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Analysis of oligouronates by reversed-phase ion-pair h.p.l.c.: Role of the mobile phase

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ABSTRACT

The separation of oligouronic acids has been performed by the h.p.l.c. ion-pair method on a C_{18} reversed-phase column using various divalent-cation salt solutions containing 0.05% dodecyltrimethylammonium chloride. Oligogalacturonic, oligoguluronic, and oligomannuronic acids have been studied. In each series, the separation is dependent on the ionic strength of the eluent. By changing the cation, it is possible to significantly modify the resolution, and the system has been shown to be very useful, not only in the studies of selective interaction between divalent cation and oligouronates, but also in structural studies of alginates.

INTRODUCTION

Liquid chromatography on bonded-phase silica gel and on ion-exchange resins has become a common and valuable method for the fractionation of neutral oligosaccharides, and several reports have been published[1–3]. Conversely, the high-performance liquid chromatography of oligouronic acids is more complex. Only a few papers of interest have appeared that principally deal with the analysis of oligogalacturonic acids[4–8]. Different systems based on anion-exchange[4,7,8], cation-exchange[6] or ion-pair chromatography[4,5] have been described for the separation of saturated and unsaturated oligogalacturonic acids. Good resolution has been obtained for a broad range of degree of polymerization (d.p) among oligomers the results depending on the specific procedure used. The best results have been recently reported by Hotchkiss and Hicks[8], with the separation of oligogalacturonic acids with a d.p. ranging from 2 to 50 by high-performance anion-exchange chromatography on pellicular anion-exchange resin stationary phases coupled with pulsed amperometric detection.

H.p.l.c. has also been used with success in analysis of alginate lyase-generated products, and separations of unsaturated oligomannuronans have been described[9–11]. In a recent paper[12] dealing with the structural characterization of alginates by liquid chromatography, we have proposed the use of divalent-cation salts in the eluent in an ion-pair method to analyze the composition of oligomers obtained by acid hydrolysis of heterogeneous blocks of alginates. The purpose of this paper is (*i*) to discuss the mechanism of separation involved on the basis of specific ionic interactions between divalent cations and oligouronides and (*ii*) to define the critical parameters involved in the separations.

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EXPERIMENTAL

Materials. — Chemicals were analytical grade. All aqueous solutions were prepared with distilled water. Alginates were commercial samples from Satia or Sobalg. Polygalacturonic acid from orange and pectinases (EC 3.2.1.15) from *Aspergillus niger* were obtained from Sigma Chemical Co.

Preparation of substrates. — Alginates were hydrolysed in three different blocks according to a procedure previously described[12]. The different oligosaccharides were obtained by hydrolysis of these blocks in hydrochloric acid (pH 3.5, 72 h, 100°) for oligoguluronic acids $(Gul A)_n$; 48 h for oligomannuronic acids $(Man A)_n$; and 6 h for heterogeneous blocks (Man A-Gul A). The oligogalacturonic acids $(Gal A)_n$ were obtained by degrading polygalacturonic acid with an endopolygalacturonase as described by Thibault[13]. Pectic acid (1 g) was dissolved in 500 mL of acetate buffer, pH 4.2. Enzyme digestion was initiated by addition of 20 units (1 unit = 1 μmol reducing end produced/min at pH 4.0 and 25°), and the reaction was continued for 1 h with stirring at 30°. The reaction was stopped by heat inactivation for 10 min at 100°. The solution was filtered and analyzed by gel-permeation chromatography. Each mixture of different oligouronic acid series was fractionated on a preparative scale using Bio-Gel P4 columns (4 × 100 cm) eluted with 0.05M $NaNO_3$ at a rate of 30 mL/h. Desalting was performed on a Bio-Gel P2 column (1.5 × 200 cm) eluted with water. Detection was achieved with a refractive-index detector. Each oligomer was characterized by ^{13}C-n.m.r. spectroscopy and by fast-atom bombardment mass spectroscopy.

^{13}C-n.m.r. spectroscopy. — The spectra were recorded at 60° with a Bruker AC 300 spectrometer operating in the Fourier-transform mode. The samples were dissolved in D_2O in a 0.5-cm diameter tube (2% w/v). Experiments were performed with ^{13}C- and broad-band proton-decoupling at 75 MHz under the following conditions: sweep width, 16.6 KHz; pulse width, 4 ms; acquisition time, 1.5 s on 8K data points; 4000 transients. Chemical shifts (p.p.m.) were measured relative to internal acetone.

Fast-atom bombardment mass spectrometry (f.a.b.m.s.). — Spectra were determined at 8 KV using argon gas in a quadripolar R-1010 C Nermag mass spectrometer model 2000, interfaced with a PDP 11/73 computer. The samples were generally dissolved in water and added to a drop of glycerol on a copper target.

H.p.l.c. of oligouronides. — A nucleosil C_{18} 5μm, prepacked column (4.6 × 250 mm) from S.F.C.C. (France) was used. Detection was achieved with an IOTA refractive-index detector (r.i.) in series with a polarimetric detector (Perkin-Elmer model 241, equipped with a microflow cell (100 mm pathlength, 80 μL volume)[5]. The retention of each oligomer is characterized by the capacity factor k', defined as the ratio $(v_e - v_s)/v_s$, (v_e, being the solute elution volume and v_s the elution volume of a simple salt).

RESULTS AND DISCUSSION

Let us first consider the different systems described in the literature and summarized in Table I. The different methods appear to be rapid and versatile, and whatever

TABLE I

Methods proposed for the fractionation of oligouronides

System	*Column*	*Oligouronates*	*Eluent*	*Elution mode*	*Detection*	*Ref.*
Strong anion-exchange columns	Nucleosil 10SB (Chrompack)	Oligogalacturonides and unsaturated oligomers	Sodium acetate buffers	isocratic	r.i. or u.v.	4
	Zobax SAX (Dupont)	Oligogalacturonides and unsaturated oligomers	Sodium acetate buffers	isocratic	r.i. or u.v.	4
	Partisil SAX 10 μm (Perkin–Elmer)	Unsaturated oligomers from alginates	Potassium phosphate buffers	linear concentration gradient	u.v.	10
	TSK DEAE 2-SW (Beckman)	Oligogalacturonides after derivization	Acetate buffers	linear concentration gradient	u.v.	7
	Carbopack PA1 Ion Pack 4S4A (Dionex)	Oligogalacturonides	Acetate or oxalate buffers	non-linear concentration gradient	p.a.d.	8
Weak anion-exchange columns	Lichrosorb 10 NH_2 (Merck)	Oligogalacturonides and unsaturated oligomers	Sodium acetate buffers	isocratic	r.i. or u.v.	4
	μ-Bondapak NH_2 (Waters)	Unsaturated oligomers from polyguluronic acid	Aq. ammonium formate	isocratic	u.v.	11
Cation-exchange columns	HPX-22H (Bio-Rad Labs)	Oligogalacturonides	0.005M sulfuric acid	isocratic	r.i.	6
Reversed-phase columns	Lichrosorb 10 RP-18 (Merck)	Oligogalacturonides and unsaturated oligomers	Mixture of methanol and phosphate buffer containing tetrabutylammonium bromide	isocratic	r.i. or u.v.	4
	μ-Bondapak C_{18} (Waters)	Oligogalacturonides	Aq. sodium nitrate containing 0.05% dodecyltrimethylammonium chloride	isocratic	r.i.	5
	μ-Bondapack C_{18} 8MB 10 μm (Waters)	Unsaturated oligomers from polymannuronic acid	10% acetonitrile–10mM tetrabutylammonium hydroxide–0.1M sodium phosphate, pH 6.5	isocratic	r.i.	9

the system used, the capacity factors for oligomers have been shown depending on the molarity and/or the pH of the eluent. If it is obvious that by increasing the ionic strength of the eluent, the retention of the oligouronides on the column is decreased, no result was found on the possible role of cations in the mobile phase. As ion-exchange columns were not convenient for this study, we chose to work with ion-pair chromatography on a reversed-phase C_{18} column. Isocratic elution was the only elution mode considered, and a detector based on optical rotation was adapted to the h.p.l.c. equipment and connected in series with the differential refractometer to suppress some problems of baseline drift sometimes observed by eluting with salts of divalent cations[12].

In a previous paper[5] we describe an eluent for the separation of oligogalacturonates. A 0.1N sodium nitrate solution containing 0.05% (v/v) dodecyltrimethylammonium chloride was shown to provide an interesting system for the resolution of the first five d.p.s. By increasing the ionic strength, a decrease in retention time was observed (Fig. 1), indicating that separations of higher d.p. are indeed feasible. Similar results are obtained with oligoguluronides and oligomannuronides as shown on Fig. 2.

The mechanism of retention in reversed-phase ion-pair chromatography has been the subject of considerable discussion in the literature[11–15]. We propose an ionic interaction between the uronate and the ion-pairing reagent. The pseudo neutral complex interacts by its alkyl chain with the alkyl chain of the C_{18} phase. An increase in the ionic strength (I) with a simple salt leads to both a decrease of the uronate dodecyltrimethylammonium form by ion-exchange and a decrease of retention time. Considering a

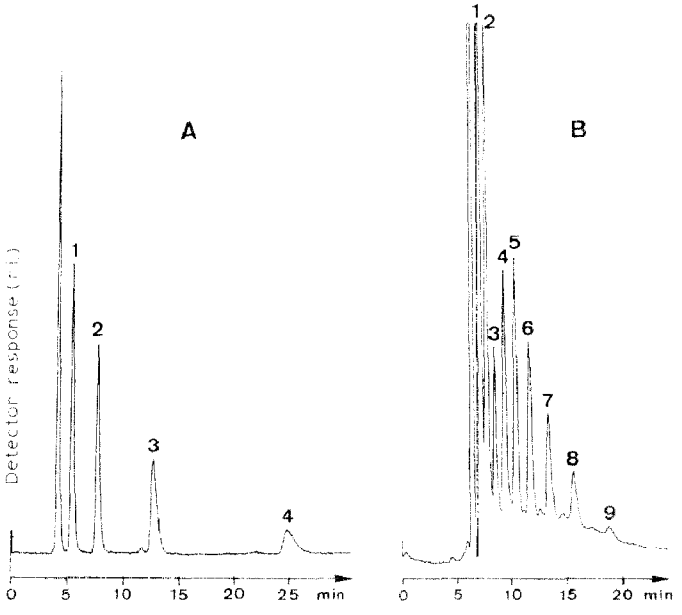

Fig. 1. Separation of oligogalacturonates. (A) eluent, 0.1M $NaNO_3$ + 0.05% (v/v) dodecyltrimethylammonium chloride; flow rate, 0.6 mL/min. (B) eluent, 0.2M $NaNO_3$ + 0.05% (v/v) dodecyltrimethylammonium chloride; flow rate 0.4 mL/min; detector, refractive-index (r.i.). The degree of polymerization (d.p.) is indicated by numbers over the peaks.

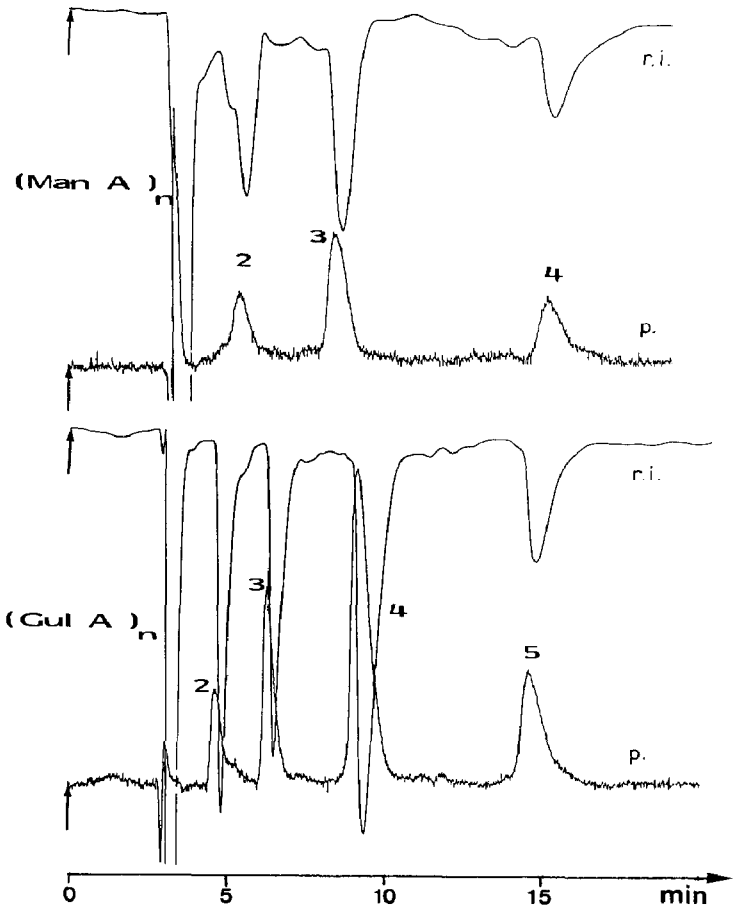

Fig. 2. Separation of alginate oligomers. $(Gul\ A)_n$ oligoguluronates; $(Man\ A)_n$ oligomannuronates. Experimental conditions: eluent, 0.2M $NaNO_3$ + 0.05% (v/v) dodecyltrimethylammonium chloride; flow rate, 0.6 mL/min; detectors: polarimeter (p.) and refractive-index detector (r.i.). The degree of polymerization (d.p.) is indicated by numbers over the peaks.

particular d.p. in the three different series, there is a similar behavior in regard to ionic strength, but a great difference in capacity factor is observed with the given conditions of elution. Fig. 3 shows the relationship between the capacity factor and different d.p. of the uronate series in 0.15N NaCl eluent. There is considerable similarity among capacity factors of oligoguluronates and oligogalacturonates; however, the values are higher with the oligomannuronates. This difference may be due, not only to the difference in the dissociation of the complex controlled by the ionic strength, but also to the difference in the value of the selectivity coefficients for the dodecyltrimethylammonium–sodium ion-exchange reaction.

Considering the original polysaccharides (except the polymannuronate), it is well known that the polyguluronate and the polygalacturonate are able to form gels in the

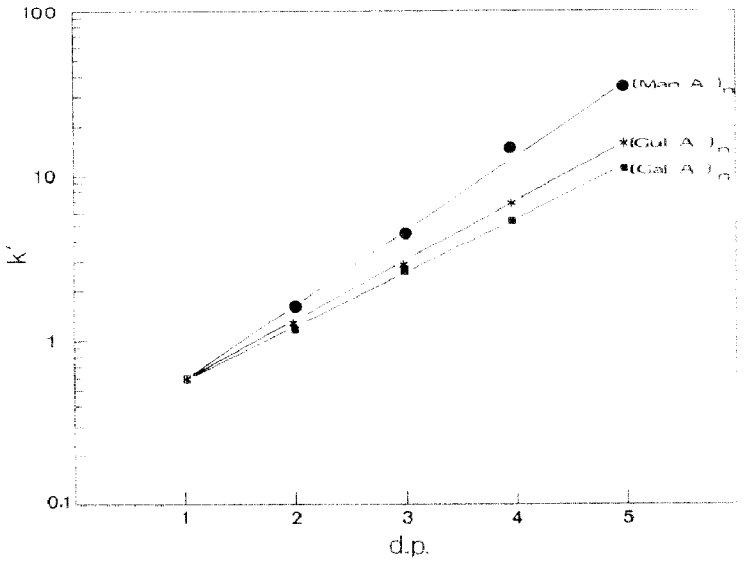

Fig. 3. Dependence of the capacity factor k' on the d.p. for the three series of uronates. Eluent: 0.15N NaCl +0.05% (v/v) dodecyltrimethylammonium chloride. (Man A)$_n$ oligomannuronates; (Gul A)$_n$, oligoguluronates; (Gal A)$_n$ oligogalacturonates.

presence of divalent cations such as calcium, due to the remarkable affinity of the two latter polyuronates for calcium ions[17,18]. This behavior has been related to their polymeric nature[19]; nevertheless, we thought it would be possible to modify the capacity factors of oligomers by eluting with divalent-cation salts in place of sodium nitrate.

Calcium, barium, and magnesium chlorides were assessed as eluents, and these are compared in Fig. 4 with results obtained using sodium chloride. As was expected, whatever the cation, a small difference is observed in the mannuronate series[20]. Except with barium, the values of the slopes are very similar, indicating a lack of selectivity. The difference in k' for a particular d.p. is related to the difference in the stability constant of the oligouronide–dodecyltrimethylammonium complex in the presence of the particular cation. In the same way, the capacity factors of oligogalacturonides are slightly modified in this range of d.p., which is in agreement with the literature data[19]. The activities of the calcium and barium ions decrease slowly in a continuous manner with increasing degree of polymerization, and purely electrostatic interactions of counterions with the carboxyl group of oligomers are involved[20,21]. On the other hand, this effect is very important on oligoguluronates, and it is therefore reasonable to assume that cations such as calcium or barium interact specifically with low d.p. (< 10 units). These results differ significantly from the literature data assuming no original behavior in selectivity up to d.p. 18 with oligoguluronides[22]. With the polymer, the increase of the affinity for the calcium or barium cation has been related to a cooperative association according to the "egg-box" model[23]. Considering this model, at least four units are necessary, and a change in the slope of the plot k' *vs.* d.p. would be observed. In the range

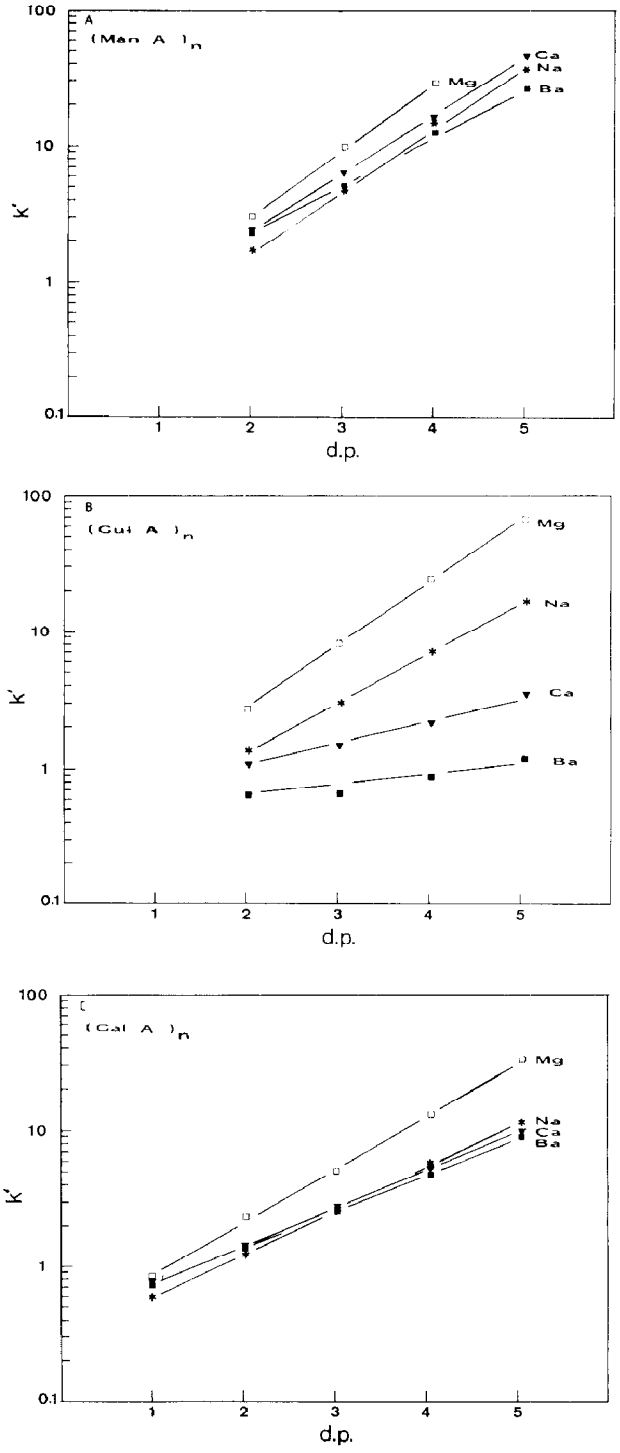

Fig. 4. Influence of the cations on the elution of the three series considered in a 0.15 ionic strength of eluent (A) $(Man\ A)_n$, oligomannuronates; (B) $(Gul\ A)_n$, oligoguluronates; (C) $(Gal\ A)_n$, oligogalacturonates.

of d.p.s studied, there is no change in the behavior, so the high selectivity is not due to the length of the oligomer, but must be related to the intrinsic properties of the guluronic acid unit[24]. From these results, we can assume a great difference in the behavior of galacturonides and guluronides and a long sequence of guluronate units may not be essential for alginate gelification. In regard to divalent cations, information about the order of selectivity can also be deduced. Thus the lower the selectivity value, the higher the k' value, which corresponds to a shift in the equilibrium shown below:

Oligouronide–dodecyltrimethylammonium ⇄ oligouronide–cation.

In all three series, the k' values of magnesium are higher. Assuming a similarity in affinity of oligouronides for sodium and magnesium[25,26], the effect of the divalent cation

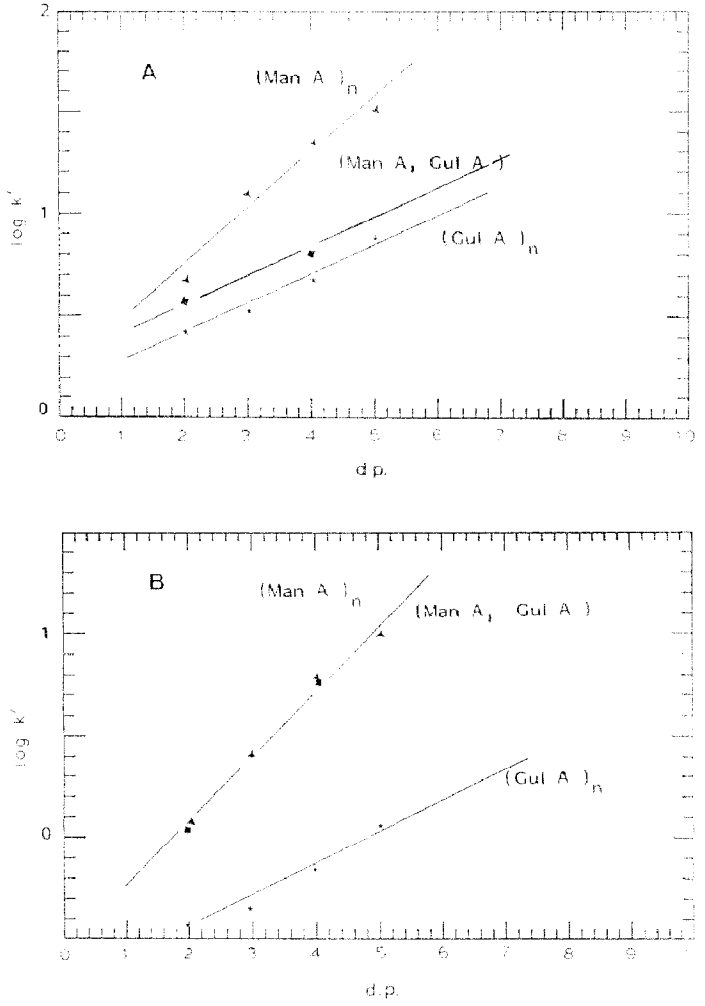

Fig. 5. Influence of the divalent cation on the elution of alginate oligomers. Experimental conditions: ionic strength, 0.2; flow rate, 0.6 mL/min; detector, polarimeter. (A) eluent, $CaCl_2$ + 0.05% dodecyltrimethylammonium chloride; (B) eluent, $BaCl_2$ + 0.05% dodecyltrimethylammonium chloride.

on the stability of the complex is less important than that of a monovalent cation. Due to a higher affinity of calcium and barium, the stability of the complex decreases, as do the k′ values. The role of calcium and barium is closely similar in the case of the galacturonides, but a great difference is observed in the case of the guluronides, and the selectivity increases in the following order: Mg < Ca < Ba. The same classification has also been proposed by Haug and Smidsrod[25,26] for alginates.

Among problems of ion selectivity, another of interest is the study of the composition of oligomers resulting from hydrolysis of the alternating structure (Man A–Gul A). In Fig. 5, the different capacity factors for alginate oligomers obtained by eluting the column with $CaCl_2$ and $BaCl_2$ are shown. It is interesting to note the behavior of these types of oligomers. When $CaCl_2$ is used (Fig. 5a), the binding properties of calcium are consistent with the results reported above. It is possible to fractionate the (Man A-Gul A) oligomers from the (Man A) oligomers due to the selectivity of the guluronate unit for Ca^{2+}. With $BaCl_2$ (Fig. 5b), this high selectivity is lost, and we can observe the same behavior with the (Man A-Gul A) oligomers and the (Man A) oligomers. Thus, it seems reasonable to conclude that there is a different mechanism governing the interactions of

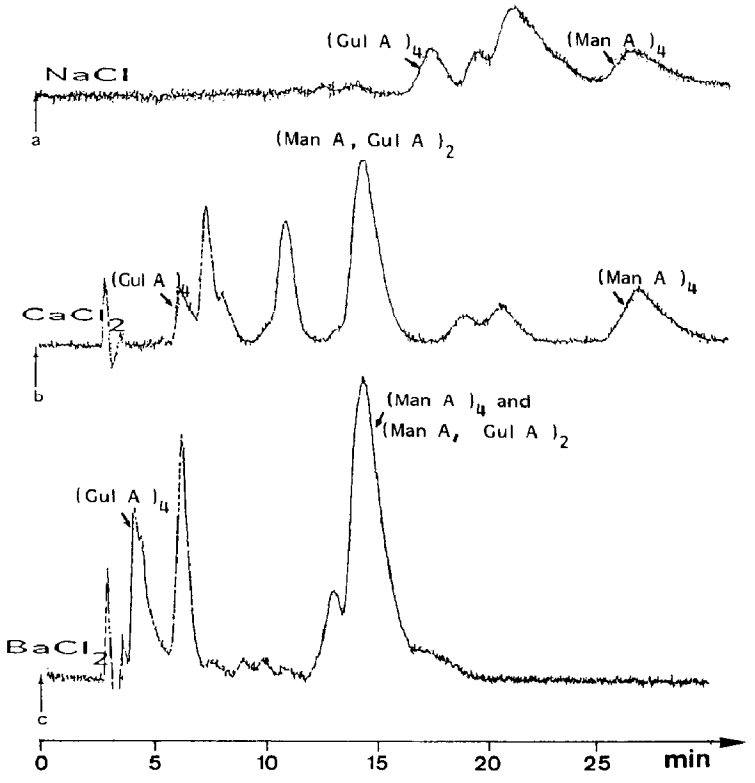

Fig. 6. Chromatograms showing the influence of the nature of divalent cations on the fractionation of tetramers obtained by acid hydrolysis of alternated alginate blocks. Ionic strength, 0.2; flow-rate, 0.6 mL/min; detector, polarimeter.

the calcium and barium cations. The fixation of barium in enhanced by a regularity of the structure whatever the sequence $(Gul A)_n$ or $(Man A)_n$. These particularities are very useful in the study of a particular d.p. isolated from acid or enzymic hydrolysis of alginates, as shown on the chromatograms of Fig. 6. By a judicious choice of cation, it may be possible to resolve a complex mixture of different structures[12-14].

CONCLUSIONS

The technique of reversed-phase ion-pair h.p.l.c. allows rapid separation of oligouronates through the appropriate choice of ionic strength and type of eluent. With a monovalent-cation salt, the separation is primarily controlled by electrostatic forces, and poor separation is obtained among the different series considered. By using divalent-cation salts, specific ionic interactions can be generated depending on the oligouronate structure and the nature of the cation. Ion-pair chromatography then becomes a powerful approach for the study of cation binding to oligomeric fragments of pectin and alginates. A similar behavior of oligogalacturonides and oligomannuronides is observed with a slight and continuous increase of the affinity for the cation when Ca^{2+} and Ba^{2+} are used. The interactions are important with oligoguluronides, and a selectivity between the cations can be deduced. For the range of d.p. studied (1→6), these interactions do not depend on the length of the oligomer but are attributed to an intrinsic properties of the uronate unit. Nevertheless, the role of the barium ion is peculiar and must be related to a structural factor. The affinity of Ba^{2+} for the carboxylic group increases with the regularity of the oligomer structure.

Independent of the ion-selectivity studies, knowledge of the different parameters governing the elution of the oligouronides allows one to propose an interesting chromatographic system. By adjusting the ionic strength and the type of cation, it is now possible to resolve complex mixtures. The method has been shown to be very useful in the study of oligomers obtained by the acid hydrolysis of heterogeneous blocks in alginate chains and should also provide a valuable tool for determining the action pattern of pectin- and alginate-degrading enzymes.

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