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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF URONIC ACIDS AND OLIGOGALACTURONIC ACIDS

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SUMMARY

The analysis of uronic acids such as D-galacturonic acid, D-glucuronic acid, D-mannuronic acid and L-guluronic acid and the analysis of oligogalacturonic acids by high-performance liquid chromatography was studied. The uronic acids were separated on a strong anion-exchange column with acetic acid as eluent. D-Galacturonic acid and L-guluronic acid could not be separated.

A mixture of unsaturated oligogalacturonic acids obtained by enzymatic degradation of pectic acid (pectic acid lyase) was successfully separated by three different systems based on ion exchange or ion-pair chromatography. The influence of the eluent composition on the performance of these systems was investigated. Also normal oligogalacturonic acids could be separated.

The methods were applied to the characterization of alginate samples with respect to their uronic acid composition and to the analysis of digests of enzyme-degraded pectins. The methods also proved to be very helpful in the elucidation of the fine structure of pectin.

INTRODUCTION

Uronic acids such as D-galacturonic acid, D-glucuronic acid, D-mannuronic acid and L-guluronic acid are commonly found in plants as components of gums and cell walls. Plant biochemists and food scientists are interested in methods for determining the uronic acid composition of polysaccharides, in order to identify the polysaccharides, to establish their fine structure and to understand their physiological and biochemical roles in plants and physico-chemical functions in food. The first step is the chemical or enzymatic degradation of the polysaccharides to monomers or oligomers. The mixtures of monomers and/or oligomers in digests can then be analysed by paper or thin-layer chromatography, by conventional ion-exchange chromatography or by gas–liquid chromatography. For gas chromatographic analysis the uronic acids have to be converted into volatile derivatives. For this purpose Raunhardt[1] prepared trimethylsilyl esters, and Jones and Albersheim[2] developed a method in which the uronic acids were first reduced to aldonic acid, then lactonized and

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further reduced to alditols, and finally converted into alditolacetates. Mergenthaler and Scherz[3] released the uronic acids as the methyl esters of the methyl glycosides by methanolysis of the polysaccharides, and these products were reduced with borohydride to methylglycosides and then converted into aldonitrile acetates.

A different aproach is the reduction of the uronic acid residues in the polymer to their corresponding neutral sugar residues prior to hydrolysis.

The above derivatization procedures are elaborate and the recovery of the uronic acids is often low; trimethylsilylation gives multiple peaks representing the anomers of the pyranose and furanose forms of the uronic acids.

Pure and well characterized enzymes can be used as powerful tools in the elucidation of the fine structure of polysaccharides. They split specific glycosidic linkages and form polymeric and/or oligomeric fragments or even monomers. These fragments can be characterized more easily. The preparative separation of oligogalacturonides has been performed successfully by anion-exchange chromatography[4,5] and by thin-layer chromatography[6]. Automated anion-exchange chromatography systems for uronic acids have been developed by Johnson and Samuelson[7], Spiro[8] and Sinner *et al.*[9]. Lee and Tieckelmann[10], Delaney *et al.*[11] and Baenziger and Natowisz[12] have described ion-exchange high-performance liquid chromatographic (HPLC) systems for the separation of anionic (sulphated) oligosaccharides.

In this paper we describe some possibilities for the analysis of uronic acids and oligogalacturonic acids by HPLC. Because of their sensitive and versatile UV detection, unsaturated oligogalacturonic acids (double bond between C_4 and C_5 of the terminal non-reducing galacturonic acid unit) were used to develop and test various HPLC systems.

EXPERIMENTAL

Reference samples

D-Galacturonic acid monohydrate and D-glucuronic acid were obtained from Fluka (Buchs, Switzerland). A mixture of D-mannuronic and L-guluronic acid was prepared by acid hydrolysis of an alginate sample; 50 mg were dissolved in 2.5 ml of 80% (w/w) sulphuric acid by stirring for 17 h at 20°C. The sulphuric acid was then diluted with distilled water to 2 *N* and hydrolysis was conducted at 100°C for 3 h. The hydrolysates were neutralized with calcium carbonate. The bulky precipitate was filtered off and washed three times with distilled water and the washings were combined with the filtrate. This pool was evaporated to dryness and redissolved in 5 ml of distilled water.

Pure normal and unsaturated oligogalacturonic acids were prepared in this laboratory by fractionation of oligomer mixtures. These mixtures were obtained by degrading pectic acid with endo-polygalacturonase (PG; E.C. 3.2.1.15) or endo-pectic acid lyase (PAL; E.C. 4.2.2.2) as described by Voragen[13] and Rombouts *et al.*[14]. Fractionation was performed by ion-exchange chromatography according to the procedure of Hatanaka and Ozawa[5].

Alginate preparations were obtained from Proton and Fagertun (Drammen, Norway) and Kelco (San Diego, CA, U.S.A.). They were converted into their composite uronic acids by hydrolysis with sulphuric acid[15]. Sodium pectate and Brown ribbon apple pectin (75% esterified) were made available by Obipectin (Bischofszell,

Switzerland). Polygalacturonic acid was obtained from Fluka. Pectins were hydrolysed with sulphuric acid as described for alginates and enzymatically with PAL after cold alkaline saponification[14,15].

Chromatographic apparatus and materials

A Spectra-Physics SP 8000 liquid chromatograph equipped with a Schoeffel 770 variable-wavelength detector or a Waters R-401 refractive index (RI) detector was used. For the separation of uronic acids strong anion-exchange columns were used (Nucleosil 10 SB, Chrompack, Middelburg, The Netherlands; Zorbax SAX, DuPont, Wilmington, DE, U.S.A.).

Aqueous acetic acid solutions were used as the eluent. For the analysis of normal and unsaturated oligogalacturonic acids three different systems were studied: (i) a strong anion-exchange column (Nucleosil 10 SB or Zorbax SAX) with sodium acetate buffers as eluent; (ii) a weak anion-exchange column (LiChrosorb 10 NH_2) combined with a Vydax 501SC guard column (100 × 2.1 mm I.D., 30–44 μm; Chrompack) and also with sodium acetate buffers as eluent; and (iii) a reversed-phase column (LiChrosorb 10 RP-18) combined with a Co:Pell ODS guard column (100 × 2.1 mm I.D., 37–44 μm; Chrompack) applying ion-pair chromatography, using as the eluent a mixture of methanol and phosphate buffer containing tetrabutylammonium bromide as ion-pair reagent.

Unsaturated oligogalacturonic acids were detected by their UV absorbance at 235 nm; uronic acids and normal oligogalacturonic acids were detected with the RI detector.

All analytical columns used were 250 × 4.6 mm I.D. LiChrosorb 10 NH_2 and LiChrosorb 10 RP-18 were obtained from Merck (Darmstadt, G.F.R.) and the columns were made in our laboratory by the upward slurry packing method. All chemicals and solvents were of analytical-reagent grade; water was purified with the Millipore purification system (Milli-Q water).

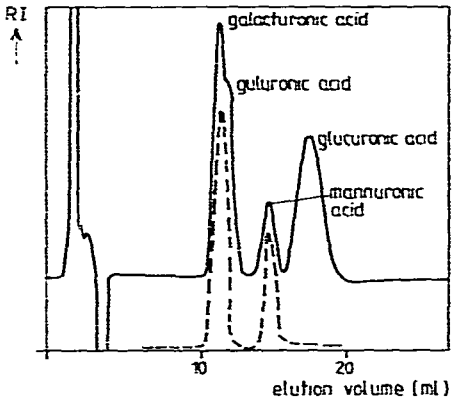
Volumes of 20 μl of aqueous solutions of the uronic acids, normal oligogalacturonic acids (0.1–0.5% each) and unsaturated oligogalacturonic acids (*ca.* 0.005–0.1% each) were injected. The interstitial volumes (V_0) of the columns were determined from the centre of the band of non-retained compounds (*e.g.*, glucose) following sample injection.

RESULTS

Uronic acids

Fig. 1 shows the separation of a standard mixture of uronic acids and an alginate hydrolysate (broken line) on Nucleosil 10 SB. The column was eluted with acetic acid (0.7 *N*) at a flow-rate of 1.4 ml/min. To obtain symmetrical peaks it was necessary to keep the column at a temperature above 30°C; we chose 40°C. The separation shown was achieved in less than 20 min; for D-galacturonic acid and D-glucuronic acid a resolution (R_s) of 3.3 was calculated, for D-galacturonic and D-mannuronic acid R_s was 2.1 and for D-mannuronic and D-glucuronic acid R_s was 1.4. We were not able to obtain a separation between D-galacturonic acid and L-guluronic acid. With Zorbax SAX almost identical results were obtained.

A drawback of the anion-exchange columns is their low stability, which causes



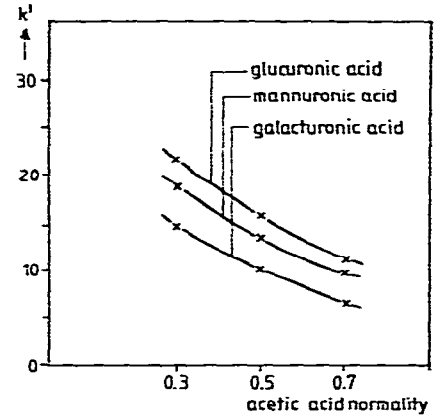


Fig. 1. Separation of a reference mixture of uronic acids (——) and an alginate hydrolysate (– – –) on a Nucleosil 10 SB or Zorbax SAX column. Eluent, 0.7 *N* acetic acid; flow-rate, 1.4 ml/min; column temperature, 40°C; detection, RI.

Fig. 2. Relationship between capacity factors (k') of uronic acids and acetic acid concentration in eluent. Conditions as in Fig. 1.

a gradual decrease in retention of the uronic acids with time; their lifetime is limited to about 6 months. The loss in retention time can be compensated for by lowering the acetic acid concentration in the eluent; this has no effect on the separation. Fig. 2 shows the relationship between the capacity factors (k') of the uronic acids and the acetic acid concentration, illustrating that in the concentration range studied the capacity factors are affected in the same way.

We were not able to separate the uronic acids on the LiChrosorb 10 NH_2 column or on the LiChrosorb 10 RP-18 column. Various systems as described for the oligomers were tested without success.

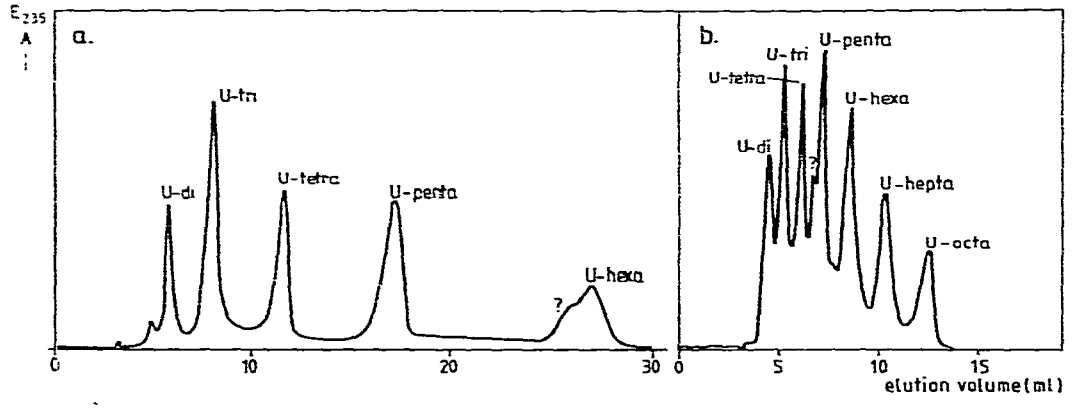


Fig. 3. Separation of unsaturated oligogalacturonic acids on a Nucleosil 10 SB or Zorbax SAX column. Flow-rate, 1 ml/min; column temperature, 40°C; detection, absorbance at 235 nm. (a) Separation of oligomers up to hexamers with 0.3 *M* sodium acetate buffer (pH 5.4) as eluent; (b) separation of oligomers up to octamers with 0.4 *M* sodium acetate buffer (pH 5.4) as eluent. U-di = unsaturated digalacturonic acid; U-tri = unsaturated trigalacturonic acid, etc.

Oligogalacturonic acids

The separation of unsaturated oligogalacturonic acids on Nucleosil 10 SB is shown in Fig. 3. The oligomers were eluted with sodium acetate buffers of pH 5.4 at a flow-rate of 1 ml/min and a column temperature of 40°C. With a 0.3 *M* buffer (Fig. 3a) oligomers up to hexamers were well separated in about 35 min, and with a 0.4 *M* buffer (Fig. 3b) oligomers up to octamers could be separated in about 14 min. Also in this system a decrease in retention times was observed owing to the instability of the ion exchanger. This could be compensated for by decreasing the sodium acetate concentration and/or the buffer pH. This is demonstrated in Fig. 4a and b, which show the influence of the buffer molarity and the buffer pH, respectively, on k' of unsaturated di- and trigalacturonic acid. In the pH range 6–8 the capacity factors were fairly constant: below pH 6 the k' values increased with decreasing pH. By increasing the molarity the difference in the capacity factors becomes smaller; at 0.4 *M* and pH 7.8 the k' values for the di- and trimer are almost identical.

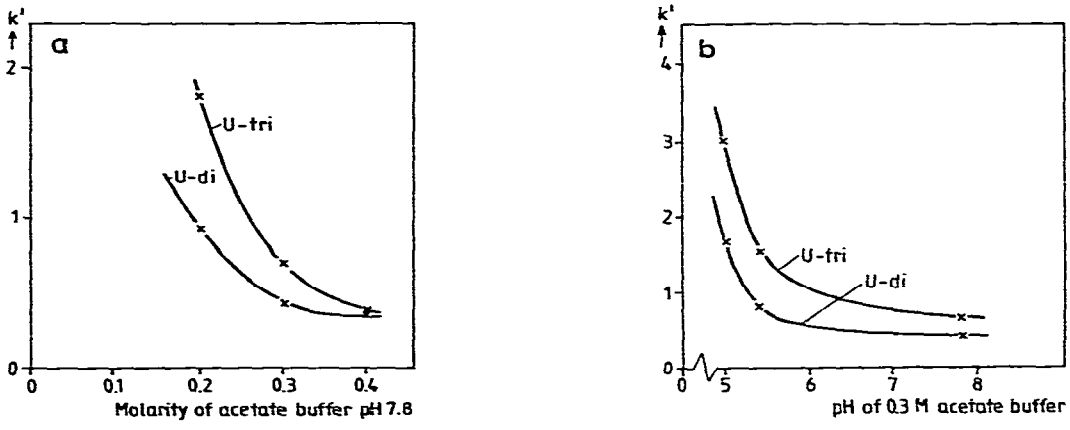


Fig. 4. Relationship between capacity factors (k') of unsaturated di-(U-di) and trigalacturonic acid (U-tri) and eluent composition. Conditions as in Fig. 3. (a) k' as a function of buffer molarity; (b) k' as a function of buffer pH.

Good separations were also obtained on the amino-bonded silica column under the following conditions: eluent, sodium acetate buffer, 0.04–0.11 *M*; pH range, between 4 and 7.5; flow-rate 1-2 ml/min; and temperature, 40°C. The influence of buffer molarity and buffer pH on the capacity factors of unsaturated dimer and trimer are shown in Fig. 5. By increasing the buffer molarity and/or the pH the retention of the oligomers on the column is decreased. This is demonstrated in Fig. 6a and b, which show respectively the separation of an enzyme digest (PAL) of pectate with 0.11 *M* sodium acetate (pH 7.5) as eluent and the separation of the PAL digest of esterified pectin [5 mg/ml pectin in sodium carbonate buffer (pH 6.9), containing 0.25 m*M* calcium chloride and 1 U/ml PAL[14], incubated for 20 h at 30°C] with 0.04 *M* sodium acetate (pH 5) as eluent. Under both conditions baseline separations were obtained; elution with 0.04 *M* sodium acetate (pH 5) gives, however, longer retention times and this permits the separation of partially esterified oligogalacturonides, which can be observed as three peaks between the peaks for unsaturated di- and trigalactu-

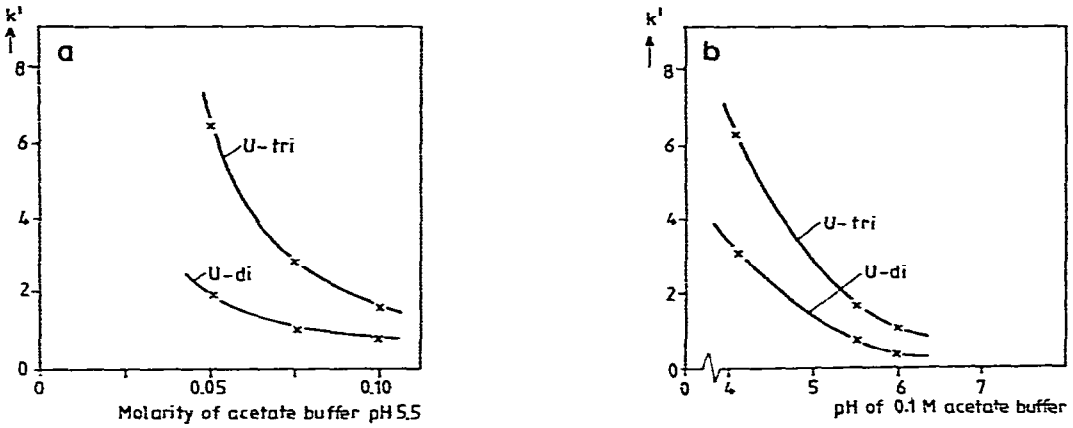


Fig. 5. Relationship between capacity factors (k') of unsaturated di(U-di) and trigalacturonic acid (U-tri) and eluent composition obtained for chromatography on a LiChrosorb 10 NH_2 column. Eluent, sodium acetate buffer; flow-rate, 1.5 ml/min; column temperature, 40°C; detection, absorbance at 235 nm. (a) k' as a function of buffer molarity; (b) k' as a function of buffer pH.

ronic acid. When these peaks were collected separately, saponified with alkali and re-chromatographed, it was found that each peak represented a mixture of unsaturated oligogalacturonides. It can be concluded that the mechanism involved in this separation is ion exchange; the system appeared to be very flexible.

Amino-bonded silica columns are widely used for the analysis of neutral sugars

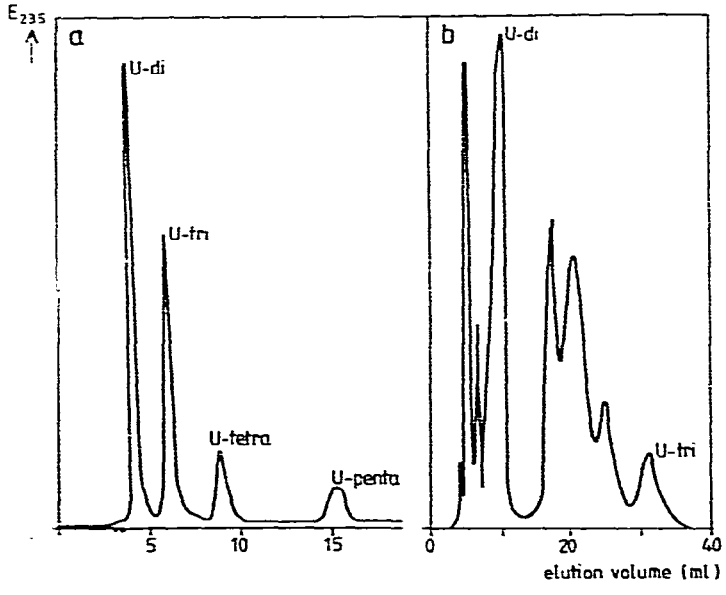


Fig. 6. Separation of unsaturated oligogalacturonic acids on a LiChrosorb 10 NH_2 column. Conditions as in Fig. 5. (a) Separation of enzyme (PAL) digest of pectate with 0.11 *M* sodium acetate buffer (pH 7.5) as eluent; (b) separation of enzyme (PAL) digest of esterified pectin with 0.04 *M* sodium acetate buffer (pH 5) as eluent.

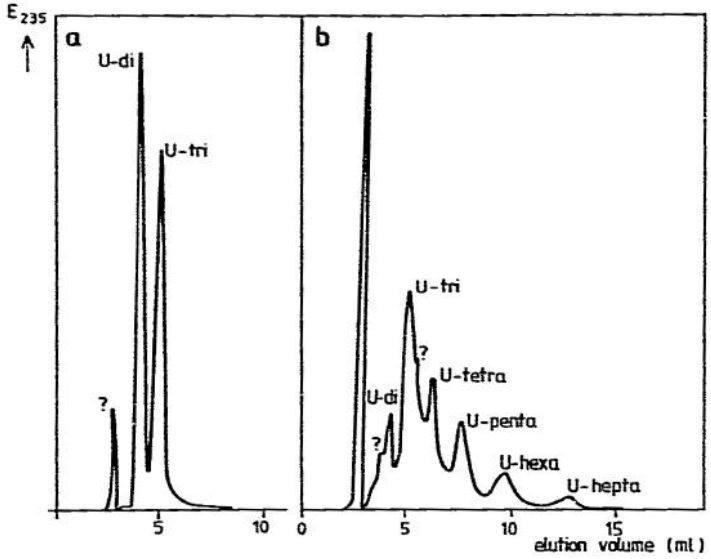


Fig. 7. Separation of unsaturated oligogalacturonic acids by ion-pair chromatography on a reversed-phase column (LiChrosorb 10 RP-18). Eluent, methanol–0.05 *M* phosphate buffer (pH 7) (30:70) containing 25 m*M* tetrabutylammonium bromide; flow-rate, 1 ml/min; column temperature, 40°C; detection, absorbance at 235 nm. (a) Separation of unsaturated di- and trigalacturonic acid; (b) separation of enzyme (PAL) digest of pectate.

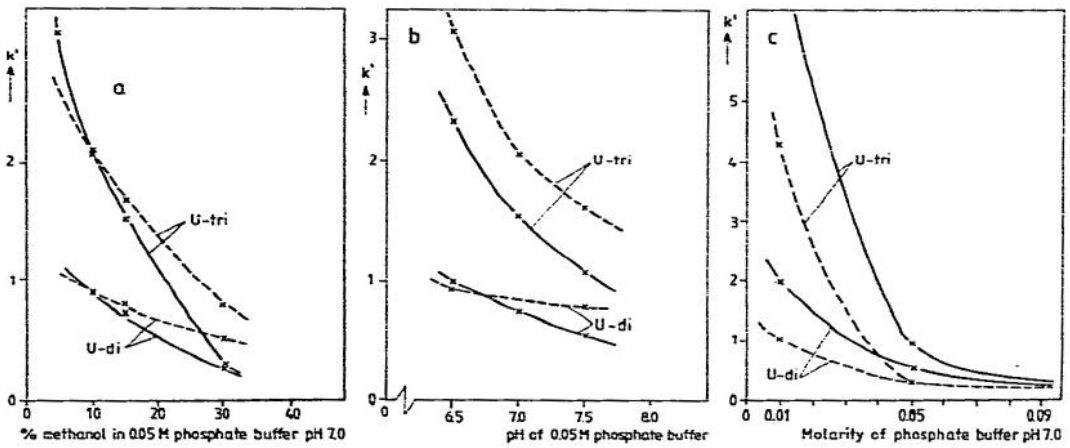


Fig. 8. Relationship between capacity factors (k') of unsaturated di- and trigalacturonic acid and eluent composition. Conditions as in Fig. 7. (a) k' as a function of methanol: buffer ratio at two tetrabutylammonium bromide concentrations; – – –, 0.025 *M*; ——, 0.005 *M*; (b) k' as a function of buffer pH at two methanol–buffer ratios; – – –, 20:80; ——, 10:90; (c) k' as a function of buffer molarity at two methanol–buffer ratios: – – –, 20:80; ——, 30:70.

with acetonitrile–water mixtures as eluent (carbohydrate column). It was found that in these applications the columns have a limited lifetime because of the instability of the packing material. In our system the column appeared to be more stable, and the small loss of efficiency could be compensated for by changing the buffer molarity or pH.

A third system tested was ion-pair chromatography on a reversed-phase column (LiChrosorb 10 RP-18). Unsaturated di- and trimer could be separated successfully as shown in Fig. 7a. The eluent was a mixture of methanol and 0.05 *M* phosphate buffer (pH 7) (30:70) containing 25 m*M* tetrabutylammonium bromide. The flow-rate was 1 ml/min and the column temperature was 40°C. Fig. 7b shows the chromatogram of a PAL digest of pectate in which two unknown peaks can be observed. The retention times of these peaks were very sensitive to small changes in the molarity and pH of the buffer. The optimal eluent composition can be derived from Fig. 8, which shows the influence of the methanol buffer ratio at two tetrabutylammonium bromide concentrations (a), the buffer pH (b) and the buffer molarity (c) on the capacity factors of unsaturated dimer and trimer. The column appeared to be very stable.

The separation of the normal oligogalacturonic acids in the three systems is shown in Fig. 9. Because these oligomers do not show UV absorbance, their separation was recorded with an RI detector which has a low sensitivity. Therefore, more concentrated samples (0.1–0.5%) had to be injected. The separation conditions depend on the age of the column, the intensity of the signal for non-retained com-

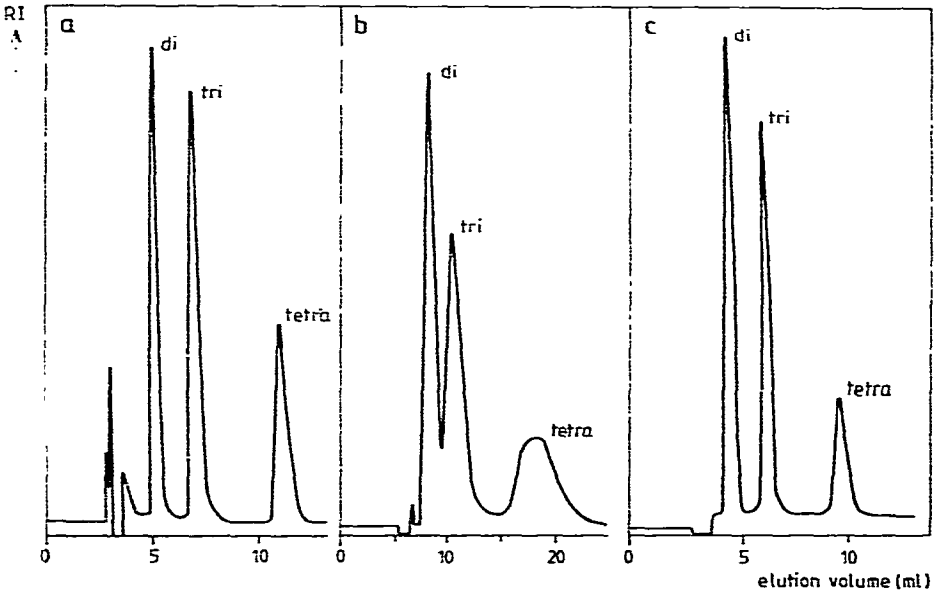


Fig. 9. Separation of normal oligogalacturonic acids on (a) a Nucleosil 10 SB or Zorbax SAX column; conditions as in Fig. 3a; detection, refractive index; (b) a LiChrosorb 10 NH_2 column; eluent, 0.075 *M* sodium acetate buffer (pH 5.5); flow-rate, 2 ml/min; column temperature, 40°C; detection, RI; (c) a LiChrosorb 10 RP-18 column; eluent, methanol–0.05 *M* phosphate buffer (pH 7) (10:90) containing 25 m*M* tetrabutylammonium bromide; flow-rate, 1 ml/min; column temperature, 40°C; detection, RI.

pounds in the sample which may disturb RI detection, the composition of the oligomer mixture and the desired separation.

DISCUSSION

The results show that four common uronic acids can be analysed by HPLC on strong anion exchangers (Nucleosil 10 SB, Zorbax SAX). As the occurrence of L-guluronic acid and D-mannuronic acid is restricted to the cell walls of brown marine algae, it is not a serious drawback for the analysis of plant polysaccharides that D-galacturonic acid and L-guluronic acid cannot be separated. In the analysis of gum samples that contain both pectins and alginates it is possible to degrade specifically either pectin or algin by specific enzymes such as pectic acid lyase or alginate lyase, thus omitting elaborate separation techniques. This system was used for the characterization and analysis of commercial alginate samples. In general, recoveries of around 60% for uronic acids were obtained, and the ratio between the amounts of mannuronic and guluronic acids for the preparations analysed varied between 0.6 and 3.9.

For the analysis of oligogalacturonic acids three systems were successfully tested using the UV absorbing unsaturated oligogalacturonic acids as reference compounds. These methods appeared to be rapid and versatile; conventional anion-exchange chromatographic methods, however, give better separations[4–9]. For saturated oligogalacturonides similar results were obtained; corresponding saturated and unsaturated oligomers cannot be separated completely, as can be derived from the results shown. However, the specific and sensitive UV absorbance of unsaturated oligogalacturonic acids makes it possible to distinguish between normal and unsaturated oligomers.

The HPLC methods presented here make it possible to analyse specifically pectins after enzymic degradation as oligomers with high recoveries (80–100%). This is in contrast to acid hydrolysis methods as described for alginates, which gave about 30% recovery as galacturonic acid. These methods are also helpful in the study of the action of pectin-depolymerizing enzymes and for the elucidation of the fine structure of pectin as demonstrated in Fig. 6b[16].

REFERENCES

1 O. Raunhardt, *Gas-chromatographische Untersuchungen an Uronsäuren und Ihren Umwandlungsprodukten, Dissertation E.T.H.*, Zürich, 1968, No. 4161.
2 T. M. Jones and P. Albersheim, *Plant Physiol.*, 49 (1972) 926.
3 E. Mergenthaler and H. Scherz, *Z. Lebensm.-Unters.-Forsch.*, 162 (1976) 159.
4 C. W. Nagel and T. M. Wilson, *J. Chromatogr.*, 41 (1969) 410.
5 C. Hatanaka and J. Ozawa, *J. Agr. Chem. Soc. Jap.*, 46 (1972) 417.
6 Y. K. Liu and B. S. Luh, *J. Chromatogr.*, 151 (1978) 39.
7 S. Johnson and O. Samuelson, *Anal. Chim. Acta*, 36 (1966) 1.
8 J. Spiro, *Anal. Biochem.*, 82 (1977) 348.
9 M. Sinner, M. H. Simatupang and H. H. Dietrichs, *Wood Sci. Technol.*, 9 (1975) 307.
10 G. J.-L. Lee and H. Tieckelmann, *J. Chromatogr.*, 195 (1980) 402.
11 S. R. Delaney, H. E. Conrad and J. H. Glaser, *Anal. Biochem.*, 108 (1980) 25.
12 J. U. Baenziger and M. Natowisz, *Anal. Biochem.*, 112 (1981) 357.
13 A. G. J. Voragen, *Characterization of Pectin Lyases on Pectins and Methyl Oligogalacturonates, Agric. Res. Rep. No. 780*, Pudoc, Wageningen, The Netherlands, 1972.

14 F. M. Rombouts, C. H. Spaansen, J. Visser and W. Pilnik, *J. Food Biochem.*, 2 (1978) 1.
15 A. G. J. Voragen, H. A. Schols and W. Pilnik, in G. O. Phillips, D. J. Wedlock and P. A. Williams (Editors), *Proc. Int. Conference on Gums and Stabilizers for the Food Indstry, Wrexham, Wales, July 13–17, 1981*, Pergamon Press, Oxford, in press.
16 J. A. de Vries, F. M. Rombouts, A. G. J. Voragen and W. Pilnik, *Carbohyd. Polym.*, submitted for publication.