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Isocratic separations of closely-related mono- and disaccharides by high-performance anion-exchange chromatography with pulsed amperometric detection using dilute alkaline spiked with barium acetate

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Abstract

Mixtures of closely related mono- and disaccharides may be efficiently separated by high-performance anion-exchange chromatography (HPAEC) only when relatively dilute alkaline eluents are employed (i.e., <20 m*M* NaOH). The main drawbacks of these eluent solutions are (i) column regeneration between runs, (ii) poor reproducibility of the retention times, and (iii) the need for post-column base addition for enhancing sensitivity. Here, we describe some examples of isocratic separations of carbohydrates by HPAEC coupled with pulsed amperometric detection (PAD) accomplished by carbonate-free alkaline eluents (i.e., 5–20 m*M* NaOH) obtained upon addition of $Ba(OAc)_2$ (1–2 m*M*). These separations include aldohexoses (i.e., galactose, glucose, and mannose), aminohexoses (i.e., glucosamine and galactosamine) and their *N*-acylated derivatives (i.e., *N*-acetylglucosamine and *N*-acetylgalactosamine) along with some isomeric disaccharides (i.e., lactose, lactulose and epilactose). The separation of closely related isomers of trehalose, α,α, α,β, and β,β, is also presented. It is recommended to add $Ba(OAc)_2$ to NaOH solutions several hours before using the alkaline eluent (i.e., 12–24 h) to ensure complete barium carbonate precipitation in the eluent reservoir. Adopting such a simple strategy can be especially useful for performing carbohydrate separations under isocratic conditions in which no regeneration and or re-equilibration of column between runs is required. Excellent repeatability of retention data throughout a three-day working session was observed, with relative standard deviations ranging from 2.0 to 3.7%, and from 0.5 to 2.0%, as day-to-day and within-day values, respectively. In addition, there was no need for postcolumn addition of strong bases to the eluent, and successful applications of the present approach confirmed its validity and practicability with detection limits of simple carbohydrates in the picomole range.

Keywords: Mobile phase composition; Monosaccharides; Disaccharides; Hexoses; Lactuloses

1. Introduction

Carbohydrates and carbohydrate-related compounds are widely distributed in nature and play significant roles in various biochemical processes and activities [1–3]. The knowledge of monosac-

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charide composition and sequence, linkage position, and branch point, as well as anomeric configuration of each glycosidic bond is often of crucial importance in pharmaceutical, agriculture, biotechnology, cellulose, and food industries [4]. The significance in all these fields is that there is a need for robust, sensitive and rapid methods for carbohydrate determination.

With the advent of high-pH tolerant columns, high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC–PAD) has proven to be a very useful method for the compositional analysis of carbohydrates without the need of sample derivatisation and using simple sample preparation [4–19]. An inherent difficulty for the separation of epimeric and closely-related carbohydrates is their co-elution because their retention behaviour as oxyanions does not differ very much. The selectivity of the separation system, however, can be enhanced significantly by lowering the pH of the alkaline eluent to a value that is comparable to the pK_a of the sugar molecules, namely pH 12. Unfortunately, under such experimental conditions there is yet a major drawback, which is related to a poor repeatability of the retention times [20]. Highly reproducible separations of carbohydrates cannot be indeed obtained because the adsorption of carbon dioxide and subsequent production of carbonate ion progressively reduces the number of anion-exchange sites available to eluting sample compounds [21]. Efforts to solve this problem may rely on the use of a novel but expensive system for the electrochemical generation of potassium hydroxide eluents which is not affected by carbonate interference [22].

Here, we describe the application of a very useful means for obtaining reliable results when dilute alkaline solutions (e.g., pH 12) are employed. As previously described, efficiency and reproducibility of the chromatographic results may be significantly improved by addition of barium ions to the mobile phase, using for instance barium acetate [20]. This was speculated to be the result of carbonate removal from the eluent solution with corresponding formation of barium carbonate ($pK_s=8.3$, at 25°C [23]), and its consequent precipitation at the bottom of the eluent bottle. The separation of aldohexoses, aminohexoses (i.e., D-galactosamine, and D-glucosamine) and acylated-aminohexoses (i.e., *N*-acetylgalactosamine, and *N*-acetylglucosamine), as well as isomeric disaccharides including lactose, lactulose, and epilactose, was accomplished upon optimisation of the chromatographic conditions. Retention data were compared in terms of day-to-day and within-day repeatability. Retention times, sensitivity, stability of response, detection limits, and linearity of working curves are presented. We anticipate that adopting the addition of barium acetate to alkaline solutions will be especially useful for performing routine carbohydrate determinations under conditions which otherwise imply the need of column regeneration between runs.

2. Materials and methods

2.1. Chemicals

Sodium hydroxide, 50% solution in water (1.515 g/ml), $Ba(OAc)_2$ 99%, α-D-lactose monohydrate 97%, α,α-trehalose dihydrate 99%, α,β-trehalose 98%, β,β-trehalose 98%, 2-deoxy-D-ribose 97%, 2-deoxy-D-glucose 99%, L-fucose 98%, D-galactose, D-glucose, D-mannose, D-sorbitol 99+%, lactitol monohydrate 98%, *myo*-inositol, L(+)-rhamnose, D-ribose were purchased from Aldrich (St. Louis, MO, USA), lactulose >98% (4-O-β-D-galactopyranosyl-D-fructofuranose) was from Fluka (Buchs, Switzerland), NaN_3, sucrose >99.5%, D-fructose 99%, D-arabinose 99%, epilactose 95% (4-O-β-D-galactopyranosyl-D-mannopyranose), D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine were from Sigma (Steinheim, Germany). Other chemicals were purchased from Carlo Erba (Milan, Italy) and were used as received. Doubly distilled, deionized water was used throughout for preparing solutions. Sodium hydroxide solutions used as the eluents and were prepared by diluting of a carbonate-free 50% (w/w) NaOH solution in water, previously filtered with 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. Carbohydrate standard solutions to be injected were prepared fresh daily by dilution of the stock solutions.

2.2. HPAEC–PAD system

Carbohydrates analyses were performed using a metal-free isocratic pump (Dionex, Sunnyvale, CA, USA), Model IP20, a Dionex pulsed amperometric detector (Model ED40), and a Dionex metal-free rotary injection valve with a 10-μl injection loop. Two anion-exchange columns in separate experiments were evaluated for the separation of carbohydrates: (i) a Dionex CarboPac PA1 (250×4 mm I.D.) plus guard column (50×4 mm I.D.), and (ii) a Dionex CarboPac PA10 (250×4 mm I.D.) plus guard column (50×4 mm I.D.). The analytical and guard columns were regenerated at the beginning of each working day by washing with 200 m*M* NaOH for ca. 30 min. The flow-through detection cell (Dionex) contained a gold working electrode (1.0-mm diameter) and an Ag/AgCl reference electrode; the counter electrode is the titanium cell body across the 25-μm thin-layer channel from the working electrode. All chromatography data acquisition and processing were performed using the Chrom-Card for Windows from CE Instruments (ThermoQuest, Milan, Italy). Pulsed amperometric detection was carried out with the following pulse potentials and durations: $E_{OX}=+0.80$ V ($t_{OX}=180$ ms), $E_{DET}=+0.05$ V ($t_{DEL}=200$ ms, and $t_{INT}=240$ ms), and $E_{RED}=-0.30$ V ($t_{RED}=360$ ms). The response time was 1 s. Detection potential was chosen such that carbohydrates could be detected with the lowest background current. Following a delay during which charging current decays, oxidation current is measured by integration for a fixed sampling period, t_{INT}, of 240 ms, giving rise to a charge signal (coulombs). All experiments were carried out at ambient temperature under isocratic elution using a flow-rate of 1.0 ml min^{-1}.

2.3. Mobile phase preparation

As previously proposed [20], it is strongly recommended to make the addition of barium acetate to the alkaline solution the day before using the eluent. This allows the precipitation of barium carbonate in the eluent reservoir, thus avoiding wear either of pistons or piston seals and injection valve rotor seals as well. Modified alkaline eluents were kept in plastic bottles and a Dionex eluent organiser (EO1) was used to saturate them with helium or nitrogen to minimise CO_2 adsorption, and to allow the almost complete precipitation of the low amount of barium carbonate in the eluent reservoir. Excellent results can be obtained preparing two litres of eluent solution and using them for two or three daily working sessions.

3. Results and discussion

3.1. The alkaline eluent choice

Good resolution of commonly occurring carbohydrates in HPAEC can be obtained upon optimisation of the alkaline eluent composition. The use of alkaline eluents having hydroxide concentration higher than the minimum value (i.e., 5–10 m*M*) is demanded for the separations of neutral carbohydrates on anion-exchange columns. Such conditions are necessary for ionisation of sugar hydroxyl groups ($pK_a \cong 12-14$) to produce the required anion that interacts with the positively charged stationary phase. Indeed, when the pH of mobile phase is lowered to a value corresponding to the pK_a of carbohydrates, even small differences in the dissociation behaviour contribute to the separation. Shown in Fig. 1 is the chromatogram of a standard mixture of carbohydrates including α,α-trehalose, fucose, 2-deoxyribose, 2-deoxyglucose, arabinose, galactose, glucose, mannose, fructose, and lactose. The separation was accomplished with a CarboPac PA1 column. Baseline separation was achieved in a short analysis time, eluting isocratically with 10 m*M* NaOH spiked with 2 m*M* $Ba(OAc)_2$ at a flow-rate of 1.0 ml/min. Under these chromatographic conditions, even galactose, glucose, and mannose, which differ only in the axial–equatorial configuration of their hydroxyl groups, are baseline resolved (see peaks 6, 7, and 8 in Fig. 1).

Previous work has suggested that the addition of barium acetate to sodium hydroxide eluents ensures carbonate-free solutions [20], as proven also in the

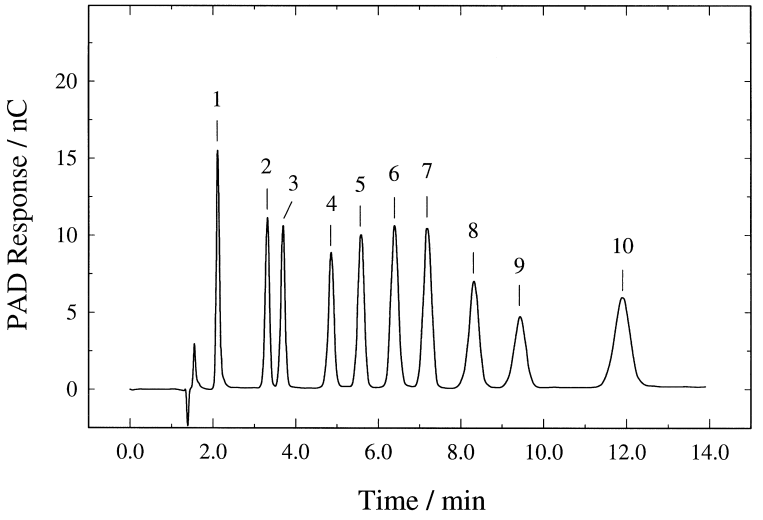

Fig. 1. Separation and detection of a carbohydrates mixture by HPAEC with pulsed amperometric detection. Peak and concentration: (1) α,α-trehalose, 10 μ*M*; (2) L-fucose, 20 μ*M*; (3) 2-deoxyribose, 40 μ*M*; (4) 2-deoxyglucose, 20 μ*M*; (5) D-arabinose, 20 μ*M*; (6) D-galactose, 20 μ*M*; (7) D-glucose, 20 μ*M*; (8) D-mannose, 20 μ*M*; (9) D-fructose, 20 μ*M*; (10) lactose, 20 μ*M*. Eluent, 10 m*M* NaOH+2 m*M* $Ba(OAc)_2$ at a flow-rate of 1.0 ml/min. Column, CarboPac PA1 plus guard (Dionex). Detection potential at the gold working electrode, E_{DET} = +0.05 V vs. Ag/AgCl.

next section. Moreover, the residual amount of Ba^{2+} ions [0.5–0.7 m*M* upon addition of 1 m*M* $Ba(OAc)_2$] may be involved in selective complex formation with carbohydrates possessing an axial–equatorial–axial sequence of three hydroxyl groups on the six-membered ring. This is specifically the case for D-ribose, D-allose, and D-talose [24]. Although selective complexation may be beneficial to separation of carbohydrates, the stability constants (β) with alkaline-earth metal ions in water are reported to be not greater than two [25]. Alduronate ions, like all α-hydroxy acid anions, form much stronger complexes with cations than neutral sugars [24]. Indeed, modifying the alkaline eluent with Ba^{2+}, Sr^{2+} or Ca^{2+}, considerable changes in retention times and peak intensity have been observed for uronic acids [26]. In this work, however, our goal was to demonstrate the positive effects related to barium–acetate addition to dilute sodium hydroxide solutions employed for the separation of closely related mono and disaccharides. To assess the validity of our proposal, the chromatographic separations were accomplished in isocratic mode.

3.2. Evaluation of within-day and day-to-day retention repeatability

It is well recognised that the reproducibility of chromatographic data in HPAEC is strongly affected by the interference of carbonate. Such a divalent ion is always present in sodium hydroxide solutions in spite of the precautions used during the eluent preparation. Accordingly, carbonate ions tend to progressively occupy the active sites of column, thereby progressively decreasing the retention of sugar molecules. The problem is particularly striking when relatively dilute alkaline mobile phases are employed (i.e., ≤20 m*M* NaOH), since OH^- does not effectively remove carbonate ions from the ion-exchanging sites. When NaOH eluent solutions were modified and used as described above with a millimolar concentration of $Ba(OAc)_2$, the following results were obtained. A standard solution of carbohydrates was injected repeatedly to determine the relative capacity factors as a function of time. The reliability of chromatographic data was judged by (i) retention times consistency during a daily working

session, (ii) retention times consistency between consecutive days using the same alkaline eluent, and (iii) amperometric response stability determined by peak areas measured after repeated injections of the same sample solution.

In Fig. 2 are plotted the normalised capacity factors (k'/k'_0) as a function of time of the most retained compounds (five) separated in the chromatogram of Fig. 1. The k'_0 represents the value of each compound evaluated by the initial run of a comprehensive set of measurements. The capacity factors obtained after column regeneration and using 10 m*M* NaOH plus 4 m*M* NaOAc as eluent are also compared in the same Fig. (open circles). The distinct difference between these results is immediately apparent for all compounds investigated. The use of conventional eluent leads to a gradual diminution of the retention, with k'/k'_0 ratios lower than 0.7 after 3–4 h for all compounds. When 10 m*M* NaOH upon addition of 2 m*M* $Ba(OAc)_2$ as eluent was employed, excellent within-day repeatability of retention was obtained (solid squares). The capacity factors upon about 6 h decreased only up to 2–4% of the initial value for all investigated compounds. Each data point in Fig. 2 represents the mean value of six measurements accomplished over the corresponding six days. It is quite remarkable that the k'/k'_0 ratio of lactose (plot E in Fig. 2), with a retention time= 11.90 min, is lowered to less than 6% of its initial value after 7.5 h of chromatographic runs. Of more importance is the fact that acetate counter ion contributes towards shortening the retention of carbohydrates without any adverse effects on the baseline. These findings demonstrate that the use barium acetate as an eluent component of dilute NaOH solutions is a potentially useful means for the isocratic separation of sugar molecules by HPAEC.

To further support the consistency of chromatographic data using dilute NaOH solutions spiked with millimolar amounts of barium acetate, the same eluent was employed over three consecutive daily working sessions. Chromatographic performance characteristics are summarised in Table 1. The initial and final retention times are listed along with the daily average, the average retention over three days plus the corresponding standard deviation. There was a very high repeatability of the retention for all sugar molecules; the relative standard deviations (RSDs) range from 2.0 to 3.7%. Constant retention can be maintained over a comprehensive set of chromatographic determinations because the within-day repeatability was even better, that is 0.5–2.0% for eight replicated injections. According to data reported in Table 1, comparable agreement was obtained when experiments were repeated over the ensuing days using the same eluent solution. These findings signify the ability of barium acetate to ensure carbonate-free elution conditions, thereby allowing the separation of closely related carbohydrates without the need of time-comsuming regeneration of the column between runs and post-column base addition as well. This last observation is addressed in the next section.

3.3. Calibration data, limits of detection and precision

Representative chromatograms for the separation of some of the sugars studied in this work are shown in Fig. 3. The separation was accomplished by a CarboPac PA10 column, which is especially suited for the separation of mono- and disaccharides [27]. Compared to a CarboPac PA1 column, the retention times are slightly longer. Linearity was determined by a series of two–three injections of six standard solutions whose concentrations spanned from 2 to 100 μ*M*, curves a–f, respectively. These chromatograms reflect a comparison of the retention behaviour of common carbohydrates under the same elution conditions. Three important disaccharides, lactose, lactulose, and epilactose are also well-separated in the same chromatogram, peaks 5, 6, and 7, respectively. Therefore, quantification of each sugar was readily attained with good linearity for peak area calibration plots; the relevant data are summarised in Table 2. The linearity extends over a roughly three-orders-of-magnitude range above the limit of detection, with correlation coefficients (*r*) in the range of 0.996–0.999. Limits of detection evaluated with a signal-to-noise ratio of three were near the pmol level. It is especially interesting to note that these data were obtained using the common PAD waveform parameters (see Experimental section), which guarantee rapid baseline stabilisation within ca. 15

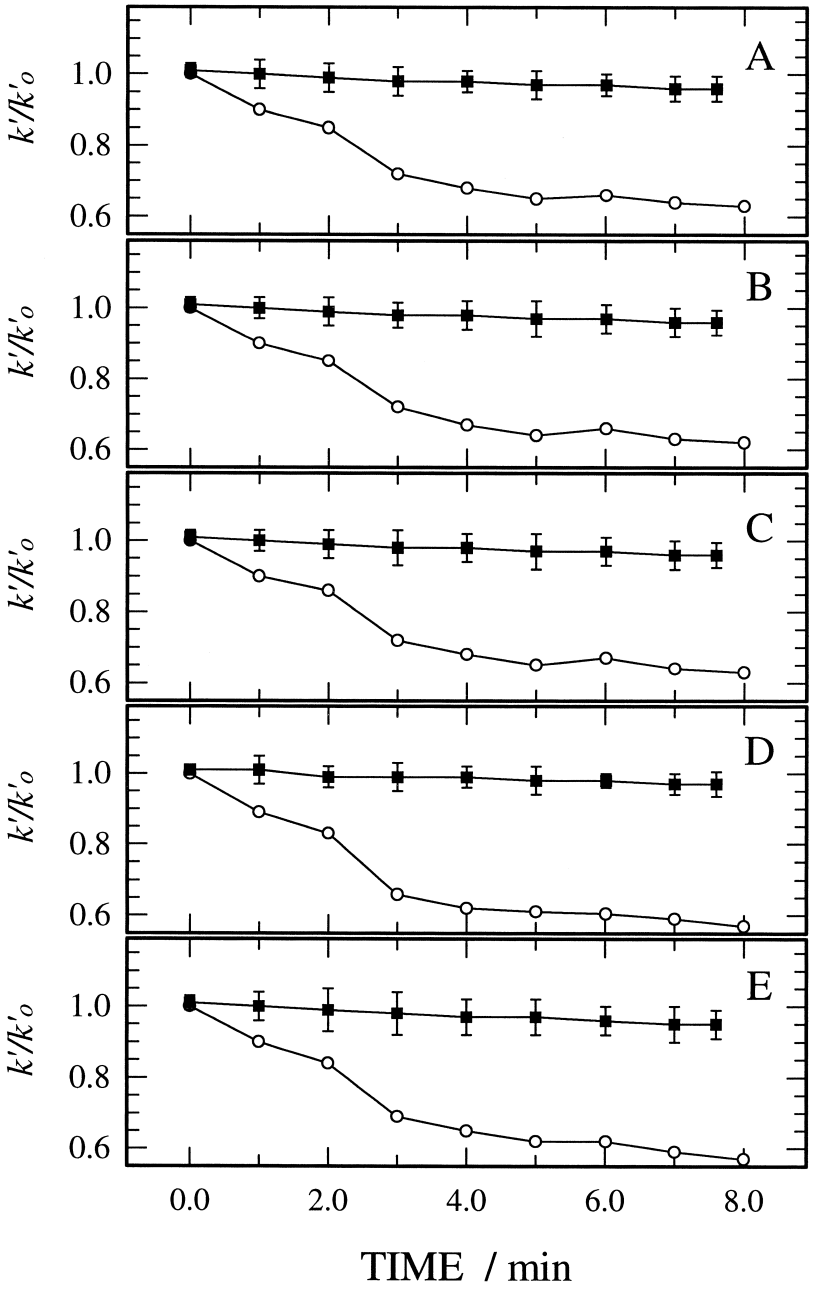

Fig. 2. Time-dependence of the relative capacity factor (k'/k'_0) of D-galactose (A), D-glucose (B), D-mannose (C),) D-fructose (D), and lactose (E) using a conventional 10 m*M* NaOH+4 m*M* NaOAc (open circles), and 10 m*M* NaOH+2 m*M* $Ba(OAc)_2$ (solid squares) as eluents. The error bars represent the difference between the highest and lowest value obtained. Experimental conditions as in Fig. 1.

min. The average noise signal, measured before and after sample injections, was found to be about 30 pC. Five replicate injections were carried out to determine the precision of response. The repeatability of the peak heights at concentrations ten times greater than the limits of detection was comprised

Table 1
Retention times, t_R, evaluated by HPAEC–PAD during repetitive injections of a sample mixture on three consecutive days using 10 m*M* NaOH spiked with 2 m*M* $Ba(OAc)_2$ as eluent[a]

Compound	Running day									$t_R \pm s$[c]	RSD(%)[d]
	1st Day			2nd Day			3rd Day				
	2nd Hour	8th Hour	Ave. t_R[b]	2nd Hour	8th Hour	Ave. t_R[b]	1st Hour	8th Hour	Ave. t_R[b]		
dGlu	5.40	5.33	5.37	5.56	5.38	5.47	5.62	5.56	5.59	5.47±0.11	2.0
Gal*N*	6.10	6.05	6.07	6.33	6.11	6.22	6.38	6.30	6.34	6.21±0.14	2.3
Gal	7.10	7.20	7.15	7.45	7.25	7.35	7.50	7.46	7.48	7.33±0.17	2.3
Glu	8.20	8.25	8.23	8.65	8.31	8.48	8.65	8.55	8.60	8.44±0.19	2.2
Gal*N*Ac	9.35	9.40	9.38	9.84	9.50	9.67	9.90	9.76	9.83	9.63±0.23	2.4
Fru	10.25	10.35	10.30	10.80	10.46	10.63	10.80	10.70	10.75	10.56±0.23	2.2
Lactose	13.90	14.04	13.97	15.10	14.30	14.70	15.10	14.90	15.00	14.56±0.53	3.6
Lactulose	16.00	16.16	16.08	17.35	16.35	16.85	17.30	17.10	17.20	16.71±0.57	3.4
Epilactose	18.20	18.40	18.30	19.80	18.70	19.25	19.74	19.60	19.67	19.07±0.70	3.7

[a] Column, CarboPac PA10 with guard column; flow-rate, 1.0 ml/min. Each working day the column was initially flushed for ~30 min with the commercial carbonate-free 200 m*M* NaOH solution before equilibration. Other experimental conditions are reported in Fig. 1.
[b] Average daily retention times in min.
[c] Data are expressed as the mean value±standard deviation.
[d] Relative standard deviation for the retention time evaluated over three days of chromatographic operation.

between 1.2% for galactose and 3.1% for epilactose. Although, elution of the same analytes may be also accomplished by similarly concentrated NaOH solutions, using column regeneration after each chromatographic run, at least two further predictable disadvantages should be mentioned. These drawbacks include low reproducibility of retention data and relatively longer analysis times, as chromatographic run time, column washing with a concentrated base (i.e., 200 m*M* NaOH) and its subsequent re-equilibration are inevitable steps [21].

The above results are noteworthy in one additional respect. It is general knowledge that sensitivity of detection and especially the response stability at a Au electrode in PAD may be substantially improved when the electrocatalytic oxidation process occurs in strongly alkaline solutions [9,12,19]. Under conditions of incomplete oxide reduction the baseline signal exhibits a continuous drift, which is especially evident at high detection sensitivity. Barium–acetate addition to freshly prepared alkaline eluents allows the chromatographic operation to be greatly simplified as both sensitivity and stability of response reported here were obtained without post-column addition of high-pH solutions. All these inherent shortcomings, we believe, should make the present method of eluent modification very attractive for many applications involving the analysis of carbohydrates and related compounds in a variety of complex samples. These applications include in vivo fermentation studies [28], urine samples [29,30], sweet whey permeate [31], hydrolysate of glycoconjugates [32], and hydrolysate of non-starch polysaccharides [33]. To illustrate the capability of dilute NaOH eluents to efficiently perform separations of simple carbohydrates and closely related compounds, four specific examples are illustrated below.

3.4. Selected examples

Depending on the particular problem, the adaptability of elution-conditions in HPAEC enables adequate resolution of relevant sugar compounds. We demonstrate that excellent separations can be obtained under optimum chromatographic conditions, which imply the addition of barium acetate at the alkaline eluent. As discussed by Townsend [34], HPAEC with PAD may be particularly useful in quantifying the sugar compounds of glycoproteins after acidic hydrolysis. A mixture of sugar components, typically found in the hydrolysates of glycoproteins [32], is separated in the chromatogram of Fig. 4. In this example, the mobile phase consists of 20 m*M* NaOH spiked with 1 m*M* $Ba(OAc)_2$; all components of the sample were eluted within 12.5

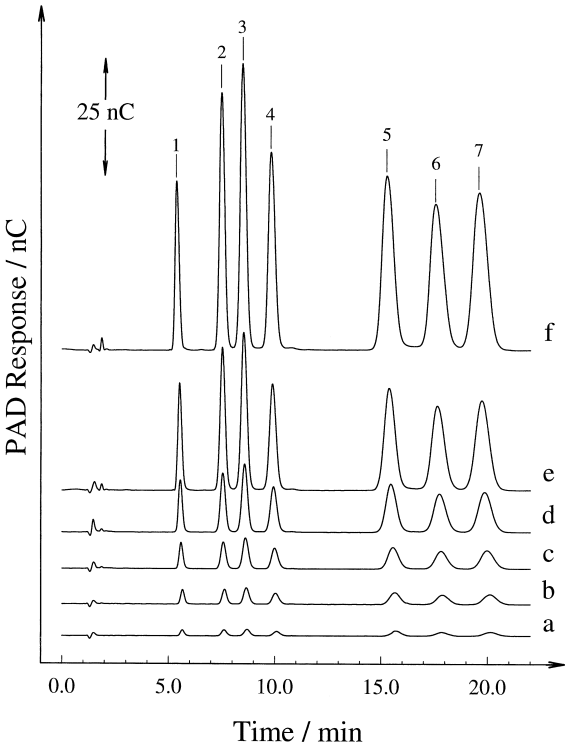

Fig. 3. Isocratic separations by HPAEC–PAD of mixed standard solutions containing: (1) 2-deoxyglucose, (2) D-galactose, (3) D-glucose, (4) D-mannose, (5) lactose, (6) lactulose, and (7) epilactose. Standard solution concentrations, 2, 5, 10, 20, 50, and 100 μ*M*, chromatograms a, b, c, d, e, and f, respectively. Eluent, 10 m*M* NaOH+2 m*M* $Ba(OAc)_2$ at a flow-rate of 1.0 ml/min. Column, CarboPac PA10 plus guard (Dionex). Other conditions as in Fig. 1.

min. From all investigated monosaccharides, fucose, glucose, galactose, and mannose play an important role. Mannose is bound in structure polysaccharides of microorganisms and plants. Glucose, galactose, and mannose are the three most common hexoses involved in mammalian physiology and, therefore, were chosen in this example for their biological relevance. Under the conditions employed, these analytes are separated baseline resolved. Of more importance is the fact that no post-column addition of base was employed, as it is normally required for enhancing carbohydrate response and to maintain a stable baseline and low signal noise as well [19,21].

An interesting example of separation is given by the trehalose's family. Trehaloses are non-reducible disaccharides consisting of two glucose residues: α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside, α-D-glucopyranosyl-(1→1)-β-D-glucopyranoside, β-D-glucopyranosyl-(1→1)-β-D-glucopyranoside, also known as α,α-, α,β-, and β,β-trehalose, respectively. The most common seems to be α,α-trehalose, which is widely distributed in nature, for example in bacteria, fungi and invertebrates, but no studies are reported on the isomeric α,β, and β,β forms [35-38]. As seen in Fig. 5, complete separation of trehalose isomers was obtained. A standard equimolar solution (i.e., 30 μ*M*) of each trehalose was injected. The order of elution and sensitivity are different. While the most retained is the β,β isomer of trehalose, the pulsed amperometric response was higher in the case of the α,β isomer, probably as a result of a favourable sterical disposition of the electroactive groups toward the gold electrode surface. Therefore, it is possible to make an assessment of the relevance of individual trehaloses in the corresponding samples as enzymatic methods based on trehalase are not suitable in this respect [39].

It was also possible to separate and detect the two most common aminohexoses, namely glucosamine and galactosamine, along with *N*-acetylgalactosamine and *N*-acetylglucosamine as evidenced by the chromatogram of Fig. 6. Baseline separation of acylated compounds was not possible using 10 m*M* NaOH mobile phase, whether or not modified with barium acetate. The increase of the NaOH concentration up to 20 m*M* plus 2 m*M* $Ba(OAc)_2$ leads to an acceptable separation of both glucosamine from galactosamine and *N*-acetylgalactosamine from *N*-acetylglucosamine. With the applied method, galactosamine and galactose as well as *N*-acetylgalactosamine, mannose and xylose coeluted. By changing either anion-exchange column or eluent concentration the resolution of one of these pairs could not be improved.

In addition to the above examples, HPAEC–PAD was applied to separate a standard mixture of sugar compounds using 5 m*M* NaOH plus 1 m*M* barium acetate. Shown in Fig. 7 is a representative chromatogram in which alditols and sugar compounds are efficiently separated. Considering that no post-column base addition was done, good baseline stability along with the absence of peak tailing was observed. Therefore, routine isocratic separations can be successfully accomplished by the use of dilute NaOH

Table 2
Quantitative parameters of some carbohydrates determined by HPAEC with PAD using 10 m*M* NaOH spiked with 2 m*M* $Ba(OAc)_2$ as eluent[a]

Compound	Linear portion of the calibration graph[b] $y=a+b\times C$[c]				
	$a\pm t_{95}\times s_a$ (nC min)	$b\pm t_{95}\times s_b$ (nC min/μ*M*)	*r*	LOD[d] (pmol)	Repeatability RSD (%)[e]
dGlu	0.4±0.6	0.08±0.01	0.996	1.6	1.5
Gal	0.1±0.3	0.15±0.01	0.999	1.5	1.2
Glu	0.2±0.5	0.19±0.01	0.999	1.4	1.7
Man	0.1±0.3	0.15±0.01	0.999	2.1	1.6
Lactose	0.5±1.2	0.23±0.02	0.999	2.0	2.3
Lactulose	0.4±0.9	0.22±0.02	0.998	2.6	2.5
Epilactose	0.7±1.6	0.23±0.04	0.998	2.5	3.1

[a] Column, CarboPac PA10 with guard column; flow-rate, 1 ml/min; sample loop, 10 μl; other conditions are reported in Fig. 3.
[b] Each data point ($n=6$) was generated from at least two separate injections. Peak area signal corresponding to nC min.
[c] *C* represents the concentration in μ*M*; slope and intercept evaluated at the 95% confidence level.
[d] Limits of detection, expressed in picomole, were evaluated as the concentration of carbohydrate in the injected sample that would result in a peak height of three times the noise level ($S/N=3$). Noise measurements over 2–5 min periods of flat baseline in different chromatograms gave a mean value of 30 pC.
[e] RSDs evaluated at a concentration ten times greater than the LOD of each compound.

eluents. The presented examples clearly demonstrate the usefulness of barium addition in the low-concentrated alkaline eluent solutions, and, therefore, it may ultimately be possible to use these eluents to characterise the sugar content of a broad combination of real samples.

4. Conclusions

The addition of barium acetate to low-concentrated NaOH eluents for carbohydrate separations in HPAEC–PAD by virtue of its inherent shortcomings, is a possible solution to many problems related to

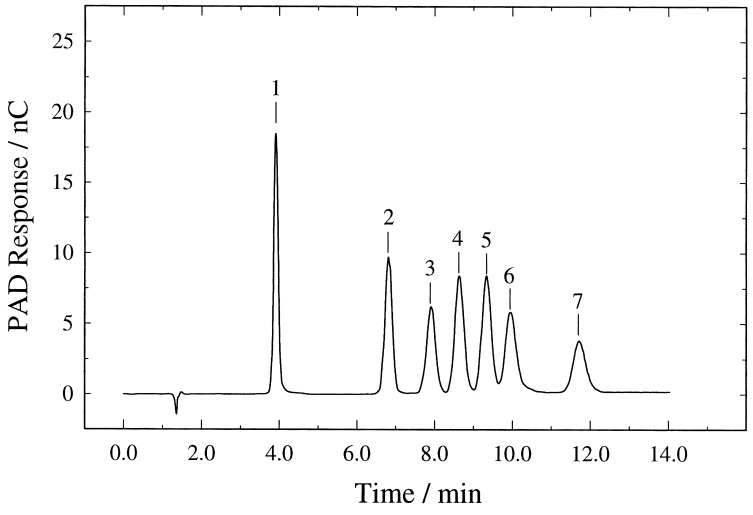

Fig. 4. HPAEC with pulsed amperometric detection of (1) L-fucose, 40 μ*M*; (2) D-galactosamine, 10 μ*M*; (3) D-glucosamine, 10 μ*M*; (4) D-galactose, 20 μ*M*; (5) D-glucose, 20 μ*M*; (6) D-mannose, 20 μ*M*; and (7) D-fructose, 20 μ*M*. Eluent, 20 m*M* NaOH+1 m*M* $Ba(OAc)_2$ at a flow-rate of 1.0 ml/min. Column, CarboPac PA1 plus guard. $E_{DET}=+0.05$ V vs. Ag|AgCl.

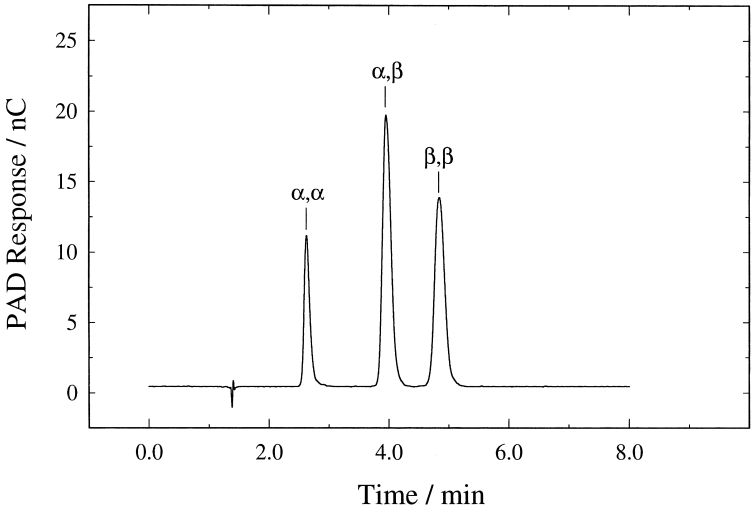

Fig. 5. Separation by HPAEC–PAD of a standard solution containing α,α-trehalose, α,β-trehalose, and β,β-trehalose, 30 μ*M* each. Eluent, 10 m*M* NaOH+1 m*M* $Ba(OAc)_2$ at a flow-rate of 1.0 ml/min. Other conditions as in Fig. 1.

presence of carbonate in the alkaline mobile phases. The significance of the present strategy is that it provides excellent relative standard deviations for both elution time and response. The above results suggest that predominantly alkaline eluents spiked with barium ions should be used to retain the advantages discussed in this study in terms of good detection stability and high precision of retention as well as short analysis times. Expected component sugars present in the hydrolysate of glycoconjugates can be well separated and assigned under the proposed elution conditions. We wish also to mention that weakly alkaline conditions are better suited to prevent undesirable alkali-mediated side reactions of

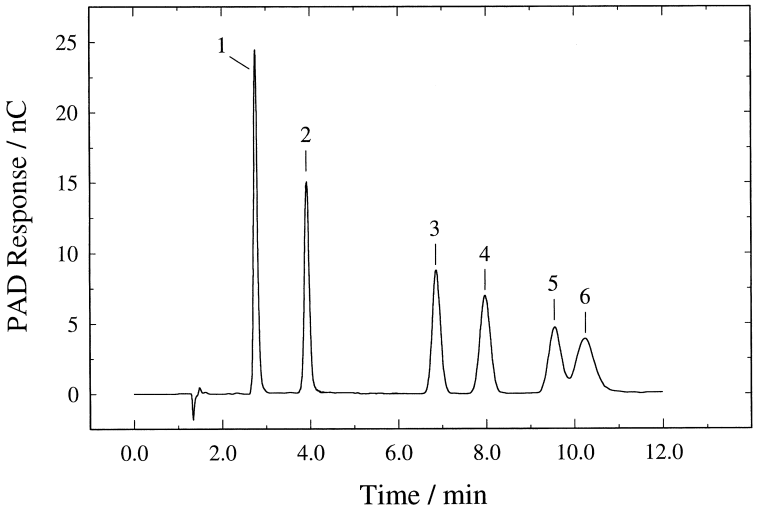

Fig. 6. HPAEC with pulsed amperometric detection of (1) α,α-trehalose, 20 μ*M*; (2) L-fucose, 30 μ*M*; (3) D-galactosamine, 10 μ*M*; (4) D-glucosamine, 10 μ*M*; (5) *N*-acetylglucosamine, 20 μ*M*; (6) *N*-acetylgalactosamine, 20 μ*M*. Eluent, 20 m*M* NaOH+1 m*M* $Ba(OAc)_2$ at a flow-rate of 1.0 ml/min. A steady baseline was observed without post-column base addition. Other conditions as in Fig. 4.

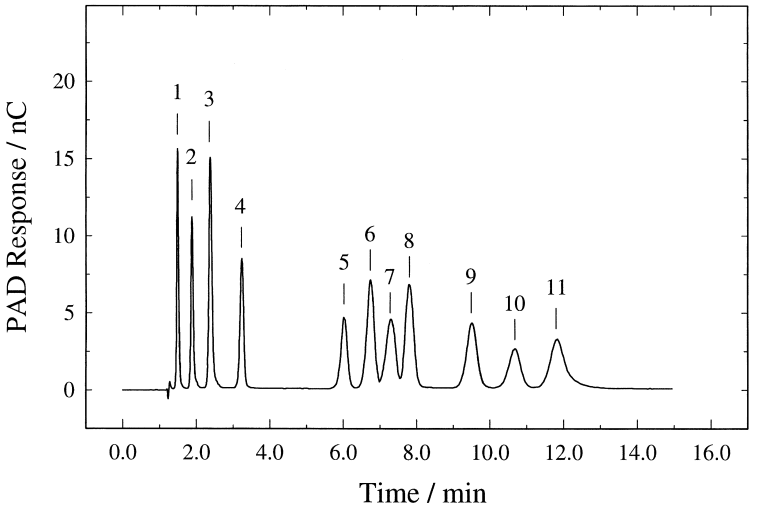

Fig. 7. Separation by HPAEC–PAD of a mixed standard solution containing: (1) *myo*-inositol, 10 μ*M*; (2) D-sorbitol, 10 μ*M*; (3) lactitol, 10 μ*M*; (4) L-fucose, 20 μ*M*; (5) rhamnose, 20 μ*M*; (6) D-galactose, 20 μ*M*; (7) D-glucosamine, 10 μ*M*; (8) D-glucose, 20 μ*M*; (9) D-mannose, 20 μ*M*; (10) D-fructose, 20 μ*M*; and (11) D-ribose, 20 μ*M*. Eluent, 5 m*M* NaOH+1 m*M* $Ba(OAc)_2$ at a flow-rate of 1.0 ml/min. Other conditions as in Fig. 4.

carbohydrates including epimerization and degradation.

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