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REVIEW

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# Carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection: The potential is still growing

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**Abstract** This article reviews recent advances of carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection. Starting from the paper of Dennis C. Johnson [1] in which the great analytical promise of such a technique was anticipated, a multitude of exciting new research possibilities have recently emerged. The great attractiveness of high-performance anion-exchange chromatography is largely due to its compatibility with such a sensitive, selective and reliable detection method as pulsed amperometry. This very good match between liquid chromatography and electrochemical detection has allowed the determination of carbohydrates in a variety of complex matrices, for instance, foods, beverages, diary and biotechnological products, vegetal tissues, and also in the area of clinical diagnostics. For this reason, the introduction of HPAEC-PAD into regulated methods is becoming increasingly accepted. A comprehensive collection of applications to carbohydrates and samples of interest is given, with special focus on the separation of closely related sugar compounds using dilute alkaline eluents. Advances in pulsed potential waveforms are also discussed, and a comparison with other liquid chromatographic methods addressed.

**Abbreviations** HPAEC, high-performance anion-exchange chromatography; PAD, pulsed amperometric detection; DP, degree of polymerization; Glc, D-glucose; dGlc, 2-deoxy-D-glucose; Glc*N*, D-glucosamine; Glc*N*Ac, *N*-acetyl-D-glucosamine; Glc*N*-4-P, D-glucosamine-4-phosphate; 3-*O*MeGlc, 3-*O*-methyl-D-glucose; Gal, D-galactose; Gal*N*, D-galactosamine; Gal*N*Ac, *N*-acetyl-D-galactosamine; Fru, D-fructose; Man, D-mannose; Ara, D-arabinose; Rha, L-rhamnose; Fuc, L-fucose; Xyl, D-xylose; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; KDN, 2-keto-3-deoxy-D-glycero-D-galactonononic acid; KDO, 2-keto-3-deoxyoctulosonic acid; FOS, fructooligosaccharides; GF5, GF6, and GF7, oligofructans; Hib, *Haemophilus influenzae* type b; FAB, fast atom bombardment; ESI, electrospray ionization; MALDI-TOF, matrix assisted laser desorption ionization-time of flight.

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## 1 Introduction

At the beginning of the eighties a novel methodology was developed with the precise intent to provide a highly sensitive detection and efficient separation tool for carbohydrate analysis. It was the result of a very happy marriage between high-pH (or high-performance) anion-exchange chromatography (HPAEC) and pulsed amperometric detection (PAD) in which the use of strongly alkaline solutions was successfully exploited. While the separation is based on the weakly acidic properties of sugar molecules, detection is favorably performed by taking advantage of their electrocatalytic oxidation mechanism at the gold working electrode in basic media. Indeed, the high detection sensitivity, which inherently characterizes pulsed amperometry, is exactly what is required for the analysis of sugar compounds in their native state.

It is recognized that the anion-exchange separation of carbohydrates owes its popularity to the development of pulsed amperometry which has been profusely investigated in the laboratory of Dennis C. Johnson at Iowa State University [1–5]. The title of one of his contributions "Carbohydrate detection gains potential", published in Science in 1986 [1], anticipated the great promise of pulsed electrochemical detection in carbohydrate analysis. From a chromatographic point of view, the relevant advantages of HPAEC were already manifest in the original paper of Rocklin and Pohl in 1983 [6] where the effects of eluent composition and column temperature on the separation of alditols and simple carbohydrates using a pellicular anion-exchange resin were described. From the beginning, HPAEC-PAD has stimulated a phenomenal amount of research in many different fields. In the following two decades such a technique has assumed a significant role, thus becoming increasingly established as one of the ma-

jor tools for carbohydrate analysis and being also the subject of extensive reviews [7–17]. Such a considerable interest has been demonstrated probably because of the ubiquity and relevant role of carbohydrates in many biological processes as well as in biotechnology and food science.

The purpose of this paper is to briefly review the basic concepts of HPAEC-PAD, outlining new developments and the state of the art with special focus on column and eluent selectivity, separation of selected sugar compounds and also electrode sensitivity. We report on the major developments in carbohydrate analysis that have occurred over the past few years. Emphasis has been placed on applications that use the common modes of solvent preparation and isocratic separation using dilute alkaline eluents, which are ideally suited for the separation of closely related mono- and disaccharides. Recent work on some aspects of detection in PAD at gold working electrodes is also considered. The applications section gives a brief examination of the sugar compounds for which HPAEC-PAD represents the best method of choice, thus demonstrating some of the most frequent analyses of real samples. A survey of the literature shows that HPAEC-PAD has been applied to many more matrices than are covered in this review, but we have focused either on situations in which the determination of carbohydrates has proven successful for routine analysis or those in which routine application is expected.

## 2 Basic concepts of HPAEC-PAD

It is well known that in basic solution sugars behave as weak anions [18]. Until some years ago such a property could not be exploited for the separation of carbohydrates, as the available columns for anion-exchange chromatography were packed with silica particles, which are notoriously unstable in alkaline solutions (i.e., at pH > 8.5). The introduction of polymer-based stationary phases, that affords the advantage of stability over a wide pH range, has contributed to the development of a versatile and efficient separation technique for carbohydrates and related compounds. The combination with pulsed amperometry at a gold working electrode, whose surface is able to catalyze the electrooxidation of –COH containing compounds in high pH solutions, has provided a selective and sensitive tool for the determination of both reducing and nonreducing sugars, alditols, oligosaccharides and closely related compounds, including methylated aldoses, deoxysugars, amino sugars, *N*-acetylated amino sugars, acidic sugars, etc.

### 2.1 Chromatographic behavior of sugar compounds in HPAEC

A monosaccharide molecule possesses several potentially ionizable hydroxyl groups with the following hierarchy of acidity: 1-OH > 2-OH ≥ 6-OH >3-OH > 4-OH. Taking the glucose molecule as a reference, the influence of the acidities of these hydroxyls on the retention times in HPAEC has been studied [19]. Glucose was O-methylated at each ring position, and the chromatographic behavior of the corresponding O-methylated derivatives was investigated. The 1-O-methylated compound was poorly retained, while all the other derivatives exhibited higher retention times; moreover, they eluted with very similar velocities. These results indicate that the elution characteristics are mainly due to the presence of an anomeric hydroxyl group at carbon C1, in which the relatively high acidity, caused by the inductive effect of the ring oxygen, prevails on the other hydroxyl groups. Notably, the presence of an anomeric hydroxyl group is not an essential requisite for the anion-exchange separation. A very illustrative example is given by the reduced form of carbohydrates, that is alditols (pKa > 13.5), which have been successfully separated by HPAEC [20–25]. Aldoses and alditols exhibit an anion-exchange affinity in which their acidity trend is followed. The practical consequence is that alditols elute earlier than their unreduced counterparts as they give rise to weaker interactions with the stationary phase.

When dealing with oligosaccharides, the retention times are not directly related to the hierarchy of acidity of the monosaccharide units, as clearly shown by several studies [10, 11, 26]. While initially great attention was focused on the detection optimization of carbohydrates, there is now a growing interest both on improving the chromatographic conditions and altering the selectivity of separation relevant to all the different classes of sugar molecules.

### 2.2 Electrochemical detection at the gold working electrode

Though a large number of compounds exhibit suitable electrooxidation activity, being amenable to determination with high sensitivity by amperometric detection in flowing liquids [27], there are a large number of important analytes (e.g. carbohydrates, amino acids, amines, etc.) that were considered scarcely electroactive at noble electrode materials. An interesting review of pulsed electrochemical detection at gold and platinum working electrodes emphasized that the main problem with the detection of polar aliphatic compounds lies mainly in electrode surface poisoning by intermediate and/or final oxidation products and not in the electrochemical reactivity of the abovementioned compounds [9, 15]. The gold electrode surface is able to catalyze the oxidation of polar aliphatic compounds in alkaline media and is the best choice for the detection of carbohydrates. Several review articles highlight the basic aspects of pulsed electrochemical detection, and a book by LaCourse details the most relevant aspects of pulsed amperometry and represents a valuable source of information and applications [27].

Aided by pulsed amperometric detection, coupling of electrochemistry and liquid chromatography grew out of the need for sensitive, selective and relatively inexpensive detection of sugar compounds at trace levels. According

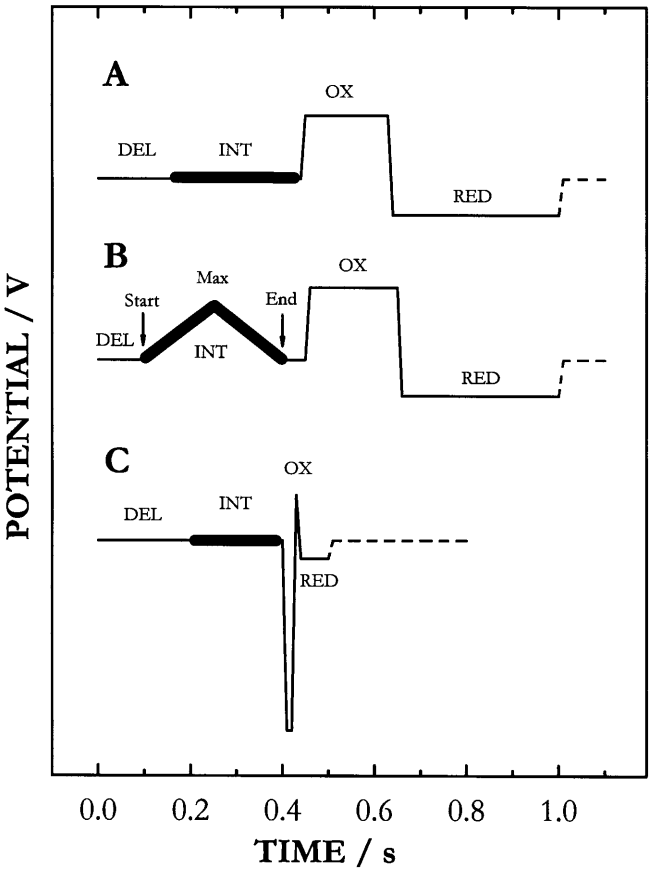


**Fig. 1** Typical waveforms for pulsed-potential amperometric detection in alkaline solutions at a gold working electrode. (*A*) standard three-potential steps waveform; (*B*) integrated voltammetric detection waveform (IPAD); (*C*) four-potential steps waveform. Legend: $t_{DEL}$, and $t_{INT}$, delay and integration times, respectively, at the detection potential ($E_{DET}$); $E_{OX}$ and $E_{RED}$, oxidation and reduction potentials; Start-Max-End, voltammetric scan during which the current integration is performed

to the relevant mechanism, it is possible to distinguish between different detection modes in the whole family of pulsed electrochemical detection. The most frequently used potential waveform (Fig. 1 A), called mode I detection, involves direct oxidation at the oxide-free electrode surfaces ($E_{DET}$) followed by a cleaning procedure consisting of two steps, one at a higher potential ($E_{OX} > E_{DET}$), necessary to fully oxidize the surface ($E_{OX}$), and the other one at a potential low enough to strip away the previously formed gold-oxide film ($E_{RED}$). Such a repeating pattern of surface oxidation and reduction is necessary to maintain a highly reproducible state of activity of the electrode surface. In the study of LaCourse and Johnson [29, 30] it was established that the background signal, which originates mainly from double-layer charging, can be minimized by delaying the integration time before current integration (that means a relatively long delay time, $t_{DEL}$). As discussed in the following section, three potential values along with their time settings have to be optimized in pulsed electrochemical detection, and different procedures have been developed for this purpose. Mode II detection comprises amine- and sulfur-based compounds and differs from the previous mode, as the oxidation is catalyzed by oxides present on the electrode surface. Accordingly, a detection potential favoring oxide formation has to be selected. Since the electrode surface must be at least partly oxidized, the relevant currents strongly affect the background signal. Significant differences exist between the detection parameters of mode I and II [9, 27]. For instance, mode II comprises relatively high potential values of $E_{DET}$ compared to mode I, a shorter delay time, a reduction potential selected to favor analyte adsorption, and a longer $t_{RED}$ [31].

In pulsed amperometry the signal output is recorded as a charge since the current integration is normally accomplished, and in our earlier work [24, 25] the acronym integrated pulsed amperometric detection (IPAD) was used. However, IPAD is simply referred to as a mode of detection (mode II) and the relevant waveform is depicted in Fig. 1 B [32]. A very rapid triangular potential scan is applied during oxide formation instead of a constant detection potential. As a matter of fact, the integration period starts and ends at a potential at which surface oxide is not formed and consists of a potential scan into the region where carbohydrate oxidation occurs. IPAD is excellent in overcoming the drawback of mode II detection, as it succeed in subtracting the current signal due to oxide formation.

Very recently, Rocklin et al. [33] demonstrated that under the continuous application of a standard three-potential waveform peak area decreases ranging from 22 to 32% occur due to working electrode recession. This phenomenon was related to gold dissolution during the high positive potential, and a four-potential waveform was devised and optimized. As carbohydrate oxidation products could be flushed out of the electrode at high negative potentials, such a four-potential waveform comprised a negative potential step lasting 10 ms immediately before applying the activation potential, at which gold oxide formation occurs (Fig. 1 C). A further outcome of this protocol was the reduction of waveform duration without detrimental effects on the signal output.

Mention should also be made of the great improvement described by Clarke and colleagues [34] for the detection of amino acids and amino sugars employing a waveform with six potentials (not shown), thus allowing electrochemical detection without any pre- or postcolumn derivatization. This integrated amperometric method comprised a new procedure for electrode cleaning and activation which did not cause a significant loss of gold from the electrode surface and its recession. Moreover, a shorter adsorption step was adopted without limiting the linear range of the basic amino acids.

A novel development that overcomes the use of two or more working electrodes might be the use of multiplex PAD (MPAD), in close analogy to a “multi-wavelength” UV absorbance detection. LaCourse [27] demonstrated the possibility to selectively detect analytes having different detection modes at a single gold working electrode, and within a single run, by a waveform which incorpo-

rates two detection steps (and dual signal outputs) in one potential cycle. It was suggested, for example, that a detection potential for carbohydrates at +50 mV and a second one at +350 mV for oxide catalyzed analytes (e.g., compounds containing amine groups) could be used.

## 3 Experimental methodology

### 3.1 Anion-exchange columns for carbohydrate separations

The polymer-based matrices used in anion-exchange chromatography are characterized by high mechanical and chemical stability; moreover, they are able to impart distinctive selectivity to the stationary phase. Depending on the category of compounds, suitable columns can be chosen, differing in capacity, resin composition, cross-linking, and organic solvent compatibility [21, 35]. For monosaccharides, disaccharides and some oligosaccharides, the Dionex Company has designed a general-purpose column, called CarboPac[1] PA1, packed with a polystyrene/divinylbenzene substrate agglomerated with a Microbead™ quaternary amine functionalized latex, having an ion-exchange capacity equal to 100 μeq per 250 × 4 mm i.d. [36]. The superiority of HPAEC-PAD compared to other HPLC or GC methods becomes evident as soon as one needs to separate multicomponent mixtures containing monosaccharides, disaccharides and amino acids. In Fig. 2 the simultaneous separation of carbohydrates along with alanine and proline in about 20 min is shown as an example.

A column specifically designed for mono- and disaccharide analysis is the CarboPac PA10 [37], which is similar to PA1 but holds a higher percentage of divinylbenzene. Another pellicular anion-exchange column, the CarboPac PA100, is packed with a macroporous resin obtained from the copolymerization of ethylvinyl- and divinylbenzene. Its ion-exchange capacity is 90 μeq per column and it possesses a much higher compatibility with organic solvents [38]. Such a column is better suited to oligosaccharide separation, also allowing the analysis of monosaccharides, as will be demonstrated in the applications section. Furthermore, there is commercially available a stationary phase especially designed for the separation of alditols, the CarboPac MA1 [39]; it is a column packed with a macroporous polymeric resin, which has an ion exchange capacity 45 times greater than that of the PA1 column. Although alditols are generally poorly retained with pellicular columns, they have been successfully separated using PA1 or PA100 column provided that dilute alkaline eluents are employed [40, 41].

### 3.2 Eluent composition

The mobile phase composition in HPAEC significantly influences the selectivity and rapidity of separation as well as the sensitivity of detection. Sodium hydroxide solutions are normally employed, and the $OH^-$ concentration to be used depends on the class of compounds under investigation. For instance, the analysis of sugar acids and oligosaccharides is carried out with strongly alkaline eluents (i.e., $[OH^-] \geq 0.1$ M) as these compounds give rise to strong interaction with the stationary phase. To further shorten the run time, acetate or nitrate "pushers" are generally added to the mobile phase in suitable percentages. As these anions are able to interact much more strongly than hydroxide with the anion-exchange sites, the retention times can be drastically decreased [10, 21]. Acetate ion is also employed in gradient elution, where the separation of complex mixtures of saccharides with different sizes and acidities has been carried out. It should be emphasized that the high pH eluents employed in HPAEC do not induce Lowbry de Bruyn-van Ekenstein transformations (e.g. epimerization and tautomerization) [42] of monosaccharides. Indeed, these compounds always give rise to single and sharp peaks under the experimental conditions pertinent to HPAEC. The same is true for *N*-acetylated amino sugars, which do not undergo any degradation reaction even at high hydroxide concentrations, probably

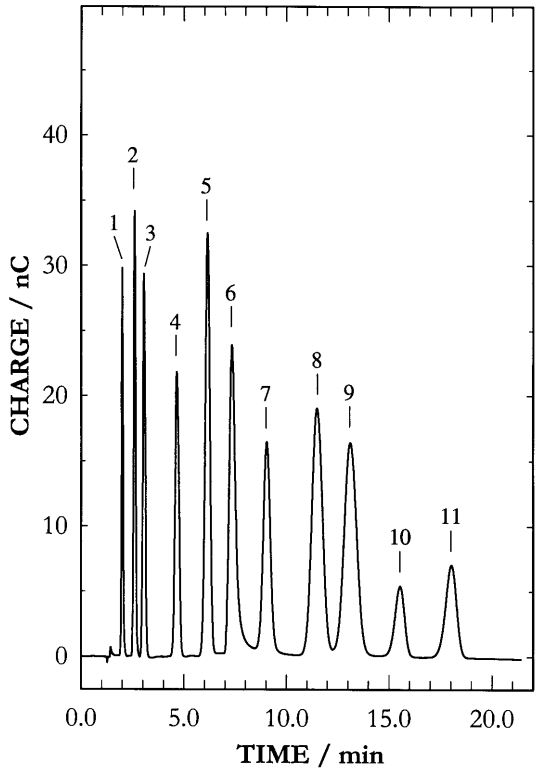


**Fig. 2** HPAEC-PAD of a standard mixture of carbohydrates. (*1*) α,α–trehaloses, 25 μM; (*2*) α,β–trehaloses, 25 μM; (*3*) β,β–trehaloses, 25 μM; (*4*) dGlc, 70 μM; (*5*) Gal, 60 μM; (*6*) tagatose, 120 μM; (*7*) Fru, 70 μM; (*8*) lactose, 60 μM; (*9*) lactulose, 60 μM; (*10*) alanine, 120 μM; (*11*) proline 120 μM. Eluent, 10 mM NaOH and 2 mM $Ba(OAc)_2$ at a flow rate of 1.0 mL/min. Column, CarboPac PA1 plus guard (Dionex). Detection potential at the gold working electrode, $E_{DET}$ = +0.25 V vs. Ag|AgCl. Column temperature, 20 °C

[1] CarboPac is a registered trademark of the Dionex Corporation, Sunnyvale, CA.

because the separation time is relatively short. Usually, oligosaccharides are more retained than monosaccharides and disaccharides and are reported to exhibit 0 to 15% epimerization, especially in the case of sialylated compounds. Since alditols do not epimerize in alkali, such a drawback can be ruled out by reducing the sugar to the corresponding sugar-alcohol.

When employing a mobile phase with $[OH^-] \geq 0.1$ M, the separation of some relevant isomeric monosaccharides, for instance galactose, glucose and mannose, cannot be achieved, as they exhibit very similar retention behavior. Upon decreasing the $OH^-$ concentration to a value lower than 20 mM, these compounds are more likely to interact with the stationary phase, thus better demonstrating their differences in ion-exchange behavior. Much research work in our group has been dedicated to improving the capability of HPAEC, increasing the number of components capable of being separated in a single run. To be functional, a column–eluent combination must necessarily impart a constant retention upon repetitive injections of a sample mixture. In anion-exchange chromatography, the quality of a separation strongly depends on the alkaline running eluent, which typically possesses an unknown amount of carbonate. Such an ion occupies the anion-exchanging sites of the column, thus hindering them to interact with the sugar anions. Carbonate contamination is always encountered when preparing any alkaline solution, as this ion is formed through atmospheric carbon dioxide; under normal conditions (25 °C and 1 bar), the saturation concentration of $CO_2$ in water is equal to 33 mM [43].

To obtain a stable retention, which allows a more definite identification of the sugar compounds, carbonate-free sodium hydroxide solutions are necessary. In addition, the advantages of performing isocratic separations using dilute sodium hydroxide eluents enable the use of HPAEC in many separations of closely related monosaccharides with short analysis times and high repeatability of retention. Considering that in HPAEC-PAD the retention time is the most important parameter for the identification of a sugar compound, many efforts have been devoted to the minimization of carbonate contamination. This can be partially accomplished by preparing eluents with carbonate-free 50% (w/v) sodium hydroxide solutions [21]. During the chromatographic runs, it is also recommended to keep an inert gas atmosphere ($N_2$ or He) on the eluent solution. Even using these precautions, the effect of carbonate ions on retention times and column performances is still quite relevant, especially when dilute alkaline mobile phases are employed [44]. Indeed, a novel system based on on-line electrochemically generated alkaline eluent which minimizes the carbonate interference is now on the market [45]. However, as most recent developments indicate, this is no longer a critical issue. By using barium or strontium ions in alkaline eluents, we have observed an outstanding improvement in the reproducibility of retention and separation as a whole [40, 44, 45]. The reason for such a phenomenon is that the cited cations are able to form carbonate salts ($pK_s$ ($BaCO_3$) = 8.30; $pK_s$ ($SrCO_3$) = 9.03 [43]), which precipitate in the eluent container, thus simply providing a chemical means for the removal of carbonate ions. Figure 3 shows a series of repetitive chromatograms of a mixture containing deoxyglucose, galactose, glucose, mannose, fructose, lactose, lactulose, and epilactose. The column was eluted with 10 mM NaOH and 2 mM $Ba(OAc)_2$. As can be seen a good selectivity was achieved without relinquishing an excellent retention reproducibility. Twelve repetitive injections were made and the relative standard deviation (RSD) of repeatability was < 3%. Note that the mobile phase was prepared with carbonate-free 50% (w/w) NaOH solution, using carefully degassed water, and maintaining an inert gas pressure on the eluent solutions, just as traditionally recommended. The net advance of performances attained with dilute alkaline eluents modified with barium acetate has encouraged their use in many analytical samples of industrial and biological interest, as will be shown later on in this review. Moreover, we highlight the fact that barium, calcium and strontium are able to influence not only the reproducibility but also the selectivity of separation through formation of weak complexes with sugar compounds [40, 47].

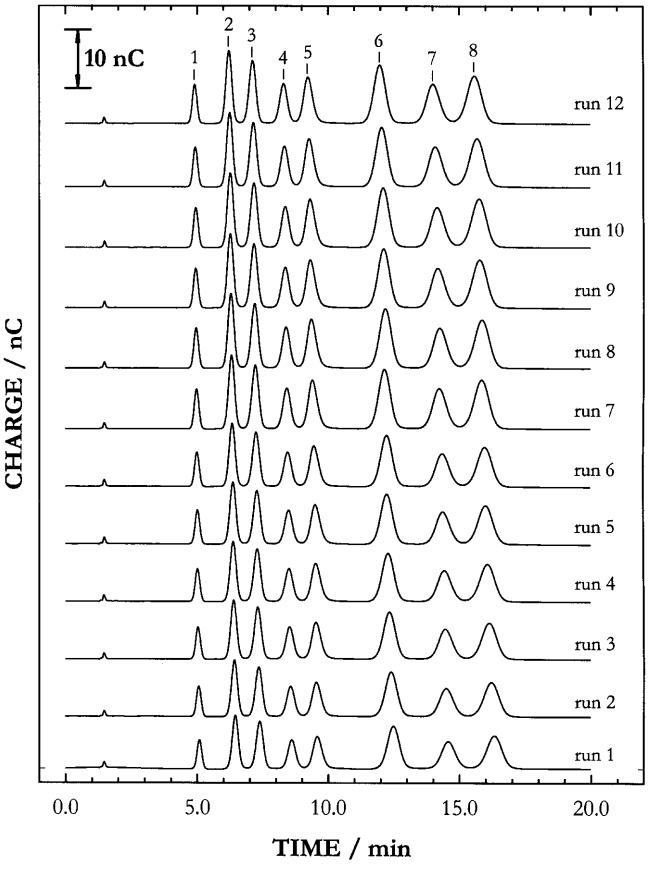


**Fig. 3** Repetitive injections of a standard mixture of carbohydrates during a typical day session using 10 mM NaOH and 2 mM $Ba(OAc)_2$ as mobile phase; injections at intervals of roughly 25–30 min. No column regeneration between runs was employed. Mixture containing: (*1*) dGlc, (*2*) Gal, (*3*) Glc, (*4*) Man, (*5*) Fru, (*6*) lactose, (*7*) lactulose, and (*8*) epilactose. All sugars were at a concentration of 30 μM except Fru, 60 μM. Column, CarboPac PA10 plus guard (Dionex); flow rate of 1.0 mL/min

### 3.3 Temperature effects

The role of column temperature on the chromatographic behavior of carbohydrates separated by HPAEC has not been emphasized enough in the literature, though this parameter may considerably affect the retention and selectivity of separation [6]. This phenomenon is more evident for oligosaccharides and to a lesser extent for disaccharides and simple sugar molecules. Although retention times decrease when the temperature is raised, temperatures higher than 45 °C should not be employed, as anion-exchange materials are subject to Hoffman degradation [6]. Changes in the ambient temperature can induce significant variations in the chromatographic results when the column is not thermostated. Van Riel and Olieman, for instance, observed that resolution between galactose, glucose and sucrose decreased on increasing the column temperature from 22 °C to 40 °C. Moreover, deterioration of some carbohydrates was observed at temperatures above 30 °C [49]. A recent study on the influence of column temperature on the separation effects of neutral and sialylated oligosaccharides has demonstrated a considerable impact on retention times, as well as a switch in the elution order of individual compounds [50].

### 3.4 Optimization of potential waveforms

The popularity of carbohydrate analysis afforded by pulsed amperometry is mainly attributable to the selectivity and sensitivity of detection. Johnson, LaCourse and co-workers have carried out a great deal of work on the potential waveform optimization for carbohydrate analysis by pulsed amperometry. The traditional method used for establishing the best potential waveform is cyclic voltammetry (CV). Typical voltammetric profiles of a gold rotating electrode in deaerated 0.1 M NaOH are reported in Fig. 4 [29]. The dotted curve represents the electrochemical behavior of gold in aerated supporting electrolyte while the dashed line was recorded in the presence of 0.2 mM glucose. Observing the voltammetric profiles in the supporting electrolyte, some relevant electrodic processes are present during the positive scan from –0.8 V to +0.8 V vs. Ag|AgCl; wave B corresponds to oxygen evolution at a potential higher than +0.7 V, wave A is due to the formation of a film of gold oxide, which is dissolved during the negative sweep giving rise to wave C, between +0.3 V and –0.1 V. Using aerated supporting electrolyte (dotted line) oxygen reduction occurs between –0.1 V and –0.8 V (wave D). Upon glucose addition, the prominent anodic peak starting at –0.6 V is attributable to the oxidation of the aldehydic group to carboxylate (wave E), while the oxidation of alcoholic and aldehydic groups between +0.4 V and +0.6 V gives rise to wave G. The sudden current signal decrease observed when increasing the potential is due to gold oxide formation. During the opposite scan, there is no signal between +0.8 V and +0.2 V, but when the gold oxide is dissolved (wave C) the electrode surface is cleaned and its electrocatalytic activity is restored. Accordingly, the aldehydic and alcoholic groups of glucose can be oxidized, giving rise to wave H. The potential values corresponding to current maxima for analyte oxidation, gold oxide formation, and its reductive dissolution, $E_{DET}$, $E_{OX}$, and $E_{RED}$, respectively, are chosen for the pulsed waveform in flowing stream amperometric detection.

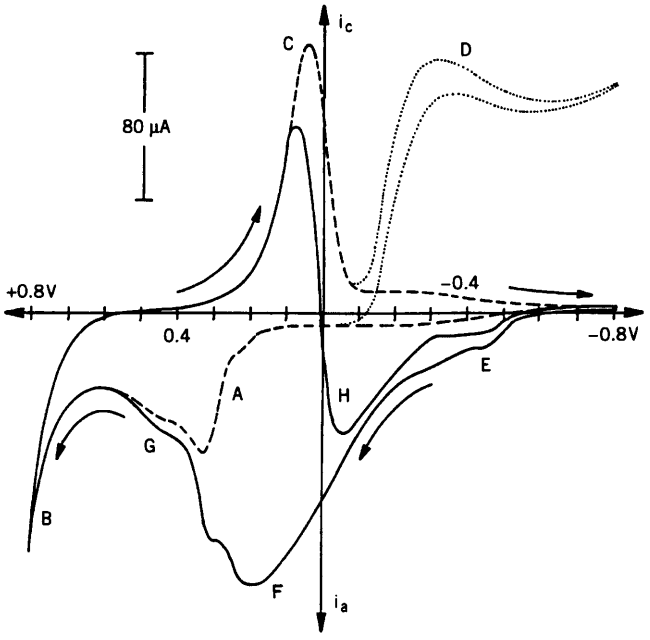


**Fig. 4** Voltammetric response (*i–E*) for glucose at a Au rotating disk electrode (RDE). Conditions: rotation speed, 1000 rpm; scan rate, 200 mv/s; and Ag|AgCl reference electrode. Solutions: (·····) 0.1 M NaOH with dissolved oxygen; (——) 0.1 M NaOH, deaerated; and (– – –) 0.2 mM Glc, deaerated. Reprinted from LaCourse WR, Johnson DC (1991) Carbohydr Res 215:159–178, with kind permission of Elsevier Science

In the study of Andrews and King [51] voltammetric experiments were employed to select the oxidation, gold oxide formation, and reductive gold oxide dissolution potentials, but disregarding any strategy for optimizing timing parameters. LaCourse and Johnson carried out a more comprehensive investigation [29, 30]. Significant differences in the reaction process were found among carbohydrates by voltammetric experiments during which rotation velocity and potential scan rate were varied. These authors also demonstrated that the duration of anodic and cathodic pulses could not be considered as independent of the choice of $E_{OX}$ and $E_{RED}$. In this respect, LaCourse and Johnson [30] showed that pulsed voltammetry (PV) was the ultimate method of time-potential setting validation, as both potential and time optimization could be addressed. Indeed, PV uses the repeated application of a pulsed waveform at a hydrodynamic electrode with small incremental changes of the time and potential parameters. As far as carbohydrate analysis is concerned, both reducing and nonreducing sugars may be detected with a waveform of $E_{DET}$ = +200 mV, $t_{DEL}$ = 240 ms, $t_{INT}$ = 200 ms (shorter times caused baseline drift and poor S/N ratios), $E_{OX}$ = +800 mV, $t_{OX}$ = 180 ms (potential and time required to build up a monolayer of surface oxide), $E_{RED}$ = –300 mV,

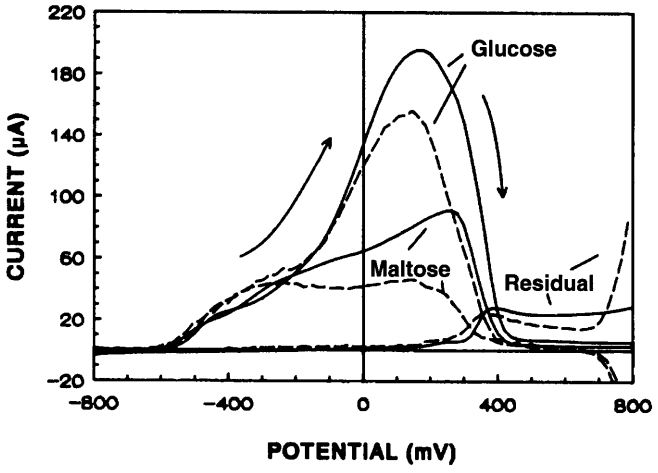


**Fig. 5** Comparison of pulsed voltammetry response ($i$–$E_{DET}$ (——) and cyclic voltammetric response ($i$–$E$) (– – –) for 0.4 mM Glc and 0.4 mM maltose at the Au RDE in 0.1 M NaOH. Conditions: 900 rpm rotation speed. In cyclic voltammetry the scan rate was 50 mV/s. In pulsed voltammetry an optimized pulsed amperometric waveform was used. Reprinted with permission from LaCourse WR, Johnson DC (1993) Anal Chem 65:50–55. Copyright (1993) American Chemical Society

$t_{RED}$ = 360 ms. Such a relatively long reduction time is needed to remove surface oxide and to allow sugar analyte adsorption onto the electrode surface. While this last potential-time setting has been demonstrated as a general-purpose waveform, different modifications can be devised, mainly due to the interdependence of the oxide formation and dissolution rates and applied potential. The reliability of PV is evident from inspection of Fig. 5, where a comparison between PV (optimized waveform) and CV profiles, solid and dashed lines, respectively, is shown for maltose and glucose (positive scans). Although PV and CV exhibit a virtually equivalent response for glucose, the same is not true for maltose, as the potential corresponding to current maximum is not clearly defined under CV conditions.

The most commonly used cell design for amperometric detectors is the thin-layer configuration. In pulsed amperometry signal magnitude depends on several factors, two of which are controlled by cell design: the surface area of the working electrode and the mobile phase flow-rate. With the aim of maximizing the signal-to-noise ratio, Rocklin et al. [52] investigated the role of the most relevant cell parameters: working electrode area and thickness of the thin-layer channel. As expected, the greater the surface area, the higher the signal, while on decreasing the channel thickness the signal increase became smaller because of the thickness of diffusion layer. As noise experiences similar behavior, theoretical and practical limitations were studied and it was found that a 25 μm × 1.3 mm thin-layer channel and a 1-mm diameter working electrode led to high sensitivities and minimum detection limits in the low femtomole range.

Two different routes have been followed to improve detector response with a three-fold objective of high sensitivity, reasonable selectivity, and durable performances: (i) application of complex potential waveforms and (ii) alkaline eluent modification. Wong and Jane [53] compared the effects of nitrate and acetate as pushing agents on the separation and detection of amylopectin; they found that the former was more sensitive and offered lower detection limits in starch analysis. No explanations were given to justify such a behavior. The addition of divalent nonelectroactive cations such as $Ba^{2+}$ and $Sr^{2+}$ was revealed to be more effective [24, 54]. Most likely, the rapid desorption from the electrode surface of the oxidation products in the presence of these ions (millimolar amounts) and, possibly, the inhibition of gold oxide formation resulted in a marked increase in sensitivity, which was approximately 50% using 1 mM $Sr^{2+}$ in the mobile phase with pulsed amperometry. The failure of calcium was investigated and related to the higher degree of suppression relevant to the onset of gold oxide formation. When dealing with uronic acids, the signal improvement was even more significant, with a 300% increase upon addition of 2 mM $Ba(NO_3)_2$ to the mobile phase, and 250% in the presence of 2 mM $Ba(OAc)_2$ [48].

The issue of fast waveforms has been examined in much greater detail following the demand of a higher sampling rate, as the 1 Hz standard waveform is not adequate for use with microbore columns and capillary electrophoresis. Lu and Cassidy [55] described a 2.6 Hz waveform optimized for the detection of carbohydrates upon their separation in a 60 cm × 10 μm capillary system. Roberts and Johnson [56–58] investigated the formation-dissolution of hydrous gold oxides; they found that a monolayer of AuOH is formed within 20 ms at $E_{OXD}$ = +0.5 V in 0.1 M NaOH and is stripped away roughly in 20 ms at a negative potential $E_{RED}$ < –0.50 V. Hence, they went on to apply faster waveforms to detect carbohydrates by HPLC-PAD with a sampling frequency of up to 6.2 Hz. Recently, a great improvement was described by Jensen and Johnson [59], who tuned up a fast potential waveform for pulsed electrochemical detection. Employing voltammetric ring-disk experiments, they discovered that fouling substances produced by glucose oxidation in alkaline medium could be reductively desorbed. Thus, upon stepping the disk potential from +0.5 to –1.0 V, a cathodic peak at –0.77 V vs. SCE in 1 M NaOH could be detected at the ring electrode in a millimolar glucose solution. On this basis, the authors were able to decrease the time periods for oxidative and reductive reactivation significantly, thereby appreciably increasing the sampling frequency. Subpicomole glucose detection limits with a linear dynamic range extending over three decades were yielded with a 6.7 Hz potential waveform of $E_{RED}$ = –1.00 V ($t_{RED}$ = 10 ms), $E_{OXD}$ = +0.60 V ($t_{OXD}$ = 10 ms) and $E_{DET}$ = +0.10 V ($t_{DET}$ = 50 ms, $t_{INT}$ = 50 ms).

## 4 Applications: HPAEC-PAD holds promise

The great sensitivity and selectivity of analysis available from HPAEC-PAD, as well as its reliability, makes this analytical tool attractive for a growing range of applica-

**Table 1** Applications related to carbohydrate analysis by HPAEC-PAD

| Sugar compounds | Mobile phase composition and elution mode | Applications | Reference |
|---|---|---|---|
| Monosaccharides, alditols | 8.0 mM $Ba(OH)_2$ and 0.5 mM HOAc, and 1.0 mM $Ba(OH)_2$ and 0.125 mM HOAc | Standard mixtures | [1] |
| Monosaccharides and mono-deuterated glucose isomers | Column washed with 100 mM NaOH and eluted with water | Standard mixtures | [61] |
| Monosaccharides | Inverse gradient with 15.4 mM NaOH followed by water elution and final column regeneration with 0.46 M NaOH | Characterization of polysaccharide degradation by colonic bacteria | [64] |
| Monosaccharides | 2.5 and 15 mM NaOH | Marine particulate matter | [70] |
| Monosaccharides | 16 mM NaOH and postcolumn base addition | Pamiteplase | [95] |
| Monosaccharides | Water and postcolumn base addition | Soluble coffee | [60] |
| Monosaccharides | 22 mM NaOH and column washing with 0.2 M NaOH | Composition of *N*-linked glycopeptides and oligosaccharides | [79] |
| Monosaccharides | 0.5 M NaOH followed by gradient elution with NaOAc and column regeneration with 0.2 M NaOH | Potato, sweetpotato and cassava | [167] |
| Mannose | 22 mM NaOH and column washing with 0.2 M NaOH | Purity of recombinant HSA | [161] |
| Myo-Inositol | 1.0 M NaOH isocratic | Infant formulae and clinical products for enteral feeding | [169] |
| D-Glycero-D-manno-heptopyranose | NaOAc and water in a gradient elution (pH 6.0) | Lipopolysaccharides | [160] |
| Mannitol, lactulose and glucose | 480 mM NaOH | Urine samples | [152] |
| Cellobiose and rhamnose | 10 mM $H_2SO_4$ and post column base addition | Urine samples | [147] |
| Glucose, fructose and sucrose | NaOH and acetate gradient | Sugar processing | [165] |
| Glucose, fructose and sucrose | 100 mM NaOH | Fruit juices | [148] |
| Amino sugars | 12 mM NaOH and 1 mM $Ba(OAc)_2$ | Seawater samples | [69] |
| Monosaccharides, lactulose, and lactose | 10 mM NaOH and 2 mM $Ba(OAc)_2$ | Commercial milks with different thermal treatments | [46] |
| Lactose | Not reported | Hydrolysis of lactose in milk | [77] |
| Glucose, fructose, sucrose | 50 mM NaOH | Tobacco products | [62] |
| Mono- and disaccharides | 150 mM NaOH and acetate gradient | Infant formulas | [66] |
| Monosaccharides | Acetate reverse gradient elution with water, followed by 0.170 M NaOAc in 0.2 M NaOH | Compositional analysis of lignocellulosic samples | [63] |
| Mono- and disaccharides | 0.1 M NaOH and 1 mM $Ba(OH)_2$ or $Ba(OAc)_2$; 0.016 M NaOH and 1 mM $Ba(OH)_2$ or $Ba(OAc)_2$ | Standard mixtures | [24] |
| Mono- and disaccharides, alditols, aminosugars | 5, 10 or 20 mM NaOH and 1–2 mM $Ba(OAc)_2$ | Standard mixtures | [44] |
| Alditols, mono and disaccharides | Water and postcolumn base addition | Wines and instant coffees | [149] |
| Monosaccharides and aminosugars | 12 mM NaOH, 16 mM NaOH, and 240 mM NaOH | Characterization of polysaccharides and humic bound carbohydrates in natural organic matter | [35] |
| Monosaccharides and oligosaccharides | Water for monosaccharides; NaOH and acetate gradient for oligosaccharides | Kraft pulps by various cellulases | [119] |
| Alditols | 0.5 M NaOH | Apple juice, chewing gum, dietetic candy | [22] |
| Ribitol | 650 mM NaOH | Glycoconjugate vaccines | [96] |
| Alditols and sugars | 0.6 or 0.45 M NaOH | Chewing gum, tabletop low calorie sweetener | [23] |
| Alditols and sugars | 0.50 M NaOH and 1 mM $Sr(OAc)_2$, $Ba(OAc)_2$ or $Ca(OAc)_2$ | Standard mixtures | [24] |
| Alditols and sugars | 0.58 M NaOH and 2 mM $Ba(OAc)_2$ | Fruit juices, celery, dietetic cherry jam, plums, cauliflower, fennel | [25] |

**Table 1** (continued)

| Sugar compounds | Mobile phase composition and elution mode | Applications | Reference |
|---|---|---|---|
| Alditols of mono- and disaccharides | 0.5–0.6 M NaOH; 40 mM NaOH and $Ba(OAc)_2$; 75 mM NaOH and $Ba(OAc)_2$ | Roasted malt, chicory, dietetic candies, chocolate, cream and sponge cake | [41] |
| Monosaccharides and alditols | NaOH gradient | Composition of mucin oligosaccharide alditols | [83] |
| Monosaccharide and sialic acids | 50 mM NaOH and 50–175 mM acetate | Gangliosides | [45] |
| NANA, GlcNAc, Gal, Man | 16 mM NaOH | Human plasma α-1-acid glycoprotein | [91] |
| Mono- and oligosaccharides | NaOH and acetate; NaOH and acetate gradient (oligosaccharides) | Standard mixtures and yeast mannan | [10] |
| Alditols, mono- and oligosaccharides | 0.1 M NaOH and NaOH and acetate gradient | Wine, fruit juices, milk oligosaccharides, honey, corn syrup, nonstarch polysaccharides, rice, corn, potato, grape musts | [17] |
| Monosaccharides and oligofructans | NaOH and acetate gradient | Transgenic potato tubers | [168] |
| Mono- and oligosaccharides | NaOH and acetate gradient | Commercial FOS preparations, FOS and inulin in dietetic samples | [118] |
| Mono- and oligosaccharides | NaOH and acetate gradient | Sugar industry, juices and liquors | [156] |
| Mono- and oligosaccharides | NaOH and acetate gradient | Fruit yogurt, candies, infant formula | [49] |
| Mono- and oligosaccharides | NaOH and acetate gradient, and postcolumn base addition | Honey samples (alfaalfa, alsike, canola, and trefoil) | [145] |
| Mono- and oligosaccharides | NaOH and acetate gradient | Beers | [154] |
| Mono- and oligosaccharides | NaOH and acetate gradient | Carbohydrates in fruit juices and honey | [155] |
| Mono- and oligosaccharides | NaOH and acetate gradient | Adulteration of grapefruit juice, orange juice, honey | [115, 116] |
| Mono- and oligosaccharides; aminosaccharides, alditols | 0.1 M NaOH, 30 mM NaOH and 0.2 M NaOH/20% acetonitrile | Standard mixtures | [29] |
| Mono- and oligosaccharides | NaOH and acetate gradient | Adulterated grapefruit juices | [146] |
| Oligosaccharides | 15 mM – 200 mM NaOH | Milk oligosaccharides and oligosaccharide alditols derived from mucin glycoproteins | [82] |
| Oligosaccharides | 100 mM NaOH and acetate gradient | In-line desalting system with a membrane | [98] |
| Oligosaccharides | 100 mM NaOH and acetate gradient | Human milk | [150] |
| Neutral-sugar and oligosaccharides | 16 mM NaOH, 150 mM NaOH and acetate gradient | Porcine intestinal gangliosides | [159] |
| Alditols, mono- and disaccharides, oligogalacturonic acids and maltooligosaccharides | KOH, LiOH, $LiNO_3$ and NaOH eluents | Separations on a macrocycle-based stationary phase | [158] |
| Oligosaccharides *O*-linked alditols | NaOH and acetate gradient | Delipidated sheep erythrocyte ghosts | [85] |
| Oligosaccharides *O*-linked | NaOH and acetate gradient | Bovine fetuin and bovine submaxillary mucin | [84] |
| Linear and branched glucans | 150 mM NaOH and acetate gradient | Amylopectins from various botanical sources (waxy rice, wheat, rice, barley, arrowhead, sweetpotato, normal maize, lotus, potato, edible canna, and yam) | [121] |
| Linear and branched glucose oligosaccharides | NaOH and acetate gradient | Waxy maize starch and pullulan | [143] |
| Maltooligosaccharides | NaOH and acetate gradient | Monitoring hydrolysates of starch by microdialysis sampling | [73, 74] |
| Maltooligosaccharides | NaOH and acetate gradient (glucoamylase reactor between column and detector) | Corn syrup | [144] |
| Debranched amylopectin | NaOH and acetate or nitrate gradients | Tapioca, corn and wheat | [53] |
| Polysaccharides | 612 mM NaOH | Carrageenans and agars | [163] |

**Table 1** (continued)

| Sugar compounds | Mobile phase composition and elution mode | Applications | Reference |
|---|---|---|---|
| Polysaccharides | 25 mM NaOH and 100 mM NaOAc | Tetanus toxoid-conjugate vaccine | [166] |
| Polysaccharides | NaOH and nitrate gradients (amyloglucosidase reactor between column and detector) | Branched-chain length distribution in starches | [157] |
| Polysaccharides | NaOH and nitrate gradient | Maize starch | [122] |
| Polysaccharides | 0.15 M NaOH and acetate gradient | A- and B-type maize starches | [123] |
| Polysaccharides | 0.15 M NaOH and acetate gradient | Wheat, potato and pea starches | [124] |
| Monosaccharides and uronic acids | NaOH and acetate gradient | Thickening agents: hydrolysates of gums, xanthan, pectins, carrageenan | [109] |
| Mono- and oligosaccharides, alditols, sulfated and phosphorylated saccharides | NaOH and acetate gradient | Glycoproteins, enzymatic reaction products | [16] |
| Mono- and oligosaccharides | NaOH and acetate gradient | Carbohydrate characterization of glycoproteins | [94] |
| *N*-linked oligosaccharides | NaOH and acetate gradient | Human IgG | [81] |
| Neutral, phosphorylated and sialylated oligosaccharides | NaOH and acetate gradient | Monitoring of dephosphorilation and desialylation of some oligosaccharides | [86] |
| Glucosamine and glucosamine-4-phosphate | NaOH and acetate gradient | Lipid A | [97] |
| Oligosaccharides and glycopeptides | NaOH and acetate gradient | Monitoring of deglycosilation of a fetuin tryptic glycopeptide | [26] |
| Glycoalkaloids: tomatine and dehydrotomatine | 20% MeCN, 15% MeOH citric acid and phosphate buffer at pH 3 | Tomatoes | [100, 101] |
| Glucose and gluconic acid | 150 mM NaOH | Glucose oxidase reaction | [76] |
| Uronic acids | NaOH and acetate gradients | Non-starch polysaccharides | [114] |
| Glycuronic acids | NaOH and acetate gradient | Microbial polysaccharides, plant residues, soil, sewage sludge | [110] |
| Phosphorylated sugars | 4 mM NaOH and acetate gradient (50–800 mM) | Plant tissues | [162] |
| 2-Keto-3-deoxyoctulosonic acid (KDO), Neu5Ac | NaOH and acetate gradient | Stability and hydrolysis of KDO | [153] |
| 2-Keto-3-deoxy-D-*glycero*-D-galactononononic acid (KDN) and deaminoneuraminic acid | NaOH and acetate gradient | KDN residues in glycoprotein and glycolipid fractions of rat tissues and human lung cancer cells | [151] |
| Neu5Ac, Neu5Gc, and KDN | NaOH and nitrate gradient | Colominic acid, polysialoglycoprotein from salmon eggs, deaminated neuraminic acid-containing glycoprotein from rainbow trout ovarian fluid | [93] |
| Monosaccharides, aminosugars and glucuronic acids | NaOH and acetate gradient | Poultry manure, sewage sludge, straw, alfaalfa (hydrolyzates) | [111] |
| Oligo- and polysialic acids | NaOH and nitrate gradient | Glycoconjugates in living organisms | [92] |
| Monosaccharides and glucuronic acids | 65 mM NaOH and 100 mM NaOAc and postcolumn base addition | Characterization of pectic substances from fruits and vegetables | [112] |
| Xylose oligomers and monosaccharides | 150 mM NaOH and acetate gradient | Xylan from corncorb holocellulose | [113] |
| Uronic acids, *N*-acetylneuraminic acid, muramic acid | 0.1 M NaOH and nitrate or acetate salts of barium, strontium and calcium | Standard mixtures | [48] |
| Muramic acid and glucosamine | NaOH and acetate gradient | Peptidoglycan analysis (*Escherichia coli* and *Provindencia stuartii*) | [90] |

**Table 1** (continued)

| Sugar compounds | Mobile phase composition and elution mode | Applications | Reference |
|---|---|---|---|
| Sialic acids | NaOH and acetate gradient | Glycoproteins (bovine fetuin, bovine and human transferrin) | [87, 88] |
| Oligogalacturonic acids | 0.1 M NaOH and acetate gradient | Strawberry juice | [164] |
| Hydroxyproline-oligosaccharides (arabinosides) | 150 mM NaOH and acetate gradient | Extensin characterization in melon callus cell walls | [67] |
| Sugar phosphates | NaOH and acetate gradients (100 mM ≤ [NaOAc] ≤ 500 mM; 54 mM ≥ [NaOH] ≥ 75 mM) | Cell extracts from *Saccharomyces cerevisiae* | [71] |
| Glycinamide ribonucleotide | NaOH and acetate gradient | Activity measurement of glutamine-5-phospho-ribosyl-1-pyrophosphate amidotransferase | [102] |

tions in biotechnology, medicine, agriculture, food and environmental samples. A comprehensive overview of analytical applications is given in Table 1. To make it easier for the reader to follow the relevant application for the compound of interest, the corresponding mobile phase composition and sample matrices are reported. Considering the current state of carbohydrate analysis, it is conceivable that many more analytical determinations will be set up and an increasing number of laboratories are on the verge of becoming involved, thus fully exploiting the direct and sensitive detection of sugar compounds without any derivatization. We will now discuss some interesting examples of carbohydrate analysis by HPAEC-PAD.

### 4.1 Alditols, mono- and disaccharides

Analysis of mono- and disaccharides is best performed employing sparingly alkaline eluents (i.e., $[OH^-] \leq 50$ mM). As mentioned earlier, the lower the alkalinity, the higher is the selectivity of the separation; to achieve this objective many authors report the use of pure water as the mobile phase (see Table 1). Under such conditions, however, it is necessary to regenerate the column with concentrated NaOH after each run in order to restore its full ion-exchange capacity. Moreover, a postcolumn addition of base is needed to guarantee the electrochemical activity of gold toward the oxidation of carbohydrates. Prodolliet et al. [60] performed the analysis of simple sugars in soluble coffees by employing water as the eluent, and a column cleanup after each elution run; the postcolumn addition was accomplished by 0.3 M NaOH at a flow rate of 0.6 mL/min. A complete carbohydrate profile was obtained (see Fig. 6), but a very long run time was required, especially considering post run conditioning and column reequilibration. Similar results were reported by Rohrer and Olechno [61] and Zook et al. [62] on deuterated glucoses and sugars in tobacco products, respectively. A different approach was adopted by Davis [63], who proposed a simple strategy to shortening analysis times during monosaccharide composition assays of lignocellulosic samples; water was used as the eluent followed by a rapid regeneration of the column with a stronger eluent composed of 200 mM NaOH and 170 mM NaOAc.

It must be emphasized that the separation of xylose and mannose can be achieved only with water [60, 64] or eluents with $[OH^-]$ concentrations as low as 2 mM. Indeed, Johnson could achieve a good resolution of these monosaccharides by the use of 1.0 mM $Ba(OH)_2$ and 0.125 mM acetic acid [1]. In one of his extensive reviews on carbohydrate analysis by HPAEC-PAD [10], Lee showed the separation of fucose, *N*-acetylgalactosamine, galactose, *N*-acetylglucosamine, glucose, xylose and mannose with the eluent 1 mM NaOH and 0.03 mM sodium acetate. Campbell et al. [65] reported the monosaccharide fingerprint of a dietary fiber in some fibrous ingredients and foodstuffs, employing a linear gradient of NaOH from a concentration of 10 mM to pure water. The same authors compared the performances of HPAEC-PAD with gravi-

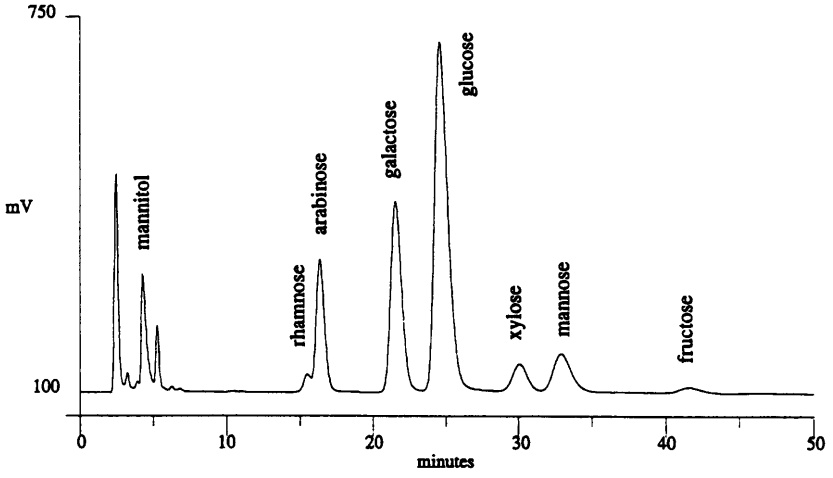


**Fig. 6** Carbohydrate profile by HPAEC–PAD of a soluble coffee containing coffee husks or parchments and cereals. Reprinted from Predolliet J, Bruelhart M, Lador F, Martinez C, Obert L, Blanc M B, Parchet J-M (1995) J AOAC Internat 78:749–761. Copyright, (1995), by AOAC International

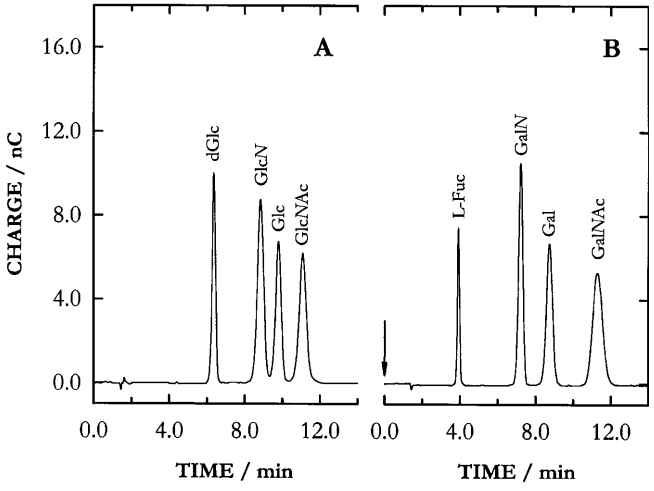


**Fig. 7** HPAEC-PAD of two standard mixtures: (**A**) dGlc, Glc*N*, Glc, and Glc*N*Ac; (**B**) l-Fuc, Gal*N*, Gal, and Gal*N*Ac. Eluent, 10 mM NaOH and 1 mM $Ba(OAc)_2$ at a flow rate of 1.0 mL/min. Column, CarboPac PA1 plus guard (Dionex). Detection potential at the gold working electrode, $E_{DET}$ = +0.05 V vs. Ag|AgCl

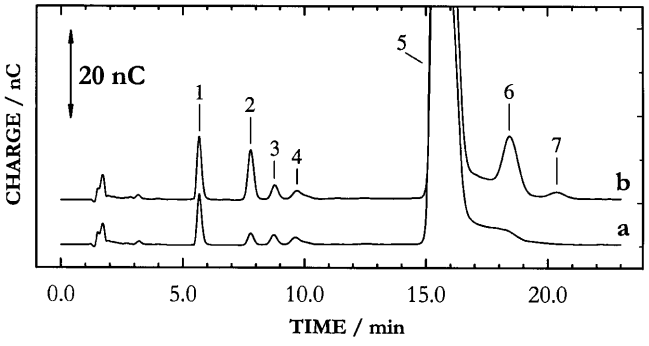


**Fig. 8** Carbohydrate profiles by HPAE-PAD of milk samples (a) pasteurized, and (b) in–container sterilized. Samples diluted 100 times with water. Peak identification: (*1*) (dGlc, IS), (*2*) Gal, (*3*) Glc, (*4*) Gal*N*Ac, (*5*) lactose, (*6*) lactulose, and (*7*) epilactose. Eluent, 10 mM NaOH and 2 mM $Ba(OAc)_2$ at a flow rate of 1.0 mL/min. Column, Dionex CarboPac PA10. Detection potential at the gold working electrode, $E_{DET}$ = +0.10 V vs. Ag|AgCl

metric, enzymatic-gravimetric and enzymatic-chemical methods.

Retention time constancy in the separation of carbohydrates in sample matrices is especially important so that the analyte is not misidentified as the nearby peaks. Cataldi and co-workers described a very straightforward method for the removal of carbonate from the mobile phase [40, 44]. When a dilute alkaline mobile phase is used (i.e. 5–20 mM), the addition of barium acetate in millimolar concentrations allows the rapid and reproducible separation of closely related mono- and disaccharides. Typical examples are given in Figs. 2 and 3. With a mobile phase consisting of 10 mM NaOH and 2 mM $Ba(OAc)_2$ no regeneration of the column between runs was needed. Moreover, under such elution conditions, good baseline stability along with the absence of peak tailing was achieved. The benefit of these eluent solutions for the determination of sugar isomers is illustrated in Fig. 7, which refers to the separation of two families of saccharides: fucose, galactosamine, galactose, and *N*-acetylgalactosamine (panel A) and 2-deoxyglucose, glucosamine, glucose and *N*-acetylglucosamine (panel B). Comparing these chromatograms with those obtained with water as an eluent, it can be noted that the selectivity of separation is very satisfactory.

Dilute alkaline mobile phases modified with barium acetate have been successfully applied to several real samples. Depending upon the disparate levels of carbohydrates present in the matrix, it is rather difficult to detect a small peak in the presence of a close big one. Thus, an example that cannot remain outside the scope of this discussion is related to milk samples. Lactulose (*O*-*β*-D-galactopyranosyl-(1→4)-D-fructose) is a disaccharide formed by heat-induced isomerization of lactose (*O*-*β*-D-galactopyranosyl-(1→4)-D-glucose) during milk sterilization. The main difficulty in accurate quantification of lactulose is due to the presence in milk of high levels of lactose. However, the sensitive and accurate quantification of lactulose by HPAEC-PAD has been demonstrated and it makes the differentiation between sterilized and pasteurized milks possible (Fig. 8) [45]. It is also of great impor-

tance to be able to determine whether or not a milk-based formula is substituted for soy or protein hydrolyzate. Kaine and Wolnik [66] developed a method to profile the carbohydrates of various milk-based, soy-based, and protein hydrolyzate infant formulas; adulteration or relabeled formulas were examined and compared to authentic foodstuffs.

The main problem associated with the determination of sugar compounds in real samples is the complexity of the matrix. The many advantages of HPAEC–PAD, such as speed, sensitivity, and relatively low purity requirements, make it a valuable analytical tool for carbohydrate analysis of vegetal tissue extracts, without requiring extensive sample cleanup. For example, HPAEC-PAD was successfully employed for determining the glycosylation pattern of hydroxyproline in extensin, which is the main hydroxyproline-rich glycoprotein in the plant cell wall [67]. Hydroxyproline and hydroxyproline-arabinosides were released from plant materials upon hydrolysis with 0.22 M $Ba(OH)_2$, which cleaves only peptidyl bonds.

The controlling function of simple sugars and alditols in the metabolism of olive plants has been reported, but little is known on the role of these compounds as osmoregulators and osmoprotectants during water drought conditions. As carbohydrates are likely to play a central function, we have demonstrated that anion-exchange chromatography coupled with pulsed amperometry is very suitable for the determination of these solutes in plant tissues which does not require the use of radioactive substrates [68]. Figure 9 shows the rapid and selective carbohydrate analysis of a water extract from olive roots. Very recently, Kaiser and Benner [69] reported that high salt concentrations (i.e., marine samples) severely compromise sensitivity of detection and interfere with the separation of amino sugars by HPAEC-PAD. It was mandatory, therefore, to remove inorganic salts prior to sample analysis, thus overcoming the separation problems previously reported for the determination of marine monosaccharides [70]. A cleanup method based on solid-phase extraction (SPE) has been developed by Smits et al. [71] to render biological extract of *Saccharomyces cerevisiae* suitable for the analysis of sugar phosphates. In-situ microdialysis probe fitted with dialysis membranes was demonstrated very useful in accomplishing both sampling and sample cleanup [72]. Torto et al. [73, 74] used an online microdialysis system coupled to HPAEC-PAD to characterize the enzymatic mannanase hydrolyzates of ivory nut mannan; the same group studied the performances of some microdialysis membranes with respect to extraction and permeability factors, temperature stability and interaction with enzymes [75]. The combination of microdialysis sampling with HPAEC-PAD was also employed for monitoring glucose conversion during glucose oxidase reaction [76], and hydrolysis of lactose catalyzed in vitro by β-galactosidase [77].

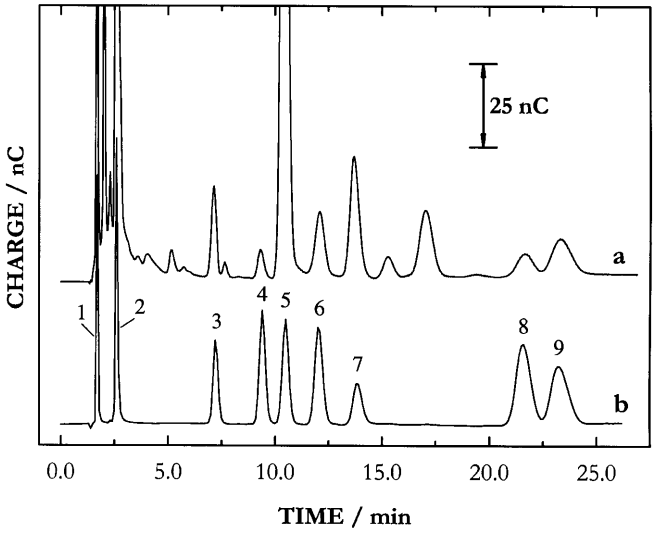


**Fig. 9** (*a*) Carbohydrates in a root extract of olive plant separated by HPAEC-PAD at a gold working electrode. (*b*) Standard solution containing, (*1*) *myo*-inositol, 8 μM; (*2*) mannitol, 20 μM; (*3, IS*) 3-omeGlc, 40 μM; (*4*) Gal, 20 μM; (*5*) Glc, 20 μM; (*6*) sucrose, 20 μM; (*7*) Fru, 20 μM; (*8*) raffinose, 20 μM; (*9*) stachyose, 20 μM. Eluent, 12 mM NaOH and 2 mM $Ba(OAc)_2$. Chromatographic conditions: CarboPac PA1, analytical and guard columns at a flow rate of 1.0 mL/min and temperature of 22 °C. Detection potential at the gold working electrode, $E_{DET}$ = +0.25 V vs. Ag|AgCl

Alditols are weaker acids than the corresponding carbohydrates and are generally well separated with macroporous anion-exchange columns using strongly alkaline mobile phases (i.e., 0.4–0.6 M NaOH). These compounds are widely present and distributed in nature and are increasingly being employed to sweeten foods or drinks. Corradini et al. [23] reported the separation of a tabletop sweetener extract containing sorbitol, mannitol and fructose using a CarboPac MA1 column. The feasibility of this approach was demonstrated by its application to the analysis of plum juice syrups (see Fig. 10). Another interesting application of HPAEC-PAD is for so-called low-calorie foods containing relatively large amounts of mannitol, such as in dietetic cherry jams [25]. It is striking that whereas the whole-sugar profile of fruit juices can be obtained, the analysis time using such a column is relatively long and the selectivity is not very satisfactory.

### 4.2 Glycoconjugates and sugar acids

Glycoconjugates are involved in several important aspects of cell metabolism, structure, assembly, and recognition as well as being carriers of information through biological interactions. Compositional analysis of the carbohydrate moiety of the glycoconjugates is of fundamental importance in structural studies of these compounds. In this respect, HPAEC-PAD analysis of hydrolyzates from glycoconjugates has been successfully employed, as extensively reviewed by Townsend [78], to identify some of their key features by carbohydrate screening and quantification. Structural analysis of carbohydrates present in glycopeptides is routinely preceded by removal of the protein portion accomplished by chemical or enzymatic methods.

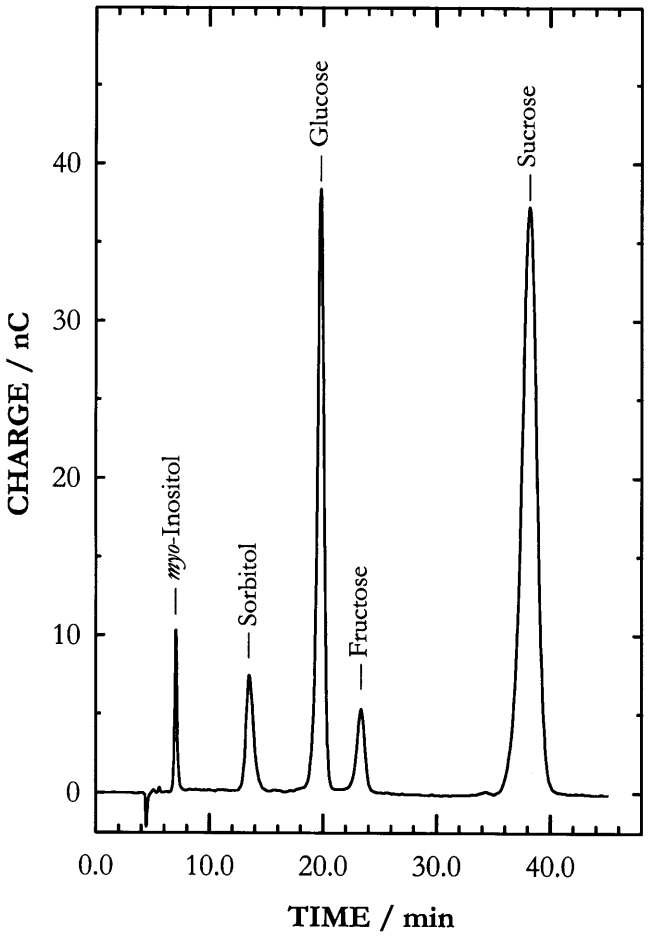


**Fig. 10** HPAEC-PAD of a sample of plum juice. Dionex CarboPac MA1, analytical and guard columns at a flow rate of 0.50 mL/min. Eluent, 0.50 M NaOH. Detection potential, $E_{DET}$ = +0.00 V vs. Ag|AgCl

Hardy et al. [79] described one of the first applications of carbohydrate analysis of some glycoproteins: asialofetuin, fetuin, orosomucoid and fibrinogen. The separation and quantification of galactose, mannose, galactosamine, and glucosamine of the relevant hydrolyzates was achieved with 22 mM NaOH as eluent. The whole run also comprised post-run column conditioning with 0.2 M NaOH and a postcolumn addition of 0.3 M NaOH. The same authors demonstrated the HPAEC-PAD capability of separation of glycopeptides differing only in a single Gal$\beta$(1,3) versus a Gal$\beta$(1,4) linkage [26]. It has been supposed that the Gal$\beta$(1,3)-GlcNAc-glycopeptide, because of its well-defined hydrophilic side, interacts more strongly with the anion exchange sites than the Gal$\beta$(1,4)-GlcNAc-containing structure. However, Wang and colleagues [80], while studying the analysis of sialic acid-containing oligosaccharides, found evidence for the pitfalls of the above hypothesis and pointed out the different factors influencing the acidity of the oligosaccharides compared to monosaccharides. These factors seems to be the following: (i) formation of hydrogen bonds with either vicinal or remote hydroxyls, (ii) acidity increase due to a statistical effect, and (iii) a cooperative interaction of the ring hydroxyls with the ionized solid phase. McGuire et al. [81], using a pool of seven neutral diantennary *N*-linked oligosaccharides, found that the influence of monosaccharide substitution on the separation process was identical to that observed for sialylated structures. Baseline resolution of neutral *N*-linked oligosaccharides was achieved with an isocratic mobile phase of 250 mM NaOH and 5 mM NaOAc. Reddy and Bush [82] isolated oligosaccharide alditols by alkaline borohydride degradation of *O*-linked mucin glycoproteins. A mixture of three oligosaccharide alditols was eluted with 15 mM NaOH. Interestingly, the linear alditol (L7) eluted earlier than the branched tetrasaccharides, in contrast to the chromatographic behavior of neutral and sialylated oligosaccharides, whose retention time is shortened in the presence of a branching at the pyranose residue [26, 80]. It should be noted that the multiplicity of factors influencing the elution order of oligosaccharides along with their pulsed amperometric response enhances the whole analytical selectivity even though it makes fairly critical the attribution of unknown peaks.

Compositional analysis of mucin oligosaccharides was accomplished by Karlsson and Hansson [83]. After release from mucin glycopeptide, oligosaccharides were hydrolyzed and re-acetylated in order to enhance the selectivity of the separation, carried out with a CarboPac MA1 column. Identification of *O*-linked oligosaccharides derived from glycoproteins was also performed by Hayase and co-workers [84]; they analyzed sialyl-oligosaccharide alditols obtained from β-elimination with reduction of bovine submaxillary mucin and bovine fetuin employing suitable gradients of NaOH/NaOAc. The same authors developed a pre-column *N*-succynylation technique for improving the HPAEC separation of desialylated oligosaccharides. Kotani and Takasaki [85] pointed out that β-elimination of glycoproteins in the presence of sodium borohydride can also cleave peptide bonds, leading to the formation of small peptides and glycopeptides which can be detected by pulsed amperometry. As interference with *O*-linked oligosaccharides was expected, they studied both the oligosaccharides derived from purified glycoproteins and crude glycoprotein samples.

As far as response factors are concerned, Hardy et al. [86] compared the PAD response of sialylated and phosphorylated oligosaccharides along with their neutral counterparts; as the response factor of these compounds was strongly affected by the acidic residue position, the conversion of sugar compounds to their neutral counterparts using alkaline phosphatase and neuraminidase to perform an accurate quantitative analysis with electrochemical detection was suggested.

There is a growing interest in the quantitative determination of *N*-acetylneuraminic acid (NANA) present at the terminal position of many glycoprotein and glycolipid oligosaccharides due to its influence on half-life and activity [87, 88]. NANA belongs to a large family of *N*- and *O*-substituted neuraminic acids, also known as sialic acids whose determination by HPAEC-PAD has been very recently reviewed by Rohrer [89]. As an example, the con-

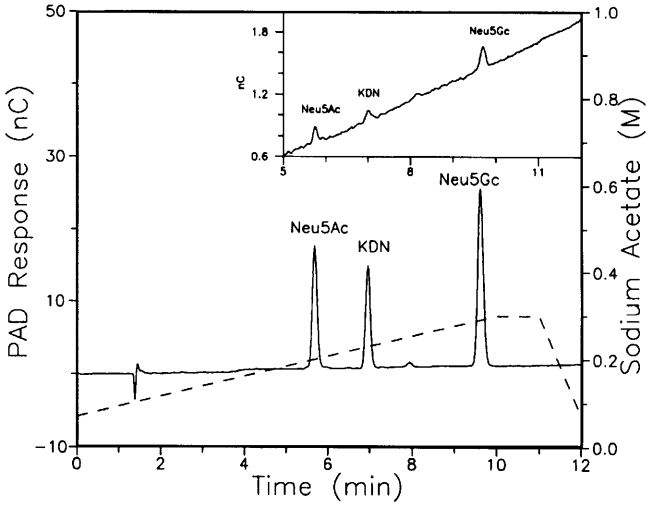


**Fig. 11** HPAEC separation of Neu5Ac, KDN, and Neu5Gc (200 pmol each). The inset shows a separation of 1 pmol of each using a new working electrode. Reprinted from Rohrer JS, Thayer J, Weitzhandler M, Avdalovic N (1998) Glycobiology 8:35–43, by courtesy of Oxford University Press

tents of NANA and *N*-glycolylneuraminic acid in bovine transferrin, bovine fetuin and human transferrin were measured, thus demonstrating the suitability of HPAEC-PAD for the direct routine analysis [87] (Fig. 11). Interestingly, the chromatographic behavior of NANA can be strongly affected by the presence of divalent ions in the eluents, especially calcium, due to the capability of such a compound to form complexes [48]. Clarke [89] was the first to report the determination of muramic acid in peptidoglycan hydrolyzates by HPAEC-PAD; he also compared the anion-exchange chromatographic method with the conventional one normally used for amino acid analysis, employing a cation-exchange column and postcolumn ninhydrin addition. A successful separation of glucosamine, muramic acid, and nine amino acids in various peptidoglycans was reported.

An alteration of the carbohydrate moiety of α-1-acid glycoprotein (AAG) occurs in several diseases such as renal insufficiency, myocardial infarction, rheumatoid arthritis and acute inflammation. Kishino et al. [91] compared the compositional analysis of the carbohydrate moiety in the AAG of patients with renal insufficiency with those of healthy subjects. They observed that in the subjects with renal insufficiency the mean concentrations of GlcNAc, Gal, and Man were significantly higher than those in the healthy people. Polysialic acids are carbohydrate residues of glycoconjugates found in different living organisms having potential roles in cell growth, differentiation and neuropathogenicity. HPAEC-PAD [92, 93] could differentiate polysialic chains having a degree of polymerization (DP) even greater than 70, as illustrated in Fig. 12 [92]. In particular, as no derivatization is needed, for polysialic chains having DP higher than 25 the method was more sensitive and specific than HPAEC coupled with fluorimetric detection.

One of the main reasons for the wide application of HPAEC-PAD in the compositional analysis of carbohydrates of pharmaceutical recombinant glycoproteins is related to the sensitivity of detection. Spellman [94] reviewed the characterization of recombinant glycoprotein carbohydrates and described the common approaches to investigate the primary structure of the glycoprotein-derived oligosaccharides. He emphasized the convenience of HPAEC-PAD in glycoconjugates mapping, due to its sensitivity to molecular size, linkage position of the monosaccharide units and anomeric configuration. Yokota et al. [95] reported the determination of neutral and amino sugars from hydrolyzed pamiteplase, which is a recombinant modified human tissue plasminogen activator. Since the pamiteplase formulation contains sucrose, it was removed prior to analysis. Determination of fucose, glucosamine, galactose and mannose was accomplished upon acid hydrolysis with TFA performed at 100 °C for 4 h. Bardotti et al. [96] discussed a method to determine free and total saccharides of *Haemophilus influenzae* type b (Hib) in the formulated Hib-CRM conjugate vaccine. As quantification of free Hib saccharide using nuclear magnetic resonance (NMR) was not possible due to a great deal of interference, a method based on acidic hydrolysis with TFA followed by chromatographic separation and quantification of ribitol on a CarboPac MA1 column was developed. The separation of ribitol is illustrated in Fig. 13; the method requires only very small amounts of sample, thereby ensuring that free saccharide can be accurately monitored both in the formulated Hib-CRM vaccine alone as well as in combination with other vaccines.

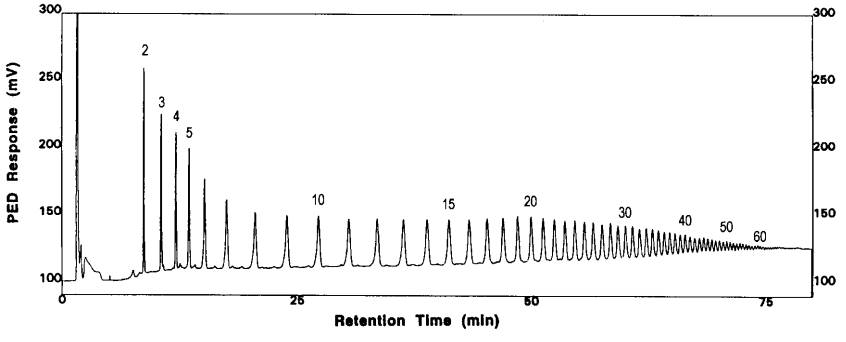


**Fig. 12** Separation of α-2–9-linked oligo/polyNeu5Ac after acid hydrolysis on a CarboPac PA100 column. Peaks are labeled with DP. Reprinted from Lin SL, Inoue Y, Inoue S (1999) Glycobiology 9:807–814, by courtesy of Oxford University Press

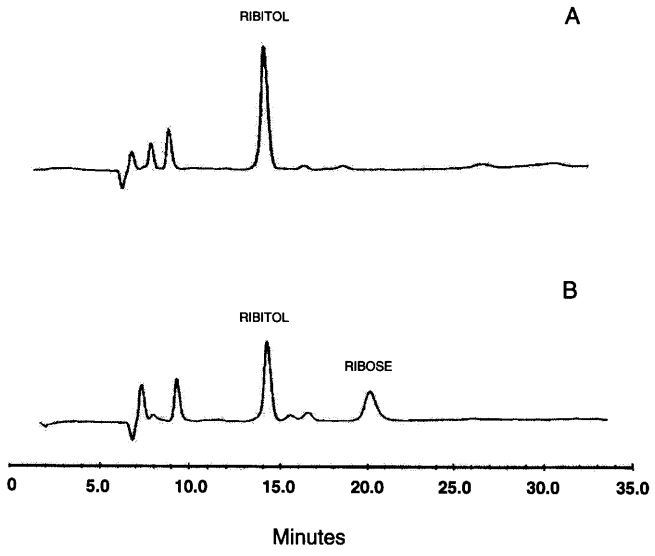


**Fig. 13** HPAEC-PAD of samples after acid hydrolysis. (*A*) Ribitol standard; (*B*) *Haemophilus influenzae* type b (Hib) in the formulated Hib-CRM vaccine. Reprinted from Bardotti A, Ravenscroft N, Ricci S, D'Ascenzi S, Guarnieri V, Averani G, Constantino P (2000) Vaccine 18:1982–1993, with kind permission of Elsevier Science

Glycolipids have also been successfully analyzed by HPAEC-PAD. For example, Lee and co-workers [97] described the simultaneous determination of Glc*N* and Glc*N*-4-phosphate released by acidic hydrolysis of the lipopolysaccaride Lipid A, and the total glucosamine content could be obtained either by correcting for the incomplete hydrolysis of Glc*N*-4-phosphate or by summing the two yields. Because sialylated and phosphorylated sugars are strongly retained on the stationary phase of an anion exchanger, larger amounts of sodium acetate, which has a much higher elution strength compared to sodium hydroxide, are added to the mobile phase; similar conditions were employed by Smits et al. [71], who reported the study of sugar phosphates in cell extracts of *Saccharomyces cerevisiae*. In these cases, NaOH serves predominantly to adjust the pH to that required for electrochemical detection.

Another area of analysis demanding glycoconjugates is represented by glycans or proteoglycans. They have linear chains of repeating carbohydrate units. Wang et al. [45] developed a method for the identification and quantification of inositol isomers and monosaccharides in inositol-containing glycans. After acid hydrolysis with TFA, samples are eluted in a cation exchange column (Aminex HPX87C) at 50 °C and detected by PAD. The four natural inositols were separated and detected in amounts as low as 10 pmol. Thayer et al. [98] pointed out the possibility of obtaining structural information for glycans using linkage-specific glycosidase in combination with HPAEC-PAD and mass spectrometry (MS). In particular, the inline use of a carbohydrate membrane desalter and a post-collection evaporator permitted one to perform all the measurements in a single run.

The biological effects of potato and tomato glycoalkaloids on cell membranes, cholesterol, pathogens, and in the human diet are strongly influenced by the nature of carbohydrate side chain attached to the 3-OH position of the steroidal part of the molecule [99]. The growing interest in glycoalkaloids and the problems inherent in their detection, as most of them do not absorb visible or ultraviolet light, are reflected in the diverse analytical approaches. The absence of chromophoric functional groups has led to development of methods based on refractive index detection and gas chromatographic methods. Friedman has suggested a different route in which a reversed-phase separation with a $C_{18}$ column was coupled with PAD and used to measure the content of tomato glycoalkaloids in tomatoes, tomato plant parts, and processed tomato products [100, 101]. Under such elution conditions, the detection requirement of high pH was satisfied by post-column addition of alkaline solutions.

An HPAEC-PAD method for the evaluation of the catalytic activity of glutamine-5-phopsphoribosyl-1-pyrophosphate (GPAT) has been proposed by Taha and Deits [102]. Such a procedure consists in the analysis of glycinamide ribonucleotide, a stable compound formed by the unstable product (phosphoribosylamine) of the GPAT enzymatic reaction. The authors pointed out that such a method was able to overcome the lack of specificity related to the traditional assays, i.e., measure of glutamate or NAD formation.

It is important to highlight that structural elucidation of complex carbohydrates is a challenge, as it involves characterization of sugar sequence, branching, linkage between the constituting monosaccharides and anomeric configuration. In this respect, NMR constitutes a very important analytical tool for the determination of the structure of carbohydrates when milligrams of sample are available [103]. Particularly powerful are also the recently developed soft ionization MS techniques, such as FAB, ESI and MALDI-TOF, which allow rapid and sensitive analysis of complex samples including oligosaccharides from sub-nanomolar amounts of material [104–106]. Indeed, the determination of the native molecular mass and the elucidation of the carbohydrate sequence can be relatively simple.

### 4.3 Oligosaccharides and polysaccharides

One of the most demanding aspects regarding the analysis of dietary carbohydrates is food authentication. Adulteration normally involves replacing natural carbohydrates with other saccharides from less expensive sources [107, 108]. A typical example is given by the addition of pectin to juices. Koswig et al. [109] analyzed the monosaccharide composition of natural pectin from apples and citrus, guar gum, gum arabic and xanthan, all used as thickening agents to obtain stable mixtures and food formulations. As seen in the case of glycoconjugates, the compositional analysis of monosaccharides is the first step to reach the complete characterization of a complex polysaccharide

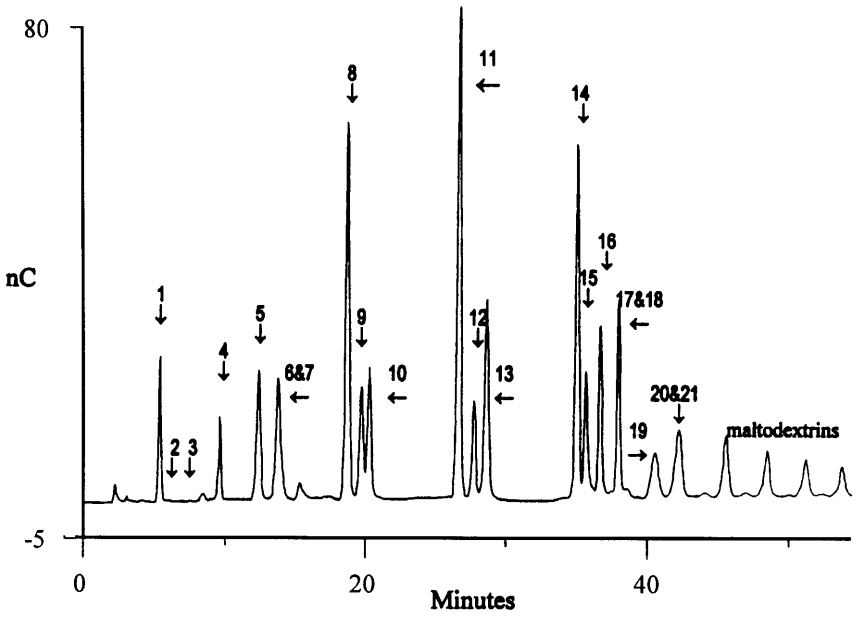


**Fig. 14** HPAEC-PAD of a mixture of maltodextrin and "Raftilose-P95®". Peak identity, (*1*) Glc, (*2*) Fru, (*3*) lactose, (*4*) sucrose, (*5*) 1-ketose, (*6*) maltose, (*7*) inulobiose, (*8*) nystose, (*9*) maltotriose, (*10*) inulotriose, (*11*) fructosylnystose, (*12*) maltotetraose, (*13*) inulotetraose, (*14*) GF5, (*15*) maltopentaose, (*16*) inulopentaose, (*17*) GF6, (*18*) maltohexaose, (*19*) inulohexaose, (*20*) GF7, (*21*) maltoheptaose. Reprinted from Durgnat J-M, Martinez C (1997) Sem Food Anal 2:85–97, with permission of Aspen Publisher

[110–113]. Quigley and Englyst evaluated the uronic acid content in non-starch polysaccharides such as pectin and sugar beet, extracted from fruits, vegetables and cereal products [114].

In many instances it may be important to identify the oligosaccharide composition of a given matrix. Low [115] pointed out that many inexpensive sweeteners or syrups contain the same major carbohydrates present in authentic foods and beverages; as these oligosaccharides also exhibit very similar ratios between the homologous series, their compositional analysis is not conclusive. Hence, food adulteration revealed by HPAEC-PAD through the recognition of fingerprint oligosaccharides is possible, but limited [115, 116]. Another class of carbohydrate used for food-labeling purposes is represented by fructooligosaccharides (FOS), which are low-molecular weight fractions of inulin. These polymers have been characterized by enzymatic hydrolysis of fructose chains followed by glucose, fructose and sucrose quantification [117]. While such a method is not very convenient for the identification of different FOS in the matrix, HPAEC-PAD has proved to be very suitable. Figure 14 shows the fingerprint of a commercial FOS preparation of "Raftilose P-95", obtained by treatment of chicory root inulin with endo-inulinase [118]. Soluble oligosaccharides of different origin (pulp and paper making process) have also been analyzed by HPAEC-PAD after enzyme hydrolysis to cello-, xylo- and mannooligomers [119].

Starch is the major storage polysaccharide of many plants and it accumulates to high levels in seeds or tubers as insoluble granules [120]. Starch is a mixture of two α-D-glucose polymers, amylose (~20–30%) and amylopectin (~70–80%). Both amylose and amylopectin are linear α-D-(1→4) linked glycosil units but they have 0.1 and 4% of α-D-(1→6) branch points, respectively. The interest in the characterization of starches with regard to plants or plant growing conditions has been successfully challenged by means of HPAEC-PAD analysis upon enzymatic hydrolysis. Hanashiro et al. [121] analyzed the chain length distributions of amylopectins from eleven different plant sources, whereas Lu et al. [122] have been able to distinguish the fine structure of maize starch grown at different temperatures. As reported by Gérard et al. [123], the structural features of two starch samples constituted solely of amylopectin, an A-type from normal and a B-type from tuber starches, have also be obtained by HPAEC-PAD analysis of the products obtained from two consecutive hydrolyses involving α- and β-amylases. The effect of annealing on wheat, potato and pea starches has been investigated by Jacobs et al. [124]. The authors found a higher ratio of singly branched to short linear chains for the annealed compared with the corresponding native starches. As a result, the acid hydrolysis step after annealing was more difficult. A similar approach demonstrated the investigation of alginate using alginate-lyase to degrade the polymer; the relevant oligosaccharides were quantified by HPAEC-PAD [125].

### 4.4 Comparison with other HPLC methods

HPLC is characterized by a relatively high resolving power, and sample analysis and pre-treatment are easily accomplished. Thus, many liquid chromatographic systems have been described for the analysis of carbohydrates in food and biological samples. The first example incorporated refractive index (RI) detection with reversed-phase separation and plain water used as the mobile phase. The earliest column packings for carbohydrate separation were based on silica derivatized with cyanopropyl, amino or aminoalkyl groups. However, the difficulty of using eluent gradients and the relatively poor sensitivity associated

with refractometry has stimulated the search for alternative methods. Considering that photometric detection is hindered by the lack of a strong inherent chromophore, the possibility of detection either by UV absorbance after suitable derivatization or at low wavelengths has been evaluated [126–129]. By employing $-NH_2$ stationary phases it has been possible to successfully resolve glucose oligosaccharides up to a DP number of 30–35 [130], where the DP signifies the number of monomeric units, maltose oligosaccharides, cyclodextrins [131], and oligosaccharides derived both from glycoproteins [132] and human milk [133]. Despite these interesting results, the use of amino-phases has a noticeable drawback related to the tendency of glycosamine formation between reducing sugars and amino groups of the stationary phase. Such a phenomenon causes a severe loss of column performance.

The use of octadecylsilica column packings for the separation of underivatized carbohydrates employing an aqueous eluent has also been reported [128, 134]. In this case, the retention behavior of oligosaccharides is probably related to van der Waals interactions with the long alkyl chains of the column. Although the sample treatment is relatively simple, this approach has some disadvantages; first of all, monosaccharides elute first and are not well resolved [134]. Moreover, separation of the anomeric forms is observed with a pair of peaks for each more retained compound. Some attempts have been made to accelerate mutarotation and to attain a higher efficiency, e.g. by increasing the column temperature up to 60 °C, the use of alkaline eluents (e.g. 1 mM triethylamine in water, pH 10) and sugar reduction with sodium borohydride [128]. However, these methods generally lead to loss in resolution or are not effective for oligosaccharide profiling. In fact, this chromatographic method is only useful when dealing with oligosaccharides which do not possess anomeric centers. Better results can be obtained upon derivatization of carbohydrates to adduct molecules containing both hydrophobic and chromophoric groups [134, 135]. In this way, it is possible to attain two objectives: photometric detection, which is much more sensitive than RI, and selective reversed-phase HPLC separation. In addition to these analytical determinations, derivatization can be useful for structure elucidation. For instance, the analysis of derivatized oligosaccharides with liquid chromatography coupled with chemical ionization MS detection, and further analysis of the collected fractions by gas chromatography (GC) and electron impact MS has been helpful in sequencing the glycosyl residues of complex carbohydrates [136]. Despite the above mentioned advantages, however, the derivatization step is labor intensive and time consuming.

Cation-exchange columns loaded with metal ions, including calcium, lead, and silver, can be used to separate underivatized carbohydrates with water as the eluent [137–140]. The retention mechanism is mainly based on the formation of weak complexes between saccharides and metal ions: the higher the affinity to the immobilized cations, the longer the retention time. Stefansson and Westerlund [141] extensively reviewed ligand-exchange chromatographic methods for carbohydrates and glycoconjugates. The main drawbacks of such an approach are related to an early elution of oligosaccharides immediately after the void volume, often making resolution of these compounds extremely difficult, and the use of a non-selective and low-sensitivity RI detection. Liquid chromatographic analysis of carbohydrates can also be accomplished through anion-exchange of borate complexes, which are negatively charged [142]. The chromatographic behavior is again governed by the different stabilities of adducts formed during the analysis. Despite its usefulness in achieving the separation of saccharides, the borate-polyol equilibrium induces peak broadening, with a consequent loss of resolution. The analysis time is also rather long.

## 5 The future

With the advent, growth, and now widespread use of HPAEC-PAD the analytical chemist has a valuable means for sensitive and selective determination of carbohydrates and sugar-related compounds. This ultimate combination between separation and detection has created an analytical research tool with both remarkable capabilities and exciting opportunities, which have yet to be fully exploited. Thus, if you are looking for an analytical separation technique to resolve complex carbohydrate mixtures and to quantify each sample molecule with high sensitivity, then you should first take a look at HPAEC-PAD. From the authors' perspective, it can be predicted that additional sensitive applications can be expected in this area of research in the near future. The challenge in HPAEC-PAD is to extend or even to enhance the potential of the system for glycoconjugate analysis, and more generally for sugar-related compounds. What will the future bring? The application of HPAEC-PAD in the analysis of carbohydrates is just one example demonstrating the power of such a combination. As electrochemical detection is ideally suited for miniaturization purposes, the next expected step is related to narrow-bore, capillary columns and LC systems: a guarantee for even more exciting applications of carbohydrate analysis.

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