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Review

Detection of carbohydrates in capillary electrophoresis

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Abstract

This review focuses on recent developments in sensitive detection modes for carbohydrates after separation by capillary electrophoretic methods. To bring detection sensitivity for carbohydrates analysis in line with current methods in protein sequencing, concentration detection limits of 10^{-6} molar or better are required. A discussion of mass detection limits and concentration detection limits is followed by an overview of detection modes for natural and labeled carbohydrates. Amperometric detection and UV and laser-induced fluorescence detection after reductive amination, in particular with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), are discussed in more detail. Finally, the paper outlines developments to be expected in the near future, focusing on the needs in glycobiology such as improved sensitivity and selectivity.

Contents

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1. Introduction

Carbohydrates are ubiquitous in the living world. In plants and animals they act as energy and carbon sources and are therefore central to metabolism. Additionally they are important structural elements in plant cell walls as well as in the extracellular matrix of animal and human tissues. As integral components of glycoproteins and glycolipids, carbohydrates are involved in many biological processes. Cell surface glycoproteins have been shown to play important roles in intracellular recognition and adhesion, in cell development and differentiation, as receptors for hormones and as mediators of immunological specificity. Secreted glycoproteins, on the other hand, include hormones, immunoglobulins, and serum transfer factors [1–3]. A number of therapeutic proteins produced by recombinant techniques in mammalian cells are expressed with glycan moieties. Since glycosylation affects the biological activity, lifetime, cellular uptake, and specificity of these proteins [4], carbohydrates are an important area of analytical biotechnology. Polymeric carbohydrates such as starch, cellulose, and pectin play an important role as nutrients and food additives. Consequently, the manufacture, processing, and stability of polycarbohydrates are the focus of considerable attention in the food sciences.

The growing carbohydrate-related research in the above-mentioned areas is also generating increased interest in suitable analytical techniques for carbohydrates. There is a wide variety of problems associated with the detection and quantification of carbohydrates, including monosaccharide determination in any possible matrix, assessing the different glycoforms of a glycoprotein (different oligosaccharides attached to the same peptide backbone of the protein [5]), oligosaccharide mapping, and separation of highly polymeric polysaccharides and proteoglycans.

The main difficulties in the analysis of carbohydrates and glycoconjugates arise from their enormous number of possible isomeric forms due to the various possible configurations of the monosaccharides, their variable linkage forms, the α- and β-anomerity of the glycosidic linkages as well as their different branching patterns. While there are only two distinct chemical structures for a dipeptide, two identical hexose units can form 11 distinct chemical structures. And whereas the linkage of three different amino acids results in only six tripeptides, three different carbohydrates can form more than 700 trisaccharides [2,6].

Finally, carbohydrates are also diversified by the presence of nonglycosyl substituents, such as acetyl, methyl, and sulfate esters and ethers, or amine and phosphate groups. And, last but not least, the very polar and nonvolatile nature of these compounds, the absence of chromophoric or fluorophoric groups, and the lack of specific functional groups for chemical modification further complicate their chemical analysis.

The last 10–20 years have seen a steady development of analytical methods for carbohydrate determination, most notably in gas chromatography (GC) and high-performance liquid chromatography (HPLC). GC of permethylated carbohydrates, a method developed by Hakomori in 1964 [7], exhibits good sensitivity. The formation of stereochemical isomers during the derivatization resulted in very complex chromatograms, but this problem could be solved by analyzing the carbohydrates as their alditol acetates. The full potential of GC analysis has only been realized since the advent of capillary columns and the coupling of GC with mass spectrometry [8].

As in HPLC separations a number of stationary phases, among them alkylated or aminoalkylated silica gels, have been used in combination with refractive index (RI) and UV–Vis detection. Furthermore, ion-exchange resins have been proposed for carbohydrate separations [8,9]. In combination with pulsed amperometric detection (PAD), high-performance anion-exchange chromatography (HPAEC) represents a selective and sensitive method for determining natural carbohydrates. The fact that HPAEC–PAD requires no derivatization in the sample pretreatment is an enormous advantage, making it currently the method of choice in carbohydrate analysis [10]. HPAEC–PAD does, however, have two drawbacks: limited choice of buffer conditions and an only moderate separation efficiency, which are of particular concern in the determination of higher and complex oligosaccharides.

Electrophoretic analysis schemes for both simple and complex carbohydrates have also been evaluated using polyacrylamide slab gels [11,12] and other supporting media such as paper [13] and silylated glass-fiber paper [14].

Capillary electrophoresis (CE) has already demonstrated its capability of fast, automated, miniaturized, and highly efficient separations peptides, proteins, and nucleic acids [15,16]. More recently, CE has developed into a promising alternative for the analysis of mono- and oligosaccharides, as well as glycoproteins and glycoprotein fragments, as documented in two recently published review articles [17,18].

The main purpose of this review is to assess the different approaches taken to address sensitive detection of carbohydrates and glycoconjugates in CE and to outline some general trends. Problems concerning different separation mechanisms and changes in selectivity in the CE-based separation systems are also touched upon.

2. Detection limits

With the continuing miniaturization of analytical instrumentation and growing biological insight into ever more selective systems, highly sensitive detection remains an important issue. Up-to-date protein sequencers need roughly 1–10 pmol protein material to yield unambiguous sequence information. For an average protein with a molecular weight of 50 000, this translates into an absolute mass of 500 ng of sample. Since glycoproteins are usually found with a 10–60% glycosylation by weight [19], this results in 50–300 ng of an oligosaccharide mixture, if all glycan chains can be completely released. Assuming further ten different oligosaccharides in the glycoprotein with an average molecular weight of 2000, roughly 5–30 ng or 2.5–15 pmol in absolute mass units for each oligosaccharide are available for analysis. Reproducible injection in CE, as well as reproducible derivatization, requires sample volumes of around 10 μl. Dissolving the 2.5–15 pmol of carbohydrate in such a volume, results in sample concentrations of $(0.25–1.5)\cdot 10^{-6}$ M. Therefore, even under optimum conditions, the detection limit of the analytical method needs to be in the submicromolar range.

In the discussion of a detection principle, two different types of detection limit have to be clearly distinguished: the concentration and the mass detection limit. Unfortunately, the two are often confused. The concentration detection limit is the minimum concentration that can be detected via a given detection principle, typical values being 10^{-6} M for UV detection, 10^{-8} M for electrochemical and fluorescence detection, and 10^{-11} M for optimized laser-induced fluorescence (LIF) detection of fluorescein derivatives (Table 1). The mass detection limit is the absolute mass subjected to the analytical separation. In the case of CE, absolute masses are rather small, in the pico- to attomole range (see Table 1). This is due to the fact that CE itself is a miniaturized system with total capillary volumes of less than 10 μl and injection volumes in the range of a few nanoliters or even only picoliters. With extremely small capillaries (<10 μm I.D.) mass detection limits of some hundred molecules have been obtained [20]. However, concentration detection limits in CE

Table 1
Injection volumes and mass detection limits in capillary electrophoresis

Capillary I.D. (μm)	Capillary volume (μl)	Injection volume (nl)	Mass detection limits (mol)			
			10^{-6} M (UV)	10^{-8} M (electrochemical, fluorescence)	10^{-11} M (laser-induced fluorescence)	
200	15.7	160	160×10^{-15}	1600×10^{-18}	1600×10^{-21}	
100	3.9	40	40×10^{-15}	400×10^{-18}	400×10^{-21}	
75	2.2	20	20×10^{-15}	200×10^{-18}	200×10^{-21}	
50	1.0	10	10×10^{-15}	100×10^{-18}	100×10^{-21}	
25	245×10^{-3}	2450×10^{-3}	2500×10^{-18}	25×10^{-18}	25×10^{-21}	
10	40×10^{-3}	400×10^{-3}	400×10^{-18}	4×10^{-18}	4×10^{-21}	2400 molecules
5	10×10^{-3}	100×10^{-3}	100×10^{-18}	1×10^{-18}	1×10^{-21}	600 molecules
2	1.5×10^{-3}	15×10^{-3}	15×10^{-18}	150×10^{-21}	150×10^{-24}	90 molecules
1	0.4×10^{-3}	4×10^{-3}	4×10^{-18}	40×10^{-21}	40×10^{-24}	20 molecules

Capillary length: 50 cm; injection plug length: 0.5 cm.

are similar to those obtained in other separation methods.

3. Detection of non-derivatized carbohydrates

3.1. Direct UV detection

Since UV detection of non-derivatized and labeled carbohydrates will be discussed in more detail elsewhere in this issue, it is only summarized in the present paper.

3.1.1. Glycosaminoglycans

UV detection is the most versatile detection method in CE, and is implemented in every commercial CE system. Its use for the analysis of carbohydrates is restricted, however, because of their lack of conjugated π-systems and consequently the extremely low extinction coefficients. Only a few carbohydrates, among them acidic disaccharides released from glycosaminoglycans (e.g. chondroitin, heparin, or hyaluronic acid) by enzymatic digestion, exhibit a considerable absorbance in the low UV region. These saccharides carry unsaturated uronic acid residues, which allow UV detection at 232 nm at a micromolar (10^{-6} M) level after separation in a sodium dodecyl sulfate (SDS) containing borate buffer, pH 8.8 [21,22].

Since most glycosaminoglycans and their enzymatic cleavage products are sulfated and therefore carry intrinsic charges, they can also be separated in acidic buffers, such as phosphoric acid. This has been demonstrated for a mixture of non-, mono-, di-, and trisulfated disaccharides [23] as well as for natural and synthetic low-molecular-weight heparin fragments [24]. In general, charged solutes show improved resolution in acidic buffer systems due to narrow Gaussian peak shapes.

3.1.2. On-column complexation with borate

On-column complexation of native carbohydrates with borate allows direct UV detection at 195 nm [25]. A 2- to 20-fold increase in UV absorbance compared to non-borate-complexed carbohydrates can be observed. Nevertheless, only nanogram amounts of non-derivatized carbohydrates, corresponding to a concentration sensitivity of 10^{-3} M, could be detected in this way. Although this approach is therefore in general not attractive for carbohydrate determination, the potential of borate complexation for sugar analysis by CE was clearly demonstrated. Neutral carbohydrates acquire a partial negative

charge by complexation with the tetrahydroxyborate ion rather than the boric acid [26], allowing their migration in an electrical field. The fraction of the negative charge is determined by the equilibrium constant and therefore by the stability of the complex. According to the law of mass action the complex concentration increases with increasing borate concentration. Due to higher concentrations of borate ions in alkaline solutions the formation of sugar–borate complexes is favored at higher pH.

Furthermore, the stability of the sugar–borate complex depends very much on the configuration, the number of hydroxyl groups and substituents in the carbohydrate molecule, as well as on the temperature. For mono- and disaccharides separation efficiency, resolution, and analysis time were improved at higher temperature [25], as illustrated in Fig. 1.

In contrast, a decrease in resolution and number of theoretical plates with increasing temperature was observed for nonreducing sugars, such as sucrose and oligosaccharides of the raffinose family [α-(1,6)-galactosides linked to the glucose moiety of sucrose], due to an increase in longitudinal diffusion at higher temperatures [27].

Borate complexation also made possible the separation of a series of homologous oligosaccharides such as α-(1,6)-linked isomalto-, α-(1,3)-linked laminara- and β-(1,4)-linked cello-

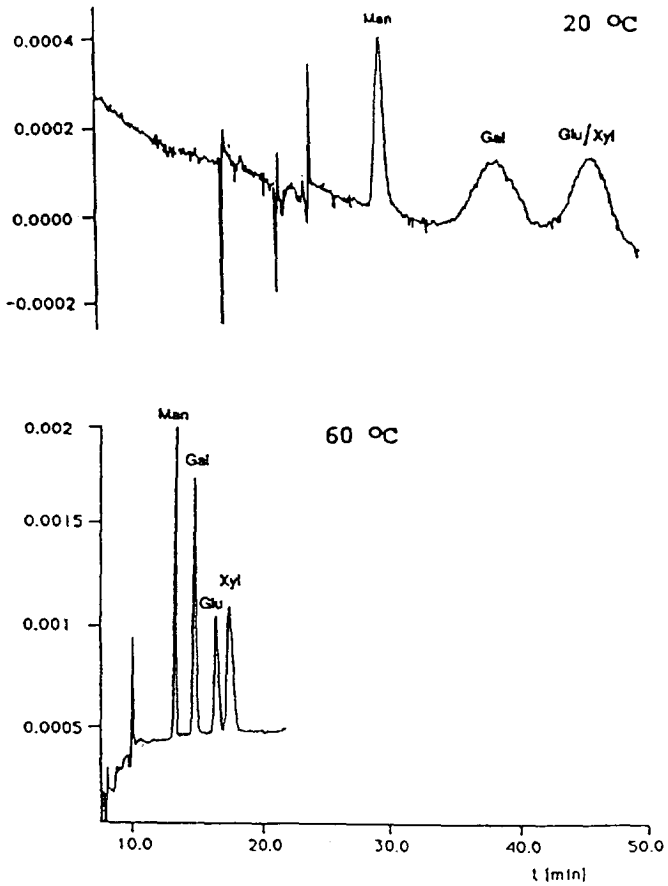


Fig. 1. Effect of temperature on the CE of underivatized monosaccharides. Operating conditions: BGE 50 mM tetraborate, pH 9.3; capillary 94 cm (87 cm to the detector) × 75 μm I.D.; voltage 20 kV; temperature 20 or 60°C; UV detection, 195 nm. Sample: mannose (Man), galactose (Gal), glucose (Glu), and xylose (Xyl). Reproduced from Ref. [25].

oligoglycans by complexation of the outermost glucose residue [28]. Since all oligosaccharide fragments carry the same charge and their electrophoretic mobility depends on their charge-to-mass ratios, size separations are possible, with the mono- and disaccharides migrating in front of the larger oligosaccharide fragments.

3.1.3. UV detection of glycopeptides and glycoproteins

UV detection of glycopeptides is straightforward since the peptide bond gives rise to sufficient absorbance at 200 nm. CE separation of the major glycoforms of ovalbumin [29] and erythropoietin [30] was achieved in 100 m*M* borate buffer, pH 8.5, and a triscine buffer, pH 6.2, respectively. For glycopeptides, the electrophoretic mobility depends more on the charged peptide moiety than on the glycan part. The buffer choice is therefore solely dictated by the isoelectric point of the peptide fragment.

CE with UV detection at 190–200 nm has been used to investigate the carbohydrate-mediated microheterogeneity of glycoproteins such as the recombinant tissue plasminogen activator (r-tPA) [31] or the α1-acid glycoprotein (AGP) [32]. High-resolution separation of the hybrid- and complex-type oligosaccharides of the r-tPA according to their sialic acid contents was achieved in a phosphate or triscine buffer containing 2.5 m*M* putrescine as a cationic additive.

Only 10 ng of an AGP–glycan pool is necessary for a CE separation, compared to 40 μg for HPAEC–PAD analysis [32]. This low mass sensitivity allowed Hermentin et al. [33] to evaluate CE methods with respect to their suitability for establishing a carbohydrate-map database, from which structural analysis of carbohydrates by comparison of migration times should be possible. With mesityl oxide and sialic acid as internal standards in the separation system and using a triplicate correction method, extremely accurate and highly reproducible migration times with R.S.D. values smaller than 0.20% were obtained for a number of sialylated N-glycans. At present, the database consists of approximately 80 different sialylated N-glycans of known structure.

3.2. Indirect UV detection

Indirect detection methods are a viable alternative for compounds lacking a chromophore. The detection principle is based on the displacement of the chromophore in the background electrolyte (BGE) by the analyte molecule, resulting in negative peaks. To ensure adequate detection limits, a carrier electrolyte anion with a high molar absorptivity and an effective electrophoretic mobility close to the mobilities of the analytes is required. Since carbohydrates have a pK_a of 12 or above, an electrophoretic analysis is only possible between pH 12 and 14, when the solutes lose a proton and thus become charged.

Indirect UV detection using sorbic acid has been applied to the determination of a number of mono-, di- and trisaccharides [34], as well as of all monosaccharides present in the glycan moieties of glycoproteins (Fig. 2) [35]. The detection limit with indirect UV using sorbic acid is $2 \cdot 10^{-4}$ *M*.

Lower detection limits could be achieved with carbohydrates carrying intrinsic negative charges. Compared to neutral compounds, these solutes displace more chromophores in the BGE. In a 6-m*M* sorbate BGE, pH 5, D-galactonic and D-gluconic acid were baseline-resolved with a detection limit of approximately $2 \cdot 10^{-6}$ *M* [36]. Even lower detection limits could be obtained for synthetic heparin fragments containing multiple carboxylic acids and sulfate groups [37].

The drawbacks of indirect UV detection under strongly alkaline conditions also apply to indirect LIF detection. Using coumarin 343 as the fluorescence emitter, concentration detection limits of $5 \cdot 10^{-6}$ *M* were found for sucrose, glucose, and fructose [38]. Since coumarin 343 degrades above pH 11.5, the resolution in this separation system is inferior to that in the sorbic acid system. The separation of high-molecular-weight polysaccharides is possible using fluorescein in the BGE [39].

3.3. Amperometric detection

The success of HPAEC–PAD for carbohydrate analysis clearly established amperometric detec-

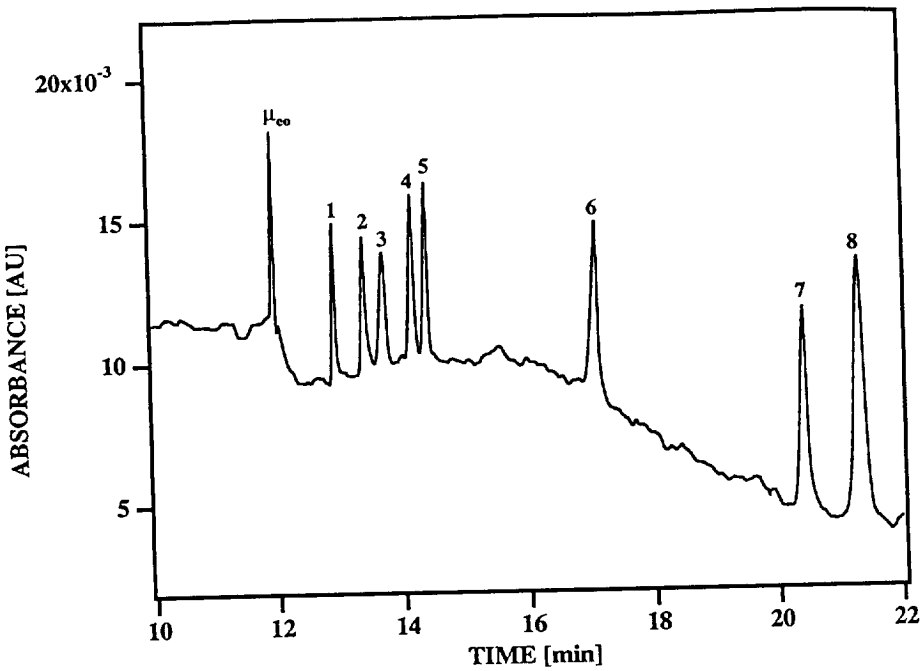


Fig. 2. Capillary zone electrophoresis of carbohydrates with indirect UV detection. Operating conditions: BGE 6 m*M* sorbate, pH 12.2; capillary 90 cm (83 cm to the detector) × 50 μm I.D.; voltage 20 kV; temperature 15°C; 1-s injection; indirect UV detection, 256 nm. Carbohydrates: 0.97–1.55 m*M*; 1 = fucose, 2 = galactose, 3 = glucose, 4 = N-acetylgalactosamine, 5 = N-acetylglucosamine, 6 = N-acetylneuraminic acid, 7 = galacturonic acid, 8 = glucuronic acid. Reproduced from Ref. [35].

tion as a highly sensitive and selective detection method for non-derivatized sugars [40,41]. Not surprisingly, amperometric detection in CE was developed as an alternative to unsatisfactory photometric methods. Since neither amperometry nor conductivity depends on path length, very narrow capillaries can be used.

Zare and his group demonstrated a separation of a mixture of 15 carbohydrates at pH 13 in less than 45 min with efficiencies of up to 200 000 theoretical plates. Detection was accomplished by a cylindrical copper electrode with amperometric detection at constant potential (Fig. 3) [42]. Concentration detection limits of $5 \cdot 10^{-6}$ *M* were achieved with linear calibration plots over three orders of magnitude. The high pH improved the selectivity of the separation system and therefore the resolution of the saccharides.

A slightly lower detection limit of 10^{-6} *M* (for glucose) was achieved using pulsed amperometric detection at a gold wire electrode [43]. The problem of electrode fouling was overcome by alternating anodic and cathodic polarization to clean and reactivate the electrode surface. Consequently, a uniform and reproducible electrode activity allowed the sensitive detection of glucose in human blood and of biologically important carbohydrates, such as glucosamine and its derivatives. However, the selectivity of the buffer system used did not allow the separation of the three neutral carbohydrates sucrose, glucose, and fructose, which can, though, be resolved according to other studies [34,35]

Cassidy and his group used a 10-μm disk electrode for pulsed amperometric detection. In a 10-μm capillary with a 0.1 *M* NaOH BGE, eight monosaccharides were determined at the 10^{-6} *M* level [44]. Initial separation efficiencies of 100 000–200 000 decreased after a few days of operation. The increased peak tailing led to the assumption that the properties of the capillary wall changed with time, and that interactions of the carbohydrates with the wall would result in a loss of efficiency.

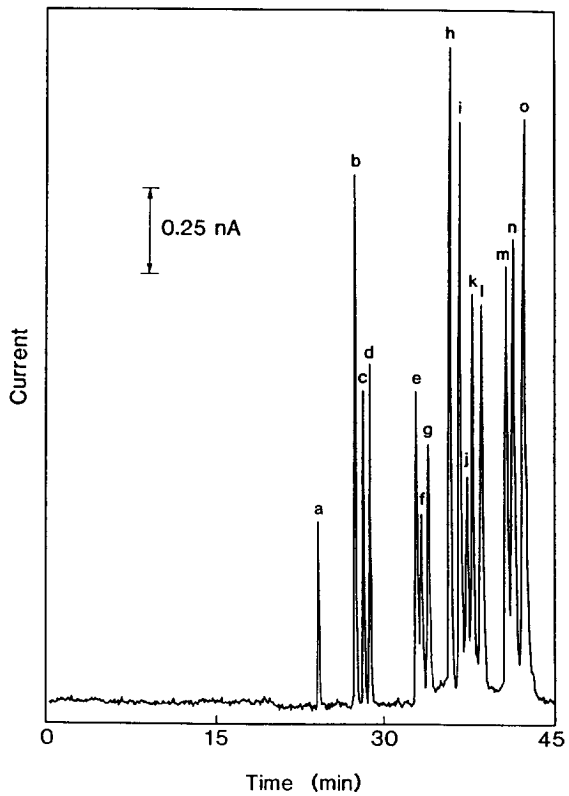


Fig. 3. Capillary zone electrophoresis with amperometric detection of a mixture containing 15 different carbohydrates (80–150 μM). Operating conditions: BGE 100 mM NaOH; capillary 73 cm × 50 μm I.D.; voltage 11 kV; injection 10 s by gravity (10 cm height); peak assignment: a = trehalose, b = stachyose, 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. Reproduced from Ref. [42].

The same group attempted to combine the selectivity of a borate buffer system with the sensitivity of amperometric detection through a post-capillary reaction system [45]. After the separation of carbohydrates in a borate buffer system, 0.4 M NaOH was added before the solutes reached the electrode. Although the sensitivity in this post-capillary reaction system was only one-tenth of that of direct amperometric detection in a strongly alkaline BGE, this system did allow the separation and detection of larger carbohydrates, such as cyclodextrins and maltoheptaose, which did not migrate in a NaOH electrolyte.

The wall-jet electrochemical detector, allowing the use of normal-size working electrodes (diameter > 100 μm), represents a third approach to amperometric detection in capillaries [46]. A disk-shaped electrode, consisting of a copper wire with only its tip cross-section exposed, was positioned immediately in front of the much smaller capillary opening (25 μm). In the fluid stream, exiting the capillary and flowing radially across the face of the electrode, detection of 10^{-6} M mono- and disaccharides was accomplished without significant post-capillary-zone peak broadening and loss in separation efficiency. The major advantages of this approach are the use of larger, more rugged electrodes and the better reproducibility of the electrode–capillary alignment.

As discussed in the HPLC literature, amperometric detection suffers from three general drawbacks independent of the detector design [10]. The amperometric detector is nondiscriminatory, and positive responses originating from amino acids, peptides, and organic acids can confuse peak assignment. More important, detector response is not uniform within a class of compounds, requiring standard curves for each solute before quantification. Thirdly, the high pH required for the proper working of the electrodes limits the choice of separation conditions. There is also the concern that strongly alkaline conditions can induce epimerization and degradation. This is known to reduce carbohydrates, especially if the reducing terminus is a 2-acetamido-2-deoxy sugar (N-acetylhexosamines). One has to keep these drawbacks in mind when comparing amperometric detection with other alternatives.

3.4. Refractive index (RI) detection

Recently, RI detectors based on interferometry have been developed for on-column detection in CE and micro-HPLC [47–49]. In one design, the interferometric pattern is generated at the capillary wall [47]; in a second design, a hologram is used [48]. Both RI detectors allowed the detection of carbohydrates at a 10^{-4} M level after separation in an alkaline borate

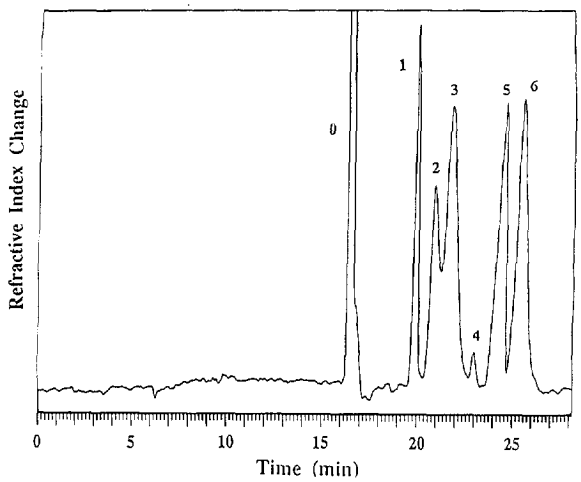


Fig. 4. Electropherogram of a mixture of five underivatized saccharides. Operating conditions: BGE 100 m*M* tetraborate, pH 9; capillary 70 cm (55 cm to the detector) × 50 μm I.D.; voltage 14 kV; thermocooler temperature 27°C; injection 7 s at 12 kV; RI detection, interference fringe, $n = 2$; carbohydrates (1% each, sucrose = 0.5%): 1 = sucrose, 2 = N-acetylglucosamine, 3 = cellobiose, 4 = impurity, 5 = N-acetylgalactosamine, 6 = lactose. Peak 0 resulted from the BGE. Reproduced from Ref. [47].

buffer (Fig. 4). Since the overall sensitivity of the RI detector depends mainly on the diameter of the capillary, higher sensitivity should be obtained with a 100-μm instead of a 50-μm capillary. Unfortunately, the extremely high currents and the Joule heat levels generated inside the 100-μm capillary at pH 13 inhibit its use. Therefore, this approach is only suitable for narrow capillaries with 10–50 μm I.D.

4. Detection of carbohydrates after labeling

4.1. Pre-column derivatization

The most appropriate method for converting a solute with no or only slight detector response into a derivative with enhanced detector properties is pre- or post-column derivatization. Pre-column derivatization is often preferred in both LC and CE because then the derivatization reaction is independent of the mobile phase or the BGE, respectively, and the reaction kinetics are not a limiting factor [50]. Additionally, pre-column derivatization may improve the selectivity and the resolution of the overall method by changing the chemical or structural characteristics of the analyte.

Three different chemistries have been described in the literature to label carbohydrates prior to CE analysis with suitable chromophores or fluorophores:

(1) reductive amination [51],
(2) condensation with 1-phenyl-3-methyl-5-pyrazolone (PMP) [28],
(3) condensation of carboxylated (acidic) saccharides with amines [52].

Depending on the label characteristics, derivatization chemistry (1) and (3) can be applied for both UV and fluorescence detection (Tables 2 and 3).

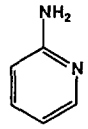
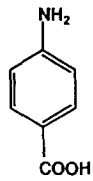
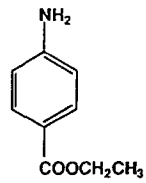
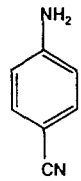
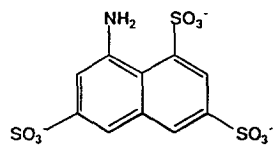
4.2. UV detection

4.2.1. UV labeling by reductive amination

The most frequently used method for the pre-column derivatization of carbohydrates is reductive amination. Reducing carbohydrates exist in solution in either an annular form or in the open-chain aldehyde form. The carbonyl group can react with the amino group of a label, forming a Schiff base. However, this is an equilibrium reaction with the thermodynamics favoring in most cases the educts. Therefore, in a second step, the Schiff base is reduced with sodium cyanoborohydride to a stable secondary amine (Fig. 5) [53,54]. The formation of the Schiff base is the rate-limiting step; the reduction proceeds rapidly. To shift the initial equilibrium more into the direction of the condensation and to prevent a further reaction of the final secondary amine with the carbonyl compounds, at least a five-fold excess of amine is necessary. Although the reductive amination works best in a pH range of 6 to 8, the reaction has been shown to work successfully at a pH as low as 4 and as high as 10 [55].

Honda and his group were the first to demonstrate the feasibility of pre-column labeling of carbohydrates for CE analysis with 2-aminopyridine (2-AP) [51], a label previously developed for paper electrophoresis [56] and

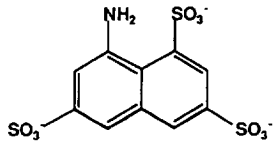
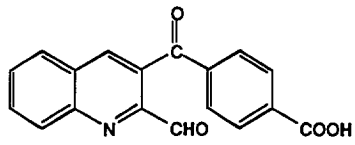
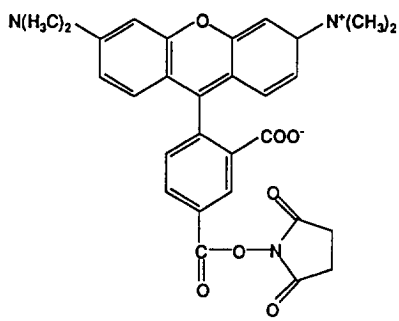
Table 2
UV labels for reductive amination of carbohydrates

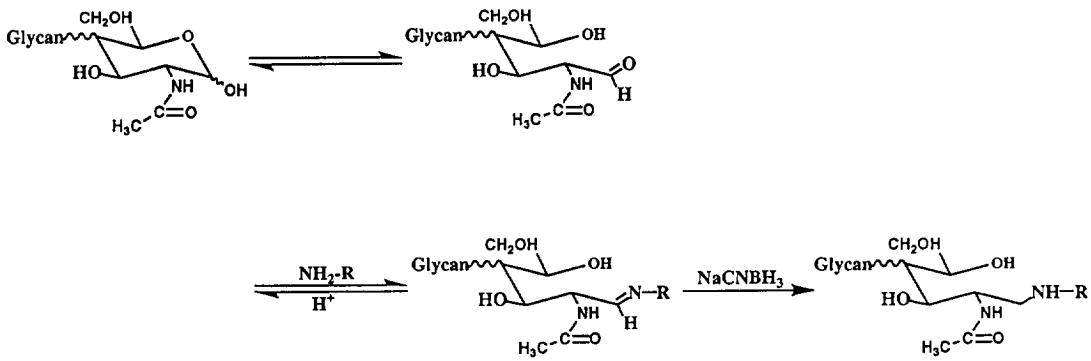
Label	Structure	Wavelength (nm)	L.O.D. (*M*)	Ref.
2-Aminopyridine		240	8×10^{-6}	[51,58–62]
p-Aminobenzoic acid		285	4×10^{-6}	[63,64]
4-Aminobenzoic acid ethyl ester		305	2×10^{-6}	[65]
4-Aminobenzonitrile		285	3×10^{-7}	[66]
8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS)		223	5×10^{-7}	[67,68]

HPLC [57]. A separation of 12 monosaccharides was completed within 25 min using a 200 m*M* borate buffer, pH 10.5 (Fig. 6). With UV detection at 240 nm, a concentration detection limit of $8 \cdot 10^{-6}$ *M* 2-AP-labeled glucose could be achieved (Table 2) [58].

A series of 2-AP-labeled maltooligosaccharides with four to seven glucose units was separated in a 100 m*M* phosphate BGE, pH 3–4.5 [59]. With a pK_a of 6.7, pyridylamino derivatives are positively charged under the acidic CE conditions. With the low electroosmot-

Table 3
LIF labels for reductive amination of carbohydrates

Label	Structure	Excitation/emission (nm/nm)	L.O.D. (*M*)	Ref.
8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS)		325/520 He–Cd laser	5×10^{-8}	[67,75–77]
		257/520 Ar-ion laser	1×10^{-9}	[78]
3-(4-Carboxybenzoyl)-2-quinoline carboxyaldehyde (CBQCA)		457/552 Ar-ion laser	3×10^{-9}	[80–83]
5-Carboxytetramethylrhodamine succinimidyl ester (TRSE)		543/580 He–Ne laser	5×10^{-11}	[20]



Schiff Base

Fig. 5. Reaction scheme for the reductive amination of carbohydrates.

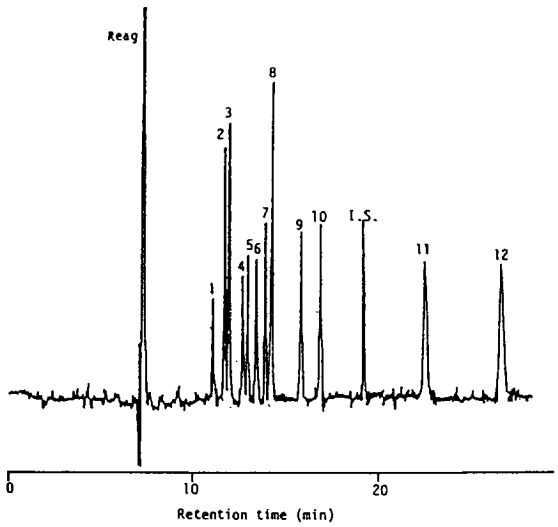


Fig. 6. Separation of N-2-pyridylglycamines derived from various monosaccharides. Operating conditions: BGE 200 m*M* borate, pH 10.5; capillary 65 cm × 50 μm I.D.; voltage 15 kV; UV detection, 240 nm. Peak assignment: Reag = reagent, 1 = N-acetylgalactosamine, 2 = lyxose, 3 = rhamnose, 4 = xylose, 5 = ribose, 6 = N-acetylglucosamine, 7 = glucose, 8 = arabinose, 9 = fucose, 10 = galactose, I.S. (internal standard) = cinnamic acid, 11 = glucuronic acid, 12 = galacturonic acid. Reproduced from Ref. [51].

ic flow (EOF), migration order depends on the charge-to-mass ratio and is a linear function of the number of glucose residues in the homologous series, with smaller oligomers eluting first.

Glycoprotein-derived monosaccharides failed to separate completely after 2-AP labeling under acidic conditions, but could be resolved in an alkaline borate buffer [60].

Ovalbumin-derived oligosaccharides were 2-AP-labeled and detected by UV at 240 nm with a HPLC fluorescence detector modified in-house [61]. The labeled ovalbumin glycans were separated in two different buffer systems: in a pH 2.5 phosphate BGE, according to their degree of polymerization (7–15 saccharide units), and in a borate buffer, which allowed additional separation according to the structural differences of the glycans. Combining acidic and borate buffer conditions, Suzuki et al. developed a 2D map of relative electrophoretic mobilities, showing three distinct domains for highly mannose-, complex-, and hybrid-type oligosaccharides, respectively [62].

Recently, a number of aminobenzene derivatives were introduced as carbohydrate labels for reductive amination, comprising 4-aminobenzoic acid [63,64], ethyl-4-aminobenzoate [65], and 4-aminobenzonitrile [66]. These labeling reagents allowed complete derivatization of the carbohydrates within only 2 h at 50°C, and in case of the 4-aminobenzonitrile within only 15 min. UV detection of the labeled compounds at 285 and 305 nm, respectively, provided detection limits of $4 \cdot 10^{-6}$–$3 \cdot 10^{-7}$ *M* (Table 2). Unlike 2-AP, these new labels are able to react with ketoses, such as fructose and sorbose.

All reported separations of carbohydrates labeled with aminobenzene derivatives were achieved in 175 or 150 m*M* borate buffers of pH 10–10.5 in open-tube capillaries, and were found to be dependent on the stability of the carbohydrate–borate complexes.

A completely different selectivity was obtained when 4-aminobenzonitrile derivatives were separated on the basis not of borate complexation but of differences in their solubilization in SDS micelles [66]. Generally, carbohydrates are too hydrophilic to be solubilized in ionic surfactants. But pre-column derivatization with 4-aminobenzonitrile rendered them hydrophobic enough to permit their separation due to the different distribution of the derivatives between the aqueous mobile phase and the micellar phase. Fig. 7 demonstrates the capillary zone electrophoresis (CZE) separation of a mixture of 16 mono- and oligosaccharides in a borate buffer, pH 10.5 (Fig. 7a) in comparison to the micellar electrokinetic chromatographic (MEKC) separation of the same saccharide mixture in a 25 m*M* Tris–phosphate BGE, pH 7.5, containing 100 m*M* SDS (Fig. 7b).

The last in the series of UV labels for carbohydrates is 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS). ANTS was originally developed for use in polyacrylamide slab gel electrophoresis [11] and has recently been adapted to CE systems [67,68]. It exhibits a high molar absorption coefficient of 68 350 M^{-1} cm^{-1} at 238 nm in a pH 2.5 phosphate electrolyte. Coupling of ANTS

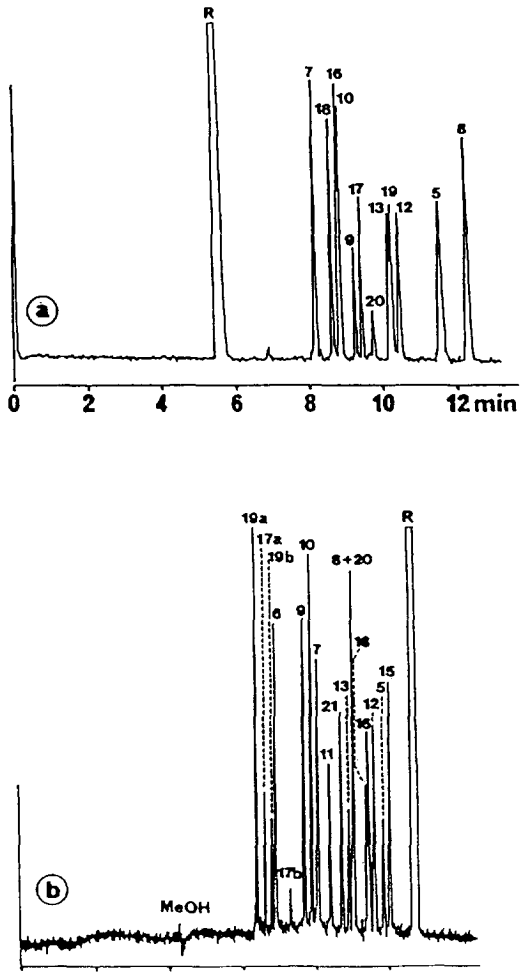


Fig. 7. Capillary zone electrophoresis (a) and micellar electrokinetic chromatography (b) of standard mixtures of mono- and oligosaccharides derivatized with 4-aminobenzonitrile. Operating conditions: BGE (a) 175 m*M* borate, pH 10.5, (b) 25 m*M* Tris–phosphate, pH 7.5, 100 m*M* SDS; capillary: (a) 72 cm (55 cm to the detector) × 75 μm I.D., (b) 75 cm (55 cm to the detector) × 50 μm I.D.; voltage: (a) 24 kV, (b) 30 kV; temperature 30°C; injection: vacuum, (a) 1 s, (b) 0.5 s; UV detection, 285 nm. Peak assignment: 5 = D-fucose, 6 = lactose, 7 = maltotriose, 8 = D-galactose, 9 = melibiose, 10 = cellobiose, 11 = maltose, 12 = L-arabinose, 13 = D-glucose, 15 = D-xylose, 16 = D-lyxose, 17 = L-sorbose, 18 = L-rhamnose, 19 = D-fructose, 20 = D-ribose, 21 = D-mannose. Reproduced from Ref. [17].

to a carbohydrate shifts the absorption maximum to 223 nm and allows sensitive UV detection of the labeled carbohydrate with a detection limit of $5 \cdot 10^{-7}$ *M* [67]. Using glucose as a model carbohydrate a derivatization efficiency of more than 99% could be achieved [68]. However, the reaction rate decreases for larger oligosaccharides, especially for those with N-acetylglucosamine residues at their reducing end, as in the case of glycoprotein-derived complex oligosaccharides.

Since the ANTS label carries three sulfonic acid groups which are negatively charged over a wide pH range, the ANTS conjugates can be subjected to CE at alkaline as well as acidic pH, as demonstrated with the separation of ANTS-derivatized maltooligosaccharides (Fig. 8). Since the net charge is the same for all the derivatives and the migration velocity depends on their charge-to-mass ratios, their effective migration velocity decreases as the number of glucose units increases. In the case of the alkaline BGE, where the negatively charged ANTS conjugates migrate against the strong EOF, the largest oligosaccharides reached the detector first and the glucose last, with glucose having the highest charge-to-mass ratio. In the acidic phosphate BGE, where only a small EOF is generated, the elution order was reversed, with the ANTS glucose eluting immediately after the ANTS peak, followed by the larger oligosaccharide fragments [68].

By adding triethylamine to the pH 2.5 phosphate BGE, a masking agent for silanol groups, the EOF inside the capillary could be eliminated. This way, extremely fast, and highly efficient separations were realized. For instance, ANTS-derivatized maltose, maltotetraose, and maltohexaose were separated within 30 s [68].

4.2.2. UV labeling by condensation with 1-phenyl-3-methyl-5-pyrazolone

The second derivatization scheme, developed by Honda and co-workers, involves the condensation between the active hydrogens of 1-phenyl-3-methyl-5-pyrazolone (PMP) or 1-(*p*-methoxy)phenyl-3-methyl-5-pyrazolon (PMPMP) with the carbonyl group of the reducing carbohydrate under slightly basic conditions, resulting in bis-PMP and bis-PMPMP derivatives [28,69–72].

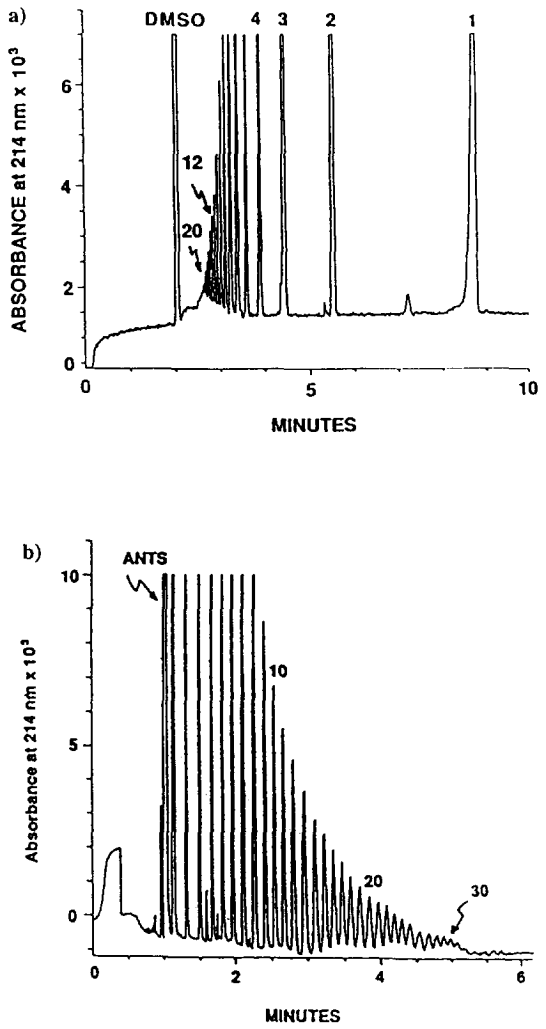


Fig. 8. Capillary zone electrophoretic separations of maltooligosaccharides derivatized with ANTS under (a) alkaline and (b) acidic conditions. Operating conditions: BGE: (a) 50 m*M* sodium phosphate, pH 9.0, (b) 50 m*M* sodium phosphate, 10.8 m*M* triethylamine, pH 2.5; capillary: (a) 37 cm (30 cm to the detector) × 50 μm I.D., (b) 27 cm (20 cm to the detector) × 50 μm I.D.; voltage: (a) 17 kV, (b) −22 kV; temperature 25°C; UV detection, 214 nm. Sample: (a) 11 ng of Dextrin 15 (Fluka Chemie, Buchs, Switzerland) injected at the anodic end, (b) 80 ng of Maltrin M040 starch hydrolyzate (Grain Processing, Muscatine, IA, USA) injected at the cathodic end. Reproduced from Ref. [68].

Since the procedure requires only slightly alkaline conditions (pH 8.3), both labels are especially attractive for the derivatization of sialylated oligosaccharides because no loss of sialic acid occurs [70].

Whereas PMPMP has only been used for carbohydrate analysis in HPLC, PMP was recently adapted for use in CE. Mixtures of PMP-aldopentoses and -hexoses as well as PMP-derivatized homologous oligoglycans of the isomalto, laminara, and cello series could be well separated in a 100 m*M* borate buffer, pH 9.5 [28]. The detection limit for the PMP carbohydrates was in the range of 10–50 fmol, corresponding to a concentration sensitivity of $(1–5)\cdot 10^{-6}$ *M* [71,72].

Honda et al. [72] have developed a separation method, an alternative to the usual borate complexation, which is based on the interaction of PMP carbohydrates with bivalent metal ions. When calcium ions were added to an acetate BGE, five PMP monosaccharides were resolved, which co-migrated in a single peak in a sodium acetate BGE. Separation of PMP pentoses in electrolyte solutions carrying different bivalent metal salts such as calcium, barium, and strontium acetate resulted in varying migration times and peak resolutions, as depicted in Fig. 9. The authors attributed this to differences in the ease of complexation due to differences in electronegativity and valence angles among the metal nuclei.

4.2.3. UV labeling by condensation of acidic saccharides with amines

Mechref and El Rassi [52] recently introduced a new, specific derivatization for acidic monosaccharides, which allowed separation and sensitive detection by CE. The derivatization reaction involved the condensation between the amino group of sulfanilic acid (SA) or 7-amino-naphthalene-1,3-disulfonic acid (ANDSA) and the carboxyl group of the acidic monosaccharide to form a peptide link in the presence of a water-soluble carbodiimide (Fig. 10). This proton-catalyzed derivatization procedure not only provides the chromophore or fluorophore for the detection, but also replaces a weak carboxylic group of the analytes by a stronger acidic group (sulfonic acid). The labeled carbohydrates carry negative charges, independent of

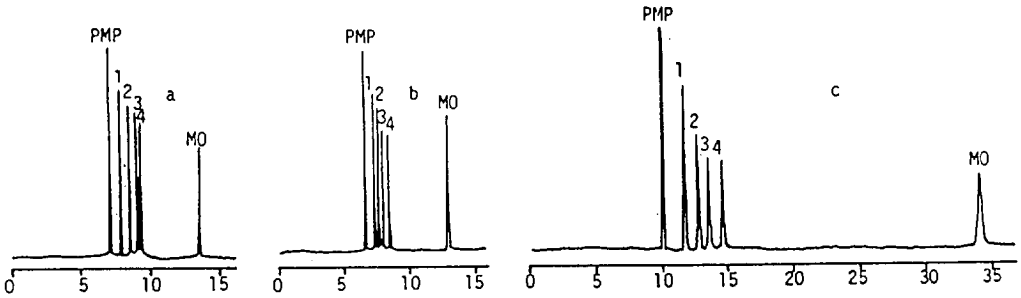


Fig. 9. Separation of pentose-PMPs in 100 m*M* aqueous solutions of calcium acetate (a), barium acetate (b), and strontium acetate (c). Other operating conditions: capillary 49 cm × 50 μm I.D.; voltage 10 kV; UV detection, 245 nm. Peak assignment: 1 = ribose-PMP, 2 = lyxose-PMP, 3 = arabinose-PMP, 4 = xylose-PMP, MO = mesityl oxide (internal neutral marker). Reproduced from Ref. [72].

the pH, allowing the separation of closely related SA- or ANDSA-labeled acidic monosaccharides in a low- or high-pH BGE. Separation efficiency varied from 50 000 theoretical plates per meter in the alkaline to 250 000–400 000 in the acidic electrolyte.

UV detection at 247 nm resulted in mass detection limits of 30 fmol for the SA and 15 fmol for the ANDSA derivatives, which corresponds to concentration detection limits of $2 \cdot 10^{-5}$ and $1 \cdot 10^{-5}$ *M*, respectively. Thus, surprisingly, the sensitivity in the case of the ANDSA derivatives is more than one order of magnitude less than that obtained with the ANTS derivatives. From the experimental data it is not clear whether this is due to the slight

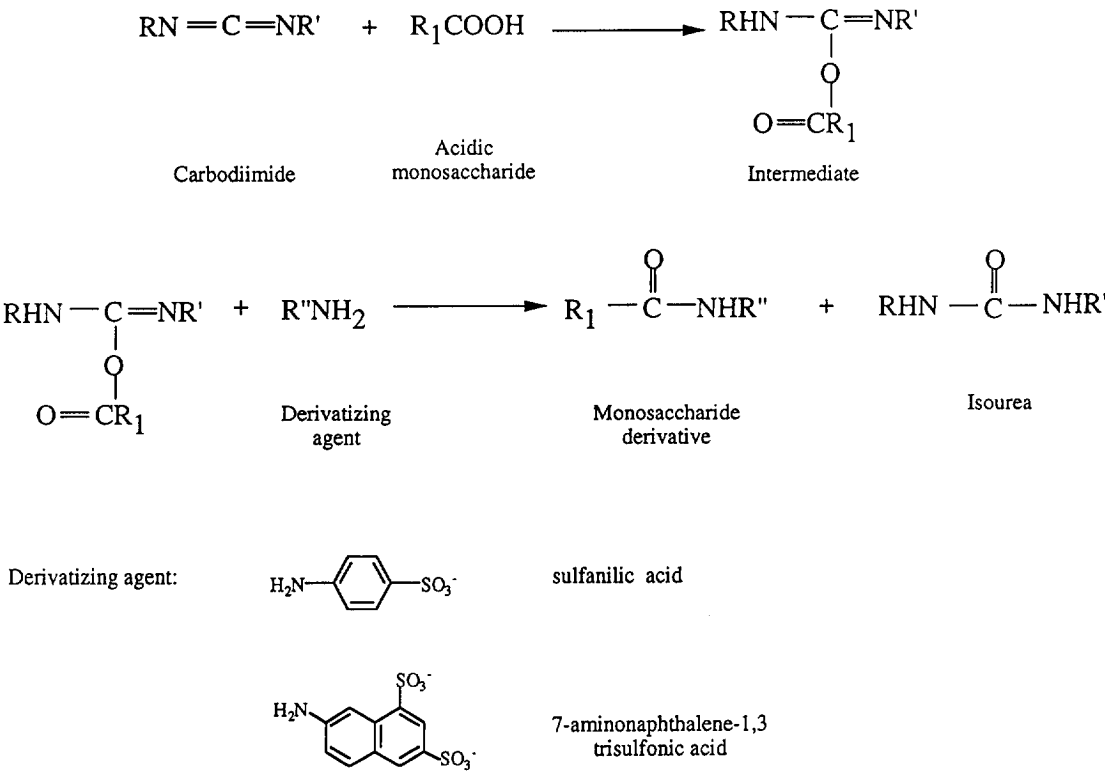


Fig. 10. Derivatization reaction for acidic monosaccharides with SA and ANDSA.

difference between the two labels (ANTS carries one more sulfonic acid group than ANDSA and has the amino group in the 8- instead of the 7-position) or to the difference in the derivatization mechanisms.

One crucial point in the present derivatization procedure is the amount of carbodiimide added to the reaction solution. To avoid the formation of side products, the molar amount of carbodiimide should not exceed that of the reacting carboxyl groups.

4.3. Fluorescence detection

According to Lambert–Beer's law, UV detection sensitivity in small-bore capillaries is limited by the small path length. For most solutes, concentration detection limits in the range of 10^{-5} to 10^{-7} *M* are found with on-column UV detection [73]. In micro-separation, fluorescence is generally expected to achieve lower detection limits than UV for two reasons: first, fluorescence is not path-length-dependent and, second, it is experimentally easier to detect a few photons in an essentially black background than to compare a large reference with a sample beam where a few photons are missing due to absorption. Using conventional deuterium, mercury or xenon lamp light sources for capillary fluorescence detection, only marginal gains in sensitivity of approximately one order of magnitude could be achieved. This is mainly due to the difficulties in focusing a sufficient amount of light from a divergent source onto an extremely small detection area with only a nanoliter volume, while light scattering from the capillary wall has to be minimized [73].

Therefore only a few applications of conventional fluorescence detection in CE have been published. With a xenon–mercury lamp as radiation source, Mechref and El Rassi [52] detected as little as $4 \cdot 10^{-7}$ *M* ANDSA-labeled acidic carbohydrates, using an excitation wavelength of 315 nm and monitoring the fluorescence emission at 420 nm. Compared to UV detection limits, a 25-fold improvement in concentration detection limit could be achieved.

Conventional fluorescence detection has also been used for the quantitation of chitooligosaccharides which are formed during the chitin breakdown by chitinase [74]. Six N-acetylglucosamine oligosaccharides with different degrees of polymerization ($n = 1–6$) were labeled with ANDSA and subsequently treated with the chitinase. The digestion products were analyzed by CE in an alkaline borate buffer. Fluorescence detection using an excitation wavelength of 250 nm and measuring the emission at 420 nm allowed the determination of single chitooligosaccharides; at the same time the enzyme activity was determined by following the kinetics.

4.4. Laser-induced fluorescence detection (LIF)

Compared to conventional light sources, narrow focusing of excitation light onto the capillaries is easily accomplished with lasers. Today, CE–LIF instrumentation is commercially available, adding to the attraction of this technique. Since lasers lack the flexibility of conventional broad-band light sources in terms of wavelength selection, a CE interface allowing the connection to various laser sources is required to maximize the versatility of the instrument.

Various laser sources and excitation lines have already been used for CE–LIF applications, with the majority of these applications being performed with the easy-to-use and relatively low-cost Ar-ion and He–Cd-laser [73]. The 488 nm line of the Ar-ion laser matches very closely the 490 nm absorption maximum of popular fluorescein-based labels such as fluorescein isothiocyanate and fluorescein succinimidyl ester. The 325-nm line of the He–Cd laser is suitable for the detection of *o*-phthalaldehyde (OPA) and dansyl-labeled compounds, while the 442-nm line is compatible with the naphthalene-2,3-dicarboxylic acid (NDA) and 3-(4-carboxybenzoyl)-2-quinoline carboxyaldehyde (CBQCA) labels [73].

4.4.1. Oligosaccharides labeled with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)

The ANTS label exhibits intrinsic fluorescence and should therefore also be amenable to LIF detection. To achieve the lowest detection limits, fluorescence should be excited close to the ab-

sorption maximum of 223 nm. However, laser sources around 220 nm are not feasible for economic reasons. Since ANTS and its carbohydrate derivatives exhibit a second, though lower, excitation maximum at 360 nm with an emission at 520 nm, a He–Cd laser with a 325-nm line is the optimal choice for LIF detection with commercial instrumentation [67]. Concentration detection limits of $5 \cdot 10^{-8}$ *M* were achieved for both small carbohydrates such as maltose [67] and complex oligosaccharides with molecular weights of 2000–3000 [75]. Compared to UV detection, LIF detection of ANTS-labeled carbohydrates provides only a modest gain in sensitivity of one order of magnitude.

Stefansson and Novotny [76,77] demonstrated the suitability of ANTS derivatization for the separation and detection of various oligo- and polysaccharides, differing in the degree of polymerization. The separations were carried out in polyacrylamide-coated capillaries using 100 to 200 m*M* Tris–borate buffers, pH 8.65. The authors stress the importance of using wall-coated capillaries to eliminate the EOF. In uncoated fused-silica capillaries, the EOF is much higher than the electrophoretic mobility of the labeled oligosaccharides, and in the opposite direction. With increasing size, the net velocity approaches the electroosmotic flow velocity asymptotically, thereby decreasing resolution with size. Moreover, peak efficiencies have been found to be considerably higher in coated capillaries [76]. The combination of coated capillaries and a charged label resulted in highly efficient, extremely fast separations of oligo- and polysaccharides.

Since fluorescent labeling with ANTS does not affect enzymatic cleavage, ANTS derivatization seems to be a valuable tool for kinetic studies of enzymatic degradation processes. Additionally it could be used for the structural characterization of polysaccharides, with CE oligosaccharide maps being compared before and after controlled enzymatic fragmentation [76,77].

ANTS derivatization also proved to be a valuable tool for the analysis of glycoprotein-derived complex oligosaccharides [75]. In contrast to homologous oligosaccharides, complex carbohydrates display heterogeneity in composition and structure. Absolute amounts of 25 to 50 pmol of high-mannose- and complex-type oligosaccharides could be labeled with ANTS in a total reaction volume of as little as 2 μl and subsequently detected using a He–Cd laser. By carrying out the derivatization at 40°C overnight, even sialylated oligosaccharides could be labeled without significant loss of sialic acid.

Fast separations of the labeled oligosaccharides within 6 to 10 min with excellent resolution were achieved in a pH 2.5 BGE. In the case of the more homologous high-mannose-type oligosaccharides the separation was found to depend on the differences in the charge-to-mass ratios of the solutes. Since the high mannoses themselves are neutral, carrying only the three negative charges originating from the ANTS label, they migrated in order of increasing molecular weight. In contrast, the complex-type oligosaccharides, which can be neutral or charged, showed some deviation from this linear relationship between electrophoretic mobility and the charge-to-mass ratio. It was assumed that the distinct three-dimensional structure of the carbohydrates, determined by the stereochemical position of the sialic acid residues, the glycosidic linkages, the α/β-anomerity, and the variety of branching, caused these deviations. Because of this additional influence of the carbohydrate structure, even small differences in molecular weight were found to be sufficient to give baseline resolution for the complex oligosaccharides.

The authors could also demonstrate that the ANTS derivatization is suitable for the fast generation of oligosaccharide patterns derived from various glycoproteins. Fig. 11 shows the separation of oligosaccharide mixtures derived from human immunoglobulin G and bovine fetuin.

Using a frequency-doubled Ar-ion laser with an emission wavelength of 257 nm, detection limits as low as 10^{-9} *M* could be achieved for ANTS maltose [78]. This is due to the higher absorption of the ANTS molecule at 257 nm compared to the absorption at the 325-nm line of the He–Cd laser. Fig. 12 demonstrates the separation of a mixture of a 100 n*M* solution of neutral complex-type oligosaccharides in an

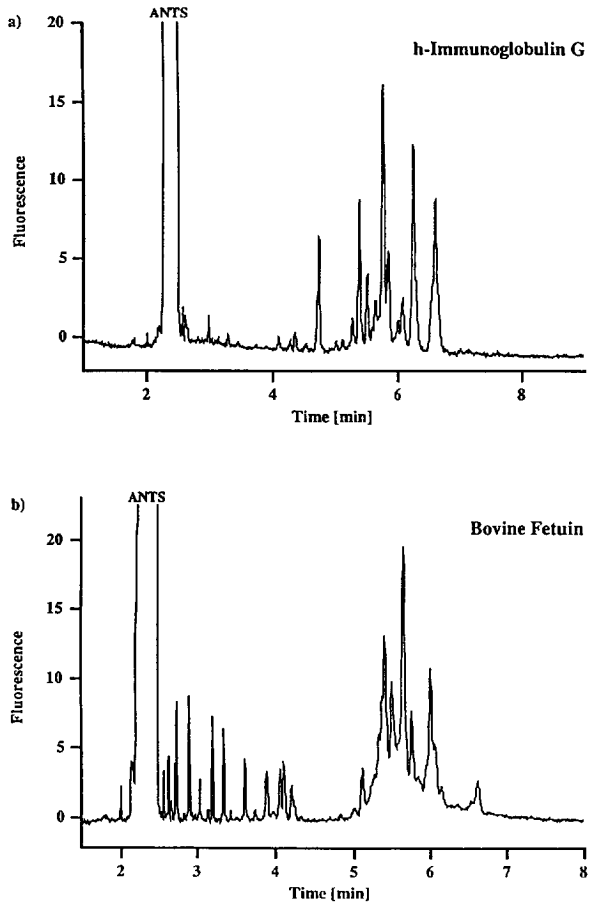


Fig. 11. Separation of ANTS-labeled oligosaccharide libraries derived from human immunoglobulin G (a) and bovine fetuin (b). Operating conditions: BGE 50 m*M* sodium phosphate, pH 2.5; capillary 27 cm (20 cm to the detector) × 50 μm I.D.; voltage −10 kV; temperature 25°C; injection 3 s at the cathodic end; LIF detection, He–Cd laser, 325 nm excitation, 520 nm emission.

acidic phosphate BGE. Because the instrumental setup required a separation capillary of 75 cm long, all peaks are very broad due to increased diffusion.

4.4.2. Oligosaccharides labeled with 3-(4-carboxybenzoyl)-2-quinoline carboxyaldehyde (CBQCA)

CBQCA (Table 3), a fluorogenic compound that was originally developed for the high-sensitivity analysis of amino acids and peptides [79], can be used as an alternative to ANTS in highly sensitive LIF detection. Fluorescent CBQCA derivatives of carbohydrates containing no amino group were obtained by reductive amination with ammonia leading to the formation of 1-amino-1-deoxyalditols that readily reacted with CBQCA in the presence of potassium cyanide within 1 h at room temperature. In this way mono- and homologous oligosaccharides, as well as glycoprotein-derived complex carbohydrates and glycosylaminoglycans were derivatized [80–83]. The CBQCA derivatives were detectable with either an Ar-ion or a He–Cd laser. On-column fluorescence detection with the 457-nm line of an Ar-ion laser, with the emission at 552 nm being monitored, resulted in detection limits of $3 \cdot 10^{-9}$ *M* for CBQCA-labeled monosaccharides with a linear range over four orders of magnitude [81].

A major drawback of this derivatization method is the closely defined molar ratio of CBQCA and potassium cyanide to carbohydrate at which a maximum yield of CBQCA derivative can be obtained. At a one- to two-fold molar excess of CBQCA the fluorescence yield for galactosamine was highest, while it decreased to one-tenth at a 0.2- and five-fold molar excess, respectively [80]. Another disadvantage is the simultaneous labeling of other amines usually present in glycoprotein hydrolyzates, that may interfere with the analysis. On the other hand, it is not necessary to remove the excess of CBQCA, as the unreacted compound does not fluoresce.

CBQCA-labeled oligosaccharides have been separated in open-tube capillaries and in capillaries filled with highly concentrated (30% T and 3% C) polyacrylamide gels [83]. Fig. 13 shows the separation of a polygalacturonic acid hydrolyzate after derivatization with CBQCA. Since in a wall-treated, gel-filled capillary no EOF occurs, electromigration of the labeled oligosaccharides depends only on their size and charge. The separation of CBQCA-labeled polydextrans with molecular weights ranging from 8000 to 2 000 000 was achieved in an entangled polyacrylamide solution with applied pulsed-field alteration. At constant potentials of 500 and 300 V/cm, the polysaccharides could not be size-separated due to molecular stretching. By applying a potential gradient along the separation

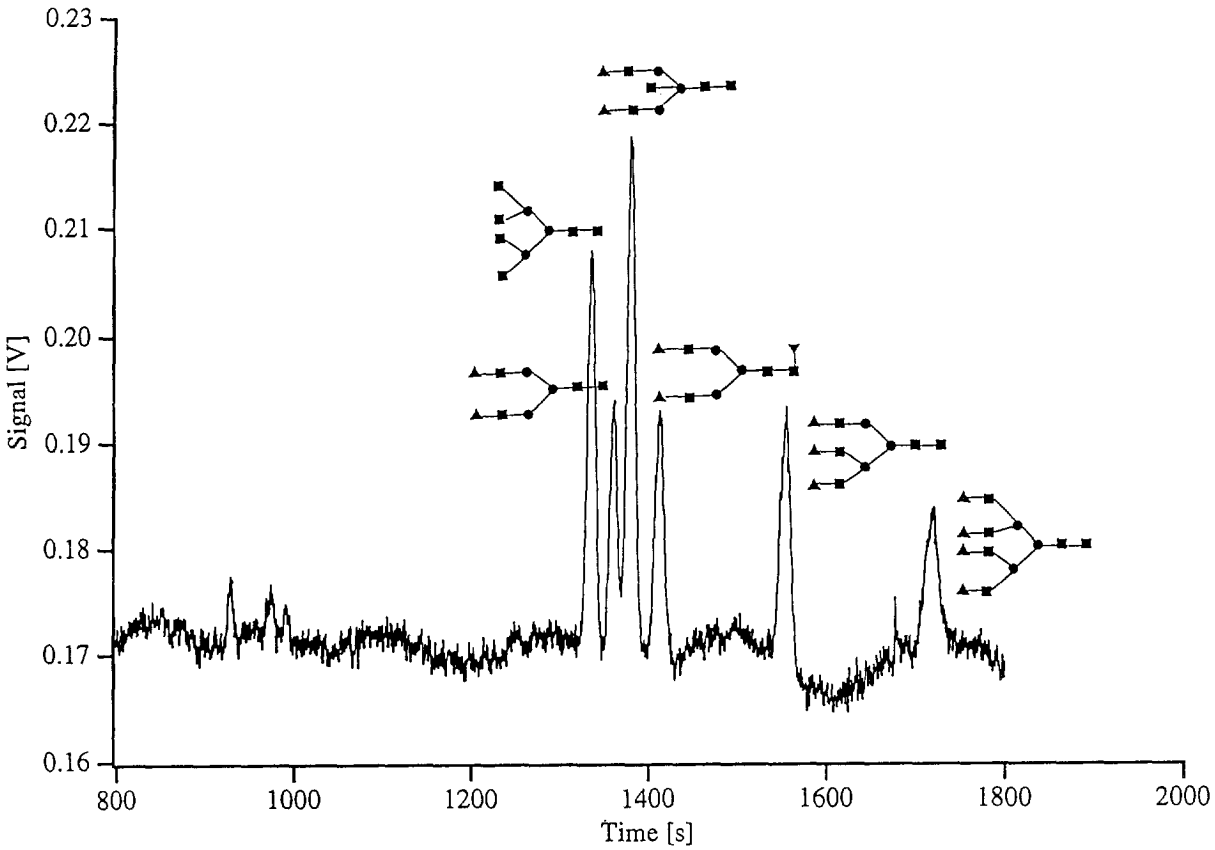


Fig. 12. Detection of neutral complex-type oligosaccharides with a frequency-doubled Ar-ion laser, after capillary electrophoretic separation. Operating conditions: BGE 50 m*M* sodium phosphate, pH 2.5; capillary 70 cm (50 cm to the detector) × 50 μm; voltage −20 kV; injection 9 s, 100 mbar; LIF detection, Ar-ion laser, 257 nm excitation, 520 nm emission. Samples: neutral complex oligosaccharides, 50–70 n*M*; ▲ = galactose, ■ = N-acetylglucosamine, ● = mannose, ▼ = fucose. (Unpublished results.)

capillary, which is periodically inverted at an angle of 180°, the reptation behavior of the polysaccharides could be overcome, as the molecules undergo shape transition, and the separation could be accomplished [84].

4.4.3. Carbohydrates labeled with 5-carboxy-tetramethylrhodamine succinimidyl ester (TRSE)

Zhao et al. [20] recently introduced another two-step derivatization procedure for carbohydrates. In the first step, carbohydrates containing no amino group were transformed to the corresponding 1-amino-1-deoxyalditols, while hexosamines were reduced with sodium borohydride to the 2-amino-2-deoxyalditols. These aminated carbohydrates were subsequently labeled with TRSE (Table 3). With this procedure the six major hexoses found in mammalian glycoproteins: glucose, galactose, mannose, fucose, N-acetylglucosamine, and N-acetylgalactosamine, could be derivatized and separated in an alkaline borate–phosphate–SDS BGE. The addition of phenyl boronic acid was found to have a great impact on the resolution of the labeled saccharide isomers. A He–Ne laser was used for LIF detection of the TRSE derivatives, with an excitation wavelength of 543.5 nm and an emission wavelength of 580 nm. With this setup a concentration detection limit of $5 \cdot 10^{-11}$ *M* was achieved, corresponding to a mass detection limit of $4 \cdot 10^{-22}$ mol (injection volume 9 pl) or 260 analyte molecules. This is the lowest con-

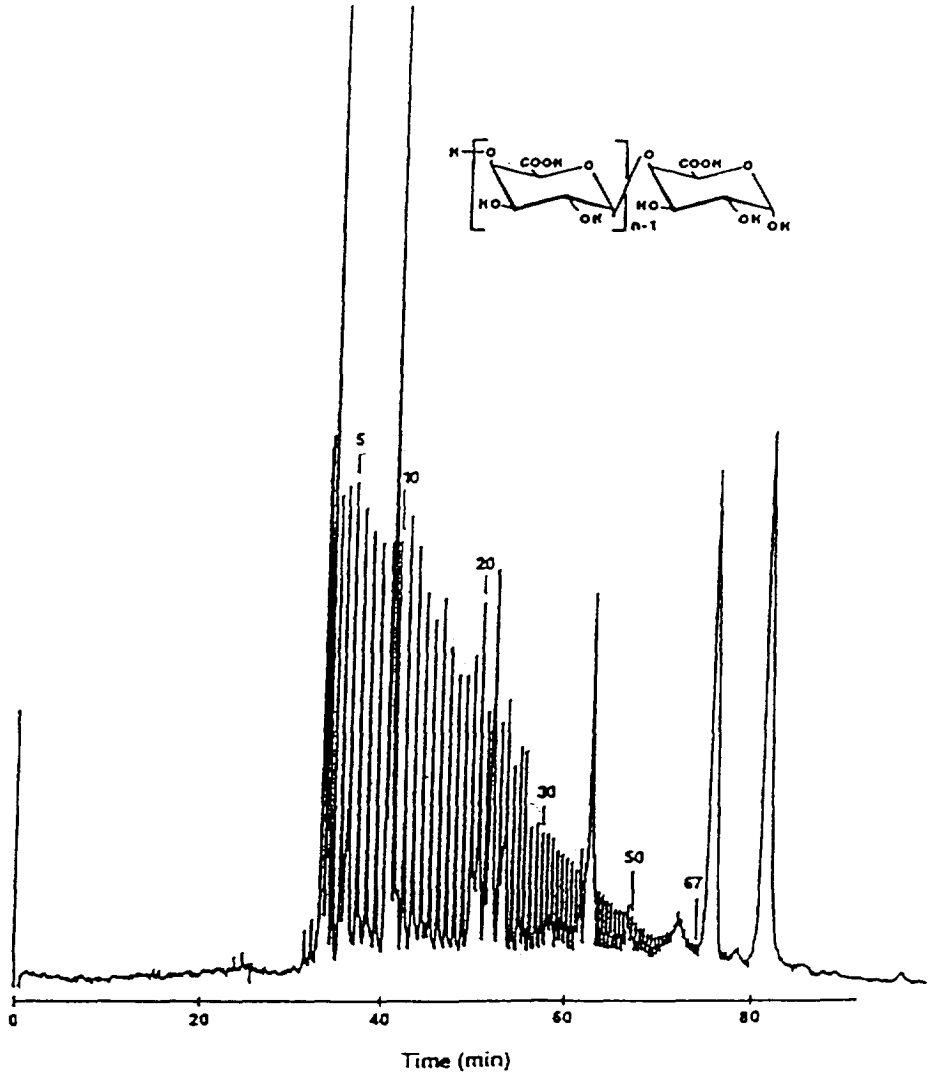


Fig. 13. Capillary gel electrophoresis of oligomers derived from an autoclave hydrolysis of polygalacturonic acid. The numbers indicate estimated degree of polymerization repeated by a monosaccharide unit. Operating conditions: BGE 0.1 *M* Tris–0.25 *M* borate–2 m*M* EDTA, pH 8.48; capillary 32 cm (23 cm to the detector) × 50 μm I.D.; polyacrylamide gel concentration 18% T, 3% C; voltage 7.5 kV; electrokinetic injection, 5 kV, 25 s. Reproduced from Ref. [83].

centration detection limit reported for carbohydrates in CE.

The main drawback of this derivatization procedure is the poor stability of the succinimidyl ester in a basic buffer, resulting in the generation of more than one peak for the dye itself in the electropherogram. This may cause an overlapping of analyte- and dye-related peaks when more complex mixtures of TRSE-labeled carbohydrates are separated.

4.4.4. Fluorescent cyclodextrin inclusion complexes

The tendency of cyclodextrins (CDs) to form inclusion complexes can be used for their separation and detection. Since CDs are known to

dramatically alter the fluorescence quantum efficiency of fluorophores which form inclusion complexes, complexation with 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) allowed visualization of the CDs [85]. Using an Ar-ion laser operating at 363.8 nm, α-, β- and γ-CD could be determined with a sensitivity of 62, 2.4, and 24 μM, when the fluorescence emission was monitored at 424 nm. Additionally the 2,6-ANS molecule imparted a charge to the neutral CDs, allowing their separation according to differences in the binding constants for the CD–2,6-ANS complexes. For the separation of derivatized CDs with different degrees of substitution, a modification of the separation conditions was required in terms of increasing electrophoretic mobility of the CDs and reducing the EOF.

4.5. Thermooptical absorbance detection (TOA)

In TOA an intense laser pulse irradiates the sample repeatedly with a wavelength matching an absorption system in the sample. The absorbed light, which is converted into heat, increases the solvent temperature in the illuminated region [86,87]. Since the refractive index (RI) of a material is a function of the temperature, this change in temperature can be monitored by a laser-based RI detector. Using the TOA detector developed by Krattiger et al. (Fig. 14), the carbohydrate samples were optically pumped on-column by the 257-nm line of a frequency-doubled Ar-ion laser and probed by a hologram-based RI detector [88].

Although absorption of ANTS-labeled carbohydrates was only one-fifth, at 257 nm, of that at 223 nm (where UV absorption was usually measured), it was still at least a factor of five higher than at the 325-nm line of the He–Cd laser. Therefore sensitive detection of the ANTS derivatives with this TOA device should be expected. With a detection limit for ANTS maltose of 10^{-7} M, only a five-fold increase in sensitivity was gained compared to conventional UV detection. But it can be assumed that with laser pumping the ANTS derivatives at a lower wavelength (240–220 nm), the detection limits can be further improved. The TOA detector could be used for monitoring glycoprotein-derived complex oligosaccharide mixtures. Fig. 15 shows the separation of the oligosaccharide chains of white hen-egg ovalbumin [78]. An identical pattern was obtained by He–Cd laser-based LIF detection [75].

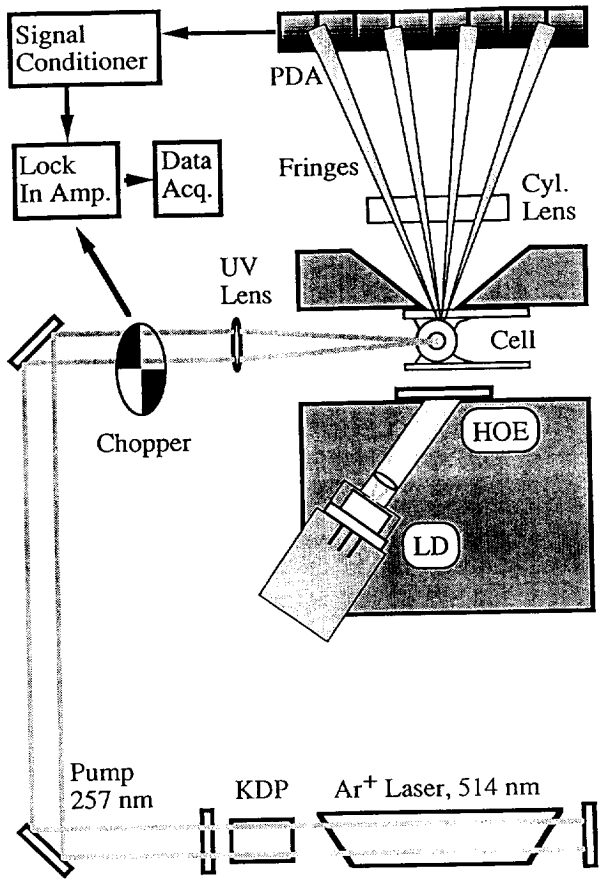


Fig. 14. Experimental setup for the TOA detector: KDP = potassium dihydrogen phosphate frequency-doubling crystal; LD = laser diode; HOE = holographic optical element; PDA = photodiode array.

5. Conclusions

During the last five years, carbohydrates have been added to the classes of compounds that are being intensively studied by CE. Since sample

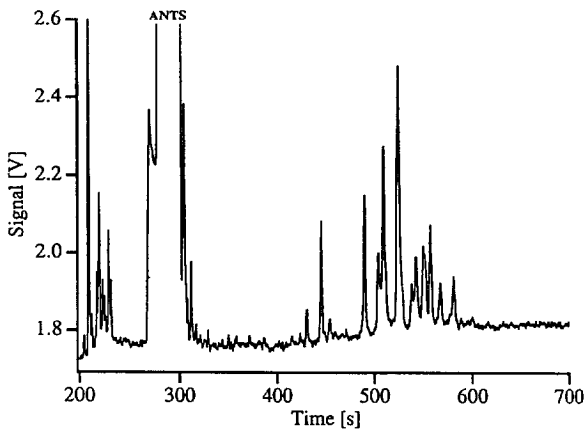


Fig. 15. Detection of the ANTS-labeled ovalbumin-derived oligosaccharide ladder by TOA. Operating conditions: BGE 50 m*M* sodium phosphate, pH 2.5; capillary 70 cm (50 cm to the detector) × 20 μm I.D.; voltage −30 kV; injection 0.94 min, 100 mbar. Pumping power 10 mW; lock-in detection sensitivity 10 mV. (Unpublished results.)

quantity is a restricting element in glycobiology, highly sensitive detection methods are being developed. At the same time, there is a need for knowing how to manipulate selectivity in CE carbohydrate separations. Because of their lack of chromophores, detection limits of naturally occurring carbohydrates are in the millimolar to high micromolar range. Amperometric detection is one real possible alternative. However, reported detection limits are only in the low micromolar range. In addition, amperometric detectors are in the research phase and not commercially available.

Developments using the common UV or LIF detector have met greater acceptance. It is being recognized that appropriate labeling strategies could lead to new methods for glycobiology analysis. Early efforts with UV labels such as 2-aminopyridine and *p*-aminobenzoic acid have been encouraging but are still short of fulfilling the sensitivity requirements of modern glycobiology. LIF detection can provide the means if a reactive label with a laser-matching emission line and good electrophoretic characteristics can be found. To date, the available lasers are Ar-ion, He–Cd, and He–Ne laser with laser wavelengths of 325 to 645 nm.

In the future there will be a trend towards the use of smaller, easy-to-use, inexpensive laser diodes, integrated in optical systems. Potentially, pre-column derivatization of mono- and oligosaccharides, as well as glycoprotein-derived complex carbohydrates with highly fluorescent labels, could push concentration detection limits into the nanomolar range. However, neither instrumentation nor the labels that are the limiting factors but the chemistry of the labeling reaction. For larger oligosaccharides and complex carbohydrates in particular, the small diffusion rates have to be taken into account. New micromethods will have to be developed that work on a microliter scale while at the same time ensuring quantitative reaction at the dilute concentrations of both solute and label.

Parallel to the improvement of detection limits, much effort is being expended on the introduction of charged labels in the CE of neutral carbohydrates. The charged labels, such as ANTS, allow extremely fast and highly efficient separations of homologous oligosaccharides and complex carbohydrates with excellent resolution, under conditions where EOF is almost negligible. In this way fast screening of the oligosaccharide patterns of various glycoproteins could be demonstrated. CE, with its outstanding features of speed of analysis, resolution, and reliable quantitation in combination with a highly sensitive LIF-based detection system, will become an effective and valuable tool in the structural elucidation of glycoproteins and the quality control of novel glycoconjugates to be used as pharmaceutical drugs.

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