4:2:3, v/v). Development: ascending elution, twice. Spray reagents: guide spots (both sides) were sprayed first with 0.1 *M* NaOH, then with mixed indicator (thymol blue + bromothymol blue + methyl red).

TABLE IV

QUANTITATION OF THE END PRODUCTS OF HYDROLYSIS DETERMINED BY TLC SPOT ASSAY WHEN PGA-S (DP = 134) WAS HYDROLYSED BY RAPG FOR DIFFERENT TIMES

Oligomer DP	*Oligogalacturonic acids (%, w/w) at different reaction times*								
	20 min	*40 min*	*60 min*	*80 min*	*120 min*	*180 min*	*240 min*	*300 min*	*20 h*
1	0	0	0	0.18	0.73	0 96	1.27	1.73	2.41
2	0.49	0.65	1.61	1.88	2.16	3.73	4.99	6.17	14.19
3	0.73	1.02	1.39	2.22	4.48	10.88	15.27	19.81	50.93
4	0.52	0.37	0.59	2.50	6.39	8.75	13.42	14.20	15.59
5	0.65	0.56	0.96	3.70	7.56	9.15	10.19	10.31	6.73
6	1.24	1.57	3.02	8.70	11.17	10.29	6.83	5.65	4.75
7	1.62	6.50	12.96	22.07	14.81	7.56	4.34	4.63	0
Total (DP $\leq$ 7)	5.25	10.67	20.53	41.75	47.30	51.32	56.31	62.50	94.60

DISCUSSION

Oligogalacturonic acids are not available commercially and workers who need them for research purposes have to use either paper chromatography or column chromatography to separate them from the enzymatic hydrolysate of pectic acid. Obviously they are limited to very small amounts. The method described here could be used for mass production on a commercial scale.

TLC has been found to be useful for the identification of oligogalacturonic acids. This study has shown that the solvent system ethyl acetate–acetic acid–water (4:2:3, v/v/v) gives the best separation on an Eastman E-13255 cellulose plate when developed twice at 25° in an ascending manner. Compared with paper chromatography, TLC has the advantages of better separation and faster operation. TLC has been applied to the quantitative analysis of sugars in food materials[16], but there are no reports of the quantitative assay of oligogalacturonic acids by TLC. The present TLC assay has been used to determine the quantitative changes in the end products during the hydrolysis of pectic acid. It was found very helpful to study the mechanism of enzyme action in the hydrolysis process.

Non-uronide residues have been reported to be incorporated in the structure of pectic substances. Aspinall and Canas-Rodriquez[17] and Barrett and Northcote[18] stated that small amounts of neutral sugars may be attached either to the main chain or in the side-chain of polygalacturonic acid. This heteropolysaccharide character has also been indicated by the present investigation. TLC of the hydrolysate of pectic (DP = 70) revealed that both galactose and arabinose were involved, as shown by TLC on an Eastman E-13255 cellulose plate and eluting with ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v) at 25°. Moreover, two other unidentified components were also observed in the hydrolysate of pectic acid. Two small peaks appeared on the chromatogram (Fig. 1) when the hydrolysate was separated on a DEAE-Sephadex column. They were the first two peaks found in the elution profile and were not homologs of the oligogalacturonic acids as examined by TLC. They were tentatively identified to be pseudo-aldouronic acids (a disaccharide containing one neutral sugar

and one galacturonic acid). According to Aspinall and co-workers[19–23], these pseudo-aldouronic structures are usually 1,3-glycosidic linkages, which were not attacked by the pectic enzymes and therefore remained in the hydrolysate. The detailed structure of pectic acid is still not clearly understood and TLC will play an important role in solving this problem.

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