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Study of sugar acids separation by high-performance anion-exchange chromatography–pulsed amperometric detection using alkaline eluents spiked with Ba^{2+}, Sr^{2+}, or Ca^{2+} as acetate or nitrate salts

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Abstract

The influence of Ba^{2+}, Ca^{2+} and Sr^{2+} ions on the anion-exchange chromatographic separation of some carboxylated sugar acids such as D-gluconic acid, *N*-acetylneuraminic acid, muramic acid and D-galacturonic, D-mannuronic and D-glucuronic acids was investigated. The chromatographic technique was coupled with pulsed amperometric detection using gold as a working electrode. Since acidic carbohydrates are strongly retained on the anion-exchange column, acetate and nitrate as counterions were used to regulate retention within 30 min. The addition of alkaline-earth metal ions at a millimolar concentration to the alkaline eluents does impart a noticeable decrease in the retention, especially for *N*-acetylneuraminic and uronic acids. Complexation of these compounds with free divalent metal ions presumably occurs in the alkaline eluent. The extent of decreased retention is related to each divalent metal ion, and a good correlation was found between the retention modulus ($\eta = k'/k'_o$) and the stability constant of each sugar–metal ion complex. As expected, calcium ion induced a slightly greater retention compared to strontium and barium ions, and this is consistent with the fact that alduronate–calcium complexes are relatively more stable ($\eta < 1.00$). We demonstrate also that upon the addition of Ba^{2+} and Sr^{2+} ions in the alkaline eluent, but the same cannot be claimed for calcium-containing eluents, there is a gain in sensitivity for all compounds investigated. The increment on the peak height when the column was eluted with NaOH spiked with $Ba(NO_3)_2$ was generally higher (5–75%) than that with $Ba(OAc)_2$.

Keywords: Mobile phase composition; Electrochemical detection; Carbohydrates; Organic acids; Sugar acids; Alkaline-earth cations

1. Introduction

Acidic carbohydrates, including D-gluconic acid, *N*-acetylneuraminic acid (NANA), muramic acid and D-galacturonic, D-mannuronic and D-glucuronic acids (Fig. 1) are implicated in a wide variety of cellular interactions. Besides, they are largely present in many plant and animal polysaccharides, representing very often the major carboxylated-sugar components. Uronic acids, for instance, are the most important sugar compounds of pectins, gums and alginates, and several analytical methods have been proposed for

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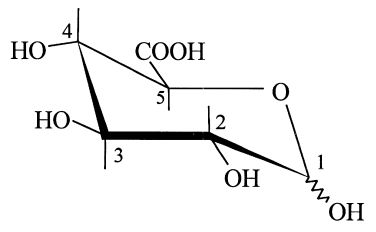

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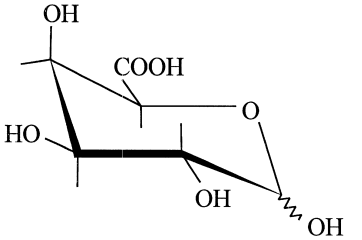

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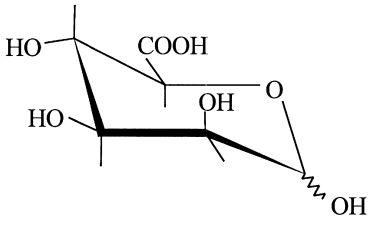

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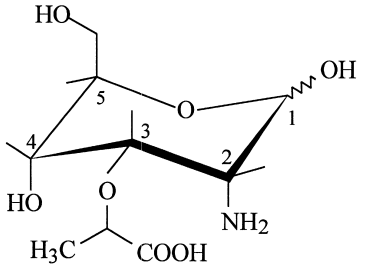
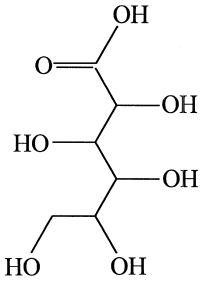
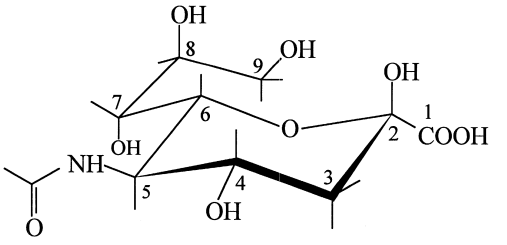


Muramic Acid



D-Gluconic Acid



N-Acetyl-β-Neuraminic Acid

Fig. 1. Structures of sugar acids studied.

their quantitative determination [1–9]. NANA is the most common sialic acid present in the human serum; sialic acids are fundamental components of sialylglycoproteins and sialylglycolipides [10–12]. Muramic acid is an amino sugar specific of bacterial peptidoglycan [13,14], which has been used as a biomarker of bacteria in various matrices of environmental and clinical relevance [15,16].

Alduronate ions, like all α-hydroxy acid anions, form much stronger complexes with cations than neutral sugars because carboxylate group is able to chelate together to the oxygen atom present on the anomeric cycle. The earlier works on complexes of metal ions with sugar molecules were summarised in the fine review of Angyal [17]. Various analytical techniques have been employed in the studies of the

intrinsic complexing ability of carbohydrates and related compounds, especially nuclear magnetic resonance (NMR), ligand-exchange chromatography and thin-layer chromatography. High-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) is nowadays recognised as a very selective and sensitive technique for the analysis of neutral and acidic carbohydrates [17–25]. In our laboratory, the effect of Ba^{2+}, Sr^{2+} and Ca^{2+} on the separation and detection of neutral sugars and alditols by HPAEC–PAD has been recently investigated [26–28]. Our main aim was the removal of carbonate ions from sodium hydroxide eluents through the addition of barium, strontium or calcium ions, and consequent formation of insoluble salts in the eluent bottle. Yet, we noted that residue free divalent cations in the mobile phase positively affect both the sensitivity of detection and the separation of sugar molecules [29]. While the last effect was ascribed to their selective complexing interactions with certain carbohydrates, the electrocatalytic oxidation process on the gold electrode surface appeared to be positively influenced by presence of barium and strontium ions. Calcium ions exhibited, however, an adverse effect on the anodic currents.

Here, we describe the influence of barium, strontium and calcium ions on the HPAEC–PAD analysis of some relevant carboxylated sugar acids. Note that complex formation between cations and neutral carbohydrates is weak, whereas the ease of complexation of acidic carbohydrates is recognised. Indeed, gels formed by the interaction of Ca^{2+} with alginates are largely exploited in biotechnology to immobilise cells [30] and uronic acids present in plant cells activate the solubilisation of metal ions through complex formation [4,31]. Hence, because sugar acids are strongly retained on the anion exchanger stationary phase, additional counterions were added to the sodium hydroxide mobile phase. Nitrate and acetate ions were used as they posses much higher elution strength compared to hydroxide, and are PAD-inactive. For a direct comparison between chromatographic data, all separations were carried out under isocratic conditions. The analytical implications of the degree of complex formation and selectivity of complexation have been also addressed.

2. Experimental

2.1. Chemicals

Sodium hydroxide, 50% (w/w) solution in water (1.515 g ml^{-1}), $Ba(OAc)_2$ 99%, $Sr(OAc)_2$ 99.995%, $Ca(OAc)_2 \cdot H_2O$ 99.99%, $NaNO_3$ 99+%, $Ba(NO_3)_2$ 99+%, $Sr(NO_3)_2$ 99+%, $Ca(NO_3)_2 \cdot 4H_2O$ 99%, *N*-acetylneuraminic acid were purchased from Aldrich (St. Louis, MO, USA), NaOAc 99% was from Carlo Erba (Milan, Italy), D-galacturonic acid 99% and D-glucuronic acid >97% were from Fluka (Buchs, Switzerland), D-mannofuranono-6,3-lactone, D-gluconic acid >99% and muramic acid >98% were from Sigma (Steinheim, Germany) and were used as received. Doubly distilled, deionized water was used throughout for preparing solutions. Sodium hydroxide solutions used as the eluents were prepared by diluting of a carbonate-free 50% (w/w) NaOH solution in water, previously filtered with a 0.45-μm membrane and degassed for 30 min with N_2 gas. The exact concentration of hydroxide ions in the mobile phase was determined by titration with a standard solution of hydrochloric acid and phenolphthalein as indicator. Stock solutions of sugars were prepared in pure water and were stabilised with 0.1% sodium azide to prevent microbial growth. Sugar acid standard solutions to be injected were prepared fresh daily by dilution of the stock solutions.

2.2. HPAEC–PAD

Sugar acids analysis was performed using a metal-free isocratic pump (Dionex, Sunnyvale, CA, USA) Model IP20, a pulsed amperometric detector (Model ED40), and a metal-free rotary injection valve with a 10-μl injection loop. An anion-exchange column, Dionex CarboPac PA1 (250 mm×4 mm I.D.) coupled with a guard CarboPac PA1 column (50 mm×4 mm I.D.) was used for the separations. The flow-through detection cell (Dionex) contained a 1.0-mm diameter gold working electrode and a Ag/AgCl reference electrode with the titanium cell body serving as the counter electrode. Acquisition and processing of chromatographic data were done by a personal computer equipped with the Kontron PC Integration Pack software (Kontron Instruments, Milan, Italy). The pulsed amperometric detector

settings were as follows: (i) $E_{OX}=+0.80$ V ($t_{OX}=$ 180 ms), $E_{DET}=+0.20$ V ($t_{DEL}=200$ ms, and $t_{INT}=$ 240 ms), and $E_{RED}=-0.30$ V ($t_{RED}=360$ ms). The response time was set to 1 s, and the current signal was integrated in nanocoulombs (nC) during the t_{INT} sampling period. All experiments were carried out at ambient temperature with isocratic elution using a flow-rate of 1.0 ml min^{-1}. Sodium hydroxide eluents were kept in plastic bottles, and a nitrogen headspace was maintained on the solutions with a Dionex eluent organiser (EO1). The alkaline mobile phases modified by the addition of barium, strontium or calcium salts were prepared some hours before using the eluent solution and stored under nitrogen gas. As already described, it is strongly recommended to make the addition of divalent ions the day before using the eluent solution in order to prevent wear of the piston and piston seals and injection valve rotor seals as well [26].

2.3. Evaluation of chromatographic data

The capacity factor, k', was calculated according to the expression $k'=(t_R-t_M)/t_M$, where t_R is the retention time and t_M is the column dead time, measured from the front disturbance in the chromatogram. According to Horváth et al. [32] the retention modulus, η, was evaluated as the ratio between the capacity factor of the metal ion–sugar complex (k') and that of the uncomplexed sugar acid (k'_o).

3. Results and discussion

3.1. Effect of Ba^{2+}, Sr^{2+} and Ca^{2+} using nitrate as a counterion

Owing to the high affinity of sugar acids toward the stationary phase, the separation by anion-exchange chromatography requires stronger solution conditions than those employed with neutral sugars. This is generally accomplished by adding nitrate, or more often acetate counterions to the sodium hydroxide mobile phase. Recently, Wong and Jane [33] reported the use of nitrate as a ''pusher'' anion in the HPAEC–PAD determination of debranched amylopectins. The nitrate ion is able to interact very strongly with the anion-exchange sites, so drastically reducing the retention of those compounds having high affinity with the stationary phase. In our experiments, the employment of 100 m*M* NaOH+4 m*M* $NaNO_3$ (control) as eluent allowed the isocratic separation in less than 30 min of gluconic acid, NANA, muramic acid and uronic acids (Fig. 2A). Note that the retention time of galacturonic acid (peak 4), which is the more weakly retained compound among uronic acid, is greater than 1 h using 200 m*M* NaOH as eluent, thus making the isocratic elution not very useful for practical applications.

Marked differences were found when alkaline-earth metal ions were added to the alkaline eluent. Typical isocratic separations obtained with 100 m*M*

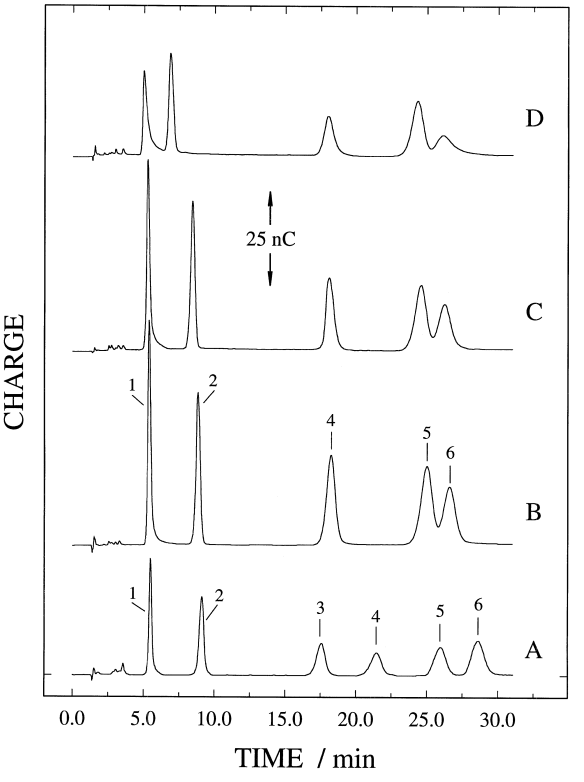


Fig. 2. Isocratic separations of sugar acids by HPAEC–PAD using the following eluents: (A) 100 m*M* NaOH+4 m*M* $NaNO_3$ (control), (B) 100 m*M* NaOH+2 m*M* $Ba(NO_3)_2$, (C) 100 m*M* NaOH+2 m*M* $Sr(NO_3)_2$, and (D) 100 m*M* NaOH+2 m*M* $NaNO_3$+1 m*M* $Ca(NO_3)_2$. Peaks: (1) D-gluconic acid, (2) *N*-acetylneuraminic acid, (3) muramic acid, (4) D-galacturonic acid, (5) D-glucuronic acid, (6) D-mannuronic acid, at 100 μ*M* each. CarboPac PA1 plus guard column; flow-rate: 1.0 ml $min;^{-1}$ loop: 10 μl.

NaOH upon the addition of 2 m*M* $Ba(NO_3)_2$, and 2 m*M* $Sr(NO_3)_2$ are illustrated in Fig. 2B and C, respectively. With regard to Fig. 2D, it was obtained with 100 m*M* NaOH+2 m*M* $NaNO_3$+1 m*M* $Ca(NO_3)_2$ as mobile phase. We employed a relatively lower concentration of calcium because the solubility of $Ca(OH)_2$ in the alkaline solution is lower than that of the corresponding barium and strontium hydroxides (the solubility products, pK_s, in water at 25°C of $Ca(OH)_2$, $Sr(OH)_2$, and $Ba(OH)_2 \cdot 8H_2O$ are 4.2, 3.8 and 1.6, respectively [34]). In all these cases, the divalent ions are present in large excess with respect to the sugar acids (i.e., 100 μ*M* injected with a loop of 10 μl). Indeed, we have recently reported that the concentration of free barium in a sample of alkaline eluent is about 0.5–0.7 m*M* [26]. The capacity factors (k') evaluated from each chromatogram, along with that of muramic acid, are summarised in Table 1; for comparison the k'_o values for the control eluent are also reported. The isocratic mode of separation was chosen to isolate the sole effect of cations, and to make a direct comparison with the experiments accomplished with acetate (see below). Although the elution order stayed the same upon varying the nature of the divalent ion, the chromatographic impact of barium, strontium and calcium ions can be effectively appreciated on the basis of two main effects. The retention of each investigated compound is decreased, and the signal response of almost all compounds is enhanced. This last effect will be described in more detail in the next section. Data on retention times may be considered as unexpected because the nitrate content is the same in all chromatograms reported in Fig. 2. As carbonate depletion occurs upon the addition of each metal ions, instead of a general decrease of the retention, there should be a more or less pronounced increase of the retention times [26]. Of more importance is that the lowering of retention is not homogeneous among all sugar acids, but its extent is related to each compound. For instance, the decrease of retention is more pronounced for mannuronic acid compared to glucuronic acid (see Table 1), with consequent less resolved peaks. Moreover, muramic acid exhibits a slight variation of the retention time, while among uronic acids the retention of galacturonic acid is strongly affected, leading to overlapped peaks. For this reason, muramic acid was not added to the standard mixture of the chromatograms illustrated in Fig. 2B–D.

A plausible explanation for the behaviour of carboxylated sugar acids is presumably related to complexation of divalent metal ions in the alkaline solution. Since complexation gives rise to positive charge around the metal nucleus of the complexes, it causes a reduction in total negativity. Consequently there is a general decrease of retention, and the difference in the ease of complexation is at least a cause of difference in retention. Table 2 reports for each compound the retention modulus (η) after the addition of Ba^{2+}, Ca^{2+} or Sr^{2+} to the mobile phase. As defined by Horváth et al. [32], the retention modulus is the ratio between the capacity factors of the metal ion–sugar complex (k') and the uncomplexed sugar acid (k'_o), both measured under otherwise identical elution conditions. Note that whether complexation of sugar compounds occurs, η values

Table 1
Summary of capacity factors of sugar acids in nitrate-containing alkaline eluents[a]

	Gluconic acid	NANA	Muramic acid	Galacturonic acid	Glucuronic acid	Mannuronic acid
				k'_o		
100 m*M* NaOH+4 m*M* $NaNO_3$	2.94	5.56	11.64	14.45	18.10	19.60
				k'		
100 m*M* NaOH+2 m*M* $Ba(NO_3)_2$	2.88	5.34	11.60	12.14	17.07	18.20
100 m*M* NaOH+2 m*M* $Sr(NO_3)_2$	2.78	5.06	11.29	11.95	16.69	17.86
100 m*M* NaOH+1 m*M* $Ca(NO_3)_2$ +2 m*M* $NaNO_3$	2.58	3.92	11.09	11.93	16.48	17.78

[a] Dionex CarboPac PA1 plus guard column; flow-rate, 1.0 ml min^{-1}; back pressure, 98 bar; t_M=1.39 min.

Table 2
Effect of barium, strontium and calcium on the retention modulus of sugar acids in nitrate-containing alkaline eluents[a]

	Gluconic acid	NANA	Muramic acid	Galacturonic acid	Glucuronic acid	Mannuronic acid
η_{Ba}	0.980	0.960	0.997	0.840	0.943	0.929
η_{Sr}	0.946	0.910	0.970	0.827	0.922	0.911
η_{Ca}	0.878	0.705	0.953	0.826	0.910	0.907

[a] Retention modulus evaluated as $\eta = k'/k'_o$ using the eluent solutions reported in Table 1.

have to be smaller than unity. Among alduronate ions, galacturonate exhibits the smaller retention modulus, 0.840, 0.827 and 0.826 in the presence of barium, strontium and calcium ions, respectively, thus suggesting the formation of relatively stable complexes, followed by mannuronate and glucuronate (see Table 2). This agrees well with the previous findings based on electrophoretic data reported by Bettler et al. [35]. *N*-Acetylneuraminic acid, in addition to a carboxylate group is characterised by several hydroxyl groups available for complex formation [17]. Indeed, especially in the presence of calcium the retention modulus is relatively low (0.705), thus confirming our interpretation of complex formation during the elution run. There are no reported data concerning complexation of gluconic acid with alkaline-earth cations. Our results show that this compound gives rise to weak interactions with Ca^{2+} and to a lesser extent with Ba^{2+} and Sr^{2+} because a more moderate decrease of retention in the presence of these last two ions was observed. Similar considerations might explain retention modulus close to unity for muramic acid, which possess a structure less favourable to complexation than alduronate ions. Apparently, muramic acid behaviour is similar to that observed previously for some carbohydrates, like ribose, allose and talose, analysed by HPAEC–PAD with a mobile phase modified with Ba^{2+} [26]. These compounds exhibited a constant retention time upon addition of barium, while the other investigated carbohydrates were comparably more retained because of the depletion of carbonate ions in the eluent occurred. The formation of weak complexes between Ba^{2+} and ribose, allose and talose, was invoked to explain their retention only slightly affected; these sugars possess, indeed, sequences of hydroxyl groups favourable to complexation [17]. Most likely, the unchanged retention of muramic acid in the presence of alkaline-earth metal ions suggests the formation of complexes as weak as those formed with ribose, allose and talose.

3.2. Effect of Ba^{2+}, Sr^{2+} and Ca^{2+} using acetate as a counterion

The effect of barium, strontium and calcium on the chromatographic separations of carboxylated sugar acids using acetate as the counterion was almost analogous to that described above for nitrate. Acetate is the most convenient anion used to increase the elution strength of sodium hydroxide solutions for the separation of di- and polysaccharides by HPAEC [18]. The acetate ion is weaker than nitrate, and to achieve a rapid elution has to be used at relatively higher concentrations (50–500 m*M*). Thus, unlike nitrate, the retention behaviour in the presence of acetate offers the advantage of being amenable to fine-tuning retention, thereby allowing for the optimisation of the sugar separation.

Fig. 3A illustrates the separation of a standard mixture of sugar acids using 100 m*M* NaOH+75 m*M* NaOAc as the eluent, while the corresponding k'_o values are reported in Table 3. The effect of Ba^{2+} and Sr^{2+} ions can be noted from the inspection of chromatograms in the same Figure. The following elution conditions were employed: 100 m*M* NaOH+71 m*M* NaOAc plus 2 m*M* $Ba(OAc)_2$, or plus 2 m*M* $Sr(OAc)_2$, Fig. 3B and C, respectively, while the eluent of Fig. 3D was 100 m*M* NaOH+73 m*M* NaOAc+1 m*M* $Ca(OAc)_2$. For comparison of chromatograms B, C and D with A (control) the evaluated capacity factors (k') are collected in Table 3. In the presence of divalent ions, there is a noticeable decrease of retention, which is more pronounced for galacturonic acid, followed by mannuronic and glucuronic acids. Again, the present data may be interpreted as being due to complex formation in the

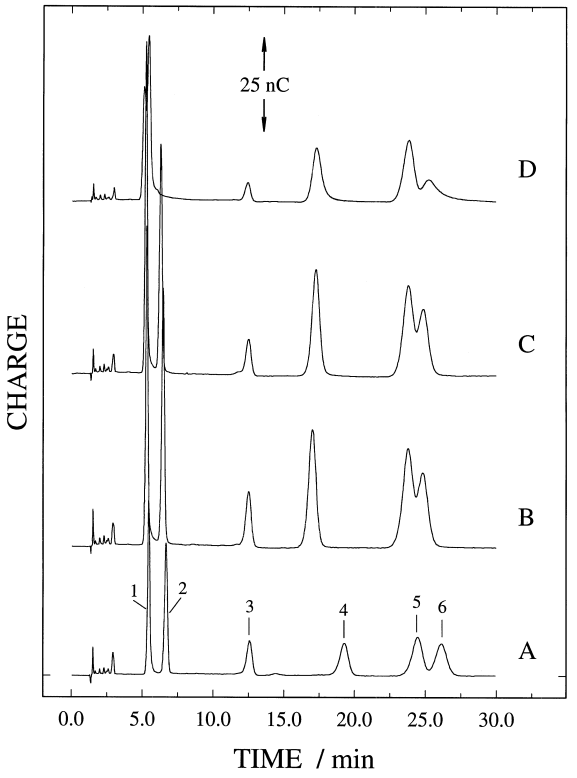


Fig. 3. Isocratic separations of sugar acids by HPAEC–PAD using the following eluents: (A) 100 m*M* NaOH+75 m*M* NaOAc (control), (B) 100 m*M* NaOH+71 m*M* NaOAc+2 m*M* $Ba(OAc)_2$, (C) 100 m*M* NaOH+71 m*M* NaOAc+2 m*M* $Sr(OAc)_2$, and (D) 100 m*M* NaOH+73 m*M* NaOAc+1 m*M* $Ca(OAc)_2$. Peaks: (1) D-gluconic acid, (2) *N*-acetylneuraminic acid, (3) muramic acid, (4) D-galacturonic acid, (5) D-glucuronic acid, (6) D-mannuronic acid. Other conditions as in Fig. 2.

eluent solution. Note that using acetate as the counterion, peaks of muramic and galacturonic acids are not superimposed even though the retention of muramic acid is decreased slightly in magnitude. As mentioned above the decrease of retention is more striking in the presence of calcium. The stability constant of the complexes between *N*-acetylneuraminic acid (β-anomer) and Ca^{2+} in water is about 121 M^{-1}, and such a value is greater than that of galacturonate with the same divalent ion (70 M^{-1}) [17]. Indeed, the η values of galacturonate in the presence of Ca^{2+}, under both elution conditions, are smaller than those of NANA (see Tables 2 and 4). The reasonable agreement with these data demonstrate the usefulness of the alkaline-earth cations in establishing the relative stability of complexes with sugar acids analysed by HPAEC, where the combination of NaOH and $Ba(OAc)_2$ is able to work better than the other ones. Moreover, the precision of retention times was very good (e.g., the RSD of the barium–mannuronate peak was lower than 1.2% for $n=5$) under both eluent solutions.

3.3. Pulsed amperometric response of sugar acids upon addition of divalent ions

Besides the decrease of retention, there is another aspect that needs to be emphasised concerning the determination of sugar acids by HPAEC–PAD. As can be seen in Figs. 2 and 3 there is a significant signal improvement upon addition of divalent metal ions to the alkaline solution. The distinct difference

Table 3
Summary of capacity factors of sugar acids in acetate-containing alkaline eluents[a]

	Gluconic acid	NANA	Muramic acid	Galacturonic acid	Glucuronic acid	Mannuronic acid
				k'_o		
100 m*M* NaOH +75 m*M* NaOAc	2.96	3.86	8.15	13.00	16.79	17.99
				k'		
100 m*M* NaOH+71 m*M* NaOAc +2 m*M* $Ba(OAc)_2$	2.87	3.71	8.13	11.42	16.35	17.10
100 m*M* NaOH+71 m*M* NaOAc +2 m*M* $Sr(OAc)_2$	2.80	3.56	8.05	11.51	16.24	17.00
100 m*M* NaOH+73 m*M* NaOAc +1 m*M* $Ca(OAc)_2$	2.72	2.94	8.02	11.54	16.28	17.26

[a] Experimental conditions as in Table 1.

Table 4
Effect of barium, strontium and calcium on the retention modulus of sugar acids in acetate-containing alkaline eluents[a]

	Gluconic acid	NANA	Muramic acid	Galacturonic acid	Glucuronic acid	Mannuronic acid
η_{Ba}	0.970	0.961	0.998	0.878	0.974	0.951
η_{Sr}	0.946	0.922	0.988	0.885	0.967	0.945
η_{Ca}	0.919	0.762	0.984	0.888	0.970	0.959

[a] Retention modulus evaluated as $\eta = k'/k'_o$ using the eluent solutions reported in Table 3.

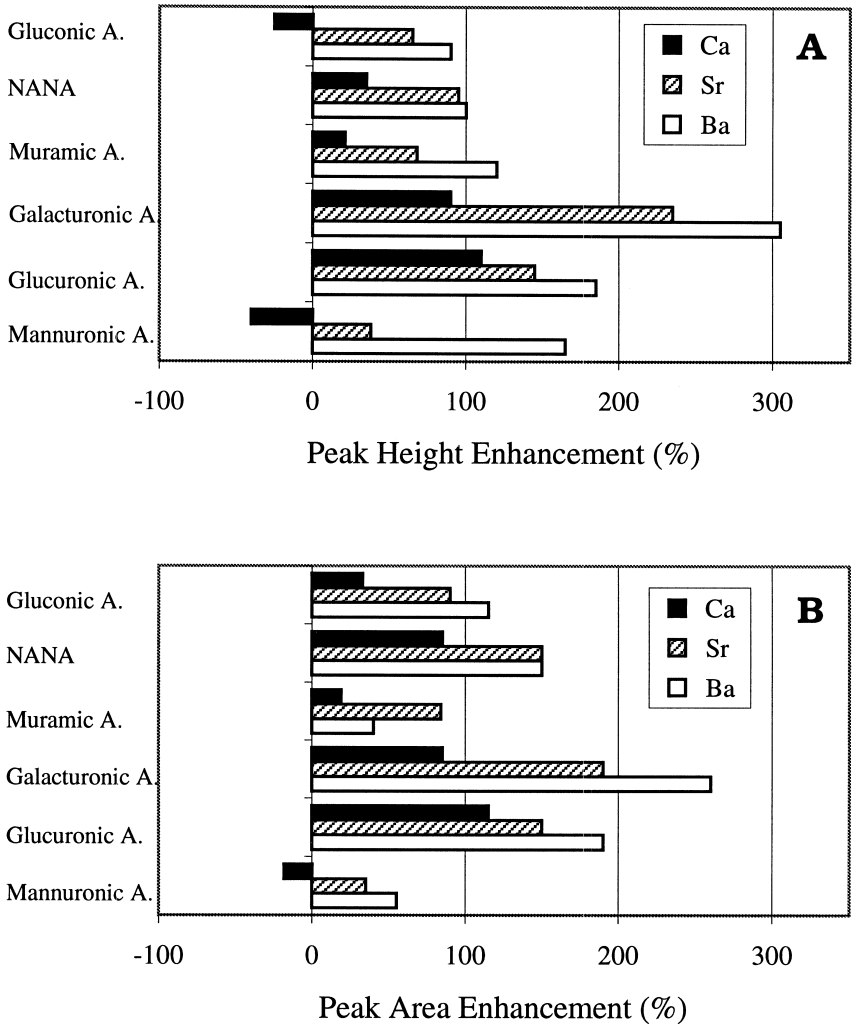


Fig. 4. Peak height (A) and peak area (B) enhancements (%) of sugar acids observed in HPAEC–PAD upon addition of divalent metal ions as nitrate salts. The corresponding peak enhancement percentages were calculated as follows: {[peak signal$_{(M^{2+})}$-peak signal$_{(M^{2+}=0)}$]/peak signal$_{(M^{2+}=0)}$}×100, where M^{2+}, and $M^{2+}=0$ correspond to the presence and absence of Ba^{2+}, Sr^{2+}, and Ca^{2+} in the eluent solution, respectively. Other conditions as in Fig. 2.

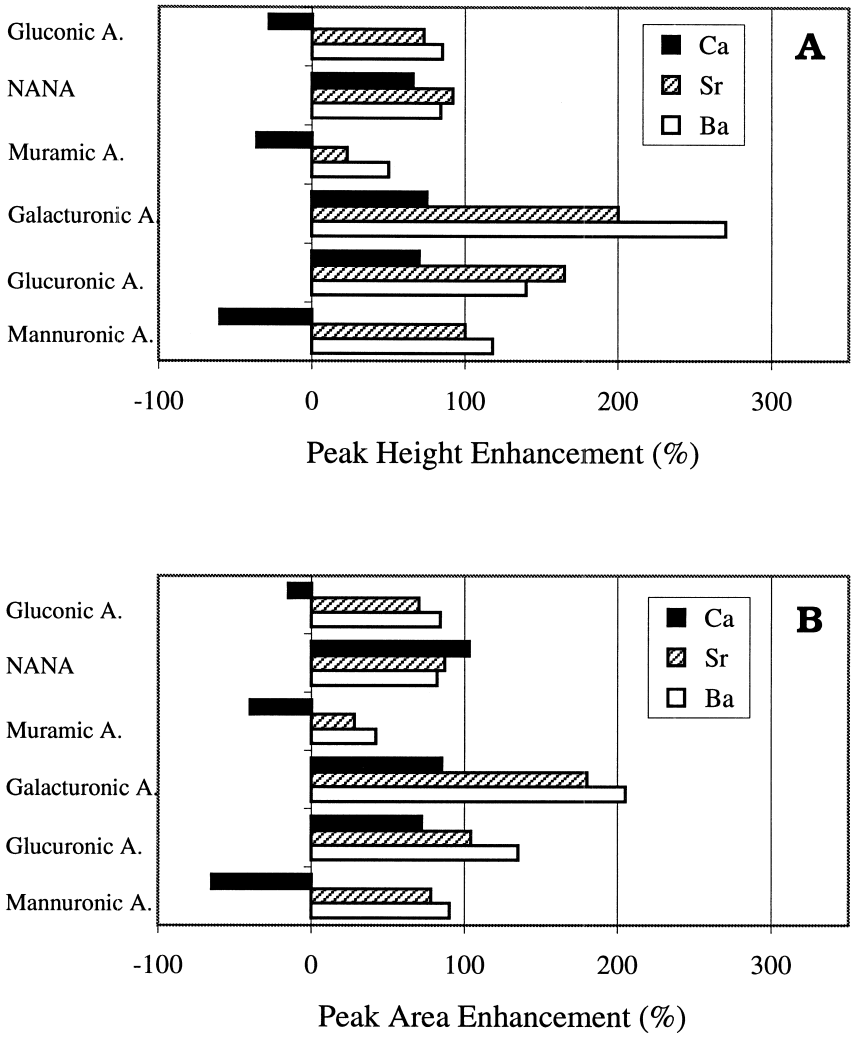


Fig. 5. Peak height (A) and peak area (B) enhancements (%) of sugar acids observed in HPAEC–PAD upon addition of divalent metal ions as acetate salts. Other conditions as in Fig. 3.

between panel A and panels, B, C and D is immediately apparent for all investigated compounds. The relative charge signal enhancement in terms of peak height (A) and peak area (B), employing nitrate and acetate-containing eluents, is shown in Figs. 4 and 5, respectively. Generally, the detector response was slightly greater in the presence of nitrate than acetate ion, either in terms of peak height or peak area. Interestingly, in the presence of Ba^{2+} and Sr^{2+} a signal gain is evident for all the sugar acids investigated. For instance, galacturonic acid exhibited a percentage peak height enhancement equal to 305% and 235%, upon $Ba(NO_3)_2$ and $Sr(NO_3)_2$ addition to the sodium hydroxide eluent, respectively. Yet, the same effect is less pronounced when Ca^{2+} was present in the alkaline eluent, and even a signal decrease of mannuronic and gluconic acids was observed. Moreover, the increment on the peak height when the column was eluted with NaOH plus $Ba(NO_3)_2$ was generally higher than that with $Ba(OAc)_2$, with changes of increment comprised between +5% for gluconic acid and +70% for

muramic acid. No satisfactory explanation can be given for these observations; currently, we are investigating the exact mechanism by which such an effect may be induced.

The increase of response is phenomenon similar to that previously described with neutral sugars and alditols [26,28]. This event seems to be the result of changes in the adsorption features of the gold electrode surface and to the restrained formation of gold oxide [29]. Under the present experimental conditions, the magnitude of signal enhancements is persistent after several chromatographic injections but is not comparable for all the investigated compounds; galacturonic acid, for instance, exhibits the highest enhancement of response accompanied by the lowest retention modulus among uronic acids. Hence, there is no simple correlation between increment of response and the extent of complexation. Actually, there should be most likely a current signal attenuation for those compounds involved in complexation equilibria because a change in the charge distribution occurs and less favourably oxidisable species are formed. Perhaps, the effect of calcium is more consistently explained by invoking its involvement within the electrocatalytic oxidation process of sugars and sugar-related compounds. Unfortunately, at the moment no direct evidences are available. We wish to emphasise, however, that the primary reason for conducting the present studies has been illustration and explanation of the effects taking place in HPAEC–PAD of carboxylated-sugar acids in the presence of alkaline-earth metal ions, and not the development of an analytical method.

4. Conclusions

In the separation of sugar acids by HPAEC, advantage can be taken of the complex formation between metal ions present in the eluent and the sugar molecules to be separated. The selectivity of complexation can be judged from alterations in retention times and peak intensity. Although the use of nitrate as an additional counterion is advantageous with retention times comparably shorter at millimolar concentration, acetate salts are better performing because elution conditions can be more conveniently adjusted. To explain the dramatic changes in the retention of NANA in the presence of calcium acetate or calcium nitrate, the formation of relatively strong complexes in the alkaline eluent solution has been suggested. This because complexation with negatively charged alduronates imparts a reduction in total negativity and lower interactions with the stationary phase. Although not uniform, Ba^{2+} and Sr^{2+} ions produce a significant improvement in the amperometric signal of all compounds investigated. The distinctive behaviour of each sugar acid in the presence of divalent ions may prove to have considerable utility for their characterisation in complex matrices like those deriving from hydrolysates of polysaccharides.

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