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SEPARATION OF TETRAMETHYLRHODAMINE LABELED MONO- AND OLIGOSACCHARIDES BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY AND CAPILLARY ELECTROCHROMATOGRAPHY COUPLED WITH LASER-INDUCED FLUORESCENCE DETECTION

BY

HANS D. OSTHOFF ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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September 25th, 2000

UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Separation of tetramethyl-rhodamine labeled mono- and oligosaccharides by micellar electrokinetic chromatography and capillary electrochromatography coupled with laser-induced fluorescence detection submitted by Hans D. Osthoff in partial fulfillment of the requirements for the degree of Master of Science.

Dr. N. J. Dovichi

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Date: Sept 18, 2000

ABSTRACT

Tetramethylrhodamine labeled mono- and oligosaccharide standards were separated by micellar electrokinetic chromatography and detected using an in-house built laser-induced fluorescence detection system, based on a sheath flow cuvette. Optimal separation conditions were obtained using sodium dodecyl sulfate, methanol and boric acid as buffer ingredients at pH 9. The effects of adding a series of monoamines are presented and discussed. At pH 12, ethylamine was found to be most advantageous amine in the series. Several derivatives of boric acid, including phenylboronic and 4-carboxyphenylboronic acid, were evaluated in this separation scheme. Alternative surfactants, such as triton X, tetradecyltrimethylammonium bromide, and octanesulfonic acid were found to be inferior to sodium dodecylsulfate. The same standards were size-separated by capillary electrochromatography using aminopropylsilica packed capillaries. Octadecylsilica packed capillaries were not found to be useful. These experiments are the first work on capillary electrochromatography with laser-induced fluorescence detection based on a sheath flow cuvette.

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TABLE OF CONTENTS

Chapter 1: An introduction to carbohydrate analysis

Chapter 2: The effects of amine modifiers carbohydrate separation by MEKC

2.2 Experimental

2.3 Results

2.4 Discussion

Chapter 3: Effects of boronic acids and organic modifiers in the analysis of carbohydrates by MEKC

3.2 Experimental

3.3 Results and Discussion

3.3.4 Alternative surfactants

Chapter 4: Application of CEC to the separation of carbohydrates

4.2 Experimental

4.2.3 Preparation of specific capillaries

4.3 Results and Discussion

Chapter 5: Future work and conclusions

5.1 Future work

LIST OF TABLES

CHAPTER 1

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF ABBREVIATIONS

Abbreviation	**Full name**
ACN	AcetoNitriie
ANDSA	7-AminoNapththalene-1,3-DiSulfonic Acid
ANTS	8-AminoNapththalene-1,3,6-TriSulfonic acid
APTS	9-AminoPyrine-1,4,6-TriSulfonic acid
2-AP	2-AminoPyridine
6-AQ	6-AminoQuinoline
ATP	Adenosine TriPhosphate
CBQCA	3-(p-CarboxyBenzoyl)-Quinoline-2-CarboxyAldehyde
CE	Capillary Electrophoresis
CEC	Capillary ElectroChromatography
CGE	Capillary Gel Electrophoresis
CMC	Critical Micelle Concentration
CTAB	CetylTrimethylAmmonium Bromide
CZE	Capillary Zone Electrophoresis
DDAB	DidodecylDimethylAmmonium Bromide
EOF	ElectroOsmotic Flow / ElectroOsmotic Force
HPLC	High Performance Liquid Chromatography
I.D.	Inner Diameter
IEF	IsoElectric Focusing
ITP	IsoTachoPhoresis
LIF	Laser Induced Fluorescence
MEKC	Micellar ElectroKinetic Chromatography
O.D.	Outer Diameter
ODS	OctaDecylSilica
ODSS	OctaDecyl Sulfonated Silica
PEEK	PolyEther Ether Ketone

PMP	1-Phenyl-3-Methyl-5-Pyrazolone
p.s.i.	Pounds per Square Inch (≈ 6870 Pa)
SCX	Sulfonated Cation eXchanger
SDS	Sodium Dodecyl Sulfate
TMR	TetraMethylRhodamine
TRIS	TRIS(hydroxymethyl)aminomethane
TTAB	TetradecylTrimethylAmmonium Bromide
UV	UltraViolet

CHAPTER 1

AN INTRODUCTION TO CARBOHYDRATE ANALYSIS

1.1 Analyte description

1.1.1 Biological significance

Carbohydrates, proteins, nucleic acids and lipids are the four major classes of biomolecules. Carbohydrates are the most abundant of these by mass. Their biological functions are: (a) Energy stores, fuels and metabolic intermediates, for example in the form of starch in plants, glycogen in animals or the well-known universal energy carrier adenosine triphosphate (ATP), (b) backbone to the genetic code in the form of ribose in RNA and desoxyribose in DNA, (c) structural element in the cell walls of bacteria and plants, for example as cellulose, and (d) recognition sites on lipids, proteins and cells [1].

1.1.2 Chemical structures and isomerisms

Carbohydrates can be divided into mono-, oligo- and polysaccharides. The later two are large carboyhydrate structures that can be digested into monosaccharides. Monosaccharides are aldehydes or ketones that have two or more hydroxyl groups. Depending on the number of carbons, they are referred as trioses (or three-carbon aldoses), tetroses, pentoses, hexoses, etc.

Carbohydrates are chiral molecules and exhibit stereoisomerism. For example. pentoses have three asymmetric centers, for which there are $2^3 = 8$ stereoisomers. In general, the number of stereoisomers for a monosaccharide lacking a plane of symmetry and bearing n asymmetric carbons is 2^n. Stereoisomerism gives rise to optical activity. Depending on the direction in which light is rotated through a dilute solution of the stereoisomer, the letters D (for Dextro, right) and L (for Levo, left) are used. The symbols D and L in carbohydrate nomenclature refer to the absolute configuration of the asymmetric carbon farthest from the aldehyde or ketogroup. For example, there are 4 pentoses in the D-series, 4 pentoses in the L series, 8 hexoses in the D-series, and 8 hexoses in the L-series, and so forth. Monosaccharides that only differ by the stereochemistry at one single carbon atom are called epimers. For example, D-glucose and D-galactose are 4-epimers, because they only differ in the stereochemistry at carbon number four.

Pentoses and hexoses have the ability to cyclize by forming intramolecular hemiacetals or hemiketals. In aqueous solution, both the open and closed forms co-exist. Upon formation of a hemiacetal or –ketal, stereoisomerism occurs at the so-called anomeric carbon, the former aldehyde- or ketogroup. This form of isomerism is given the designations α or β. The designation α means that the hydroxyl group attached to the anomeric carbon is below the plane of the ring; β means that it is above the plane of the ring [2]. An example is given in figure 1.1.

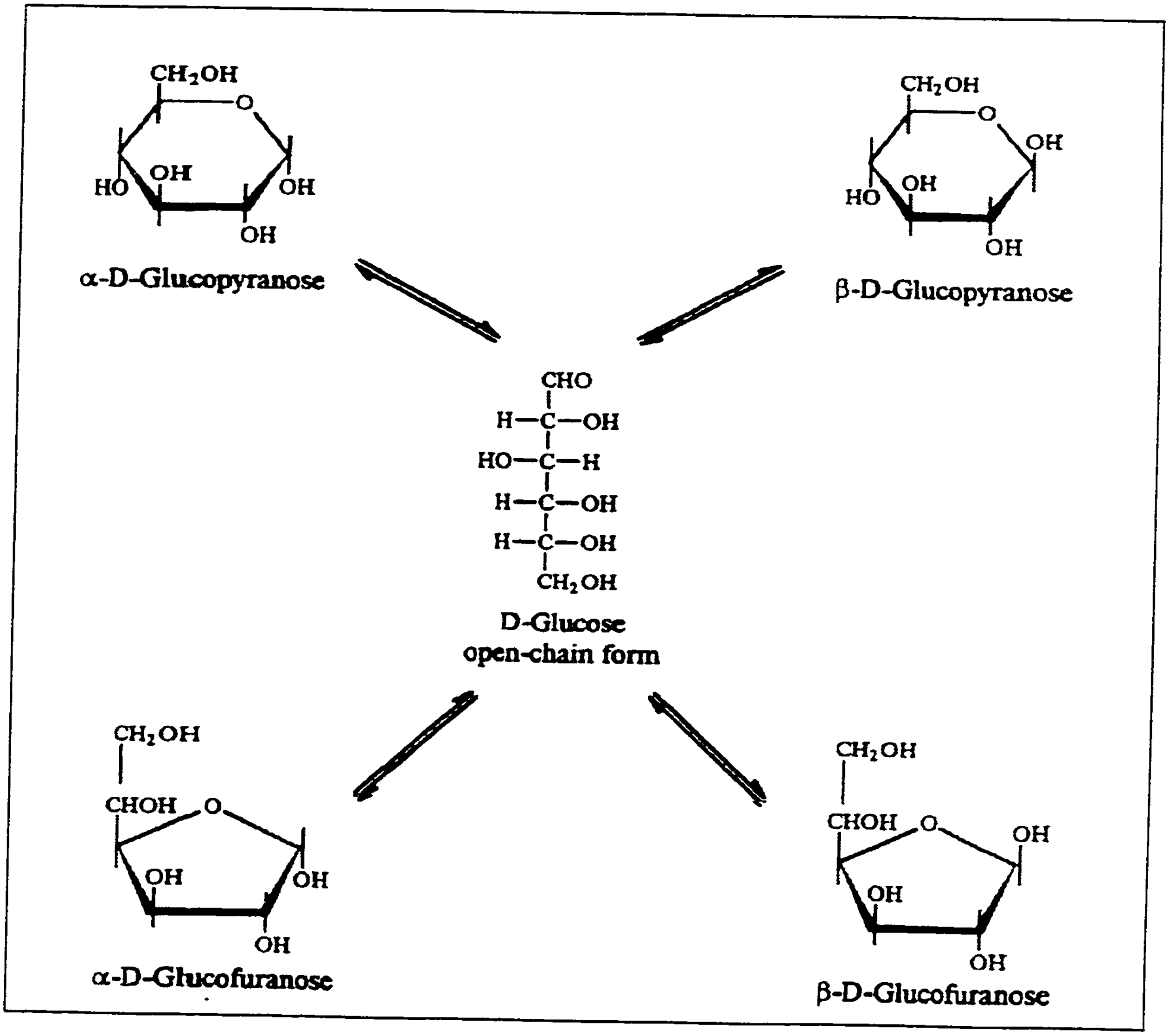

Figure 1.1. Isomerisms arising from cyclization of D-glucose in water

Adapted from: Aran Paulus and Antje Klockow-Beck *Analysis of Carboyhdrates by Capillary Electrophoresis* Chromatographia CE series **1998**.

Monosaccharides are joined to other molecules bearing hydroxyl or amine groups by either O- or N-glycosidic bonds. Again, there is the possibility for isomerism: Linkages can be 1→2-, 1→3-, 1→4-, or 1→6-, depending on which hydroxy groups are involved in the bond formation. The stereochemistry at the anomeric carbon (α or β) is, of course, retained. For example, cellulose is a β-1→4-polymer of D-glucose. Most N-glycosidic linkages, for example in adenosine (ribose linked to the nucleic acid adenine), have the β-configuration [3].

Once monosaccharides are linked to other saccharides by O-glycosidic bonds, an acetal has formed at the anomeric carbon. Unlike hemiacetals, acetals are stable in neutral and basic solutions, and hydrolyze only under acidic conditions. Thus, the ring-opening equilibrium (mutarotation) observed for monosaccharides is not observed in larger oligosaccharides. The readily oxidized keto or aldehyde form ("free reducing group") at the terminating sugar unit plays in important role in biochemistry [4].

Because of the large number and types of possible isomerisms, the number of structurally different oligosaccharides increases exponentially with the number of sugar units. For example, whereas 4 different amino acids may form 24 different tetrapeptides, 4 different hexoses may form thousands of different tetrasaccharides. The diversity of oligosaccharides may be further amplified by methylation, acetylation, or deoxygenation [1-3].

1.1.3 Common analytical techniques

1.1.3.1 Sample preparation and derivatization

Carbohydrates are usually water-soluble and are therefore easily extracted with water from biological samples. Larger oligosaccharides can be hydrolyzed with hydrochloric acid, or digested enzymatically. Methylation often complements acid hydrolysis and gives structural information on the position of glyosidic linkages [5]. Methylation is done in the following manner: A strong base is used to deprotonate all free hydroxy groups, which then readily react with methyl iodide [6]. Enyzmatic digestion is more specific. It allows, for example, the differentiation between α and β configuration in glycosidic linkages [7].

Oligosaccharides can be cleaved from glycoproteins in various ways. The reaction of alkali in the presence of sodium or potassium borohydride liberates the reduced glycans, which were O-glycosidically linked to the amino acids serine, threonine, hydroxylysine or hydroxyproline. N-Glycosidically linked glycans are usually linked to asparagine residues. They can be removed by hydrazinolysis or alkaline cleavage under harsher conditions than for O-glycosidially linked glycans in the presence of sodium borohydride [8]. Many glycans cleaved from glycoproteins also can contain sialic acid residues, which can be cleaved enzymatically or by mild acid hydrolysis [9].

If a particular detection technique (e.g. UV, LIF, see below) requires the presence of a chromo- or fluorophore, it can be introduced by reductive amination, performed in mild acid followed by treatment with sodium cyanoborohydride [10].

1.1.3.2 Separation and detection

For the purification and determination of oligosaccharides colorimetric assays, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography and capillary electrophoresis (CE) have been used. Due to the large number of possible isomers, the separation of carbohydrate mixtures can be quite challenging. Detection and structure determination can be performed by mass spectrometry (MS), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), laser-induced fluorescence (LIF) and ultraviolet spectroscopy (UV/Vis) [11]. For sequencing of complex oligosacharides mass spectrometry based techniques, especially MS^n methods using ion-trap or FTMS mass spectrometers, are the preferred methods of analysis [12].

1.2 Capillary electrophoresis (CE)

1.2.1 Basic principles of capillary zone electrophoresis (CZE)

Electrophoresis is the separation of ions in (usually aqueous) solution by an applied electric field. Capillary zone electrophoresis (CZE) was first demonstrated by Stellan Hjertén in 1967 [13]. James Jorgenson and Krynn Lukacs [14-15] pioneered fast separation of fluorescently tagged amines and amino acids in thin glass tubes using capillary zone electrophoresis. Briefly, the ions separate on the basis of their electrophoretic mobility μ_{ep}. The electrophoretic velocity v_{ep} is defined by

$$v_{ep} = \mu_{ep} \frac{V}{L} \tag{1}$$

V is the applied voltage and L is the length of the capillary in S.I. units.

The force on an ion arising from the electric field is opposed by frictional drag. The force induced by frictional drag is given by Stoke's law:

$$F = 6 \pi \eta r v \tag{2}$$

F is the frictional force, η is the viscosity, r is the ionic radius, and v is the velocity of the ion relative to the mobile phase. A well-known equation is:

$$F = q\,E = q\,\frac{V}{L} \qquad (3)$$

E is the electric field strength and q is the charge of the ion.

It is trivial to show that

$$\mu_{ep} = \frac{q}{6\pi\eta r} \qquad (4)$$

The electrophoretic mobility thus depends on the charge-to-size ratio (q/r) of the ion [16]. Neutral molecules are therefore difficult to separate by CZE.

In CZE, the ionic mobility is the sum of electrophoretic (μ_{ep}) and electroosmotic (μ_{eo}) mobilities. Electroosmotic flow (EOF) arises from the surface charge of the capillary walls. Exposed at the surface are silanol groups, whose pK_a typically ranges from 2 to 5. Electroosmotic flow gives rise to a plug-like flow profile, as opposed to a parabolic (laminar) flow profile which occurs in pressure-driven flow. The electroosmotic mobility μ_{eo} is equal for all molecules and is given by the Smoluchovsky equation:

$$\mu_{eo} = \frac{\varepsilon \varepsilon_0 \varsigma}{\eta} \qquad (5)$$

ε and ε_0 are the dielectric coefficient and the permittivity of free space, and ζ is the zeta-potential of the capillary walls. The zeta-potential is the potential at the plane of shear of the capillary surface. It is affected by the pH of the buffer solution and proportional to $\frac{1}{\sqrt{I}}$, where I is the ionic strength [17]. Ionic strength and buffer pH need to be strictly controlled in order to obtain a reproducible EOF.

In CZE, the overall mobility of an ion is thus

$$v = v_{eo} + v_{ep} = (\mu_{eo} + \mu_{ep})\, E = \left(\frac{\varepsilon\varepsilon_0 \varsigma}{\eta} + \frac{q}{6\pi\eta r} \right) \frac{V}{L} \qquad (6)$$

The zeta-potential ζ and the viscosity η and therefore the EOF can be modified by the addition of organic modifiers to the running buffer [18-19]. Commonly employed are alcohols (methanol, ethanol, isopropanol, etc.) and aprotic solvents (acetonitrile, dimethylsulfoxide, acetone, tetrahydrofuran, etc.) [20].

Plate numbers in CZE are higher (typically above 10^5) than in HPLC. The classical causes for bandbroadening in HPLC are absent, namely resistance to mass transfer in the mobile phase (because of the absence of laminar flow), resistance to mass transfer to and in the stationary phase (because there is no stationary phase and ideally no interaction with the capillary walls), and multipath bandbroadening, which is sometimes called eddy diffusion (because there is no packing). Separations are therefore more efficient than in HPLC [21]. However, bandbroadening may arise from Joule heating, which causes a non-parabolic flow profile, from the injection, from analyte-wall-interactions, or from longitudinal diffusion. Bandbroadening from longitudinal diffusion is given by the Einstein equation:

$$\sigma_L{}^2 = \frac{2DL^2}{\mu V} \tag{7}$$

$\sigma_L{}^2$ is the variance of the broadened band and D is the diffusion coefficient of the solute [22].

Typical operating conditions in CZE are 5 to 30 kV applied voltage and 10-75 μm diameter fused-silica capillaries, which are surrounded by a polyimide coating. Arcing and the quality of the power supply usually limits this technique. Because of the small column diameters used, very low mass sensitivity detection limits can be achieved. Detectors that have been used include UV/Vis, fluorescence, conductivity, amperometric detectors, and MS [23]. Exquisite detection limits can be achieved by coupling CE with laser-induced fluorescence (CE-LIF) [24].

1.2.2 Micellar electrokinetic chromatography (MEKC)

In CZE, molecules separate on the basis of their charge. In order to separate neutral molecules by electrophoresis, charge must be introduced to them, usually by complexation with a charged ligand.

One method commonly employed involves the use of surfactants and is called MEKC. Surfactants typically are molecules with a long hydrophobic tail and a polar head group. Above the so-called critical micelle concentration (CMC) the surfactant molecules form aggregates called micelles. The CMC in water varies slightly with the ionic strength in the solution. In practice, one operates well above the CMC. For example, Terabe *et al.* [25] used sodium dodecyl sulfate (SDS) to separate a series of neutral aromatic alcohols. For SDS, the CMC in water is about 8 mM. Other surfactants commonly used are cationic surfactants, such as cetyltrimethylammonium bromide (CTAB) [26]. Since cationic surfactants adsorb strongly to the fused silica surface of the inner capillary walls, the electroosmotic flow (EOF) is reduced or its direction reversed. The addition of bile salts [27], cyclodextrins [28] or crown ethers [29] allows chiral separations if desired.

The separation mechanism in MEKC is similar to retention in liquid chromatography. It is based on the distribution of a solute between two discrete phases, namely between the hydrophobic micelles and the polar buffer solution. The equation for the partitioning coefficient must account for the fact the pseudostationary phase is actually moving under the influence of the applied electric field, in contrast to conventional chromatography, where the stationary phase is not moving. The equation for the partitioning coefficient $\tilde{k}'$ in MEKC is

$$\tilde{k}' = \frac{t_R - t_0}{t_0 (1 - t_R / t_{mc})} \tag{8}$$

t_R, t_0 and t_{mc} are the migration times of the neutral analyte, an unretained compound and the micelles, respectively [30]. The separation efficiency in MEKC is affected by longitudinal diffusion, sorption-desorption kinetics, intermicelle mass transfer, Joule heating or micellar heterogeneity [31].

1.2.3 Capillary electrochromatography (CEC)

CEC is a combination of liquid chromatography (LC) and capillary electrophoresis (CE). It is an electrokinetically driven separation method, yet contains a packed bed as a stationary phase in the separation column or capillary. CEC was pioneered in 1974 by Pretorius *et al.* [32] and revived in 1981 by Jorgenson and Lukacs [33]. The main advantages of CEC over LC are the low-pressure, pulse-free and plug-flow characteristics, which theoretically produce higher separation efficiency. However, the electroosmotic flow is difficult to control, since it is affected by temperature, pH, viscosity, organic solvents, ionic strength and retention of the analytes [34]. The performance of CEC columns and their theoretical limitations have been discussed in several recent publications [35-37].

Retention in electrochromatography must reflect the concurrence of chromatographic and electrophoretic processes. The electrochromatographic retention factor k* is given by [38]:

$$k^* = k' + k'\left(\frac{\mu_{ep}}{\mu_{eo}}\right) + \left(\frac{\mu_{ep}}{\mu_{eo}}\right) \tag{9}$$

For a neutral solute, where $\mu_{ep}=0$, the electrochromatographic retention factor equals the chromatographic retention factor, k* = k'.

For the preparation of columns, it is important to have both a significant amount of stationary phase to accomplish a large column capacity (e.g. octadecyl silica, ODS) as well as charged sites (e.g. underivatized silanols) to achieve a robust EOF. Charged surface sites can be created by preparing octadecyl-sulfonated silica (ODSS) [39], by segmented capillaries, which have a significant portion packed with bare silica post the on-line detector [40], by the use of propylamino derivatized silica [41], by the use of strong cation-exchangers such as propanesulfonic acid (SCX) derivatized silica [42], or SCX derivatized capillaries coated dynamically with cetyltrimethylammonium bromide (CTAB) [43]. In addition to ODS, C8 [44] and C30 [45] reversed phases have been used.

For reversed phase packings, the mobile phase in CEC usually contains an organic modifier, for example acetonitrile in water. Analogous to HPLC, the eluent strength increases with organic modifier content. It has been reported [46] that the EOF, measured using an electroosmotic flow marker such as thiourea, also increases with increasing acetonitrile content. Analogous to HPLC [47], strong tailing is observed in the analysis of basic (= nitrogen bearing) molecules with reversed phases. The problem is usually dealt with by addition of a competing base to the mobile phase, for example triethylamine [48-49] or triethanolamine [50], or by lowering the buffer pH to 2.5 [48].

In order to get proper performance and reduce bubble formation in CEC, the mobile phase should be thoroughly degassed, preferably by a combination with helium and applied vacuum [35]. Often, both the inlet and outlet sides of the column are pressurized to avoid bubble formation during a run, in a mode termed "pressurized CEC".

Proper inlet and outlet frit fabrication is crucial. In addition to mechanical strength considerations, improperly designed frits can promote bubble formation. Frits are often prepared by sintering wet silica particles with a Bunsen burner flame or a hot metal wire. Some alternative techniques are silicate polymerization [51], methacrylate photopolymerization [52] or tapering of the capillary inlet [53].

1.2.4 Other electrophoretic methods

Other commonly employed electrokinetic separation methods are isoelectric focussing (IEF), isotachophoresis (ITP) and capillary gel electrophoresis (CGE). In CGE the analytes size-separate by sieving through polyacrylamide, agarose, methylcellulose or dextran polymer [54]. In IEF, biomolecules migrate along a pH gradient generated by so-called carrier ampholytes in a polyacrylamide or agarose gel, until the local pH matches the pI of the analyte. After focussing, the analytes are mobilized chemically, hydrodynamically, or electroosmotically [55]. In the analysis by ITP the sample is sandwiched between a high-mobility leading electrolyte and a low-mobility terminating electrolyte. The analytes separate depending on their electrophoretic mobility in so-called isotachophoretic zones, which are in contact with each other. Sample components with lower mobility and conductivity will generate a greater field strength across their respective zone, so that at equilibrium all zones migrate at the same velocity [56]. A comparison of the different CE modes is shown in figure 1.2. .

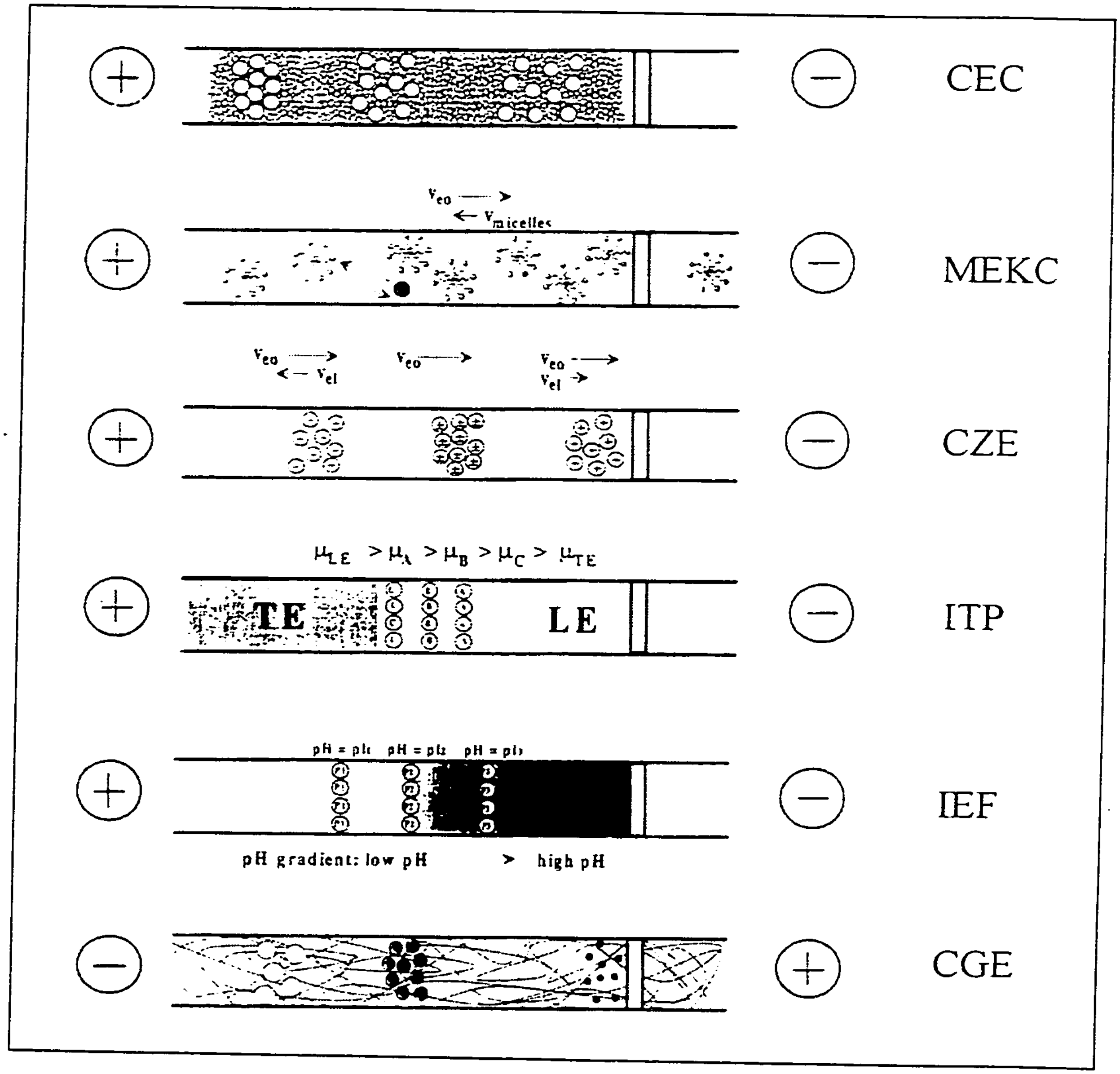

Figure 1.2. Illustration of different modes of CE. Adapted from Aran Paulus and Antje Klockow-Beck *Analysis of Carboyhdrates by Capillary Electrophoresis* Chromatographia CE series **1998**.

1.3 Application of CE to the separation of carbohydrates

A variety of methods have been used for the analysis of inherently neutral oligosaccharides by capillary electrophoresis and have been the subject of many reviews [57-64]. These methods include the use of strong alkali, precapillary conversion to ions. *in situ* conversion to ions, micellar electrokinetic chromatography (MEKC). and size exclusion methods in packed beds [61].

1.3.1 Complexation and labeling reagents

In situ conversion of oligosaccharides to ions is traditionally done by complexation of cis-diols with boric acid or divalent metal ions such as alkaline earth metal ions [65] or copper ions [66]. Some of these reagents, for example dansylaminophenylboric acid [67], may be fluorescent. Tris-(hydroxymethyl)-aminomethane (TRIS) [68], diethylamine [69], triethylamine [70], or tetrabutylammonium phosphate [71] have been used as ion-pairing reagents to negatively charged borate complexes or sialic acid residues as well as to modify the analyte migration times by dynamically coating the inner capillary walls.

Precapillary conversion to ions is often the same step as derivatization for UV or LIF detection. Some frequently used precapillary derivatizing reagents are 2-aminopyridine (2-AP) [72], 1-phenyl-3-methyl-5-pyrazolone (PMP) [73], 6-aminoquinoline (6-AQ) [74], p-aminobenzoic acid [75], 7-aminonapthalene-1,3-disulfonic acid (ANDSA) [76], and 8-aminonapthalene-1,3,6-trisulfonic acid (ANTS) [77]. ANTS can be used as a dye in CE-LIF in conjunction with a He-Cd-laser (λ_{ex} = 325 nm). 8-aminopyrene-1,3,6-trisulfonic acid (APTS), which is synonymous to 9-aminopyrene-1,4,6-trisulfonic acid [78-79], has been employed for CE-LIF using an argon-ion laser (λ_{ex} = 488 nm). Other fluorescent precapillary oligosaccharide labeling reagents are 3-(p-carboxybenzoyl)-quinoline-2-carboxyaldehyde (CBQCA) [80-82], and tetramethylrhodamine (TMR, see section 1.3.4). The structures of these derivatization agents are shown in figure 1.3. With the exception of PMP, these dyes are coupled to carbohydrates *via* reductive amination using sodium cyanoborohydride.

1.3.2 MEKC and buffer additives

Many procedures employ micellar reagents such as sodium cholate, CTAB [83] or SDS. Often, the fluorescent or UV absorbing dye attached for detection is less polar than the carbohydrate analyte, increasing the partitioning coefficient of the analyte molecules into the pseudostationary phase. Organic modifers, such as butanol and octanol [84] have been used to further modify this partitioning coefficient.

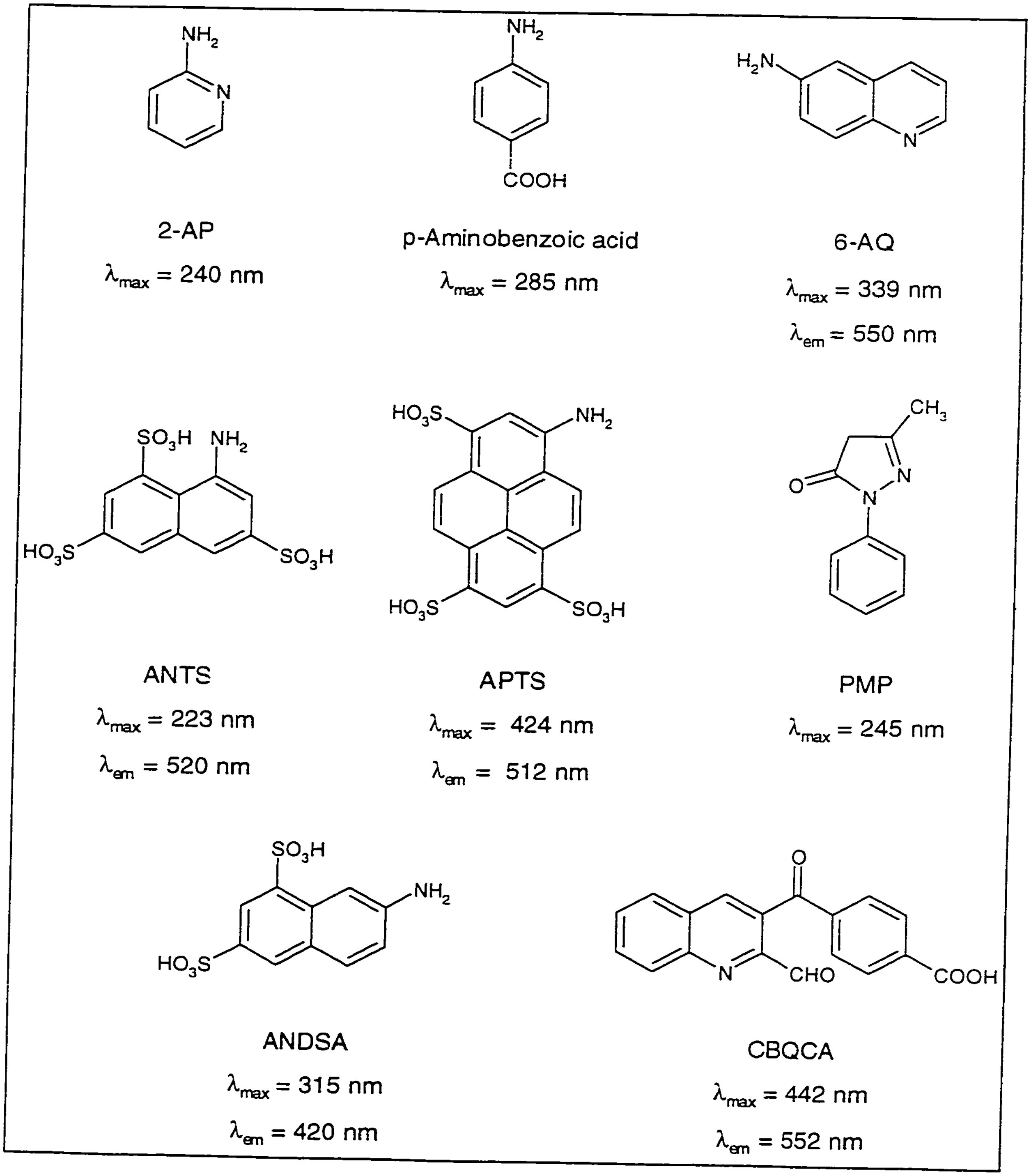

Figure 1.3. Structures of commonly employed carbohydrate labeling reagents

1.3.3 Capillary electrochromatography (CEC)

The analysis of oligosaccharides by CEC has recently been reviewed by Ziad El Rassi [85] for the period of 1997 to 1999. He writes:

> "In summary, although only three papers [86-88] on CEC of carbohydrates have been published during the period covered by this review article, the high separation efficiency and unique selectivity of CEC make it very suitable for the analysis of carbohydrates. Thus, the exploitation of its full potential is yet to come."

In two articles covered by the review, fused-silica capillaries were filled with partially derivatized octadecyl silica particles. Separations were performed in the presence of 20% to 40% acetonitrile (ACN) in the running buffer [86-87]. The authors of the third paper [88] employed a macroporous polyacrylamide/poly(ethylene glycol) column derivatized with C4 alkyl chains and vinylsulfonic acid.

In a recent paper, Suzuki *et al.* [89] derivatized silica particles packed in capillary columns with octadecyltrimethoxysilane, 3-aminopropyltrimethoxysilane and dimethyloctadecyl-trimethoxysilylpropylammonium chloride and separated several PMP derivatives of aldopentose isomers.

1.3.4 Tetramethylrhodamine (TMR) labeled mono- and oligosaccharides

1.3.4.1 Usage as substrates for metabolic cytometry

TMR-labeled oligosaccharides have been used extensively in work done on metabolic cytometry, in which the uptake, biosynthesis, and biodegradation of these standards in single cells were monitored [90-91]. Standards were synthesized (see section 1.3.4.2), and enzymatic products measured by coelution in the CE-LIF electropherogram. The dye molecule attached to the linker arm is also injected to check for possible degradation products.

TMR-labeled oligosaccharides are ideal probes in glycosyltransferase assays. These enzymes are responsible for transferring monosaccharide units from nucleotide donor sugar molecules to other molecules. Examples of such transformations are illustrated in figure 1.4. For TMR-labeled oligosaccharides the limits of detection are below 100 molecules [92-95].

The use of TMR-derivatives has several advantages, among them: Stability of the derivatives to hydrolysis, high fluorescent quantum yields, and an excitation maximum around 550 nm ($\varepsilon = 65000$ L mol^{-1} cm^{-1}) that matches the output of the green helium-neon laser (543.5 nm). Tetramethylrhodamine itself is known to be relative photostable and is often the dye of choice for single molecule detection [92-95].

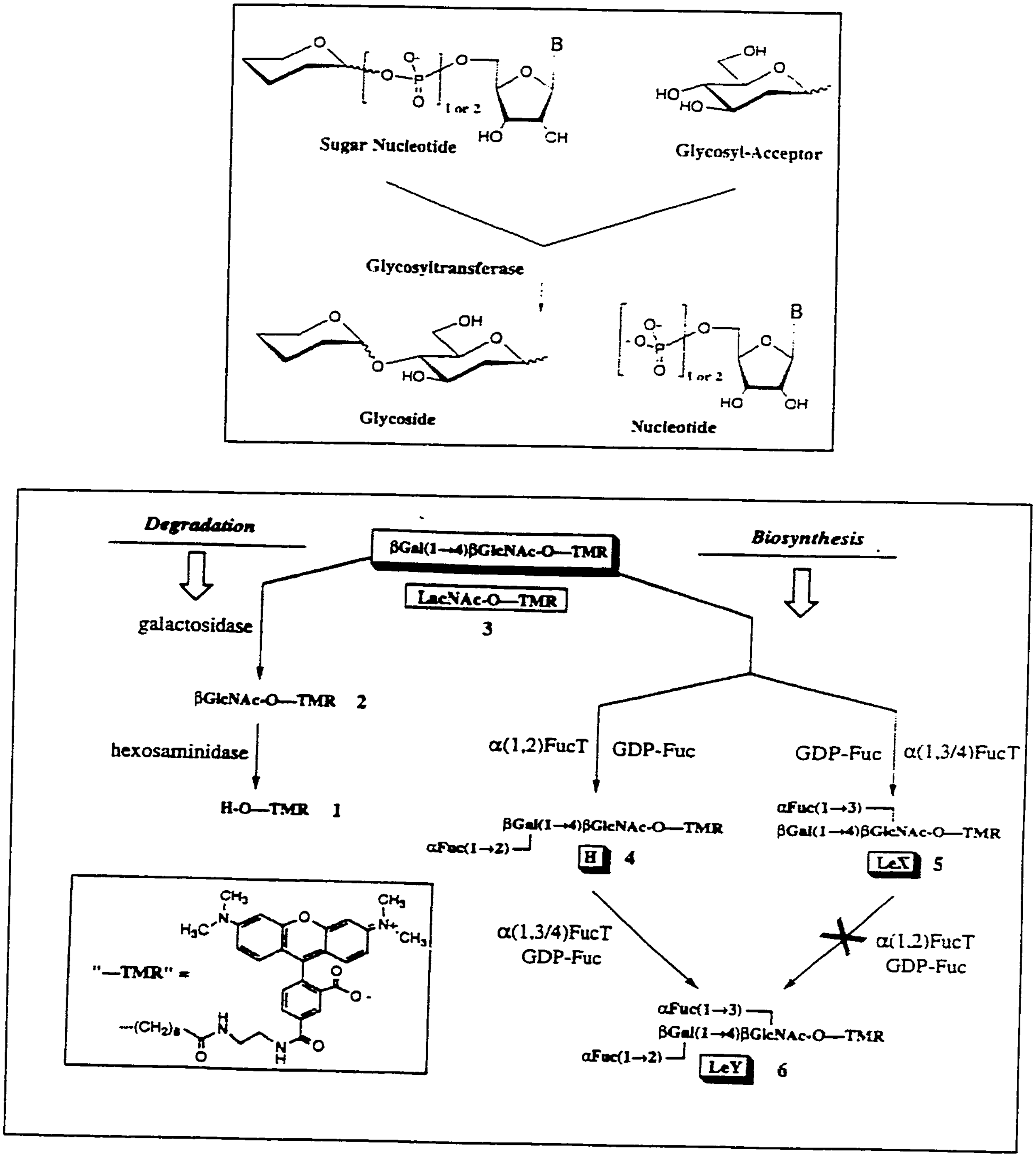

Figure 1.4. Top: The general reaction catalyzed by glycosyltransferases.

Bottom: Some potential biosynthetic transformations of LacNAc-O-LTMR.

Adapted from *Anal. Biochem.* **1995**, 227, 368

1.3.4.2 Structures and Synthesis

The names and structures of the TMR-labeled oligosaccharides used in this thesis are shown in table 1.1 and figure 1.5. They were a gift by Monica Palcic's group, who had synthesized them as published in the literature [93, 96]. Briefly, sugar standards were reacted with a linker arm, 8-methoxycarbonyloctanol, to form the corresponding glycoside, which was treated with ethylenediamine to form sugar-$O(CH_2)_8CO-NHCH_2CH_2NH_2$. This compound was coupled with the commercially available N-hydroxysuccinimide ester of tetramethylrhodamine (TMR). None of the sugar units studied have a free reducing group (a keto- or aldehyde group obtained through ring-opening). In this thesis, the abbreviation sugar–O-LTMR is meant to imply tetramethylrhodamine and the linker arm attached to a particular sugar unit.

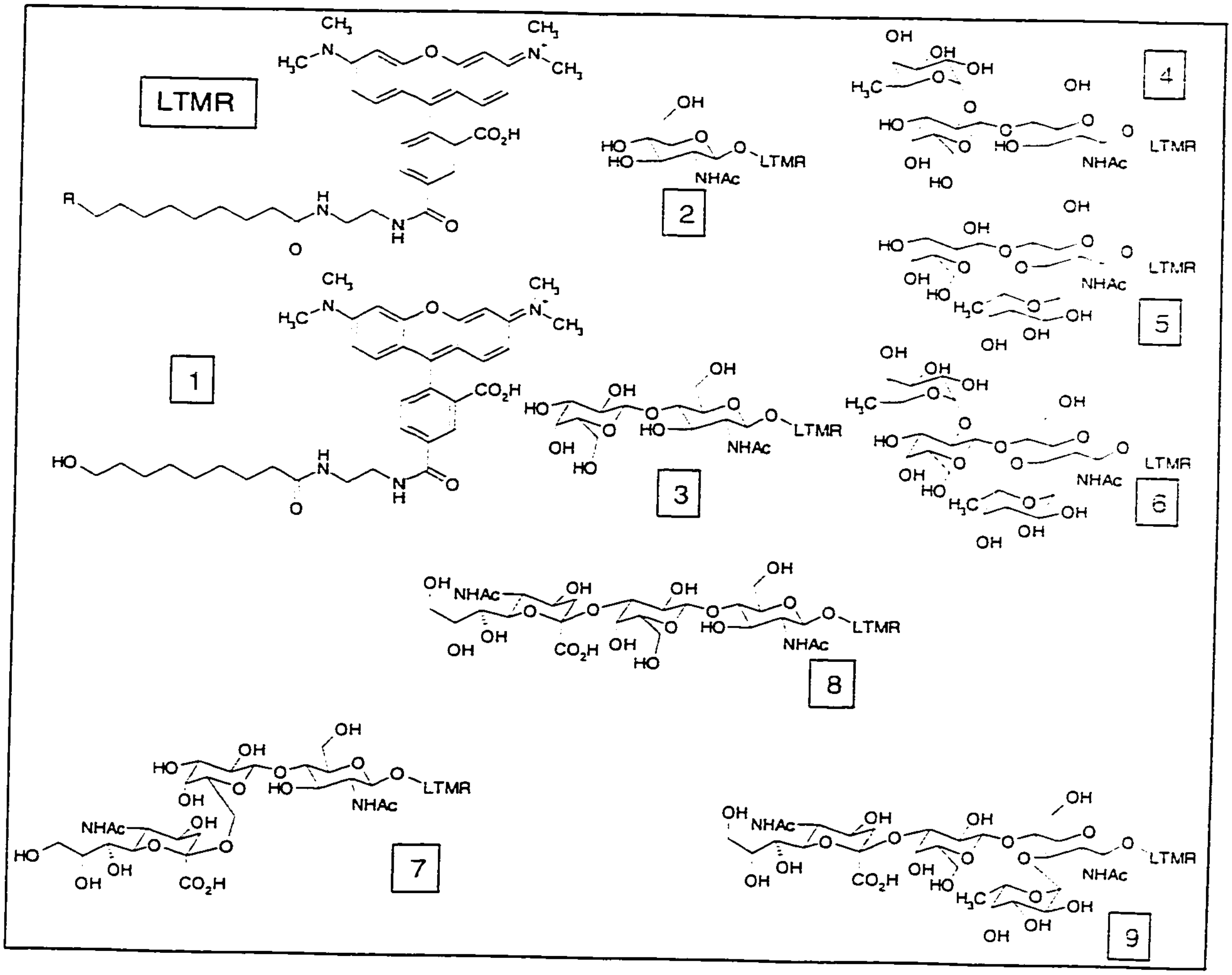

Figure 1.5. Structures of tetramethylrhodamine (TMR) labeled mono- and oligosaccharide standards

Number	Structure	Trivial name
1	HO-LTMR	Linker arm
2	βGlcNAc-O-LTMR	GlcNAc-LTMR
3	βGal(1→4)βGlcNAc-O-LTMR	LacNAc-LTMR
4	αFuc(1→2)βGal(1→4)βGlcNAc-O-LTMR	H-type II-LTMR
5	βGal(1→4)[αFuc(1→3)]βGlcNAc-O-LTMR	Lewis^X-LTMR
6	αFuc(1→2)βGal(1→4)[αFuc(1→3)] βGlcNAc-O-LTMR	Lewis^Y-LTMR
7	αNana(2→6)βGal(1→4)βGlcNAc-O-LTMR	2,6-sialylated LacNAc-LTMR
8	αNana(2→3)βGal(1→4)βGlcNAc-O-LTMR	2,3-sialylated LacNAc-LTMR
9	αNana(2→3)βGal(1→4)[αFuc(1→3)] βGlcNAc-O-LTMR	Sialylated Lewis^X-LTMR

Table 1.1. Names of tetramethylrhodamine (TMR) labeled mono- and oligosaccharide standards

1.4 CE-LIF instrumentation

Capillary electrophoresis was performed with an in-house constructed instrument [97-99]. A schematic of the instrument is shown in figure 1.6. The instrument contains the standard components of any CE instrument: Injection vial, high voltage power supply with a platinum electrode, separation capillary and detector.

Detection limits of single molecules have been achieved with this instrument [24]. Detection was performed with a locally constructed ultrasensitive laser-induced post-column fluorescence detector, based on a sheath-flow cuvette (Figure 1.6). A laser beam was focussed onto the cuvette. Fluorescence was collected at right angles with a 60×, 0.7 NA microscope objective, filtered with a 590DF35 band-pass filter from Omega Optical (Brattleboro, VT, USA), imaged onto an iris to block stray light and detected with a Hamamatsu R1477 photomultiplier tube usually operated at 1000V. The separation voltage was set by a CZE 1000R high voltage power supply (Spellman, Plainview, NY, USA). Data were digitized with a data acquisition board and collected on a Macintosh computer, controlled by Labview (Version, 3.0.1, National Instruments, Austin, TX, USA). The sensitivity of the instrument was limited by the noise introduced due the light scattering of the solvent flowing along the capillary.

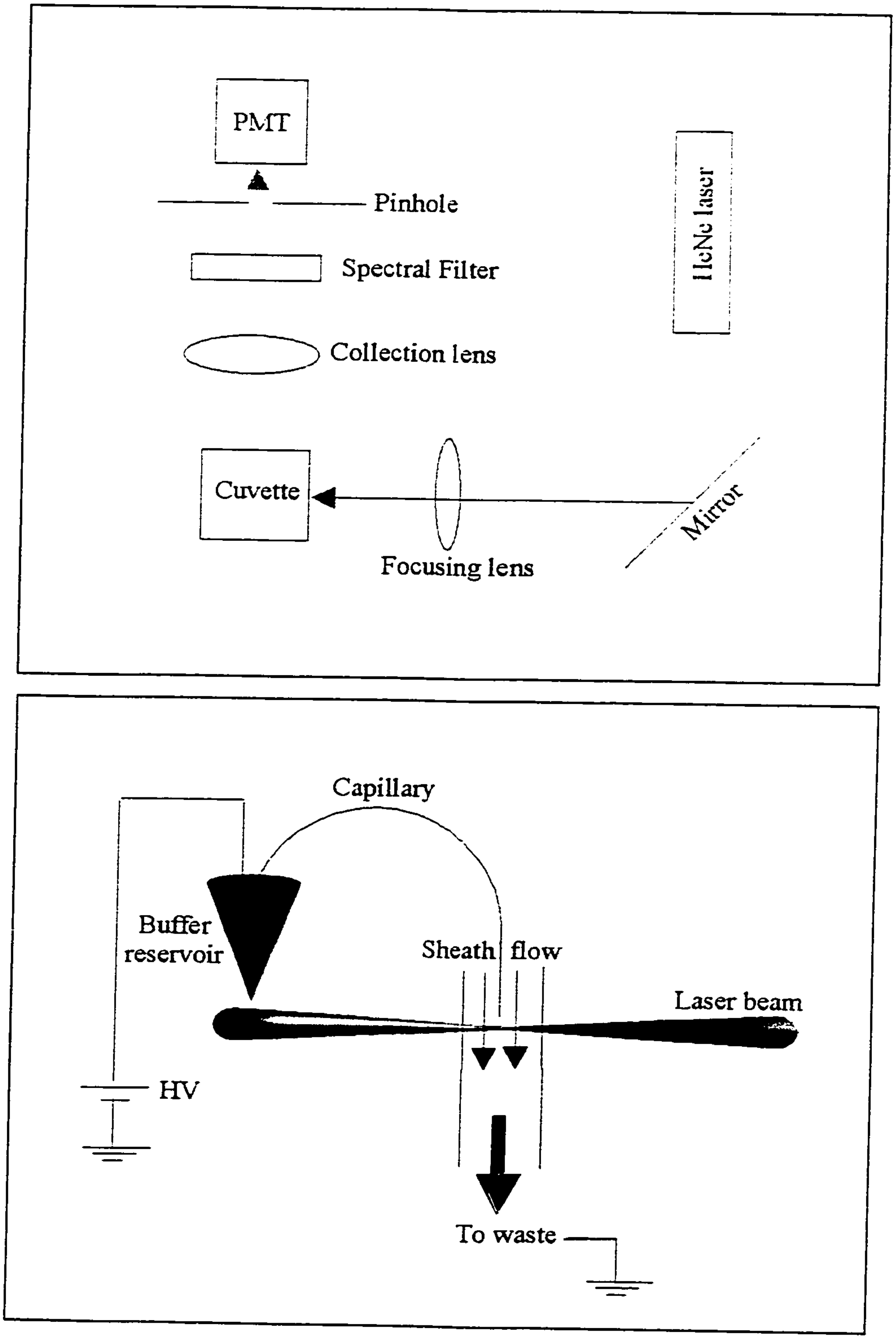

Figure 1.6. Schematic diagram of CE-LIF instrumentation

1.5 Thesis Summary

The separation of carbohydrates by MEKC and CEC is an important analytical technique. The next three chapters present the separation of tetramethylrhodamine labeled mono- and oligosaccharides by MEKC and CEC coupled with laser-induced fluorescence detection.

Chapter 2 presents an improved separation method of TMR-labeled mono- and oligosaccharides by adding ethylamine to the separation buffer consisting of borate, phenylborate, and SDS.

In chapter 3, micellar separation of TMR-labeled mono- and oligosaccharides is investigated further by studying the role of borate and various aromatic boronic acids as well as organic modifiers and alternative surfactants.

Chapter 4 presents several approaches to the separation of TMR-labeled mono- and oligosaccharides by CEC. The applicability of several stationary phases, including ODS and propylamino dervivatized silica, is examined.

Chapter 5 describes some possible future work and concludes the thesis. The applicability of *in situ* modified silica packed capillaries as well as pressurized CEC is discussed, and likely experimental designs are presented.

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Chapter 2: The effects of amine modifiers on carbohydrate separation by MEKC*

* A shorter version of this chapter has been accepted for publication by the Journal of Chromatography A and is currently in press.

2.1 Introduction

The addition of amines to running buffers in oligosaccharide analysis is a frequently used technique. For example, Lamb *et al.* [1] observed improved separation of carbohydrates by CZE upon addition of diethylamine and acetonitrile to the running buffer. Under alkaline conditions (above pH 12.0), underivatized carbohydrates begin to ionize by deprotonation, and separation based on their electrophoretic mobilities becomes possible. However, the pH is traditionally set with sodium hydroxide, which makes the buffer exceedingly conductive at high pH. The high conductivity causes Joule heating, baseline noise, and bandbroadening. Using diethylamine, Lamb *et al.* [1] were able to achieve lower currents at higher pH than previously possible, because the mobility and thus conductivity of sodium is larger than that of the amine. Apart from lower buffer conductivity, the authors identified as favourable factors dynamic coating of the inner capillary walls, which reduced the EOF, and ion-pairing of diethylammonium ion to the carbohydrate anions. The addition of acetonitrile to the running buffer reduced the EOF even further.

In a preceding paper, Scapol *et al.* [2] used triethylamine and acetonitrile as buffer additives and came to the same conclusions as Lamb *et al.* [1].

Zemann *et al.* [3] dynamically reversed the EOF with a polycationic surfactant hexadimethrine bromide to separate a series of monosaccharides at high pH. The main advantage of EOF reversal is separation speed: Negatively charged sugars now migrate in the same direction as the EOF, towards the anode. Zemann *et al.* [3] also added acetone to the running buffer to reduce the net velocity of the analytes with slight changes in selectivity, which were attributed to changes to solvation shells and thus effective ionic radii. Borate was used as additional buffer ingredient.

Stefansson *et al.* [4] used CTAB above its CMC and the polycation polybrene at high pH in the presence of organic modifiers methanol or acetonitrile to separate several glycosides. They thus performed MEKC with a positive surfactant under reversed EOF conditions.

The work presented in this chapter got started with an observation by Keiko Sujino. In her experiments she observed that the separation of TMR-labeled mono- and oligosaccharide standards significantly improved upon addition of ethylamine to the running buffer. In this chapter, her observations were reinvestigated: TMR-labeled mono- and oligosaccharides were separated in a buffer containing the negatively charged SDS as surfactant and ethylamine as amine modifier. The polarity was altered as a function of pH. The effects of adding a variety of other simple amines were also investigated.

2.2 Experimental

2.2.1 Materials

250 mM stock solutions including sodium tetraborate (J.T. Baker, Philipsburg, NJ, USA), disodium phosphate (Fisher, Fair Lawn, NJ, USA), sodium dodecyl sulfate (BDH Biochemicals, Toronto, ON, Canada) and phenyl boronic acid (Sigma, St. Louis, MO, USA) were prepared using deionized water (Barnstead NANOpure water system) and filtered using 0.22 µm pore size filters (Biorad, Mississauga ON, Canada). Phenylboronic acid was dissolved with an equivalent amount of NaOH. The amines added were either pure (propyl- and butylamine) or aqueous solutions (methyl- and ethylamine) supplied by Aldrich (Milwaukee, WI, USA). The pH of the buffers was adjusted using 0.10 M NaOH or 0.50 M HCl solutions. Sample solutions were 2.5 nM in running buffer.

2.2.2 Capillary electrophoresis

Separations were performed in a 31-cm long, 10-µm I.D., and 150-µm O.D. fused-silica capillary (Polymicro, Phoenix, AZ, USA) at ambient temperature. The separation buffers typically contained 10 mM disodium phosphate, 10 mM SDS, 10 mM borate and 10 mM phenylborate. Samples were injected hydrodynamically (10 cm height difference, 90 s unless stated otherwise). Separations were performed at 400 V/cm. Data were analyzed using the software package Matlab (The Mathworks, Natick, MA, USA) and convoluted with a 0.15 s standard deviation Gaussian filter before presentation.

2.3 Results

2.3.1 Separation in the absence of ethylamine at pH 12.0

In the absence of amine modifier all peaks elute in a 2-minute window (Figure 2.1, top trace). Similar separation behavior was observed at lower pH (not shown). Larger, less hydrophobic (#7 - #9) molecules elute first. The variation in peak height is due to variation in analyte concentration.

2.3.2 Separation in the presence of ethylamine at pH 12.0

A sample electropherogram at pH 12.0 with an ethylamine buffer is shown in the bottom trace of Figure 2.1. The elution order of two of the sialylated compounds is reversed. In general, the elution times increased when ethylamine was added. Migration times in the presence of ethylamine depended on capillary history, varying over time (data not shown).

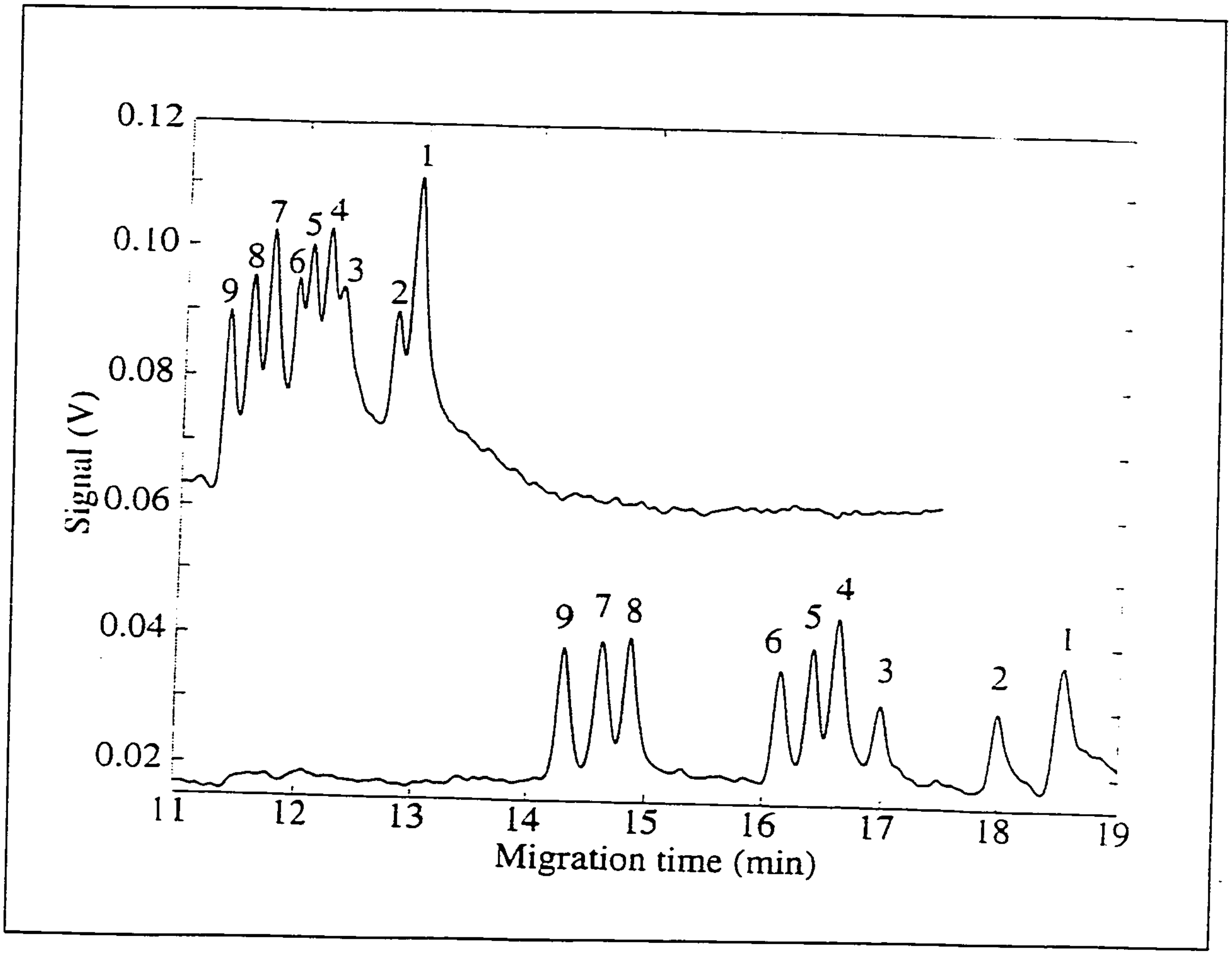

Figure 2.1. Separation of 2.5 nM mono- and oligosaccharides in 10 mM borate. 10 mM phenylborate, 10 mM SDS, 10 mM Na_2HPO_4, pH 12.0 buffer. Separation was in a 31 cm long, 10 µm I.D., 150 µm O.D., bare silica capillary at 400 V/cm. Current was 1.7 µA. Detection was by LIF, excitation at 543 nm and emission at 580 nm. Top trace: No ethylamine added. Bottom trace: 360 mM ethylamine added. Numbers in the Figure correspond to compounds listed in Table 1.1.

2.3.3 Separation in the presence of ethylamine at pH 10.2 (reversed polarity)

A typical trace generated at pH 10.2 is shown in Figure 2.2. Similar electropherograms with shorter migration times were obtained at pH 9.0 and pH 9.8 (data not shown). Migration times were generally more reproducible at pH 10 than at pH 12. The elution order reversed compared with pH 12 data – more hydrophobic compounds elute first. Most carbohydrate-bearing compounds show satellite peaks at slightly faster migration times.

2.3.4 Comparison of methyl-, ethyl-, propyl- and butylamine at pH 12.1

The electropherograms generated at pH 12.1 are shown in Figure 2.3. Addition of methylamine yields sharper but less resolved peaks than the addition of ethylamine. The buffer containing propylamine yields broader, faster migrating peaks, with some loss of separation. The resolution breaks down completely when butylamine is added.

2.3.5 Effect of setting pH with NH_3 instead of NaOH

In figure 2.4, the results of setting the pH to 11.9 using the bases NaOH and NH_3 are compared. The data in figure 2.4 were analyzed using the software package Igor Pro (Wavemetrics, Lake Oswego, OR, USA) and smoothed with a Savitzky-Golay 4th order filter (25 data points).

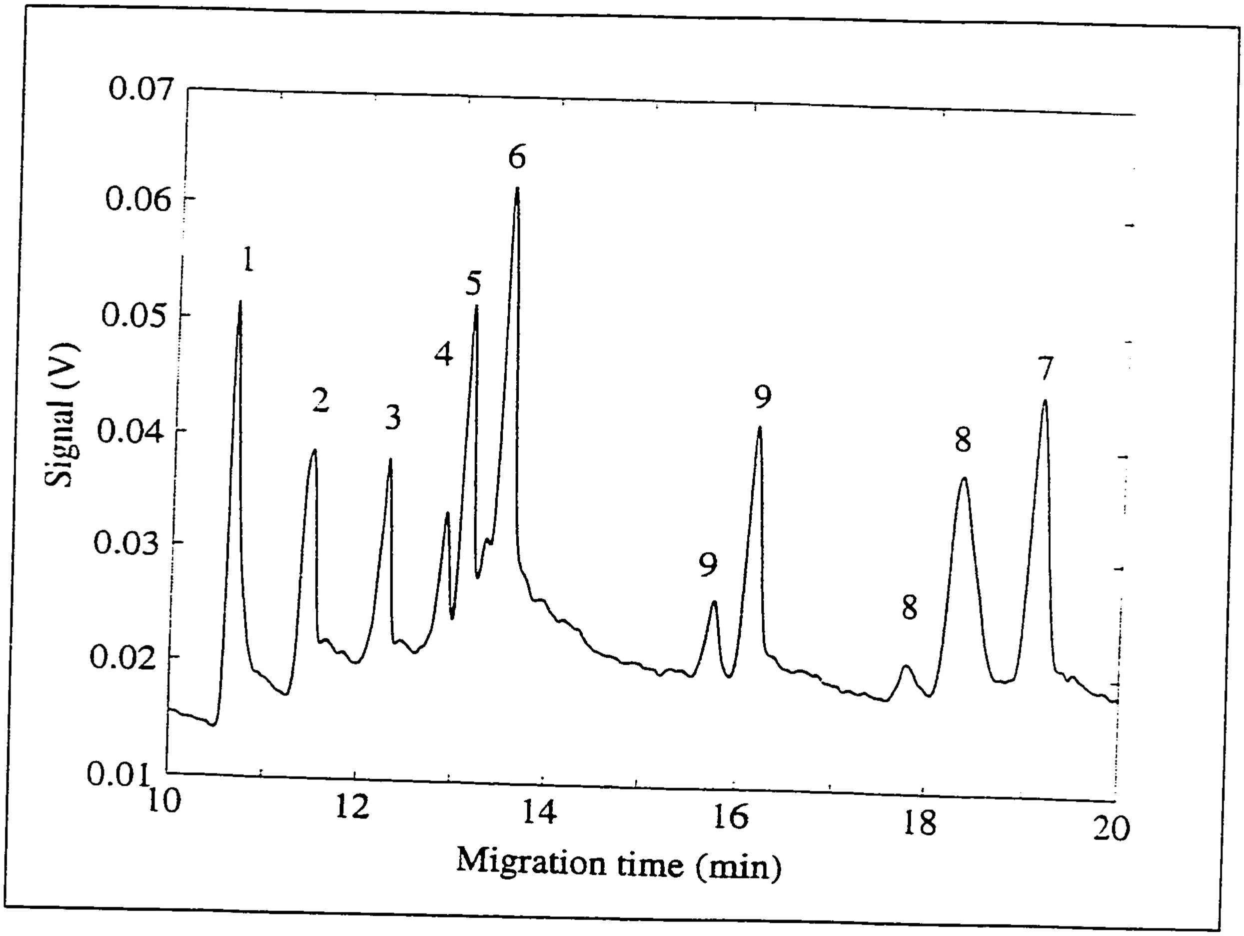

Figure 2.2. Separation in reversed polarity mode of 2.5 nM mono- and oligosaccharides in 10 mM borate, 10 mM phenylborate, 10 mM SDS, 10 mM Na_2HPO_4, 360 mM $EtNH_3Cl$, pH 10.2 buffer adjusted with 0.10 M NaOH. Separation was at 400 V/cm. Current was 10.3 μA. Injection was by siphoning, 10 cm height difference, for 360 s.

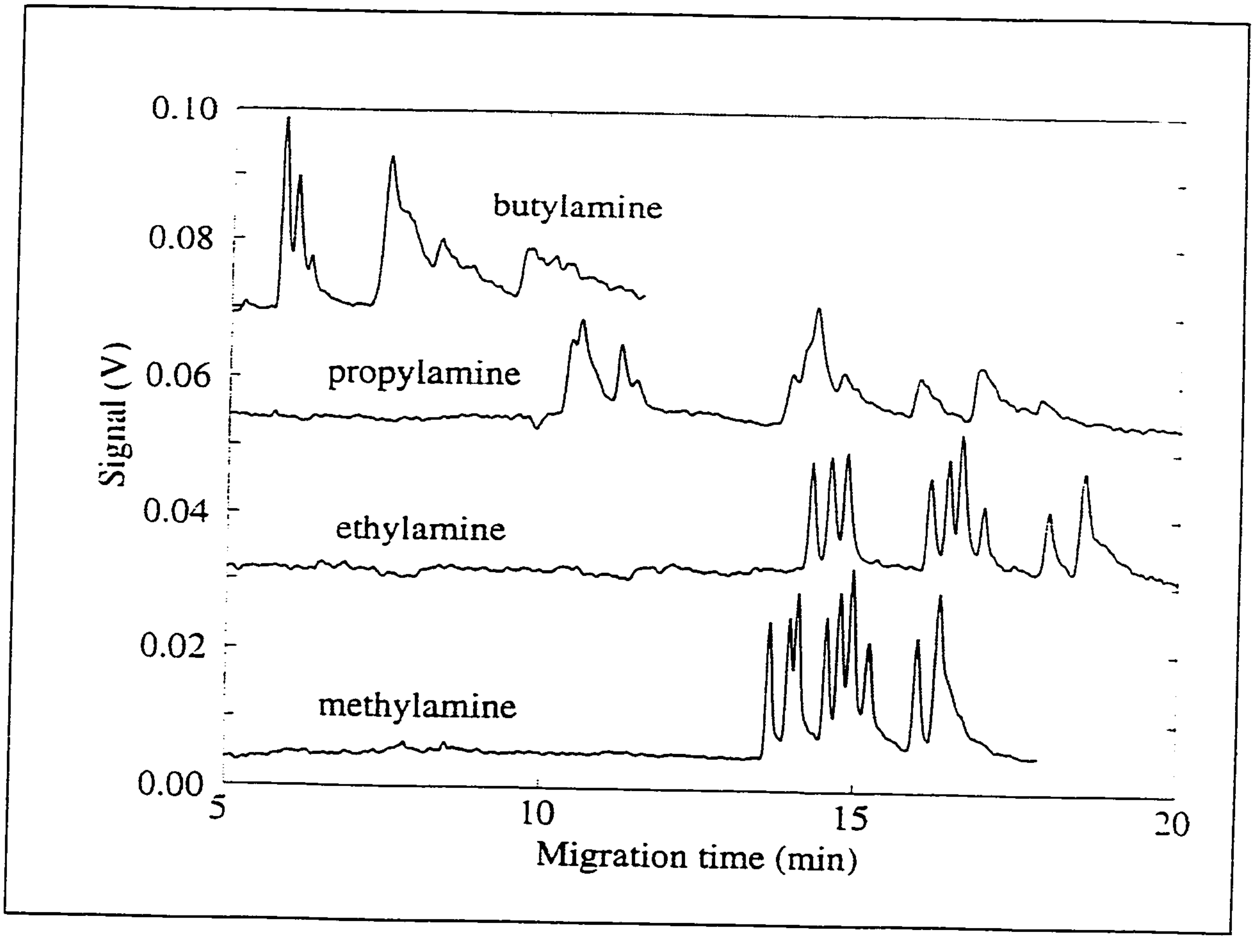

Figure 2.3. Effect of adding equal concentrations of a series of monoamines to the running buffer at pH 12.1. Conditions are otherwise the same as in Figure 2.1.

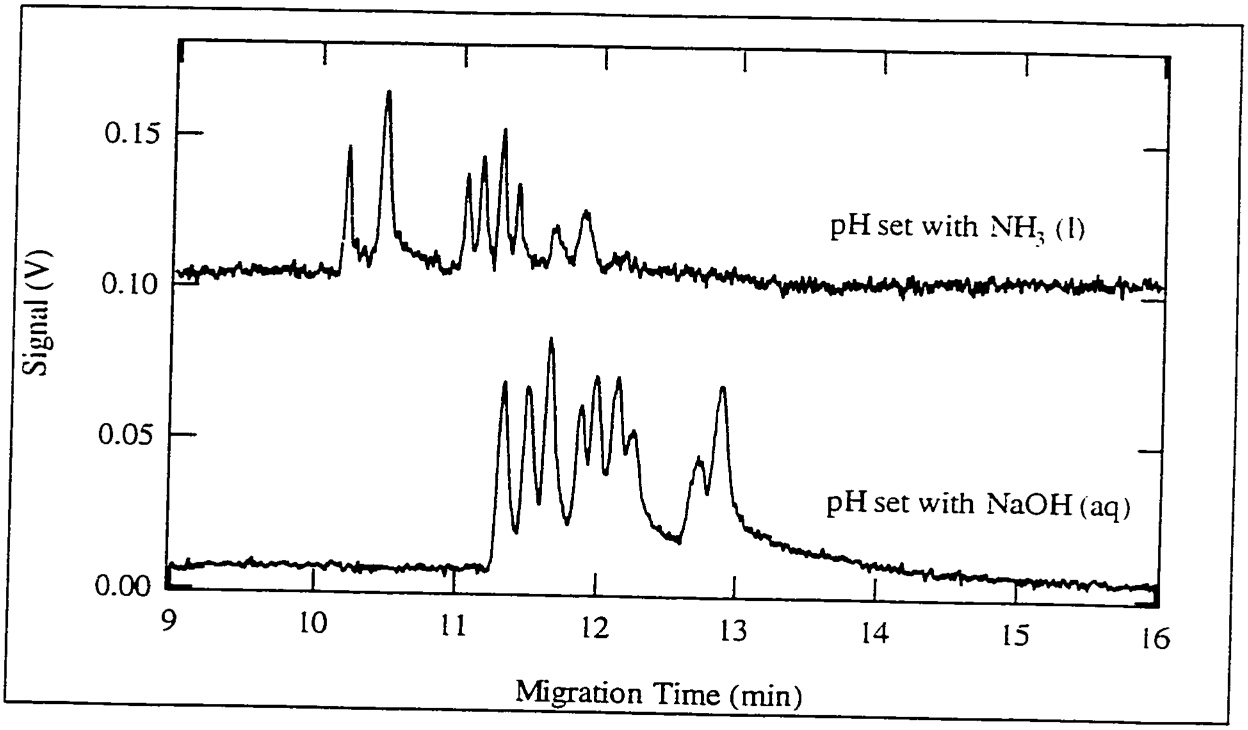

Figure 2.4. Effect of setting the pH with NH_3 instead of NaOH. Conditions are the same as in figure 2.1, top trace. The pH was set to 11.9.

2.4 Discussion

2.4.1 Dynamic coating of capillary wall

Ethylammonium cation has a pKa of 10.8. At pH 12.0, roughly 10% of the compound is in the acid form. The capillary walls are dynamically coated with the residual ethylammonium ion. Ammonium cations adsorbing to the capillary wall are known to affect the EOF [5]. The dynamic nature of the coating process and the dissolution of the capillary wall explain the observed drifts in the migration time. We measured the migration time of a neutral marker by injecting a short plug of 15% methanol in running buffer. Migration of the methanol into the fluorescence detector causes a refractive index perturbation that scatters the laser beam, which generates a peak in the fluorescence signal. The observed migration times were 168 seconds and 184 seconds in the absence and presence of ethylamine, respectively. This increase in migration time is too small to account for the improved resolution observed in Figure 2.1. However, the capillary walls become more hydrophobic in the presence of amine.

At pH 10.2, the majority of the additive is present as the weak acid, and sufficient ethylammonium is adsorbed to the capillary wall so that the EOF is reduced to near zero. In Figure 2.2, the net movement of the analytes is in same direction as the movement of the SDS micelles. The order of elution is therefore reversed compared to higher pH.

2.4.2 Increased partitioning coefficient into pseudostationary phase

As stated above, the change in EOF alone does not account for the change in resolution of the analytes. The improved resolution in the presence of ethylamine (Figure 2.1) can be explained by changes in the partitioning coefficient into the SDS micelles. We discuss several hypotheses:

2.4.2.1 Amine-analyte ion pair mechanism

It is plausible that improved resolution is due to an ion-pairing mechanism as was postulated for the buffer additives diethylamine [1], triethylamine [2], TRIS [6] and tetrabutylammonium ion [7]. When the pH of the running buffer was adjusted with NH_4OH (aq) instead of NaOH (aq) in the absence of ethylamine (Figure 2.4), compounds 7 and 8 co-migrate, indicating that some ion-pairing to the sialic acids occurs. In the presence of ammonia, a slightly lower EOF is expected due to wall interactions. On the other hand, the analytes elute earlier (Figure 2.4), indicating a charge reduction of the negative borate complexes. The more negatively charged, the slower the analytes' movement towards the detector. In the presence of ethylamine the analytes elute later. This is because the pK_a is much higher for ammonia than for ethylamine. At pH 12, most of the ammonia is present as the ammonium cation, and most of the ethylamine is present as the conjugate base. In addition, the breakdown of resolution in the presence of propyl- and butylamine indicates that ion-pairing alone does not provide a sufficient explanation for the improved resolution with ethylamine.

2.4.2.2 Amine-micelle partitioning competes with analyte-micelle partitioning

The amines, being present at relatively high concentration, likely partition into the micelles, causing the micelles to swell. Charge reduction of the micelles by the amines was not a significant factor, since the migration times of the analytes increased in the presence of the small amines. However, competitive partitioning between the analyte and the amine is consistent with the observation that the smaller, less polar analyte (compounds 1 and 2), which partitioned into the micelles more efficiently, are better resolved than the larger, more polar molecules. In the presence of more hydrophobic propyl- and butylamine the partitioning then would become so inefficient for the analytes that the resolution breaks down.

2.4.2.3 Amine-borate complexation changes hydrophobicity of borate-analyte complex

Boric and phenylboronic acids require the presence of hydroxide ion in order to efficiently complex the cis-diols occurring in oligosacchararides [8]. In other words, the complexing species is the borate or phenylboronate anion (Figure 2.5, structure I). It is known [9] that amines form complexes with borates as depicted in Figure 2.5, structure III. This complex may also form an ester with a cis-diol, giving rise to structures IV and V in Figure 2.5. The presence of the hydrophobic R group increases the overall hydrophobicity of the borate-analyte complex. Therefore, the partitioning coefficient increases. We speculate that the neutral species (structure IV) is the dominant species at pH 10. This molecule is expected to partition preferentially into the micelles. where it would be protected from exchange with polar hydroxy ion.

2.4.3 Satellite peaks

Addition of ethylamine to the separation buffer caused the formation of small leading peaks for the largest oligosaccharides. The origin of these satellite peaks is not clear. but may reflect the presence of structural isomers of the labeling dye molecule.

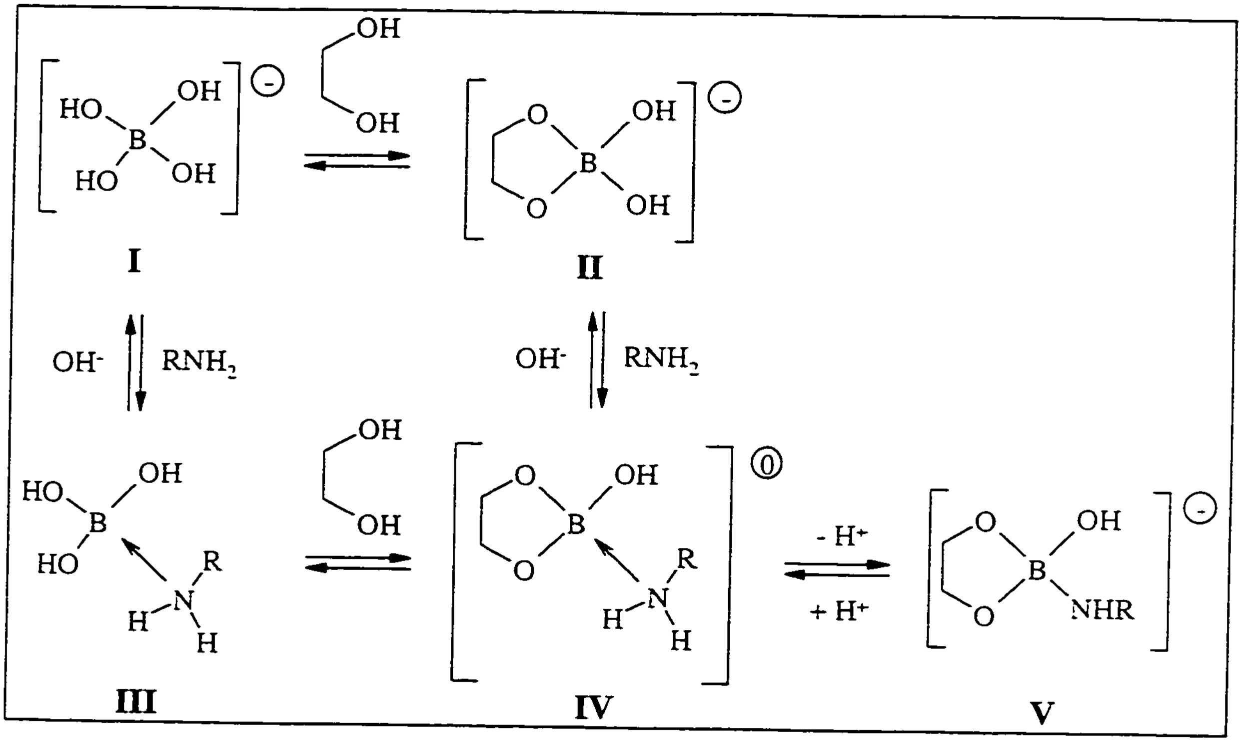

Figure 2.5. Postulated mechanism for the complexation of borate with cis-diols in the presence of an amine

2.5 Conclusions

The addition of ethylamine to the running buffer significantly improved the separation. Unfortunately, the buffer make-up is quite complex, and attempts to eliminate some of the buffer components resulted in loss of resolution. We find it undesirable to operate at pH 12 and to employ dynamic coatings because of fluctuating migration times. The poorer separation in the presence of propylamine in contrast to ethylamine suggests that the use of the more nonpolar and substituted diethyl-, dipropyl-, triethylamine, etc. as modifiers would not improve the separation.

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CHAPTER 3: EFFECTS OF BORONIC ACIDS AND ORGANIC MODIFIERS IN THE ANALYSIS OF CARBOHYDRATES BY MEKC

3.1 Introduction

In capillary electrophoresis, molecules are separated on the basis of their electrophoretic mobility in an applied electric field. In order to separate neutral molecules, charge must be introduced to them. This is usually done by adduct formation, by micelles, or both. For example, it is well known that neutral oligosaccharides bearing cis-diol groups can be complexed *in situ* with borate, which induces charge to the molecules [1]. In a previous publication from this research group, we observed that the addition of phenylboronic acid to a separation buffer containing 10 mM SDS as well as 10 mM boric acid enhanced the resolution of an oligosaccharide mixture of type βGal(1→4)βGlcNAc-O-LTMR. It was hypothesized that the hydrophobicity of the phenyl group increased the partitioning coefficient of the resulting complex into the SDS micelles [2-3].

The analyte-micelle partitioning coefficient and therefore the migration time window size is known to be affected by the addition of organic modifiers [4-6] or by varying surfactant concentration and identity. This is because all neutral analytes inevitably elute in a window bracketed by compounds with $\tilde{k}' = 0$ and $\tilde{k}' = \infty$, i.e. fully unretained and fully retained compounds. The migration time window size can be quantified by the ratio of the micelle migration time t_{mc} divided by the migration time of an unretained compound t_0 [4]. It is trivial to show that

$$\frac{t_{mc}}{t_0} = \frac{1}{1 - \frac{\mu_{ep.mc}}{\mu_{eo}}} \qquad (10)$$

In a recent study [4], it was found that the logarithm of the partitioning coefficient $\tilde{k}'$, the micellar electrophoretic mobility $\mu_{ep.mc}$ and the electroosmotic mobility μ_{eo} are generally inversely proportional to the organic modifier content. The mobility ratio R. which is defined as the ratio of $\mu_{ep.mc}$ over μ_{eo}, was found to be proportional to organic modifier concentration. Thus, by increasing the organic modifier content. the migration time window can be enlarged.

In this chapter, the hypothesis on the effect of phenylboronic acid was reinvestigated by studying the effects of adding other boronic acids on the separation of the oligosaccharide standards, in particular napthaleneboronic acid and 4-carboxyphenylboronic acid. If the original hypothesis were correct, the increased hydrophobicity of naphthalene- in comparison to phenylboronic acid would be expected to result in an increased micelle partitioning coefficient, which in turn would drastically affect the separation. In addition, the effects of adding organic modifiers to the running buffer were investigated. It was also attempted to improve the separation by switching to the alternative surfactants octanesulfonic acid, tetradeyltrimethylammonium bromide (TTAB), and Triton X.

3.2 Experimental

3.2.1 Materials

A set of 250 mM and 50 mM stock solutions including sodium tetraborate (J.T. Baker. Philipsburg, NJ, USA), sodium dodecyl sulfate (BDH Biochemicals. Toronto. ON. Canada), phenylboronic acid (Sigma, St. Louis, MO. USA), sodium octanesulfonate (Aldrich, Milwauki, WI, USA), TTAB (Sigma, St. Louis, MO, USA) and 1-napthaleneboronic acid (Lancaster, Windham, NH, USA) was prepared using deionized water and filtered using 0.22 μm pore size filters (Biorad, Mississauga, ON. Canada). The pH of the buffers was adjusted to 9.0 by addition of NaOH solution before the addition of organic modifiers. Because of solubility issues, napththaleneboronic acid solutions have a pH of ≥10. Triton X (Sigma, St. Louis, MO, USA) was added in neat form to the running buffer. The TMR-labeled oligosaccharides were dissolved in running buffer at 25 nM (unlesss stated otherwise).

3.2.2 Capillary electrophoresis

Capillary electrophoresis was performed in 30-cm long, 10-μm I.D., and 150-μm O.D. fused-silica capillaries (Polymicro, Phoenix, AZ, USA) at ambient temperature. Samples were injected hydrodynamically (10 cm height difference, 60 s unless stated otherwise). The separation voltage was 400 V/cm. Data were analyzed with the software package Igor Pro (Wavemetrics, Lake Oswego, OR, USA) and presented without modification.

3.3 Results and Discussion

3.3.1 Separation in the presence of 10 mM SDS

In previous studies [3,7] separation was achieved by employing a mixture of 10 mM boric acid and 10 mM phenylboronic acid at slightly higher ionic strength. As can be seen in this study, changing from 10 mM boric acid to 10 mM phenylboronic acid and naphthaleneboronic acid in the presence of 10 mM SDS results only in minor changes in the migration times and no improvement in resolution (Figure 3.1). Peak-tailing due to analyte adsorption to the inner capillary walls sometimes becomes a problem under these separation conditions (data not shown).

The electroosmotic flow (EOF) was measured at 2.5 mm sec^{-1} by spiking the sample with methanol. The change in light scattering due to the organic modifier can be detected with the sheath flow detection system employed. Migration times are slightly higher in the presence of naphthaleneboronic acid, which is most likely due to the difference in ionic strength.

In the presence of 4-carboxyphenylboronic acid the migration times are increased, because of the formation of a doubly negatively charged complex. Also, the EOF is slightly lowered to 2.3 mm sec^{-1}, because the ionic strength at pH 9.0 is obviously higher for the difunctional buffer. The resolution in the presence of 4-carboxyphenylboronic acid is improved in comparison to the other boronic acids.

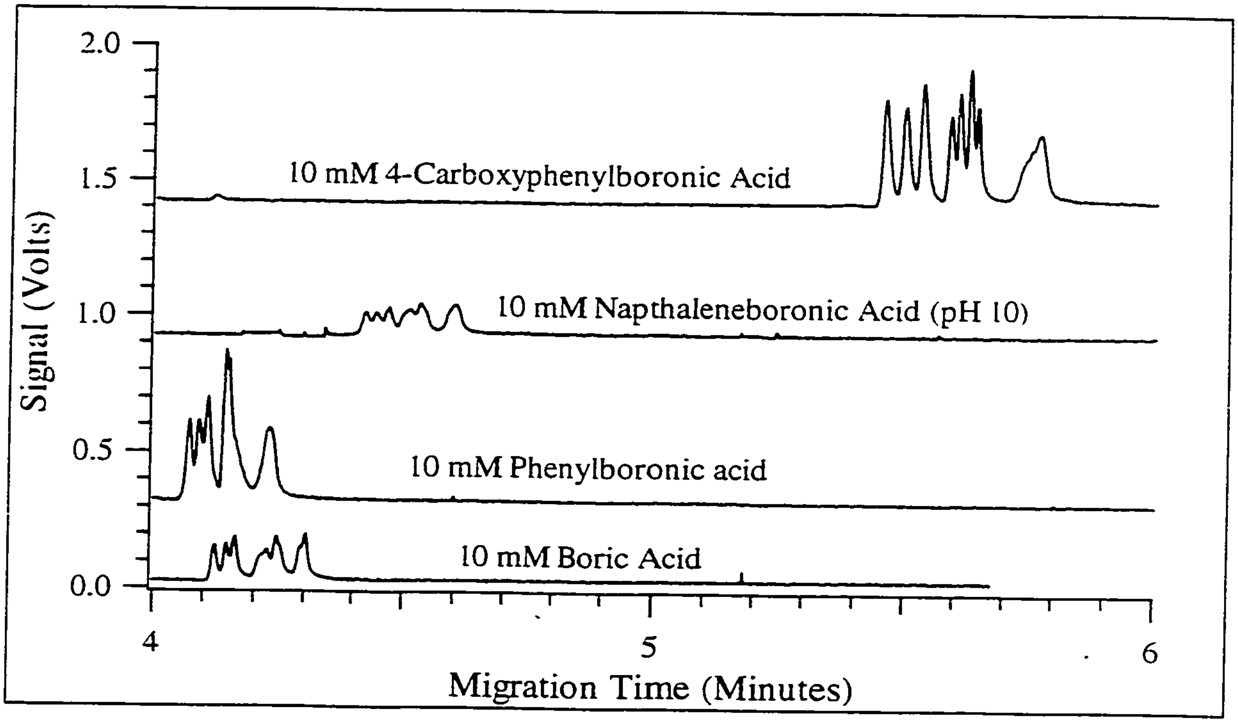

Figure 3.1. Separation of nine 25 nM oligosaccharide standards in 10 mM SDS, pH 9.0 buffer, containing the compound indicated. Separation was in a 30 cm long, 10 µm I.D., 150 µm O.D. bare silica capillary at 400 V/cm. Detection was by LIF, excitation at 543 nm and emission at 580 nm. Elution order is the same as in figure 2.1.

3.3.2 Separation in the presence of 50 mM SDS

In the presence of 50 mM SDS all the migration times are increased and the resolutions are improved (Figure 3.2). However, the change from boric to phenylboronic and napththaleneboronic acid has very little impact on the resolution and selectivity. The improvement is due the presence of more micelles, which yields a better phase ratio. The EOF is decreased only marginally, even though the ionic strength is increased almost five-fold.

3.3.3. Separation in the presence of 50 mM SDS and 10% (v/v) methanol

Greatly improved resolution was achieved when methanol was added to the separation buffer (Figure 3.3). In the prescence of 10% methanol, the EOF is reduced to 1.8 mm sec^{-1}. Similar results were obtained with ethanol. The effects of adding organic modifiers in MEKC are well-documented in the literature [4-6]. Organic modifiers are known to reduce EOF by changing the zeta-potential and increasing the buffer viscosity, and to expand the migration time window. The elutropic strength order of organic modifiers in MEKC has recently [4] been determined to follow urea < methanol < acetonitrile < isopropanol < dioxane. The strength of the modifiers to suppress EOF follows the order urea < methanol, acetonitrile < isopropanol, dioxane. Separations were attempted with the modifiers dioxane and isopropanol at low concentrations. However, migration times became very long and the selectivity, i.e. the partitioning coefficient $\tilde{k}'$, was not significantly improved (data not shown).

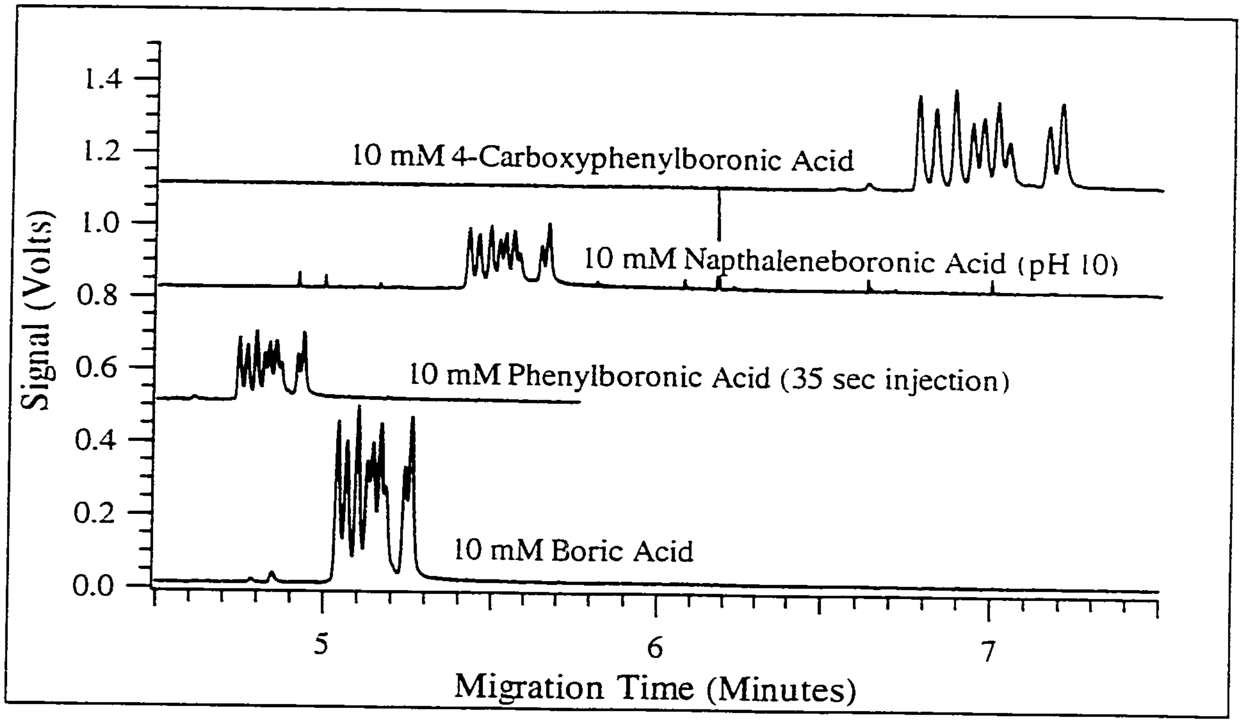

Figure 3.2. Separation of 25 nM oligosaccharides in 50 mM SDS. Spikes on the traces are due to small bubbles eluting in the running buffer. Other conditions were the same as in the previous figure.

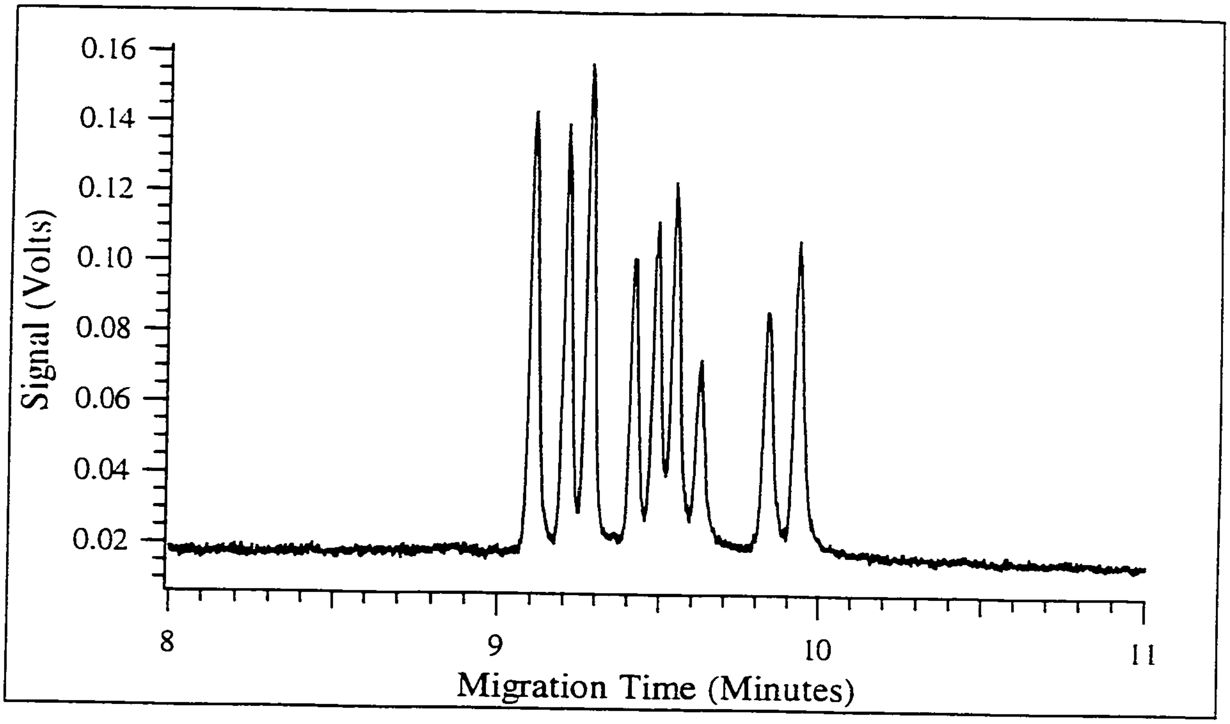

Figure 3.3. Separation of 25 nM mono- and oligosaccharide standards in 10 mM borate, 50 mM SDS, pH 9.0 buffer, containing 10% (v/v) methanol. Average efficiency 1.5 million plates per meter.

3.3.4 Alternative surfactants

3.3.4.1 Separation in the presence of 50 mM octanesulfonic acid

The electropherograms obtained in 10 mM borate and 50 mM octanesulfonic acid at pH 9.2 exhibit poor resolution and tailing (Figure 3.4, top trace). The compounds eluted faster than in a comparable SDS buffer. The sialic acid bearing carbohydrates are separated from the remaining molecules, due to their higher charge-to-size ratio. The poor resolution is consistent with reduced micellar retention. The shorter chain length of octanesulfonic acid should cause the formation of smaller micelles, and therefore drastically reduce the partitioning coefficient of analyte molecules into these micelles.

Better separation was achieved when the EOF was reduced by the presence of 100 mM borate and 20% methanol (Figure 3.4, center trace). Compared to the use of SDS, the order of migration is reversed. This is again consistent with relative poor micellar partitioning. Unfortunately, the reproducibility of the separation using octanesulfonic acid in terms of migration times was generally poor, so that not all peaks could be identified. Still, there is an apparent change in selectivity. It can be interpreted as faster migration of Lewis^X (#5) and Lewis^Y (#6) relative to the other compounds. The reason for this behaviour is not understood.

In the absence of sodium octanesulfonate (Figure 3.4, bottom trace), much stronger peak tailing was observed, proving that partitioning into octanesulfonic acid micelles took place in the previous runs.

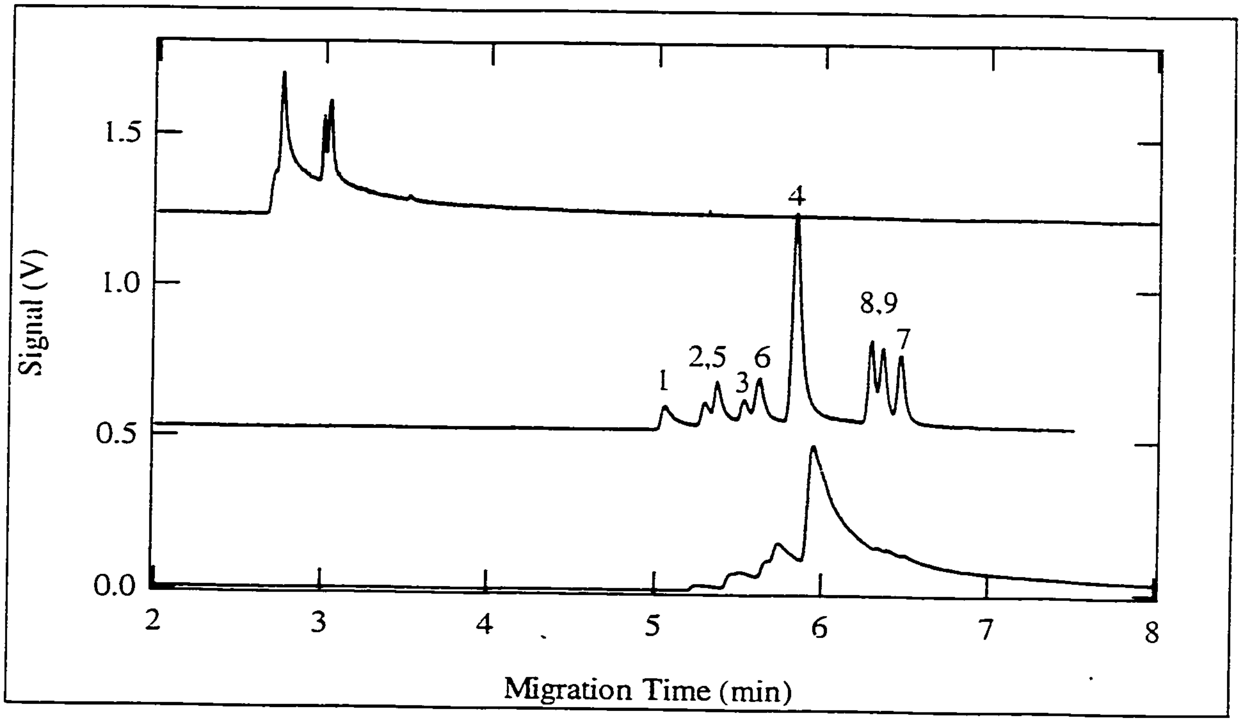

Figure 3.4. Effect of octanesulfonic acid on the separation of 25 nM mono- and oligosaccharide (#4: 100 nM) standards at 400 V/cm in a 30 cm, 10 μm I.D., 150 μm O.D. fused-silica capillary. Top trace: Injection 3 min. Buffer: 10 mM borate, 50 mM sodium octanesulfonate, pH 9.2. Center trace: Injection 2 min, Buffer: 100 mM borate, 20% MeOH, pH 9.2. 50 mM sodium octanesulfonate in sheath flow. Compounds were identified by spiking. Bottom trace: Same as centre trace but no sodium octanesulfonate in sheath flow buffer. Compounds 3-5 spiked to 100 nM.

3.3.4.2 Separation in the presence of triton X

In the presence of a 5% aqueous solution of the neutral surfactant Triton X containing 50 mM borate at pH 9.2, all standards coeluted in one strong tailing peak (data not shown). This is not surprising, since the neutral micelles should move at the same speed as the EOF, and therefore do not act as a pseudostationary phase.

3.3.4.3 Separation in the presence of TTAB

In reversed polarity mode, the separation of the standards in 25 mM Borate and 50 mM TTAB buffer (pH 9.2) was similar but not as efficient as in SDS (Figure 3.5). Due to strong ion-pairing, the analyte-micelle exchange kinetics is possibly slow, which leads to broader peaks and some loss in selectivity. Due to the dynamic nature of capillary coating, migration time reproducibility was poorer than in SDS buffer. Because of EOF reversal, the positive micelles electrophorese in opposite direction than the negative analyte-borate complexes, which, in contrast to the separation in SDS, electrophorese towards the detector. This has implications for the migration time window size: Smaller sugar compounds, which have a larger electrophoretic mobility due to increased charge-to-size ratio, are more strongly retained by the micelles, because they contain fewer polar sugar units. Thus, the two effects partially cancel each other, and the separation is not improved in comparison to SDS. In SDS buffer, the stronger retained smaller compounds electrophorese faster away from the detector, widening the gap to the larger compounds.

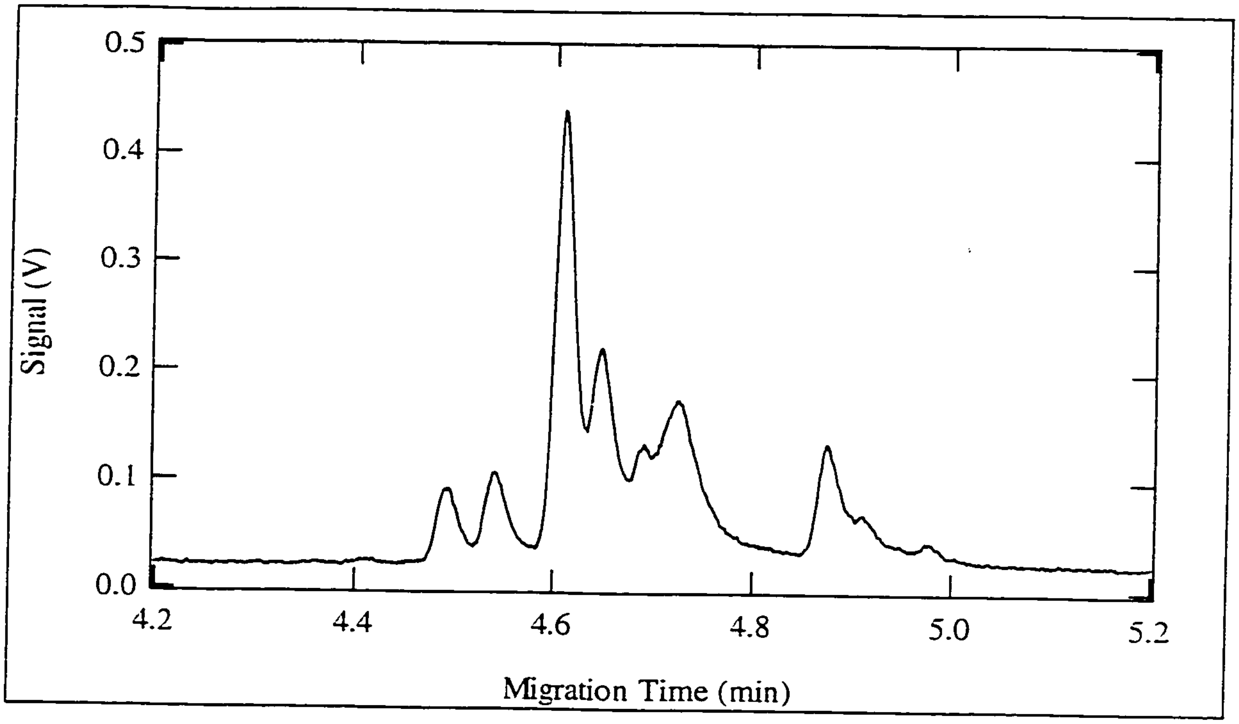

Figure 3.5. Separation at 400 V/cm (reversed polarity) of 6.8 nM mono- and oligosaccharide standards (#4 – 32 nM) in 50 mM TTAB and 25 mM borate, pH 9.2. Injection 2 min.

3.4 Conclusions

In contrast to a previously published hypothesis by the same research group, the addition of aromatic boronic acids to the running buffer *per se* does not improve separation of oligosaccharides by MEKC. Baseline resolution could be achieved with only boric acid as complexing agent. 4-Carboxyphenylboronic acid appears to be a viable alternative to boric acid for the complexation of sugars. 4-Carboxyphenylboronic acid bears the advantage of introducing a doubly negative charge to the complex, which increases the charge-to-size ratio and widens the separation window. Another advantage is an increased buffering range because of the presence of both a benzoate and borate functional group. The aromatic complexing agent may prove useful in methods of analysis that employ UV detection.

The separation window could easily be widened by the use of organic modifiers. Optimal separation in a reasonable amount of time was obtained when 10% methanol was added to the running buffer. The use of modifiers with larger elutropic strengths, such as dioxane or isopropanol, was not advantageous.

SDS was confirmed as the best surfactant for the analysis of TMR-labeled oligosaccharides, when compared to TTAB, Triton X, or octanesulfonic acid.

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CHAPTER 4

APPLICATION OF CEC TO THE SEPARATION OF CARBOHYDRATES

4.1 Introduction

Capillary electrochromatography is currently a very popular analytical separation technique, especially for the separation of neutral analytes. In this chapter. methodology for the analysis of TMR-labeled mono- and oligosaccharides by capillary electrochromatography coupled with laser-induced fluorescence (CEC-LIF) is presented. To the best of my knowledge, this represents the first work done on CEC-LIF using a sheath flow cuvette.

Since the separation mechanism in CEC is based on the analytes' partitioning coefficient between the mobile and stationary phase, the basic principles of high performance liquid chromatography (HPLC) apply, for example how to control retention and resolution of analytes by varying mobile phase composition. Using HPLC, carbohydrates have been separated by size using reversed-phase (ODS) packings with water as eluent [1-2], or cyano- and aminopropyl bonded phases using acetonitrile/water mixtures [3]. In cases where column performance was lost over time due to interactions with free reducing sugars, *in situ* modification of silica with a di- or polyamine have been used [4].

However, the combination of mobile and stationary phases used in CEC must produce a robust EOF. Flow generation should be the first priority in CEC method development. Once flow is achieved, one can gradually vary the mobile phase composition to control retention behavior. EOF and retention control are separate but interdependent problems and must be solved simultaneously.

Many issues associated with the manufacture and operation of CEC-LIF columns had to be resolved, for example column packing techniques, frit fabrication, capillary alignment, buffer viscosity, operating voltage and current range. Rhodamine was used in many cases as a probe molecule to address these issues.

4.2 Experimental

4.2.1 Materials

Stock solutions of buffers were prepared using deionized water (Barnstead NANOpure water system). Phosphate buffers were made from disodium phosphate (Fisher, Fair Lawn, NJ, USA) or phosphoric acid (Fisher, Fair Lawn, NJ, USA). The pH was set by adding 100 mM NaOH solution before addition of organic modifiers. Organic modifiers including methanol (Caledon, Georgetown, ON, Canada), isopropanol (BDH, Toronto, ON, Canada), acetone (ACP, Montreal, PQ, Canada) and acetonitrile (BDH, Toronto, ON, Canada) were added using Eppendorf pipettors. Additional buffer ingredients were putrescine dihydrochloride (Sigma, St. Louis, MO, USA) or tetrabutylammonium bromide (Sigma, St. Louis, MO, USA).

4.2.2 General capillary packing and frit fabrication procedures

The capillary packing and frit fabrication procedures were those developed by Ming Qi in our research group [5] with minor modifications. Briefly, the CEC columns were prepared using 50 μm I.D., 178 μm O.D., fused-silica capillaries (Polymicro, Phoenix, AZ, USA). Before packing, a temporary frit was created by dippping one end of the capillary into a 5 μm silica paste. After several hours of drying, this end was heated in a Bunsen burner flame, until the polyimide coating had burned off and the silica paste had begun to glow red briefly. The temporary frit was tested for porosity by a gentle flush of water using a syringe. An HPLC pump (Waters 515) with a maximum pressure of 6000 p.s.i. was used for slurry packing. The slurry was made by mixing the packing material (10-30 mg) in about 1 mL of He-purged solvent and sonicating for at least 5 minutes. After sonication, the slurry was transferred to an empty HPLC column (70 mm long, 4.6 mm I.D.) serving as a slurry reservoir. The capillary was connected to the reservoir with a polyether ether ketone (PEEK) sleeve tubing (1.6 mm O.D., 180 μm I.D.) and column fittings. The particles were pumped into the capillary at pressures up to 5000 p.s.i. The packing process could be monitored by holding the capillary against the light from a desk lamp. During packing, best results were achieved when the reservoir was positioned with the outlet towards the ground and was occasional inverted and tapped. Particles that were settling out could be pushed into the capillary this way. After the packing reached the desired length (between 20 and 30 cm) it was sonicated for 5 minutes. This procedure reduced the length of the packing by up to 10%. Finally, the pressure was slowly released.

Frits were produced using a fiber optic splicer (Orionics FW-301, Scarborough, Canada). During frit fabrication, water was pumped through the capillary at pressures of 1000 to 3000 p.s.i. The arc formed between the two electrodes first expelled solvent in both directions, then burned off the polyimide coating and turned the packing material white. The formation of the frit was monitored under a microscope. The frits obtained this way were about 5 mm in length. After frit fabrication, the pressure was slowly released. When the inlet pressure reached about 300 p.s.i., the temporary frit was removed by cutting the capillary at the end of the outlet frit, and the capillary was mounted in the sheath flow cuvette detector to prevent air from getting into the capillary. After the pressure at the pump had dropped to zero, the capillary was cut at the inlet frit, and the inlet immediately immersed in a buffer vial in the injection carousel. If the cut at the outlet was undesirable (i.e. irregularly shaped), the capillary was reversed.

The mobile phase was introduced into the capillary either electrokinetically or with the HPLC pump. The use of a syringe pump seemed less practical, since the pressure applied could not be monitored. Because of air pockets introduced upon assembly of the pump–capillary interface the later approach was time-consuming. In case no EOF was observed, the HPLC pump was the only reliable way of delivering the mobile phase into the column.

4.2.3 Preparation of specific capillaries

4.2.3.1 Octadecyl silica (ODS) packed capillaries

The capillaries were packed with spherical 3 μm C18 derivatized particles. Both regular (Spherisorb 3 μm ODS-1) and endcapped (Spherisorb 3 μm ODS-2) batches were used. He-purged acetonitrile was used as packing liquid and water for frit preparation.

4.2.3.2 Propylamino derivatized silica packed capillaries

Two propylamino bonded phases were used: An older batch of 5 μm particles (Spherisorb 5μm NH_2), and a new batch of 3 μm particles (Waters 3μm NH_2). The 5 μm particles were harder to pack into the capillary because of the larger size but the resulting columns were less susceptible to bubbles. Initially, acetonitrile was used as the packing liquid. However, the propylamino particles quickly settled out of the suspension in acetonitrile. Better results were achieved with water or methanol. Water was found to be superior during frit fabrication.

4.3 Results and Discussion

4.3.1 General

The are four pressing issues in the daily operation of CEC-LIF: EOF generation. bubble prevention, inlet frit and column stability, and long run times.

4.3.1.1 Verification of EOF generation

In practice, it is difficult to know whether enough EOF is generated. Sometimes one can see flow through the microscope at the capillary tip, if the make-up of the sheath flow liquid is different from the running buffer or if there are bubbles eluting from the capillary. Both of the later are, of course, undesirable. In addition, there is no such thing as a neutral EOF marker molecule in CEC-LIF. The molecule used most as EOF marker in CE coupled with UV detection is thiourea, which cannot be detected by LIF. Tricks such as injection of a plug of methanol, employed in open-tubular CE-LIF to measure the EOF, do not work in CEC-LIF: Solvent plugs alter the EOF along the capillary and potentially create bubbles through EOF and conductivity mismatch. One thus depends on a fluorescent dye to elute from the capillary to actually observe flow. However. fluorescent dyes are subject to retention in CEC. If one does not observe fluorescence after some time, it does not necessarily mean that the mobile phase is not flowing; the mobile phase strength could be too weak to elute the dye from the column in a reasonable amount of time. The observation of a robust current only indicates the absence of bubbles but not solvent flow.

4.3.1.2 Bubble suppression

A second problem in the operation of CEC-LIF is bubble formation. It is caused by EOF mismatch or sudden pressure and flow changes within the capillary. An EOF mismatch usually occurs at the frits: The makeup of the surface silica is different here than in the remaining column, resulting in a different EOF to be generated. Also, if the packing was overheated during fabrication of the frits, the particles may form different size pores and channels than in the remaining packing. Since the mass flow rate has to be the same throughout the column, local flow velocity and pressure drops and consequently bubbles may occur. Bubble formation is enhanced as well by high applied fields due to Joule heating [6]. Low conductivity mobile phases (e.g. 4-morpholinosulfonic acid, MES) often suppress bubble formation by preventing Joule heating. Bubble formation can also be suppressed by solvent degassing with helium and vacuum, and by changing the voltages slowly. Sudden voltage changes are known [7] to promote the formation of bubbles. In practice, during injection and operation the voltages were ramped up to the desired voltage and back down to zero. Pressurization of inlet and outlet, which is done by most researchers to suppress bubbles, is not possible with the present design.

4.3.1.3 Column stability

If an electric field is applied to a bed filled with charged particles the particles experience a force. If there are void volumes, the bed may shift in a process termed electrokinetical post packing [8-9]. If these shifts occur suddenly, higher pressure than normal is exerted on the inlet frit, which may cause it to break. It is therefore important only to gradually increase and decrease the applied voltage.

During operation, the packing is held in place mainly by the inlet frit. This is because of Newton's third law. The particles push the liquid mobile phase towards the detector with the electroosmotic force. However, the same force pushes the particles towards the inlet. In addition, the particles electrophorese towards the inlet since they are charged. CEC using only an inlet frit has been demonstrated [10]. However, outlet frits are still required when the force from the flow is stronger than the force induced by the particle at the outlet, especially when long beds are employed. This was the case here.

Both inlet and outlet frit breakage were observed during experiments. Inlet frit breakage usually manifested itself by a gradually increasing current. The resistance of the column decreases as particles are replaced by conductive mobile phase. Outlet frit breakage could be observed through the microscope –a "fuming volcano"-like cone would form at the capillary tip.

4.3.1.4 Interferences due to long run times

During longer runs, the fluorescence detection was disturbed by debris collecting at the capillary tip, where the laser beam is focussed. This debris, usually small bubbles from the sheath flow buffer or bubbles and particles eluting from the column, scatter the laser beam and disrupt the focussing effect of the sheath flow. The debris are held at the capillary tip by surface tension and could be removed by squeezing the sheath flow buffer reservoir or gentle tapping of the cuvette. Another problem with the long run times in CEC that does not occur in CE is a gradual change of buffer composition in the inlet vial due to prolonged electrolysis. Vacuum degassing of the mobile phase to prevent bubble formation is arguably pointless during long runs. The problem of gradual inlet buffer change could be overcome by using a larger injection vial and by stirring. However, in the current design of the injection carousel, this was not possible.

One cannot easily shorten the run times by increasing the applied voltage. Joule heating, bubble formation and slow retention kinetics are potential problems when high voltages are applied. It is more practical to reduce the viscosity of the mobile phase by varying the nature and percentage of the organic modifier.

4.3.2 ODS packed capillaries

The capillaries packed with endcapped trimethylsilyl chloride endcapped ODS failed to generate any considerable EOF. This is consistent with the generally accepted notion that endcapping is undesirable in CEC. The decrease of the density of the silanol group causes reduction of the velocity of the EOF due to the decrease of the negative charge on the particle surface [11].

Better results were obtained with non-endcapped ODS packed capillaries. A decent signal eluting in a reasonable time was achieved with a mobile phase containing 94% methanol, 1% isopropanol, and 5% phosphate (10 mM, pH 7.9). Peak tailing was observed (see figure 4.1, top trace), indicating that some of the tetramethylrhodamine molecules strongly interact with secondary sorption sites. These strongly-sorbing sites were suspected to be the residual silanol sites. Tetramethylrhodamine contains two basic nitrogen atoms, and basic compounds are known [12-13] to strongly sorb to residual silanols. In order to obtain faster elution from the column, acetone was added to the mobile phase. Water-acetone mixtures have relative low viscosity [14]. It was also suspected that the combination of methanol and acetone better solvates tetramethylrhodamine.

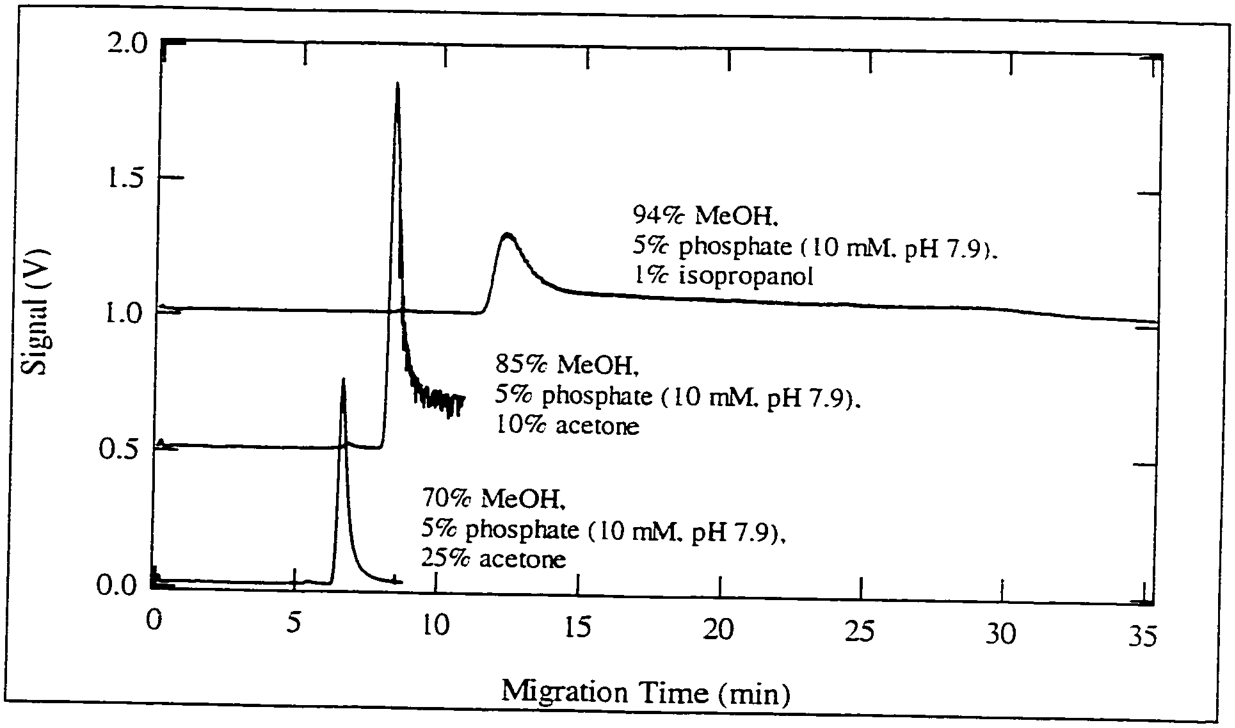

Figure 4.1. Top trace: Injection of 25 nM rhodamine B onto a 20 cm, 50 μm I.D., 178 μm O.D. capillary packed with 3μm Phenomenex ODS-1. Injection was ramped up to 500 V/cm and back down to 0 V/cm in 30 sec each, which gives an average of 250 V/cm for 60 sec. The separation field strength was 1000 V/cm. Buffer composition: 94% MeOH, 1% isopropanol, 5% phosphate (10 mM, pH 7.9). The retention time stayed within 1% in repeated trials. Center trace: Injection of 130 nM rhodamine B at 250 V/cm, 40 sec effective time. Mobile phase: 85% MeOH, 10% Acetone, 5% phosphate (10 mM, pH 7.9). Other conditions same as top trace. Bottom trace: Injection of 130 nM rhodamine B at 250 V/cm, 25 sec effective time. Mobile phase: 70% MeOH, 25% Acetone, 5% phosphate (10 mM, pH 7.9). Other conditions same as top trace.

To test whether or not the second band originated from sorption of tetramethylrhodamine to strong silanol sites, a lower pH buffer and the competing base tetrabutylammonium bromide were added to the mobile phase (Figure 4.2). Surprisingly, the symmetry of the peaks and the tailing did not improve under these conditions. This could be because the band is moving too fast for desorption from the stationary phase to occur. In other words, the desorption kinetics is slow. In contrast, at lower separation voltage (i.e. at lower separation speed), better peak shapes were obtained (Figure 4.2, top trace).

In spite of the relative low pH and the amine modifier the EOF does not appear to be significantly affected. This is in agreement with a report in the literature [13]. However, this effect can only happen when the buffer pH and the pH of the charged sites underneath the hydrocarbon layer are not in equilibrium with each other. The failure to achieve equilibrium is consistent with the poor peak shapes obtained (Figure 4.2).

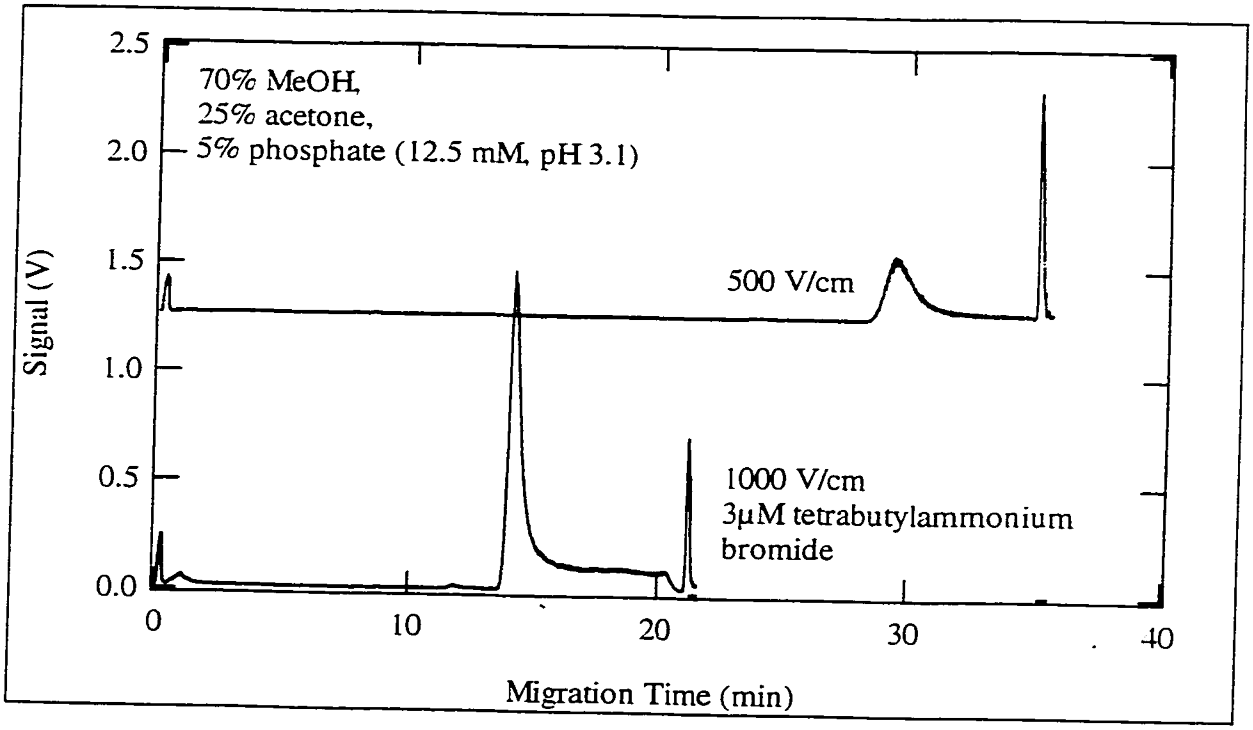

Figure 4.2. Top trace: Injection of 130 nM Rhodamine B at 500 V/cm, 30 seconds effective time, onto the same column as in figure 4.1. Separation was at 500 V/cm. Buffer: 70% Methanol, 25% Acetone, 5% phosphate (12.5 mM, pH 3.1) Bottom trace: Same conditions as top trace, 3 μM tetrabutylammonium bromide added. Separation at 1000V/cm. Rhodamine elutes faster in the bottom trace because of the increased field strength.

The test mixture employed in the two previous chapters was injected onto the column. Unfortunately, the standards failed to separate under these acidic, high percent organic modifier conditions (Figure 4.3).

A common mobile phase used in CEC is a mixture of accetonitrile and aqueous phosphate at neutral pH. An example of the separation of the standards is shown below (Figure 4.4). Again, poor separation was observed. The poor separation is due to rather dramatic bandbroadening of individual peaks. This was verified by injecting a spiked sample (data not shown).

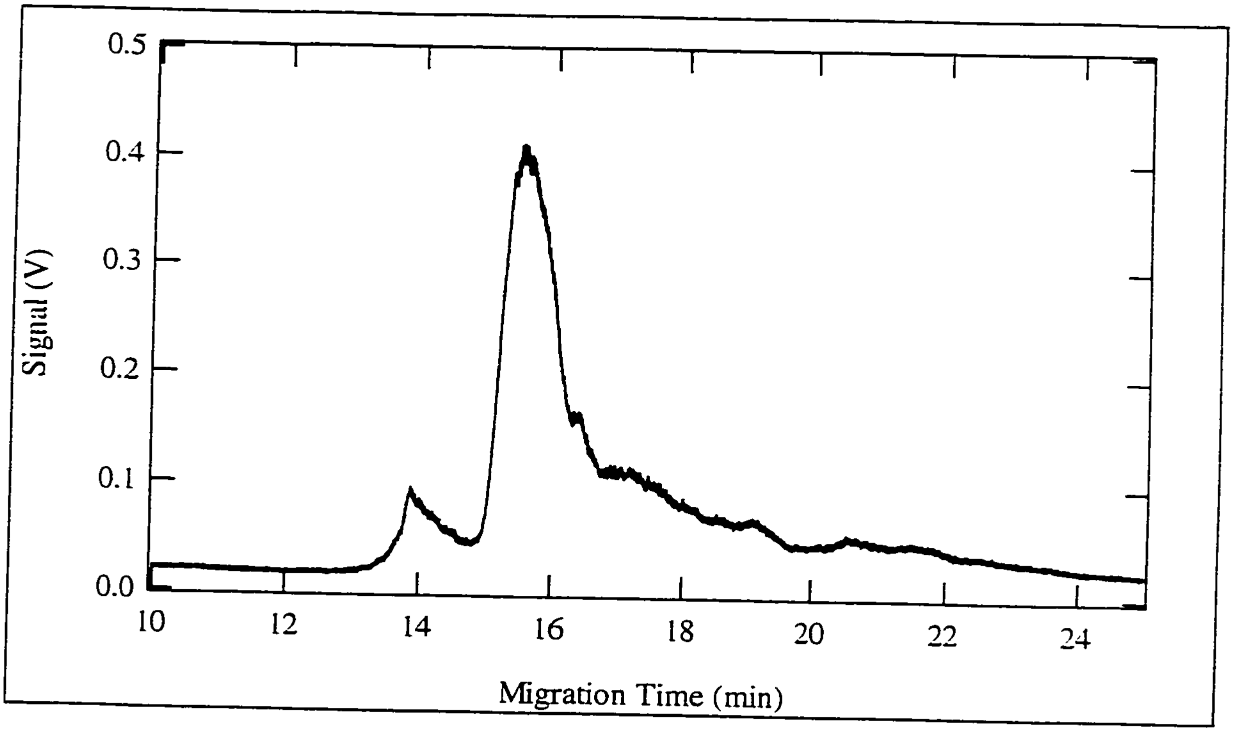

Figure 4.3. Attempted separation of 25 nM TMR-labeled mono- and oligosaccharide standards. Injection 500 V/cm, 40 seconds effective time. Separation conditions were the same as in figure 4.2, bottom trace.

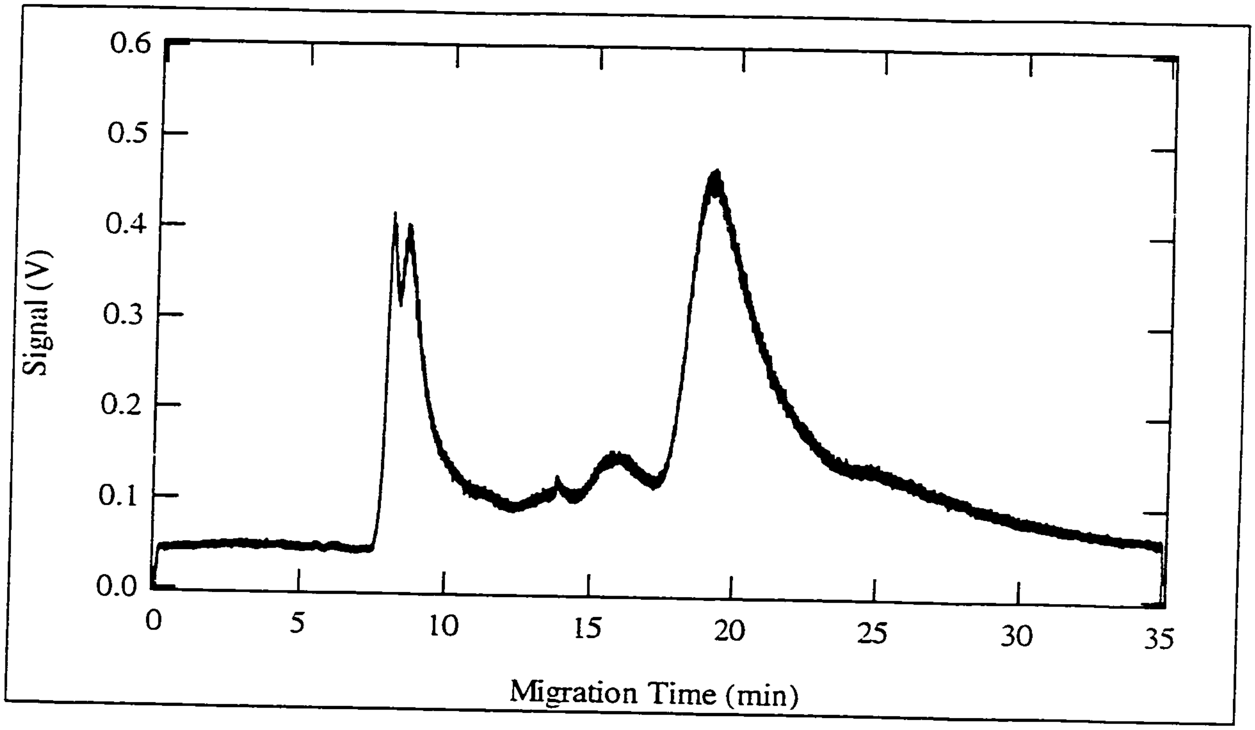

Figure 4.4. Attempted separation of 25 nM TMR-labeled mono- and oligosaccharides on a 23 cm, 50 μm I.D., 178 μm O.D. capillary packed with 3μm Phenomenex ODS-1. Separation was at 400 V/cm. Injection was 10 sec at 400 V/cm. Buffer: 75% Acetonitrile, 25% Phosphate (5 mM, pH 7.0).

4.3.3 Propylamino derivatized silica packed capillaries

Rather than further trying to improve the separation on ODS phases by modifying the mobile phase, it appeared more logical to alter the stationary phase. ODS can be predicted to strongly retain the hydrocarbon linker arm and the rhodamine dye. but not the polar sugar units. Therefore, more polar propylamino derivatized silica was employed as the stationary phase.

The results obtained with the new stationary were promising. Initially. a buffer consisting of 60% acetonitrile and 40% phosphate (pH 7.0, 2.5 mM) was employed. As can be seen from the chromatogram (Figure 4.5) the nine standards separate in a relative short time. However, peak size and shape still needed improvement.

In the present column design, the inner capillary walls consist of bare silica. which have a net negative charge. The walls thus create EOF opposite that of the particles, which causes an undesirable flow pattern within the capillary. Also. the presence of large amounts of bare silica constitutes a second type of sorption site. which increases bandbroadening. To overcome this problem, the walls (and free silanols on the particles) were dynamically coated by adding putrescine dihydrochloride [15] to the mobile phase. The chloride salt was chosen because of putrescine's odor. However. at higher organic modifier concentration, precipitation of the chloride on the amino packing may become a problem. Nevertheless, neutral TMR-labeled mono-, di-, tri- and tetrasaccharides could be separated under these conditions (Figure 4.6).

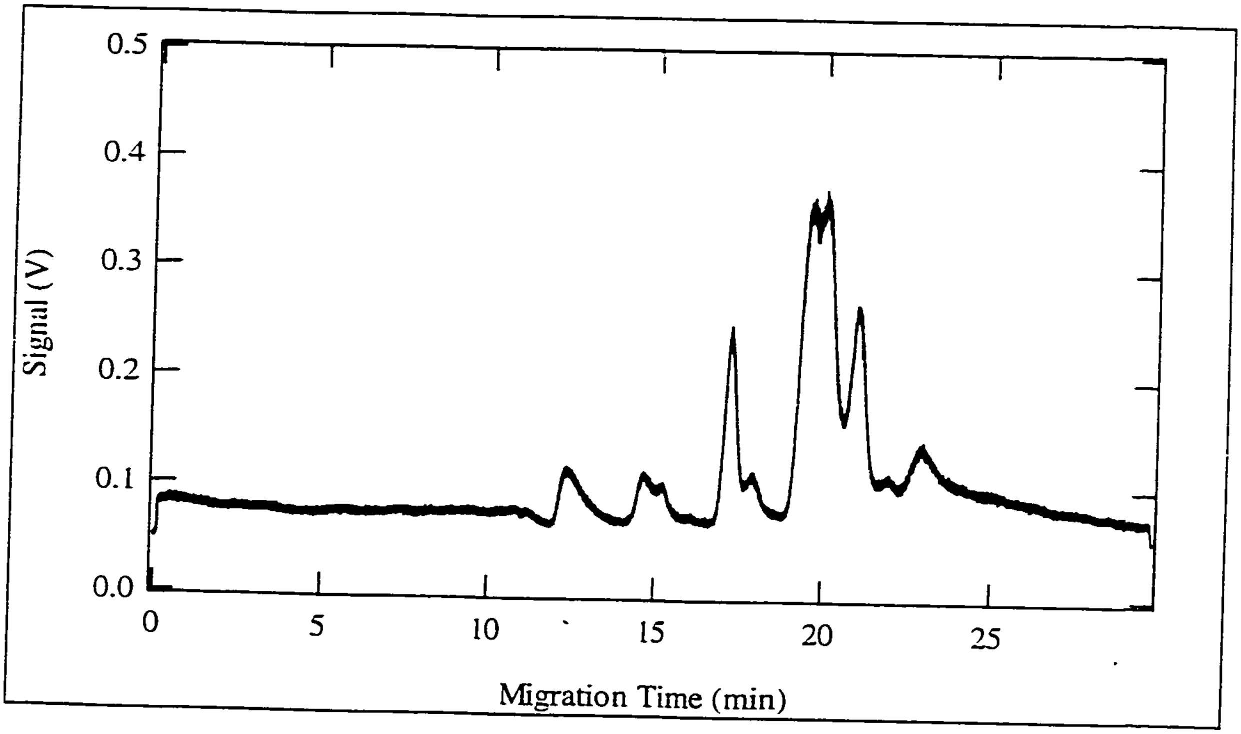

Figure 4.5. Separation of nine TMR-labeled mono- and oligosaccharide standards (25 nM) at 400 V/cm on a 19.2 cm, 178 μm O.D., 50 μm I.D. capillary filled with 5 μm Spherisorb Amino particles. Injection was at 200 V/cm, 10 seconds effective time. Buffer: 60% acetonitrile, 40% phosphate (pH 7.0, 2.5 mM).

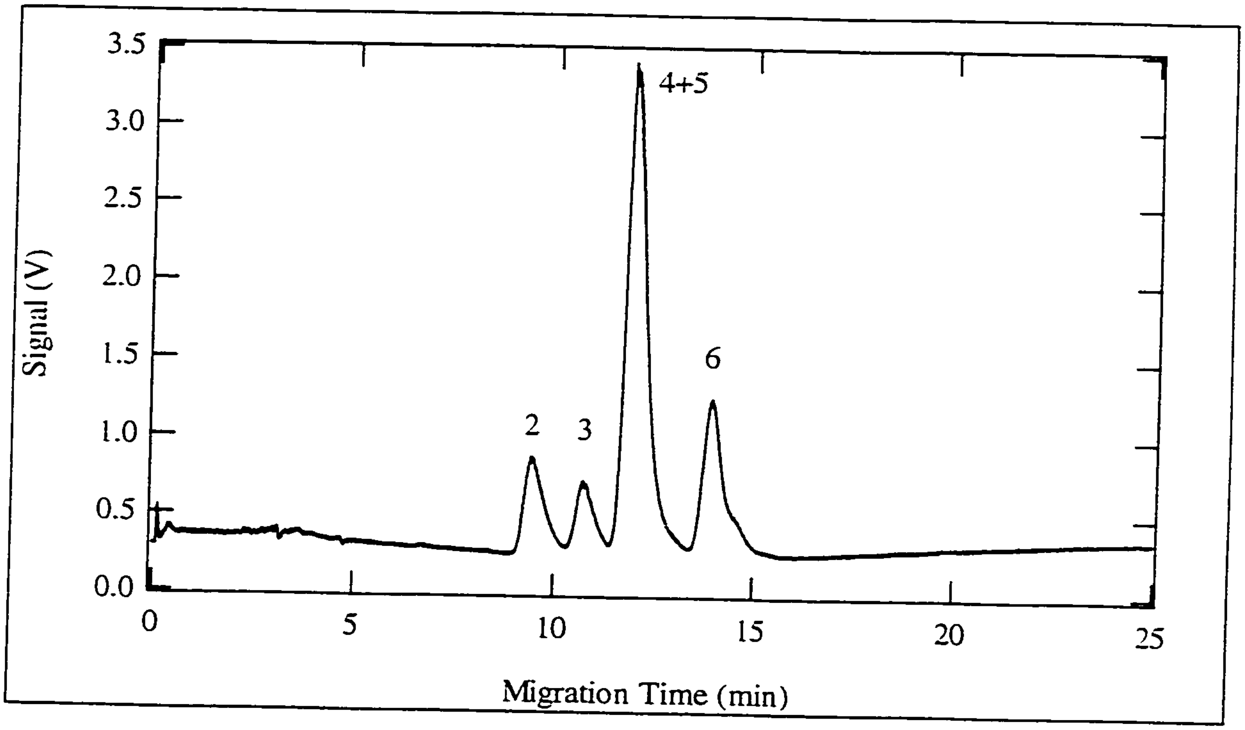

Figure 4.6. Separation of neutral mono- and oligosaccharides. Five compounds at 100 nM were injected at 240 V/cm, 10 seconds effective time. Separation conditions were the same as figure 4.5, with 0.025% (w/v) putrescine dihydrochloride added to the running buffer.

4.4 Conclusions

Among the advantages of CEC-LIF is the relative ease with which neutral molecules can be separated in an electrokinetically driven system. Potentially, CEC gives selectivity that cannot be achieved by either HPLC, CZE or MEKC. However, the main advantages of CEC-LIF still need to be demonstrated: These are the expected improved separation efficiency compared to HPLC and MEKC and the simultaneous separation of neutral, positively and negatively charged compounds.

However, the disadvantages of CEC-LIF still outnumber the advantages. CEC-LIF is not yet a mature technique - separation efficiency and speed still have room for improvement. A significant amount of time and effort is spent reducing bubble formation during the long run times encountered. Column performance needs to become more reproducible. In comparison to open-tubular CE, CEC-LIF is rather slow.

In this chapter it has been shown that CEC-LIF can be used for the analysis of TMR-labeled mono- and oligosaccharides. However, optimization of CEC-LIF for the analysis of CEC-LIF is by no means accomplished. The technique itself could be improved by developing a sheath liquid detector that is pressurized. Many variables, including ideal concentrations and identity of organic modifier, amine and buffer as well as operating voltage also still need to be optimized.

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CHAPTER 5

FUTURE WORK AND CONCLUSIONS

5.1 Future work

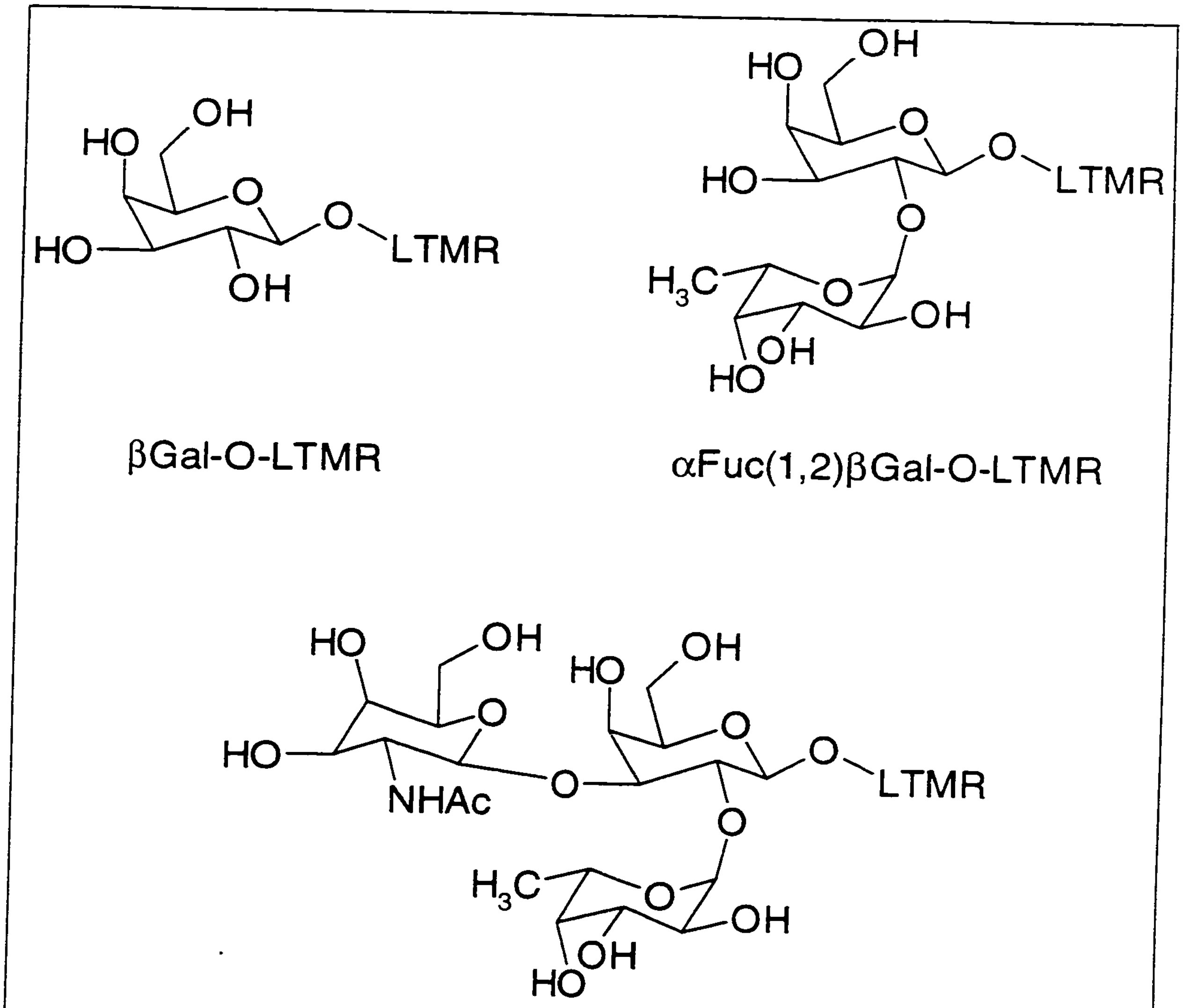
5.1.1 *In situ* modified silica packed capillaries

Permanently derivatized silica stationary phases do not tolerate pH values higher than about 8. At high pH, the bonded phase is removed from the silica. and the column is destroyed. However, carbohydrate analysis by CE is usually done at slightly alkaline pH. anywhere from 7 to 12. A stationary phase more appropriate for carbohydrate analysis would be (a) tolerant to basic conditions, and better still (b) self-regenerating. An example of such a stationary phase would be silica dynamically coated with an amine. These *in situ* modified packings have the advantage that the inner capillary walls would have the same surface chemistry and history as the remaining packing. The more homogenous the inside of the capillary the better the separation efficiency becomes. The high pH tolerance also has the advantage that the charge-inducing complexing agent boric acid, which has a pK_a of about 9, can be employed.

In order to be useful in CEC, these columns should produce a decent EOF. Simple amines, for example triethylamine, will only adsorb up to the point of neutralization of the surface charge. Further adsorption will not occur due to electrostatic repulsion. Polymeric materials, such as hexadimethene bromide (polybrene), or tetraethylenepentaamine, may be able to reverse the surface charge, as it has been demonstrated for open-tubular columns. Smaller diamines, e.g. 1,4-diaminobutane (putrescine), do not reverse the EOF [1]. The EOF could be reversed by cationic surfactants, for example CTAB or the double-chained surfactant didocyldimethyl-ammonium bromide (DDAB). Above the CMC these surfactants condense on the surface as micellar aggregates, putting a large positive charge on the surface [2]. However, the surfactant must be added to the running buffer for long run times. There is the danger of increased bandbroadening due to mixed-mode retention: Part of the retaining phase is sorbed to the packing and is stationary, and the other part is moving. If the mobility of the micelles is not matched to the EOF, broad bands may be obtained.

In order to prepare *in situ* modified silica packed capillaries, they would be packed with 3 μm spherical silica particles using water as packing fluid. After frit fabrication, the excess packing material would be removed from the packing reservoir, and the reservoir filled with an aqueous, degassed amine solution (e.g. 100 mM TTAB) at slightly alkaline pH, perhaps buffered with borate. This solution could be pumped through the capillary until pH indicator paper turns colour at the capillary outlet.

These stationary phases could be employed in the size separation of oligosaccharide standards that do not bear cis-diol groups. Examples of such molecules, which were recently synthesized in Monica Palcic's laboratory, are shown in figure 5.1. Attempted separation of this mixture using borate or SDS/borate buffers yield a single peak (data not shown).



βGal-O-LTMR

αFuc(1,2)βGal-O-LTMR

αGalNAc(1,3)[αFuc(1,2)]βGal-O-LTMR

Figure 5.1. Some TMR-labeled saccharides not separable by CZE and MEKC

5.2 Pressure-assisted CEC

Most recent publications on CEC employ commercial instruments. which have the capability of pressurizing the inlet and outlet vials. Pressurization is an easy way to suppress bubble formation inside the column. By applying a pressure differential. separations using a different separation mode called pressure-assisted CEC. a hybrid of CEC and HPLC, can be performed.

In the present instrument design, pressurization at the outlet is not possible. Recently, an inlet pressurization bomb was made in the machine shop out of teflon (Figure 5.2). The bomb is designed to tightly fit a 4 mL glass vial. A 400 μL Eppendorf tube fits inside the vial. The inside of this bomb is accessed through the lid by three plastic srews, which fit 16/1000'' tubing or polyether ether ketone (PEEK) sleeves. The screws are inserted at a slight angle towards the centre. One inlet holds the tubing connected to a helium gas tank, which is used for pressurization. Another tightly holds a platinum electrode, and the third the capillary. The bomb has been tested for pressures up to 120 p.s.i.

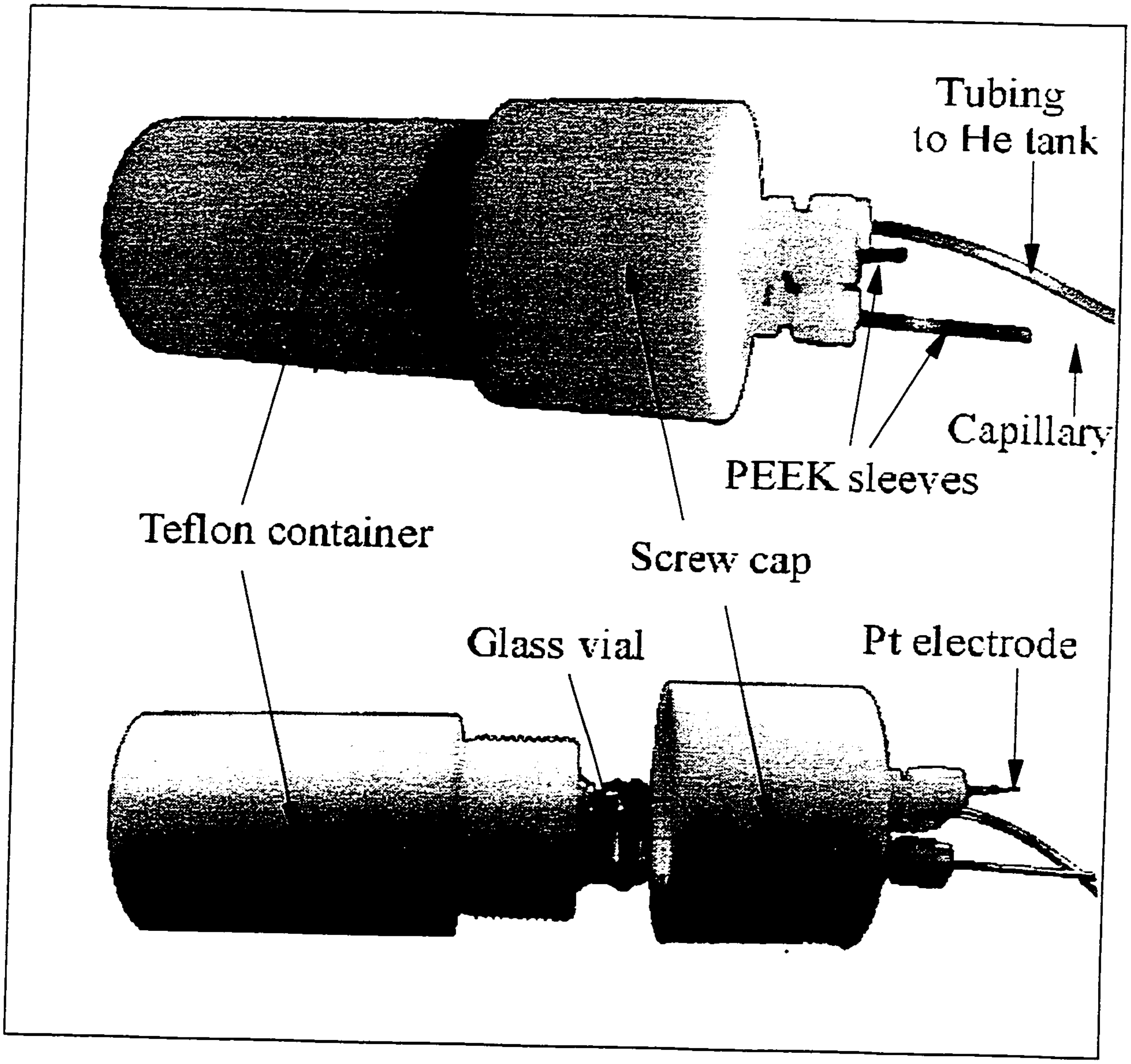


Figure 5.2. Sketch of the in-house developed inlet pressurization bomb

In preliminary tests several disadvantages of the present design have become apparent: A pressure of up to 120 p.s.i. is often not sufficient to push solvent through the column. One cannot easily look inside the bomb to check if the capillary and electrode are submerged in buffer solution. This can be a problem, as the buffer solution appears to evaporate faster than in ambient pressure. For injection, the bomb must be depressurized and be taken apart. The bomb itself takes some time to assemble, which is not a big problem because long run times are involved anyways. Unfortunately, using the bomb at pressures up to 120 p.s.i. does not appear to reduce bubble formation (data not shown), indicating that it is important to pressurize the outlet vial as well.

5.2 Conclusions

In this thesis several electrokinetically driven in-capillary separations of TMR-labeled mono- and oligosaccharides using both pseudostationary and stationary phases were presented. In chapter 2, an improved separation based on adding ethylamine to the running buffer was described. In chapter 3, the role of the buffer components used in the original separation was reinvestitaged. It was found that only SDS and borate were necessary buffer components. This is a significant simplification compared to previous work published by this research group [3-4]. Alternative buffer systems were also discussed. The best resolution was obtained using high SDS concentration and boric acid (or 4-carboxyphenylboronic acid) in either normal or reversed mode, the later achieved by adding ethylammonium ion. In chapter 4, several approaches on the separation of TMR-labeled mono- and oligosaccharides by CEC were presented. These approaches are not yet optimized. The *in situ* modified silica packed capillaries described in chapter 5 establish a realistic concept for future separations of carbohydrates by CEC.

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