High Performance Capillary Electrophoresis of Carbohydrates

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About the Author

Ziad El Rassi is an Associate Professor at the Department of Chemistry, Oklahoma State University. His current research includes the development of HPCE and HPLC methods for important biological and environmental species and the miniaturization of immobilized enzyme reactors for use in analytical separations. He has published over 90 papers and 11 book chapters, and presented several invited lectures at national and international meetings and locations. He has edited

(i) a book entitled *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis* for Elsevier Science Publishers, Amsterdam, The Netherlands, 1995,

(ii) a symposium paper, 211 pages, for the journal *Electrophoresis*, 1995, on "CE of Amino Acids, Peptides and Proteins" and

(iii) a symposium paper, 162 pages, for the journal *Electrophoresis*, 1996 on "CE of Carbohydrate Species."

He is currently editing another symposium paper on "CE of Pollutants and Toxicants." He is a member of the scientific committee of the Frederick Conference on Capillary Electrophoresis, the International Organizing Committee of the International Symposium of Asia-Pacific Capillary Electrophoresis, and the editorial boards of the *Journal of Liquid Chromatography*, *Journal of Chromatography*, *Journal of Microcolumn Separations* and *Journal of Capillary Electrophoresis*. He is the Associate Editor of the international journal *Electrophoresis*.

Acknowledgments

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Front Cover

The illustration on the cover depicts a computer-generated rendition of the N-terminal domain of a "variant surface glycoprotein" from *T. brucei*. This is a dimer, with a trisaccharide (NAG-NAG-MAN) attached to each monomer. The protein is shown as a ribbon, color coded by structural domain; the two trisaccharides are shown, one in "space-filling" representation, and the other as "liquorice bonds." Courtesy of Don Gregory, Molecular Simulations, San Diego, CA.

Other Beckman primers (Volumes I through VII) on capillary electrophoresis:

Title	Beckman Part Number
Introduction to Capillary Electrophoresis	360643
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Micellar Electrokinetic Chromatography	266924
Introduction to the Theory and Applications of Chiral Capillary Electrophoresis	726388
Separation of Proteins and Peptides by Capillary Electrophoresis: Application to Analytical Biotechnology	727484
Introduction to Quantitative Applications of Capillary Electrophoresis in Pharmaceutical Analysis	538703
Separation of DNA by Capillary Electrophoresis	607397

Acronyms and Symbols Used

2-AA	2-aminoacridone
ACN	acetonitrile
ADCP	amperometric detection at constant potential
AGP	α_1-acid glycoprotein
AHNS	4-amino-5-hydroxynaphthalene-2,7-disulfonic acid
3-ANDA	3-aminonaphthalene-2,7-disulfonic acid
ANDSA	7-aminonaphthalene-1,3-disulfonic acid
2,6-ANS	2-anilinonaphthalene-6-sulfonic acid
2-ANSA	2-aminonaphthalene-1-sulfonic acid
5-ANSA	5-aminonaphthalene-2-sulfonic acid
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
2-AP	2-aminopyridine
APTS	9-aminopyrene-1,4,6-trisulfonic acid
6-AQ	6-aminoquinoline
CBQCA	3-(4-carboxybenzoyl)-2-quinolinecarboxyaldehyde
CD	cyclodextrin
CE	capillary electrophoresis
CIF	capillary isoelectric focusing
CZE	capillary zone electrophoresis
C_6MetBr	hexamethonium bromide
C_6MetCl	hexamethonium chloride
C_{10}MetBr	decamethonium bromide
DAB	1,4-diaminobutane
DAP	diaminopropane
DM-β-CD	2,6-di-O-methyl-β-CD
ED	electrochemical detection
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EOF	electroosmotic flow

GAGs	glycosaminoglycans
GlcNAc	*N*-acetylglucosamine
hCG	human chorionic gonadotropin
HPAEC-PAD	high-performance anion exchange chromatography-pulsed amperometric detection
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
IFN-γ	interferon-γ
LIF	laser-induced fluorescence
MECC	micellar electrokinetic capillary chromatography
MEGA 10	decanoyl-*N*-methylglucamide
MS	mass spectrometry
NMR	nuclear magnetic resonance
PAD	pulsed amperometric detection
PAGE	polyacrylamide slab gel electrophoresis
PMP	1-phenyl-3-methyl-5-pyrazolone
PMPMP	1-(*p*-methoxy)phenyl-3-methyl-5-pyrazolone
rFVIIa	human recombinant factor VIIa
rhBMP-2	recombinant human bone morphogenetic protein 2
rHuEPO	recombinant human erythropoietin
SA	sulfanilic acid
TEA	triethylamine
TRSE	5-carboxytetramethylrhodamine succinimidyl ester

I. Introduction

Carbohydrates are polyhydroxylated aldehyde or ketone compounds that make up most of the organic matter on earth because of their involvement in many life processes. Carbohydrates have the following important functions:

(i) serve as energy stores, fuels, and metabolic intermediates,
(ii) are structural elements in the cell walls of bacteria and plants and in the exoskeletons of arthropods,
(iii) are integral parts of glycoproteins and glycolipids, and
(iv) play key roles in cell-cell recognition processes.[1]

Carbohydrates encompass a wide spectrum of compounds, many of which are isomers or slightly different from each other. While monosaccharides are divided into several classes (*e.g.*, aldoses, ketoses, alditols, aldonic acids, etc.) and subclasses (*e.g.*, trioses, pentoses, and hexoses), oligo- and polysaccharides which are composed of various combinations of monosaccharides are even more diverse in structures than their monosaccharide constituents, forming linear, cyclic, or branched polymeric species. Several oligosaccharide variations can be formed from a small number of monosaccharide units. For instance, while two amino acids can form only two different dipeptides, two monosaccharides can be joined together in as many as 32 different disaccharides since the linkage can (i) occur at any of the four hydroxyl groups per monosaccharide, (ii) exist in either of two anomeric forms, and (iii) involve either furanose or pyranose rings. Most oligosaccharides occur as side chains attached to lipids in glycolipids and to polypeptides in glycoproteins and proteoglycans. Furthermore, since the carbohydrate chains of glycoproteins are enzymatically generated by processing enzymes that are generally not available in sufficient quantities to yield uniform products, the composition of carbohydrates at any of the glycosylation sites of a given glycoprotein may vary substantially, yielding what is known as microheterogeneity.

Because of the multilateral roles of carbohydrates, their analysis has come to have increasing importance. The complexity of carbohydrate solutes has most often engendered the need for an arsenal of analytical techniques and the use of several chemical and biochemical tools and processes to bring about their separation and structural characterization. The most widely used physicochemical methods in the analysis of carbohydrates include nuclear magnetic resonance (NMR), mass spectrometry (MS), gas-liquid chromatography (GLC), polyacrylamide gel electrophoresis (PAGE), traditional liquid chromatography (*i.e.*, low-pressure) and high-performance liquid chromatography (HPLC), and more recently HPCE. NMR and MS are indispensable tools for the structural elucidation of carbohydrates. With the development of the ion-

ization methods, electrospray ionization mass spectrometry (ESIMS) and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS), intact carbohydrates (*e.g.*, oligosaccharides, glycoproteins, glycopeptides, and other glycoconjugates) can be readily measured at the picomole to the femtomole levels. In addition, ESI is interfaced relatively easily with HPLC or CE, thus facilitating the simultaneous separation and determination of minute amounts of carbohydrates. However, both NMR and MS as well as CE- and LC-MS involve expensive equipment which generally restrict their use to a few specialized laboratories. Furthermore, while NMR still requires relatively large amounts of samples which most often are difficult to obtain from biological sources, MS usually gives no information about linkage positions and anomeric configurations. Due to these limitations, most often the analysis of carbohydrates is accomplished by chromatographic and electrophoretic methods in conjunction with complimentary biochemical tools such as the use of specific lectins or monoclonal antibodies as well as specific exo- and endoglycosidases. While lectins and monoclonal antibodies (for instance, in the form of lectin chromatography or immuno-chromatography or blotting), serve as biomolecular probes in the tentative structure elucidation of carbohydrates, exo- and endoglycosidases provide information about the type of saccharide liberated and anomericity of the cleaved glycosidic bond as well as the position to which cleaved sugar residue is linked.

Because of the inherent hydrophilic nature of carbohydrates, aqueous-based separation methods including HPLC, PAGE, and HPCE are very suitable for their analysis. In this regard, HPCE seems to possess several advantages over HPLC and PAGE by (i) offering higher separation efficiencies, (ii) yielding shorter analysis time, (iii) requiring small sample amounts, and (iv) consuming lower amounts of expensive reagents and solvents.

However, to realize the full benefits of the many sound features of HPCE including, among other things, its intrinsically high resolving power in the separation of complex carbohydrate samples, two major difficulties have to be surmounted. First, with the exceptions of few naturally charged mono- and oligosaccharides, most carbohydrate molecules lack readily ionizable charged functions, a condition that excludes their direct differential migration and, in turn, separation in electrophoresis. Second, most carbohydrate species neither absorb nor fluoresce, hindering their sensitive detection by modern analytical separation techniques including HPCE.

Various approaches have been introduced to render carbohydrates amenable to separation and detection by HPCE. These approaches have exploited many of the inherent properties of carbohydrates including (i) the ease with

which these molecules can be readily converted *in situ* to charged species by complex formation with other ions such as borate and metal cations which then ensure their differential electromigration and, in turn, separation in an electric field, and (ii) the reactivity of the reducing end and other functional groups of the sugar molecules (*e.g.*, carboxylic acid groups and amino groups) which can be readily labeled with UV-absorbing or fluorescent tags, thus providing the centers for sensitive detection. In addition, the electrochemical oxidation of carbohydrates at the surface of metallic electrodes provides another means by which underivatized carbohydrates can be sensitively detected.

The aims of this primer are to describe the basic aspects of the electrolyte systems used in HPCE of carbohydrates, to discuss the advantages and disadvantages of the approaches and concepts that are most useful in the separation and detection of carbohydrates by HPCE, and to review important applications.

For recent and detailed reviews of the various aspects of the capillary column technology, the interested reader may consult References 2 and 3. Furthermore, a special issue of the journal *Electrophoresis* on "Capillary Electrophoresis of Carbohydrate Species" will appear at the same time as this primer.[4]

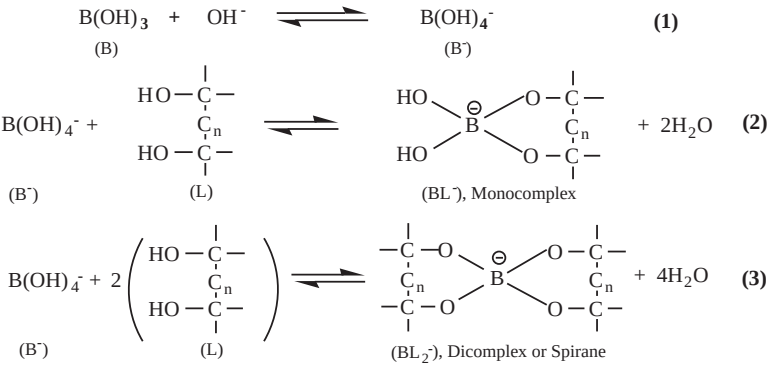
II. Electrolyte Systems

Only some saccharides possess charged functional groups in their structures which would allow their differential electromigration and eventually separation. These saccharides are the aldonic acids, uronic acids, sialic acids, amino sugars (glucosamine, galactosamine), and compositional sulfated sugars of chondroitin, dermatan, keratan, and heparin. On the other hand, the separation of "neutral" carbohydrates by HPCE has often required the *in situ* conversion of these polyhydroxy compounds into charged species via complex formation with other ions such as borate and metal cations. Furthermore, because of the ionization of the hydroxyl groups of the sugars at extremely high pH, highly alkaline pH electrolyte solutions are also useful for the electrophoresis of carbohydrates.

Thus far, most HPCE separations of "neutral" carbohydrates have been achieved by borate complexation and to a lesser extent by ionization at alkaline pH. Only one paper has appeared on the HPCE of sugars as alkaline earth metal ion complexes.[5]

A. Borate-Based Electrolytes

Polyhydroxy compounds including carbohydrates can reversibly form anionic complexes with borate;[6] for recent reviews, see References 2 and 3. In these complexation reactions, it is the tetrahydroxyborate ion, $B(OH)_4^-$, rather than boric acid that undergoes complexation with the polyols. This is because boric acid in aqueous media acts as a Lewis acid to form the tetrahedral anion $B(OH)_4^-$, and at alkaline pH, *i.e.*, pH 8-10, where the complexation is most effective, equilibrium (1) is to a very large extent shifted to the right. In equilibria (1), (2), and (3), BL^- and BL_2^- are the mono- and diesters, respectively, L is the polyol, and n = 0 or 1. Equilibrium (2) is situated very much to the right whereas Equilibrium (3) is dependent upon the position of the hydroxyl groups in the polyol.



According to the above equilibria, carbohydrates could form cyclic borate esters with either five or six atom rings when n = 0 or 1, respectively (*i.e.*, with hydroxyl groups on adjacent or alternate carbon atoms, respectively).

The complex formation, as described by reactions (2) and (3), is possible only if two hydroxyl groups in the polyol molecule are favorably situated. In this regard, borate forms more stable complexes with *cis*- than with *trans*-oriented pairs of hydroxyl groups. Also, cyclic forms of sugars react less strongly with borate ions than do those having open chains (acyclic). In other words, alditols form stronger complexes with borate than do their counterpart aldoses under otherwise identical conditions. Increasing the number of hydroxyl groups increases the strength of borate complexation. This is because two hydroxyl groups will be more and more favorably situated with reference to one another as the number of adjacent hydroxyl groups increases. The carbohydrate-borate complex formation is largely influenced by the presence of substituents in the polyol molecule as well as by their charges, locations, and anomeric linkages. Methylated sugars complex less than the parent unsubstituted sugars, and consequently the electrophoretic mobilities of methylated sugars in zone electrophoresis are much lower. Also, the complexation is stronger with methyl-β- than methyl-α-D-glucopyranoside and consequently the mobility of the α anomer, compared to that of the β anomer, is lower in zone electrophoresis with alkaline borate. This may be due to the fact that in the α anomer the glycosidic methoxyl group occupies an axial position and will interact strongly with the axial hydrogen atoms on C3 and C5, thus destabilizing the borate complex. This is not the case for the β anomer where the substituent occupies an equatorial position and consequently is free from strong, non-bonded interactions. Another parameter that must be considered in corroborating mobilities in borate systems is the presence of charged substituents in the polyhydroxy molecule. Generally, a decrease of the stability of a borate ester is observed as a result of Coulombic repulsion between a negatively charged substituent (*e.g.*, COO^-) and BO_4^- moieties.[7]

Usually, mono- and dicomplex (or spirane) borate esters coexist in aqueous solutions, and their molar ratio, among other things, is affected by the relative concentration of borate ions and sugar molecules. Spirane complexes predominate at a high sugar:borate ratio. In CE of carbohydrates, usually 0.1-0.2 M borate is added to the running electrolyte and small plugs of 10^{-4}-10^{-5} M sugar samples are introduced into the separation capillary. Under these conditions, anionic monocomplexes (*i.e.*, BL^-) are likely to predominate and thus migrate differentially under the influence of an applied electric field. It should be noted that, whether the injected sugar samples form BL^- or BL_2^- or both while migrating in a borate medium, all sugar molecules will be associated with a negative charge since mono- and dicomplex formations are dynamic. The magnitude of the charge will be influenced by the position of the

equilibrium and therefore by the stability of the complex. According to equilibria (1), (2), and (3) at constant sugar concentration, the amount of complex increases with borate concentration according to the law of mass action and also with pH due to a higher concentration of borate ions.

In HPCE, at a given pH, resolution among various sugars increases with increasing borate concentration. Usually, there is an optimum borate concentration at which maximum resolution is obtained for a multicomponent mixture[8] (see Figure 1). As seen in Figure 1b, 75 mM borate allowed the full resolution of the seven sialooligosaccharides derived from gangliosides and derivatized with 7-aminonaphthalene-1,3-disulfonic acid. Also, at constant borate concentration, the complex formation as a function of pH varies among various carbohydrate species with an optimum in the pH range 10-11 for most monosaccharides.[9]

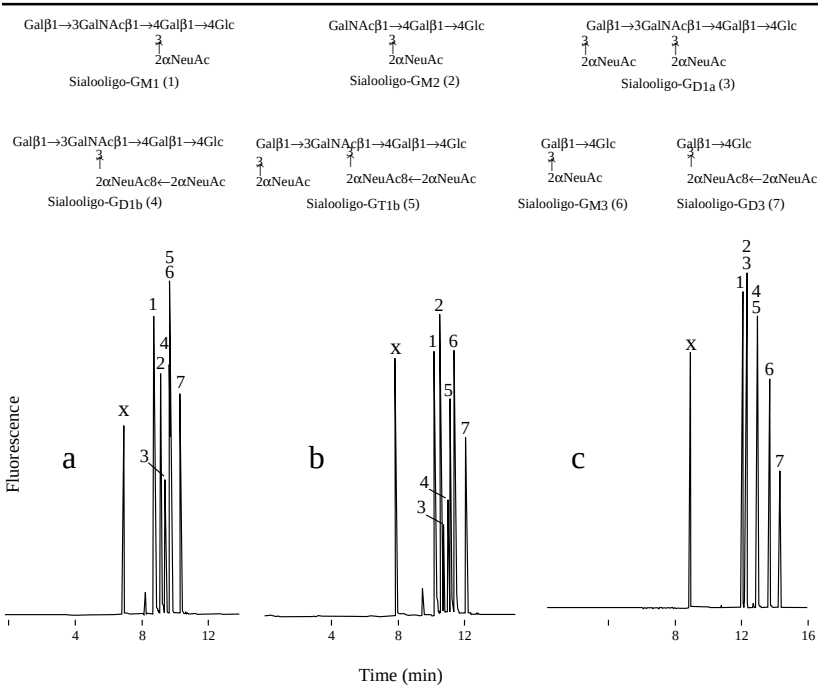


Figure 1. *Electropherograms of ANDSA derivatives of sialooligosaccharides. Capillary, fused-silica, 50 cm (to detection point), 80 cm (total length) × 50-μm i.d.; running electrolytes, borate buffer of (a) 50, (b) 75, and (c) 125 mM, pH 10.0; voltage, 20 kV. Solutes, X = byproduct, 1 = sialooligo-G_{M1}, 2 = sialooligo-G_{M2}, 3 = sialooligo-G_{D1a}, 4 = sialooligo-G_{D1b}, 5 = sialooligo-G_{T1b}, 6 = sialooligo-G_{M3}, 7 = sialooligo-G_{D3}. Reprinted with permission.[8]*

In summary, under a given set of conditions, various sugars (whether charged or neutral) would undergo varying degrees of complexation with borate leading to differences in the electrophoretic mobilities of the complexed solutes and hence separation. Thus, the use of alkaline borate is definitely an elegant approach for the high selectivity separation of saccharides.

B. Highly Alkaline pH Electrolytes

The differential electromigration of carbohydrates in alkali-metal hydroxide solutions, such as lithium, sodium, or potassium hydroxide is presumably due to the ionization of the hydroxyl groups of saccharides at highly alkaline pH, yielding negatively charged species called alcoholates.[10] The ionization constants for carbohydrates are in the range of 10^{-12} to 10^{-14}, *i.e.*, pK_a = 12-14. The pK_a values of some typical sugars are listed in Table 1. Usually, reducing sugars (*e.g.*, glucose, galactose, mannose, etc.) are the most easily ionized while straight-chain alditols (*e.g.*, glucitol, mannitol) have, on the average, about the same acidity as cyclitols (*e.g.*, inositols) and glycosides (*e.g.*, methyl-glucopyranosides) of similar molecular weight and hydroxyl content. The higher acidity of reducing sugars is caused by the higher lability of the hydrogen atom of the hemiacetal (anomeric) hydroxyl group, a condition that apparently stems from an electron-withdrawing polar effect (inductive effect) exerted upon this group by the ring oxygen.

Table 1. Ionization Constants (Hydroxyl Group) of Carbohydrates in Water at 25°C [10]

Compound	pK_a
D-Glucose	12.35
2-Deoxyglucose	12.52
D-Galactose	12.35
D-Mannose	12.08
D-Arabinose	12.43
D-Ribose	12.21
2-Deoxyribose	12.67
D-Lyxose	12.11
D-Xylose	12.29
Lactose	11.98
Maltose	11.94
Raffinose	12.74*
Sucrose	12.51
D-Fructose	12.03
D-Glucitol	13.57*
D-Mannitol	13.50*
Glycerol	14.40

* Measured at 18°C.

One important feature of Table 1 is that the greater the number of hydroxyl groups, the greater the acidity. Glycerol has the same acidity as water; lactose and maltose, which are reducing disaccharides, seem to be somewhat more acidic than aldopentoses (arabinose, ribose, lyxose and xylose).

Recently, highly alkaline electrolyte solutions such as lithium, potassium, or sodium hydroxide at pH greater than 12 have been shown to be useful in the separation of underivatized saccharides by CZE.[11] As expected, the resolution among the various saccharides increased when moving from pH 12.3 to pH 13.0 due to increasing ionization of the separated analytes[11] (see Figure 2). Also, the nature of the alkali-metal influences the resolution of the sugar analytes.[11] It should be noted that separations at extremely high pH can only be performed on naked fused-silica capillaries since most coated fused- silica capillaries will undergo hydrolytic degradation under such basic conditions.

C. Carbohydrate-Metal Cation Complexes

In the complex formation between metal cations and carbohydrates, the hydroxyl groups of carbohydrates are thought to from coordinate bonds with the metal cation. Usually, strong complexing occurs between cations and a contiguous axial (a), equatorial (e), axial sequence of hydroxyl groups in carbohydrates as was ascertained from the electrophoretic movement of compounds containing this sequence, and the immobility of many others lacking such an arrangement.[12]

For acyclic alditols, when three consecutive carbon atoms have the *threo-threo* configuration, the complex is most favored. An *erythro-threo* configuration is less favored for complex formation, and an *erythro-erythro* arrangement does not give rise to any noticeable complexation with cations. The more *threo* pairs of hydroxyl groups there are in the alditol, the stronger will be its complexes.[13] If one of the three hydroxyl groups is replaced by a methoxy group, complexing becomes weaker. If all three hydroxyl groups are methylated, complex formation becomes negligible.[13] This draws similarities to borate complex formation.

The complex formation involving cyclic monosaccharides yields tridentate complexes (see Figure 3), whereby no more than three oxygen atoms can coordinate to one cation as shown in the following structure, where M^+ is the metal cation.

Only in a few cases involving disaccharides, complexation was reported to occur at more than three oxygen atoms, and tetra- and even pentadentate complexation have been described.[13] Further discussion regarding metal cation-carbohydrate complexation can be found in References 2, 3, and 13.

Recently, the use of electrolyte systems containing alkaline-earth metals for the separation of neutral carbohydrates by CZE has been reported.[5] Separations in these media are mainly based on differences in the extent of com-

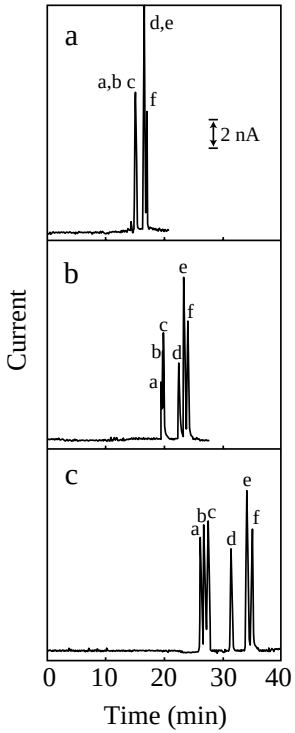


Figure 2. *Electropherograms of six saccharides in different concentrations of NaOH: (a) 20 mM, (b) 50 mM, and (c) 100 mM. The sugars are (a) stachyose, (b) raffinose, (c) sucrose, (d) lactose, (e) galactose, and (f) glucose (concentrations between 80 and 150 μM). The fused-silica capillary dimensions are 50-μm i.d. and 70 cm in length. The separation voltage is 10 kV. Injection is 10 s by gravity (10-cm height); the ADCP is performed at 0.6 V (vs. Ag/AgCl). Reprinted with permission.*[11]

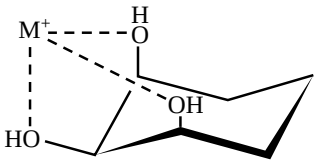


Figure 3. *Metal-carbohydrate complex.*

plexation of the divalent metals with the carbohydrate solutes and, to a lesser extent, on the bulk and shape of the molecule. Although these systems provided a different selectivity than that achieved with borate buffers, the resolution was in general inferior to that of borate buffers.

III. Detection Systems and Precolumn Derivatization

Most carbohydrates lack chromophores in their structures, a condition that hinders their detection at low concentrations. To circumvent this impediment, several detection strategies have been developed including indirect UV and fluorescence detection, electrochemical detection, dynamic labeling via complexation with absorbing or fluorescing ions, and precolumn derivatization with a suitable chromophore and/or fluorophore.

A. Detection of Underivatized Carbohydrates

A.1. Direct UV Detection

UV detectors, which are the workhorses of HPCE, have been used only occasionally in HPCE of underivatized carbohydrates. The major drawback of this approach is the limited sensitivity associated with the inherent low molar absorptivities of most carbohydrates.

Recently, Hoffstetter-Kuhn and co-workers[14] performed spectral measurements of several simple carbohydrates and found that the addition of borate yielded a two- to fifty-fold increase in the molar absorptivities at 195 nm for mono- and oligosaccharides as their borate complexes. This increase was attributed to the fact that borate complexation shifts the equilibrium between carbonyl and cyclic sugar forms toward the carbonyl form or to the presence of additional functional elements such as oxygen bridges between boron and carbon. However, in that work the sugars were detected from relatively concentrated solutions, thus rendering the borate complexation not an attractive detection approach for underivatized carbohydrates.

Only a few carbohydrates exhibit more or less significant absorbance in the low UV. Oligosaccharides containing *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid residues (*i.e.*, glycans) can be detected at 200 nm[15-17,18] or 185 nm.[19] The glycosaminoglycan hyaluronan could be detected at 200 nm at a rather modest sensitivity.[20] This is facilitated by the presence of a repeat unit of one glucuronic acid residue and one *N*-acetylglucosamine residue, linked by glycosidic bonds. Also, low-molecular-mass heparins and heparins could be detected at moderate sensitivity at 200 nm.[21] However, such low UV wavelengths impose serious restrictions on the choice of the composition of the running electrolytes by not allowing the use of many useful additives that may absorb extensively in the low UV.

Another class of oligosaccharides which can be detected directly in the UV consists of acidic di- and oligosaccharides derived from glycosaminoglycans (GAGs). These saccharides, which result from the enzymatic depolymerization of the large GAGs, bear unsaturated uronic acid residues at the nonreducing end that allow their direct UV detection at 232 nm.[21,22-27]

Synthetic oligosaccharide fragments of heparin including di-, tetra-, penta-, and hexasaccharides which lack the double bond in the uronic acid residue were detected at 214 nm but the limit of detection was one order of magnitude lower than that reached by indirect UV[25] (see below for further discussion).

A.2. Indirect UV and Fluorescence Detection of Underivatized Carbohydrates

Indirect detection schemes are universal and can be used for compounds which do not possess the necessary physical properties for direct detection, *i.e.*, chromophores or fluorophores. Indirect detection eliminates the need for pre- or postcolumn derivatization to convert the analyte of interest into a species that yields an acceptable detector response. In indirect detection, the analyte is thought to displace a component of the running electrolyte which may be a chromophore or fluorophore. Usually, a co-ion containing detectable functions is added to the running electrolyte. Since charge neutrality must be maintained, an analyte of the same charge as the co-ion will therefore displace the detectable co-ion. A general scheme illustrating the principles of indirect detection as was reported by Yeung[28] is shown in Figure 4.

As can be seen in Figure 4A, the detectable co-ion provides a constant background signal and, as the analyte zone, which is deficient in the absorbing co-ion, passes through the detector, a decrease in the background signal occurs (see Figure 4B). When the analyte has completely passed through the detection point, the detector response returns to the original baseline (see Figure 4C). Thus, the resulting peak is derived from the detectable background co-ion rather than from the analyte itself. On these bases, almost any detection scheme in HPCE can be made to function in the indirect mode by altering the composition of the running electrolyte and not the actual instrumentation used for direct detection.

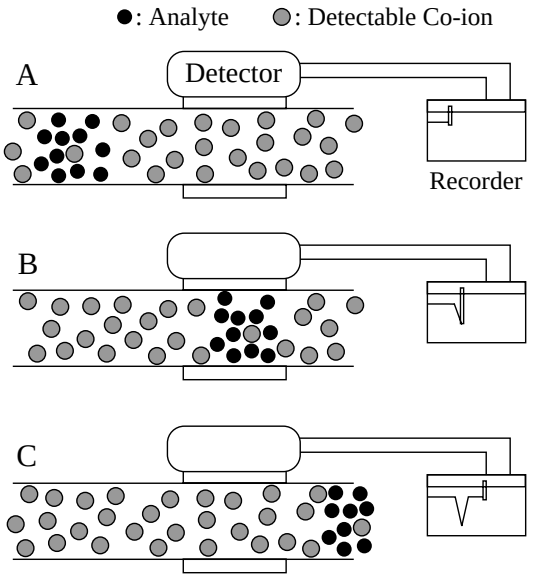


Figure 4. *Illustration of the displacement mechanism for indirect detection.*

The mechanism of displacement in indirect detection was described by Yeung.[28] The number of electrolyte co-ions displaced (or replaced) by one analyte molecule is defined as the transfer ratio (*TR*). Since a large background signal is required, the instability of the background signal can have tremendous effects on the dynamic reserve (*DR*). *DR* is defined as the ability to measure a small change on top of a large background signal. The *DR* is essentially the ratio of the background signal to the background noise. The concentration limit of detection (C_{lim}), expressed in concentration units, is given by[29]

$$C_{lim} = \frac{C_M}{DR*TR} \qquad (1)$$

where C_M is the concentration of the detectable co-ion which generates the background signal. For a given system, the more stable the background signal (larger *DR*), the smaller the fractional change one can detect. Likewise, the more efficient the displacement process (large *TR*), the lower the C_{lim}. Also, the lower the C_M is, the greater the fractional change will be. It is desirable that the value of *TR* be close to unity.[29] It has been shown that the best detection sensitivity is achieved when the analyte ions have an effective mobility close to that of the detectable co-ion.[30] When optimizing C_{lim}, it must be taken into account that the three parameters are not necessarily independent. For example, decreasing C_M will increase *TR*, but at the expense of decreasing *DR*.

It should be noted that the *TR* is not necessarily the same for all analytes. For in depth discussion of this mode of detection, the interested reader is advised to consult recent reviews.[28,29,31]

Both indirect photometric and fluorometric detection modes have found use in CE of carbohydrates. In both types of indirect detection, one of the critical factors is the selection of the detectable co-ion which should meet several criteria including: (i) a high molar absorptivity at the detection wavelength and excitation wavelength used in UV and fluorescence, respectively, (ii) a high quantum efficiency in fluorescence, preferably as close to unity as possible, (iii) compatibility with the solvent system, *i.e.*, it must be soluble and inert, (iv) non interactive with the capillary wall, and (v) must be charged, preferably with a charge identical to that of the analyte being displaced. The last criterion will ensure a value close to unity for the transfer ratio, *TR*. To realize the full benefits of indirect LIF detection in terms of limit of detection, the stabilization of the laser power is of primary importance because it greatly improves the dynamic reserve, *DR*, by decreasing fluctuations in the background signal.

The first application of the principle of indirect detection to the area of HPCE of carbohydrates was demonstrated by Garner and Yeung[32] employing laser-induced fluorescence (LIF) detection. In that work, Coumarin 343 was used as the background fluorescing co-ion for indirect LIF. Coumarin 343 was selected because of its good solubility, high quantum efficiency, and high molar absorptivity (ε = 20,000) at 442 nm, which matches the 442 nm line of a helium-cadmium (He-Cd) laser. Using this indirect LIF detection system, 640 femtomoles of three simple sugars could be separated using 1 mM Coumarin 343, pH 11.5, as the running electrolyte and a capillary of 18 μm i.d. The high pH used ensured the partial ionization of the neutral carbohydrates, rendering them amenable to electrophoretic separation as well as to indirect detection. In indirect detection, the sensitivity is a function of the fraction of the analyte that is ionized. This means that, when using indirect detection with sugars, the pH of the running electrolyte must be approaching 12 to have any substantial fraction, α, of the sugar solute in the ionized form. When the pH of the running electrolyte approaches pH 12, the concentration of hydroxide ions is no longer negligible relative to the concentration of the detectable co-ion. The effect of the hydroxide ion can be approximated[33] by:

$$TR_{tot} = \frac{\alpha[\text{sugar}]}{[\text{FL}] + [\text{OH}^-]} \qquad (2)$$

where TR_{tot} is the total transfer ratio, α [sugar] is the fraction of the sugar ionized, [FL] is the concentration of the detectable fluorophore, and [OH^-] is the hydroxide ion concentration. As can be seen in equation (2), at constant fluorophore (or chromophore) and sugar concentrations, α in the numerator

and [OH^-] in the denominator are competing functions of pH. The total transfer ratio goes through a maximum when plotted as a function of pH. This maximum is the most sensitive pH for detection of a given sugar. The optimum detection pH for simple sugars was found to be 11.65, but 11.5 was used for detection because the rate of degradation of the fluorophore (*i.e.*, Coumarin 343) was decreased without any appreciable decrease of detection efficiency. Using the detection system described above, the absolute limit of detection was 2 femtomoles for fructose when using 5 μm i.d. capillaries with exceptional separation efficiencies.[33]

Very recently, detection limits in the picogram range are possible for high-molecular-weight polysaccharides (*e.g.*, dextran, amylose, amylopectin, etc.) when laser-excited indirect fluorescence detection is employed.[34] An argon-ion laser source operating at 488 nm was used for excitation. Because of the highly alkaline electrolyte (pH 11.5) needed to ionize the polysaccharides, 1 mM fluorescein has to be added to the running electrolyte as the fluorophore in order to overcome the competition with the high concentration of hydroxide ions and in turn to detect the polysaccharides (see Figure 5). Also, because of the weak ionization of polysaccharides at high pH, only 9 out of 11 polysaccharides studied could be detected.[34]

Although the above indirect detection schemes showed exceptional results in both sensitivity and efficiency, they involved the use of expensive laser equipment not available in most separation facilities. Indirect UV has been shown to be feasible for the detection of underivatized carbohydrates. Bonn and co-workers[35,36] have demonstrated the use of 6 mM sorbic acid at pH 12.1 as both the electrolyte and the detectable co-ion in the separation and indirect detection of several simple sugars. The alkaline pH ensured ionization of the sugars and, hence, their detection by means of charge displacement. Sorbic acid has a high molar absorptivity ($\varepsilon = 27800\ M^{-1}cm^{-1}$ at 256 nm), and carries a single charge, thus ensuring enhanced detectability and a favorable transfer ratio, respectively. Under these conditions, a detection limit of 2 picomoles was obtained for glucose.

More recently, Bergholdt *et al.*[37] employed indirect UV combined with HPCE for the separation and detection of two aldonic acids. Since these analytes are naturally ionized, extreme pH is not required in order to ionize the sugars. The running electrolyte was 6 mM sorbic acid. Optimum resolution was obtained at pH 5.0. The detection limit under these conditions was determined to be 18 femtomoles. The lower detection limits, when compared to the previous example, can be attributed to the lower pH which allows for a larger *TR*. Moreover, it was shown recently that the use of high pH background electrolyte resulted in a rapid increase of the low-frequency noise and baseline instability. This was found to be related to the joule heat production and insufficient

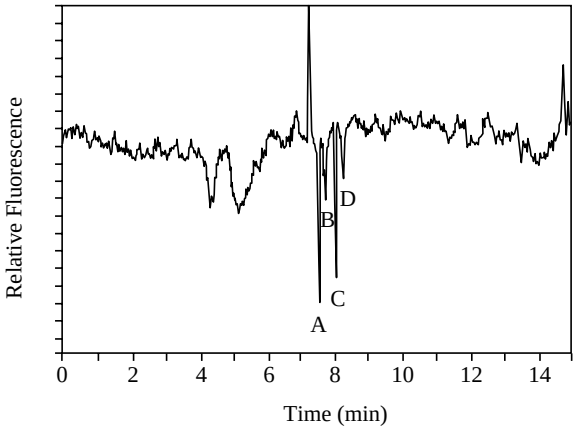


Figure 5. S*eparation and detection of polysaccharides in CE by indirect fluorescence. Capillary, 80 cm total length, 65 cm to detection window × 26-μm i.d., 140-μm o.d.; injection, electromigration 1 s at 15 kV; run at 15 kV with 1 mM fluorescein, pH 11.5. Solutes: A, dextran; B, comb-dextran; C, hydroxyethylamylose; D, amylose. Reprinted with permission.[34]*

thermostating of the capillary tubing.[38] Detection could be greatly improved by using narrow (25 μm i.d.) capillaries and low voltages, and by thermostating the surrounding of the capillary column to allow a uniform heat dissipation along the capillary.[38] In addition, the noise was influenced by the composition of the background electrolyte. For instance, riboflavin and lithium were found to be the best chromophore and counterion, respectively, giving the best performance in terms of higher signal-to-noise ratio. This was due to the fact that both compounds had the lowest mobility compared to the compounds tested. By optimizing all these factors, the pH range for the separation could be extended to 13 with a limit of detection of 50 μM[38] which is one order of magnitude lower than previously reported values.[35] Using a riboflavin-NaOH electrolyte system, the detection limit was improved by approximately 25 times at pH 12.3.

Recently, the results of HPCE experiments involving the determination of carbohydrates in fruit juices by the HPCE-indirect UV mode of detection were compared with those obtained by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).[39] In that work, potassium sorbate was chosen as the background electrolyte (pH 12.2-12.3) and chromophore for indirect UV detection at 256 nm. HPAEC-PAD yielded detection limits of 2-3 orders of magnitude lower than those with HPCE-indirect UV. However, the comparison was in favor of HPCE in terms of mass

detection. The absolute amount detectable for HPAEC-PAD was 25-50 pmol, while it was 0.9-1.1 pmol for HPCE-indirect UV. In the application of HPCE-indirect UV to the analysis of sugars in fruit juices, the detection sensitivity is not an issue since the sugar concentration is relatively high (100 gL^{-1}) and the sample must be diluted 1:50 prior to HPCE-indirect UV analysis.

Other carbohydrates were detected by HPCE-indirect UV including eight heparin disaccharides and some synthetic sulfated disaccharide and oligosaccharide fragments of heparin.[25] HPCE-indirect UV was achieved by using either 5 mM 5-sulphosalisylic acid, pH 3.0, or 5 mM 1,2,4-tricarboxylbenzoic acid, pH 3.5, as the running electrolyte and chromophore. In contrast to direct UV detection, with indirect UV detection, the signal obtained for various synthetic pentasaccharides is nearly independent of their molecular structure and the sensitivity is at least one order of magnitude higher than that of direct UV detection. Again, because of the low pH where the transfer ratio is at its optimum value, the limit of detection of synthetic pentasaccharide heparin fragments was below 5 fmol when performing the detection at 214 nm using 5 mM 5-sulphosalicylic acid, pH 2.5.[25] Even for heparin disaccharides possessing unsaturated uronic acid residues at the nonreducing end, HPCE-indirect UV at 214 nm in the presence of 5 mM 1,2,4,-tricarboxybenzoic acid at pH 3.5 yielded higher sensitivity than HPCE-direct UV at 230 nm when employing 200 mM sodium phosphate, pH 2.5, as the running electrolyte (see Figure 19).

Although the principle of indirect UV (also fluorescence) detection appears relatively simple and is significantly more sensitive than direct low wavelength UV of underivatized carbohydrates, several drawbacks can be pointed out. First, the instability of the detection system results in drift or disturbances of the baseline. Second, an indirect detection system requires working at a low concentration of background electrolyte in order to have efficient transfer ratios, a condition that results in lower efficiencies at higher sample concentrations and the possibility of solute-wall interactions. A third disadvantage imposed by indirect detection in HPCE is the limitation in the selection of the composition and pH of the background electrolyte. In other words, there is not much room to manipulate selectivity and optimize separations. In fact, "neutral" carbohydrates are only partially ionized at the optimum pH normally used in indirect detection, *i.e.*, pH~12, a condition that does not favor their high-resolution separation. Fourth, according to equation (2), quantification can become a significant problem with indirect detection methods, especially if the analytes possess significantly different electrophoretic mobilities. Finally, another disadvantage is the limited linear dynamic range, typically under two orders of magnitude. Therefore, this mode of detection should be used only for fairly concentrated samples or whenever analytes are not easily derivatized or cannot be detected otherwise. It should be noted, however, that indirect UV detection is perhaps the best approach described so far for CE of low-molecu-

lar-weight ionic species such as anions, cations, and organic acids[40] where more than 30 ions have been separated in less than three minutes. This is because these ionic species are charged in their natural environment and, therefore, selection of pH and ionic strength of the running electrolyte may not be as critical for the outcome of the separation as when dealing with weakly ionized analytes, *e.g.*, neutral sugars.

A.3. Electrochemical Detection

Electrochemical techniques have proven to be useful methods for the detection of underivatized carbohydrates. In addition, electrochemical detection (ED) is an ideal method of detection for microcolumn-based separation systems. This is because detection is based on an electrochemical reaction at the surface of the working electrode so that cell volumes can be made very small with no loss in sensitivity. This is contrary to optical detectors where response is dependent on path length. In particular, amperometric methods are among the most sensitive approaches currently available for the detection of underivatized sugars. Amperometric detection is based on the measurement of current resulting from the oxidation or reduction of analytes at the surface of an electrode in a flow cell (for detailed discussions, see Reference 41).

Recently, two approaches for ED have been reported as useful methods for carbohydrate detection, namely amperometric detection at constant potential (ADCP)[11,42,43] and pulsed amperometric detection (PAD).[44-46] The principles of PAD have been described in detail by Johnson and LaCourse.[47]

The metallic electrodes most widely used to date for the detection of carbohydrates and related species after HPLC separations have been the platinum (Pt) and gold (Au) electrodes.[47] The success of Pt and Au electrodes is due largely to the tendency of sugars to adsorb on their surfaces where the sugars readily undergo electrochemical reactions at low potentials.[48] Unfortunately, these same adsorption phenomena also constitute one of the major disadvantages of Pt and Au in that accumulation of oxidation products generally leads to electrode poisoning and, unless overcome experimentally, a rapid decrease in analyte response occurs. As a result, anodic detection schemes using Pt and Au electrodes typically include routine desorption and conditioning steps in order to provide a stable and reproducible response. Most frequently, these measures consist of a continuous pulsing of the surface to extreme positive and/or negative potentials. This technique is known as pulsed amperometric detection (PAD).

Recently, O'Shea *et al.*[44] introduced the PAD concept to the detection of carbohydrates after CE separation in an off-column detection format with a gold wire microelectrode. The PAD has been successful because the multistep waveform solves the problem of electrode poisoning typically found with the

oxidation of carbohydrates at Au electrodes in the direct amperometric detection mode. This HPCE-PAD allowed a detection limit of 22.5 fmol. However, this detection limit was 1-2 orders of magnitude lower when a 10-μm disk gold electrode was utilized for the detection of carbohydrates separated in a 10-μm i.d. capillary.[45] Figure 6 shows the separation and detection of eight sugars using a gold working electrode. A linear working range was observed from 10^{-6} to 10^{-4} M for inositol.[45] Recently, Weber *et al.*[46] reported the applicability of PAD to the detection of complex carbohydrates such as glycopeptides with a detection limit of 2 μM (S/N = 3). Although this approach has solved the problem of electrode poisoning usually encountered with the oxidation of carbohydrates on Pt or Au electrodes in the direct amperometric detection mode, the PAD detection system requires specialized pulse sequences, thus entailing expensive instrumentation. Also, other major drawbacks of PAD include problems involving charging currents and surface changes associated with potential pulsing which do not allow the ultimate detectability to be achieved.

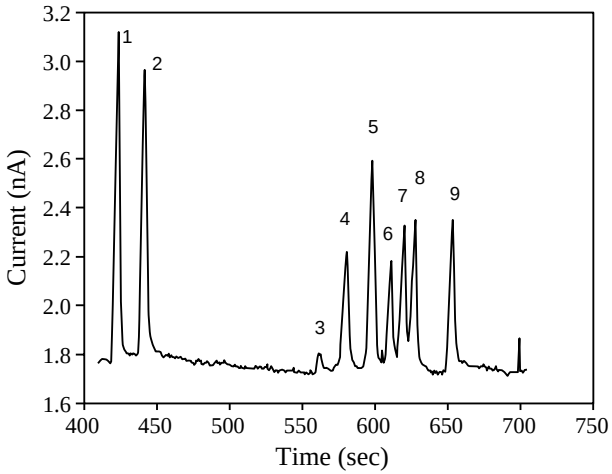


Figure 6. *Electropherogram of carbohydrates with PAD. Experimental conditions: separation voltage, 30 kV over 10 μm i.d. × 60 cm capillary; electrode, 10 μm (in diameter) Au disk; electrode potential, 300 mV (vs. SCE) for 165 ms (sampling at 111-165 ms), 1200 mV for 55 ms, and -1000 mV for 165 ms; electrolyte, 0.1 M NaOH; electromigration injection, 30 kV for 3 s; sample concentrations, 1×10^{-4} M for inositol and 2×10^{-4} M for others. Peaks: 1 = inositol, 2 = sorbitol, 3 = unknown, 4 = maltose, 5 = glucose, 6 = rhamnose, 7 = arabinose, 8 = fructose, and 9 = xylose. Reprinted with permission.[45]*

An alternative to PAD is using amperometric detection at a constant potential (ADCP). ADCP has long been proven a useful approach for the detection of electroactive species at the trace level using carbon electrodes. The principal difficulty encountered in this approach is that carbohydrates exhibit a large overpotential for oxidation at the carbon electrodes used in conventional liquid chromatography with electrochemical detection. This phenomenon drastically increases the potential required for the oxidation and thereby compromises both the selectivity and sensitivity of the detection. To overcome this problem, attention has been focused on the development of new electrode materials[49] that permit the oxidation of carbohydrates at high pH and at relatively low potentials to provide optimum detector performance. In general, the Cu electrode was found to provide superior detection capabilities in terms of its range of response, detection limits, and especially stability, even in the 0.10 M NaOH at which the studies were performed. The Cu electrode had a detection limit of 3×10^{-8} M for glucose at +0.58 V (vs. Ag/AgCl) with a linear response range over four decades.[49]

Zare and co-workers[11] employed ADCP with a Cu microelectrode for the detection of carbohydrates after separation by HPCE. The separation of sugars was performed in strongly alkaline solutions (*i.e.*, pH 13) without prior derivatization or complexation with borate ions. The Cu microelectrode at +0.6 V (vs. Ag/AgCl) could be employed for hundreds of runs without deterioration. Because the pKs of most sugars are in the vicinity of 12-13, they are ionized at high pH and separated by HPCE under such conditions. Figure 7 illustrates the separation and detection of 15 different carbohydrates.

The limits of detection were calculated to be below 50 femtomoles for the 15 sugars studied with a linear dynamic range that extended over 3 orders of magnitude (*e.g.*, mM-mM). However, the reproducibility of this system is very low due to the difficulty associated with the electrode/capillary alignment during an electrophoresis run and from run-to-run. Recently, Ye and Baldwin[42] reported the design and characterization of a simple wall-jet electrochemical detector which allows the use of normal size working electrodes, thus increasing the reproducibility of amperometric detectors without introducing significant peak broadening. In this approach, a disk-shaped electrode consisting of metal wire with only its tip cross section exposed was positioned immediately in front of the capillary outlet. Detection was performed on the solution exiting the capillary and flowing radially across the face of the copper electrode. This design was shown to exhibit 50 times improvement in detection limit (ca. 1 fmol) over the conventional ED, and 5-6 times reproducibility improvement.[42]

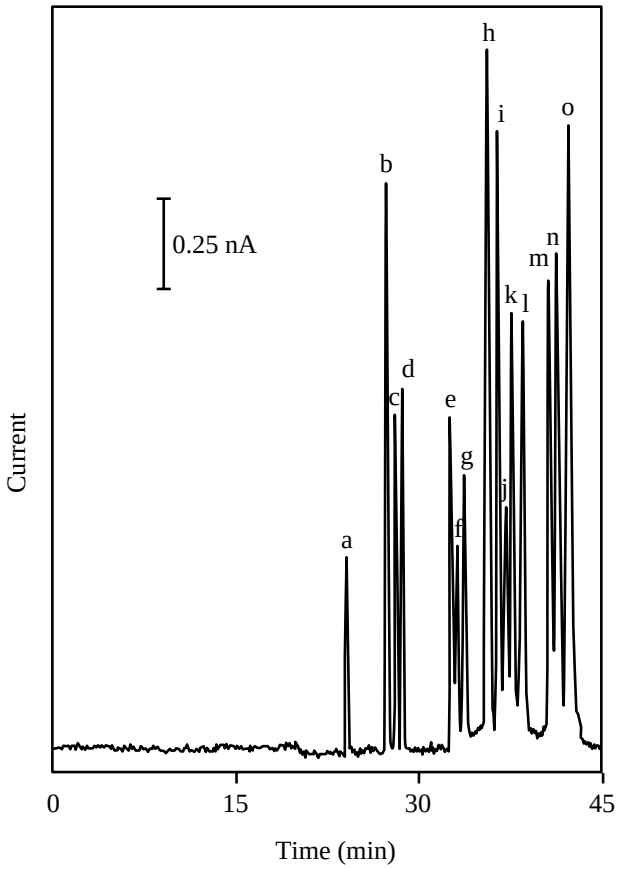


Figure 7. *CZE/ADCP electropherogram of a mixture containing 15 different carbohydrates (80-150 μM). Electrolyte, 100 mM NaOH; capillary, fused-silica, 73 cm (total length) × 50-μm i.d.; 10 s hydrodynamic injection, 10-cm height; voltage, 11 kV; analytes, a = trehalose, b = stachyose, c = raffinose, d = sucrose, e = lactose, f = lactulose, g = cellobiose, h = galactose, i = glucose, j = rhamnose, k = mannose, l = fructose, m = xylose, n = talose, o = ribose. Reprinted with permission.*[11]

Regardless of whether the PAD or ADCP approach is used for the amperometric detection of carbohydrates, both approaches suffer from (i) the limitations imposed by the alkaline conditions needed for sensitive detection and differential electromigration which restrict the useful pH to a very narrow range, *i.e.*, pH >12, and (ii) the non-discriminative nature of amperometric detection which is known to yield a response not only for carbohydrates but

also for other analytes including amino acids, peptides, organic acids, simple alcohols, and aliphatic amines,[47] a condition that may render peak assignment difficult and may lead to less accurate quantitative measurements. Nevertheless, HPCE-ED permitted the rapid determination of sugars in beverages,[11,43] the detection of glycopeptides,[46] the determination of glucose in human blood,[44] and the monitoring of the enzymatic oxidation of glucose with time.[43] Unfortunately, at the present time this type of detector is not available from a commercial source. This may become a reality as soon as further improvements are realized in the design of electrochemical detectors.

B. Detection of Labeled Carbohydrates

B.1. Dynamically Labeled Carbohydrates

Recently, mixtures of α-, β- and γ-cyclodextrins (CDs) were separated and detected by HPCE-LIF using 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) as the background electrolyte.[50] This scheme exploits the ability of cyclodextrins to form inclusion complexes with 2,6-ANS, thus allowing their simultaneous differential electromigration and LIF detection. As the fluorophore complexed with the hydrophobic cavity of CDs, its fluorescence was enhanced, and consequently the CD-2,6-ANS adducts could be detected by the fluorescence increase as positive peaks. The LIF detection was performed with an argon ion laser operating at 363.8 nm for excitation and the emission was collected at 424 nm. Under these conditions, the detection limits were determined to be 62, 2.4 and 24 μM for α-, β- and γ-cyclodextrins, respectively. This may reflect that β-CD forms the strongest complex with 2,6-ANS. In fact, they eluted and separated in the order of increasing strength of complexation (see Figure 8).

Very recently, large polysaccharides such as amylopectin and amylose were shown to complex with iodine which provides both the charge needed for differential electromigration and the chromophore necessary for direct visible detection at 560 nm.[51] The primary basis for this process is iodine binding affinity to carbohydrates which can be manipulated through control of temperature and iodine concentration (see section IV.C.2 for more details).

B.2. Precolumn Derivatization

Carbohydrates are generally tagged with a suitable chromophore or fluorophore to allow their detection at low levels. As the tagging process brings about dramatic changes in the structure of the carbohydrate analytes, it is wise to carefully select the tag not only to allow the sensitive detection of the derivatized carbohydrates but also to produce the changes needed for the subsequent separation step. In other words, it is preferred that the tag also supplies

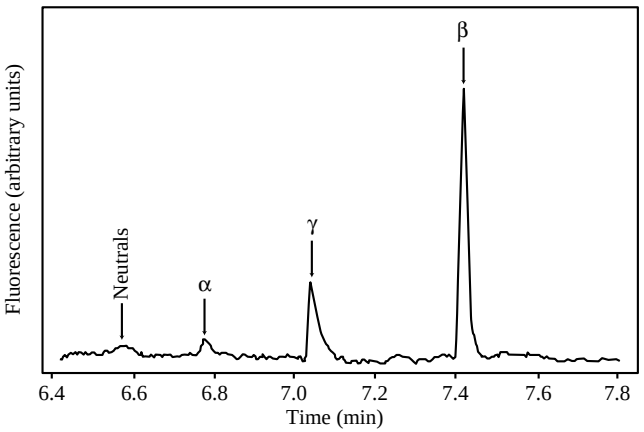


Figure 8. *CE separation of α-, β- and γ-CD. Analysis buffer: 40 mM phosphate, pH 11.76, 1 mM 2,6-ANS. The analysis was carried out in a capillary of dimensions 50-μm i.d., 360-μm o.d. and 1 m in length in a field of 300 Vcm^{-1}. The sample was introduced into the capillary by electrokinetic injection; 5 kV for 2 s from a sample containing 1.44 mg/mL α-CD, 0.017 mg/mL β-CD, and 0.24 mg/mL γ-CD. Detection was by fluorescence excited at 363 nm and monitored at 424 nm. Reprinted with permission.*[50]

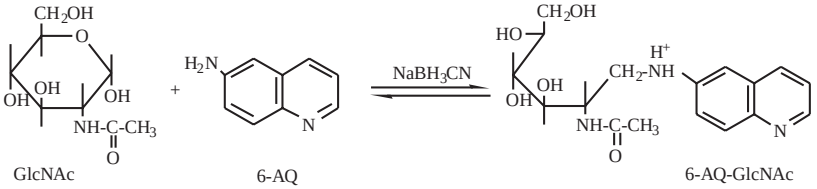
the charge necessary for electrophoresis over a relatively wide range of pHs or that the tag imparts a hydrophobic character to the derivatives so that the principles of MECC can be applied to the separation of derivatized carbohydrates. Other essential criteria for a successful precolumn derivatization include (i) high yields, (ii) the formation of a single product for each species, (iii) no detectable side products, (iv) minimum sample workout and cleanup, and (v) no cleavage of an essential sugar residue, *e.g.*, sialic acid residue. In precolumn derivatization reactions, it is generally preferred that the tagging occurs at only one reactive functional group of the analyte and should be complete so that a single derivative is obtained in high yields. The polyhydroxy nature of sugars is attractive as far as the attachment of a tag to the molecule is concerned. This route to derivatization has been used extensively in gas-liquid chromatography in order to increase the volatility of carbohydrates and consequently facilitate their separation. However, derivatizing the hydroxyl groups would lead to multiple tagging of an analyte and, because hydroxyl groups vary in their relative reactivities, a distribution of derivatives rather than a single product would be obtained. In order to prevent multiple derivatization, other functional groups on the sugar molecule must be considered. The most popular sites for

tagging include (i) the carbonyl group in reducing sugars, (ii) amino group in amino sugars, and (iii) the carboxylic moiety in acidic sugars. To produce a single product in a given precolumn derivatization, the tag must possess only one reactive site for attachment to the analyte. For UV detection, the tagging agent should exhibit a high molar absorptivity at a given wavelength with minimal interferences from the running electrolyte to ensure highly sensitive detection of the derivatized carbohydrates. Likewise, in fluorescence a tagging agent should exhibit high quantum efficiencies at a given excitation wavelength. These requirements become crucial when dealing with extremely small sample volumes encountered in nano-scale separation techniques such as HPCE.

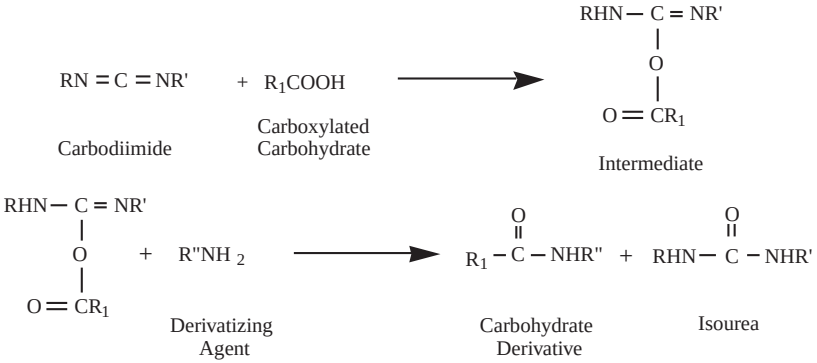
Thus far, five different precolumn derivatization schemes have been introduced for the tagging of carbohydrates: (1) reductive amination (the most widely used)[52,53] (see Scheme I); (2) condensation of carboxylated carbohydrates with aminated tags in the presence of carbodiimide[8,54-56] (see Scheme II); (3) base-catalyzed condensation between the carbonyl group of reducing carbohydrates and the active hydrogens of 1-phenyl-3-methyl-5-pyrazolone (PMP) or 1-(p-methoxy)phenyl-3-methyl-5-pyrazolone (PMPMP), forming bis-PMP and bis-PMPMP derivatives, respectively[5,9,57,58] (see Scheme II); (4) reductive amination of reducing carbohydrates with amines to yield 1-amino-1-deoxyalditols followed by reaction with 3-(4-carboxybenzoyl)-2-quinolinecarboxyaldehyde (CBQCA) in the presence of potassium cyanide[59] (see Scheme IV); (5) reductive amination of reducing carbohydrates with amines to yield 1-amino-1-deoxyalditols followed by reaction with 5-carboxytetramethylrhodamine succinimidyl ester (TRSE)[60] (see Scheme V).

With the exception of precolumn derivatization in Scheme III which yields UV absorbing derivatives, all other precolumn derivatization schemes can produce both UV-absorbing and fluorescing derivatives depending on the spectral properties of the tag used. It should be noted that the precolumn derivatization according to Scheme III will yield fluorescing derivatives if a fluorescent tag similar to PMP in terms of chemical reactivity becomes available. While precolumn derivatization according to Schemes IV and V necessitates two or more distinct workout steps, the remaining are much simpler, requiring only one derivatization step.

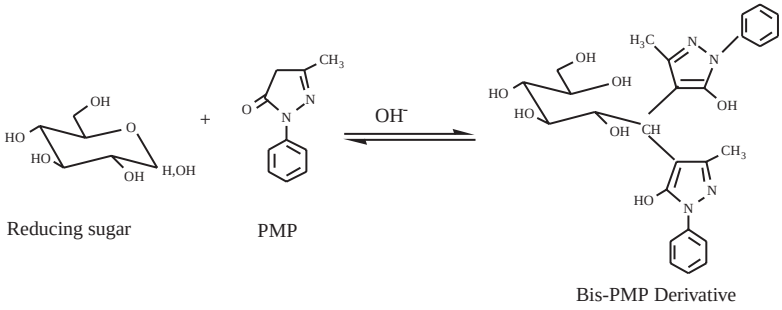
Charts 1 and 2 list the most important tags for the derivatization of carbohydrates. Those which yield neutral or ionizable sugar derivatives are listed in Chart 1 while those yielding permanently charged sugar derivatives are listed in Chart 2.



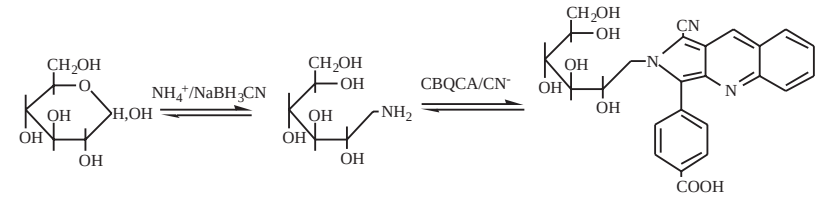
Scheme I. *Illustration of reductive amination where the reducing sugar is N-acetylglucosamine (GlcNAc) and the tag is 6-aminoquinoline (6-AQ).*[61]



Scheme II. *Illustration of the selective precolumn derivatization of carboxylated carbohydrates via a condensation reaction between the carboxylic group of the saccharide and the amino group of the derivatizing agent in the presence of carbodiimide.*[54]

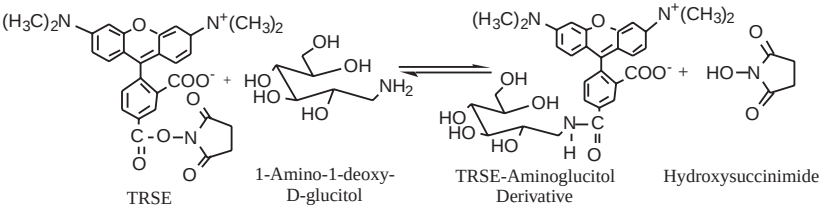


Scheme III. *Illustration of condensation reaction with PMP.*[58]

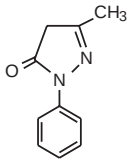


Reducing Sugar | 1-Amino-1-deoxyalditol | CBQCA-Sugar Derivative

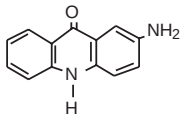
Scheme IV. *Illustration of precolumn derivatization with CBQCA.*[59]



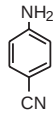
Scheme V. *Illustration of the derivatization of aminated sugar with TRSE. Since it was not provided in the pertinent reference,* i.e., *60, this scheme was inspired from Reference 62 which describes the linking of peptides to activated methoxy polyethylene glycol succinimidyl succinate.*



(I)
1-Phenyl-3-methyl-5-pyrazolone (PMP)
λ_{max} = 245 nm

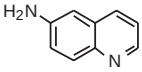


(II)
2-aminoacridone (2-AA)
λ_{exc} = 425 nm, λ_{em} = 520 nm
Argon ion laser 488 nm

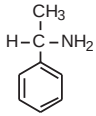


(III)
p-Aminobenzonitrile
λ_{max} = 285 nm

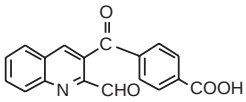
(IV)
2-Aminopyridine (2-AP)
λ_{max} = 240 nm
λ_{em} = 375 nm
He-Cd laser 325 nm



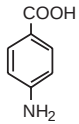
(V)
6-Aminoquinoline (6-AQ)
λ_{max} = 270 nm
λ_{em} > 495 nm
He-Cd laser 325 nm



(VI)
S-(-)-1-Phenethylamine
λ = 200 nm

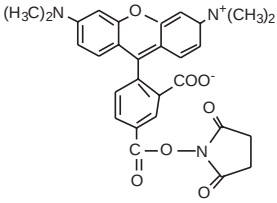


(VII)
3-(-4-Carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA)
λ_{em} = 552 nm
Argon ion laser 457 nm or 488 nm
He-Cd laser 442 nm



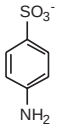
(VIII)
p-Aminobenzoic acid
λ_{max} = 285 nm

Chart 1. *Structures, names and abbreviations of tags yielding neutral and ionizable sugar derivatives. The spectral data were taken from Ref. 58 for tag I; from Refs. 138 and 110 for tag II concerning the fluorescence properties and the use of LIF detection, respectively; from Ref. 68 for tag III; from Refs. 9 and 65 for tag IV regarding UV detection and LIF detection, respectively; from Refs. 61 and 65 for tag V concerning UV detection and LIF detection, respectively; from Ref. 69 for tag VI; from Refs. 77, 79, and 82 for tag VII; from Ref. 67 for tag VIII. The spectral values listed in the chart correspond to the sugar derivatives of the indicated tags. As can be seen in this chart, most of the derivatives were excited at a wavelength dictated by the line of the laser source available, where in most cases the laser line yields only a fraction of the maximum absorption of the derivatives. The interested reader is advised to consult the listed references to find more details concerning UV and LIF detection of the various derivatives.*



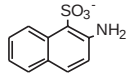
(IX)

5-Carboxytetramethylrhodamine succinimidyl ester (TRSE)
λ_{em} = 580 nm
He-Ne laser 543 nm



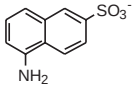
(X)

Sulfanilic acid (SA)
λ_{max} = 247 nm



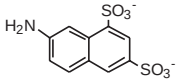
(XI)

2-Aminonaphthalene-1-sulfonic acid (2-ANSA)
λ = 235 nm



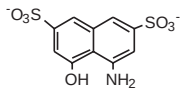
(XII)

5-Aminonaphthalene-2-sulfonic acid (5-ANSA)
λ = 235 nm
λ_{em} = 475 nm
He-Cd laser 325 nm



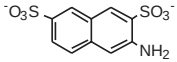
(XIII)

7-Aminonaphthalene-1,3-disulfonic acid (ANDSA), λ_{max} = 247 nm
λ_{exc} = 315 nm λ_{em} = 420 nm
Xenon-Mercury Lamp



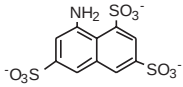
(XIV)

4-Amino-5-hydroxynaphthalene-2,7-disulfonic acid (AHNS)
λ_{em} = 475 nm
He-Cd laser 325 nm



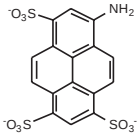
(XV)

3-Aminonaphthalene-2,7-disulfonic acid (3-ANDA)
λ = 235 nm



(XVI)

8-Aminonaphtha-lene-1,3,6-trisulfonic acid (ANTS)
λ_{exc} = 370 nm, λ_{em} = 520
He-Cd laser 325 nm



(XVII)

9-Aminopyrene-1,4,6-trisulfonic acid (APTS)
λ_{exc} = 455 nm, λ_{em} = 512 nm
Argon ion laser 488 nm

Chart 2. *Structures, names and abbreviations of tags yielding permanently charged sugar derivatives. Spectral data were taken from Refs. 60 and 84 for tag IX; from Ref. 54 for tag X; from Ref. 98 for tag XI; from Refs. 98 and 65 for tag XII concerning UV and LIF detection, respectively; from Ref. 54 for tag XIII; from Ref. 65 for tag XIV; from Ref. 98 for tag XV; from Refs. 74 and 97 for tag XVI; from Refs. 70 and 71 for tag XVII. As in Chart 1, the spectral values listed in the chart correspond to the sugar derivatives of the indicated tags. For more details, see the listed references. Here also most of the derivatives were excited at a wavelength dictated by the line of the laser source available, where in most cases the laser line yields only a fraction of the maximum absorption of the derivatives.*

Besides their different spectral characteristics in terms of molar absorptivities and quantum efficiencies, and whether they are UV absorbing and/or fluorescing tags, the different tags listed in Charts 1 and 2 will yield sugar derivatives of varying electrophoretic mobility, separation efficiency, and selectivity. Limiting our discussion to labeling only "neutral" carbohydrates with the various tags, and distinguishing between the various schemes of precolumn derivatization listed above, we can state the following guidelines. PMP (tag I), which can only label carbohydrates according to Scheme III, usually yields neutral sugar derivatives which become negatively charged in aqueous basic solutions due to the partial dissociation of the enolic hydroxyl group of the PMP tag. Usually, the weakly ionized derivatives do not separate well. Thus, the sugar derivatives of tag I will require borate-based electrolytes and/or micellar phases such as SDS to bring about differential electromigration and, in turn, separation. Tags II and III will lead to neutral sugar derivatives via Scheme I, thus requiring borate-based electrolytes and/or micellar phases for separation. Using Scheme I, tags IV, V, and VI will lead to sugar derivatives that can acquire a positive charge at acidic pH. In addition, tag VI is an enantiomeric reagent that allows the separation of sugar enantiomers. Sugar derivatives of tags IV, V, and VI obtained via Scheme I will electrophorese in borate buffers at alkaline pH. Since the sugar derivatives of tags IV, V, and VI are neutral at basic pH, one can envision that they can be electrophoresed by MECC at basic pH. CBQCA (tag VII), which is exclusively used in precolumn derivatization according to Scheme IV, will yield derivatives that can become negatively charged via the ionization of the carboxylic group. TRSE (tag IX) used for labeling carbohydrates according to Scheme V will produce sugar derivatives that can acquire a net positive charge at acidic pH and become a zwitterion at neutral and basic pH. Tag VIII is an amphoteric solute and, therefore, when used to tag carbohydrates via Scheme I, will give derivatives which will charge positively at $pH < 3.0$ and negatively at ≥ 3.8. Using Scheme I, all the other tags (X to XVII) will produce sugar derivatives that are negatively charged over a wide range of pH due to their strong sulfonic acid groups and the very weak ability of their amino groups to become protonated (pK_a values ≤ 3). Thus, under a given set of conditions and for a given set of saccharides, different tagging agents will lead to sugar derivatives with different electrophoretic behavior and selectivity.

It should be emphasized that the sugar derivatives of all the tags will, of course, electrophorese at high pH in the presence of borate buffers. This will become clear as this discussion progresses and more details are provided in Part IV. Due to the presence of permanently ionized strong sulfonic acid groups in the tags listed in Chart 2, their sugar derivatives will electrophorese over a wide range of pH. Although they do not require borate complexation to undergo differential electromigration, the sugar derivatives of the sulfonic

acid-based tags will certainly exhibit different electrophoretic behavior as borate complexes and, in turn, different selectivity may be obtained.

Due to the inherent high sensitivity of fluorescence, it is not surprising to see that laser-induced fluorescence (LIF) is the current trend for high-sensitivity detection of labeled carbohydrates after HPCE separation. In addition, fluorescence is characterized by a good specificity and a large linear dynamic range. The specificity of fluorescence is the result of two main factors. First, only a small percentage of compounds fluoresce because not all compounds that absorb radiation are capable of emitting radiation upon returning to the ground state. In fact, fewer than 10% of all absorbing compounds will emit radiation via any luminescence scheme. Second, two wavelengths are used in fluorometry, but only one in spectrophotometry. Two compounds that absorb radiation at the same wavelength are not likely to emit radiation at the same wavelength unless they are structurally very similar. Likewise, two compounds that emit radiation at the same wavelength will probably absorb radiation at two different wavelengths. Another important advantage is the extended linear dynamic range. It is not unusual to encounter six to seven orders of magnitude linearity with fluorescence spectrometry, but only two to three orders of magnitude with spectrophotometric procedures.

One major drawback of fluorescence detection is that most analytes do not possess satisfactory fluorophores and must be chemically tagged in order to achieve high-sensitivity detection. This is especially true when working with carbohydrates. Also, another principal disadvantage of fluorescence as an analytical tool lies in its serious dependence on environmental factors such as temperature, pH, ionic strength, and the presence of dissolved oxygen. Photochemical decomposition is rarely a problem when using fluorescence as a mode of detection because the analyte is only briefly exposed to the intense excitation radiation. Quenching, which is the reduction of fluorescence by a competing deactivating process resulting from the specific interaction between a fluorophore and another substance present in the system, can introduce significant errors when using fluorescence as an analytical technique. The most noticeable forms of quenching in fluorescence detection are temperature, oxygen, and analyte concentration. As the temperature is increased, the fluorescence decreases. This is due to the resulting increase in molecular motion and collisions which rob a molecule of energy through collisional deactivation. The change in fluorescence is typically 1% per 1°C. However, for some compounds, such as tryptophan or rhodamine B, it can be as high as 5% per 1°C. In HPCE, changes in the buffer composition, pH, ionic strength, and the operating voltage can all lead to large changes in temperature within the capillary. For quantitative analysis, using fluorescence as the mode of detection, care must be taken such that the temperature at which the separation is carried out is

similar to that at which calibration was performed. Oxygen is another source of quenching. Oxygen present in solutions at a concentration of 10^{-3} M normally reduces fluorescence by 20%. In HPCE, oxygen quenching can be reduced or eliminated by degassing solvents and sample solutions before use. Concentration quenching causes many problems during fluorometric determinations. In order for fluorescence to be observed, absorption must occur first. When the absorption is too high, light can not pass through the entire flow cell to cause excitation. In order for a linear relationship to be observed between fluorescence and concentration, the absorbance must be kept below 0.05, otherwise a decrease in fluorescence can be observed. For a detailed description of the instrumentation used in fluorescence detection with HPCE, see recent reviews by Amankwa *et al.*[63] and Li.[64]

So far, precolumn derivatization by reductive amination (Scheme I) has been the method of choice for the labeling of reducing saccharides.[65-71] This has been facilitated by the availability of a large number of chromophores with amino functions. Typically, 2-aminopyridine (2-AP, tag IV) derivatives of sugars formed via reductive amination[52] are usually detected in the UV at 240 nm at the 10 pmol level. They also can be detected by fluorescence. Also, 6-aminoquinoline (6-AQ, tag V)[61] is an efficient tag for linear and branched oligosaccharides and the subsequent separation of the 6-AQ derivatives in HPCE. The 6-AQ derivatives showed a maximum absorbance at 270 nm, and the signal obtained was 8 times higher compared with 2-AP derivatives under otherwise identical conditions.[61] The removal of excess 2-AP and 6-AQ is not required in HPCE since the derivatized carbohydrates elute after the excess tag or, in the case of acidic sugars,[72] the derivatives and the derivatizing agent move in opposite direction. Linhardt and co-workers[73] have described the derivatization via reductive amination of *N*-acetylchitooligosaccharides with a negatively charged tag, 7-aminonaphthalene-1,3-disulfonic acid (ANDSA, tag XIII). The ANDSA-derivatives were excited at 250 nm using an arc-lamp and the fluorescence was collected at 420 nm with detection limits in the femtomole range. UV detection was also described for sugar derivatives of ANDSA.[73] ANDSA has a fairly strong absorbance at 247 nm ($\varepsilon = 3100\ M^{-1}\ cm^{-1}$), but more importantly it supplies the charge necessary for rapid electrophoretic analysis of sugars in HPCE. Chiesa and Horváth[74] utilized a similar tag, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS, Tag XVI), for the derivatization of malto-oligosaccharides separated in HPCE which was first demonstrated in the derivatization of sugars separated in polyacrylamide slab gel electrophoresis.[75] ANTS not only supplies a strong chromophore but also provides multiple negative charges even at low pH, which allow rapid electrophoretic analysis. Using ANTS-derivatized glucose, Chiesa and Horváth[74] were able to detect as little as 15 fmol at 214 nm. Using HPCE-LIF with a

He-Cd laser at 325 nm, the limits of detection lie in the low attomole range, three orders of magnitude lower than that of the UV detection. Other aminonaphthalene sulfonic acid-based tags were introduced for the derivatization of carbohydrates by reductive amination.[65] They were the fluorescent tags 5-aminonaphthalene-2-sulfonic acid (5-ANSA, tag XII) and 4-amino-5-hydroxynapthalene-2,7-disulfonic acid (AHNS, tag XIV). A very recent development in fluorescent labeling of carbohydrates has been the introduction of APTS (tag XVII)[70,71,76] for the HPCE-LIF of mono- and oligosaccharides (see Part IV for more discussions).

The selective precolumn derivatization according to Scheme II, which was introduced recently by El Rassi's research group for the derivatization of carboxylated carbohydrates,[8,54,56] offers several important features including (i) the formation of a stable amide bond between the amino group of the derivatizing agent and the carboxyl group of the carbohydrate molecule by acid catalyzed removal of water in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), (ii) the selective precolumn derivatization of sialylated saccharides at room temperature, thus avoiding the cleavage of the sialic acid residue from the carbohydrate molecule being derivatized, (iii) quantitative yield as deduced from mass spectrometry data,[54] and (iv) the replacement of the weak carboxylic acid group of the saccharide by one or more strong sulfonic acid groups when tagging with sulfanilic acid (SA, tag X) or 7-aminonaphthalene-1,3-disulfonic acid (ANDSA), a condition that allows the electrophoresis over a wider range of pH. Furthermore, using UV absorbance at 247 nm, the detection limit of acidic monosaccharides labeled with ANDSA or SA was 15 fmol or 30 fmol, respectively. For ANDSA derivatives, as low as 0.6 fmol could be detected with on-column fluorescence detection using a detector operated with a 200 W xenon-mercury lamp.[54]

The labeling procedure with CBQCA (Scheme IV, tag VII) which was first introduced by Novotny and co-workers[77-80] allows the detection of submicromolar concentration of CBQCA-sugar derivatives by LIF. CBQCA-sugar derivatives have an excitation maximum at 456 nm, which conveniently matches the 442 nm line of a He-Cd laser, and an emission maximum near 552 nm. Using CBQCA, attomole levels of amino sugars have been analyzed by LIF-HPCE.[81] Very recently, Zhang *et al*.[82] utilized CBQCA for the labeling of aminated monosaccharides. The CBQCA-sugar derivatives were detected by LIF at the nanomolar concentration level, three orders of magnitude lower than the limit of detection reported by Novotny and co-workers. The nanomolar limit of detection was achieved by utilizing a detection scheme that was based on a low scattering sheath flow cuvette as a postcolumn detector and two photomultiplier tubes that mutually exclude wavelength ranges to prevent the water Raman band from contributing to the background system.

Such an arrangement had a limit of detection of 75 zeptomoles of fluorescently labeled 1-glucosamine.[83] One of the virtue of CBQCA derivatization is that the excess derivatizing agent does not have to be removed since the fluorogenic CBQCA does not interfere with the analysis.

The derivatization according to Scheme V was used to label the six most abundant hexoses (*e.g.*, glucose, galactose, mannose, fucose, glucosamine, galactosamine) found in mammalian carbohydrates with TRSE, tag IX.[60] The detection limit of these derivatives was 100 molecules utilizing a postcolumn laser-induced fluorescence detection in a sheath flow cuvette.[60] Although TRSE is a useful fluorescent label for aminated sugar monomers, the labeling reaction requires high sugar concentration to overcome the competition between the labeling reaction and the hydrolysis of the dye, thus rendering the analysis of some real sugar samples not feasible. In fact, the dye yielded two hydrolysis products as was reported by the authors.[60] In another report, six structurally similar oligosaccharides were labeled according to the aforementioned procedure and were baseline resolved by CE. Laser-induced fluorescence detection of these derivatized oligosaccharides allowed a detection limit of 50 molecules.[84]

IV. Separation Approaches and Selected Applications

A. Monosaccharides

A.1. Underivatized Monosaccharides

In general, intact monosaccharides are separated by CZE using either alkaline borate buffers or high pH electrolyte systems. The borate electrolyte systems have been shown to be compatible with direct UV detection at 195 nm[14] and RI detection.[85] The high pH electrolyte systems allowed indirect detection of monosaccharides by fluorescence[32] or UV[35,36,38,39] and also direct detection by amperometry.[11,42-45]

As discussed above (see part II), ED of monosaccharides requires highly alkaline pH electrolyte systems (*e.g.*, alkali-metal hydroxide solutions, pH 12-13) for achieving high sensitivity detection.[11,42-45] At this high pH, the monosaccharides become negatively ionized due to their weakly acidic properties (pK_a of most sugars is ca. 12-13). The concentration and nature of the alkali-metal hydroxide largely affected the analysis time and the resolution of underivatized saccharides.[11] This separation approach was successfully used in the determination of sugars in two common beverages.[11] When using electrochemical detection for the sensing of underivatized saccharides, the addition of borate to bring about a better separation of closely related sugars was reported to decrease the anodic response which might be due to the reduced availability of oxidation sites present on carbohydrates.[11]

Again, and for achieving high sensitivity detection of underivatized monosaccharides by ED, electrolyte solutions at highly alkaline pH were used even for the HPCE of charged monosaccharides including glucosaminic acid, glucosamine-6-sulfate, and glucosamine-6-phosphate.[44] Separation and detection were achieved by using a 10 mM sodium hydroxide solution containing 8 mM sodium carbonate, pH 12. This electrolyte system was shown useful in the determination of glucose in biological samples as complex as human blood after 1:50 dilution (85 μM); the only sample pretreatments were centrifugation and filtration.[44] Ye and Baldwin[43] reported the separation of mixtures containing glucose and galactose as well as their respective alditol and aldonic, uronic, and aldaric acid derivatives using 50 mM sodium hydroxide and ED at a copper disk electrode. A higher sodium hydroxide concentration (250 mM), however, was used in order to achieve a baseline resolution of a set of eight alditols whose pK_a values are in the 13-14 range. These separation and detection approaches have been applied to the analysis of the sugar contents of

commercial apple juice as well as for monitoring the activity of glucose oxidase enzyme.[43]

Although they adversely affect the sensitivity of indirect UV or fluorescence detection, highly alkaline pH electrolytes ought to be used in the HPCE-indirect photometric or fluorometric detection of underivatized saccharides to ensure optimum differential electromigration. The relatively low detection sensitivity of HPCE-indirect UV detection at highly alkaline pH is not an issue when the concentration of the saccharides to be determined is relatively high, such as the determination of the sugar contents of fruit juices.[39]

While with borate buffers the separation of underivatized sugars is based on differences in the extent of complexation among the various solutes, the separation in the presence of high-pH electrolyte systems can be explained by the lability of the proton of the monosaccharides and by the charge-to-mass ratio. Using aqueous sodium hydroxide solution, pH 11.5, as the running electrolyte,[32] sucrose (pK_a = 12.51) was detected first followed by glucose (pK_a = 12.35) and then fructose (pK_a = 12.03). This is the expected elution order when using positive polarity (anode to cathode). The stronger acid, fructose, is moving at a higher velocity upstream against the EOF, thus eluting last. Glucose, a weaker acid, eluted before fructose. Sucrose, a disaccharide, moves upstream against the flow at a lower velocity than glucose due to its higher molecular weight, thus eluting first. The influences of acidity and size of the molecule were also observed with a mixture of raffinose, deoxyribose, galactose, glucose, and mannose.[35] They eluted in the order of increasing acidity: raffinose (a trisaccharide, pK_a =12.74) < deoxyribose (pK_a = 12.52) < galactose (pK_a = 12.35) < glucose (pK_a = 12.35) < mannose (pK_a =12.08).

Using the proper pH for ionized acidic monosaccharides, even two chiral 4-epimers, D-galactonic acid and D-gluconic acid, in an underivatized form could be separated with a resolution of 1.2 at pH values of 4.1 to 5.0. The addition of a chiral selector (β-cyclodextrin) did not improve the separation of the two aldonic acids.

A. 2. Derivatized Monosaccharides

The separation of closely related derivatized monosaccharides by HPCE may require the use of borate buffers even when the tag contains ionizable functional groups. For instance, twelve monosaccharides derivatized with 2-aminopyridine (2-AP) were separated as anionic borate complexes at pH 10.5 in ca. 25 min[52] (see Figure 9). Obviously, the migration velocity of each derivative was primarily affected by the extent of complexation with borate. As typical examples, arabinose and ribose with *cis*-oriented hydroxyl groups at C3/C4 positions were more retarded than lyxose and xylose with *trans*-oriented hydroxyl groups.

The same behavior was also observed with aldohexoses, *e.g.*, galactose (*cis*) and glucose (*trans*), and hexuronic acids, *e.g.*, galacturonic acid (*cis*) and glucuronic acid (*trans*). However, *N*-acetylhexosamines, *e.g.*, *N*-acetylgalactosamine (*cis*) and *N*-acetylglucosamine (*trans*), showed the reverse effect concerning the 3,4-orientation of hydroxyl groups. This was attributed to the contribution of the *N*-acetyl substituent at the C2 position.[52] The borate buffer system was useful in the quantitative determinations of the monosaccharide composition of various di- and oligosaccharides such as lactose, melibiose, rutin, digitonin, and arabic gum, which were in good agreement with the theoretical values.[52]

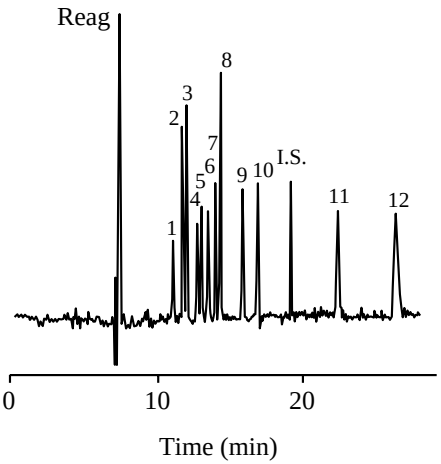


Figure 9. *Separation of* N-*2-pyridylglycamines derived from various reducing monosaccharides. Electrolyte, 200 mM borate buffer, pH 10.5; applied voltage 15 kV; detection UV at 240 nm. Peak assignment of parent saccharides: Reag, reagent (2-AP), 1 =* N-*acetylgalactosamine; 2 = lyxose; 3 = rhamnose; 4 = xylose; 5 = ribose; 6 =* N-*acetylglucosamine; 7 = glucose; 8 = arabinose; 9 = fucose; 10 = galactose; I.S. (internal standard), cinnamic acid; 11 = glucuronic acid; 12 = galacturonic acid. Reprinted with permission.[52]*

Monosaccharides derivatized with 2-AP can be migrated in electrophoresis at low pH in the presence of noncomplexing buffer (*i.e.*, direct CZE) since the 2-AP tag imparts the analytes with a positive charge arising from the protonation of the amino group of the 2-AP-sugar derivative. However, due to equal charge-to-mass ratios, the 2-AP tagging of closely related monosaccharide isomers would only bring about group separation of the derivatives by direct CZE, *i.e.*, in the absence of complex formation with a suitable complexing ion.[86] The positively charged 2-AP derivatives of galactose (Gal), mannose (Man), *N*-acetylglucosamine (GlcNAc), and *N*-acetylgalactosamine (GalNAc)

were separated into groups using phosphate buffer containing small amounts of tetrabutylammonium bromide, pH 5.0. In other words, 2-AP-Gal and 2-AP-Man, differing only in the position of hydroxyl groups, emerged together as one peak separated from the single peak of 2-AP-GalNAc and 2-AP-GlcNAc, which have in their structures an additional acetyl group. The poor resolution of 2-AP-sugar derivatives at pH 5.0 may be due to the partial ionization of these rather weak bases. The 2-AP-sugar mixture was resolved when a 200 mM borate buffer, pH 10.5, was used as the running electrolyte. At pH 10.5, the 2-AP sugar derivatives become neutral, thus requiring a relatively high borate concentration in order to sufficiently complex them with borate and, in turn, allow their differential migration under the influence of an applied electric field.

Although monosaccharides derivatized with PMP may be charged negatively in aqueous basic solutions due to the dissociation of the enolic hydroxyl group, the PMP derivatives of isomeric aldopentoses or aldohexoses could not be separated because they have the same charge-to-mass ratio.[58] With a running electrolyte containing alkaline borate, the PMP derivatives of aldohexoses or aldopentoses were readily converted to anionic borate complexes and separated on the basis of the extent of complexation with the borate. The separation efficiency was quite high with a plate height of 4.1 μm for PMP-xylose. The mechanism of CZE separation of the PMP-derivatives was the same as that of the 2-AP derivatives, although the optimum pH for borate complexation was shifted to 9.5 as opposed to 10.5 in the case of the 2-AP-derivatives, presumably due to the participation of the PMP substituent group in the complexation, perhaps through its hydroxyl groups. An electropherogram depicting a typical separation of a mixture of six PMP-aldohexoses of the D-series is illustrated in Figure 10. When run alone, the aldopentoses were also well separated. However, the peaks of a few species of the pentose and hexose derivatives overlapped when a mixture of pentoses and hexoses was analyzed by CZE.

Thirteen monosaccharides derivatized with CBQCA were separated by HPCE-LIF in less than 22 minutes with separation efficiencies that ranged from 100,000 to 400,000 per meter (see Figure 11).[77] Besides tagging the sugar molecule with a fluorogenic group, the derivatization of monosaccharides with CBQCA provides each sugar derivative with an ionizable weak carboxylic acid group, thus allowing their electromigration. Therefore, the inclusion of only 10 mM borate in the running electrolyte (pH 9.4) was enough to magnify small steric differences between closely related isomers and bring about their separation by CZE. Also, despite the fact that the introduction of a negatively charged group would weaken the complex formation by virtue of Coulombic repulsion, the extent of borate complexation with the hydroxyl groups of CBQCA sugar derivatives was still enough to produce differential migration among the various derivatized monosaccharides.

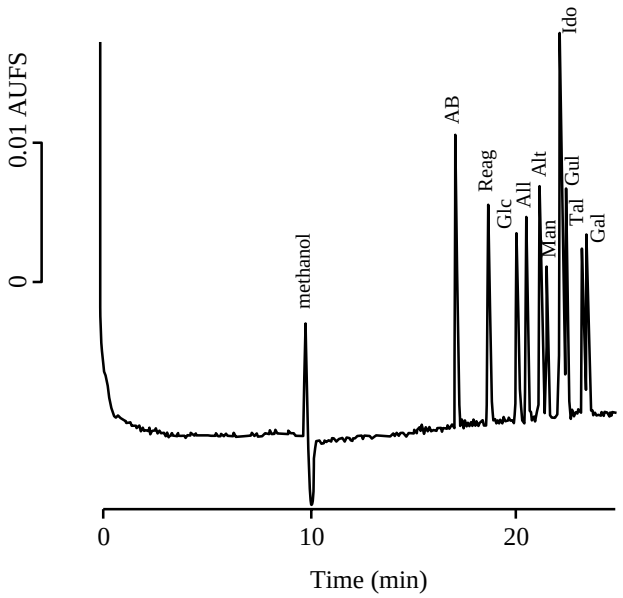


Figure 10. *Separation of PMP-aldohexoses by CZE. Capillary, fused silica tube, 63 cm (to the detection point), 78 cm total length × 50-μm i.d.; electrolyte, 0.2 M borate solution, pH 9.5; voltage, 15 kV; detection, UV at 245 nm. AB, amobarbital (internal standard); Reag, excess reagent (PMP); Glc, glucose; All, allose; Alt, altrose; Man, mannose; Ido, idose; Gul, gulose; Tal, talose; Gal, galactose. Reprinted with permission.*[58]

The concentration of borate in the running electrolyte required to bring about the separation of a given set of monosaccharide derivatives varies with the nature of the sugar derivatives being separated. For instance, the separation of six ANDSA-monosaccharides was best achieved in the presence of a 0.10 M borate buffer, pH 10, see Figure 12.[55] The presence of two strong sulfonic acid groups in each derivative would weaken borate complexation to a much larger extent than in the preceding case (*i.e.*, CBQCA-sugar derivatives) due to a higher Coulombic repulsion. Under this condition, higher borate concentration is needed to overcome Coulombic repulsive forces and bring about sufficient complexation with borate. The ANDSA-sugar shown in Figure 12 was obtained by the new and specific precolumn derivatization reaction for acidic monosaccharides which was recently introduced by El Rassi's laboratory, Scheme II.[8,54,56] In addition to the improved detection sensitivity, the derivatization offered the advantage of replacing the weak carboxylic acid group of the sugar by the stronger sulfonic acid group of the tag, which is fully

ionized at all pHs. This allowed the electrophoresis of the sugar derivatives over a wide pH range and permitted the determination of acidic carbohydrates at the low femtomole levels by UV and fluorescence detection.[54]

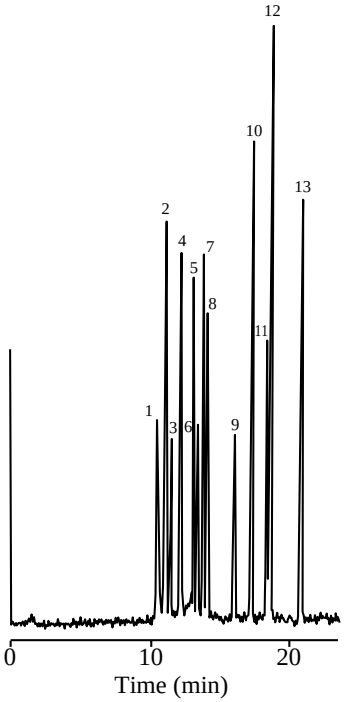


Figure 11. *Electrophoretic separation of a derivatized monosaccharide mixture. The derivatizing agent was CBQCA. Sample concentrations were 6.2 μM for glucosamine and galactosamine, 5.5 μM for galacturonic acid, and 4.4 μM for other sugars. Peaks: 1 =* D*(+)-glucosamine; 2 =* D*(+)-galactosamine; 3 =* D*-erythrose; 4 =* D*-ribose; 5 =* D*-talose; 6 =* D*-mannose; 7 =* D*-glucose; 8 =* D*-galactose; 9 = impurity; 10 =* D*-galacturonic acid, 11 =* D*-glucuronic acid, 12 =* D*-glucosaminic acid; 13 =* D*-glucose-6-phosphate. Electrophoretic conditions were: buffer, 10 mM* Na_2HPO_4*/10 mM* $Na_2B_4O_7.10H_2O$*, pH 9.40; capillary 50-μm i.d. × 88 cm (58 cm effective length; applied voltage, 20 kV. Reprinted with permission.*[77]

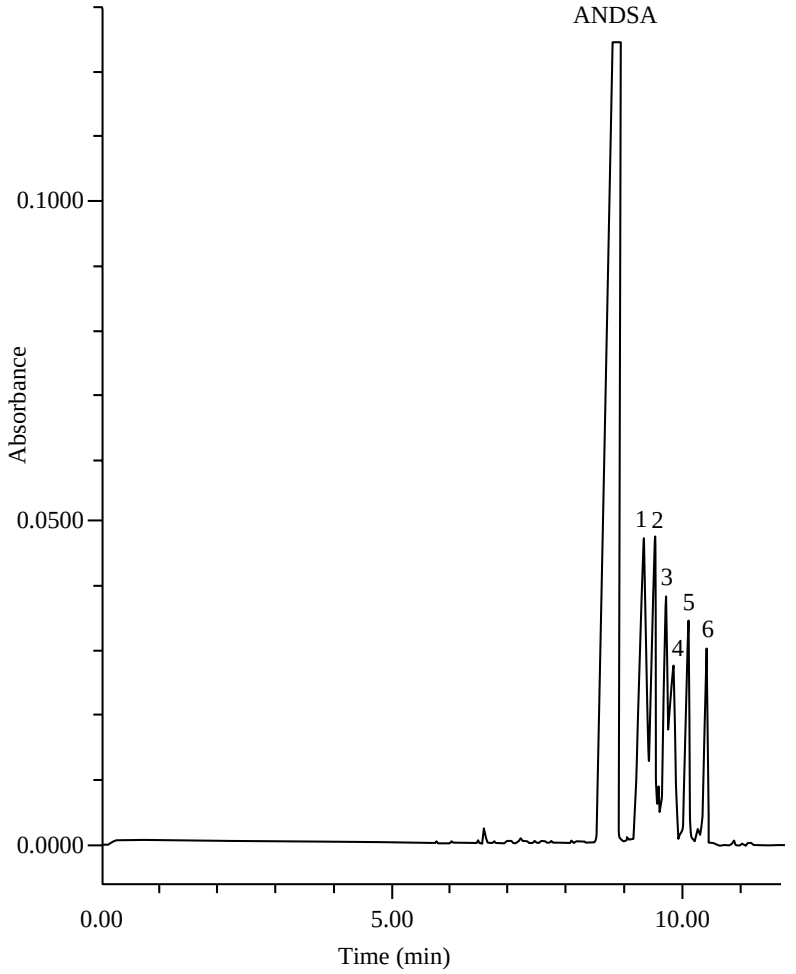


Figure 12. *Electropherogram of ANDSA derivatives of acidic monosaccharides obtained on a dextran 150 kDa-coated capillary. Capillary, 47 cm total length (40 cm effective length) × 50-μm i.d.; electrolyte: 0.10 M borate, pH 10.0; pressure injection, 1 s; applied voltage, -15 kV; detection UV at 250 nm. Samples: 1 =* D*-glucuronic acid, 2 =* D*-glyceric acid, 3 =* D*-galactonic acid, 4 =* D*-galacturonic acid, 5 =* D*-gluconic acid, 6 =* N*-acetylneuraminic acid. Reprinted with permission.*[55]

A different situation was encountered in terms of the separation requirements when the derivatized monosaccharides had a sizable triply charged polyaromatic tag. This was the case of ten monosaccharides including *N*-acetylgalactosamine, *N*-acetylglucosamine, rhamnose, mannose, glucose, fructose, xylose, fucose, arabinose, and galactose which were derivatized with APTS by the standard reductive amination procedure (Scheme I) and subsequently separated by HPCE-LIF.[70,71] The various APTS-monosaccharide derivatives were more or less fully separated by four different buffer systems but, of course, with different migration patterns and selectivities. The four buffer systems include: 135 mM borate, pH 10.2; 100 mM acetate buffer, pH 5.0; 120 mM Mops buffer, pH 7.0; and 50 mM sodium phosphate, pH 7.4.[70] Using 135 mM borate, pH 10.2, did not resolve APTS-arabinose and APTS-fucose. These two APTS derivatives were separated at much higher borate concentration but at the expense of much longer separation time. Again, the formation of anionic borate complexes with the APTS sugar derivatives may be inhibited by the three negatively charged sulfonic acid groups of the APTS tag. With the exception of the borate buffer where separation among the different APTS-monosaccharides is due to difference in borate complexation, the separation in the other buffers are based on the difference in the relative stereochemistry of the hydroxyl groups in aldoses which ultimately determines the hydrodynamic radius of the derivatized species which is inversely proportional to the electrophoretic mobility. Increasing the Mops buffer concentration was shown to improve the resolution substantially; this might be due to the decrease in the EOF caused by increasing buffer concentration. As shown in Figure 13, the order of migration of the APTS derivatives with the Mops buffer (positive or normal polarity) was opposite to that observed with the acetate buffer (negative or reverse polarity) and the migration of the analytes toward the detector (at the cathode end) was due to the high cathodal EOF which was strong enough to overcome the electrophoretic mobility of the analytes in the opposite direction. Phosphate buffer provided a good resolution and the migration order paralleled that obtained with the Mops buffer. In all cases, the analysis time in these buffer systems was shorter than that of the borate buffer system.[71] The tagging of sugars with APTS (tag XVII) is an interesting development in the area of fluorescent labeling of carbohydrates for LIF detection.[70,71] Due to the higher aromaticity and number of fused rings in APTS, APTS-sugar derivatives have substantially higher molar absorptivity and quantum efficiency than most of the commonly used fluorophore sugar derivatives. Also, the presence of three negatively charged groups seems to ensure the separation of closely related structures such as those in Figure 13.

In general, and as discussed above, borate buffers find wide applicability in the separation of derivatized monosaccharides. An important development in the area of HPCE of monosaccharides has been the separation of enantio-

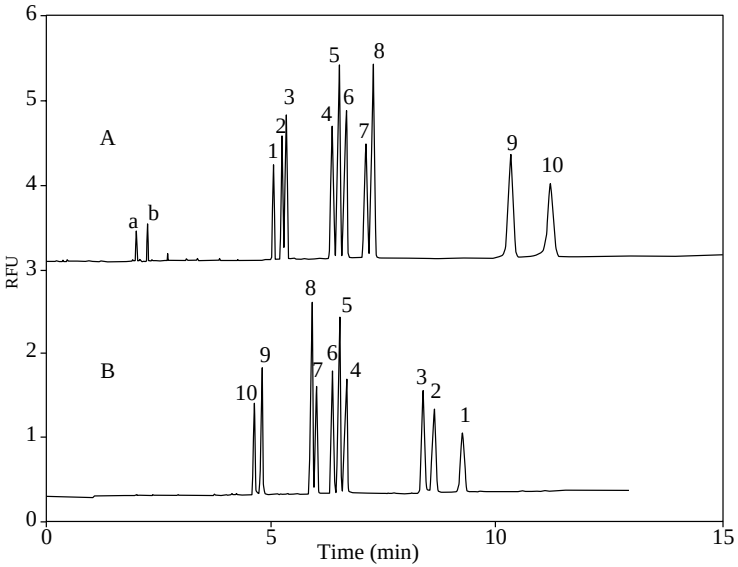


Figure 13. (A) *Electropherograms of 10 APTS-derivatized monosaccharides. Conditions: fused-silica capillary, 20-μm i.d. × 27 cm; laser source, 488 nm argon-ion, 2.5 mW; emission filter, 520 ± 20 nm and a notch filter at 488 nm; buffer, 100 mM sodium acetate, pH 5.0; outlet, anode; applied potential, 25 kV/14 μA; peak identification, 1 = xylose, 2 = arabinose, 3 = ribose, 4 = fucose, 5 = rhamnose, 6 = glucose, 7 = galactose, 8 = mannose, 9 =* N*-acetylglucosamine, 10 =* N*-acetylglucosamine.* ***(B)*** *Electropherogram of 10 APTS-derivatized monosaccharides. Conditions; same as in (A). Buffer, 120 Mops, pH 7.0; outlet, cathode; applied potential 25 kV/19 μA; peak identification, same as in (A). Reprinted with permission.*[70]

meric sugars using the principle of borate complexation in the presence of chiral selectors. D- and L-monosaccharides derivatized according to Scheme I with different fluorophores, namely 2-aminopyridine (2-AP, tag IV), 5-aminonaphthalene-2-sulfonic acid (5-ANSA, tag VII) and 4-amino-5-hydroxynaphthalene-2,3-disulfonic acid (AHNS, tag XIV), were enantiomerically separated by capillary electrophoresis as borate complexes in the presence of linear or cyclic dextrins.[65] 5-ANSA was shown to be the most suitable tag for the enantiomeric separation of the of D- and L- forms of the carbohydrate derivatives when β-CD was used as the chiral selector.[65] Systematic studies on the effects of different CDs and modified β-CDs revealed the importance of the hydroxyl groups of the CD for chiral recognition since enantioselectivity was only observed with the underivatized CDs and hydroxypropyl-β-CD.

In another report, monosaccharides were also enantiomerically separated in a borate buffer after their derivatization with *S*-(-)-1-phenylethylamine[69] (tag VI). The derivatization followed the general reductive amination procedure (Scheme I) and the derivatives were detected in the UV at 200 nm. The enantiomeric separation was based on the formation of diastereoisomers by the derivatization. Optimum enantiomeric separation of 16 aldohexoses was achieved in 50 mM borate, pH 10.3, 23% acetonitrile and 30 kV separation voltage (see Figure 14).[69]

Other avenues have been explored in the HPCE separation of derivatized monosaccharides in order to provide an alternative to borate complexation. For instance, five PMP-monosaccharide derivatives, namely arabinose, ribose, galactose, glucose, and mannose were separated by CZE as complexes with divalent metal ions.[5] The five PMP-monosaccharides, initially coeluting using 100 mM sodium acetate as the background electrolyte, were fully resolved in an electrolyte system containing 20 mM calcium acetate. The separation was presumably due to the relative ease of complexation of these derivatives with the metal ion. In fact, the PMP-pentoses eluted in the order of increasing complexing ability with the metal ion which depends on the orientation of their hydroxyl groups. Ribose-PMP with *erythro-erythro*-oriented hydroxyl groups eluted first, followed by lyxose-PMP (*erythro-threo*) and arabinose-PMP (*threo-erythro*) and then xylose-PMP (*threo-threo*). This interaction gives rise to a positive charge around the metal nucleus of the sugar-metal complexes and, consequently, causes a relative reduction in the total negativity of the sugar derivatives, a condition that favors their migration toward the anode at a much slower rate than the unreacted reagent PMP. However, divalent metal ions have the tendency to adsorb electrostatically on the negatively charged fused-silica wall, leading to a gradual inversion in the direction of the electroosmotic flow, from cathodal to anodal passing by zero EOF, as the capillary surface charge changes from negative to positive passing by neutral with time.[5] With anodal flow (cathode to anode), the electrophoretic migration of the derivatives is in the same direction as the EOF, a condition that leads to rapid separation. The inversion of EOF is rather slow and can take up to six hours to occur. To speed up the inversion and to ensure reproducible separations, the capillary column should be preconditioned before use with a more concentrated metal ion solution than the one used as the separation electrolyte.[5] Other alkaline earth metal salts including barium, strontium and magnesium acetate were also investigated.[5] While the elution order of selected PMP-aldopentoses stayed the same upon varying the nature of the metal ion in the electrolyte, as expected, the electrophoretic mobility of the sugar-metal complexes was slightly higher with Ba^{2+} than with Sr^{2+} and Ca^{2+}, which is consistent with the fact that Ba^{2+} has a slightly larger ionic radius.

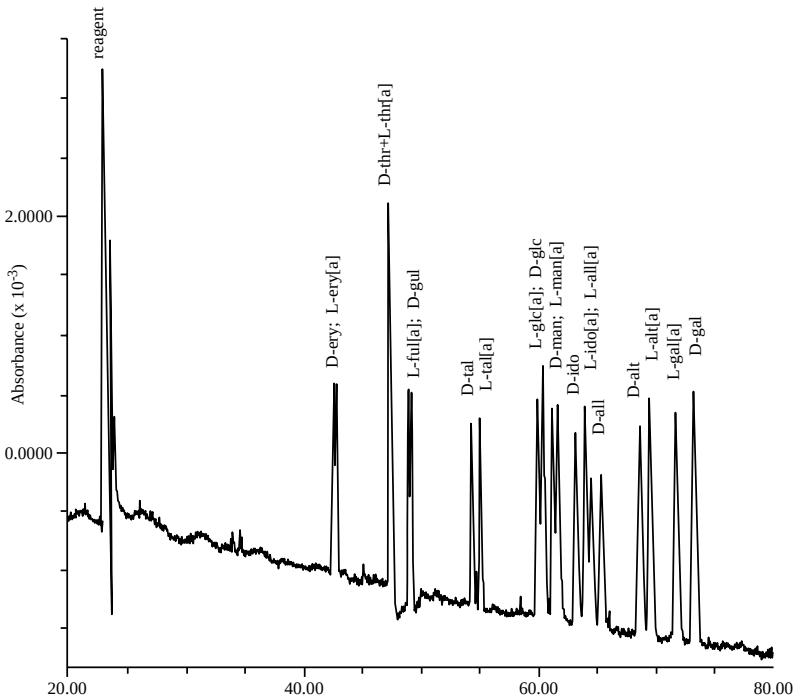


Figure 14. *Separation of a mixture of eight* D*-aldohexoses and two aldotetroses with* rac*-1-phenylethylamine. Capillary: fused-silica, 107 cm (total length), 100 cm (to detector) × 50-μm i.d.; electrolyte, 50 mM borate, pH 10.3, and 23% acetonitrile; voltage 30 kV; temperature, 25°C; UV detection, 200 nm; injection, 3.0 s by pressure (3.45 kPa). In the electropherogram the signals of derivatives of* D*-sugars with* R*-(+)-1-phenylethylamine are assigned as* L*-sugars marked with [a]. They are enantiomers of the derivatives of* L*-sugars and* S*-(-)-1-phenylethylamine and have the same electrophoretic mobility in the separation system. Reprinted with permission.*[69]

The separation efficiencies decreased in the following order: $Ba^{+2} > Ca^{+2} > Sr^{+2} >> Mg^{+2}$.[5] Also, it seems that the binding of Ba^{2+} ions to the capillary surface is slightly stronger than that of Ca^{2+}, while the binding of Sr^{2+} is the weakest. This was manifested by a higher anodal flow in the presence of barium acetate electrolyte and, consequently, the separation was faster.

A given precolumn derivatization of saccharides is usually performed to allow the high-sensitivity detection of the analytes. Often, precolumn labeling may also impart the saccharide with a charge and, in addition, improve its

compatibility with certain electrolyte systems. For instance, monosaccharides are generally too hydrophilic to be solubilized in ionic surfactant-based micellar systems; however, their labeling with hydrophobic tags allows their separation by MECC. In fact, eight different 2-aminoacridone- (2-AA, tag II) derivatized sugars (Scheme I), namely *N*-acetylglucosamine, galactose, mannose, fucose, glucose, *N*-acetylgalactosamine, ribose, and lyxose were separated by MECC in the presence of an electrolyte consisting of sodium taurodeoxycholate and sodium borate.[66] The mechanism of migration of the 2-AA labeled monosaccharides in the MECC system was described as complex and may involve partition equilibria of the neutral species into the micellar surface and electrophoretic movement of these molecules complexed with borate. This same system was also employed in the enantiomeric separations of 2-AA-galactose, -fucose and -ribose enantiomers by the addition of β-CD to the sodium taurodeoxycholate and sodium borate buffer system. However, baseline resolution was only achieved for fucose enantiomers and the analysis time was long (ca. 43 min). The replacement of taurodeoxycholate by a nonchiral surfactant such as sodium dodecyl sulfate (SDS) did not cause the enantiomeric separation.[66]

Another precolumn derivatization, which seems to impart hydrophilic saccharides with sufficient hydrophobicity to allow their separation by MECC, is PMP labeling (Scheme III, tag I).[87] This precolumn labeling, which was first introduced by Honda *et al.*,[9] yields bis-PMP adducts that absorb strongly in the UV. Various PMP derivatives of monosaccharides were shown to separate quite nicely in MECC in the presence of SDS at pH 7.5 using Tris-phosphate buffer. The applicability of the SDS micellar system was extended to the identification and quantitation of monosaccharides obtained from carbohydrate hydrolyzates from glycoproteins.[87]

Fourteen different saccharides including monosaccharides were derivatized by reductive amination (Scheme I) with 4-aminobenzonitrile (a hydrophobic derivatizing agent, tag III) and subsequently separated in about 5 min by MECC using an SDS micellar phase[68] (see Figure 15). The separation is based on the differential distribution of the neutral derivatives between the aqueous mobile phase and the micellar phase. While hydrophilic carbohydrate derivatives partitioned slightly inside the micelle and migrated first, hydrophobic derivatives such as deoxyaldohexoses partitioned strongly in the micelle and migrated very slowly toward the detector. Derivatives with intermediate hydrophobicity migrated within this migration time window. The final migration order exhibited the strength of partition in the micellar phase and it increased in the order ketohexoses < aldohexoses < aldopentoses < deoxyaldohexoses (see Figure 15).[68]

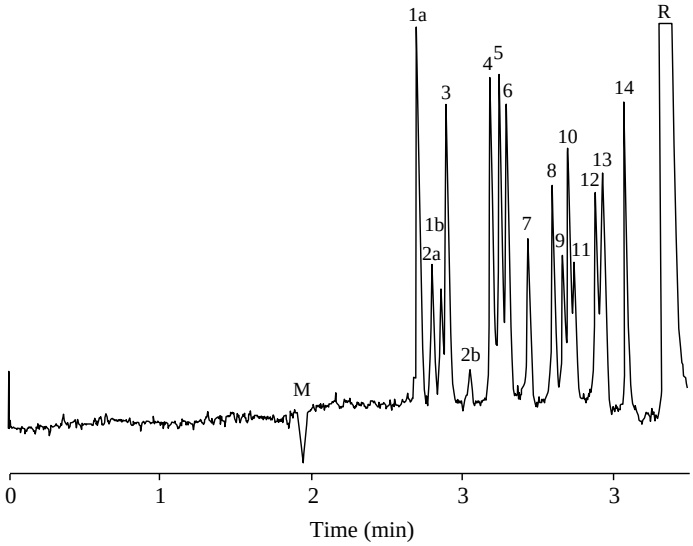


Figure 15. *High-speed analysis of 4-aminobenzonitrile derivatives by means of MECC. Capillary, fused silica, 35 cm to detection, 55 cm total length × 50-μm i.d.; electrolyte, 215 mM Tris-phosphate, pH 7.5, 100 mM SDS; voltage, 30 kV; current, 55 μA; detection. UV at 285 nm; temperature, 30°C; injection, vacuum, 1 s. Peaks: M = methanol; 1a/b = fructose, 2 a/b = sorbose; 3 = lactose; 4 = melibiose; 5 = cellobiose; 6 = maltotriose; 7 = maltose; 8 = mannose; 9 = glucose; 10 = galactose; 11 = ribose; 12 = lyxose; 13 = arabinose; 14 = xylose; R = reagent. Reprinted with permission.*[68]

B. Oligosaccharides

B.1. Underivatized Oligosaccharides

B.1.1. Simple Oligosaccharides

As their underivatized monosaccharide counterparts, simple and short underivatized oligosaccharides have been analyzed in CE with highly alkaline pH electrolytes.[11,38,42] Typical examples include the analysis of simple disaccharides (*e.g.*, trehalose, sucrose, lactose, lactulose, cellobiose), trisaccharides (*e.g.*, raffinose) and tetrasaccharides (*e.g.*, stachyose) by HPCE-ED using high pH electrolyte systems[11,42] (see Figure 7). In another approach, the disaccharides sucrose and maltose were analyzed at high pH by HPCE-indirect UV detection.[38]

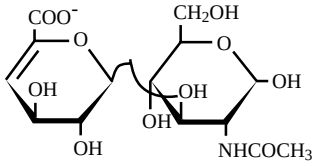
Arentoft *et al.*[88] demonstrated the separation of underivatized oligosaccharides of the raffinose family using borate complexes. These oligosaccharides are α-(1→6)-galactosides linked to C-6 of the glucose moiety of sucrose. Raffinose is the template of this homologous series with only one galactoside unit attached. By successive adding of one, two, and three additional α-galactoside units to C-6 of the terminating galactose of raffinose, the compounds stachyose, verbascose, and ajugose are formed. These sugars were separated and detected as borate complexes at 195 nm. The influence of various separation conditions including voltage, pH, temperature, and buffer composition on resolution, separation efficiency, migration time, and quantitative aspects were examined. These oligosaccharides are synthesized in various plants and accumulate in appreciable amounts in legume seeds.

B.1.2. Glycosaminoglycan-Derived Oligosaccharides

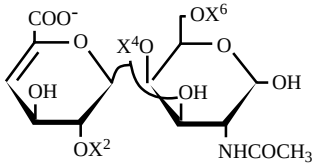
Glycosaminoglycans (GAGs), also called mucopolysaccharides, are unbranched polysaccharides of alternating uronic acid and hexosamine residues. They occur naturally in cartilage and other connective tissues, which are collectively called the ground substance. GAGs exhibit a variety of biological functions and can be altered in disease states. In GAG, while the amino group of the hexosamine residue is either *N*-acetylated or *N*-sulfated, the uronic acid may be either D-glucuronic acid or L-iduronic acid. Moreover, the repeating disaccharide units (*i.e.*, uronic acid-glucosamine disaccharide) are *O*-sulfated to varying degrees at C6 and/or C4 of the various glucosamine residues and at C2 of the uronic acid residues. Hyaluronic acid, chondroitin sulfates (chondroitin-4-sulfate and chondroitin-6-sulfate), dermatan sulfate, keratan sulfate, heparin, and heparan sulfate are the most common GAGs.

One approach for determining structural differences among various glycosaminoglycans (GAGs) is to analyze their disaccharide constituents. This involves first the enzymatic degradation of GAGs with polysaccharide lyases, *e.g.*, heparinases, chondroitinases, *etc.*, thus yielding disaccharides bearing unsaturated uronic acids at C4-C5 which allow their direct UV detection at 232 nm. Due to their ionic nature, the GAG-derived disaccharides are then analyzed readily by HPCE.

HPCE proved useful in the separation and quantitative determination of the disaccharides derived from chondroitin sulfate, dermatan sulfate, and hyaluronic acid.[89] Exhaustive treatment of these GAGs with polysaccharide lyases released nine different disaccharides bearing unsaturated uronic acids (Figure 16) that can be detected by UV absorbance at 232 nm without prior derivatization (molar absorptivity, $\varepsilon = 5000\text{-}6000\ M^{-1}\ cm^{-1}$).



1 [Δdi-HA]



2 [Δdi-0S] where X^2, X^4, X^6 = H
3 [Δdi-6S] where X^2, X^4 = H, X^6 = SO_3^-
4 [Δdi-4S] where X^2, X^6 = H, X^4 = SO_3^-
5 [Δdi-UA2S] where X^4, X^6 = H, X^2 = SO_3^-
6 [Δdi-S_E] where X^4, X^6 = SO_3^-, X^2 = H
7 [Δdi-S_D] where X^2, X^6 = SO_3^-, X^4 = H
8 [Δdi-S_B] where X^2, X^4 = SO_3^-, X^6 = H
9 [Δdi-triS] where X^2, X^4, X^6 = SO_3^-

Figure 16. *Disaccharides derived from chondroitin sulfates, dermatan sulfate, and hyaluronic acid by enzymatic depolymerization.*

These disaccharides, having a net charge from -1 to -4, were well resolved by CZE using a borate buffer, pH 8.8, primarily on the basis of their net charge and to a lesser extent on the basis of charge distribution. Despite the fact that the two nonsulfated disaccharides, structures 1 and 2, differed only by the chirality at the C4 in the hexosamine residue, these disaccharides were well resolved by CZE. In another study,[90] the electrophoretic behavior of chondroitin disaccharides (for structures, see Figure 16) was examined under various conditions including different pH, borate concentration, buffer ionic strength, and the inclusion of SDS micelles in the running electrolytes. Although the disaccharides are highly charged and too polar to partition in the SDS micelles, the presence of SDS in the electrolyte improved the resolution of the electrophoretic system. Also, the SDS-based electrolyte proved useful in the rapid separation (ca. 15 min) of six oligosaccharides derived from hyaluronan by digestion with testicular hyaluronidase (see Figure 17).

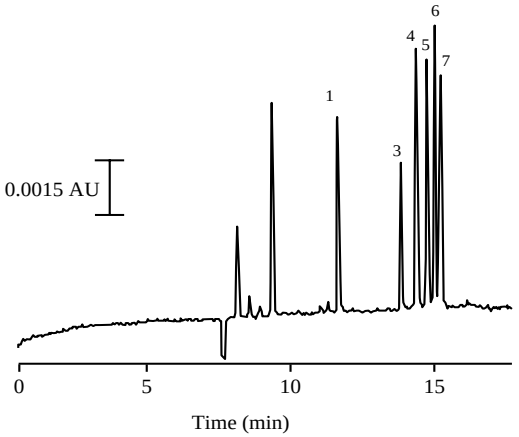
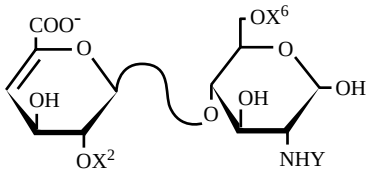


Figure 17. *Electropherogram of oligosaccharides derived from hyaluronan by digestion with testicular hyaluronidase. Applied voltage, 15 kV; temp., 40°C; electrolyte, 40 mM phosphate containing 40 mM SDS and 10 mM borate, pH 9.0; capillary, 72 cm × 50-μm i.d.; the column was monitored at 200 nm. Peak 1 is the unsaturated disaccharide of hyaluronan (Δdi-HA), peaks 3, 4, 5, and 6 are the saturated hexa-, octa-, deca-, dodeca- and tetradecasaccharide of hyaluronan, respectively. Reprinted with permission.*[90]

Also, the electrophoretic behavior of eight commercial disaccharide standards derived from heparin, heparan sulfate, and derivatized heparins of the structure ΔUA2X(1→4)-D-GlcNY6X (where ΔUA is 4-deoxy-α-L-threo-hex-4-enopyransyluronic acid, GlcN is 2-deoxy-2-aminoglucopyranose, S is sulfate, Ac is acetate, X may be S, and Y is S or Ac) were investigated in HPCE under various operating conditions.[91] Heparin and heparan sulfate are structurally similar GAGs differing primarily in their relative content of *N*-acetylglucosamine, *O*-sulfation, and glucuronic acid. Using heparinases I, II, and III as the degrading enzymes, heparin and heparan sulfate can be depolymerized through an eliminative mechanism to yield 8 different disaccharides shown in Figure 18.[91] Using a borate buffer pH 8.8, two of the standard heparin/heparan sulfate disaccharides, having an identical charge of -2, ΔUA2S(1→4)-D-GlcNAc (structure 3) and ΔUA(1→4)-D-GlcNS (structure 4), were not fully resolved. The resolution of these two saccharides could be improved by preparing borate buffer in deuterated water or eliminating boric acid. Surprisingly, baseline resolution was achieved in a micellar solution of sodium dodecylsulfate (SDS) in the absence of buffer. Since the two saccharides (structures 3 and 4, see Figure 18) are charged and polar, it is unlikely that the separation of these solutes was caused by differential partitioning in the SDS micelles. These electrophoretic systems were then applied to the determination



1. $X^2 = X^6 = H$, Y = Ac
2. $X^2 = H$, $X^6 = SO_3^-$, Y = Ac
3. $X^2 = SO_3^-$, $X^6 = H$, Y = Ac
4. $X^2 = X^6 = H$, $Y = SO_3^-$
5. $X^2 = X^6 = SO_3^-$, Y = Ac
6. $X^2 = H$, $X^6 = Y = SO_3^-$
7. $X^4 = H$, $X^6 = Y = SO_3^-$
8. $X^2 = SO_3^-$, $X^6 = H$, $Y = SO_3^-$
9. $X^2 = X^6 = Y = SO_3^-$

Figure 18. *Disaccharide fragments obtained by enzymatic depolymerization of heparin and heparan sulfate using heparinases I, II, and III.*

of disaccharide composition of porcine mucosal heparin and that of bovine kidney heparan sulfate. Both GAGs were found to have an equimolar content of disaccharide ΔUA2S(1→4)-D-GlcNAc (structure 3) and ΔUA(1→4)-D-GlcNS (structure 4).

An important application of HPCE in the area of GAGs has been in the quality control of natural and synthetic heparin fragments.[92] Because of the anti-blood-clotting activity of heparin, the production of natural and synthetic heparin fragments for pharmaceutical use relies on the availability of analytical procedures for the efficient characterization of intermediates and final products. Using a low-pH electrolyte system, namely 0.2 M phosphate, pH 4.0, and controlling the capillary column temperature at 40°C allowed the separation of the nine most common heparin disaccharides (for structures, see Figure 18), the mapping of the oligosaccharides derived from heparin after heparinase treatment, and the assessment of the quality of synthetic heparin pentasaccharide preparations.[92]

Recently, HPCE has been shown useful in determining structural differences between various GAGs. For instance, heparin and heparan sulfate are two GAGs which are structurally related species, yet heparan sulfate has a far more variable composition with a fewer *N*- and *O*-sulfate groups and more *N*-acetyl groups. The structural differences between heparin and heparan sul-

fate have been determined by HPCE analysis of the disaccharides resulting from the action of heparinases I, II, and III on the polysaccharides.[22] Using sodium phosphate buffer, pH 2.5, as the running electrolyte and a negative-polarity applied electric field, the resulting electropherograms of the digested samples were interpreted with the aid of a reference mixture of disaccharides standards derived from heparin.[22]

Another recent application of HPCE in GAG analysis has been the elucidation of the structural differences between different low-molecular-weight (LMW) heparins (*e.g.*, Fraxiparine, Fluxum, Fragmin, Sandoparin, and Enoxaparin) using oligosaccharide compositional analysis.[23] This was accomplished after complete depolymerization of heparin and LMW heparins with a mixture of heparin lyase I, II, and III followed by CE analysis using 10 mM sodium borate buffer, pH 8.81, containing 50 mM SDS. According to the authors,[23] the major mode of separation for such a system is zone electrophoresis, while MECC mode, resulting from the presence of SDS, is a minor contributor. As determined by CE, the oligosaccharide composition for the different LMW heparins varied, suggesting that LMW heparins have a significantly different proportion of antithrombin III binding sequence, which may explain their different biological activity.

As discussed above, the addition of SDS, an anionic surfactant, to the running electrolyte at a concentration above the CMC produced little change in the migration time of disaccharides from GAG and slightly improved the overall resolution. When SDS is replaced by cetyltrimethylammonium bromide, MECC seems to be a good choice for the separation of anionic GAG disaccharides [93]. Resolution improved with increasing CTAB concentration. The GAG disaccharides eluted in the order of increasing number of charged groups of the disaccharides. The method proved useful in the determination of samples of chondroitin sulfates and mink skin.

Very recently, a comparative study on compositional analysis of two sets of eight unsaturated disaccharide standards derived from heparin/heparan sulfate (see Figure 18) and chondroitin/dermatan sulfate (see Figure 16) was carried out[24] using both normal- and reverse-polarity capillary electrophoresis. Reverse-polarity CE completely resolved disaccharide mixtures into all components using a single buffer system composed of sodium phosphate, pH 3.48. At this pH, the EOF is negligible and the solutes migrate by their own electrophoretic mobilities toward the grounded anode. In the same report, the separation of 13 heparin-derived oligosaccharides of sizes ranging from di- to tetrasaccharides using both normal and reverse polarities was reported. Mixtures containing oligosaccharides primarily differing in size (*i.e.*, number of saccharide units) were better resolved by normal polarity[24] using 10 mM sodium borate buffer, pH 8.8, containing 50 mM SDS. This may be due to

some partitioning into the SDS micelles and also to the fact that the EOF is in the opposite direction to the electrophoretic mobility of the analytes, thus slowing their apparent migration and, in turn, allowing a better resolution.

More recently, variously sulfated disaccharides derived from hyaluronan and chondroitin/dermatan sulfates were analyzed by CE using phosphate buffer at low pH and negative polarity applied electric field.[27] Under these conditions, baseline separation of the nine different sulfated disaccharides was obtained while the two nonsulfated disaccharides exhibited peak splitting due to the anomeric forms of the hexosamines present in the reducing terminal of the nonsulfated disaccharides.

Other applications of HPCE in the area of GAGs include:

(i) the assay of sulfoesterase activity on sulfated disaccharides derived from chondroitin sulfate, dermatan sulfate, and heparin;[94] the high resolution of capillary electrophoresis allowed the use of the assay on impure enzyme preparations containing high protein concentrations;
(ii) The use of CE as an analytical tool for monitoring chemical reactions of trisulfated disaccharides;[26] the reactions monitored were the acylation, pivaloylation, and benzylation of hydroxyl groups on heparin-derived trisulfated disaccharides; the progress of these reactions was monitored using the borate/SDS buffer system, pH 8.8,[23,24] and UV detection at 232 nm.

While the above oligosaccharides could be readily detected at 232 nm via the unsaturated bond in the uronic acid residues, the HPCE analysis of sulfated synthetic low-molecular-weight heparin fragments necessitated the use of indirect UV.[25] This is due to the fact that the sulfated synthetic oligosaccharides exhibit low molar absorptivities as a result of the absence of the double bond in their structures. The indirect UV detection involved the use of 5-sulfosalisylic acid or 1,2,4-tricarboxybenzoic acid as the running electrolyte and background chromophore. Sulfated disaccharides with unsaturated uronic acid residues derived form heparin were also analyzed by CE at low pH with the indirect-UV mode of detection. The inherent charge possessed by most GAG disaccharides allowed CE-indirect UV detection to be conducted using buffers at low pH, thus eliminating the negative effect of the hydroxide ions.[25] The sensitivity of indirect UV detection was reported to be at least one order of magnitude higher than that of direct UV detection.[25] Due to the fact that the buffer systems used for CE-direct detection (phosphate buffer, pH 2.5) and CE-indirect UV detection (*i.e.*, 5 mM 1,2,4-tricarboxybenzoic acid, pH 3.4) were different, the resolving power of the two systems was not the same.[22] Three of the disulfated disaccharides were not totally resolved in the indirect UV system, whereas they were resolved in the direct UV system.

However, two monosulfated disaccharides coeluted in the direct UV system, while they were baseline separated in the indirect UV system (see Figure 19).

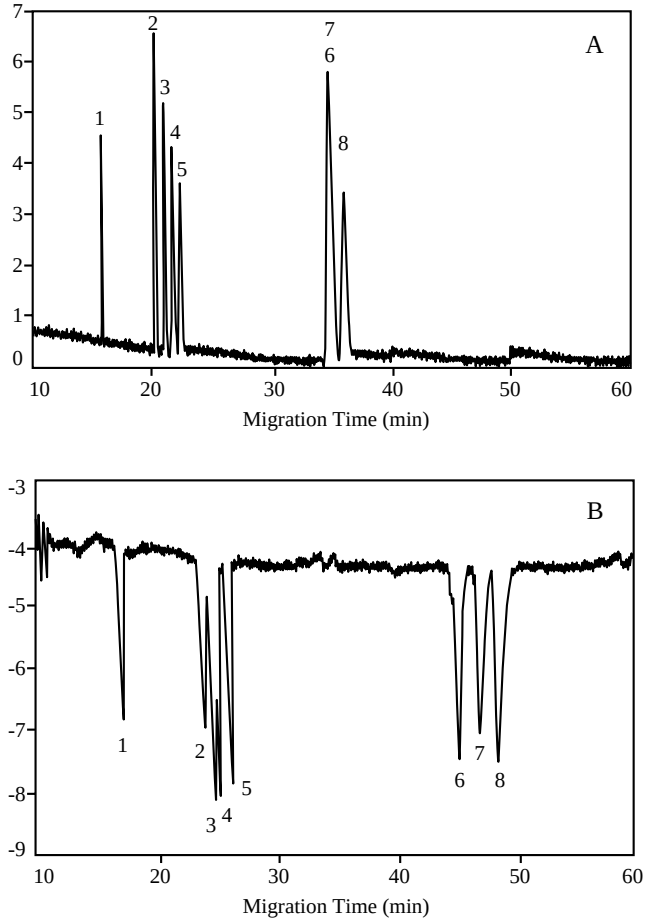


Figure 19. *Capillary electrophoresis of eight heparin disaccharides using direct (A) or indirect (B) UV detection. Electrolytes: in (A), 200 mM sodium phosphate, pH 2.5; in (B), 5 mM 1,2,4-tricarboxybenzoic acid, pH 3.5. Injections: in (A) 9 nL from a solution containing 0.16 mg/mL of each saccharide; in (B) 1.8 nL from the same solution as in (A), except that the concentration was 0.1 mg/mL for each saccharide. Applied voltages: 131.5 Vcm^{-1} in (A) and 87.7 Vcm^{-1} in (B). Temperature, 25°C. Solutes: 1 = δUA2S→GlcNS6S, 2 = δUA2S→GlcNS, 3 = δUA1→GlcNS6S, 4 = δUA2S→GlcNAc6S, 5 = δUA2S→GlcNCOEt6S, 6 = δUA2S→GlcNAc, 7 = δUA→GlcNS, 8 = δUA→GlcNAc6S. Reprinted with permission.*[25]

B.1.3. Glycoprotein-Derived Oligosaccharides

Hermentin *et al*.[95] analyzed the reducing oligosaccharides released from α_1-acid glycoprotein (AGP) using both high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and CZE with UV detection at 190 nm. According to the authors, the CZE analysis proved to be 4000 times more sensitive than HPAEC-PAD. In fact, the carbonyl function of the *N*-acetyl and carboxyl groups present in the molecules enabled their direct UV detection at concentrations in the femtomole region. This approach has the advantage of avoiding derivatization and sample clean up processes. In that study, the authors also compared the mapping profiles of AGP glycans released by conventional hydrazinolysis or by digestion with peptide-*N*-glycosidase F (PNGase F). Hydrozinolysis proved best with practically no loss of *N*-acetylneuraminic acid while the PNGase F digestion resulted in partial desialylation of the liberated *N*-glycans in the presence of SDS.[95]

N-linked oligosaccharides released from recombinant tissue plasminogen activator (rt-PA) after *N*-glycanase digestion were separated by MECC using SDS surfactant and direct UV detection at 200 nm.[15] The oligosaccharides consisted of neutral (high mannose) and mono- to tetraantennary negatively charged oligosaccharides. As one can expect, the neutral oligosaccharides separated on the basis of differential partitioning into the micelles, whereas the separation of the sialylated oligosaccharides was due mainly to differences in electrophoretic mobilities among the negatively charged glycans. The addition of a divalent ion (Mg^{2+}) to the SDS electrolyte system provided an effective means of enhancing the selectivity of separation through both an increase of the migration time window of the micellar systems and the differential complexation of carbohydrates with the divalent metal ion.[15] This electrolyte system was further utilized in the *N*-glycosylation mapping of rt-PA[16] to determine the difference between the oligosaccharides distribution of the two rt-PA variants which differ by the presence (type I) or the absence (type II) of oligosaccharides at the Asn-184 site.

N-Oligosaccharides from fetuin, tissue plasminogen activator (t-PA) and α_1-acid glycoprotein (AGP) were separated by CZE on the basis of their sialic acid content and their structures.[96] The monosialylated fraction obtained form ion-exchange chromatography eluted first, followed by di-, tri- and tetrasialylated glycans. As the number of sialic acid residues increased in the oligosaccharides, the UV absorbance at 200 nm of the underivatized analytes was greatly enhanced. Within each group of sialylated *N*-glycans a significant separation was still attainable, indicating that the separation relies not only on a charge difference but also on a structural difference between sugar chains bearing the same number of sialic acid residues. Variations in the type of linkage (α-2,4 or α-2,6) between the sialic acids and the galactose, in the oligosac-

charide size, or in the peripheral fucose residue may have facilitated the separation within each class.

Approximately eighty underivatized sialooligosaccharides derived from glycoproteins were analyzed by HPCE at 194 nm,[17] and a carbohydrate-mapping database was established which would enable a carbohydrate structural analysis by simple comparison of migration times. Reproducible migration times could be achieved (RSD < 0.20%) when mesityl oxide and sialic acid were included as two internal standards for the correction of migration time using a triple-correction method. The suitability and reliability of the database for the structural determination of sialylated *N*-glycans by comparison of corrected migration time was established by analyzing *N*-glycan pools of various glycoproteins such as recombinant human urinary erythropoietin (baby hamster kidney), bovine serum fetuin, and α_1-acid glycoprotein.[17]

Since there is no enzymatic cleaving process available for the cleavage of *O*-linked oligosaccharides, the chemical process used for their cleavage (*i.e.*, treatment with alkali in the presence of borohydride) results in reducing them to alditols. Thus, the released *O*-linked oligosaccharides lack a site for fluorescent labeling by reductive amination and their detection is only possible by measuring UV absorbance at 185 nm.[19] Using this detection approach, several *O*-glycosidically linked monosialooligosaccharides were analyzed as their alditols by HPCE.[19] Alkaline borate buffer yielded a migration profile for the oligosaccharides that was basically similar to that obtained in alkaline phosphate buffer, indicating no significant contribution of borate complex formation. However, neither electrolyte provided enough resolving power to separate *N*-acetyl and *N*-glycolylneuraminic acid containing oligosaccharide pairs. They were only resolved after the addition of 100 mM SDS to the borate buffer (see Figure 20). The separation mechanism is based on changing the conformation of these oligosaccharides, thus resulting in variation of the molecular size.[19] This buffer system was utilized in a microscale analysis of sialooligosaccharides in bovine submaxillary mucin and swallow nest material.[19]

B.2. Derivatized Oligosaccharides

B.2.1. Simple Oligosaccharides

Several disaccharides including gentibiose, maltose, lactose, cellobiose and melibiose were labeled with APTS by reductive amination and subsequently separated by CE using Mops and borate buffers.[70] In Mops buffer, the gentibiose disaccharides migrated first followed by maltose, lactose, cellobiose, and melibiose. This migration order might be governed by the differences in the hydrodynamic volume arising from varying degrees of hydration due to varying positions of hydroxyl groups in the nonreducing end pyranose.[70] In borate buffer, the migration order was gentibiose, maltose,

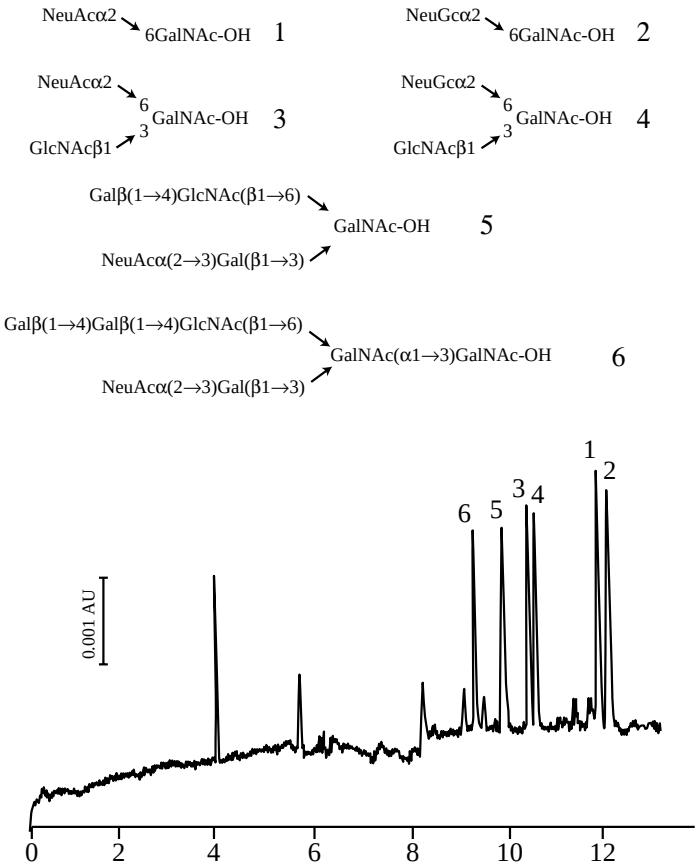


***Figure 20**. Separation of an equimolar mixture of sialooligosaccharide alditol standards. Peak numbers corresponds to the structures included with the figure. Analytical conditions: capillary, fused silica (50 cm × 50-μm i.d.); electrolyte, 200 mM borate buffer, pH 9.6, containing 0.1 M SDS; applied voltage, 17 kV; detection UV absorbance at 185 nm. Reprinted with permission.[19]*

melibiose, cellobiose, and lactose. This migration order is dictated by the magnitude of the stability constant of the disaccharide-borate complexation. In the same report, two APTS-derivatized glucose tetrasaccharide isomers differing only in one linkage at the nonreducing end were well resolved in Mops buffer but not in borate buffer. This means that borate forms a weak complex with both isomers and thus has no significant effect on the relative electrophoretic mobility of each species. These isomers were maltotetraose-APTS [glc-α-(1-4)glc-α-(1-4)glc-α-(1-4)glc-APTS] and glc-α-(1-6)glc-α-(1-4)glc-α-(1-4)glc-APTS.

Recently, HPCE-LIF was applied to monitor enzyme products formed during the incubation of yeast cells with the trisaccharide α-D-Glc(1→2)α-D-Glc(1→3)α-D-Glc-O$(CH_2)_8$CONHCH_2CH_2NHCO-tetramethylrhodamine (-TMR). TMR is the fluorescent arm attached to the trisaccharide through reductive amination of the analyte.[84] After 5 hr of incubating the yeast cells with the trisaccharide, the lysed yeast spheroplasts were injected and the components were separated and detected by HPCE-LIF. Most of this trisaccharide was converted to linker arm, intermediate disaccharide and monosaccharide (see Figure 21). This resulted from the sequential activity of α-glucosidase I and II inside the yeast cell which act specifically on α-D-Glc(1→2) and α-D-Glc(1→3) linkages, respectively.[84]

B.2.2. Homologous Oligosaccharides

The separation of derivatized homologous, ionic oligosaccharides, or homologous oligosaccharides labeled with an ionic tag by HPCE is most often accomplished in the presence of regular noncomplexing electrolytes, *e.g.*, phosphate, MES, Tris, etc. This is because the members of both types of derivatized homologous oligosaccharides will exhibit significant differences in the charge-to-mass ratios among each other, thus ensuring sufficient differential migration and, in turn, separation. This is only true up to a certain degree of polymerization.

The high resolving power of CZE in the separation of oligosaccharides was first demonstrated by Nashabeh and El Rassi[86] who reported the separation of the pyridylamino derivatives of maltooligosaccharides having a degree of polymerization (d.p.) from 4 to 7 using untreated fused-silica capillaries. The positively charged sugar derivatives migrated ahead of the EOF marker and were separated according to their size in the pH range 3.0-4.5 using 0.1 M phosphate solutions as the running electrolytes. The inclusion of 50 mM tetrabutylammonium bromide in the electrolyte solution decreased slightly the EOF, and consequently allowed the separation of the maltooligosaccharides at pH 5.0. However, as the pH approached the pK_a value of the derivatives (pK_a = 6.71), the homologues practically coeluted and moved virtually together with the EOF.

To examine the effect of the nature of the derivatizing agent on the spacing pattern between the migrating zones of homologues, a series of *N*-acetylchitooligosaccharides derivatized with either 2-AP or 6-AQ were separated by CZE[61] using the buffer system established for the maltooligosaccharides[86] as the running electrolyte, and a capillary having polyether interlocked coating (see Figure 22). As can be seen in Figure 22, since 2-Ap and 6-AQ have similar characteristic charges, the spacing between two neighboring homologues is virtually independent of the tagging agent.

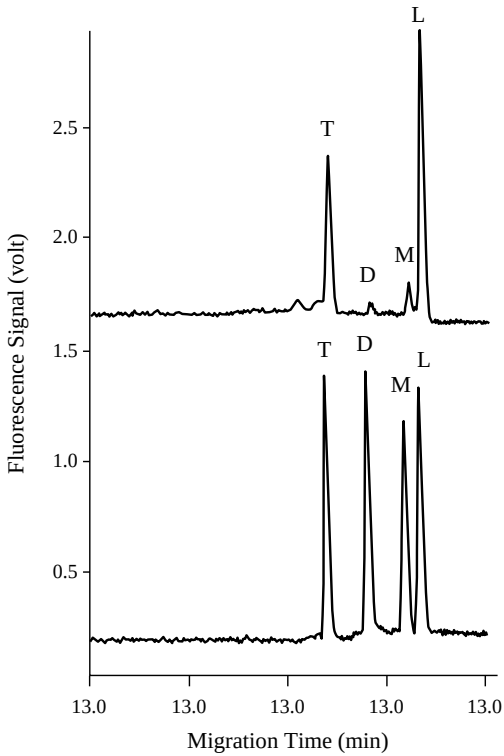


Figure 21. *Electropherograms obtained from the analysis of lysed yeast spheroplasts (top) and a standard solution containing 10^{-9} M of each component (bottom). Electrolyte, 10 mM each of phosphate, borate, phenylboronic acid and SDS, pH 9.3; capillary, 42 cm × 10-μm i.d.; voltage, 400 V/cm. Peaks:*
T = α-D-Glc(1→2)α-D-Glc(1→3)α-D-Glc-O$(CH_2)_8$CONHCH$_2$CH$_2$NHCO-TMR;
D= α-D-Glc(1→3)α-D-Glc-O$(CH_2)_8$CONHCH$_2$CH$_2$NHCO-TMR;
M = α-D-Glc-O$(CH_2)_8$CONHCH$_2$CH$_2$NHCO-TMR;
L = H-O$(CH_2)_8$CONHCH$_2$CH$_2$NHCO-TMR. Reprinted with permission.[84]

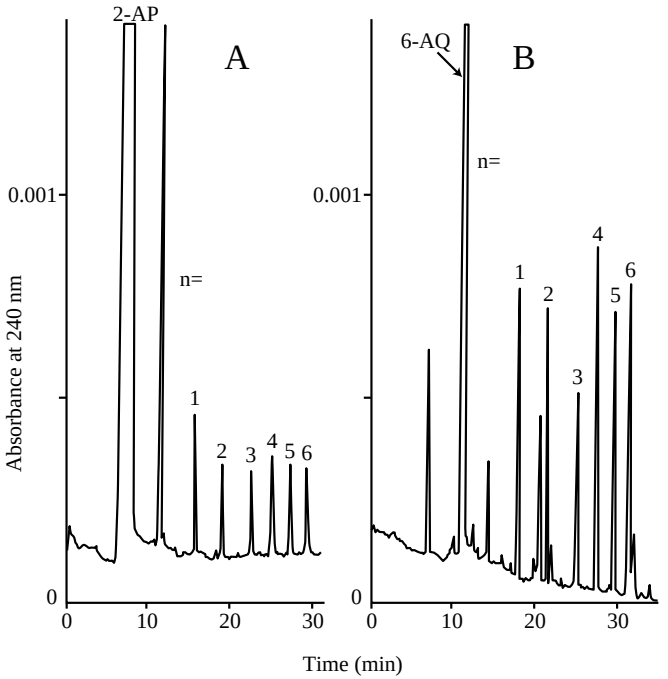


Figure 22. *Electropherograms of pyridylamino (A) and quinolylamino (B) derivatives of* N*-acetylchitooligosaccharides. Capillary, fused silica tube with polyether interlocked coating on the inner walls, 50 cm (to the detection point), 80 cm total length × 50 μm i.d.; electrolyte, 0.1 M phosphate solution containing 50 mM tetrabutylammonium bromide, pH 5.0; voltage, 18 kV. 2-Ap = 2-aminopyridine; 6-AQ = 6-aminoquinoline. Reprinted with permission.*[61]

On the contrary, when various tagging agents with different characteristic charges were used, the spacing between the oligosaccharides was largely influenced by the nature of the derivatizing agent.[97,98,99] In one study, three derivatizing agents were used in the tagging of a sample of dextran oligomers including 2-AP, 5-ANSA, and ANTS.[97] At the pH of the experiment, *i.e.*, pH 8.65, the 2-AP, 5-ANSA, and ANTS derivatives possess the negative charges of zero, one, and three sulfonic acid groups, respectively. The average migration times for the individual ANTS oligomers was roughly one-third of those observed with 2-AP derivatives, with 5-ANSA derivatives being intermediate. In addition, the ANTS derivatives exhibited narrower peaks, greater resolution, shorter analysis time, and higher peak detection.

Another study examined the effects of the structure and charge of naphthalene sulfonic acid-based derivatizing agents, such as ANTS, ANDSA, 3-aminonaphthalene-2,7-disulfonic acid (3-ANDA, tag XV), 2-aminonaphthalene-1-sulfonic acid (2-ANSA, tag XI), and 5-ANSA, on the CE analysis of their maltooligosaccharides derivatives using a running electrolyte consisting of sodium phosphate, pH 2.5, in the presence of TEA.[98] As can be seen in Figure 23a, ANTS-derivatized maltooligosaccharides were separated for up to a d.p. of more than 30 glucose units in less than 30 min. 3-ANDA-derivatized maltooligosaccharides showed the same resolution, yet the separation was only achievable up to d.p. 30 (Figure 23b). On the other hand, for 2-ANSA and 5-ANSA-derivatized maltooligosaccharides (Figure 23c and 23d, respectively), only 20 components were resolved and loss of efficiency was observed, which might be attributed to the longer analysis time (ca. 40 min). These findings show the importance of having permanent multiple charges on the tag such as ANTS and 3-ANDA. Although 2-ANSA and 5-ANSA tags are structural isomers, the migration time of 2-ANSA is almost four times lower than that of 5-ANSA (see Figures 23c and d). The pK_a value for the primary amino group of 5-ANSA is higher than that of 2-ANSA due to the fact that the sulfonic acid and amino groups are further apart. This high pK_a value decreases the net negative charge on the molecule and, in turn, its mobility. Despite its smaller size, 5-ANSA tagging agent migrated slower than the 5-ANSA oligosaccharide derivatives. This might be due to the presence of a less basic secondary amino group on the 5-ANSA oligosaccharide derivatives, thus possessing a higher net negative charge and mobility than the tagging agent.

Also, the effect of the electrical charge of the fluorescent tags on the separation of negatively charged oligosaccharides, derived from partially hydrolyzed *k*-carrageenan, was studied using capillaries coated with a layer of linear polyacrylamide.[99] This was accomplished by comparing the separation of ANTS- and 6-AQ-derivatized *k*-carrageenan oligosaccharides. When the charge-to-friction ratio of oligosaccharides is increased by the end-label (*i.e.*, ANTS), the migration order is from smaller to larger oligomers, and the separation of larger oligosaccharides is improved by using a sieving medium. The separation is based on the differences in the charge-to-mass ratio of the different oligomers. The derivatizing agent, ANTS, has three negative charges and, as a result, it will alter the charge-to-mass ratio of the analytes; however; this alteration is more significant in the case of small oligomers and diminishes as the degree of polymerization of the oligomers increases. The migration order can be entirely reversed when the charge-to-friction ratio is decreased by the end-label. This is the case of oligosaccharides tagged with 6-aminoquinoline, where the migration order starts with large oligomers and proceeds to smaller ones. This is due to the fact that the tag decreases the charge-to-mass ratio of the small oligomers more significantly than the larger ones.[99]

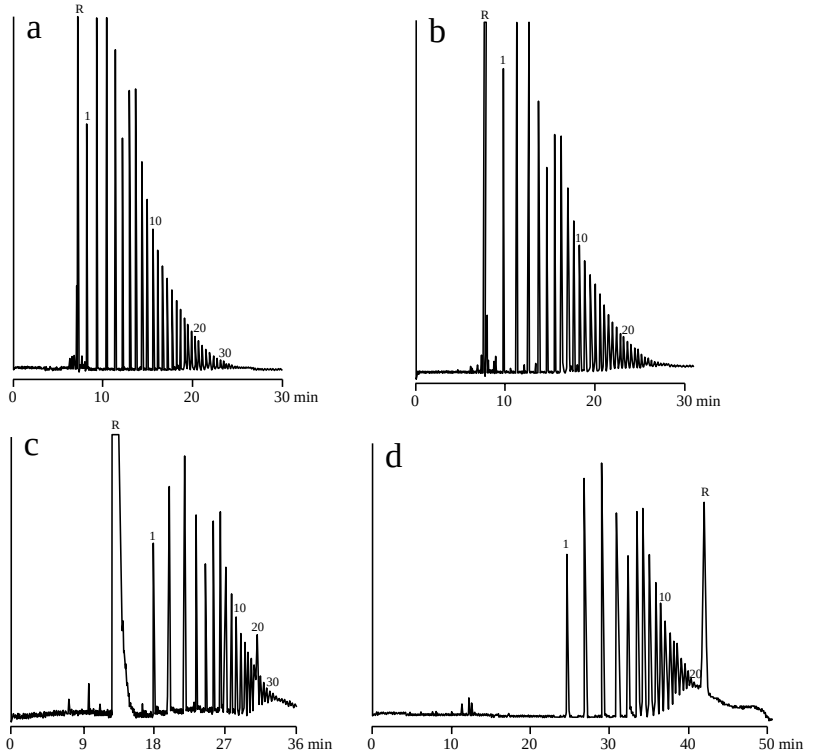


Figure 23. *Electropherograms of maltooligosaccharides labeled with (a) ANTS, (b) 3-ANDSA, (c) 2-ANSA, (d) 5-ANSA. Capillary, fused -silica, 50 cm (to detection point), 72 cm (total length) × 50-μm i.d.; running electrolyte, 50 mM phosphate, 36 mM TEA, pH 2.5; voltage, -20 kV; detection, UV, 235 nm; temperature, 25°C. Solutes, (R) reagent, in (c) the presence of a contaminant in the reagent generates a second peak, also designated as R. Numbers indicated reflect the number of glucose residues in the linear maltooligosaccharides. Reprinted with permission.*[98]

An important operating parameter that largely affects the resolution of homologous oligomers is the magnitude of the EOF. In fact, the use of coated capillaries having very low or virtually no electroosmotic flow improved the resolution of homologous oligosaccharides with a higher degree of polymerization. For instance, the separation of pyridylamino derivatives of oligogalacturonide homologous series with d.p. in the range 1 to 18 was best achieved on a coated capillary having a switchable (anodal/cathodal) EOF using 0.1 M phosphate solution, pH 6.5, as the running electrolyte since, at this pH, the

EOF is very low.[100] Similarly, pyridylamino derivatives of isomaltooligosaccharides were completely separated from each other, at least up to a d.p. of 20, using fused-silica capillaries in which the EOF was suppressed by chemically coating the capillary inner wall with linear polyacrylamide.[101]

Furthermore, in a recent study, Chiesa and Horváth[74] arrived at the conclusion that the use of polyacrylamide gel-filled capillaries does not contribute to enhancing the separation of derivatized homologous oligomers which contradicts what was first shown by Novotny and coworkers.[78,102] In their work,[74] ANTS-derivatized maltooligosaccharides were separated at pH 2.5 by open-tubular CZE and the results were compared to those reported in the literature involving ANTS-derivatized maltooligosaccharides separated by polyacrylamide slab gel[75] and CBQCA-derivatized maltooligosaccharides separated in capillaries filled with highly concentrated polyacrylamide gel according to the procedure described by Liu *et al.*[78,102] The authors concluded that the presence of the gel shows no enhancement in the resolution of oligosaccharide derivatives containing at least up to 20 glucose units.[74] On the contrary, the mobility of the ANTS derivatives appears to be lower by a factor of 22 in the cross-linked gel than in free solution, with no change in resolution. The separation of ANTS-maltooligosaccharides in open-tubular CZE was achieved with a background electrolyte of 50 mM sodium/triethylammonium phosphate buffer, pH 2.50, containing 10.8 mM triethylamine (TEA).[74] Under these conditions, nearly 30 homologues were well resolved in less than 5 min (see Figure 24). This separation electrolyte system was used elsewhere in the separation of ANTS-derivatized dextran and galacturonic acid ladders by CE.[103] The effect of the cationic additive (TEA) was mainly attributed to its interaction with the capillary wall (electrostatic binding). In fact, at sufficiently high concentrations of TEA (*e.g.*, 50 mM), an inversion in the direction of EOF from cathodal to anodal was observed.[74] Since the electrophoretic migration of the triply negatively charged ANTS-derivatives takes place in the same direction, the EOF has the beneficial effect of increasing the speed of separation. However, the resolution of the system decreased for d.p. greater than 12. This system allowed the ultrafast separation (in less than 10 s) of three short-chain maltooligosaccharides derivatized with ANTS.[74]

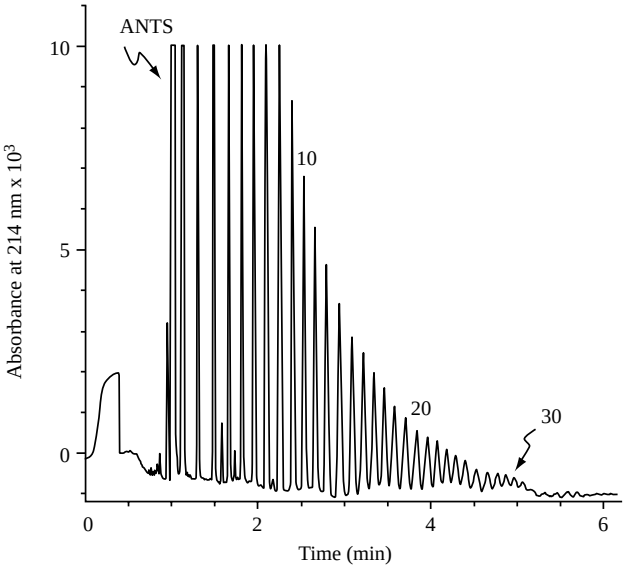


Figure 24. *Electropherogram of ANTS-derivatized maltooligosaccharides obtained with 50 mM sodium/triethylammonium phosphate buffer, pH 2.5, containing 10.8 mM TEA. Capillary, 270 mm × 50-μm i.d.; temperature, 25°C; voltage, 22 kV; 80 ng of sample. Reprinted with permission.*[74]

Very recently, Stefansson and Novotny[97] demonstrated the separation of large oligosaccharides of dextrans (although these oligosaccharides are branched, their behavior is first described here simply for the completeness of the discussion) in open-tubular capillaries coated with linear polyacrylamide (*i.e.*, zero-EOF capillaries) using running electrolytes based on Tris-borate, pH 8.65. This work has clearly demonstrated the importance of zero-flow capillaries in achieving high resolution and high separation efficiencies (excess of 1 million theoretical plates/m) for large oligosaccharides. Also in this work, open-tubular CZE with zero-flow capillaries was shown to be useful in separating the various oligomers of corn amylose as well as in preliminary examples of applications to monitoring the action of hydrolytic and synthesizing enzymes (for further discussion, see next section).

ANTS-derivatized dextran oligosaccharides were separated by CE using polymer networks under various operating conditions. As a model system, ANTS-labeled wheat starch digest was analyzed by CE using 25 mM sodium acetate buffer, pH 4.75, containing 0.5% polyethylene oxide.[104] Although the polymer concentration used was above the entanglement threshold value, separation was not based on a sieving mechanism. This was evident from Ferguson plots where the

logarithm of the electrophoretic mobility of the analytes shows no dependence on the polymer concentration in the range 0.2-1.0%. Therefore, the separation attained was caused by the charge-to-mass ratio differences of the oligosaccharides and, as was found by Chiesa and Horváth,[74] the effect of the polymer network was only to slow down the velocity of the derivatives.

As in the case of monosaccharides, oligosaccharides derivatized with neutral or weakly ionizable tags necessitate the use of high pH electrolyte solutions or borate buffers to be electrophoresed. This is the case of PMP-derivatives of homologous oligoglucans,[58] such as α-(1→3)-linked (laminara-) oligoglucans, α-(1→6)-(isomalto-) oligoglucans and β-(1→4)-(cello-) oligoglucans, which were separated as borate complexes. All the homooligoglucans eluted in the order of decreasing size and, because of the unfavorable mass-to-charge ratio at high degrees of polymerization, the resolution between the homologues decreased as the number of recurring units increased. As expected, the rate of migration varied among series since the extent of their complexation with borate is largely influenced by the orientation of hydroxyl groups, *i.e.*, by the type of interglycosidic linkage of the various oligosaccharides. In another report from the same laboratory,[5] the PMP derivatives of a series of isomaltooligosaccharides were separated by CZE using an aqueous barium salt solution as the running electrolyte. The PMP isomaltooligosaccharides were separated from each other up to d.p. of 9 as opposed to d.p. of 13 in the presence of borate.[58]

B.2.3. Branched Oligosaccharides Derived from Plants

The high resolution separation that HPCE provides for homologous linear oligosaccharides was also exploited in the separation of branched heterooligosaccharides derived from large xyloglucan polysaccharides (XGs) by enzymatic digestion.[61] Figure 25 illustrates the CZE separation of pyridylamino derivatives of xyloglucan oligosaccharides (2-AP-XG) obtained from cotton cell walls by cellulase digestion.[105] XGs possess a basic backbone identical to that of cellulose, a (1→4)-β linked D-glucan. Variations in XGs are caused by the differences in the nature and distribution of xylose, galactosyl-xylose, fucosyl-galactosyl-xylose and, in some cases, arabinosyl-xylose side chains on the glucan backbone. Cellulase, a complex of enzymes, is able to digest the backbone of XGs after any glucosyl residue which does not subtend a side chain, thus liberating fragments of the polymer that reflect its branching patterns. The peak numbering on the electropherogram (see Figure 25) reflects the elution order obtained in reversed-phase chromatography (RPC).[105] In CZE, the elution order was mainly governed by the number of sugar residues and the degree of branching, whereas in RPC the elution order was mainly influenced by the size of the oligosaccharide and the hydrophobic character of the sugar residues.

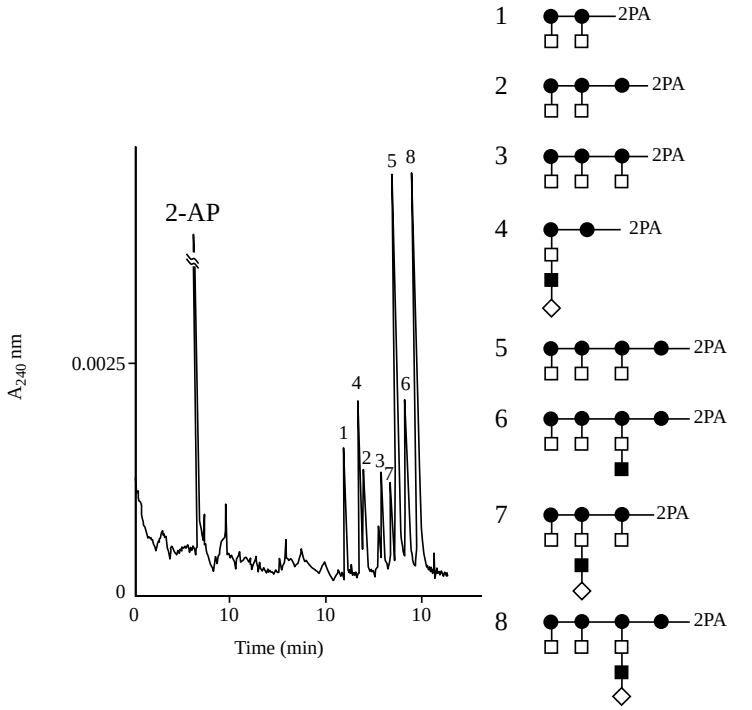


Figure 25. *Capillary zone electrophoresis mapping of pyridylamino derivatives of xyloglucan oligosaccharides from cotton cell walls. Capillary, fused-silica tube with polyether interlocked coating on the inner walls, 50 cm (to the detection point), 80 cm total length × 50-μm i.d.; electrolyte, 0.1 M sodium phosphate solution containing 50 mM tetrabutylammonium bromide, pH 4.75; running voltage, 20 kV. Symbols: 2-AP, 2-aminopyridine; ●, glucose; □, xylose; ■, galactose; ◊, fucose. Reprinted with permission.*[61]

In order to interpret the electrophoretic behavior of the various 2-AP-XG and to quantitatively describe the effects of the various sugar residues on their electrophoretic mobility, Nashabeh and El Rassi[61] have introduced a mobility indexing system for the branched xyloglucan oligosaccharides with respect to the linear pyridylamino derivatives of *N*-acetylchitooligosaccharides (2-PA-$GlcNAc_n$) homologous series, shown in Figure 22. The indexing system revealed that the addition of a glucosyl residue to the linear core chain of the oligosaccharide showed a similar change in the mobility index decrement as the addition of a xylosyl residue at the glucose loci and behaved as one half of a GlcNAc residue in terms of its contribution to the electrophoretic mobility of

the 2-AP-XG (see structures in Figure 25). However, the addition of a galactosyl residue to an already branched xylosyl residue exhibited less retardation than the addition of a glucosyl or xylosyl unit to the backbone of the xyloglucan oligosaccharide. The same observation was made about adding a fucosyl residue to a branched galactosyl residue. Thus, as the molecule becomes more branched, the addition of a sugar residue does impart a slightly smaller decrease in its mobility. This approach may prove valuable in correlating and predicting the effects of several parameters such as the nature, position, and number of sugar residues on the mobilities of complex carbohydrates.

Very recently, the ability of HPCE to distinguish complex oligosaccharides of very similar structures has been further exploited in the oligosaccharide mapping of laminarin[97] (a branched polysaccharide) after enzymatic cleavage with laminarinase, cellulase, and endoglucanases. The various ANTS-oligosaccharide maps obtained with the three different β-1,3-glucosehydrolases show that cellulase and laminarinase seem to cause more complete hydrolyses than the endoglucanases (EG I and EG II). Also, EG I and EG II appeared to differ somewhat in their structural preferences. This shows again the importance of the high sensitivity and high resolution of HPCE in the characterization of structural preferences for different enzymes in the hydrolysis of a given polysaccharide.

Finally, oligosaccharides of α-D-glucans (amylose, amylopectin and pullulan) and β-D-glucans (exemplified by lichenan) were also derivatized with ANTS and analyzed by CE to evaluate their complexity.[106] The separation capillary consisted of a fused-silica capillary coated with a linear layer of polyacrylamide. The oligosaccharide maps were obtained after selective debranching using isoamylase, laminarinase, and cellulase enzymes using various borate-based electrolyte systems. According to the authors, "complex glucan chains with numerous residual branches can potentially be assessed using this oligosaccharide mapping procedure." Since the capillary column was of the type with reduced EOF, a baseline separation of an intact amylose sample with d.p. close to 70 could be achieved.

B.2.4. Glycosaminoglycan-Derived Oligosaccharides

Although underivatized GAG-derived di- and oligosaccharides can be readily electrophoresed by HPCE and detected in the UV at 232 nm, precolumn derivatization of these species with a suitable chromophore or fluorophore will certainly improve their detectability. In addition, the tagging may provide additional properties to the GAG sugars which may enhance the separation potential.

Thus far, only two research papers have appeared on the separation of derivatized GAG-derived oligosaccharides. Unsaturated disaccharides derived from GAGs by digestion with chondroitinase AC and ABC were labeled with PMP and subsequently separated by CZE.[57] The conversion of these saccharides to their PMP derivatives improved the system sensitivity (see Figure 26). The electrophoretic system also proved suitable for the quantitative estimation of human urinary chondroitin sulfates (see Figure 26b).

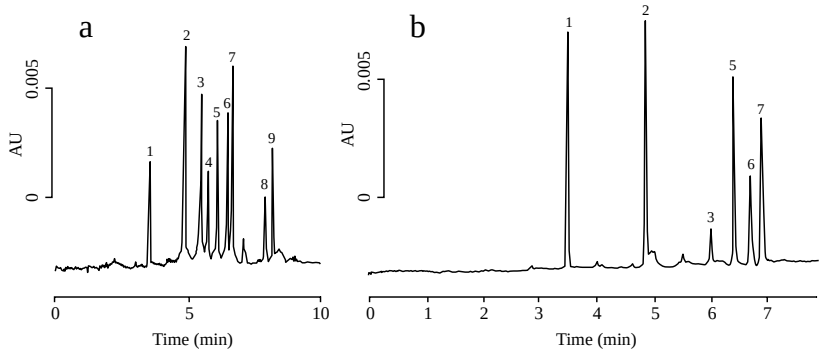


Figure 26. *(a) Electropherogram of chondroitin ABC-digested mixture of chondroitin sulfates A-E, chondroitin, and hyaluronic acid by CZE after derivatization with PMP. Capillary, fused silica (51 cm × 50-μm i.d.); electrolyte, 100 mM borate buffer, pH 9.0; applied voltage 25 kV. Peaks: 1 came from the buffer for enzymatic digestion; 2, PMP (excess reagent); 3, PMP derivative of Δdi-0S; 4, PMP derivative of Δdi-HA; 5, sodium benzoate (internal standard); 6, PMP derivative of Δdi-4S; 7, PMP derivative of Δdi-6S; 8, PMP derivative of Δdi-S_D; 9, PMP derivative of Δdi-S_E. (b) Analysis of the PMP derivatives of unsaturated disaccharides derived from the GAG fraction of a urine sample digestion with chondroitinase ABC by CZE. Conditions and peak assignment are as in (a). Reprinted with permission.[57]*

Liu *et al.*[102] demonstrated the advantages of using gel-filled capillaries with high gel concentration in the separation of enzymatically degraded hyaluronic acid from human umbilical cords. CZE with gel-filled capillaries afforded the high resolution separation of hyaluronic acid-derived oligosaccharides tagged with CBQCA.

B.2.5. Glycoprotein-Derived Oligosaccharides

HPCE is increasingly used in the separation and mapping of the oligosaccharide fragments of glycoproteins, *i.e.*, glycans. Using ovalbumin as a model protein, Honda *et al.* [101] demonstrated the separation of glycans by CZE with on-column fluorometric detection. The oligosaccharides of ovalbumin (hybrid and high mannose type) were released with anhydrous hydrazine and tagged with 2-AP after re-*N*-acetylation. As shown in Figure 27, the oligosaccharides were electrophoresed using two different electrolytes, an acidic phosphate buffer whereby the derivatized glycans are positively charged due to the protonation of the amino group of the tag (*i.e.*, direct CZE) and an alkaline borate buffer which allows the *in situ* conversion of the derivatives to anionic borate complexes (*i.e.*, indirect CZE). Because of differences in their separation mechanisms, direct and indirect CZE yielded different selectivities. As can be seen in Figure 27a, direct CZE gave good separation among the oligosaccharide derivatives that are different in their molecular size (*i.e.*, oligosaccharides having different number of monosaccharide units), but could not resolve solutes having the same degree of polymerization. On the other hand, CZE as borate complexes separated the oligosaccharide derivatives based on structural differences of the outer monosaccharide residues (Figure 27b). The greater the number of unsubstituted mannose units, the more retarded are the derivatives.[101] However, the borate system failed to resolve high mannose type oligosaccharides having the same number of outer mannose residues, such as structures h_1 and h_2 (see Figure 27). Similarly, hybrid-type oligosaccharides having the same number of peripheral mannose or galactose residues, but differing in the total number of monosaccharide units, were not resolved (see structures g_1 and g_2 in Figure 27). In both cases, however, the use of direct CZE with phosphate buffers gave satisfactory separations.

Also, capillary electrophoresis proved to be useful in elucidating the differences in glycan structures of the same glycoprotein but from different sources.[107] The CZE mapping of the pyridylamino derivatives of the oligosaccharides derived from human and bovine α_1-acidglycoprotein (AGP) yielded two different electropherograms each containing well-defined peaks and a few minor peaks, eluting after the excess 2-AP (see Figure 28). Both human and bovine AGP have been found to have the same sialic acid, galactose, and mannose content. The major differences are such that 50% of the sialic acid in bovine AGP are *N*-glycolylneuraminic acid and the fucose content is very low. These differences were unveiled by CZE mapping of both types of glycans. Based on these results, CZE will play an important role in the field of glycan separation and characterization.

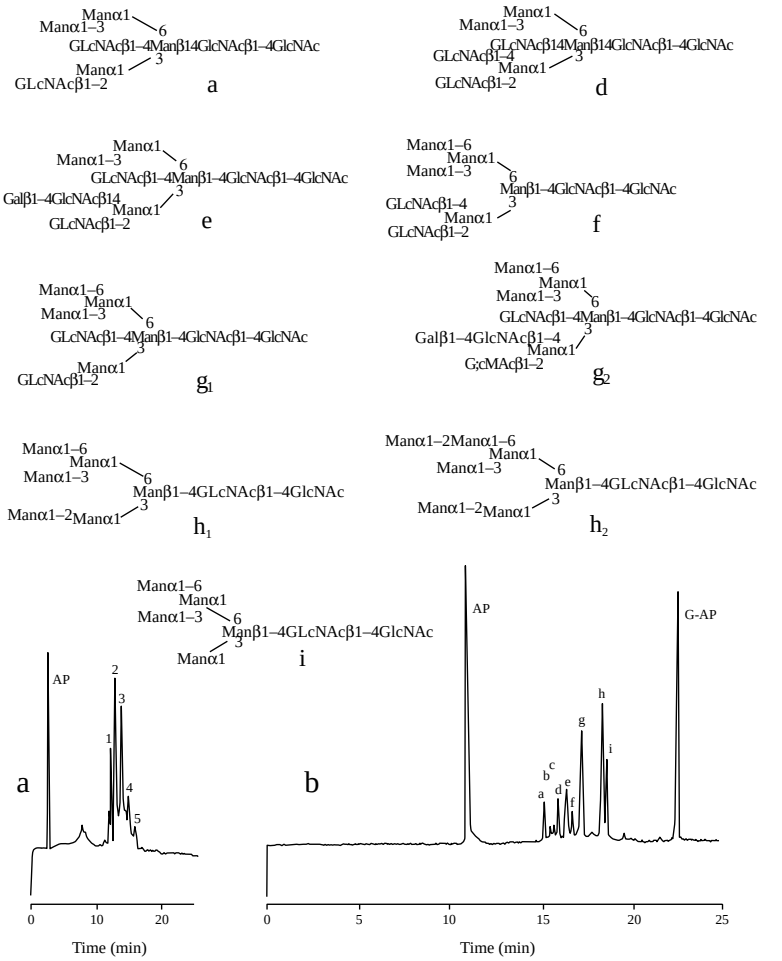


Figure 27. *Analysis of the reductively pyridylaminated oligosaccharides derived from ovalbumin (a) by direct CZE or (b) CZE as borate complexes. (a) Capillary, 20 cm × 25 μm i.d.; electrolyte, 0.1 M phosphate, pH 2.5; running voltage, 8 kV; detection, 240 nm. (b) Capillary, 95 cm × 50-μm i.d.; electrolyte, 200 mM borate buffer, pH 10.5; running voltage, 20 kV; fluorescence detection with excitation at 316 nm and emission at 395 nm. Peak assignments in (a): 1, heptasaccharide; 2, octasaccharides; 3, nonasaccharide; 4, decasaccharide; 5, undecasaccharide. Peak assignments in (b) refer to the illustration provided above the electropherograms; peaks b and c were not assigned. Reprinted with permission.*[101]

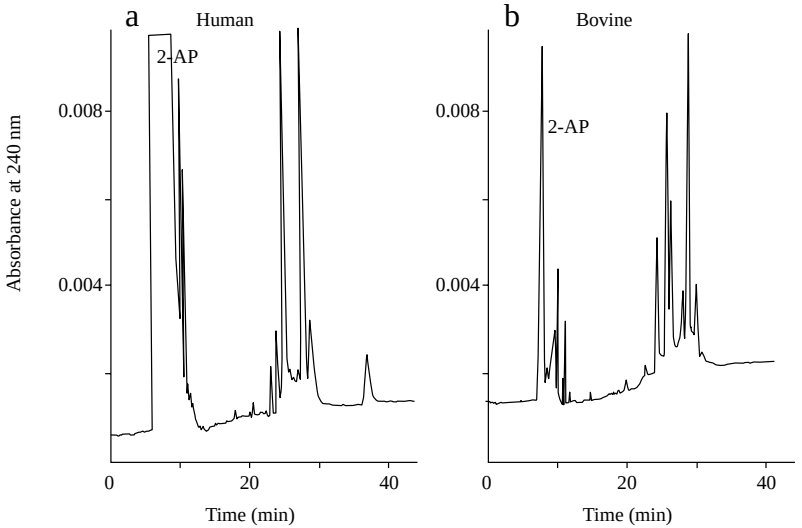


Figure 28. *Capillary zone electrophoresis mapping of pyridylamino derivatives of human (a) and bovine (b) AGP oligosaccharides. Capillary, fused-silica tube with hydrophilic coating on the inner walls, 45 cm (to the detection point), 80 cm total length × 50-μm i.d.; electrolyte, 0.1 M phosphate solution, pH 5.0, containing 50 mM tetrabutylammonium bromide; running voltage, 18 kV; current. 80 μA; injection by electromigration for 2 s at 18 kV. Reprinted with permission.*[107]

High mannose oligosaccharides released from RNase B by digesting the protein with PNGase F were also analyzed by CZE[61] as their 2-AP derivatives. One major peak on the CZE map was identified as $(GlcNAc)_2$-Man_5 using an oligosaccharide standard. The polypeptide chain of RNase B is known to have only one glycosylation site which can accommodate five different high mannose glycans. This may explain the presence of several peaks in the CZE map besides that of $(GlcNAc)_2$-Man_5 oligosaccharide which is the most predominant carbohydrate moiety of bovine RNase B.

Very recently, an electrophoretic system based on capillary gel electrophoresis was described for the profiling of oligosaccharides enzymatically derived from ribonuclease B and labeled with APTS. The capillary gel electrophoresis system involving the use of an entangled polymer network exhibited a higher resolving power than open-tubular CZE.[76] Optimum separation was attained using 25 mM acetate buffer, pH 4.75, containing 0.4% polyethylene oxide polymer and a neutrally coated capillary. This system yielded a high resolution of all the major components of the ribonuclease

N-glycan pool, and a baseline separation of the three positional isomers of mannose-7 and mannose-8 oligosaccharides (see Figure 29). According to the authors, the separation is not related to sieving effect but rather to the change in the hydrodynamic volumes of the labeled glycans and viscosity of the separation medium.[76]

To compare capillary to planar electrophoresis, various carbohydrates released from several glycoproteins including bovine fetuin, human α_1-acid glycoprotein, HIV envelope, and bovine ribonuclease B were labeled with ANTS and analyzed by HPCE-LIF and high-concentration polyacrylamide slab gel electrophoresis (PAGE). HPCE in the open-tubular format (*i.e.*, in the absence of an entangled polymer network) using acetate buffer, pH 4.75, yielded comparable results to those obtained with PAGE in terms of number of migrating bands. In both cases, high resolution separations of the released and labeled carbohydrates were achieved.[108]

Moreover, ANTS-derivatized complex oligosaccharides, both neutral and sialylated, were separated by HPCE-LIF.[109] The separation of the derivatives with good resolution was achieved in less than 8 min using phosphate buffer at pH 2.5. The linear relationship between the electrophoretic mobility and charge-to-mass ratios of the ANTS-derivatized oligosaccharides was used for peak assignment.

The effect of the structure and charge of the derivatizing agent on the CE analysis of branched oligosaccharides was also investigated.[98] High-mannose-type oligosaccharides from bovine pancreatic ribonuclease B were labeled with ANTS, ANDSA, and 2-ANSA, and separated by CZE using phosphate buffer, pH 2.5, containing TEA. A baseline resolution of the main components was obtained in all cases. The resolution of two of the three structural isomers of Man7 indicates that the mechanism of separation is not strictly based on differences in charge-to-mass ratio but also on the three-dimensional structure which also affects electrophoretic mobility.[98]

The nature of the derivatizing agent influences the choice of the HPCE mode to be used in the subsequent separation step. In fact, MECC proved useful in the analysis of complex oligosaccharides derivatized with a hydrophobic tag, 2-aminoacridone. The derivatized oligosaccharides derived from ribonuclease B, hen egg albumin, and fetuin were separated[110] using a borate buffer containing taurodeoxycholate surfactant that has been shown useful for the analysis of 2-aminoacridone-derivatized monosaccharides.[66] The separation mechanism of such a system is based more on the borate complex formation, as determined by the absence of resolution when bicarbonate buffer was used, than on the partitioning of the derivatives in the micellar phase. However, the addition of the surfactant was shown to improve the separation efficiency. The pattern of separation of the major components of the 2-AA

derivatized oligosaccharides derived from ovalbumin is similar to that obtained by gel permeation.[110]

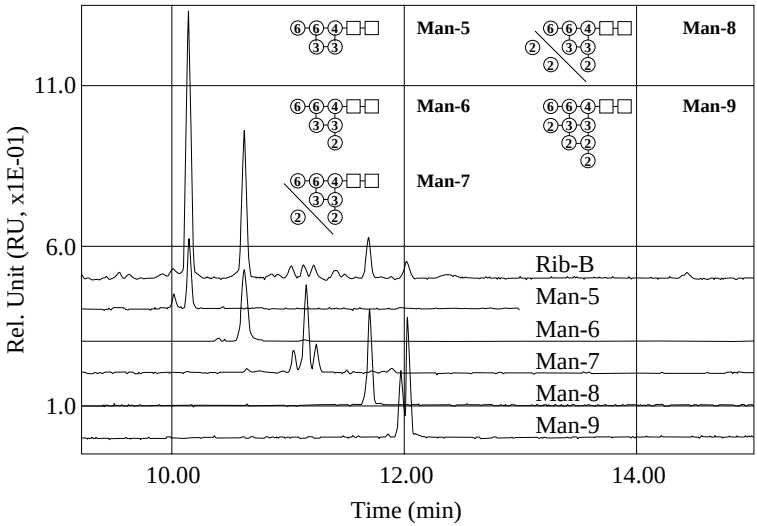


Figure 29. *CGE separation of the APTS-labeled high-mannose type oligosaccharides released from bovine ribonuclease B (upper trace) and the individual standard structures (lower traces). Inset: structural representation of the high-mannose type* N*-linked oligosaccharides: squares = GlcNAcβ1→4; circles #4 = Manβ1→4; circles #6 = Manα1→6; circles #3 = Manα1→3; circles #2 = Manα1→2. Conditions: 57 cm neutrally coated column (50 cm to detection point) ×50-μm i.d.; LIF detection: argon ion laser, excitation 488 nm, emission: 520 nm; separation buffer: 25 mM acetate buffer, pH 4.75, 0.4% polyethylene oxide, applied electric field = 500 V/cm.; i = 19 μA; 20˚C. Reprinted with permission.*[76]

Additional CZE analysis of glycans was reported by Liu *et al.*[77] *N*-linked oligosaccharides from bovine fetuin were released through hydrazinolysis and then derivatized with CBQCA. With on-column LIF detection, this tag permitted the CZE of subpicogram amounts in a phosphate-borate buffer, pH 9.5. Four major peaks as well as few minor peaks were well resolved.

Finally, to provide an HPCE methodology that makes it possible to draw inferences about structural characteristics of complex glycans and, in turn, expedite subsequent structural analyses, a novel method for identifying and quantifying 2-AP derivatives of desialylated *N*-glycosidically linked oligosaccharides in glycoproteins was introduced. It is based on two-dimensional map-

ping of different oligosaccharides by HPCE[111,112] using two different electrolyte systems so that the individual modes of separation are as different from each other as possible.

B.2.6. Glycolipid-Derived Oligosaccharides

Very recently, Mechref *et al*.[56] reported the most suitable conditions for the selective precolumn derivatization of sialooligosaccharides, derived from gangliosides, and the subsequent separation of the derivatives by HPCE. Seven sialooligosaccharides, whose structures and abbreviations are illustrated in Figure 1, were cleaved from gangliosides by ceramideglycanase and derivatized with ANDSA according to Scheme II.[8,54] This precolumn derivatization, which involves the formation of a stable amide bond between the amino group of the ANDSA tag and the carboxylic acid group of the analyte, is very attractive for the labeling of sialooligosaccharides since it is readily achieved in an aqueous medium and at room temperature. Sialooligosaccharides are prone to desialylation at high temperature and in very acidic media. The ANDSA-sialooligosaccharide derivatives, which fluoresce at 420 nm when excited at 315 nm, were readily detected in HPCE at the low femtomole levels using an on-column lamp-operated fluorescence detector. The various ANDSA-sialooligosaccharide derivatives are charged at all pH due to the fact that the precolumn derivatization with ANDSA replaces each weak carboxylic acid group of the parent sugar by two strong sulfonic acid groups. Using 100 mM sodium phosphate, pH 6.0, as the running electrolyte and an untreated fused-silica capillary allowed the resolution of six of the sialooligosaccharides investigated. The two structural isomers sialooligo-G_{D1a} and sialooligo-G_{D1b} (see Figure 1) were not resolved, suggesting that their hydrodynamic volumes are not significantly different. The separation of the seven ANDSA-sialooligosaccharides with an uncoated fused-silica capillary was best achieved when 75 mM borate, pH 10.0, was used as the running electrolyte (see Figure 1).[56] In another report by Mechref and El Rassi,[55] the seven ANDSA-sialooligosaccharides were perfectly separated in a dextran-coated capillary using 100 mM sodium phosphate buffer, pH 6.0, and a negative polarity (see Figure 30). The dextran-coated capillary used in that study exhibited a reduced EOF with respect to an untreated fused silica capillary and the reduced EOF was in the opposite direction to the intrinsic electrophoretic mobility of the analytes. This condition could have favored a better differential migration of the two structural isomers.

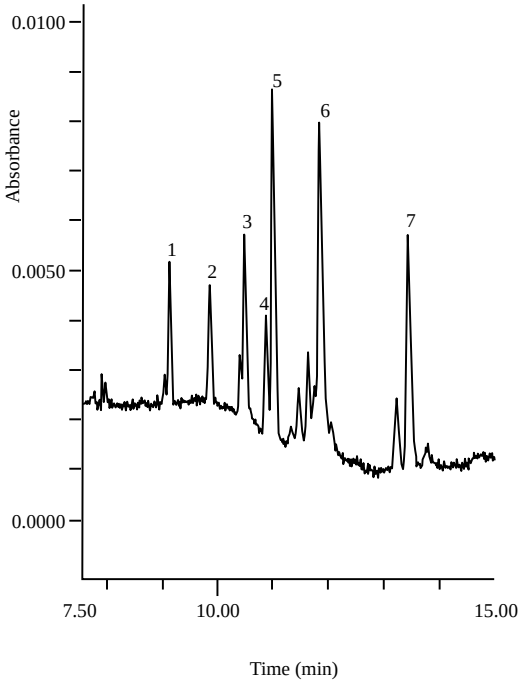


Figure 30. *Electropherogram of ANDSA derivatives of sialooligosaccharides derived from gangliosides obtained on a dextran 150 kDa-coated capillary. Capillary, 47 cm total length (40 cm effective length) × 50-μm i.d.; running electrolyte: 0.10 M phosphate, pH 7.0; pressure injection, 1 s; applied voltage, -15 kV; detection, UV at 250 nm. Sample: 1 = Sialooligo-G_{D3}; 2 = Sialooligo-G_{M3}; 3 = Sialooligo-G_{T1b}; 4 = Sialooligo-G_{D1b}; 5 = Sialooligo-G_{D1a}; 6 = Sialooligo-G_{M2}; 7 = Sialooligo-G_{M1}. Reprinted with permission.*[55]

C. Polysaccharides

C.1. Underivatized Polysaccharides

Thus far, only a few attempts have been made for the application of HPCE to polysaccharides. Recently, Richmond and Yeung[34] reported the HPCE separation and detection of high-molecular-weight native polysaccharides (see Figure 5). To partially ionize the various analytes and in turn achieve differential electromigration, an electrolyte of pH 11.5 was used. Detection was made possible by laser-excited indirect fluorescence detection. In general, migration times were reproducible to 0.05 min for consecutive injections.[34]

HPCE was utilized for the quantitative analysis of the glycosaminoglycan hyaluronan in human and bovine vitreous with UV detection at 200 nm.[20] A running electrolyte consisting of 50 mM disodium hydrogen phosphate, 10 mM sodium tetraborate, and 40 mM SDS, pH 9.0, was found to be optimum for assaying hyaluronan and its oligomers. This alkaline electrolyte ensures that hyaluronan migrates as a polyanion and minimizes the possibility of wall adsorption of both protein and polysaccharide components of the vitreous humor, while SDS binds to the proteins which then migrate away from hyaluronan. The signal corresponding to hyaluronan was confirmed by depolymerization of the native mucopolysaccharide by hyaluronidase. This resulted in the loss of the hyaluronan peak and the appearance of several new peaks corresponding to the oligomeric fragments which had shorter migration times (see Figure 31).

C.2. Derivatized Polysaccharides

Sudor and Novotny[80] reported the separation of neutral polysaccharides (*e.g.*, chitosan, dextran, various water-soluble cellulose derivatives, etc.) labeled with CBQCA using capillaries coated with polyacrylamide and filled with an appropriate polymer solution such as linear polyacrylamide. In the presence of borate, the derivatized polysaccharides migrated readily in open-tubular CZE but showed little tendency to separate. Due to their large molecular size, polysaccharides were not amenable to sieving using gel capillary electrophoresis as they did not penetrate the gel network. This hindrance was solved by causing the polysaccharides to migrate through solutions of entangled polymers. But the extent of separation in such sieving media was complicated by reptation effect. This was overcome by using pulsed-field conditions where a potential gradient along the separation capillary was periodically inverted at a 180° angle which brought about shape transitions and, in turn, favored the separation of polysaccharides according to molecular size. This approach resembles what is known as pulsed-field electrophoresis or field-inversion gel electrophoresis which was originally introduced for the separation of large DNA fragments in traditional gel slab electrophoresis.

The electrophoretic migration of neutral and highly charged polysaccharides, such as chemically modified celluloses and heparins labeled with CBQCA, was regulated by secondary thermodynamic equilibria during capillary electrophoresis by using suitable buffer additives.[113] Electrophoretic migration of uncharged chemically modified cellulose was induced by the adsorption of hydrophobic and charged detergents onto the analyte components, and the process could be described by Langmuir adsorption isotherms. Different polymers were found to contain different adsorption sites, including multilayer formation. On the other hand, reduction of a high electrophoretic mobility of highly charged heparin was attained by the addition of ion-pairing

reagents to the running electrolyte. Under these conditions, migration velocity and selectivity were influenced by the concentration and number of charges of the additive.[113]

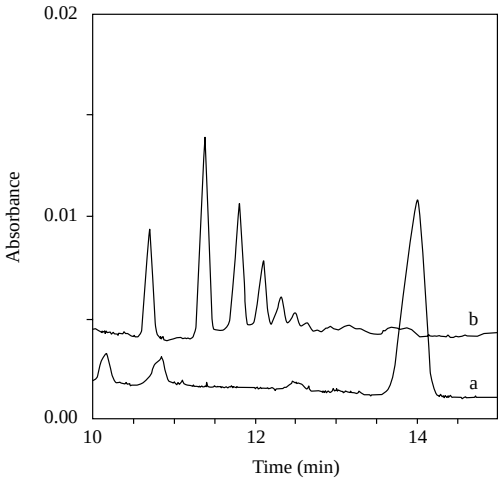


Figure 31. *Electropherogram of (a) bovine vitreous, compared with (b) hyaluronidase digest of Hyaluronan. Peaks labeled according to the number of disaccharide units in the oligomer. Separation buffer: 50 mM disodium hydrogen phosphate, 40 mM SDS, 10 mM sodium tetraborate; capillary, 50 cm × 75-μm i.d.; voltage, 15 kV; detection at 200 nm. Reprinted with permission.[20]*

Another approach for the electrophoresis and sensitive detection of polysaccharides has been the dynamic or *in situ* labeling of the analytes. In fact, underivatized starch components were separated and detected by CE as their iodine complexes.[51] Iodine complexation with carbohydrates imparts charge and optical detection sensitivity at 560 nm. The starch-iodine complex consists of a helix of sugar residues surrounding a linear I^{-5} core.[114,115] Unlike borate complexation, iodine binding is a cooperative interaction which exhibits strong chain-length dependence in both complexation and optical properties. The iodine binding constant increases nearly exponentially with glucan chain length, reaching a plateau at approximately 125 residues.[116] Moreover, the wavelength of maximum absorbance exhibits a red shift with increasing chain length.[117] These facts could be utilized to reveal information on the size and structure of the analytes independent of their electrophoretic behavior. Amylopectin and amylose were well resolved from each other in less than 10 min using iodine-containing buffers in unmodified capillaries. Amylopectin electrophoretic mobility is dependent on the iodine concentration as

well as on the separation temperature, while that of amylose is not. This reflects the fact that the long chains of amylose are essentially saturated with iodine even at low iodine concentration. This system was also shown to be useful in the analysis of potato starch, amizo V, corn starch, and maltodextrin.[51]

D. Glycopeptides and Glycoproteins

D.1. Glycopeptides

The high resolving power and unique selectivity of CZE were also exploited in the separation and characterization of peptide and glycopeptide fragments of glycoproteins. Figure 32 illustrates the CZE mapping of the tryptic peptide fragments of human α_1-acid glycoprotein (AGP) as well as the submapping of its glycosylated and nonglycosylated fragments.[107] Prior to CZE runs, the whole digest was first fractionated into peptide and glycopeptide fragments by high-performance affinity chromatography on a silica-bound concanavalin A (Con A) column. Three pooled fractions were obtained, the first two being Con A non-reactive and Con A slightly reactive eluted with 20 mM phosphate pH 6.5 containing 0.1 M NaCl, while the third fraction interacted strongly with the Con A column and eluted with the heptanic sugar, *i.e.*, methyl-α-D-mannopyranoside. The three fractions were then analyzed by CZE using a fused-silica capillary with fuzzy polyether coating and 0.1 M phosphate, pH 5.0, as the running electrolyte. As seen in Figure 32, the CZE mapping of the whole digest reveals the microheterogeneity of the glycoprotein as manifested by the excessive number of peaks.

One of the unique aspects of the primary structure of the polypeptide chain of pooled human AGP is its peculiar structural polymorphism. Substitutions were found at 21 of the 181 amino acids in the single polypeptide chain, which is responsible in part for multiple peptide and glycopeptide fragments in the tryptic digest. Another source of multiple fragments in the tryptic digest is the microheterogeneities of the oligosaccharide chains attached, which can be noticed by inspecting Figure 33. In fact, the variation in the terminal sialic acid causes charge heterogeneity in the glycopeptide fragments cleaved at the same location by trypsin, the differences in the extent of glycosylation among a population of the protein molecules lead to fragments having the same peptide backbone but with or without carbohydrate chains, and the variation in the nature of the oligosaccharide chains at each glycosylation site yields several glycopeptides that have the same peptide backbone but are different in their oligosaccharide structures.[107]

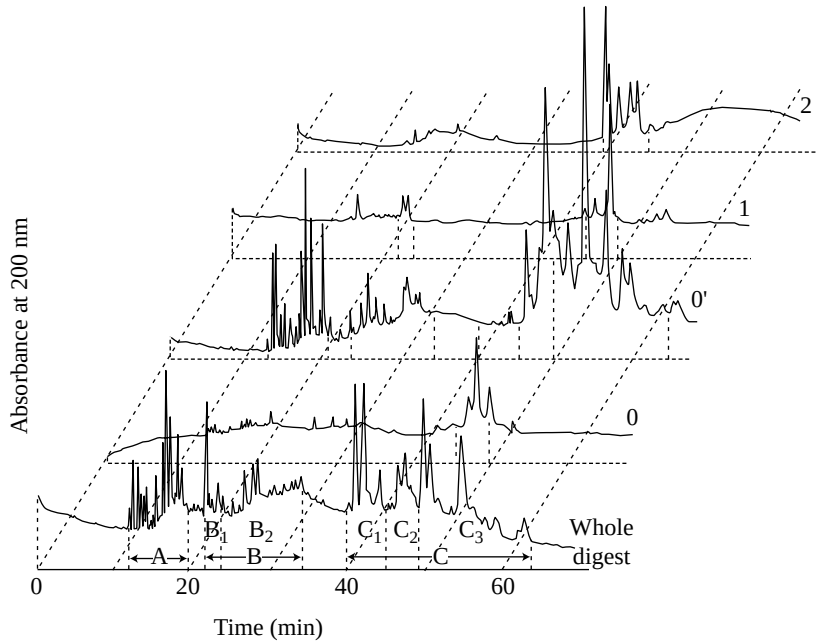


Figure 32. *Capillary zone electrophoresis tryptic mapping and submapping of human AGP. Capillary, fused-silica tube with hydrophilic coating on the inner walls, 45 cm (to the detection point), 80 cm total length ×50-μm i.d.; electrolyte, 0.1 M phosphate solution, pH 5.0; running voltage, 22.5 kV; injection by electromigration for 4 s at 22.5 kV. Symbols: fraction 0, con A non-reactive (excluded from the column); fraction 0', Con A non-reactive (unretained by the column); fraction 1, Con A slightly reactive (eluted with buffer); fraction 2, Con A strongly reactive (eluted with the haptenic sugar). Reprinted with permission.*[107]

As can be seen in Figure 32, the CZE submapping of Con A reactive peptides produced peaks that are missing from the submaps of all other collected fractions, *i.e.*, 0, 0' and 1, but whose components are found in the whole map (see area C1, Figure 32). This approach allows the monitoring of a group of peptides as well as the assessment of glycosylated fragments in the whole map. This methodology is expected to work also with other glycoproteins, and the CZE submapping of all the glycosylated tryptic fragments with different types of glycans may require the use of more than one lectin column in the prefractionation step.[107]

Also, HPCE has been shown to be useful in evaluating the glycopeptide microheterogeneity of recombinant human erythropoietin (rHuEPO) expressed from Chinese hamster ovary (CHO) cells. This was achieved by using 100 mM heptanesulfonic acid (ion-pairing agent) in 40 mM sodium phosphate buffer, pH 2.5.[118] The negatively charged heptanesulfonic acid forms ion pairs with basic amino acid residues, thus reducing analyte-wall interaction as well as altering analyte electrophoretic mobility. This led to improved peptide resolution, which allowed the evaluation of the heterogeneity of glycopeptides derived from rHuEPO. The total tryptic map exhibited two regions: nonglycosylated and glycosylated peptides. Since rHuEPO glycoprotein has three glycosylation sites, three glycopeptides are expected to result from tryptic digestion of this glycoprotein. However, the aforementioned electrophoretic system revealed the microheterogeneity of these glycopeptides by yielding at least 12 glycopeptide peaks in the peptide map.[118]

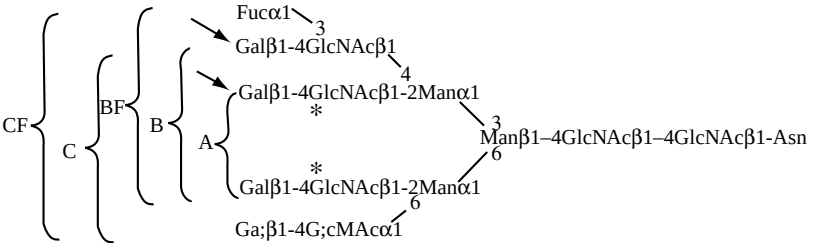


Figure 33. *Primary structure of the carbohydrate classes of AGP. There are five carbohydrate classes attached to AGP having different degrees of branching and sialylation. Classes A, B and C are the bi-, tri- and tetraantennary complex* N*-linked glycans, respectively, whereas BF and CF are the fucosylated B and C structures. Two additional glycans exist: one has two additional fucose linked to the GlcNAc residues marked with asterisks, and one has an outer chain prolonged by Galβ1-4GlcNAc at either of the Gal residues marked with an arrow.*

Very recently, Weber *et al.*[46] reported the characterization of glycopeptides from recombinant coagulation factor VIIa by HPLC and HPCE using UV and pulsed electrochemical detection (PED). The combination of the more traditional methods of HPLC-UV and HPAEC-PED with capillary electrophoresis methods based on CZE-UV and CZE-PED allowed a better characterization of the glycopeptides' heterogeneity. In addition, this report demonstrated the potential of CZE-PED in the analysis of PNGase F-treated glycopeptides where, in contrast to UV detection, both the peptides and the released carbohydrates can be detected simultaneously.[46]

D.2. Glycoprotein Glycoforms

Protein glycosylation can occur at two or more positions in the amino acid sequence, and the glycans at even a single position may be heterogeneous or may be missing from some molecules. This leads to populations of glycosylated variants of a single protein, usually referred to as glycoforms, whose relative proportions are found to be reproducible and not random. However, the glycoforms may be affected by several factors including the environment in which the protein is glycosylated, the manufacturing process, and the isolation procedures. This would affect the function of a glycoprotein, thus engendering the need for high-resolution separation methods to allow the monitoring of glycoform populations especially for genetically engineered glycoprotein pharmaceuticals.

Several HPCE approaches have been described for profiling glycoprotein glycoforms. Transferrin glycoforms were separated by CZE and CIF.[119] In both modes of HPCE, at least five components corresponding to the di-, tri-, tetra-, penta-, and hexasialo-transferrins differing from each other by one negative charge were resolved. The capillary columns used in this study were coated with a layer of linear polyacrylamide on the inner wall to suppress EOF and consequently provide better resolution and sharper focusing of the closely related glycoforms by CZE and CIF, respectively. To assess the presence of varying degree of sialylation among the various glycoforms, the action pattern of neuraminidase on the electrophoretic behavior of the various isoforms was monitored by HPCE. Neuraminidase is an exoglycosidase that liberates specifically the negatively charged sialic acids from the terminal non-reducing positions in glycans. As shown in Figure 34, the electrophoretic analyses of samples taken from the enzymatic digestion at various time intervals demonstrated the gradual removal of sialic acid as manifested by the changes in the relative proportions of the different isoforms with time. The electrophoretic pattern of the final product was completely different from the starting material and showed one main component, the asialo-transferrin. Thus, in the case of transferrin, the major source of microheterogeneity seems to be the variation in the terminal sialic acid of the glycans.

The microheterogeneity of glycoproteins results from sugar residues other than the sialic acid residues. This is the case of ribonuclease B (RNase B) whose glycans portions are of the high-mannose type. The separation of the five different glycoforms of RNase B has been achieved through the formation of anionic borate complexes with the hydroxyl groups of the glycan moiety (Figure 35).[120] The relative proportions of the various glycoforms correlated with the relative proportions of the high mannose glycan populations, *i.e.*, Man_9-Man_5, determined by other more established analytical methods, *e.g.*, mass spectrometry, high performance anion exchange chromatography (HPAEC) and size exclusion chromatography on Bio-Gel P4. To further substantiate the presence of the various glycoforms of RNase B, the time course for the diges-

tion of the protein with *A. saitoi* α(1-2) mannosidase was monitored by CZE. Mannosidase is an exoglycosidase that specifically cleaves mannose from the non-reducing end of glycans. Figure 35b shows that after 25 hrs the glycoform populations carrying Man_9-Man_6 structures were all reduced to a single population carrying Man_5. Thus, HPCE offers a direct method for analyzing glycoforms at the protein level with high resolution and precision.

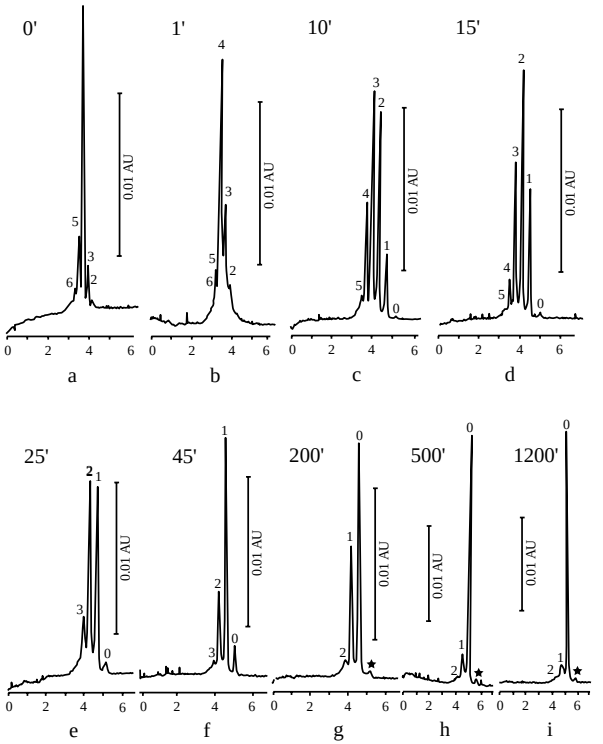


Figure 34. *CZE of iron-free transferrin following incubation with neuraminidase. Capillary, 18.5 cm × 50-μm i.d.; electrolyte, 18 mM Tris-18 mM boric acid-0.3 mM EDTA, pH 8.4; running voltage, 8 kV. The samples for electrophoresis were taken after various incubation times: (a) 0; (b) 1; (c) 10; (d) 15; (e) 25; (f) 45; (g) 200; (h) 500; (i) 1200 min. The proportions of the transferrin isoforms (asialo, mono-, di-, trisialo, etc., marked 0, 1, 2, 3, etc.) changed with time. The sample taken after 20 hr still contained transferrin molecules having one and two sialic acids. The small peak (labeled with a star) appeared after 50-80 min, but did not increase in size on prolonged incubation time (g-i). Reprinted with permission.*[119]

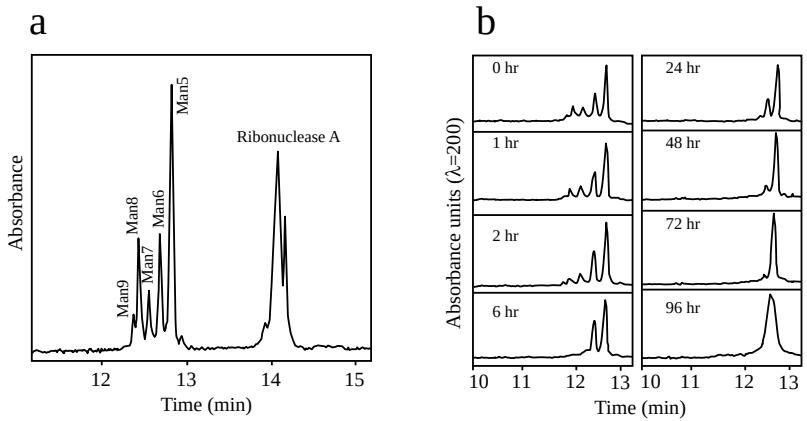


Figure 35. (a) *CZE profile of ribonuclease showing the nonglycosylated form of the protein, ribonuclease A, and the glycoforms of the same protein, collectively known as ribonuclease B. RNase A is a contaminant of RNase B as supplied by Sigma. RNase B remained unaffected during the digestion of the oligosaccharide component of RNase B with* A. saitoi *α(1-2) mannosidase.* ***(b)*** *CZE profile of RNase B showing the time course for the digestion of the glycoprotein with the exoglycosidase,* A. saitoi *α(1-2) mannosidase. Capillary, fused silica 72 cm × 75-μm i.d.; applied voltage 1 kV for 1 min and 20 kV for 19 min, temp., 30°C; detection, UV at 200 nm; injection 1.5 s; electrolyte, 20 mM phosphate containing 50 mM sodium dodecylsulfate, 5 mM borate, pH 7.2. Reprinted with permission.*[120]

Another example of high-mannose related microheterogeneity has been the various glycoforms of recombinant human bone morphogenetic protein 2 (rhBMP-2).[121] The separation of rhBMP-2 glycoforms by CE necessitated the use of simple phosphate buffer containing no additives. Under this condition, the rhBMP-2 sample yielded nine peaks which have been identified to be glycoforms of rhBMP-2. The difference between any adjacent peaks is only one mannose residue (M_r = 162). The nine peaks obtained with intact rhBMP-2 reduced into one major peak when the endo-H digested rhBMP-2 was analyzed by CE. Endo-H is an endoglycosidase specific for the cleavage of high-mannose glycans from glycoproteins. This confirms that the microheterogeneity of the glycoprotein is due to the high mannose-type carbohydrates. The migration order of the glycoforms was found to follow the increasing number of mannose residues in the analyte molecules. Mannose residue can affect the separation by providing higher friction coefficient rather than by a charge shielding effect.[121]

Phosphorylation of glycans seems to be another source of glycoprotein microheterogeneity. In fact, CE analysis of proteinase A glycoforms, both native and underglycosylated, revealed charge heterogeneities attributed to differences in the phosphorylation level of the carbohydrate moiety at Asn-68.[122] The CE analysis was performed using an electrolyte consisting of 100 mM acetate/phosphate buffer, pH 3.2. Both forms were separated into three distinct peaks that probably correspond to charge heterogeneities due to differences in carbohydrate phosphorylation.

The microheterogeneity of a glycoprotein is mostly the result of glycosylation and is largely unaffected by the presence of other functionalities in the protein. This is the case of ovalbumin, a phosphorylated glycoprotein. This protein has one asparagine residue that can accommodate at least nine different carbohydrate structures of the high mannose and hybrid type *N*-glycans. There are also two potential phosphorylation sites at two serine residues, one at position 68 and the other at position 344. The various glycoforms were separated via borate complex formation with the hydroxyl groups of the carbohydrate moieties of the protein using an untreated fused silica capillary.[123] This glycoprotein is a strongly acidic species and therefore would not undergo adsorption onto the naked capillary surface when using alkaline borate. To improve the resolution of the ovalbumin glycoforms, putrescine (*i.e.*, 1,4-butanediamine), a doubly charged cationic species, was added in small amounts (1 mM) to the borate buffer. Using these conditions, five major protein peaks were separated, indicating the presence of protein glycoforms. Upon dephosphorylation of the glycoprotein with calf intestinal alkaline phosphatase or potato acid phosphatase, the five peaks were still resolved but shifted in the position to a more rapid migration time, a behavior consistent with a loss of negative charge. Based on this observation, it was suggested that all ovalbumin glycoforms are phosphorylated to the same degree, and heterogeneity among ovalbumin isoforms resides solely in the carbohydrate structures. Also, the same electrophoretic system was shown to permit the separation of pepsin glycoforms.

Realizing the benefit of including an amine additive (e.g., 1,4-butanediamine, DAB) into the running electrolyte in achieving the separation of ovalbumin glycoforms prompted the investigation of other amine additives such as α,ω-bis-quaternary ammonium alkanes.[124] Three of the α,ω-bis-quaternary ammonium alkane additives, namely hexamethonium bromide (C_6MetBr), hexamethonium chloride (C_6MetCl), and decamethonium bromide (C_{10}MetBr), were examined and the results were compared to those obtained using DAB as a buffer additive.[124] The alkyl chain length and the cation group of α,ω-bis-quaternary ammonium alkanes strongly influence the analysis time and resolution.[124] Under identical separation conditions, C_{10}MetBr was shown to yield a better resolution and shorter analysis time than C_6MetBr. Originally, with DAB, it was thought that such an additive exerts its effect by

mainly altering the EOF.[123] The fact that the additives repressed EOF similarly but the quaternary ammonium alkane additives allowed the resolution of ovalbumin glycoforms in half the time required with DAB suggests that the mechanism of action of these additives is not solely related to EOF repression. Other mechanisms may be involved including protein-additive interactions, protein-wall interactions, additive-wall coating interactions, or any combination of these. In the same report, seven of the eight glycoforms of human chorionic gonadotropin (hCG) were resolved by CE using 1 mM C_6MetBr and 25 mM borate pH 8.4.[124] However, the eight glycoforms of the hCG were near-baseline resolved using 25 mM borate and 5 mM diaminopropane and separated in less than 50 min.[125]

Along the above separation strategies which include (i) the employment of either borate complexation or amine additives and (ii) the use of specific enzymes directed either toward the glycan moieties or other protein functionalities, HPCE was applied successfully to the analysis of recombinant glycoprotein glycoforms. In fact, the fractionation of the human recombinant tissue plasminogen activator (rt-PA) by CZE[126] was recently reported. The CZE analysis of two main glycosylation variants (type I and II) of the same glycoprotein showed different electrophoretic migration patterns. The study further elucidated the microheterogeneity of the glycoprotein as was manifested by the partial resolution of almost 15 glycoforms in a protein that has only four possible *N*-glycosylation sites. This report compared the CZE profile of an rt-PA sample to that of a desialylated rt-PA obtained through neuraminidase treatment, an approach similar to that introduced by Kilàr and Hjertén for human transferrin.[119] The desialylated rt-PA exhibited a much simpler CZE profile indicating that the glycoprotein microheterogeneity is mostly the result of different levels of sialylation. Along the same lines, Watson and Yao[127] extended the use of CZE to the separation of glycoforms of recombinant human granulocyte-colony-stimulating factor (rhGCSF) produced in Chinese hamster ovary cells. This glycoprotein contains only two *O*-linked carbohydrate moieties that differ only in having one or two sialic acid residues. Due to its relative simplicity compared to other more complex glycoproteins, the rhGCSF yielded two well- resolved and equally sized peaks using phosphate-borate buffer, pH 8.0, and an untreated fused-silica capillary. Under these conditions, the acidic protein was repelled from the negatively charged capillary wall and no apparent solute adsorption was observed. When the glycoforms were incubated with neuraminidase, a single peak was obtained eluting earlier than either of the original two sialylated glycoforms.

The high selectivity of CZE was also demonstrated in the separation of the glycoforms of recombinant human erythropoietin (rHuEPO),[128,129] a glycoprotein hormone produced in the kidney of adult mammals that acts on bone

marrow erythroid progenitor cells to promote their development into mature blood cells.

James and co-worker[130] demonstrated the usefulness of MECC in resolving recombinant interferon-γ (IFN-γ) glycoforms produced by Chinese hamster ovary cells. Separations were performed in uncoated fused-silica capillaries at alkaline pH in the presence of SDS micelles. Optimal separation was obtained with 400 mM borate buffer containing 100 mM SDS, pH 8.5. It was noted that optimum separation of IFN-γ glycoforms occurred at a pH close to the pI of the protein (8.5-9.0) since it is most susceptible to hydrophobic interaction at this pH. However, this electrolyte system did not resolve bovine serum fetuin nor α_1-acid glycoprotein glycoforms and showed partial resolution for ribonuclease B and horseradish peroxidase glycoforms.[130]

Very recently, a combination of HPLC and HPCE was found useful in the analysis of the glycoforms of human recombinant factor VIIa (rFVIIa).[131] Again the use of DAB was found to be essential for the separation of the various glycoforms. The separation is reported to be based primarily upon the different content of *N*-acetylneuraminic acid of the oligosaccharide structures of rFVIIa.

Another application of HPCE in the area of glycoforms has been the identification and determination of the isoforms of monoclonal $F(ab')_2$ fragment obtained after pepsin proteolysis of IgG.[132] The presence of these isoforms is usually attributed to post-translational modification of the IgG molecule. It was found that the variation in the pH of the background electrolyte can be effectively employed in modulating selectivity of isoform separations with optimum resolution observed at alkaline pH (9.50).

E. Glycolipids

Recently, the potentials of HPCE have also been demonstrated in the separation of gangliosides, the sialic acid-containing glycosphingolipids.[56,133,134] As shown in Figure 36, a ganglioside molecule has a hydrophilic sialooligosaccharide chain and a hydrophobic moiety, *i.e.* ceramide, that consists of a sphingosine and fatty acid. Due to this inherent structural feature, the gangliosides are amphiphilic solutes forming stable micelles in aqueous solutions with very low critical micellar concentration values (10^{-8}-10^{-10} M).[135] Furthermore, gangliosides most often exist at low concentrations and their structures lack strong chromophores. Thus, two major obstacles must be overcome when developing an HPCE method for the analysis of gangliosides, namely to be able to (i) separate them as monomers and (ii) detect them at low levels. The first obstacle has been addressed recently by Yoo *et al.*[134] and further elaborated by Mechref *et al.*[56] while the second obstacle was overcome by Mechref *et al.*[56]

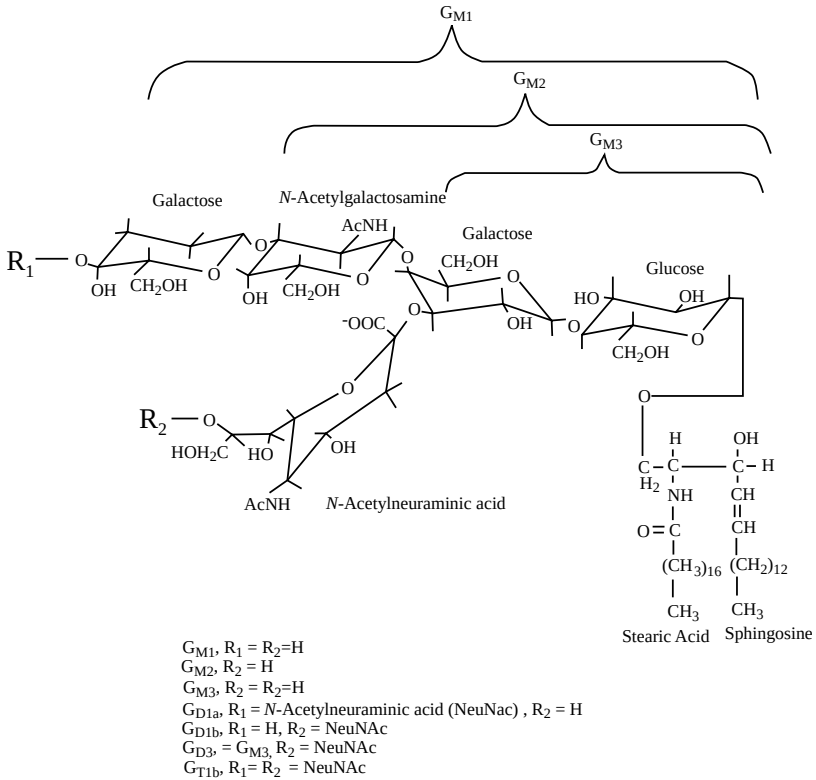


Figure 36. *Structures of the gangliosides. Reprinted with permission.*[56]

On the other hand, Liu and Chan[133] applied HPCE to the study of the behavior of gangliosides in aqueous solutions. These researchers[133] demonstrated that CZE can separate some ganglioside micelles and consequently permitted studies of the micellar properties of these amphiphilic species using untreated fused-silica capillaries and on-column direct UV detection at 195 nm. The ganglioside micelles were successfully analyzed within 10 min with mass sensitivity in the order of 10^{-11} mol. Baseline resolution of a mixture of three ganglioside micelles, namely, G_{M1}, G_{D1b}, and G_{T1b}, was achieved using 2.5 mM potassium phosphate, pH 7.40, as the running electrolyte (see Figure 37). The separation was mainly facilitated by the varying content of the sialic acid residues in the ganglioside micelles, which imparted them with different electrophoretic mobilities.

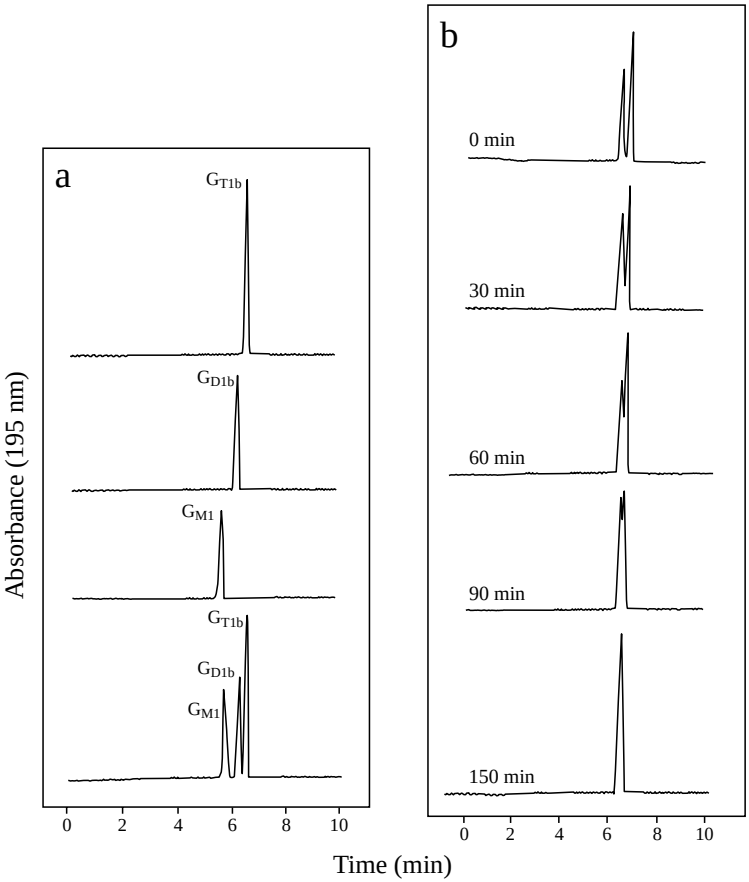


Figure 37. (a) *Capillary electrophoresis of a mixture of G_{M1}, G_{D1b}, and G_{T1b}. Individual G_{M1}, G_{D1b}, and G_{T1b} micelles and a mixture of these three gangliosides shortly after mixing were analyzed by CE. The buffer was 2.5 mM potassium phosphate, pH 7.40. Detection was by UV at 195 nm.* ***(b)*** *Time course of mixed micelle formation between G_{D1b} and G_{T1b}. Equimolar concentrations of polysialogangliosides G_{D1b} and G_{T1b} (165 μM) in 2.5 mM potassium phosphate, pH 7.40, were mixed by vortexing and incubated in a water bath at 37°C. At time intervals, the electrophoretic patterns of the ganglioside mixtures were analyzed. Electrophoretic conditions are as in (a).* ***Reprinted with permission.***[133]

However, upon incubation at 37°C, complete fusion between both micellar peaks could be observed in less than 2.5 hrs (see Figure 37b). The fusion process was accelerated by raising the incubation temperature. Also, it was found that polysialogangliosides (*e.g.*, G_{D1a} and G_{T1b}) may have higher propensities for micellar fusion than monosialogangliosides (*e.g.*, G_{M1} and G_{M2}). Thus the high resolution, high speed, and quantitative aspects of CZE were clearly demonstrated in monitoring processes that may have important implications in the distribution and function of gangliosides in biological membranes.

One of the many elegant features of HPCE is the ease with which the electrophoretic behavior of the analytes can be altered through the simple addition of specific reagents to the running electrolyte. Among the many buffer additives described so far,[136] two kinds of additives are suitable for HPCE of gangliosides, namely cyclodextrins (CDs) and acetonitrile (ACN). While CDs can alter the electrophoretic behavior of a wide variety of compounds via inclusion complexes, the extent of which is determined by the solute hydrophobicity and size, ACN improves analyte solubility as well as selectivity and controls electroosmotic flow.[136] On this basis, Yoo *et al.*[134] demonstrated the utility of CDs in the separation of native gangliosides at 185 nm. Among the various CDs, α-CD was the best buffer additive as far as the separation is concerned. This may be due to the size of the cavity of α-CD that best fit the lipid moiety of the gangliosides.

Although the gangliosides could be detected at 185 nm,[134] the sensitivity is rather low to allow their detection in biological matrices where they are normally found in minute amounts. To overcome this difficulty, Mechref *et al.*[56] have expanded the utility of the selective precolumn derivatization procedure which has been developed originally for the tagging of carboxylated monosaccharides[54] (see Scheme II) to include the derivatization of gangliosides in order to improve their detectability. Moreover, novel electrolyte systems were also introduced for the analysis of derivatized gangliosides by HPCE. The derivatization involved the tagging of gangliosides with either SA (a UV-absorbing tag) or ANDSA (a UV absorbing and also fluorescent tag). The derivatization was shown to occur uniformly on the sialic acid residues by monitoring the enzymatic digestion of SA-G_{T1b} with neura-minidase. The SA-G_{T1b} yielded three peaks upon digestion, which corresponded to be SA-G_{M1}, SA-G_{D1b} and SA-G_{T1b}, thus indicating that all three sialic acid residues of G_{T1b} were labeled.[56]

To separate the derivatized gangliosides in their monomeric forms, ACN and CD were added to the running electrolyte to break up ganglioside micelles. As can be seen in Figure 38, HPLC-grade acetonitrile at 50% (v/v) brought about the separation of three ANDSA-derivatized gangliosides in their monomeric forms while, in the absence of ACN, the three ganglioside derivatives

(*i.e.*, ANDSA-G_{M1}, ANDSA-G_{D1a}, and ANDSA-G_{T1b}) migrated as a single broad peak.[56]

The advantage of using ACN, whose UV cutoff is at 185 nm, was also illustrated in the ability to separate and detect underivatized gangliosides at 195 nm.[56] The use of ACN allowed the separation of the differently sialylated gangliosides shown in Figure 38; however, it did not provide enough selectivity to cause the separation of structural isomers ANDSA-G_{D1a} and ANDSA-G_{D1b}. Partial separation was attained using α-CD and 100 mM sodium borate, pH 10.0.[56] Other additives such as polyvinyl alcohol and hydroxypropyl cellulose brought about a better resolution of the two isomers, *i.e.*, ANDSA-G_{D1a} and ANDSA-G_{D1b}. Complete baseline separation of the two disialylated ganglioside isomers was attained by utilizing an *in situ* charged micellar system composed of decanoyl-*N*-methylglucamide (MEGA 10)/ borate in the presence of α-CD[56] (see Figure 39).

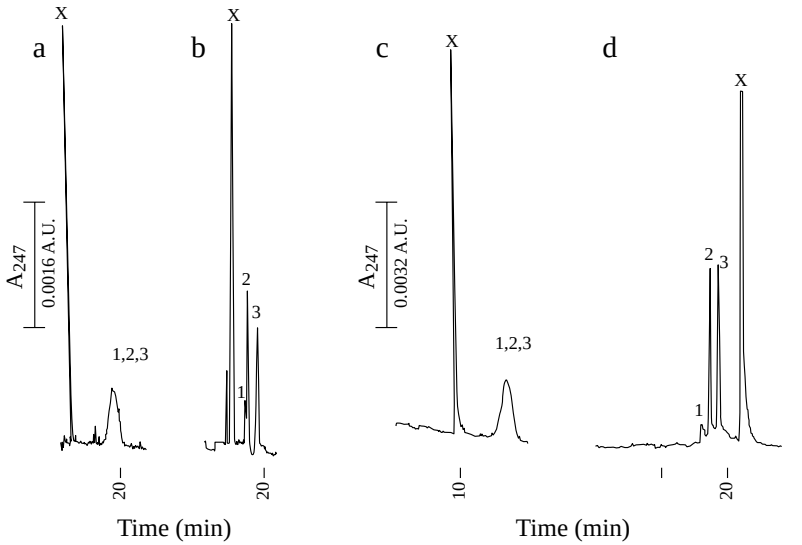


Figure 38. *Electropherograms of standard gangliosides labeled with ANDSA at neutral (a and b) and high (c and d) pH in the presence (b and d) and absence (a and c) of acetonitrile in the running electrolyte. In (a) and (b): running electrolyte, 25 mM sodium phosphate, pH 7.0, at 0% (a) and 50% v/v (b) acetonitrile; voltage, 25.0 kV. In (c) and (d): running electrolyte, 10 mM sodium phosphate, pH 10.0, at 0% (c) and 50% v/v (d) acetonitrile; voltage, 20 kV; capillary, fused-silica, 50 cm (to detection point), 80 cm (total length) × 50-μm i.d. Solutes, (1) G_{M1}, (2) G_{Da}, (3) G_{T1b}. For structures refer to Figure 36. Reprinted with permission.*[56]

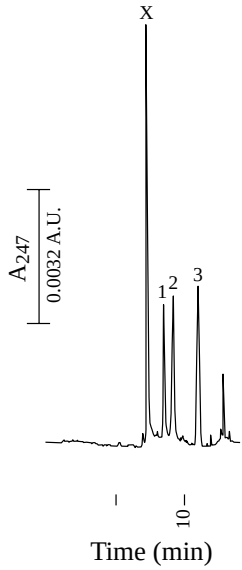


Figure 39. *Electropherogram of standard ANDSA-1. Running electrolyte, 50 mM borate, pH 6.0, containing 5.0 mM MEGA surfactant and 15.0 mM α-CD; running voltage, 18.0 kV; capillary, fused-silica, 50 cm (to detection point), 80 cm (total length) × 50-μm i.d. Peaks: 1 = G_{D1a}; 2 = G_{D1b}; 3 = G_{D3}. Reprinted with permission.*[56]

V. Conclusions

As this review reveals, HPCE in its various modes of separation and detection is suitable for the analysis of a wide variety of carbohydrate species including mono-, oligo-, and polysaccharides, glycopeptides, glycoproteins, and glycolipids. This was facilitated in part by the progress made in the capillary column technology and by the introduction of novel electrolyte systems. However, the major factors behind the advances made in HPCE of carbohydrates have been the development of various detection systems and approaches which include

(i) indirect UV and LIF detection,
(ii) electrochemical detection, and
(iii) precolumn labeling with suitable chromophores and fluorophores for the sensitive UV and fluorescence detection, respectively.

In surveying the literature, precolumn derivatization seems to be the most elegant approach for the separation and detection of carbohydrates. In fact, five different reaction schemes have been introduced for the labeling of carbohydrates with various kinds of tags. All of these tagging processes have yielded the sensitivity required for the analysis of carbohydrates at moderate and low levels. Furthermore, the tagging of carbohydrates imparted the derivatized carbohydrates with charges and/or hydrophobic functional groups that facilitated their efficient separation by various separation principles, thus leading to varying degree of selectivity. It should be noted that multiply charged tags such as ANDSA, ANTS and APTS are excellent labels for the HPCE of sugar derivatives not only because of their high detection sensitivity by either UV or LIF but also because they yield derivatives that are readily separated by HPCE. Although major progress has been made in the area of precolumn labeling of carbohydrates, there is still room for improvements regarding the introduction of other tagging agents and optimizing the existing reaction schemes.

Although noncomplexing electrolyte systems have found some use in the electrophoresis of a wide range of carbohydrate species, the bulk of HPCE separation is still accomplished primarily by borate complexation regardless whether the carbohydrates are derivatized or underivatized. Borate complexation magnifies small structural differences among closely related carbohydrates, thus leading to a better resolution for multicomponent mixtures.

The ease with which the electrolyte systems can be modified and tailored to fit a given separation problem is another important feature of HPCE. In fact, glycolipids such as gangliosides which are not compatible with purely aqueous electrolyte solutions were readily separated in their monomeric forms in hydro-organic electrolyte systems.

Furthermore, the unique selectivity and high separation efficiencies of HPCE have proved extremely useful in the separation of all kinds of carbohydrate species. The technique provides an unsurpassed resolving power for profiling and mapping closely related oligosaccharides cleaved from glycoproteins, glycolipids, and glycosaminoglycans. This high resolving power has also allowed the efficient fingerprinting of complex glycoprotein glycoforms which, in other separation techniques such as ion-exchange HPLC and traditional gel electrophoresis, would yield smeared, unresolved bands.

The advantages of HPCE over other separation techniques such as HPLC and traditional polyacrylamide gel electrophoresis reside in its higher separation efficiencies, shorter analysis time, small sample requirements and, more importantly, lower consumption of expensive reagents and solvents. With the introduction of precolumn labeling with suitable fluorescent tags for LIF detection, HPCE can reach nanomolar detection limits, thus making the technique extremely suitable for the analysis of minute amounts of carbohydrates. However, the major drawback of the technique is its limited preparative capability.

VI. References

1. Stryer, L. *Biochemistry.* 4th ed., New York, W. H. Freeman and Co., 1995.
2. El Rassi, Z. Capillary Electrophoresis of Carbohydrates. *Adv. Chromatogr. 34*, 177-250 (1994)
3. El Rassi, Z., Nashabeh, W. High Performance Capillary Electrophoresis of Carbohydrates and Glycoconjugates, in *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis,* 267-360. Z. El Rassi, Ed., Amsterdam, Elsevier, 1995.
4. El Rassi, Z. Ed. Capillary Electrophoresis of Carbohydrate Species. *Electrophoresis, Vol. 17*, pp. 275-437 (1996)
5. Honda, S., Yamamoto, K., Suzuki, Kakehi, U. M., Kakehi, K. High-Performance Capillary Zone Electrophoresis of Carbohydrates in the Presence of Alkaline Earth Metal Ions. *J. Chromatogr. 588*, 327-333 (1991)
6. Foster, A. B. Zone Electrophoresis of Carbohydrates. *Adv. Carbohydr. Chem. 12*, 81-116 (1957)
7. van Duin, M., Peters, J. A., Kieboom, A. P. G., van Bekkum, H. Studies on Borate Esters II. *Tetrahedron 41*, 3411-3421 (1985)
8. Mechref, Y., Ostrander, G. K., El Rassi, Z. Capillary Electrophoresis of Carboxylated Carbohydrates. Part 2. Selective Precolumn Derivatization of Sialooligosaccharides Derived from Gangliosides with 7-Aminonaphthalene-1,3-disulfonic Acid Fluorescing Tag. *Electrophoresis 16*, 1499-1504 (1995)
9. Honda, S., Akao, E., Suzuki, S., Okuda, M., Kakehi, K., Nakamura, J. High Performance Liquid Chromatography of Reducing Carbohydrates as Strongly Ultraviolet-Absorbing and Electrochemically Sensitive 1-Phenyl-3-methylpyrazolone Derivatives. *Anal. Biochem. 180*, 351-357 (1989)
10. Rendleman, J. A. Ionization of Carbohydrates in the Presence of Metal Hydroxides and Oxides. *Adv. Chem. Ser. 117*, 51-69 (1971)
11. Colón, L. A., Dadoo, R., Zare, R. N. Determination of Carbohydrates by Capillary Zone Electrophoresis with Amperometric Detection at a Copper Microelectrode. *Anal. Chem. 65*, 476-481 (1993)
12. Angyal, S. J., Davies, K. P. Complexing of Sugars with Metal Ions. *Chem. Commun. 10*, 500-501 (1971)
13. Angyal, S. J. Complexes of Metal Cations with Carbohydrates in Solution. *Adv. Carbohydr. Chem. Biochem. 47*, 1-43 (1989)

14. Hoffstetter-Kuhn, S., Paulus, A., Gassmann, E., Widmer, H. M. Influence of Borate Complexation on the Electrophoretic Behavior of Carbohydrates in Capillary Electrophoresis. *Anal. Chem. 63*, 1541-1547 (1991)
15. Taverna, M., Baillet, A., Baylocq-Ferrier, D. Analysis of Neutral and Sialylated *N*-Linked Oligosaccharides by Micellar Electrokinetic Capillary Chromatography with Addition of a Divalent Cation. *Chromatographia 37*, 415-422 (1993)
16. Taverna, M., Baillet, A., Schlüter M., Baylocq-Ferrier, D. *N*-glycosylation Site Mapping of Recombinant Tissue Plasminogen Activator by Micellar Electrokinetic Capillary Chromatography. *Biomed. Chromatogr. 9*, 59-67 (1995)
17. Hermentin, P., Doenges, R., Witzel, R., Hokke, C. H., Vliegenthart, J. F. G., Kamerling, J. P., Conradt, H. S., Nimtz, M., Brazel, D. A Strategy for the Mapping of *N*-Glycans by High-Performance Capillary Electrophoresis. *Anal. Biochem. 221*, 29-41 (1994)
18. Hughes, D. E. Capillary Electrophoretic Examination of Underivatized *O*-Linked and *N*-Linked Oligosaccharide Mixtures and Immunoglobulin G Antibody-Released Oligosaccharide Libraries. *J. Chromatogr. B 657*, 315-326 (1994)
19. Kakehi, K., Susami, A., Taga, A., Suzuki, S., Honda, S. High-Performance Capillary Electrophoresis of O-Glycosidically Linked Sialic Acid-Containing Oligosaccharides in Glycoproteins as Their Alditol Derivatives with Low-Wavelength UV Monitoring. *J. Chromatogr. A 680*, 209-215 (1994)
20. Grimshaw, J., Kane, À., Trocha-Grimshaw, J., Douglas, A., Chakravarthy, U., Archer, D. Quantitative Analysis of Hyaluronan in Vitreous Humor Using Capillary Electrophoresis. *Electrophoresis 15*, 936-940 (1994)
21. Malsch, R., Harenberg, J., Heene, D. L. High-Resolution Capillary Electrophoresis and Polyacrylamide Gel Electrophoresis of Heparins. *J. Chromatogr. A716*, 259-268 (1995)
22. Damm, J. B. L., Overklift, G. T., van Dedem, G. W. K. Determination of Structural Differences in the Glycosaminoglycan Chains of Heparin and Heparan Sulfate by Analysis of the Constituting Disaccharides with Capillary Electrophoresis. *Pharm. Pharmacol. Lett. 3*, 156-160 (1993)
23. Desai, U. R., Wang, H., Ampofo, S. A., Linhardt, R. J. Oligosaccharide Composition of Heparin and Low-Molecular-Weight Heparins by Capillary Electrophoresis. *Anal. Biochem. 213*, 120-127 (1993)
24. Pervin, A., Al-Hakim, A., Linhardt, R. J. Separation of Glycosaminoglycan-Derived Oligosaccharides by Capillary Electrophoresis Using Reverse Polarity. *Anal. Biochem. 221*, 182-188 (1994)

25. Damm, J. B. L., Overklift, G. T. Indirect UV Detection as a Non-Selective Detection Method in the Qualitative and Quantitative Analysis of Heparin Fragments by High-Performance Capillary Electrophoresis. *J. Chromatogr. A 678*, 151-165 (1994)
26. Kerns, R. J., Vlahov, I. R., Linhardt, R. J. Capillary Electrophoresis for Monitoring Chemical Reactions: Sulfation and Synthetic Manipulation of Sulfated Carbohydrates. *Carbohydr. Res. 267*, 143-152 (1995)
27. Karamanos, N. K., Axelsson, S., Vanky, P. Tzanakakis, G. N., Hjerpe, A. Determination of Hyaluronan and Galactosaminoglycan Disaccharides by High-Performance Capillary Electrophoresis at the Attomole Level. Applications to Analyses of Tissue and Cell Culture Proteoglycans. *J. Chromatogr. A 696*, 295-305 (1995)
28. Yeung, E. S. Indirect Detection Methods: Looking for What Is Not There. *Acc. Chem. Res. 22*, 125-130 (1989)
29. Yeung, E. S., Kuhr, W. G. Indirect Detection Methods for Capillary Separations. *Anal. Chem. 63*, 275A-282A (1991)
30. Foret, F., Fanali, S., Boceck, O. L., Bocek, P. Indirect Photometric Detection in Capillary Zone Electrophoresis. *J. Chromatogr. 470*, 299-308 (1989)
31. Yeung, E. S. Optical Detectors for Capillary Electrophoresis. *Adv. Chromatogr. 35*, 1-51 (1995)
32. Garner, T. W., Yeung, E. S. Indirect Fluorescence Detection of Sugars Separated by Capillary Zone Electrophoresis with Visible Laser Excitation. *J. Chromatogr 515*, 639-644 (1990)
33. Kuhr, W. G., Yeung, E. S. Optimization of Sensitivity and Separation in Capillary Zone Electrophoresis with Indirect Fluorescence Detection. *Anal. Chem. 60*, 2642-2646 (1988)
34. Richmond, M. D., Yeung, E. S. Development of Laser-Excited Indirect Fluorescence Detection For High-Molecular-Weight Polysaccharides in Capillary Electrophoresis. *Anal. Biochem. 210*, 245-248 (1993)
35. Vorndran, A. E., Oefner, P. J., Scherz, H., Bonn, G. K. Indirect UV Detection of Carbohydrates in Capillary Zone Electrophoresis. *Chromatographia 33*, 163-168 (1992)
36. Oefner, P. J., Vorndran, A. E., Grill, E., Huber, C., Bonn, G. K. Capillary Zone Electrophoretic Analysis of Carbohydrates by Direct and Indirect UV Detection. *Chromatographia 34*, 308-316 (1992)
37. Bergholdt, A., Overgaard, J., Colding, A., Frederiksen, R. B. Separation of D-Galactonic and D-Gluconic Acids by Capillary Electrophoresis. *J. Chromatogr. 644*, 412-415 (1993)

38. Xu, X., Kok, W. T., Poppe, H. Sensitive Determination of Sugars by Capillary Electrophoresis with Indirect UV Detection under Highly Alkaline Conditions. *J. Chromatogr. A 716*, 231-240 (1995)
39. Klockow, A., Paulus, A., Figueiredo, V., Amado, R., Widmer, H. M. Determination of Carbohydrates in Fruit Juices by Capillary Electrophoresis and High-Performance Liquid Chromatography. *J. Chromatogr. A 680*, 187-200 (1994)
40. Weston, A., Brown, P. R., Jandik, P., Heckenberg, A. L., Jones, W. R. Optimization of Detection Sensitivity in the Analysis of Inorganic Cations by Capillary Ion Electrophoresis using Photometric Detection. *J. Chromatogr. 608*, 395-402 (1992)
41. Rocklin, R., in *A Practical Guide to HPLC Detection*, 145. D. Parriott, Ed. New York, Academic Press, 1993.
42. Ye, J., Baldwin, R. P. Amperometric Detection in Capillary Electrophoresis with Normal Size Electrodes. *Anal. Chem. 65*, 3525-3527 (1993)
43. Ye, J., Baldwin, R. P. Determination of Carbohydrates, Sugar Acids, Alditols by Capillary Electrophoresis and Electrochemical Detection at a Copper Electrode. *J. Chromatogr. A 687*, 141-148 (1994)
44. O'Shea, T. J., Lunte, S. M., LaCourse, W. R. Detection of Carbohydrates by Capillary Electrophoresis with Pulsed Amperometric Detection. *Anal. Chem. 65*, 948-951 (1993)
45. Lu, W., Cassidy, R. M. Pulsed Amperometric Detection of Carbohydrates Separated by Capillary Electrophoresis. *Anal. Chem. 65*, 2878-2881 (1993)
46. Weber, P., Kornfelt, T., Klausen, N. K., Lunte, S. M. Characterization of Glycopeptides from Recombinant Coagulation Factor VIIa by High-Performance Liquid Chromatography and Capillary Zone Electrophoresis Using Ultraviolet and Pulsed Electrochemical Detection. *Anal. Biochem. 225*, 135-142 (1995)
47. Johnson, D. C., LaCourse, W. R. Pulsed Electrochemical Detection of Carbohydrates at Gold Electrodes Following Liquid Chromatography Separation, in *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis*, 391-429. Z. El Rassi, Ed. Amsterdam, Elsevier, 1995.
48. Johnson, D., LaCourse, W. Liquid Chromatography with Pulsed Electrochemical Detection at Gold and Platinum Electrodes. *Anal. Chem. 62*, 589A-597A (1990)
49. Luo, P., Zhang, F., Baldwin, R. Comparison of Metallic Electrodes for Constant-Potential Amperometric Detection of carbohydrates, Amino Acids and Related Compounds in Flow Systems. *Ana. Chim. Acta 244*, 169-178 (1991)

50. Penn, S. G., Chiu, R. W., Monnig, C. A. Separation and Analysis of Cyclodextrins by Capillary Electrophoresis with Dynamic Fluorescence Labeling and Detection. *J. Chromatogr. A 680*, 233-241 (1994)
51. Brewster, J. D., Fishman, M. L. Capillary Electrophoresis of Plant Starches as the Iodine Complexes. *J. Chromatogr. A 693*, 382-387 (1995)
52. Honda, S., Iwase, S., Makino, A., Fujiwara, S. Simultaneous Determination of Reducing Monosaccharides by Capillary Electrophoresis as the Borate Complexes of *N*-2-Pyridylglycamines. *Anal. Biochem. 176*, 72-77 (1989)
53. Hase, S. Pre- and Post-Column Detection-Oriented Derivatization Techniques in HPLC of Carbohydrates, in *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis*, 555-575. Z. El Rassi, Ed. Amsterdam, Elsevier, 1995.
54. Mechref, Y., El Rassi, Z. Capillary Electrophoresis of Derivatized Acidic Monosaccharides. *Electrophoresis 15*, 627-634 (1994)
55. Mechref, Y., El Rassi, Z. Fused-Silica Capillaries with Surface-Bound Dextran Layer Crosslinked with Diepoxypolyethylene Glycol for Capillary Electrophoresis of Biological Substances at Reduced Electroosmotic Flow. *Electrophoresis 16*, 617-624 (1995)
56. Mechref, Y., Ostrander, G. K., El Rassi, Z. Capillary Electrophoresis of Carboxylated Carbohydrates I. Selective Precolumn Derivatization of Gangliosides with UV Absorbing and Fluorescent Tags. *J. Chromatogr. A 695*, 83-95 (1995)
57. Honda, S., Ueno, T., Kakehi, K. High-Performance Capillary Electrophoresis of Unsaturated Oligosaccharides Derived from Glycosaminoglycans by Digestion with Chondroitinase ABC as 1-Phenyl-3-methyl-5-pyrazolone Derivatives. *J. Chromatogr. 608*, 289-295 (1992)
58. Honda, S. Suzuki, S. Nose, A., Yamamoto, K. Kakehi, K. Capillary Zone Electrophoresis of Reducing Mono- and Oligosaccharides as the Borate Complexes of Their 3-Methyl-1-phenyl-2-pyrazolin-5-one Derivatives. *Carbohydr. Res. 215*, 193-198 (1991)
59. Novotny, M. V., Sudor, J. High-Performance Capillary Electrophoresis of Glycoconjugates. *Electrophoresis 14*, 373-389 (1993)
60. Zhao, J. Y., Diedrich, P., Zhang, Y., Hindsgaul, O., Dovichi, N. J. Separation of Aminated Monosaccharides by Capillary Electrophoresis with Laser-Induced Fluorescence Detection. *J. Chromatogr. B 657*, 307-313 (1994)
61. Nashabeh, W., El Rassi, Z. Capillary Zone Electrophoresis of Linear and Branched Oligosaccharides. *J. Chromatogr. 600*, 279-287 (1992)

62. Lee, C. K., Sandler, S. I. Vancomycin Partitioning in Aqueous Two-Phase Systems: Effects of pH, Salts, and an Affinity Ligand. *Biotechnol. Bioeng. 35*, 408-416 (1990)
63. Amankwa, L., Albin, M., Kuhr, W. Fluorescence Detection in Capillary Electrophoresis. *Trends Anal. Chem. 11*, 114-120 (1992)
64. Li, S. F. Y. *Capillary Electrophoresis. Principles, Practice and Applications*, Ch. 3. Amsterdam, Elsevier, 1992.
65. Stefansson, M., Novotny, M. Electrophoretic Resolution of Monosaccharides Enantiomers in Borate-Oligosaccharide Complexation Media. *J. Am. Chem. Soc. 115*, 11573-11580 (1993)
66. Greenaway, M., Okafo, G. N., Camilleri, P., Dhanak, D. A Sensitive and Selective Method for the Analysis of Complex Mixtures of Sugars and Linear Oligosaccharides. *J. Chem. Soc. Chem. Commun.*, 1691-1692 (1994)
67. Huber, C., Grill, E., Oefner, P., Bobleter, O. Capillary Electrophoretic Determination of the Component Monosaccharides in Hemicelluloses. *Fresenius J. Anal. Chem. 348*, 825-831 (1994)
68. Schwaiger, H., Oefner, P., Huber, C., Grill, E., Bonn, G. K. Capillary Electrophoresis and Micellar Electrokinetic Chromatography of 4-Aminobenzonitrile Carbohydrate Derivatives. *Electrophoresis 15*, 941-952 (1994)
69. Noe, C. R., Freissmuth, J. Capillary Zone Electrophoresis of Aldose Enantiomers: Separation after Derivatization with *S*-(-)-1-Phenylethylamine. *J. Chromatogr. A 704*, 503-512. (1995)
70. Chen F., Evangelista, R. A. Analysis of Mono- and Oligosaccharide Isomers Derivatized with 9-Aminopyrene-1,4,6-trisulfonate by Capillary Electrophoresis with Laser-Induced Fluorescence. *Anal. Biochem. 230*, 273-280 (1995)
71. Evangelista, R. A., Liu, M., Chen, F. Characterization of 9-Aminopyrene-1,4,6-trisulfonated-Derivatized Sugars by Capillary Electrophoresis with Laser-Induced Fluorescence Detection. *Anal. Chem. 67*, 2239-2245 (1995)
72. Smith J. T., El Rassi, Z. Capillary Zone Electrophoresis of Biological Substance with Fused Silica Capillaries Having Zero or Constant Electroosmotic Flow. *Electrophoresis 14*, 396-406 (1993)
73. Lee, K., Kim, Y., Linhardt, R. Capillary Electrophoresis for the Quantitation of Oligosaccharides Formed Through the Action of Chitinase. *Electrophoresis 12*, 636-640 (1991)
74. Chiesa, C., Horvath, C. Capillary Zone Electrophoresis of Malto-Oligosaccharides Derivatized with 8-Aminonaphthalene-1,3,6-trisulfonic acid. *J. Chromatogr. 645*, 337-352 (1993)

75. Jackson, P. Polyacrylamide Gel Electrophoresis of Reducing Saccharides Labeled with the Fluorophore 2-Aminoacridone: Subpicomolar Detection Using an Imaging System Based on a Cooled Charge-Coupled Device. *Anal. Biochem. 196*, 238-244 (1991)
76. Guttman, A., Pritchett, T. Capillary Gel Electrophoresis of High-Mannose Type Oligosaccharides Derivatized by 1-Aminopyrene-3,6,8-trisulfonic Acid. *Electrophoresis 16*, 1906-1911 (1995)
77. Liu, J., Shirota, O., Wiesler, D., Novotny, M. Ultrasensitive Fluorescence Detection of Carbohydrates as Derivatives in Mixtures Separated by Capillary Electrophoresis. *Proc. Natl. Acad. Sci. USA 88*, 2302-2306 (1991)
78. Liu, J., Shirota, O., Novotny, M. Separation of Fluorescent Oligosaccharides Derivatives by Microcolumn Techniques Based on Electrophoresis and Liquid Chromatography. *J. Chromatogr. 559*, 223-235 (1991)
79. Liu, J., Shirota, O., Novotny, M. Sensitive, Laser-Assisted Determination of Complex Oligosaccharides Mixtures Separated by Capillary Gel Electrophoresis. *Anal. Chem. 64*, 973-975 (1992)
80. Sudor, J., Novotny, M. Electromigration Behavior of Polysaccharides in Capillary Electrophoresis Under Pulsed-Field Conditions. *Proc. Natl. Acad. Sci. USA 90*, 9451-9455 (1993)
81. Liu, J., Shirota, O., Novotny, M. Capillary Electrophoresis of Amino Sugars with Laser-Induced Fluorescence Detection. *Anal. Chem. 63*, 413-417 (1991)
82. Zhang, Y., Arriaga, E., Diedrich, P., Hindsgaul, O., Dovichi, N. J. Nanomolar Determination of Aminated Sugars by Capillary Electrophoresis. *J. Chromatogr. A 1716*, 221-229 (1995)
83. Zhang, Y., Le, X., Dovichi, N. J., Compston, C. A., Palcic, M. M., Diedrich, P., Hindsgaul, O. Monitoring Biosynthetic Transformations of *N*-Acetyllactosamine Using Fluorescently Labeled Oligosaccharides and Capillary Electrophoretic Separation. *Anal. Biochem. 227*, 368-376 (1995)
84. Le, X., Scaman, C., Zhang, Y., Zhang, J., Dovichi, N. J., Hindsgaul, O., Palcic, M. M. Analysis of Capillary Electrophoresis-Laser-Induced Fluorescence Detection of Oligosaccharides Produced from Enzyme Reaction. *J. Chromatogr. A 716*, 215-220 (1995)
85. Bruno, A. E., Krattiger, B. On-Column Refractive Index Detection of Carbohydrates Separated by HPLC and CE, in *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis*, 431-446. Z. El Rassi, Ed. Amsterdam, Elsevier, 1995.
86. Nashabeh, W., El Rassi, Z. Capillary Zone Electrophoresis of Pyridylamino Derivatives of Maltooligosaccharides. *J. Chromatogr. 514*, 57-64 (1990)

87. Chiesa, C., Oefner, P. J., Zieske, L. R., O'Neill, R. A. Micellar Electrokinetic Chromatography of Monosaccharides Derivatized with 1-Phenyl-3-methyl-2-pyrazolin-5-one. *J. Cap. Elec. 2*, 175-183 (1995)
88. Arentoft, A. M., Michaelsen, S., Sørensen, H. Determination of Oligosaccharides by Capillary Zone Electrophoresis. *J. Chromatogr. A 652*, 517-524 (1993)
89. Al-Hakim, A., Linhardt, R. J. Capillary Electrophoresis for the Analysis of Chondroitin Sulfate- and Dermatan Sulfate-Derived Disaccharides. *Anal. Biochem. 195*, 68-73 (1991)
90. Carney, S. L., Osborne, D. J. The Separation of Chondroitin Sulfate Disaccharides and Hyaluronan Oligosaccharides by Capillary Zone Electrophoresis. *Anal. Biochem. 195*, 132-140 (1991)
91. Ampofo, S. A., Wang, H. M., Linhardt, R. J. Disaccharide Compositional Analysis of Heparin and Heparan Sulfate Using Capillary Zone Electrophoresis. *Anal. Biochem. 199*, 249-255 (1991)
92. Damm, J. B. L., Overklift, G. T., Vermeulen, B. W. M., Fluitsma, C. F,. van Dedem, G. W. K. Separation of Natural and Synthetic Heparin Fragments by High Performance Capillary Electrophoresis. *J. Chromatogr. 608*, 297-309 (1992)
93. Michaelsen, S., Schroder, M.-B., Sorensen, H. Separation and Determination of Glycosaminoglycan Disaccharides by Micellar Electrokinetic Capillary Chromatography for Studies of Pelt Glycosaminoglycans. *J. Chromatogr. A 652*, 503-515 (1993)
94. Pervin, A., Kenan, G., Linhardt, R.J. Capillary Electrophoresis to Measure Sulfoesterase Activity on Chondroitin Sulfate and Heparin Derived Disaccharides. *Appl. Theor. Electrophoresis 3*, 297-303 (1993)
95. Hermentin, P., Witzel, R., Doenges, R., Bauer, R., Haupt, H., Patel, T., Parekh, R. B., Brazel, D. The Mapping by High-pH Anion-Exchange Chromatography with Pulsed Amperometric Detection and Capillary Electrophoresis of the Carbohydrate Moieties of Human Plasma α_1-Acid Glycoprotein. *Anal. Biochem. 206*, 419-429 (1992)
96. Taverna, M., Baillet, A., Biou, D., Schlüter, M., Werner, R., Ferrier, D. Analysis of Carbohydrate-Mediated Heterogeneity and Characterization of *N*-Linked Oligosaccharides of Glycoproteins by High Performance Capillary Electrophoresis. *Electrophoresis 13*, 359-366 (1992)
97. Stefansson, M., Novotny, M. Separation of Complex Oligosaccharide Mixtures by Capillary Electrophoresis in the Open-Tubular Format. *Anal. Chem. 66*, 1134-1140 (1994)
98. Chiesa, C., O'Neill, R. A. Capillary Zone Electrophoresis of Oligosaccharides Derivatized with Various Aminonaphthalene Sulfonic Acids *Electrophoresis 15*, 1132-1140 (1994)

99. Sudor, J., Novotny, M. V. End-Label, Free-Solution Capillary Electrophoresis of Highly Charged Oligosaccharides. *Anal. Chem. 67*, 4205-4209 (1995)
100. Smith, J. T., El Rassi, Z. Capillary Zone Electrophoresis of Biological Substances with Surface-Modified Fused Silica Capillaries with Switchable Electroosmotic Flow. *J. High Resolut. Chromatogr. 15*, 573-578 (1992)
101. Honda, S., Makino, A., Suzuki, S., Kaheki, K. Analysis of the Oligosaccharides in Ovalbumin by High-Performance Capillary Electrophoresis. *Anal. Biochem. 191*, 228-234 (990)
102. Liu, J., Dolnik, V., Hsieh, Y.-Z., Novotny, M. Experimental Evaluation of the Separation Efficiency in Capillary Electrophoresis Using Open Tubular and Gel-Filled Columns. *Anal. Chem. 64*, 1328-1336 (1992)
103. Klockow, A., Widmer, H. M., Amadò, R., Paulus, A. Capillary Electrophoresis of ANTS Labeled Oligosaccharides Ladders and Complex Carbohydrates. *Fresenius J. Anal. Chem. 350*, 415-425 (1994)
104. Guttman, A., Cooke, N., Starr, C. M. Capillary Electrophoresis Separation of Oligosaccharides: I. Effect of Operational Variables. *Electrophoresis 115*, 1518-1522 (1994)
105. El Rassi, Z., Tedford, D., An, J., Mort, A. High-Performance Reversed-Phase Chromatographic Mapping of 2-Pyridylamino Derivatives of Xyloglucan Oligosaccharides. *Carbohydr. Res. 215*, 25-38 (1991)
106. Stefansson, M., Novotny, M. Resolution of the Branched Forms of Oligosaccharides by High-Performance Capillary Electrophoresis. *Carbohydr. Res. 258*, 1-9 (1994)
107. Nashabeh, W., El Rassi, Z. Capillary Zone Electrophoresis of α_1-Acid Glycoprotein Fragments from Trypsin and Endoglycosidase Digestions. *J. Chromatogr. 536*, 31-42 (1991)
108. Guttman, A., Starr, C. Capillary and Slab Gel Electrophoresis Profiling of Oligosaccharides. *Electrophoresis 16*, 993-997 (1995)
109. Klockow, A., Amado, R., Widmer, H. M., Paulus, A. Separation of 8-Aminonaphthalene-1,3,6-trisulfonic Acid-Labeled Neutral and Sialylated *N*-Linked Complex Oligosaccharides by Capillary Electrophoresis. *J. Chromatogr. A 716*, 241-257 (1995)
110. Camilleri, P., Harland, G. B., Okafo, G. High Resolution and Rapid Analysis of Branched Oligosaccharides by Capillary Electrophoresis. *Anal. Biochem. 230*, 115-122 (1995)
111. Suzuki, S., Honda, S. Two-Dimensional Mapping of *N*- Glycosidically Linked Oligosaccharides in Glycoproteins by High-Performance Capillary Electrophoresis. *Trends Anal. Chem. 14*, 279-288 (1995)

112. Suzuki, S., Kakehi, K., Honda, S. Two-Dimensional Mapping of *N*- Glycosidically Linked Asialo-Oligosaccharides from Glycoproteins as Reductively Pyridylaminated Derivatives using Dual Separation Modes of High-Performance Capillary Electrophoresis. *Anal. Biochem. 205*, 227-236 (1992)
113. Stefansson, M., Novotny, M. Modification of the Electrophoretic Mobility of Neutral and Charged Polysaccharides. *Anal. Chem. 66*, 3466-3471 (1994)
114. Teitelbaum, R. C., Ruby, S. L., Marks, T. J. *J. Am. Chem. Soc. 100*, 3215-3217 (1961)
115. Rundle, R. E., French, D. The Complexation of Starch and Starch-Iodine Complex. II. Optical Properties of Crystalline Starch Fractions. *J. Am. Chem. Soc. 65*, 558-561 (1943)
116. Thoma, A., French, D. Starch-Iodine-Iodine Interaction. I. Spectrophotometric Investigations. *J. Am. Chem. Soc. 65*, 1825-1828 (1961)
117. Banks, W., Greenwood, C. T., Khan, K. M. Interaction of Linear Amylose Oligomers with Iodine. *Carbohydr. Res. 17*, 25-33 (1971)
118. Rush, R. S., Derby, P. L., Strickland, T. W., Rohde, M. F. Peptide Mapping and Evaluation of Glycopeptide Microheterogeneity Derived from Endoproteinase Digestion of Erythropoietin by Affinity High-Performance Capillary Electrophoresis. *Anal. Chem. 65*, 1834-1842 (1993)
119. Kilàr, F., Hjerten, S. Separation of the Human Transferrin Isoforms by Carrier Free High-Performance Zone Electrophoresis and Isoelectric Focusing. *J. Chromatogr. 480*, 351-357 (1989,)
120. Rudd, P. M, Scragg, I. G., Coghil, E., Dwek, R. A. Separation and Analysis of the Glycoform Populations of Ribonuclease B Using Capillary Electrophoresis. *Glycoconjugate J. 9*, 86-91 (1992)
121. Yim, K., Abrams, J., Hsu, A. Capillary Zone Electrophoretic Resolution of Recombinant Human Bone Morphogenetic Protein 2 Glycoforms. An Investigation into the Separation Mechanisms for an Exquisite Separation. *J. Chromatogr. A 716*, 401-412 (1995)
122. Pedersen, J., Biedermann, K. Characterization of Proteinase A Glycoforms from Recombinant *Saccharomyces cerevisiae. Biotechnol. Appl. Biochem. 18*, 377-388 (1993)
123. Landers, J. P., Oda, R. P., Madden, B. J., Spelsberg, T. C. High Performance Capillary Electrophoresis of Glycoproteins: The Use of Modifiers of Electroosmotic Flow for Analysis of Microheterogeneity. *Anal. Biochem 205*, 115-124 (1992)
124. Oda, R. P., Madden, B. J., Spelsberg, T. C., Landers, J. P. α,ω-Bis-Quaternary Ammonium Alkanes as Effective Buffer Additives for Enhanced Capillary Electrophoretic Separation of Glycoprotein. *J. Chromatogr. A 680*, 85-92 (1994)

125. Morbeck, D. E., Madden, B. J., McCormick, D. J. Analysis of the Microheterogeneity of the Glycoprotein Chorionic Gonadotropin with High-Performance Capillary Electrophoresis. *J. Chromatogr. A, 680*, 217-224 (1994)
126. Yim, K. W. Fractionation of the Human Recombinant Tissue Plasminogen Activator (rtPA) Glycoforms by High-Performance Capillary Zone Electrophoresis and Capillary Isoelectric Focusing. *J. Chromatogr. 559*, 401-410 (1991)
127. Watson, E., Yao, F. Capillary Electrophoretic Separation of Recombinant Granulocyte-Colony-Stimulating Factor Glycoforms. *J. Chromatogr. 630*, 442-446 (1993)
128. Tran, A. D., Park, S., Lisi, P. J., Huynh, O. T., Ryall, R. R., Lane, P. A. Separation of Carbohydrate-Mediated Microheterogeneity of Recombinant Human Erythropoietin by Free Solution Capillary Electrophoresis. *J. Chromatogr. 542*, 459-471 (1991)
129. Watson, E. ,Yao, F. Capillary Electrophoretic Separation of Human Recombinant Erythropoietin (r-HuEPO) Glycoforms. *Anal. Biochem. 210*, 389-393 (1993)
130. James, D. C., Freedman, R. B., Hoare, M., Jenkins, N. High-Resolution Separation of Recombinant Human Interferon-γ Glycoforms by Micellar Electrokinetic Capillary Chromatography. *Anal. Biochem. 222*, 315-322 (1994)
131. Klausen, N. K., Kornfelt, T. Analysis of the Glycoforms of Human Recombinant Factor VIIa by Capillary Electrophoresis and High-Performance Liquid Chromatography. *J. Chromatogr. A 718*, 195-202 (1995)
132. Vincentelli, R., Bihoreau, N. Characterization of Each Isoform of $F(ab')_2$ by Capillary Electrophoresis. *J. Chromatogr. 641*, 383-390 (1993)
133. Liu, Y., Chan, J. K.-F. High-Performance Capillary Electrophoresis of Gangliosides. *Electrophoresis 12*, 402-408 (1991)
134. Yoo, Y. S., Kim, Y. S., Jhon, G.-J., Park, J. Separation of Gangliosides Using Cyclodextrin in Capillary Zone Electrophoresis. *J. Chromatogr. A 652*, 431-439 (1993)
135. Ulrich-Bott, B., Wiegandt, H. Micellar Properties of Glycophingolipids in Aqueous Media. *J. Lip. Res. 25*, 1233-1245 (1984)
136. Issaq, J. I., Janini, G. M., Chan, K. C., El Rassi, Z. Approaches for the Optimization of Experimental Parameters in Capillary Zone Electrophoresis. *Adv. Chromatogr. 35*, 101-169 (1995)