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Review

Derivatization of carbohydrates for chromatographic, electrophoretic and mass spectrometric structure analysis

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Abstract

Carbohydrates, either alone or as constituents of glycoproteins, proteoglycans and glycolipids, are mediators of several cellular events and (patho)physiological processes. Progress in the ‘‘glycome’’ project is closely related to the analytical tools used to define carbohydrate structure and correlate structure with function. Chromatography, electrophoresis and mass spectrometry are the indispensable analytical tools of the on-going research. Carbohydrate derivatization is required for most of these analytical procedures. This review article gives an overview of derivatization methods of carbohydrates for their liquid chromatographic and electrophoretic separation, as well as the mass spectrometric characterization. Pre-column and on-capillary derivatization methods are presented with special emphasis on the derivatization of large carbohydrates.
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Keywords: Reviews; Derivatization; Structure analysis; Carbohydrates

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1. Introduction

The notion that carbohydrates in nature only serve the dual purpose of being structural components and key players of the energy metabolism is now past, in the light of findings from the field of glycobiology. Carbohydrates, either alone or as constituents of glycoproteins, proteoglycans and glycolipids, are mediators of several cellular events, such as intra- and extracellular recognition, differentiation, proliferation and even signal transduction [1–7]. Their structural complexity, far greater than that of proteins and nucleic acids, allows them to encode information for specific molecular recognition and to determine protein folding, stability and pharmacokinetics. The interest in glycosylation is rising not only out of academic curiosity to decipher their biologic roles but also due to the implication of carbohydrates in many pathologic states, such as cancer, atherosclerosis and rheumatoid arthritis.

Progress in the field of glycobiology, albeit significant in recent years, has long been hampered due to a lack of analytical tools to define carbohydrate structure and correlate structure with function. Carbohydrate structure characterization is still far from being a routine procedure, despite the advances in the analytical techniques. The dawn of the 21st century coincided with the first references to ''glycome'' and ''glycomics'' research [8–10]. Chromatography, electrophoresis and mass spectrometry (MS) are the indispensable analytical tools of the on-going research [11–14]. Carbohydrate derivatization is required for most of these analytical procedures.

Derivatization of carbohydrates serves multiple purposes [15]. Detection of intact carbohydrates can only be spectroscopic at 195 nm, amperometric or refractive index, all of which are associated with low sensitivities. The exception to this rule are the Δ-di- and oligosaccharides produced by the action of bacterial lyases on glycosaminoglycan (GAG) chains, which strongly absorb at 232 nm, due to the conjunction of the formed double bonds with the carboxyl groups in the unsaturated uronic acid residues [9,16,17]. Derivatization with UV-absorbing or fluorescent molecules significantly enhances the detection sensitivity of high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and polyacrylamide gel electrophoresis (PAGE).

The great majority of carbohydrates are hydrophilic and neutral. Derivatization with appropriate compounds changes carbohydrate properties in order to assist their resolution, i.e. endows them with charge to make easier their electrophoretic separation or hydrophobicity for their efficient resolution by reversed-phase HPLC or micellar electrokinetic chromatography (MEKC). For example, neutral sugars were converted to ionized species by dithioacetylation [18]. Two 2-mercaptoethanesulfonic acid molecules were introduced to the reducing end. Separation of diastereomers is often feasible after derivatization with chiral molecules. For instance, reductive amination of aldoses, hexuronic acids and aminosugars with a chiral derivatizing agent, *S*-1-phenylethylamine, resulted in pairs of diastereomer derivatives (D- and L-) which could be separated with CE in an achiral environment of an alkaline borate buffer [19].

Intact glycans are poor analytes for any MS procedure. In detail, intact native oligosaccharides are not ionized very efficiently by the soft ionization methods, fast atom bombardment (FAB), electrospray ionization (ESI) or matrix assisted laser desorption/ionization (MALDI), since they are polar, thermally labile and relatively non-volatile. Derivatization may offer several advantages such as less

complicated spectra and increased response. Permethylation and peracetylation among other methods have been used to enhance MS characterization of oligosaccharides, but are not described herein. Reducing carbohydrates can be derivatized with a number of compounds containing amino-groups in order to make easier their MS characterization (for a thorough presentation of MALDI-MS analysis of carbohydrates the review written by Harvey [20] is recommended). For example, after reaction of oligosaccharides with diethylenetriamine and addition of $ZnCl_2$, the carbohydrate–metal–ligand complexes are characterized in regard to the anomeric configuration and stereochemistry of constituent monosaccharides with ESI-MS–MS [21].

Derivatization may occur prior to, during or after the separation technique (HPLC or CE) and certainly prior to MS. Post-column derivatization of carbohydrates has been used after HPLC separation and was thoroughly reviewed by Honda in 1996 [22]. The principles of post-column derivatization in the publications after 1996 are similar to those described in that review and therefore post-column derivatization will not be dealt with in this review article. Hase [23] reviewed methods of pre-column derivatization of carbohydrates also in 1996, but a number of derivatizing agents and optimizations of reactions have been reported ever since. The aim of this article is to review the methods of carbohydrate derivatization before and/or during separation techniques (HPLC, CE, PAGE) and MS characterization, with special emphasis on the derivatization of large carbohydrates. Derivatization protocols developed in our laboratories are also presented here.

2. Pre-column derivatization

Several parameters must be considered before developing a pre-column derivatization procedure. These include the molecular size of the saccharides and their solubility in solvents convenient for the derivatization reaction, desired hydrophobicity of the derivatives (e.g. for reversed-phase HPLC) and the requirement for basic groups for more efficient MS analysis. Other important parameters are the extinction coefficient in the case of UV detection, the compatibility of fluorophore properties with the available detection equipment and fluorescence yield, the cost and the yield of the reaction, ease of separation of excess reagent from the product, stability and compatibility of the derivatives with downstream characterization techniques (e.g. MS and NMR).

2.1. Reductive amination

The labeling reaction starts with the attack of the lone pair of amino groups of the label to the carbon of carbonyl groups of reducing sugars, yielding a Schiff base (imine derivative) (Fig. 1A). Mild acidic

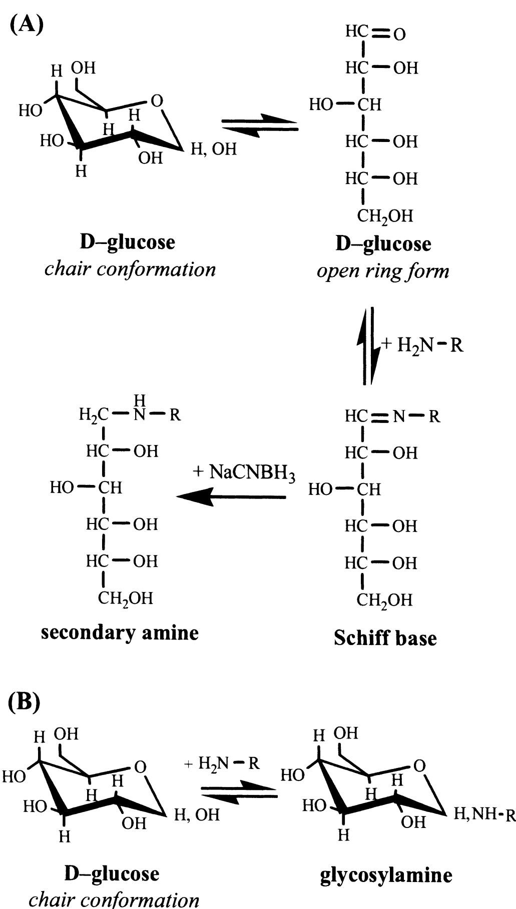

Fig. 1. Derivatization of reducing sugars (D-glucose) with amino-compounds in the presence of reducing agent (reductive amination) (A) and in the absence of reducing agent (B). The product of the former reaction is a secondary amine and of the latter one a glycosylamine.

conditions are necessary to promote sugar ring opening. The Schiff base is acid-labile and is reduced with sodium cyanoborohydride to a stable secondary amine. As shown in Fig. 1A, the stoichiometry of labeling is one label (UV-absorbing or fluorescent) per saccharide.

Because of the equilibrium nature of the first step, the reaction should be conducted in a way that maximizes the yield of labeled sugars. This can be accomplished by using excess of labeling reagent and/or varying the reaction conditions, such as labeling time, temperature as well as the pH and solvent system in the reaction mixture. Typical reaction conditions are temperature: 37 to 90 °C, time: 1 h to overnight incubation, acid: acetic acid or citric acid, reducing agent: $NaCNBH_3$, organic solvent for the labeling reagent: methanol or dimethylsulfoxide (DMSO). Optimization of these parameters is necessary for quantitative reaction yield and minimization of sample decomposition [24]. Sialic acid-containing oligosaccharides are particularly labile at the acidic conditions employed. High molecular mass oligosaccharides may be relatively insoluble in methanol at some concentrations and difficult to recover from the DMSO usually employed [25]. The reaction should then be performed in aqueous conditions, which involves the selection of a labeling reagent that can be solubilized in aqueous solution. Derivatization of ketoses seems to present a particular problem since only some of the derivatizing agents react with them and, in some cases, not quantitatively.

The excess of labeling reagent necessitates high purity of the labeling reagent preparation because impurities carrying amine groups in the preparation will also react [26]. Moreover, an excess of labeling reagent may pose a problem in the separation or MS techniques. In some separation procedures the migration or elution behavior of the labeling reagent is distinctly different from its sugar derivatives and therefore it does not interfere with the analysis. Derivatization of large amounts of glycans and dilution of the derivatization mixture prior to analysis is a common approach. Fraction collection of the sugar derivatives during liquid chromatography (LC) makes easy the off-line MS characterization. However, in many cases analysis of trace amounts of saccharides requires the removal of the excess reagent. Clean-up with extraction with organic solvents is performed when the difference in hydrophobicity between the label and the derivatives is great and when the reaction volumes are large, e.g. reactions for LC. Alternatively, the labeled glycans are separated from free dye by adsorption into a hydrophilic filter in the presence of acetonitrile and subsequent elution with water [27]. Mort et al. [28] introduced the use of Sephadex beads carrying aldehyde groups in the reaction mixture (excess reagent reacted with the ''scavenger beads'') and reported complete removal of excess reagent. Gel filtration in Sephadex G-10 and Biogel P-2 columns or packed 96-well filterplates has also been used [11,29,30].

A common problem in reductive amination procedures is the influence of salts on the derivatization reaction. Salts accumulated through the various sample treatment steps prior to labeling cause reduction of the labeling yield [31–33]. However, treatment of glycoproteins with enzymes yielded five times larger amounts of glycans than hydrazinolysis and therefore enzymic treatment is necessary [34]. Buffers in enzymic treatments that can be removed later in vacuo are often used. Charlwood et al. [34] suggested the clean-up on GlycoClean columns after enzymic release of glycans because it gave higher yield in comparison to ethanol precipitation and solid-phase extraction.

Numerous reagents have been used for sugar derivatization by reductive amination and a short description of their properties and applications in CE, LC and MS analysis is presented below. Structures and names are given in Fig. 2. Photometric and fluorometric characteristics and applications to oligo-/polysaccharide analysis of the most widely used tagging agents are presented in Table 1.

2.1.1. 2-Aminopyridine

2-Aminopyridine (2-AP) was first used for HPLC analysis of glycan derivatives by Hase et al. in 1981 [35]. Derivatization with 2-AP allows sensitive UV and fluorescence detection (Fig. 2). Neutral and acidic branched glycans released from glycoproteins were derivatized with 2-AP and 2-D and 3-D mapping of their properties was performed with HPLC [36,37]. $GalA_nGal$ oligosaccharide derivatives of 2-

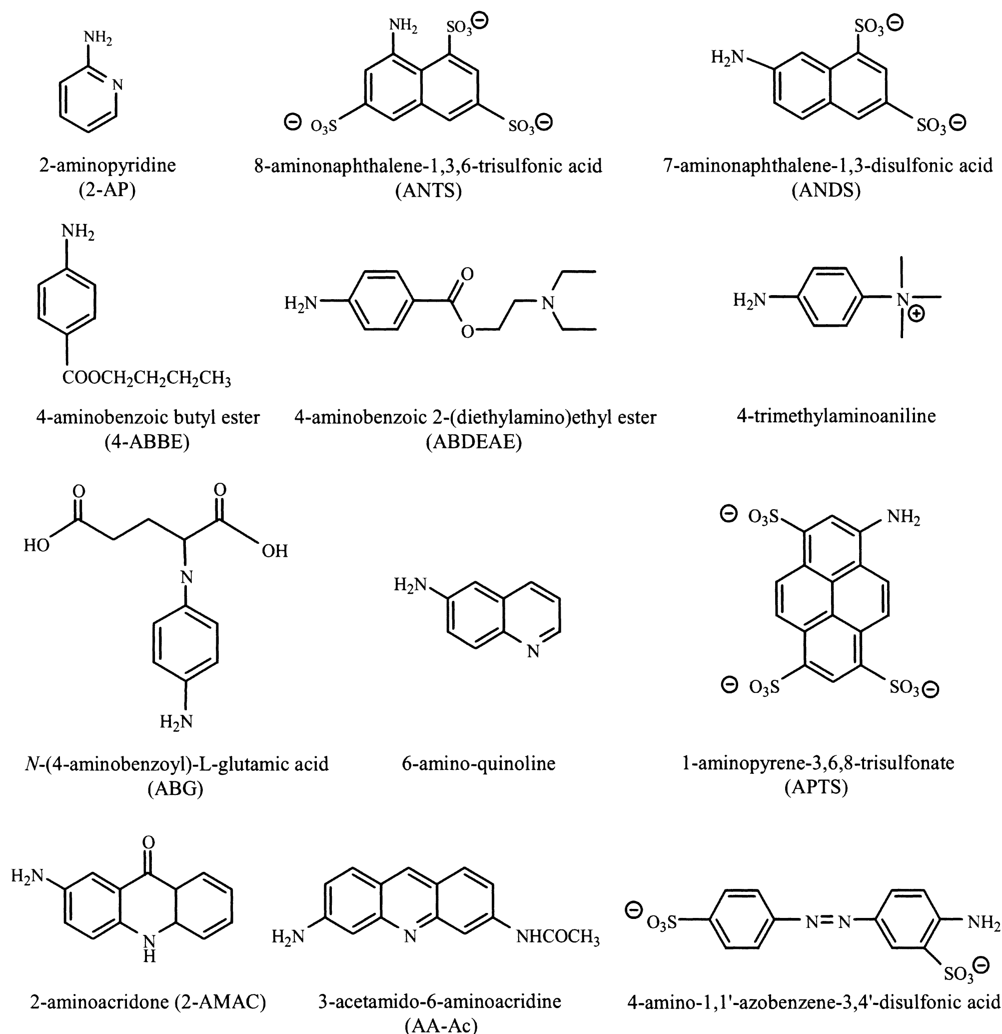

Fig. 2. Structures, names and abbreviations of the most widely used tagging agents having amino groups. These compounds are commonly attached to reducing sugars through reductive amination.

AP were separated with HPLC and detected either with a UV or fluorescence detector. Excess labeling reagent had to be removed before the separation and in the process there was a major loss of the short oligomers [38]. 2-AP has also been used for the derivatization of high molecular mass glycosaminoglycans and subsequent analysis by HPLC [32,39].

Honda et al. [40] were the first to utilize reductive amination for derivatizing 12 monosaccharides with 2-AP for CE analysis. Smith and El Rassi [41] succeeded in resolving 2-AP-labeled GalA oligomers up to 18 residues in length with CE using a coated capillary and a buffer of pH 6. Structural characterization of 2-AP derivatives of sialyloligosaccharides was performed with FAB-MS and FAB-MS–MS [42]. Recently, derivatization with 2-AP was incorporated in a strategy for analysis of the glycome [13]. 2-AP derivatives were analyzed by 2-D/3-D HPLC mapping of their chemical properties, i.e. charge, molecular size and hydrophobicity, and by reinforced frontal affinity chromatography to investigate affinity to lectins and MS [13].

Table 1
Photometric and fluorometric characteristics of the most widely used compounds for reductive amination of carbohydrates. References to reports, which utilize these tags for the electrophoretic, chromatographic or MS analysis of large carbohydrates, are also provided

Derivatizing agents Absorbance/fluorescence	Applications
2-Aminopyridine	HPLC
	N-linked oligosaccharides [13,36,37]
UV: 240 nm	$GalA_n$Gal oligosaccharides [38]
λ_{em}=375 nm	Glycosaminoglycans [32,39]
	CE
	GalA oligomers [41]
	MS
	Sialyloligosaccharides [42]
7-Aminonaphthalene-1,3-disulfonic acid	HPLC
	Maltooligosaccharides [24,173]
UV: 247 nm	CE
λ_{exc}=315 nm, λ_{em}=420 nm	Acarbose [44]
	Maltooligosaccharides [24]
8-Aminonaphthalene-1,3,6-trisulfonic acid	PAGE
	N-linked oligosaccharides [53,174–178]
UV: 220, 370 and 360 nm	MALDI-MS
λ_{exc}=360 nm, λ_{em}=515 nm	Oligosaccharides [53,54]
	HPLC
	N-linked oligosaccharides [179]
	CE–MS
	Maltooligosaccharides and N-linked glycans [50–52]
	CE–UV and CE–LIF
	Neutral and acidic GalA- and Gal-containing oligomers [55]
	Neutral branched alpha- and beta-D-glucans [58]
	Maltooligosaccharides [43,46,63]
	Hydrolyzed κ-carrageenan [61]
	Sialylated oligosaccharides [52]
	Hyaluronan-derived oligosaccharides [62]
1-Aminopyrene-3,6,8-trisulfonate	Gel electrophoresis
	N-oligosaccharides [11]
λ_{exc}=455 nm, λ_{em}=512 nm	Maltooligosaccharides [180,181]
	CE
	N-linked sialylated oligosaccharides [26,78,182,183]
	High molecular-weight carrageenans [81,184]
	Alginic acid polysaccharides [82]
	N-glycans of antibodies [31,83]
	Chitin oligosaccharides [185]
	CE–MS
	Oligosaccharides from lipoarabinomannans [85]
	MALDI-MS
	Oligosaccharides [84]
	Lipoarabinomannan oligosaccharides [186]
2-Aminoacridone	Gel electrophoresis
	Maltooligosaccharides [86]
UV: 250, 276 and 421 nm	Cell wall polysaccharides [92]
λ_{exc}=428 nm, λ_{em}=525 nm	Glycosaminoglycan disaccharides [94]
	N-linked oligosaccharides [187]

Table 1. Continued

Derivatizing agents Absorbance/fluorescence	Applications
	CE Glycosaminoglycan oligosaccharides [87–90] N-linked oligosaccharides [57,95,188] Neutral oligosaccharides [189] HPLC N-linked oligosaccharides [34,93,95,98,99] Glycosaminoglycan oligosaccharides [96] HPLC–MS N-linked oligosaccharides [190] MALDI-MS Linear and branched oligosaccharides [95] MS N-linked oligosaccharides [95,191]
3-Acetamido-6-aminoacridine UV: 272, 377 and 432 nm λ_{exc}=382 or 445 nm, λ_{em}=520 nm	HPLC, CE, CE–MS, MALDI-MS N-linked oligosaccharides [100,101]

2.1.2. Aminonaphthalene sulfonic acid isomers

Various aminonaphthalene sulfonic acid isomers have been investigated for saccharide derivatization because apart from their UV-absorbing and fluorescent properties, they also impart charge to the carbohydrates (Fig. 2). Chiesa and O'Neill [43] compared the CE separation of the derivatives of various aminonaphthalene sulfonates. They examined in detail the effects of structure and charge of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), 3-aminonaphthalene-2,7-disulfonic acid (3-ANDA), 2-aminonaphthalene-1-sulfonic acid (2-ANSA) and 5-ANSA on the CE analysis of their maltooligosaccharide derivatives using sodium phosphate, pH 2.5, in the presence of triethylamine. ANTS-derivatized maltooligosaccharides with a degree of polymerization (d.p.) of more than 30 glucose units were separated in less than 30 min. 3-ANDA-derivatized maltooligosaccharides showed the same resolution, yet the separation was only achievable up to d.p. 30. On the other hand, resolution of 2-ANSA and 5-ANSA-derivatized maltooligosaccharides was achievable only up to a d.p. of 20. The loss of efficiency might be attributed to the longer analysis time [43].

7-Aminonaphthalene-1,3-disulfonic acid (ANDS) has been used for the derivatization of the pseudo-oligosaccharide, acarbose, and its main metabolite and their CE–LIF determination in human urine [44] (Table 1). Kazmaier et al. [45] reported that quantification of maltooligosaccharides and determination of the oligomer distribution was impossible with the routine derivatization procedures previously reported. This was mainly due to the incomplete derivatization reaction and the risk of hydrolysis of higher molecular mass carbohydrates in the derivatization process [45]. Optimization of derivatization conditions of maltodextrins with ANDS has therefore been the focus of further studies [24]. Employing a Box–Behnken design, the effect of temperature, reaction time and pH on the degradation was studied. Optimal derivatization conditions for obtaining high reaction yields and low degradation were a pH value of 2.5, a temperature of 68 °C and a reaction time of 120 min [24]. The separation of ANDS-derivatives of maltodextrins with a d.p. up to 20 in an acidic buffer with CE is presented in Fig. 3. The method was applied to the quantitative analysis of oligosaccharides in coffee.

The most widely used aminonaphthalene compound is ANTS (Fig. 2, Table 1). The UV spectrum of ANTS shows a maximum at ~220 nm followed by two other maxima at 270 and 360 nm; its molar absorptivity in water at 360 nm is 5700 M^{-1} cm^{-1} [46]. As a fluorophore, ANTS has a maximum excitation wavelength at 360 nm, which shifts to 370 nm for the ANTS-derivatized maltose [46]. The

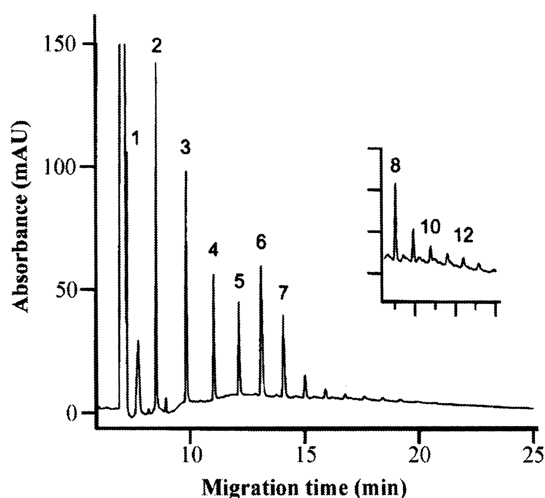

Fig. 3. Analysis of maltooligosaccharides by capillary electrophoresis after derivatization with ANDS [24]. Peak numbers indicate the corresponding degree of polymerization. Operating buffer: 100 m*M* Tris–phosphate, pH 2.0. Reversed polarity. Reprinted with permission from Vieweg Publishing.

wavelength for the maximum emission is at 515 nm. ANTS was first introduced as a fluorophore for the high resolution PAGE of reducing saccharides [47] and has since been used for LC–UV, LC–MS, CE–UV, CE–LIF and CE–MS. Due to the fact that its fluorophore properties match with the 325 nm line of He/Cd, ANTS has been extensively used for laser-induced fluorescence (LIF) detection in CE analysis. A concentration detection limit of 5×10^{-8} *M* (500 amol) for both monosaccharides and large complex oligosaccharides (molecular sizes 2000–3000) was obtained [48,49]. ESI-MS characterization of ANTS-labeled glycans is also feasible [50–52].

ANTS-labeled neutral and sialylated N-glycans were separated with PAGE, eluted from PAGE gel slices and then analyzed by MALDI-TOF-MS in negative ion mode with a sensitivity in the 2–10 pmol range [53]. Urinary oligosaccharides were monitored and characterized after coupling with ANTS, fast purification over a porous graphite carbon extraction column and MALDI-MS analysis [54].

The CE–UV and CE–LIF resolution of ANTS-labeled carbohydrates has been achieved using buffer systems containing phosphates [46,55,56], borates [56,57], tris–borates [58], citrates [56], acetate buffers [59,60], sieving buffers [61,62] and non-aqueous conditions [63]. ANTS has been used for the derivatization of both neutral and acidic GalA- and Gal-containing oligomers of up to 20 residues [55], neutral branched alpha- (amylose, amylopectin and pullulan) and beta-D-glucans (lichenan) up to 70 residues [58], neutral linear maltooligosaccharides [43,46,63], hydrolyzed κ-carrageenan [61], sialylated oligosaccharides [52] and hyaluronan-derived oligosaccharides [62].

Jackson [47] routinely used ~200-fold excess of ANTS for the derivatization. A 40-fold molar excess of ANTS sufficed for complete conversion and under such conditions no interference by excess ANTS was observed [46,55]. Klockow et al. [49] studied the miniaturization of the derivatization reaction for CE analysis and they reported a total reaction volume of 2 μl instead of the 10 μl usually employed for pre-capillary derivatization. Two labeling protocols were established, one with overnight reaction at 40 °C and the other with a 2.5-h derivatization time at 80 °C. Neutral oligosaccharides could be labeled with either protocol. However, sialylated oligosaccharides hydrolyzed when reaction took place at 80 °C [49].

2.1.3. Aminobenzene derivatives

The aminobenzene derivatives have been largely used for glycan analysis by capillary electrochromatography, CE, HPLC, PAGE, NMR and MALDI-TOF-MS. The reader interested in the photometric and fluorometric characteristics of these compounds should refer to an interesting article by Kakehi et al. [29]. In general, aminobenzene derivatives exhibit maximum absorbance at wavelengths in the region of 270 to 325 nm and maximum emission at wavelengths of 343–452 nm, which is compatible with the He/Cd laser line of LIF detectors. The wavelengths close to 300 nm are advantageous in the spectroscopic analysis of complex ‘‘real’’ samples (e.g. extracts or hydrolysates), because they are more specific than lower wavelengths.

Bigge et al. [64] extensively studied the reaction conditions for the derivatization of N-glycans with 2-aminobenzamide (2-ABM) and 2-aminobenzoic acid (2-ABA). Sato et al. [65] have optimized the reaction conditions for the derivatization of monosaccharides with 2-ABA. 4-Aminobenzene deriva-

tives (4-aminobenzoic acid, ethyl 4-aminobenzoate, 4-aminobenzonitrile) allow the derivatization of ketoses in addition to aldoses and uronic acids, in contrast to other labeling tags, such as 2-aminobenzene derivatives and 2-AP [66]. Although this research group [66] optimized the reaction conditions for the derivatization of mono- and disaccharides with 4-aminobenzonitrile, an incomplete reaction was reported. Methyl-, ethyl- and butyl-4-aminobenzoates as derivatization agents were investigated for the MEKC and reversed-phase HPLC analysis of homologous maltodextrins and oligosaccharides from human milk [67]. The butyl ester (4-ABBE) allowed the best separation [67]. 4-ABBE also allowed the online ESI-MS characterization and the off-line MALDI-TOF-MS analysis of the human milk oligosaccharide derivatives [68]. The butyl ester has also been successfully used for the reversed-phase capillary HPLC analysis of galactosaminoglycan (GalAG) disaccharides and their chromatographic separation is shown in Fig. 4.

Kakehi et al. [29] conducted a thorough study of nine different monosubstituted aminobenzene derivatives as labeling reagents of glycans. It was concluded that although aminobenzene derivatives substituted at 2- and 4-positions yield derivatives with

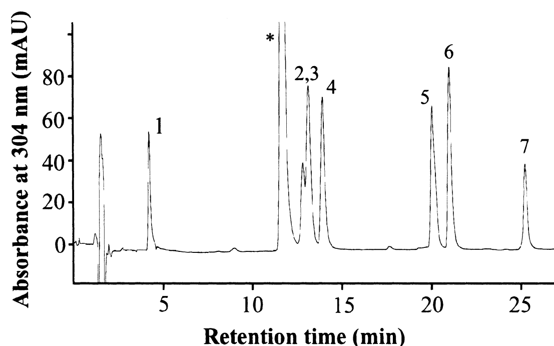

Fig. 4. Separation of seven variously sulfated galactosaminoglycan disaccharides derivatized with 4-ABBE with capillary HPLC. Derivatization reactions were performed at 45 °C for 4 h in the presence of 0.2 *M* 4-ABBE and extraction with dichloromethane followed for the removal of excess reagent. Identity of peaks: 1, Δdi-nonS$_{CS}$; 2, 3, Δdi-mono4S and Δdi-mono6S; 4, Δdi-mono2S; 5, Δdi-di(2,4)S; 6, Δdi-di(2, 6)S; and 7, Δdi-triS. The asterisk marks the excess of 4-ABBE. HPLC column, Agilent Zorbax SB-C$_{18}$, 5 μm, 150×0.5 mm. Separation conditions, 35 to 67% CH_3CN for 40 min. Aqueous phase, 10 m*M* TBA/H_3PO_4, pH 5.6 (F. Lamari, personal data).

high fluorescence intensities and molar absorptivities, 3-substituted aminobenzene derivatives (3-aminobenzamide and 3-aminobenzoic acid) show the highest reaction efficiencies. Using such derivatization conditions, sialic acids are not destructed and analysis of sialylooligosaccharides is possible. Quantitative labeling of sialylated oligosaccharides with 2-ABA with negligible or no desialylation was reported when the reaction was performed in methanol containing acetate–borate buffer (approximately pH 5.0) at 80 °C for 1 h [69].

Derivatization of oligosaccharides with 4-amino benzoic 2-(diethylamino) ethyl ester (ABDEAE) has been utilized for sensitive detection using ESI-MS [70] and MALDI-MS [71,72]. In all cases sensitivity was enhanced by a factor of 1000 over techniques with free oligosaccharides, and this sensitivity does not appear to have been matched by other derivatives. Furthermore, ABDEAE derivatives were well separated by HPLC. 4-Trimethylaminoaniline was also used for improvement in sensitivity of ESI-MS characterization of sugar derivatives, but the gain in sensitivity of MALDI-MS analysis was only a 10-fold increase [73].

N-(4-Aminobenzoyl)-L-glutamic acid (ABG) is another aminobenzene derivative that has been used for oligosaccharide derivatization and CE analysis. ABG has an absorption maximum at 273 nm and a molar absorptivity of 17 000 M^{-1} cm^{-1}, whereas the sugar derivatives exhibit a broad maximum at 291 nm [30].

Derivatization of oligosaccharides (maltoheptaose and human milk oligosaccharides) with benzylamine followed by N,N-dimethylation with methyl iodide has been used for the characterization with MALDI-TOF-MS and MALDI-post source decay-TOF-MS [74]. The derivatization imparted a positive charge at the reducing end. A 10-fold increase in sensitivity compared with underivatized oligosaccharides was reported.

2.1.4. APTS derivatives

Evangelista et al. [75] introduced APTS as a novel derivatization agent for sugars. The APTS-derivatized sugars show λ_{max} at 455 nm with significant absorption at 488 nm, while APTS itself has λ_{max} at 424 nm with a relatively low absorptivity at 488 nm (Fig. 2, Table 1). When excited with a 488 nm

Ar-ion laser, APTS sugar derivatives fluoresce with substantially higher intensity at 512 nm than the APTS emission at 501 nm [26]. Using a selective emission filter, preferential detection of the APTS derivatives can be achieved. The sugar conjugate shows 40-fold greater fluorescence than APTS does. N-Acetylamino sugars produced lower yields of detectable adducts in reductive amination with APTS than with 2-AP [76]. Evangelista et al. [77] suggested the utilization of citric acid as a catalyst and thus quantitative derivatization of N-acetylsugars was achieved. Guttman et al. in 1996 carefully studied and optimized the reaction conditions for the efficient derivatization of sialylated oligosaccharides in small reaction volumes [78]. They reported a fivefold increase of labeling efficiency when DMSO was replaced with tetrahydrofuran as solvent of the reducing agent. The optimized protocol was used for the derivatization and CE–LIF analysis of N-linked oligosaccharides [26].

Guttman [79] used two different labels, APTS and 8-aminoacridone (8-AMAC), in order to study the monosaccharide composition of glycoproteins by CE–LIF. The latter tag was used for the determination of sialic acids. It is important to note that, albeit the aminosugars (e.g. glucosamine) can be derivatized with APTS, their actual labeling efficiency was quite low ($>50\%$) and an N-acetylation step is necessary. Chen et al. [80] addressed the problem of the low yield of sialic acid derivatization with APTS by introducing treatment of the hydrolysates with neuraminic acid aldolase (EC 4.1.3.3). Thus, *N*-acetylneuraminic acid was converted quantitatively to *N*-acetylmannosamine, which was then derivatized with APTS in the same manner as the other monosaccharides.

Different protocols were used for the derivatization and CE–LIF analysis of intact high molecular mass carrageenan [81], alginic acid polysaccharides [82], and the N-glycans of antibodies [31,83]. An off-line method using CE separation, fraction collection of peaks, and subsequent MALDI-TOF-MS analysis of APTS derivatives was reported [84]. APTS-derivatized mannooligosaccharides were characterized by on-line CE–ESI-MS [85]. Up to tetramer oligomers were detected as singly and doubly charged pseudomolecular ions in the negative electrospray mode. Callewaert et al. [11] employed APTS derivatization of N-glycans derived from low picomole amounts of glycoproteins for their analysis by the standard DNA-sequencing equipment.

2.1.5. Aminoacridone derivatives

2-AMAC is a fluorescent hydrophobic molecule with λ_{exc}=428 nm and λ_{em}=525 nm (Fig. 2, Table 1). The UV–Vis absorbance spectrum of 2-AMAC is characterized by three maxima at wavelengths of 250, 276 and 421 nm. Quantitative derivatization was achieved with 100-fold excess of AMAC [86]. We have used 2-AMAC for the derivatization of acidic glycosaminoglycans-derived di- and oligosaccharides and their subsequent CE analysis [87–90]. Derivatization of GAG glycans with 2-AMAC was performed with a protocol slightly modified from that reported by Jackson [86]. Particularly, 10 nmol of each saccharide was lyophilized in a microcentrifuge tube at room temperature. A 5-μl volume of a 0.1 *M* AMAC solution in glacial acetic acid–DMSO (3:17, v/v) and 5 μl of a freshly prepared solution of 1 *M* $NaBH_3CN$ in water were added to each sample and then mixtures were centrifuged in a microfuge at 11 000 *g* for 3 min. Derivatization was performed by incubating the aliquots at 45 °C for 4 h. Finally, 30 μl of 50% (v/v) DMSO were added in the samples and aliquots were taken for CE analysis. The derivatization efficiency was studied by monitoring the absorbances at 232 and 255 nm as well as the fluorescence of standard Δ-disaccharides derivatized for different time periods (30 min, 1, 2, 3, 4 and 6 h) [89]. The results showed that heating at 45 °C for 4 h ensured the complete labeling of all Δ-disaccharides since CE analysis of every derivatized Δ-disaccharide showed the absence of any remaining non-derivatized Δ-disaccharide (detection at 232 nm). The analysis further confirmed that the reaction conditions do not cause release of any sulfate group. The stability of derivatized Δ-disaccharides was studied by preparing various mixtures of the standard Δ-disaccharides and keeping them at various temperatures (ambient temperature, 2 °C, −20 °C, −80 °C). CE analysis of these samples and measurement of the peak areas showed that no change in the composition of the mixture was observed even after 1 month at room temperature since the relative

standard deviation in peak areas were less than 2.4%. If AMAC derivatives must be stored for longer periods of time (at least 3 months), storage at −80 °C is recommended. Minimization of the reaction volume to 2 μl has also been reported [34].

Che et al. [91] have studied in detail and optimized the derivatization reaction of sialic acid with 2-AMAC. The AMAC-derivative of *N*-acetylneuraminic acid (Neu5Ac) showed low stability; nonetheless, the suggested method was successfully applied to the quantitative determination of sialic acid with CE in various glycoproteins with detection limit of 1 μ*M* (or 35 fmol). Guttman [79] used the fluorescent tag 9-aminoacridone (9-AMAC) for the CE determination of sialic acids.

AMAC characteristics have made it an attractive labeling reagent for analysis of both charged and neutral oligosaccharides with electrophoretic (PAGE and CE) and chromatographic techniques. The inclusion of borate anions in the buffer system used for PAGE or CE is necessary for the resolution of AMAC-derivatized neutral linear and branched sugars [57,86]. PAGE of AMAC derivatized glycans has been applied to analysis of plant cell wall polysaccharides [92], N-linked oligosaccharides [187] and hyaluronan and GalAG disaccharides [94]. Oligosaccharides labeled with 2-AMAC were separated with various CE modes, commonly capillary zone electrophoresis and MECK. Using MECK with an operating buffer of 500 m*M* sodium borate, pH 8.9 containing 80 m*M* sodium taurodeoxycholate, the excess of 2-AMAC was trapped in taurodeoxycholate micelles ensuring thus no interference with the analysis because it was uncharged and highly hydrophobic [57,95]. 2-AMAC derivatives of acidic oligosaccharides, i.e. CS- and HS-derived Δ-disaccharides as well as saturated and Δ-oligosaccharides [87–90,96,97], have been well resolved with CE analysis at low pH phosphate buffers and reversed polarity. Excess of derivatizing reagent does not interfere with the separation because at low pH it does not enter the capillary [87].

The hydrophobic nature of this molecule allows also its analysis by reversed- and normal-phase HPLC and subsequently by MALDI-TOF-MS, electrospray and nanospray MS [93,95,98,99]. Excess AMAC has such a difference in hydrophobicity from its sugar derivatives that it elutes later than all derivatives in reversed-phase HPLC.

A novel derivatization reagent, 3-acetamido-6-aminoacridine (AA-Ac) was introduced for the analysis of picomole levels of N-linked glycans both with normal and reversed-phase HPLC, free zone CE–LIF, CE–ESI-MS and MALDI-TOF-MS [100] (Fig. 2, Table 1). The UV–Vis spectrum of AA-Ac is characterized by maximum absorbance at 272, 377 and 432 nm; the molar absorptivities at these three wavelengths are 45 144, 10 890 and 8307 M^{-1} cm^{-1}, respectively [100]. The excitation spectrum has two maxima at wavelengths 382 and 445 nm. Exciting at either wavelength produces an emission spectrum with a maximum at the wavelength of 520 nm. AA-Ac exhibits twice the intensity of fluorescence of AMAC and has ionizable groups. AA-Ac has been used to derivatize glycan pools released from 2-D PAGE and analysis of these derivatives was carried out by MALDI-TOF-MS and/or hydrophilic interaction liquid chromatography [101].

2.1.6. Other derivatizing reagents

6-Aminoquinoline (λ_{max}=245 nm) has been utilized for the derivatization of neutral and acidic, linear and branched oligosaccharides and subsequent analysis by CE and CEC [102–104]. The oligosaccharides labeled with 6-aminoquinoline yielded eight times higher signal than those tagged with 2-AP [102].

7-Amino-4-methylcoumarin was introduced as a fluorescent agent of oligosaccharides and has been used for analysis of the branching pattern of isomaltooligosaccharides from beer [105,106].

A visible chromophore, 4-amino-1,1′-azobenzene-3,4′-disulphonic acid, has also been used for derivatization of saccharides (both aldoses and ketoses). The photometric properties of this compound, i.e. absorption maximum at 489 nm and extinction coefficient of 37 615 M^{-1} cm^{-1}, make it attractive for PAGE analysis of carbohydrates [107].

9-Aminofluorene has high molar absorptivity (14 000 M^{-1} cm^{-1} at 267 nm), is chemically stable and was introduced to quantitative labeling of linear and branched oligosaccharides. The derivatives were purified by LC and characterized by MALDI-FTMS [108].

2.2. Other derivatization reactions at the carbonyl group

Apart from reductive amination, other widely used derivatization procedures involving the reducing end of carbohydrate molecules are presented below.

2.2.1. Amination through the formation of glycosylamine

A slight modification of the reductive amination procedure has been used in the MS study of carbohydrates. The reaction of an amino-compound with the carbonyl group of a reducing carbohydrate in the absence of reducing agents yields glycosylamines instead of open ring secondary amines (Fig. 1B). Derivatization of di- and oligosaccharides with 4-ABBE and 2-AP (neutral pH, 80 °C, 6 h) enabled the determination of interglycosidic linkages of saccharides with negative-ion FAB-MS in combination with collisional-induced dissociation MS [109]. Disaccharides labeled with 4-ABA (50 °C, 6 h) were analyzed by CE–ESI-MS–MS [110]. The authors reported that in the negative ion ESI, the glycosylamine approach instead of reductive amination provides more information on linkage and anomeric configuration [110].

2.2.2. Pyrazolone derivatives of carbohydrates

The UV-absorbing tag 1-phenyl-3-methyl-2-pyrazolin-5-one (PMP) (λ_{max}=245 nm) and its methoxy analog, 1-(*p*-methoxy)-phenyl-3-methyl-5-pyrazolone have been used to derivatize reducing sugars (both aldoses and ketoses) in the presence of carbodiimide by a condensation reaction under mild conditions, which does not cause desialylation or desulfation (Fig. 5A) [111,112]. The bis-PMP derivatives behave like weak anions in aqueous basic solutions, strongly absorb UV light (λ_{max}=245 nm, 30 000 M^{-1} cm^{-1}) and can be detected electrochemically. The method has been used for the derivatization of carbohydrates from simple monosaccharides to sialylated N-glycans [113]. Derivatization of reducing mono- and di-saccharides with 4-(3-methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid was studied by Castells et al. [114].

Various CE separation modes of PMP-derivatives have been reported, including borate complexation, MECK in the presence of SDS, complexation with alkaline earth metals and CE analysis of the borate complexes in the presence of polybrene, a cationic water-soluble polymer [115–117]. Furthermore, bis-PMP derivatives of both aldose enantiomers were well resolved from each other in a 50 m*M* phosphate buffer, pH 7.0, containing *R*-*N*-dodecoxycarbonylvaline to a concentration of 50 m*M* [118]. Finally, separation of PMP derivatives of simple disaccharides was accomplished in a linear polyacrylamide-coated capillary using a phosphate buffer containing two types of lectins [119]. Alkaline borate buffers have also been used to analyze hyaluronan- and chondroitin-derived non-sulfated and variously sulfated Δ-disaccharides in urine samples using derivatization of Δ-disaccharides with PMP [120].

Oligosaccharides from thyroglobulin were analyzed by LC–ESI-MS as PMP derivatives [121]. PMP-labeling of sialylated oligosaccharides enables very clean and informative ESI mass spectra with high sensitivity [122]. Rozaklis et al. [123] optimized the conditions for PMP derivatization of oligosaccharides and their subsequent determination in biologic samples with ESI-MS–MS. MALDI-TOF-MS analysis of PMP-derivatives of oligosaccharides has been performed with success [124,125]. In a study of Shen and Perreault, derivatization of small sugars and medium-size oligosaccharides with PMP was preferred over pyridylamination (2-AP), owing to the enhanced ionization efficiency of the PMP derivatives with either FAB-, ESI- or MALDI-MS and the better separation and higher sensitivity with HPLC–UV [126].

2.2.3. NBD-tagged N-methylglycamine derivatives

Honda et al. [127] introduced a novel derivatization procedure for reducing sugars (Fig. 5B). Reducing sugars are converted to N-methylglycamines in the presence of methylamine and dimethylamine–borane complex at pH 4.5 and 40 °C for 30 min. The resultant N-methylglycamines were labeled with 7-nitro-2,1,3-benzoxadiazole 4-fluoride (NBD-F). These two reaction steps can be carried out in a one-pot fashion and proceed quantitatively within 50 min. The derivatives showed strong absorbance at 490 nm and their fluorometric properties match with the Ar-ion laser line of LIF detectors. CE–LIF analysis of monosaccharides in nanomolar concentrations was performed. The main advantage of this

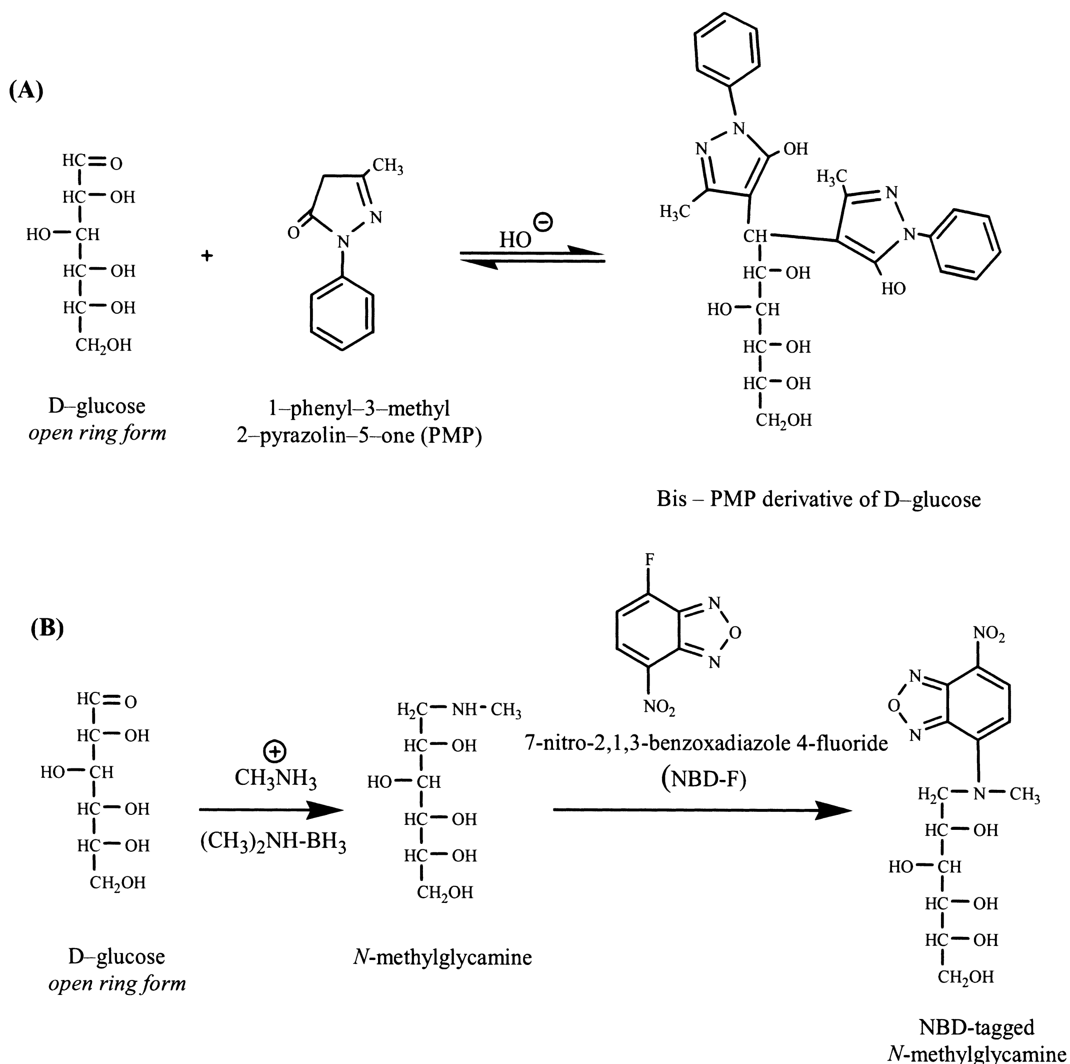

Fig. 5. Derivatization reactions of reducing sugars with PMP (A) and NBD-F (B).

scheme is that under the mild conditions of the derivatization, sialic acids are not released from sialylated glycans and can be applied to the analysis of trace amounts of sialylated oligosaccharides [127]. Labeling of amino sugars with NBD-F at pH 6.0 and subsequent analysis by microchip electrophoresis with LIF detection has been reported [128].

2.2.4. Formation of hydrazones

The carbonyl group of an aldehyde or ketone reacts with the amine group of a fluorescent or UV-absorbing hydrazine, forming a hydrazone product (Fig. 6). A drawback of this derivatization procedure is the formation of side-products or various isomers [129,130]. Derivatization of reducing sugars with dansylhydrazine (DHZ) was originally performed by Avigad [131]. Mopper and Johnson [132] have performed optimization of the reaction parameters for mono- and disaccharides. Perez and Colon have optimized the conditions for derivatization in small volumes and short times; the reaction proceeded at 68 °C for 15 min [129]. Monosaccharide-DHZ derivatives excited with a He/Cd laser at 325 nm show a maximum emission

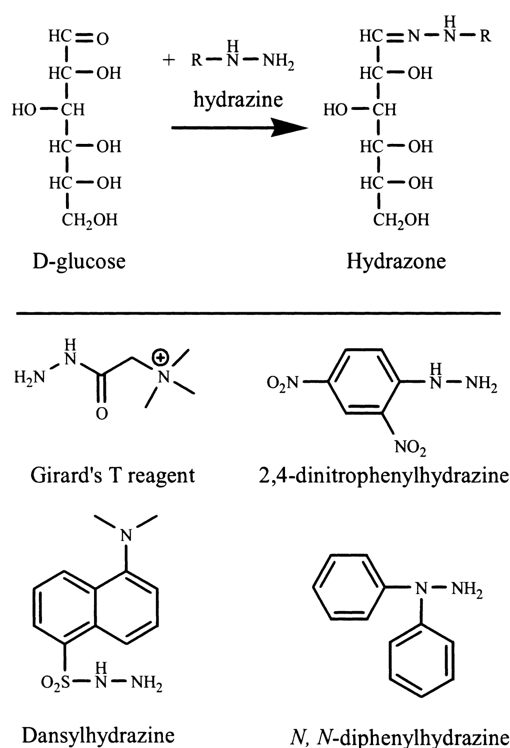

Fig. 6. Derivatization of reducing sugars (e.g. D-glucose) with hydrazine to various hydrazones. The structures of various hydrazines used for carbohydrate derivatization are also presented.

wavelength of 530 nm and can be separated with CE using borate buffer [129]. This method was also applied to the derivatization of hyaluronan- and GalAG-derived unsaturated disaccharides and analysis by isocratic HPLC [133].

Various hydrazines have been used (Fig. 6). The reaction conditions for the formation of 2,4-dinitrophenylhydrazones of the neutral sugars present in glycans/proteoglycans and glycoproteins, i.e. fucose, xylose, mannose, galactose and glucose, were optimized by our group [134]. To 10 μl of aqueous carbohydrate sample (1.5–100 nmol), 100 μl of a 1.5% w/v 2,4-dinitrophenylhydrazine in 1,2-dimethoxyethane and 100 μl of a 2% v/v trifluoroacetic acid in methanol were added and the reaction was carried out at 65 °C for 90 min. Analysis of the sugar derivatives was performed with HPLC and isocratic elution and UV detection at 352 nm [134]. *N*,*N*-Diphenylhydrazine was used by Miksik et al. [135] for the formation of mono- and bis-diphenylhydrazones of dicarbonyl and tricarbonyl sugars, which were separated by microemulsion electrokinetic chromatography and UV detection at 220 nm [135]. Labeling with *p*-hydrazinobenzenesulfonic acid of mono-, di- and trisaccharides within 10 min yielded the corresponding UV-absorbing and charged hydrazones, which were analyzed by capillary zone electrophoresis. Reaction with Girard's T reagent imparts positive charge to glycans and therefore increases the sensitivity of MALDI-MS [136].

2.2.5. Reduction with radioactive compounds

The reduction of aldehydes and ketones with sodium [^{3}H]borohydride results in the incorporation of tritium and thus monitoring with a radiochemical detector is possible [137]. In structural studies of GAG chains we have extensively used alkaline borohydride treatment of proteoglycans for the release of GAG chains and their end-labeling with tritium (0.25 *M* [^{3}H]$NaBH_4$ and 0.75 *M* $NaBH_4$ in 50 m*M* NaOH for 48 h at 45 °C). This strategy allows the structural characterization of [^{3}H]-labeled xylitole-containing linkage regions of GAGs with HPLC [138,139].

2.3. Derivatization of hydroxyl, amino and carboxyl-groups of carbohydrates

2.3.1. Derivatization of carbohydrates with α-keto carboxylic acid group

Sialic acids react with 1,2-diamino-4,5-methylenedioxybenzene (DMB) to form quinoxaline derivatives in various derivatization conditions (Fig. 7). The resultant quinoxaline derivatives show strong fluorescence at 448 nm on irradiating at 373 nm. Only an α-keto carboxylic acid group (C-1 and C-2 of the sialic acid residue) is involved in derivatization, while others are intact. Hara et al. [140] first reported the development of the reversed-phase HPLC fluorometric method. Recent studies have experimented with the introduction of internal standards in the method in order to improve the accuracy [141,142]. The acidic conditions (acetic acid or sodium hydrogensulfite) used in the DMB derivatization reaction seem to prevent hydrolysis or migration of acetyl groups from one to another hydroxyl group,

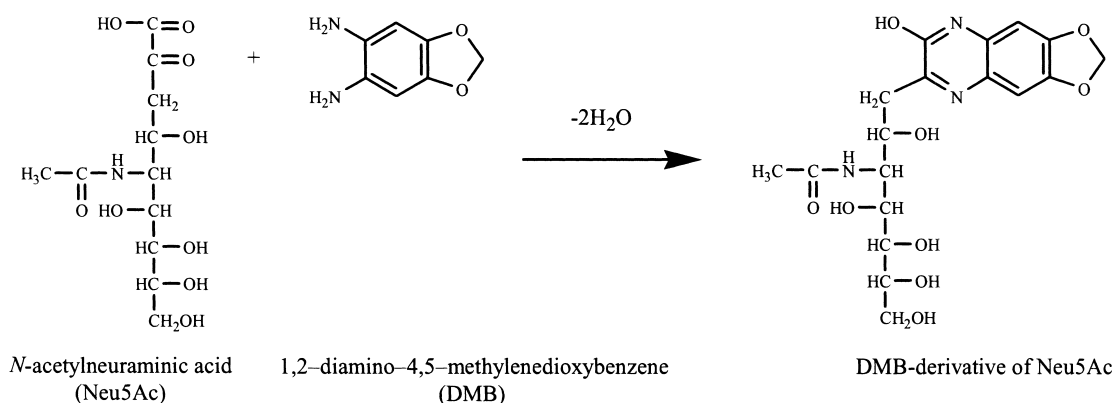

Fig. 7. Derivatization reaction of *N*-acetylneuraminic acid (it contains an α-ketocarboxylic acid group) with 1,2-diamino-4,5-methylenedioxybenzene (DMB). Carboxyl and α-keto groups participate in the formation of the third ring of the DMB derivative.

and this makes it appropriate for the analysis of O-acetylated derivatives [140,143,144].

LC–ESI-MS analysis of members of the sialic acid family derivatized with DMB allows definition of the type and position of the various O-acetyl substituents. The research group of Kakehi has developed a strategy for the simultaneous determination of about 13 members of the sialic acid family with differences in the substitution with N- or O-acetyl, glycoyl and sulfonic ester groups [144,145]. Characteristic distributions of sialic acids in the tissues of mice and rats were revealed [144], whereas Klein et al. [146] identified 28 different sialic acids.

The standard procedure for DMB labeling at 50 °C for 2 h results in significant hydrolysis of oligomers of Neu5Ac (α2→8-linked homo-oligo/poly-*N*-acetylneuraminic acid); the degree of hydrolysis is analogous to the degree of polymerization and the reaction time [147]. Changing the reaction conditions to 10 °C for 48 h in 0.02 *M* trifluoroacetic acid after careful study, a satisfactory compromise between the minimum degradation of the oligomers of Neu5Ac and a high derivatization rate was achieved [148]. The derivatization yield for Neu5Ac monomer was ca. 65% of the maximum (obtained by reaction for 2.5 h at 55 °C with the same reagent). Oligomers with a degree of polymerization of 19–28 showed 80% recovery after prolonged treatment at 10 °C [148]. This method was applied to the HPLC baseline resolution of poly(Neu5Ac) up to a d.p. of 90, colominic acid samples and poly(Neu5Ac) chains in rat brain tissues [148,149].

2.3.2. Derivatization at amino-groups

3-(4-Carboxybenzoyl-)-2-quinoline carboxyaldehyde (CBQCA) has been used for the ultrasensitive detection of aminosugars [150]. Aminosugars react readily and quantitatively with CBQCA in the presence of cyanide (toxic reagent), while neutral sugars are converted to 1-amino-1-deoxyalditols by reductive amination (Fig. 8A). The isoindole derivatives have excitation maxima near the 442 nm blue line of the He/Cd laser and the 456 nm secondary line of the argon laser. This procedure has the advantage that it is not necessary to remove the excess of the reagent, since the unreacted compound does not fluoresce. CBQCA derivative of glucosamine has been detected with CE–LIF at nanomolar (10^{-9} *M*) concentrations with mass detection limits of 75 zmol [151]. Liu et al. [152] reported dramatic separations of CBQCA-labeled GalA oligomers of up to 70 residues using a capillary containing a fixed gel of up to 30% polyacrylamide at pH 8.5. CBQCA derivatization has also been used for the CE–LIF analysis of complex oligosaccharides released from bovine fetuin [153].

Another two-step derivatization procedure for carbohydrates has been employed. In the first step, carbohydrates containing no amino group were transformed to the corresponding 1-amino-1-deoxyalditols, while hexosamines were reduced with sodium borohydride to the 2-amino-2-deoxyalditols. These aminated carbohydrates were subsequently labeled with 5-carboxytetramethylrhodamine succinimidyl ester (Fig. 8B). However, the succinimidyl ester is

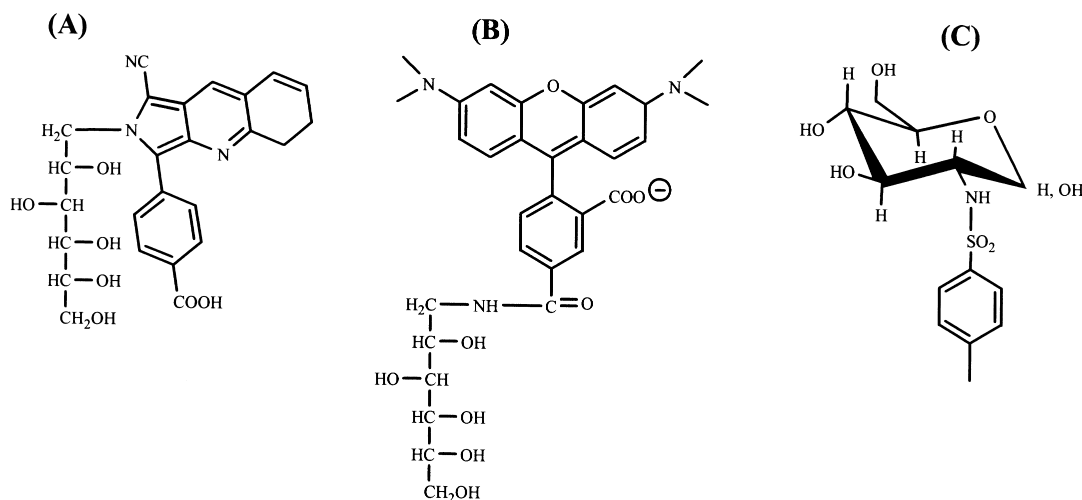

Fig. 8. Structures of CBQCA-glucose derivative (A), tetramethylrhodamine-aminoglucitol derivative (B), and N-tosyl derivative of glucosamine (C).

not stable in basic buffers yielding two hydrolysis products. This scheme was applied for the derivatization of six oligosaccharides [154]. Separation was carried out with CE and detection was performed with a home-made He/Ne laser with an excitation wavelength of 543.5 nm. With this set-up, the lowest detection limits, ever reported, were achieved. The concentration detection limit was in the order of 50–100 p*M* and the mass injection limit 8×10^{-23} mol or a few analyte molecules. This method has been also used for derivatization of both neutral and sialylated oligosaccharides [155]. Many interesting applications in metabolic cytometry, i.e. monitoring of uptake, biosynthesis and biodegradation of fluorescent labeled oligosaccharide standards, have been described [156].

The analysis of the total amount of sialic acid, glucosamine and galactosamine under the same chromatographic conditions was described by Makatsori et al. [157]. Two different hydrolytic procedures of glycoconjugates yield the hexosamines and a deacetylated decarboxylated amino-product representative of total sialic acid content. Following removal of neutral monosaccharides and amino acids by ion-exchange chromatography, derivatization with *p*-toluenesulfonylchloride (Tos-Cl) yields *N*-tosyl-derivatives (Fig. 8C), which are completely resolved on a Supelcosil LC-18 column by isocratic elution. In particular, 25 μl of aqueous sample, 37.5 μl of Tos-Cl reagent (50 mg of Tos-Cl in 10 ml acetone) and 37.5 μl of triethylamine solution (100 μl triethylamine in 10 ml of acetone) was heated at 60 °C for 40 min. The reaction was quantitative (yield >94%). Excess of reagent and non-tosylated derivatives were removed by passing the mixture through a Sep-Pak C-18 cartridge. The method shows a linearity range up to 25 nmol of sialic acids and hexosamines, has a detection limit ranging from 6 to 12 pmol, and is easily applied to the analysis of biologic samples [157].

2.3.3. Derivatization at carboxyl groups

Aminated derivatizing labels, such as ANDS (see Fig. 2), can react with acidic carboxylated sugars via a scheme different from reductive amination. The amine groups react with the carboxyl groups in the presence of carbodiimide to form a peptide bond [158] (Fig. 9). The mild reaction conditions (pH 5.0, room temperature) allow the application of this derivatization scheme to labeling of acidic monosaccharides [158], sialooligosaccharides [159,160], gangliosides [161], galactosaminoglycan disaccharides [162] for their sensitive CE–UV (at about 250 nm) or CE–LIF analysis with a He/Cd LIF detector. The reaction yields are very high; for example a yield of

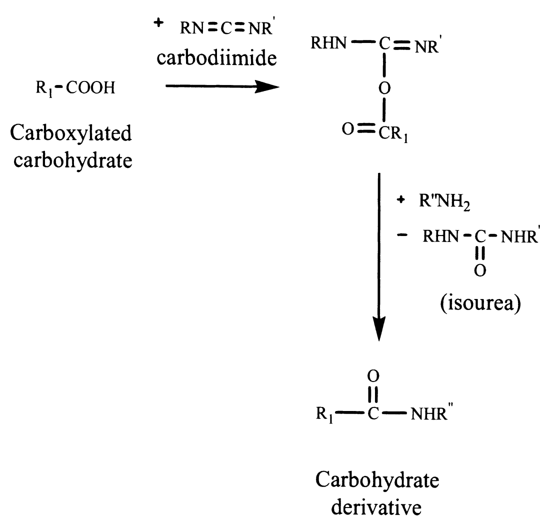

Fig. 9. Condensation reaction of carboxylated carbohydrates with aminated derivatizing agents in the presence of carbodiimide.

≥85% was reported for the derivatization of galactosaminoglycan disaccharides [162].

2.3.4. Derivatization at hydroxyl groups

Per-O-benzoylation has been extensively and successfully used by our research group for the sensitive determination of monosaccharides. Glucuronic and iduronic acid residues in GAGs were determined as per-O-benzoyl derivatives of D-glucose and 1,6-anhydro-idose, respectively, after stoichiometric reduction and depolymerization of GAG chains and per-O-benzoylation of monosaccharides [163]. Moreover, per-O-benzoylation has been used for the analysis of Neu5Ac and Neu5Gc [164]. To the dry carbohydrate sample, 100 μl of benzoylation mixture [10% (w/v) benzoic anhydrite–5% (w/v) *p*-dimethylaminopyridine in pyridine] are added and the mixture is heated at 80 °C for 20 min. The reaction is terminated by the addition of nine volumes of water and vigorous shaking. For a complete destruction of the remaining benzoic anhydrite, the mixture obtained was heated for a further 10 min at 80 °C. Excess of reagents and minute amounts of underbenzoylated derivatives are removed by solid-phase extraction on Sep-Pak C-18. Per-O-benzoylated derivatives are highly UV absorbent (maximum absorbance at 231 nm) and are nicely separated on a Supelcosil LC-18 column by isocratic elution. The method allows the determination of both sialic acid forms at the picomolar level and the calibration graphs are linear up to 160 nmol. This method has been applied to the quantitative determination of Neu5Ac and *N*-glycolylneuraminic acid with HPLC and CE in blood serum, animal and human tissues as well as for disease diagnosis and monitoring [165–168]. For details on the analysis of sialic acids and the biological significance of the results, the review in Ref. [169] is recommended.

3. On-capillary derivatization

On-capillary derivatization is a novelty introduced in CE analysis and only a few applications have been described so far [15]. Some of the major advantages of this technique are the ease of automation and the significant reduction of sample requirements. To our knowledge, only three such reports have been published in the field of carbohydrate analysis; two deal with typical on-capillary derivatization with some of the previously described chemical reactions [170,171] and the other one with two on-capillary enzymic reactions [172].

The latter study focuses on the determination of glucose in very small (<1 μl) sample volumes (tear fluid) [172]. The principle of determination is simple and depends on the selective action of two enzymes: glucose oxidase, which catalyzes the oxidation of glucose, and horseradish peroxidase, which catalyzes the oxidation of the non-fluorescent homovanillic acid (HVA) to the fluorescent 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid (HVA_{ox}) by hydrogen peroxide, produced in the first enzymic reaction. HVA_{ox} can be monitored by the He/Cd line of LIF detectors. The operating buffer contains both enzymes and HVA, and the sample is introduced by pressure. Mixing of the glucose sample with the reagents can be achieved with electrophoretic mixing or without and the authors suggest the application of a potential of 10 kV for 1 min and an incubation time of 2 min. However, two distinct zones containing the fluorescent product are formed and migrate as such during the electrophoretic separation [172]. Raising the temperature of reaction and the incubation time results in higher yield.

Both other studies involve chemical derivatization

at the inlet of the capillary by introducing the sample and reagent solutions in a sandwich or tandem mode. Taga et al. [170] studied the on-capillary condensation reaction of reducing oligosaccharides (maltooligosaccharides with a d.p. ranging from 2 to 5) with PMP. After a careful optimization, plugs of the reagent solution, the running buffer and the sample solution are introduced in this order by suction and upon voltage application are transferred to the heated portion (57 °C) of the capillary, where the plugs overlap. An incubation time of 35 min prior to electrophoretic separation was necessary for maximum derivatization yield of maltooligosaccharides. In comparison to pre-capillary derivatization the most striking difference is the amount of oligosaccharides actually required for derivatization; ~10 fmol are necessary for on-capillary derivatization in contrast to the 10 pmol necessary for pre-capillary derivatization [170]. Formation of monosaccharide hydrazones with *p*-hydrazinebenzenesulfonic acid was performed at the inlet of the capillary by Wang et al. [171]. Sample carbohydrates were introduced into the capillary tip in between two zones of labeling reagents with pressure. To prevent the labeling reagents from diffusing out of the capillary tip, a plug of running buffer was also introduced as the final zone. A reaction time of 30 min and ambient temperature were routinely used, but the reaction was not complete in this time. Although the concentration detection limits were close to those obtained by pre-capillary derivatization, once again the sample consumption is significantly decreased [171].

4. Concluding remarks

Derivatization of carbohydrates has allowed their sensitive UV or fluorescence detection in chromatographic and electrophoretic techniques and has facilitated their characterization by mass spectroscopy. Not only monosaccharides but also carbohydrates of higher molecular mass can be derivatized. Many different reaction schemes exist and the selection of the appropriate one is dictated by the composition of the sample and the requirements of the separation/characterization technique.

Carbohydrates may react with chromogenic or fluorescent molecules through their hydroxyl, carbonyl, amine or carboxyl groups. Derivatization at hydroxyl groups is not an option for large carbohydrates. Derivatization at amino groups naturally occurring or introduced to large carbohydrate molecules has been accomplished through condensation with CBQCA or the formation of tetramethylrhodamine derivatives, enabling the highest sensitivity in detection (CE–LIF). Acidic carbohydrates containing carboxylated monosaccharides have been derivatized at the carboxyl groups with aminated compounds at very mild reaction conditions. In particular, sialic acids have been studied as quinoxaline derivatives (UV-absorbing and fluorescent) and the hydrolysis of large oligomers can be minimised with careful optimization. However, the main target of derivatization reactions is the carbonyl group (reducing end). Reductive amination is by far the commonest tagging reaction due to the relatively high reaction yields, the absence of side-products and the availability of large numbers of amino compounds with a variety of properties, i.e. hydrophobicity or charge (positive or negative), absorbance in the UV–Vis or fluorescence. The lability of sialic acid-containing and/or high molecular size oligosaccharides at the reaction conditions is a point of attention. Other reactions at the carbonyl group which have been used include: (1) amination through the formation of glycosylamine with a few applications in the MS characterization, (2) formation of pyrazolone derivatives at mild reaction conditions, which strongly absorb in the UV region or can be detected electrochemically, with numerous applications in the CE, HPLC and MS characterization of oligosaccharides, (3) formation of fluorescent NBD-tagged N-methylglycamine derivatives at mild reaction conditions which has been applied to the CE–LIF analysis, (4) reaction with UV-absorbing or fluorescent hydrazines which has been used for mono-, di- and trisaccharides and has the disadvantage of side-product formation, and (5) reduction with radioactive compounds with the purpose of radiochemical detection in HPLC.

In every case, careful optimization of the reaction parameters is necessary in order to avoid sample degradation and to achieve a high and reproducible reaction yield and stability of the derivatives. The excess of labeling reagent may pose a problem in the

separation and efficient ways to remove it in a reproducible way are necessary. The applications of on-capillary derivatization in capillary electrophoresis are particularly promising since trace amounts of sample suffice for derivatization and analysis.

5. Nomenclature

AA-Ac	3-acetamido-6-aminoacridine
2-ABA	2-aminobenzoic acid
4-ABBE	4-aminobenzoic butyl ester
ABDEAE	4-amino benzoic 2-(diethylamino) ethyl ester
ABG	*N*-(4-aminobenzoyl)-L-glutamic acid
2-ABM	2-aminobenzamide
AMAC	aminoacridone
3-ANDA	3-aminonaphthalene-2,7-disulfonic acid
ANDS	7-aminonaphthalene-1,3-disulfonic acid
2-ANSA	2-aminonaphthalene-1-sulfonic acid
5-ANSA	5-aminonaphthalene-1-sulfonic acid
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
2-AP	2-aminopyridine
APTS	1-aminopyrene-3,6,8-trisulfonate
CBQCA	3-(4-carboxybenzoyl-)-2-quinoline carboxyaldehyde
CE	capillary electrophoresis
DHZ	dansylhydrazine
DMB	1,2-diamino-4,5-methylenedioxybenzene
DMSO	dimethylsulfoxide
d.p.	degree of polymerization
ESI	electrospray ionization
FAB	fast atom bombardment
GAG	glycosaminoglycan
Gal	galactose
GalA	galacturonic acid
GalAG	galactosaminoglycan
HPLC	high-performance liquid chromatography
HVA	homovanillic acid
HVA_{ox}	2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid
LC	liquid chromatography
LIF	laser-induced fluorescence
MALDI	matrix assisted laser desorption/ionization
MS	mass spectrometry
MEKC	micellar electrokinetic chromatography
NBD-F	7-nitro-2,1,3-benzoxadiazole 4-fluoride
Neu5Ac	*N*-acetyl neuraminic acid
PAGE	polyacrylamide gel electrophoresis
PMP	1-phenyl-3-methyl-2-pyrazolin-5-one
SDS	sodium dodecylsulfate
TOF	time-of-flight
Tos-Cl	*p*-toluenesulfonylchloride
UV	ultraviolet

References

[1] A. Helenius, M. Aebi, Science 291 (2001) 2364.
[2] D. Spillmann, U. Lindahl, Curr. Opin. Struct. Biol. 10 (5) (1994) 518.
[3] R.V. Iozzo, Crit. Rev. Biochem. Mol. Biol. 32 (1997) 141.
[4] M. Bernfield, M. Gotte, P.W. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, M. Zako, Annu. Rev. Biochem. 68 (1999) 729.
[5] N. Schwartz, Front. Biosci. 5 (2000) D649.
[6] K. Sugahara, H. Kitagawa, Curr. Opin. Struct. Biol. 10 (5) (2000) 518.
[7] L. Wells, K. Vosseller, G.W. Hart, Science 291 (2001) 2376.
[8] J. Hirabayashi, K. Kasai, Trends Glycosci. Glycotechnol. 12 (2000) 1.
[9] F.N. Lamari, M. Militsopoulou, T.N. Mitropoulou, A. Hjerpe, N.K. Karamanos, Biomed. Chromatogr. 16 (2002) 96.
[10] J. Turnbull, A. Powell, S. Guimond, Trends Cell. Biol. 11 (2) (2001) 75.
[11] N. Callewaert, S. Geysens, F. Molemans, R. Contreras, Glycobiology 11 (4) (2001) 275.
[12] K.R. Anumula, Anal. Biochem. 283 (2000) 17.
[13] J. Hirabayashi, K.-I. Kasai, J. Chromatogr. B 771 (2002) 67.
[14] A. Dell, H.R. Morris, Science 291 (2001) 2351.
[15] J.C.M. Waterval, H. Lingeman, A. Bult, W.J.M. Underberg, Electrophoresis 21 (2000) 4029.
[16] F.N. Lamari, M. Militsopoulou, X. Gioldassi, N.K. Karamanos, Fresenius J. Anal. Chem. 371 (2001) 157.
[17] W. Mao, C. Thanawiroon, R.J. Linhardt, Biomed. Chromatogr. 16 (2002) 77.
[18] A. Taga, M. Mochizuki, H. Itoh, S. Suzuki, S. Honda, J. Chromatogr. A 839 (1999) 157.
[19] C.R. Noe, B. Lachmann, S. Möllenbeck, P. Richter, Z. Lebensm.-Unters.-Forsch. A 208 (1999) 148.
[20] D.J. Harvey, Mass Spectrom. Rev. 18 (1999) 349.
[21] M.T. Cancilla, S.P. Gaucher, H. Desaire, J.A. Leary, Anal. Chem. 72 (2000) 2901.
[22] S. Honda, J. Chromatogr. A 720 (1996) 183.
[23] S. Hase, J. Chromatogr. A 720 (1996) 173.
[24] P. Pfaff, F. Weide, R. Kuhn, Chromatographia 49 (11/12) (1999) 666.
[25] Z. Huang, T. Prickett, M. Potts, R.F. Helm, Carbohydr. Res. 328 (2000) 77.

[26] F.-T.A. Chen, R.A. Evangelista, Electrophoresis 19 (1998) 2639.
[27] N.T. Tran, M. Taverna, F.S. Deschamps, P. Morin, D. Ferrier, Electrophoresis 19 (15) (1998) 2630.
[28] A.J. Mort, D. Zhan, V. Rodriguez, Electrophoresis 19 (12) (1998) 2129.
[29] K. Kakehi, T. Funakubo, S. Suzuki, Y. Oda, Y. Kitada, J. Chromatogr. A 863 (1999) 205.
[30] J. Plocek, M.V. Novotny, J. Chromatogr. A 757 (1997) 215.
[31] S. Ma, W. Nashabeh, Anal. Chem. 71 (1999) 5185.
[32] A.H.K. Plaas, V.C. Hascall, R.J. Midura, Glycobiology 6 (8) (1996) 823.
[33] B. Kuster, D.J. Harvey, Glycobiology 7 (2) (1997) vii.
[34] J. Charlwood, H. Birell, P. Camilleri, Anal. Biochem. 262 (1998) 197.
[35] S. Hase, T. Ikenaka, Y. Matsushima, J. Biochem. (Tokyo) 90 (2) (1981) 407.
[36] N. Takahashi, J. Chromatogr. A 720 (1–2) (1996) 217.
[37] N. Tomiya, J. Awaya, M. Kurono, S. Endo, Y. Arata, N. Takahashi, Anal. Biochem. 171 (1) (1988) 73.
[38] A.J. Mort, F. Qiu, N.O. Maness, Carbohydr. Res. 247 (1993) 21.
[39] H. Narita, Y. Takeda, K. Takagaki, T. Nakamura, S. Harata, M. Endo, Anal. Biochem. 232 (1995) 133.
[40] S. Honda, A. Makino, S. Suzuki, K. Kakehi, Anal. Biochem. 191 (2) (1990) 228.
[41] J.T. Smith, Z. El Rassi, J. High Resolut. Chromatogr. 15 (1992) 573.
[42] M. Okamoto, Biosci. Biotechnol. Biochem. 65 (11) (2001) 2519.
[43] C. Chiesa, R.A. O'Neill, Electrophoresis 15 (1994) 1132.
[44] I. Rethfeld, G. Blaschke, J. Chromatogr. B 700 (1997) 249.
[45] T. Kazmaier, S. Roth, J. Zapp, M. Harding, R. Kuhn, Fresenius J. Anal. Chem. 361 (1998) 473.
[46] C. Chiesa, C. Horvath, J. Chromatogr. 645 (1993) 337.
[47] P. Jackson, Biochem. J. 270 (1990) 705.
[48] A. Klockow, H.M. Widmer, R. Amado, A. Paulus, Fresenius J. Anal. Chem. 350 (1994) 415.
[49] A. Klockow, R. Amado, H.M. Widmer, A. Paulus, J. Chromatogr. A 716 (1995) 241.
[50] F.-Y. Che, J.F. Song, R. Zeng, K.Y. Wang, Q.C. Xia, J. Chromatogr. A 858 (1999) 229.
[51] M. Larsson, R. Sundberg, S. Folestad, J. Chromatogr. A 934 (2001) 75.
[52] L.A. Gennaro, J. Delaney, P. Vouros, D.J. Harvey, B. Domon, Rapid Commun. Mass Spectrom. 16 (2002) 192.
[53] J. Lemoine, M. Cabanes-Macheteau, M. Bardor, J.C. Michalski, L. Faye, P. Lerouge, Rapid Commun. Mass Spectrom. 14 (2) (2000) 100.
[54] A. Klein, A. Lebreton, J. Lemoine, J.M. Perini, P. Roussel, J.C. Michalski, Clin. Chem. 44 (12) (1998) 2422.
[55] A.J. Mort, E.M.W. Chen, Electrophoresis 17 (1996) 379.
[56] A. Klockow, R. Amado, H.M. Widmer, A. Paulus, Electrophoresis 17 (1996) 110.
[57] P. Camilleri, G.B. Harland, G. Okafo, Anal. Biochem. 230 (1995) 115.
[58] M. Stefansson, M. Novotny, Carbohydr. Res. 258 (1994) 1.
[59] A. Guttman, N. Cooke, C.M. Starr, Electrophoresis 15 (1994) 1132.
[60] A. Guttman, C. Starr, Electrophoresis 16 (1995) 993.
[61] M. Novotny, J. Chromatogr. B 689 (1997) 55.
[62] M. Hong, J. Sudor, M. Steffanson, M. Novotny, Anal. Chem. 70 (1998) 568.
[63] V.L. Ward, M.G. Khaledi, J. Chromatogr. A 859 (1999) 203.
[64] J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, R.B. Parekh, Anal. Biochem. 230 (2) (1995) 229.
[65] K. Sato, K. Sato, A. Okubo, S. Yamazaki, Anal. Biochem. 262 (1998) 195.
[66] H. Schwaiger, P.J. Oefner, C. Huber, E. Grill, G.K. Bonn, Electrophoresis 15 (1994) 941.
[67] D. Kratchmar, S. Wallner, M. Florenski, D. Schmid, R. Kuhn, Chromatographia 50 (9/10) (1999) 596.
[68] D. Schmid, B. Behnke, J. Metzger, R. Kuhn, Biomed. Chromatogr. 16 (2002) 151.
[69] K.R. Anumula, S.T. Dhume, Glycobiology 8 (7) (1998) 685.
[70] K. Yoshino, T. Takao, H. Murata, Y. Shimonishi, Anal. Chem. 67 (1995) 4028.
[71] T. Takao, Y. Tambara, A. Nakamura, K. Yoshino, H. Fukada, M. Fukada, Y. Shimonishi, Rapid Commun. Mass Spectrom. 10 (1996) 637.
[72] W. Mo, T. Takao, H. Sakamoto, Y. Shimonishi, Anal. Chem. 70 (1998) 4520.
[73] M. Okamoto, K.-I. Takahashi, T. Doi, Rapid Commun. Mass Spectrom. 4 (1995) 641.
[74] S. Broberg, A. Broberg, J.O. Duus, Rapid Commun. Mass Spectrom. 14 (19) (2000) 1801.
[75] R. Evangelista, M.S. Liu, F.-T.A. Chen, Anal. Chem. 67 (1995) 2239.
[76] S. Honda, S. Iwase, A. Makino, S. Fujiwara, Anal. Biochem. 176 (1989) 72.
[77] R.A. Evangelista, A. Guttman, F.-T.A. Chen, Electrophoresis 17 (1996) 347.
[78] A. Guttman, F.-T.A. Chen, R.A. Evangelista, N. Cooke, Anal. Biochem. 233 (1996) 234.
[79] A. Guttman, J. Chromatogr. A 763 (1997) 271.
[80] F.-T. Chen, T.S. Dobashi, R.A. Evangelista, Glycobiology 8 (11) (1998) 1045.
[81] M.A. Roberts, H.-J. Zhong, J. Prodolliet, D.M. Goodall, J. Chromatogr. A 817 (1998) 353.
[82] M. Stefansson, Anal. Chem. 71 (1999) 2373.
[83] T.S. Raju, J.B. Briggs, S.M. Borge, A.J.S. Jones, Glycobiology 10 (5) (2000) 477.
[84] H. Suzuki, O. Muller, A. Guttman, B.L. Karger, Anal. Chem. 69 (1997) 4554.
[85] B. Monsarrat, T. Brando, P. Condouret, J. Nigou, G. Puzo, Glycobiology 9 (1999) 335.
[86] P. Jackson, Anal. Biochem. 196 (1991) 238.
[87] F. Lamari, A.D. Theocharis, A. Hjerpe, N.K. Karamanos, J. Chromatogr. B 730 (1999) 129.
[88] T.N. Mitropoulou, F. Lamari, A. Syrokou, A. Hjepre, N.K. Karamanos, Electrophoresis 22 (2001) 2458.
[89] M. Militsopoulou, F. Lamari, A. Hjerpe, N.K. Karamanos, Electrophoresis 23 (11) (2002) 1104.
[90] O. Mastrogianni, F. Lamari, A. Syrokou, M. Militsopoulou, A. Hjerpe, N.K. Karamanos, Electrophoresis 22 (13) (2001) 2743.
[91] F.Y. Che, X.-X. Shao, K.-Y. Wang, Q.-C. Xia, Electrophoresis 20 (1999) 2930.

[92] F. Goubet, P. Jackson, M.J. Deery, P. Dupree, Anal. Biochem. 300 (2002) 53.
[93] P.M. Rudd, R.A. Dwek, Curr. Opin. Biotech. 8 (1997) 488.
[94] A. Calabro, M. Benavides, M. Tammi, V.C. Hascall, R.J. Midura, Glycobiology 10 (2000) 273.
[95] G. Okafo, L. Burrow, S.A. Carr, G.D. Roberts, W. Johnson, P. Camilleri, Anal. Chem. 68 (1996) 4424.
[96] H. Kitagawa, A. Kinoshita, K. Sugahara, Anal. Biochem. 232 (1995) 114.
[97] S. Nadakana, K. Sugahara, Glycobiology 7 (1997) 253.
[98] R. Grimm, H. Birell, G. Ross, J. Charlwood, P. Camilleri, Anal. Chem. 70 (1998) 3840.
[99] H. Birell, J. Charlwood, I. Lynch, S. North, P. Camilleri, Anal. Chem. 71 (1999) 102.
[100] J. Charlwood, H. Birell, A. Gribble, V. Burdes, D. Tolson, P. Camilleri, Anal. Chem. 72 (2000) 1453.
[101] J. Charlwood, J.M. Skehel, P. Camilleri, Anal. Biochem. 284 (2000) 49.
[102] W. Nashabeh, Z. El Rassi, J. Chromatogr. 600 (2) (1992) 279.
[103] A. Rydlund, O. Dahlman, J. Chromatogr. A 738 (1) (1996) 129.
[104] M. Zhang, H.A. Melouk, K. Chenault, Z. El Rassi, J. Agric. Food Chem. 49 (11) (2001) 5265.
[105] C. Prakash, I.K. Vijay, Anal. Biochem. 128 (1) (1983) 41.
[106] E. Vinogradov, K. Bock, Carbohydr. Res. 309 (1) (1998) 57.
[107] D.A. Westfall, R.R. Flores, G.R. Negrete, A.O. Martinez, L.S. Haro, Anal. Biochem. 265 (1998) 232.
[108] A.H. Franz, T.F. Molinski, C.B. Lebrilla, J. Am. Soc. Mass Spectrom. 12 (12) (2001) 1254.
[109] D.T. Li, G.R. Her, Anal. Biochem. 211 (1993) 250.
[110] D.T. Li, J.F. Sheen, G.R. Her, J. Am. Soc. Mass Spectrom. 11 (2000) 292.
[111] S. Honda, E. Akao, S. Suzuki, M. Okuda, K. Kakehi, J. Nakamura, Anal. Biochem. 180 (1989) 351.
[112] K. Kakehi, S. Suzuki, S. Honda, Y.C. Lee, Anal. Biochem. 199 (1991) 256.
[113] S. Suzuki, S. Honda, Electrophoresis 19 (1998) 2539.
[114] C.B. Castells, V.C. Arias, R.C. Castells, Chromatographia 56 (2002) 153.
[115] S. Honda, S. Yamamoto, S. Suzuki, M. Ueda, K. Kakehi, J. Chromatogr. 588 (1991) 327.
[116] C. Chiesa, P.J. Oefner, L.R. Zieske, R.A. O'Neill, J. Capillary Electrophor. 4 (1995) 175.
[117] S. Honda, K. Togashi, K. Uegaki, A. Taga, J. Chromatogr. A 805 (1998) 277.
[118] S. Honda, A. Taga, M. Kotani, E.R. Grover, J. Chromatogr. A 792 (1997) 385.
[119] A. Taga, Y. Yabusako, A. Kitano, S. Honda, Electrophoresis 19 (1998) 2645.
[120] S. Honda, T. Ueno, K. Kakehi, J. Chromatogr. A 608 (1992) 289.
[121] S. Suzuki, K. Kakehi, S. Honda, Anal. Chem. 68 (1996) 2073.
[122] J.A. Saba, X. Shen, J.C. Jamieson, H. Perreault, Rapid Commun. Mass Spectrom. 13 (8) (1999) 704.
[123] T. Rozaklis, S.L. Ramsay, P.D. Whitfield, E. Ranieri, J.J. Hopwood, P.J. Meikle, Clin. Chem. 48 (1) (2002) 131.
[124] D.S. Ashton, C.R. Beddell, D.J. Cooper, A.C. Lines, Anal. Chim. Acta 306 (1995) 43.
[125] J.J. Pitt, J.J. Gorman, Anal. Biochem. 248 (1997) 63.
[126] X. Shen, H. Perreault, J. Chromatogr. A 811 (1998) 47.
[127] S. Honda, J. Okeda, H. Iwanaga, S. Kawakami, A. Taga, S. Suzuki, K. Imai, Anal. Biochem. 286 (2000) 99.
[128] S. Suzuki, N. Shimotsu, S. Honda, A. Arai, H. Nakanishi, Electrophoresis 22 (18) (2001) 4023.
[129] S.A. Perez, L.A. Colon, Electrophoresis 17 (1996) 352.
[130] X. Wang, Y. Chen, Carbohydr. Res. 332 (2) (2001) 191.
[131] G. Avigad, J. Chromatogr. 139 (1977) 343.
[132] K. Mopper, L. Johnson, J. Chromatogr. 256 (1983) 27.
[133] N. Volpi, Anal. Biochem. 277 (2000) 19.
[134] N.K. Karamanos, T. Tsegenidis, C.A. Antonopoulos, J. Chromatogr. 405 (1987) 221.
[135] I. Miksik, J. Gabriel, Z. Deyl, J. Chromatogr. A 772 (1997) 297.
[136] T.J.P. Naven, D.J. Harvey, Rapid Commun. Mass Spectrom. 10 (1996) 829.
[137] H.E. Conrad, J.R. Bamburg, J.D. Epley, T.J. Kindt, Biochemistry 5 (1966) 2808.
[138] N.K. Karamanos, P. Vanky, A. Syrokou, A. Hjerpe, Anal. Biochem. 225 (1995) 220.
[139] X.M. Gioldassi, N.K. Karamanos, J. Liq. Chromatogr. Relat. Technol. 22 (13) (1997) 1997.
[140] S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, Anal. Biochem. 164 (1) (1987) 138.
[141] M. Ito, K. Ikeda, Y. Suzuki, K. Tanaka, M. Saito, Anal. Biochem. 300 (2002) 260.
[142] T. Hikita, K. Tadano-Aritomi, N. Iida-Tanaka, H. Toyoda, A. Suzuki, T. Toida, T. Imanari, T. Abe, Y. Yanagawa, I. Ishizuka, Anal. Biochem. 281 (2000) 193.
[143] P. Stehling, M. Gohlke, R. Fitzner, W. Reutter, Glycoconjug. J. 15 (1998) 339.
[144] N. Morimoto, M. Nakano, M. Kinoshita, A. Kawabata, M. Morita, Y. Oda, R. Kuroda, K. Kakehi, Anal. Chem. 73 (2001) 5422.
[145] A. Kawabata, N. Morimoto, Y. Oda, M. Kinoshita, R. Kuroda, K. Kakehi, Anal. Biochem. 283 (2000) 119.
[146] A. Klein, S. Diaz, I. Ferreira, G. Lamblin, P. Roussel, A.E. Manzi, Glycobiology 7 (3) (1997) 421.
[147] C. Sato, S. Inoue, T. Matsuda, K. Kitajima, Anal. Biochem. 266 (1999) 102.
[148] S. Inoue, S.-L. Lin, Y.C. Lee, Y. Inoue, Glycobiology 11 (9) (2001) 759.
[149] S. Inoue, Y. Inoue, Biochimie 83 (2001) 605.
[150] J. Liu, O. Shirota, M. Novotny, Anal. Chem. 63 (1991) 413.
[151] Y. Zhang, E. Arriaga, P. Diedrich, O. Hindsgaul, N.J. Dovichi, J. Chromatogr. A 716 (1995) 221.
[152] J. Liu, O. Shirota, M.V. Novotny, Anal. Chem. 64 (1992) 973.
[153] J. Liu, O. Shirota, D. Wiesler, M. Novotny, Proc. Natl. Acad. Sci. USA 88 (1991) 2302.
[154] X. Le, C. Scaman, Y. Zhang, J. Zhang, N.J. Dovichi, O. Hindsgaul, M.M. Palcic, J. Chromatogr. A 716 (1995) 215.
[155] H.D. Osthoff, K. Sujino, M.C. Palcic, N.J. Dovichi, J. Chromatogr. A 895 (2000) 285.

[156] S.N. Krylov, E.A. Arriaga, N.W.C. Chan, N.J. Dovichi, M.M. Palcic, Anal. Biochem. 283 (2000) 133.
[157] E. Makatsori, N.K. Karamanos, E.D. Anastassiou, A. Hjerpe, T. Tsegenidis, J. Liq. Chromatogr. Relat. Technol. 21 (19) (1998) 3031.
[158] Y. Mechref, Z. El Rassi, Electrophoresis 15 (1994) 627.
[159] Y. Mechref, G.K. Ostrander, Z. El Rassi, Electrophoresis 16 (1995) 1499.
[160] Y. Mechref, G.K. Ostrander, Z. El Rassi, J. Chromatogr. A 792 (1997) 75.
[161] Y. Mechref, G.K. Ostrander, Z. El Rassi, J. Chromatogr. A 695 (1995) 83.
[162] Z. El Rassi, J. Postlewait, Y. Mechref, G.K. Ostrander, Anal. Biochem. 244 (1997) 283.
[163] N.K. Karamanos, A. Hjerpe, T. Tsegenidis, B. Engfield, C.A. Antonopoulos, Anal. Biochem. 172 (1988) 410.
[164] N.K. Karamanos, B. Wilkström, C.A. Antonopoulos, A. Hjerpe, J. Chromatogr. 503 (1990) 421.
[165] S. Diamantopoulou, K.D. Stagiannis, K. Vasilopoulos, P. Barlas, T. Tsegenidis, N.K. Karamanos, J. Chromatogr. B 732 (1999) 375.
[166] E. Makatsori, K. Fermani, A. Aletras, N.K. Karamanos, T. Tsegenidis, J. Chromatogr. B 712 (1998) 23.
[167] E. Makatsori, A. Aletras, N.K. Karamanos, T. Tsegenidis, Biomed. Chromatogr. 13 (1999) 57.
[168] K. Stroussopoulou, M. Militsopoulou, K. Stagiannis, F.N. Lamari, N.K. Karamanos, Biomed. Chromatogr. 16 (2) (2002) 146.
[169] F. Lamari, N.K. Karamanos, J. Chromatogr. B (2002) in press.
[170] A. Taga, S. Suzuki, S. Honda, J. Chromatogr. A 911 (2001) 259.
[171] X.Y. Wang, Y. Chen, Z. Li, Z. Wang, J. Liq. Chromatogr. Relat. Technol. 25 (4) (2002) 589.
[172] Z. Jin, R. Chen, L.A. Colon, Anal. Chem. 69 (1997) 1326.
[173] Y.S. Kim, J. Liu, X.J. Han, A. Pervin, R.J. Linhardt, J. Chromatogr. Sci. 33 (1995) 162.
[174] Y. Friedman, E.A. Higgins, Anal. Biochem. 228 (1995) 221.
[175] K. Morimoto, N. Maeda, A.A. Abdel-Alim, S. Toyoshima, T. Hayakawa, Biol. Pharm. Bull. 22 (1999) 5.
[176] M. Bardor, M. Cabanes-Macheteau, L. Faye, P. Lerouge, Electrophoresis 21 (2000) 2550.
[177] L.Y. Frado, J.E. Strickler, Electrophoresis 21 (2000) 2296.
[178] T.L. Goins, J.E. Cutler, J. Clin. Microbiol. 38 (2000) 2862.
[179] O. Quintero, R. Montesino, J.A. Cremata, Anal. Biochem. 256 (1998) 23.
[180] M.G. O'Shea, M.K. Morell, Electrophoresis 17 (1996) 681.
[181] M.K. Morell, M.S. Samuel, M.G. O'Shea, Electrophoresis 19 (1998) 2603.
[182] A. Guttman, F.T. Chen, R.A. Evangelista, Electrophoresis 17 (1996) 412.
[183] A. Guttman, T. Pritchett, Electrophoresis 16 (1995) 1906.
[184] C.M. Mangin, D.M. Goodall, M.A. Roberts, Electrophoresis 22 (2001) 1460.
[185] C.Y. Wang, Y.Z. Hsieh, J. Chromatogr. A 979 (2002) 431.
[186] P. Ludwiczak, T. Brando, B. Monsarrat, G. Puzo, Anal. Chem. 73 (2001) 2323.
[187] P. Jackson, M.G. Pluskal, W. Skea, Electrophoresis 15 (1994) 896.
[188] G.B. Harland, G. Okafo, P. Matejtschuk, I.C. Sellick, G.E. Chapman, P. Camilleri, Electrophoresis 17 (1996) 406.
[189] J.F. Song, M.Q. Weng, S.M. Wu, Q.C. Xia, Anal. Biochem. 304 (2002) 126.
[190] P. Camilleri, D. Tolson, H. Birrell, Rapid Commun. Mass Spectrom. 12 (1998) 144.
[191] J. Charlwood, J. Langridge, D. Tolson, H. Birrell, P. Camilleri, Rapid Commun. Mass Spectrom. 13 (1999) 107.