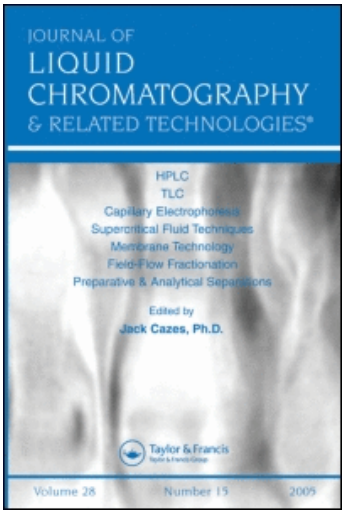

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HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS AS A POWERFUL ANALYTICAL TOOL OF GLYCOCONJUGATES

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HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS AS A POWERFUL ANALYTICAL TOOL OF GLYCOCONJUGATES

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ABSTRACT

Glycoconjugates constitute an important family of macromolecules having a key role in several cellular events. Elucidation of the fine chemical structure of their carbohydrate components is necessary for a deeper understanding of their biological functions. Capillary electrophoresis is a recently developed analytical technique with promising applications in the field of carbohydrate analysis. Various modes of CE have been used for the resolution of carbohydrates. The absence of charge from neutral carbohydrates has been circumvented by ionization of their hydroxyl groups at high pH, complexation with charged agents like borate anions and chemical derivatization. Although the absence of chromophores or fluorophores hinders a sensitive detection by direct UV or fluorescence detectors, indirect detection is a good alternative offering satisfying limits of detection. Electrochemical detectors have also been introduced. Pre-column derivatization of carbohydrates with a wide range of specific chromophores and fluorophores has offered impressive sensitivity gains especially when laser-induced fluorescence detectors are used. In this review, the separation mechanisms of

carbohydrates using various CE modes are summarized. The recent developments in detecting carbohydrates and selected applications are also presented.

INTRODUCTION

Carbohydrates are universally distributed among plants, animals, and microorganisms. They play a key role in the process of life and this is reflected in the fact that they make up most of the organic matter on earth. Besides their importance in nutrition and cellular architecture, they are vital for important functions such as cell–cell recognition, cell signaling, proliferation and migration, immunological protection, and blood coagulation.[1] Understanding these functions will never be complete unless the glycosylation processes and the carbohydrate structures are fully described. A better insight to these issues would create numerous opportunities for the design of new diagnostic techniques and powerful pharmaceuticals. The development of analytical methodologies of high sensitivity and resolving power will be, therefore, of great importance. Recently, high performance capillary electrophoresis (HPCE or CE) has emerged as a highly promising tool for carbohydrate analysis due to its high resolving power, the sensitive means of detection, the speed of analysis, the low consumption of sample and buffers and its automated simple operation coupled with its quantitative capabilities. Although its application to carbohydrate analysis has not yet reached its methodological maturity, HPCE has made remarkable achievements in the way we analyze carbohydrates as documented by El Rassi.[2,3] The progress in this field is such that it justifies the existence of a number of excellent reviews, each one of which covers different aspects of the subject.[4-9] Carbohydrate analysis presents two major problems which capillary electrophoresis has had to address. Carbohydrates comprise a great variety of molecules ranging from simple monosaccharides to structurally complex linear and branched oligosaccharides and polydisperse microheterogeneous polysaccharides. The latter are either found alone or in glycoconjugates covalently bound to proteins or lipids. The high level of complexity is ascribed to extensive isomerism due to the various possible configurations of monosaccharides, their variable linkage bonds, the α- and β-anomericity of the glycosidic linkages as well as their different branching patterns. Furthermore, they are diversified by the presence of non-glycosyl substituents, such as acetyl, sulphate, amine, and phosphate groups. Such structural complexity has no counterpart in peptide and nucleotide chemistry and poses serious problems in the development of separation technologies. Secondly, carbohydrates lack chromogenic or fluorescent groups that would enable their sensitive detection. The present review presents the basic principles that govern the separation mechanisms and the most recent developments in detection systems used for carbohydrate analysis. Our ambition is that it will serve as a short guide in the field of glycoconjugate analysis.

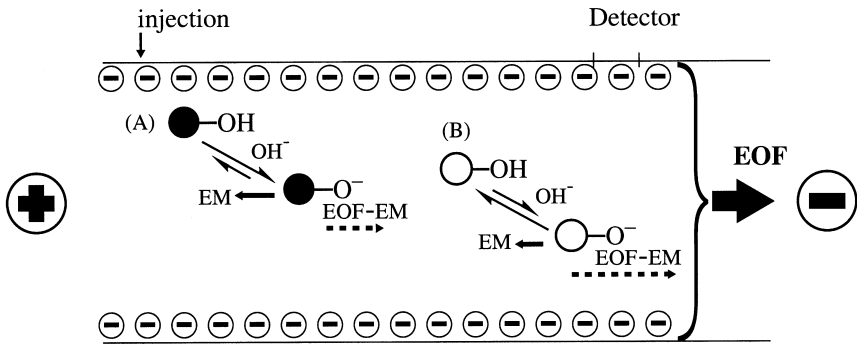


Figure 1. Separation of neutral carbohydrates with identical mass in highly alkaline conditions. EOF is large at high pH values and overwhelms the electrophoretic mobility (EM) of species so that the apparent migration is towards the negative electrode. Carbohydrates with larger ionization constants (A) migrate later than those with smaller constants (B).

SEPARATION OF NEUTRAL AND CHARGED CARBOHYDRATES

Carbohydrates can be divided in acidic carbohydrates, which have a negative charge at neutral pH, and neutral ones which carry no charge at pH lower than 12–13. CE analysis of negatively-charged carbohydrates has been reviewed by Lindhart and Pervin,[6] and of neutral ones by Honda.[4] The commonest groups responsible for the negative charge of acidic carbohydrates are carboxylate, sulphate esters, monosulphate amides, and mono–/di-phosphate esters. HPCE is principally a method applicable to ions and, thus, neutral carbohydrates have to be converted to charged species. The necessary charge can be produced by using strong alkaline conditions, by complexation of the vicinal hydroxyl groups with borate or other complexation agents, and/or chemical derivatization with a reagent possessing an ionizable group.

At highly alkaline conditions, carbohydrates behave as weak anions due to ionization of their hydroxyl groups (ionization constants in the range of 10^{-12}–10^{-14} M, i.e. pKa = 12–14). As a result, neutral carbohydrates can be separated by capillary zone electrophoresis (CZE) only at pH higher than 12. At such extremely high pH, separations can only be performed on naked fused–silica capillaries, since most of the coated fused–silica capillaries will undergo hydrolytic degradation. The weak carbohydrate anions are electrostatically attracted to the positive electrode (anodic end) but the high value of electrosmotic flow (EOF) drives the molecules to the detector at the negative electrode (cathodic end). Carbohydrates with larger ionization constants will

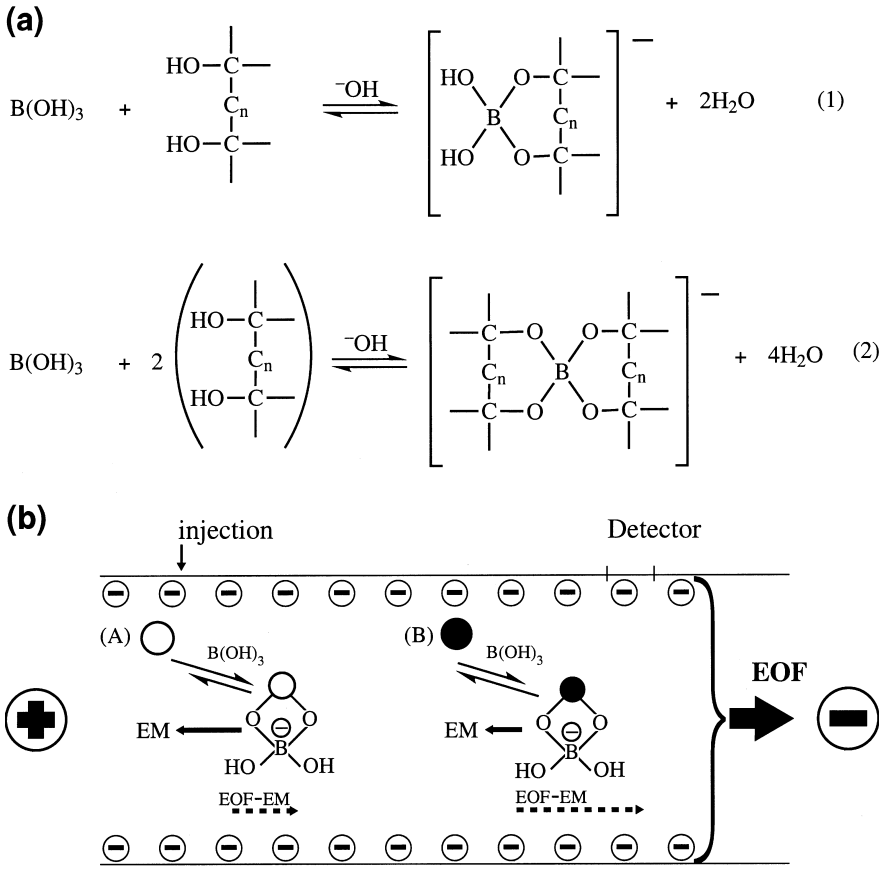


Figure 2. *In situ* conversion of carbohydrates (n=0 or 1) to borate complexes under alkaline conditions (a) and separation of carbohydrates with identical mass according to their different degree of complexation with borate anions (b). Species with larger complexation constants (A) migrate later than those with smaller ones (B).

then migrate slower than those with smaller constants (see Figure 1). In a study of Ye and Baldwin[10] glucose and galactose as well as their respective alditol and acidic derivatives were separated in a 50 mM NaOH. They migrated in the following order: alditol, aldose, aldonic acid, uronic acid, aldaric acid, which was analogous to their pKa values.

The high pH of the operating buffer was also necessary for the proper function of the copper electrode of the electrochemical detector. *In situ* conversion of carbohydrates to anions through complexation of their hydroxyl

groups with a suitable reagent is commonly used. Carbohydrates can reversibly form anionic complexes with borate under alkaline conditions (Figure 2a).

According to equilibria (1) and (2), at constant sugar concentration, the amount of complex increases with borate concentration and pH. Furthermore, the carbohydrate–borate complex formation largely depends on the structure of monosaccharide, i.e., the cis– or trans–orientation of hydroxyl groups, their number, the cyclic or open–chain conformation of the molecules, the presence of substitutes in the polyol molecule as well as by their charges, locations and anomeric linkages. Borate salts are added to the alkaline operating buffer and carbohydrate-containing samples are introduced into the capillary. Under these conditions, anionic monocomplexes are likely to predominate and, thus, migrate differentially under the influence of the applied electrical field (Figure 2b). Isomaltooligosaccharides from a partially hydrolyzed dextran sample up to a degree of polymerization of 40 derivatized with N–(4–amino–benzoyl)–L–glutamic acid were very well resolved in a 100 mM Tris-borate buffer, pH 8.8, in a coated capillary.[11]

The idea of *in situ* conversion to ions has been extended to other reaction types such as complexation with metal ions. Honda et al.[12] have reported the addition of alkaline earth metals in the operating buffer for the separation of neutral sugars. Although these systems provided a different selectivity than that achieved with borate buffers, the resolution was in general inferior to that of borate buffers.

Neutral carbohydrates can be charged through their chemical derivatization with charged molecules. The chemical reagents usually employed are either chromogenic or fluorescent, thus offering the advantage of more sensitive detection. Chemical derivatization will be dealt with, in more detail, in the chapter dealing with detection systems.

Two different modes of CZE have been used to analyze both neutral and charged species. The first and commonest, normal polarity, has already been described. Samples are introduced from the positive electrode, separated in an alkaline or neutral operating buffer, driven to the negative end by EOF and finally detected there (Figure 3a).

The second mode, known as reversed polarity, has been mainly used for the separation of acidic carbohydrates, and especially for the glycosaminoglycan derived unsaturated disaccharides.[13–15] The operating buffer used is an acidic one (pH = 3±1). At this low pH, the degree of ionization of silanol groups on the capillary wall is lower and, consequently, there is a suppression of EOF. The dominating force in this separation is the electrophoretic mobility of ions.

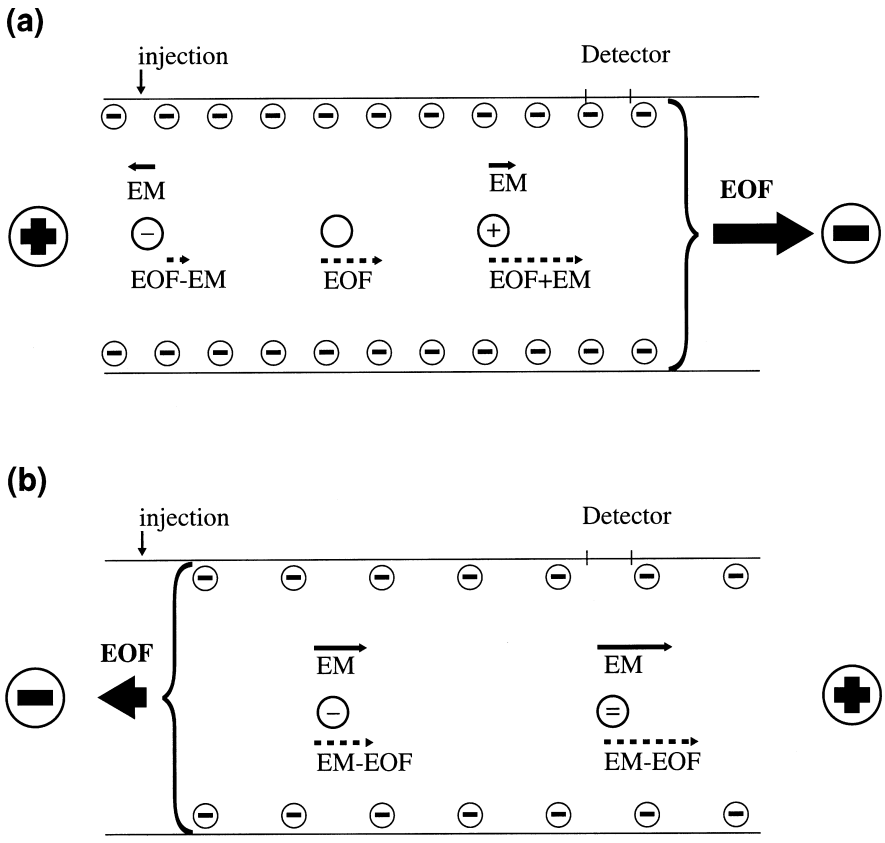


Figure 3. Migration of neutral and charged carbohydrates in CZE using basic or neutral operating buffer in a normal polarity mode (a) and low pH acidic buffer in a reversed polarity mode (b). In the normal polarity mode species migrate towards the cathode, whereas in the reversed polarity towards the anode and opposite their electrophoretic mobility.

Samples -only anionic species- are introduced from the cathodic end and detected at the positive one (Figure 3b).

An extension of CZE which has developed into a powerful method for the separation of non–ionic compounds is micellar electrokinetic capillary chromatography (MECC). It is based on solubilisation of analytes in moving ionic micelles in the carrier and separation is achieved by the difference in hydrophobicity. Although intact carbohydrates are almost non–hydrophobic, chemical derivatization with hydrophobic agents enables separation by this

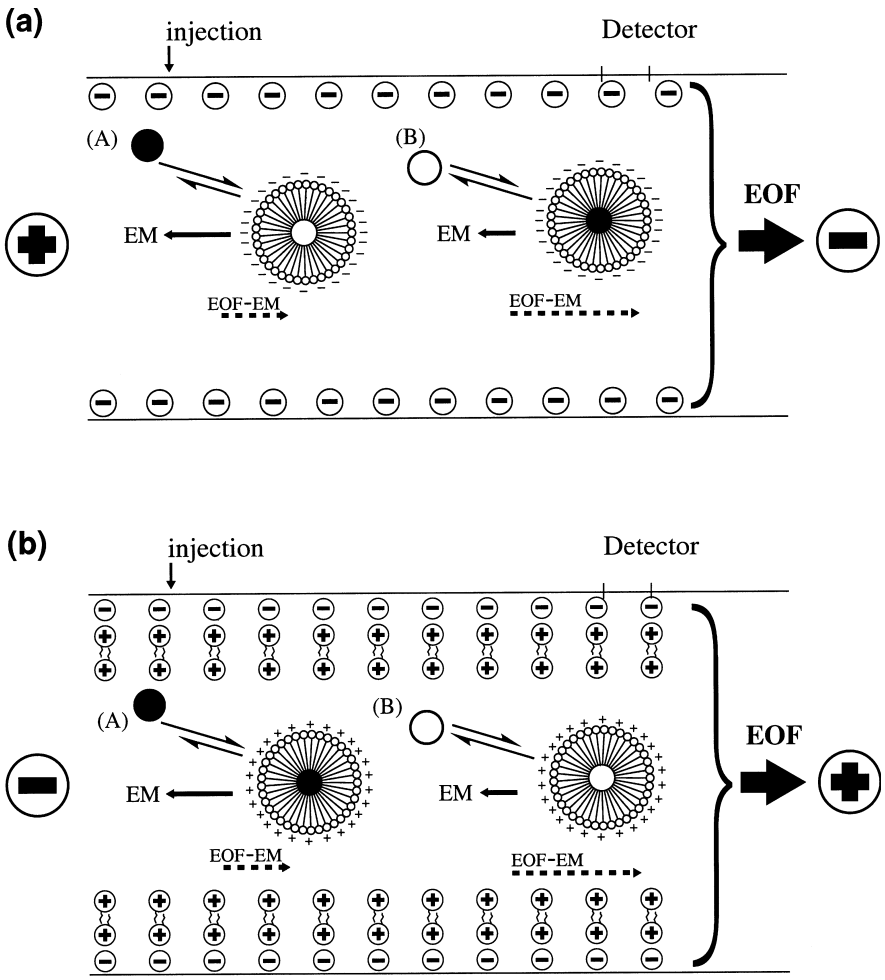


Figure 4. MECC of neutral molecules with an anionic surfactant (a) and a cationic surfactant (b). Separation is achieved through different partitioning of the solute between the micelle and the buffer.

mode (Figure 4). Derivatization of sixteen sugars with 4-aminobenzonitrile renders them hydrophobic enough to be separated in a 25 mM Tris-phosphate buffer, pH 7.5, containing 100 mM SDS.[16]

The open tubular systems with or without buffer additives (MECC or CZE) prove to be of low effectiveness for the separation of higher oligosaccharides. Gel–filled capillaries which have already been successfully used for the

separation of oligonucleotides were also employed for the separation of complex carbohydrate oligomeric mixtures. Liu et al.[17] have reported the separation of an autoclave–hydrolysed mixture of poly-galacturonic acid as isoindole derivatives in a capillary tube packed with polyacrylamide gel (18%T, 3%C) using an alkaline Tris–borate buffer and detection by laser–induced fluorescence (LIF) with Ar-source. The EOF in such gel–filled capillaries is negligible and the derivatives migrated to the positive end solely due to their electrophoretic mobility, since they are negatively charged in the basic operating buffer employed. However, for large-sized polysaccharides, the immobilized gel network is too tight and the samples do not migrate easily through the gel, concentrating instead at the capillary inlet. A convenient solution to the problem is the use of "entangled polymer matrices", i.e., viscous aqueous solutions of a linear polymer, such as polyacrylamide, dextran, or one of the water–soluble modified celluloses. Such dense polymeric media can be introduced to the separation capillaries by a syringe and be easily replaced.[18]

DETECTION SYSTEMS AND PRE-COLUMN DERIVATIZATION

The continuous interest in the miniaturization of analytical instrumentation and the rapid growing biological insight for more selective systems, leads scientists and manufacturers to the development of sensitive detection systems. Two terms exist for the description of detection limits: (a) concentration detection limit, as defined by the "minimum concentration that can be detected via a given detection principle," and (b) mass detection limit, as defined by the "absolute mass subjected to the analytical separation."[8] In the case of CE, the latter is rather small, in the pico-to-attomole level, or even yoctomole, due to the miniaturization of the system but the former is similar to that obtained with other analytical techniques, such as HPLC.

The absence of chromophores in the carbohydrate structure limits their sensitive detection. The strategies that have been developed to circumvent this obstacle are described here.

Detection of Underivatized Carbohydrates

Direct ultraviolet detection

Direct UV absorbance detection is the commonest detection method in CE, and is available for most of the commercial CE systems. However, only low wavelength UV detection (below 210 nm) can be used in analysis of underivatized carbohydrates. The detectability of the amine and carbonyl group of sugars at this wavelength is poor (approximately 1 nmol) and furthermore background absorbance of solvent causes baseline drifting and spurious peaks.

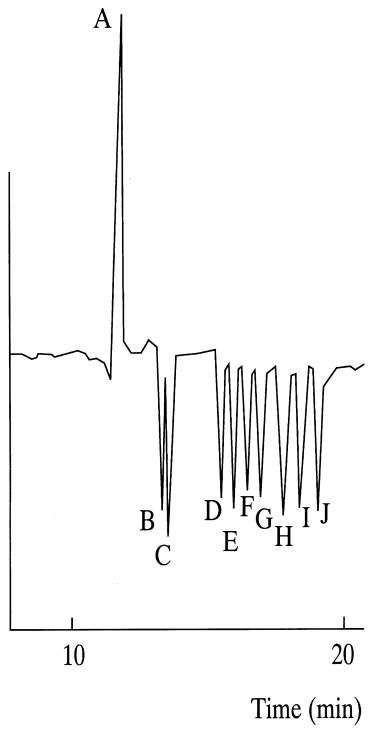


Figure 5. Separation of nine sugars at high pH and indirect UV detection, using 5.0 mM tryptophan in 50 mM NaOH at 7.0 kV. Peaks: A. System peak (EOF); B. stachyose; C. raffinose; D. melibiose; E. cellobiose; F. D–galactose; G. D–glucose; H. D–mannose; I. D–xylose; J. D–ribose. Reproduced from Ref. [20].

Only a few carbohydrates, among them acidic unsaturated disaccharides and oligosaccharides derived from proteoglycans/glycosaminoglycans by enzymic digestion, can be detected directly in the UV region of 232 nm with sufficient sensitivity (at the attomole level) due to the unsaturated uronic acid residues they carry.[13-15]

Indirect detection methods

Indirect detection methods may be an alternative for carbohydrate molecules lacking chromophores. In this detection mode, a UV–absorbing ion (marker) is added to the operating buffer, generating a high background absorbance. When a non–absorbing analyte which carries the same charge as the marker ion passes the detection window, the concentration of the marker ion

Table 1

List of the Operating Buffers that Have Been Used for Indirect UV Detection*

Operating Buffer	Molecules Separated	Wavelength (nm)	Limit of Detection (M)	Refs.
6 mM sorbate, pH 12.2-12.3	neutral mono- and disaccharides	256	$2x10^{-4}$ (0.9-1.1 pmol)	19
5 mM tryptophan in 50 mM NaOH	neutral mono- and disaccharides	280	$5x10^{-4}$ (30 fmol)	20
2mM 1-naphthylacetic acid, pH 12.2	neutral mono- and disaccharides	222	$1x10^{-4}$	21
12 mM riboflavin-63 mM LiOH, pH 13.0	neutral mono- and disaccharides	267	$5x10^{-5}$	22
6mM sorbate, pH 5.0	D-galactonic and D-glyconic acid	254	$2x10^{-6}$ (10 fmol)	23
5 mM sulphosalicylic acid, pH 3.0	heparin oligosaccharides	214	$2.7x10^{-6}$ (5 fmol)	24
4-6 mM 4-hydroxy-benzoic acid, pH 11.6-12.0	phosphorylated monosaccharides	280	$1x10^{-6}$	25

* The wavelengths of detection, the type of carbohydrate molecules analyzed and the lowest detection limits achieved are also included.

will be decreased and, thus, a negative peak will be recorded (Figure 5). A number of different marker ions have been used for indirect UV detection of various carbohydrates. The different marker ions and operating buffers used and the lowest detection limits obtained are summarized in Table 1. The differences in detection limits obtained by studies 19–22 and 23–25 are remarkable and

imply that inherently negatively charged molecules displace more marker ions in the operating buffer than neutral sugars charged at high pH and, thus, can be detected at highest sensitivity.

Based on the same principle, coumarin 343 (a fluorescent ion) has been used at high pH for indirect Laser-Induced Fluorescence (LIF) detection of sucrose, fructose, and glucose with mass detection limits of 2 fmol when excited with the 343 nm line of the He/Cd-source LIF detector.[26] Another interesting application of indirect fluorescence detection has been presented by Richmond et al.[27] High molecular weight polysaccharides, such as dextran, have been separated and detected by the 488 nm line of Ar–ion LIF detector in an operating buffer system (pH 11.5) containing 1 mM fluorescein.

Electrochemical detection

Electrochemical detection is based on the measurement of current resulting from the oxidation or reduction of analytes at the surface of an electrode cell. Very narrow capillaries can be used since there is no connection between detector response and optical path length as in the case of optical detectors. Two different approaches of electrochemical detection have been used for carbohydrate analysis, amperometric detection at constant potential (ADCP) and pulsed amperometric detection (PAD).

Fifteen reducing and non–reducing carbohydrates were separated at high pH and detected by ADCP with copper electrodes with detection limits of $5x10^{-6}$ M (below 50 fmol), but the reproducibility of the system seems to be limited.[28] A new approach to ADCP is the post–column wall–jet electrochemical detector which allows the use of normal size working electrodes (diameter larger than 100 μm) and remarkably increases the sensitivity (approximately $5x10^{-7}$ M or 1 fmol) as well as the reproducibility of the capillary–detector alignment.[10]

A common problem in electrochemical detection has been electrode fouling due to oxidation of carbohydrates at the electrodes. This can be overcome by alternating anodic and cathodic polarization (PAD) after detection, a procedure used to clean and reactivate the electrode.[29] This detection mode has been applied to the detection of molecules ranging from simple monosaccharides (determination of glucose in blood samples at levels of 22.5 fmol or 10^{-6} M)[29] to glycopeptides.[30] Generally, some PAD signals at high detection sensitivity may originate from surface oxide formation but a potential scan in the detection step, which coulometrically rejects the substantial oxide formation signal, has been shown to be effectively efficient in producing a stable baseline.[31]

Although electrochemical detection is a promising mode of carbohydrate detection, the technique suffers from (i) non–discriminatory response which may be positive not only for carbohydrates but also for amino acids, peptides, organic acids, simple alcohols, and aliphatic amines, (ii) not uniform response within a class of compounds necessitating standard curves for each solute before quantification, and (iii) the alkaline conditions required for the proper function of the electrodes. Furthermore, electrochemical detectors are not commercially available at present.

Refractive index detection

Refractive index (RI) detectors have long been used in HPLC but they are not so common in CE because heat produced by Joule effect limits its operation. Bruno et al.[32] have introduced an RI detector for on–column detection in CE in which the interferometric pattern is produced by the capillary wall. This detector allowed the detection of carbohydrates at 10^{-6} M following separation in a borate buffer.

Biosensors

An innovative approach to the detection of underivatized carbohydrates in CE has been introduced by Wei et al.[33] Two enzymes, glucose oxidase and amyloglucosidase, were immobilized on a platinum microelectrode and were used for the detection of glucose and maltose after separation in a 60 mM borate buffer, pH 9.3. The detection limits for these sugars were 1.7×10^{-4} M and 3.5×10^{-4} M, respectively.

The major drawback of this method is that it is too selective to be used for other carbohydrate molecules.

Pre-column Derivatization and Detection

The pressing need for higher detection sensitivity and improved separation of carbohydrates prompted scientists to utilize sugar reactivity so that to derivatize carbohydrates with suitable chromophores or fluorophores. Most of them were already employed in HPLC and SDS–PAGE but had to be modified. The labeling procedure should be rapid, simple, selective – which means leading to the formation of a single product without any detectable side product– and quantitative giving a high yield even for minute quantities of biological material.

The mode of separation that will be followed (CZE or MECC) and, in some cases, compatibility with demanding detection systems, such as MALDI mass spectroscopy are factors that should be taken into consideration.

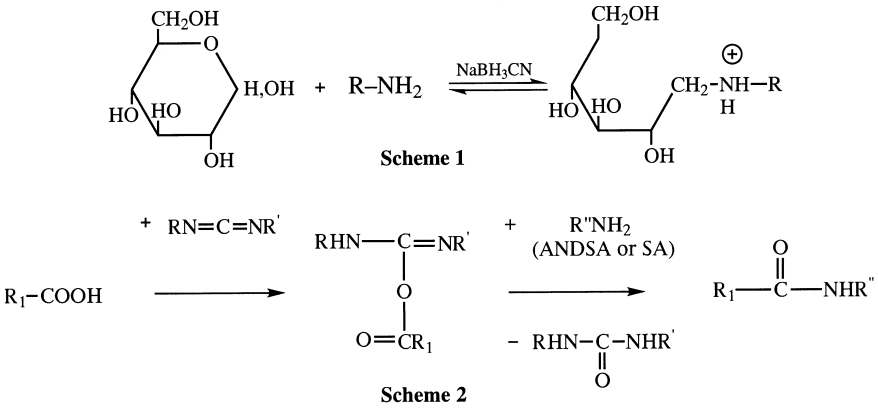


Figure 6. Common pre-column derivatization schemes followed in CE analysis of carbohydrates. Reductive amination of D–glycose is given in scheme 1 and condensation reaction of carboxylated carbohydrates with aminated derivatizing agents (SA and ANDSA) in the presence of cardodiimide is given in scheme 2.

UV detection

The chromophores that will label the carbohydrates must have a high molar absorptivity at a given wavelength at which there is no interference from the operating buffer. There are several derivatization schemes. The most widely used derivatization scheme is reductive amination. The carbonyl group of a reducing sugar reacts with the amino–group of the label and forms a Schiff base which is reduced with sodium cyanoborohydride to a stable secondary amine (Figure 6, scheme 1). Honda et al.[34] were the first that utilized this scheme for derivatizing twelve monosaccharides with 2–aminopyridine for HPCE analysis.

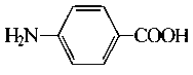
A number of studies have been performed ever since; the derivatizing agents that have so far been used and concentration detection limits obtained are summarized in Table 2. Derivatization of ketoses seems to present a particular problem since only some of the derivatizing agents react with them and, in some cases, not quantitatively.

The UV–absorbing tag 1–phenyl–3–methyl–2–pyrazolin–5–one (PMP) (λmax=245 nm) has been used to derivatize reducing sugars in the presence of carbodiimide by a condensation reaction. The bis–PMP derivatives behave like weak anions in aqueous basic solutions due to the partial dissociation of the enolic hydroxyl group of PMP. PMP-derivatives are well separated by CZE

Table 2

Common UV-Absorbing Labels Used for Carbohydrate Derivatization via Reductive Amination

Label	Structure	Wavelength (nm)	Limit of Detection (M)	Refs.
p-Aminobenzoic Acid	I	285	4×10^{-6}	35, 36
p-Aminobenzoic Acid, Ethyl Ester	II	305	2×10^{-6}	37
4-Aminobenzonitrile	III	285	3×10^{-7}	16
8-Aminonaphthalene-1,3,6-trisulfonic Acid (ANTS)	IV	214	5×10^{-7}	38, 39
6-Aminoquinoline	V	270	1×10^{-6}	40
2-Aminopyridine	VI	240	8×10^{-6}	34, 41-44
N-(4-aminobenzoyl)-L-glutamic Acid	VII	293	1.2×10^{-5}	11



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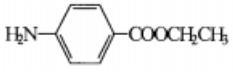

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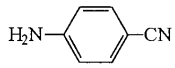

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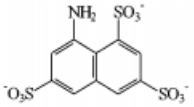

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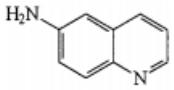

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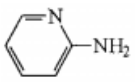

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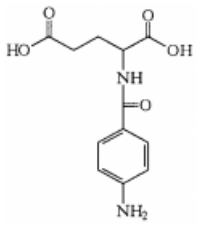

VII

using borate complexation or MECC in the presence of SDS.[45] The concentration detection limit is in the range of 1–5 x 10^{-6} M (10–50 fmol). Complexation with alkaline earth metals was also tested as described before.[12]

Carboxylated sugars react with aminated derivatizing labels such as sulphanilic acid (SA) and 7–aminonaphthalene–1,3–disulphonic acid (ANDSA) in the presence of carbodiimide via a different scheme to form a peptide bond (Figure 6, scheme 2). These derivatizing agents result in mass detection limits of 15 to 30 fmol at 247 nm. Carboxylated monosaccharides labeled with ANDSA and SA have the advantage of having fluorescence.[46]

Laser-induced fluorescence detection

Fluorescence detection is inherently more advantageous than spectroscopy because of its high specificity and sensitivity. Fluorescence is not path–length dependent unlike UV absorbance and, thus, is more sensitive. On the other hand, the limited number of fluorogenic compounds confers great specificity on the method as detection is carried out on a weak or non–existing fluorogenic background. Besides, even if two compounds absorb at the same wavelength, they are not likely to emit radiation at the same wavelength and vice versa. However, fluorescence detection of carbohydrates with conventional deuterium, mercury or xenon lamp light sources had only marginal gains in sensitivity of approximately one order of magnitude. That is ascribed to the fact that focusing of a significant amount of light on an extremely small detection area, such as the optical window of the capillary is extremely difficult.

LIF detectors have successfully addressed that problem as the monochromatic laser beam can be easily focused on a very small area. Utilization of them for carbohydrate detection resulted in ultra–high sensitivities so that a few molecules can be detected. Moreover, they have the advantage of a rather large linear dynamic range of six or seven orders of magnitude as compared to spectroscopy where only two or three orders can be obtained. There are three LIF detectors commercially available depending on the laser source; the Ar–ion, the He/Cd and the He/Ne detectors. Each one has its own distinct excitation lines at wavelengths generally smaller than 500 nm. In order to have significant sensitivity gains, the carbohydrate molecule must be labeled with a fluorogenic molecule having the same excitation wavelength as the source of the LIF detector available. Actually, this fact restricts the versatility of the instrument and a CE interface allowing the excitation of any desired wavelength is the next challenge to manufacturers.

Some of the fluorophores used were attached to the molecules via reductive amination, a one–step scheme previously described (Figure 6, scheme

1). 8–Aminonaphthalene–1,3,6–trisulfonic acid (ANTS) was reported as a UV–absorbing agent but, since it exhibits intrinsic fluorescence, it has been extensively used for LIF detection for a great variety of carbohydrates including monosaccharides, oligo– and polysaccharides. The 325 nm line of He/Cd has been used for the excitation of ANTS derivatives resulting in concentration limits of $5x10^{-8}$ M (500 amol) for both monosaccharides and large complex oligosaccharides (molecular sizes 2000–3000).[38,47] Use of a frequency doubled Ar–ion laser with an emission wavelength of 257 nm increased the sensitivity of the detection (10^{-9} M for ANTS–maltose) as this particular wavelength was closer to the absorption maximum of the molecule (λmax=223 nm).[8] Other fluorogenic compounds that have been attached to carbohydrates via reductive amination involve 9–aminopyrene–1,4,6–trisulfonic acid (APTS),[48] 2–aminoacridone (2-AA),[49,50] and 5–aminonaphthalene–2–sulfonic acid (5–ANSA).[51] In most cases, however, the excess reagent interferes with analysis and has to be removed. A very interesting approach to this problem was introduced by Okafo et al.[50] Oligosaccharides labeled with 2–AA were separated in an operating buffer, 500 mM sodium borate, pH 8.9 containing 80 mM sodium taurodeoxycholate. This MECC mode enabled excess 2–AA to be trapped in taurodeoxycholate micelles ensuring no interference with the analysis.

3–(4–Carboxybenzoyl–)–2–quinoline carboxyaldehyde (CBQCA) has been used for the ultrasensitive detection of aminosugars.[52] Carbohydrates with no amino group were derivatized according to the scheme presented in Figure 7.[17] The isoindole derivatives have excitation maxima near the 442 nm blue line of the He/Cd laser and the 456 nm secondary line of the argon laser and have been detected at nanomolar (10^{-9} M) concentrations with mass detection limits of 75 zmol of 1–glucosamine reported.[53] Furthermore, this procedure has the advantage that it is not necessary to remove the excess of the reagent – a common problem in the above mentioned cases – since the unreacted compound does not fluoresce. However, the molar ratio of reagent to carbohydrate required for a maximum yield of CBQCA derivative is very closely defined and deviations from this lead to decrease of the yield.

Another two–step derivatization procedure for carbohydrates has been employed. In the first step, carbohydrates containing no amino group were transformed to the corresponding 1–amino–1–deoxyalditols, while hexosamines were reduced with sodium borohydride to the 2–amino–2–deoxyalditols. These aminated carbohydrates were subsequently labeled with 5–carboxy-tetramethylrhodamine succinimidyl ester. This scheme was applied for the derivatization of six oligosaccharides.[54] Separation was carried out in a 10 mM phosphate–borate–phenylboronic acid–SDS buffer and detection was performed with a home–made He/Ne laser with an excitation wavelength of 543.5 nm.

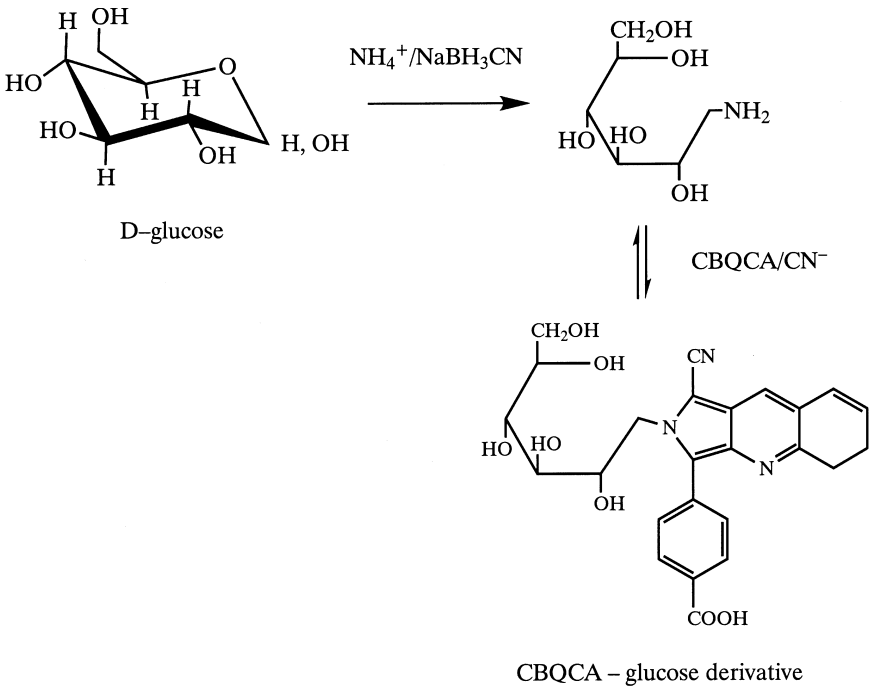

Figure 7. Precolumn derivatization of reducing sugars with the fluorescent tag CBQCA in the presence of potassium cyanide. Direct reaction with CBQCA can be used for amino sugar derivatization.

With this set–up, the lowest detection limits, ever reported, were achieved. The concentration detection limit was in the order of 50–100 pM and the mass injection limit 8 x 10^{-23} mol or a few analyte molecules. However, the succinimidyl ester is not stable in basic buffers yielding two hydrolysis products.

Finally, sialic acids, which are among the most important constituents of various glycoproteins, react with the fluorogenic reagent, 4,5–dinitrocatechol–O,O–diacetic acid (DNCDA) and give fluorescent derivatives which can be detected sensitively with the 325 nm line of the He/Cd LIF detector.[7]

APPLICATIONS OF CE IN GLYCOCONJUGATES - CONCLUDING REMARKS

The high resolving power of HPCE and its capability for ultrasensitive detection –among other merits– have made the technique an indispensable tool

for analysis of glycoconjugates, such as glycoproteins, proteoglycans, gangliosides and various pharmaceutical glycoconjugates during the last years. Glycoproteins comprise a large group of structurally complex molecules involved in a continuously increasing list of biologically significant processes. Biotechnologically produced glycoproteins, on the other hand, have recently been launched as pharmaceutical agents. Glycosylation can occur at two or more sites in the protein bone. The glycans can be attached either to Asn residues (N–linked) or to Ser, Thr, hydroxylysine, or hydroxyproline residue (O–linked). The former originate from a common pentasaccharide core and can be subdivided into three distinct groups called "high mannose type," "hybrid type," and "complex type." They are commonly branched, and these branches (antennae) are often terminated with sialic acid residues which are negatively charged.

The number and type of sialic acids varies. The glycans at even a single site of the protein core may be heterogeneous leading to populations of glycosylated variants of a single protein, widely known as glycoforms. Therefore, separation of the glycoforms seems to be the first step in the attempt to analyze them. In some cases, the technique may not be capable of a complete resolution of the many glycoforms encountered. Even in such cases, the technique serves as a rapid "fingerprinting" technique for assessing the glycosylation variants. When trying to determine the sites of incorporation of glycan moieties within the polypeptide backbone, the glycoproteins are digested with different proteases, such as trypsin, chymotrypsin, and pronase or are even chemically degraded (CNBr cleavage) at specific sites and the separation procedures lead to peptide maps.

Oligosaccharide characterization features as the next step. Oligosaccharides can be released from glycoproteins or glycopeptides in a number of different ways such as elimination for O–linked, hydrazinolysis for N–linked, or digestion with glycanases of different specificities. For the majority of cases, it is impractical to analyze all glycan species by a single separation mode. A multi–dimensional mapping of them allows the investigator to draw conclusions about their structure. It is based on the use of different electrolyte systems so that the individual modes of separation are as different from each other as possible.

In a study of Suzuki et al.,[55] 2–aminopyridine N–linked desialylated oligosaccharide derivatives were separated according to their molecular size in an acidic phosphate buffer, in capillaries where EOF was eliminated, and according to configurational differences as borate complexes in an alkaline borate buffer. As a result the linkage type (high mannose, complex or hybrid) of any N–linked oligosaccharides could be determined (Figure 8). Finally, the monosaccharide composition of glycan moieties can be determined.

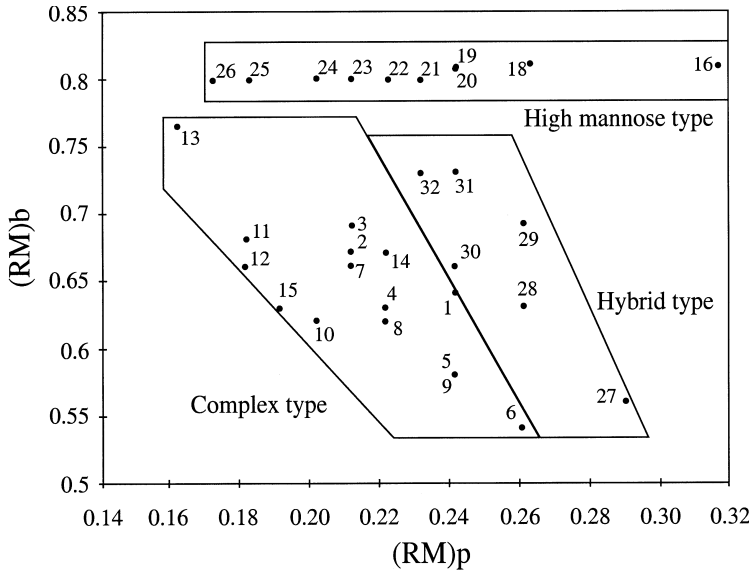

Figure 8. Two–dimensional map of 2–aminopyridine (2-AP) oligosaccharide derivatives, derived from various glycoproteins. The relative electrophoretic mobility in the phosphate buffer, $(RM)_p$, was calculated as $(t_{AP-Glc})/t$, where t and t_{AP-Glc} are the migration times of the 2–AP–oligosaccharide in question and 2-AP-glucose, respectively. The relative mobility in the borate buffer, $(RM)_b$, was calculated as $[(t_{AP-Glc})/t][(t-t_0)/(t_{AP-Glc} - t_0)]$, where t_0 is the migration time of the neutral marker. Reproduced from Ref. 55.

The potentials of HPCE have also been demonstrated by the separation of gangliosides,[56, 57] which consist of a hydrophilic sialyloligosaccharide chain and a hydrophobic moiety of a sphingosine and fatty acid, as well as macrolides[58] which are antibiotic glycoconjugates used as antimicrobial agents in humans and animals.

Oligosaccharide derivatives have been used as enzyme substrates and their HPCE analysis before and after the digestion has shed more light on the action patterns of enzymes such as chitinases, glycosidases, glycosyltransferases and polygalacturonases and has been used to monitor the presence of minor contaminating enzymes in commercial enzymes. Derivatization of oligomers of galacturonic acids with the fluorescent label ANTS may help in distinguishing between both endo– and exopolygalacturonase as well as lyase activity and it was found that both fungal and bacterial endogalacturonases need four adjacent non–esterified galacturonic acid residues in pectin to be able to act.[59]

Concluding this review on HPCE analysis of carbohydrates, the future holds many challenges for carbohydrate analysts in terms of separation modes and detection systems. Converting this technique into micro–preparative would also further help the structural determination of the carbohydrate structures. Coupling of CE to the various modes of mass spectrometry seems a highly promising tool as can be seen in the report of Duffin et al.[60] The rate of development of HPCE separation methodologies and detection systems during the last years justify one to say that its utilization in carbohydrate analysis will bring more insight to the biological processes and, thus, may signal a new era in the development of powerful pharmaceuticals.

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