Recent advances in capillary electrophoresis separation of monosaccharides, oligosaccharides and polysaccharides

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Running title

CE of carbohydrates

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Received: 07, 18, 2017; Revised: 08, 22, 2017; Accepted: 08, 23, 2017

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/elps.201700290.

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tel number: 0039 (0)59 2055543

Abbreviations used

2-AA, 2-aminobenzoic acid. AMAC, 2-aminoacridone. BGE, background electrolyte. C4D, contactless conductivity detection. CAD, charged aerosol detection. CAE, capillary affinity electrophoresis. CE, capillary electrophoresis. COS, chito-oligosaccharides. CZE, capillary zone electrophoresis. ELSD, evaporative light-scattering detection. EOF, electroendoosmotic flow. ESI, electron spray ionization. GAGs, glycosaminogycans. GC, gas chromatography. HDMB, hexadimethrine bromide. HILIC, hydrophilic interaction-liquid chromatography. 5-HMF, 5-hydroxymethylfurfural. HPEAC, high-performance anion exchange chromatography. HPLC, High pressure liquid chromatography. IMS, ion-mobility spectrometry. IR, refractive index. LC, liquid chromatography. LIF, laser induced fluorescence. MALDI, matrix-assisted laser desorption/ionization. MEKC, micellar electrokinetic capillary chromatography. MS, mass spectrometry. NMR, nuclear magnetic resonance. PA, pyridylaminated. PAD, pulsed amperometric detection. PGC, porous graphitized carbon. PMP, 1-phenyl-3-methyl-5-pyrazolone. RP, reversed phase. SDS, sodium dodecyl sulfate. SEC, size exclusion chromatography. TMT, tandem mass tags. TOF, time-of flight.

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Abstract (154 words)

This article illustrates the basis and applications of methodologies for the analysis of simple and complex carbohydrates by means of capillary electrophoresis (CE). After a description of the most common and novel approaches useful for the analysis and characterization of carbohydrates, this review covers the recent advances in CE separation of monosaccharides, oligosaccharides and polysaccharides. Various CE techniques are also illustrated for the study of carbohydrates derived from complex glyco-derivatives such as glycoproteins and glycolipids, essential for biopharmaceutical and glycoproteomics applications as well as for biomarker detection. Most glycans have no significant UV absorption and derivatization with fluorophore groups prior to separation usually results in higher sensitivity and an improved electrophoretic profile. We also discuss the recent applications and separations by CE of derivatized simple and more complex carbohydrates with different chromophoric active tags. Overall, this review aims to give an overview of the most recent state-of-the-art techniques used in carbohydrate analysis by CE.

Keywords

Monosaccharides; Oligosaccharides; Polysaccharides; Capillary Electrophoresis; laser-induced fluorescence.

1 Introduction

Carbohydrates are considered important key molecules and the largest group of biomolecules. Among these, monosaccharides are the simplest units forming complex

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macromolecules and glucose is certainly one of the most important carbohydrates for life. Furthermore, complex macromolecules include diverse subgroups such as polysaccharides, proteoglycans, glycolipids and glycoproteins. Carbohydrates are also a relatively new subject of medical research as they play important roles in biological functions such as cell-cell and cell-host interaction, regulation of the pro- or anti-inflammatory abilities of immunoglobulins according to their level of sialylation, cellular trafficking, solubility of proteins and assistance in protein folding. Alterations in the complex glycosylation machinery responsible for the biosynthesis and degradation of complex polysaccharides as well as glycoconjugates have been correlated with different conditions including inflammatory diseases, cancer, diabetes and cardiovascular diseases, lysosomal storage disease, etc. [1-3]. In addition, carbohydrates are important biomolecules in different fields such as food and beverage analysis, plant-related analysis, fermentation and metabolism studies [4].

Qualitative and quantitative elucidation of carbohydrates is a challenging task due to the lack of either chromophoric or fluorophoric functional groups and low extinction coefficients of the monosaccharide structure. Moreover, the structural complexities of glycans, along with their diversity [5], and the unique tendency towards branching, different linkages and isomerism, generally presents formidable challenges.

Capillary electrophoresis (CE) has attracted considerable attention in the recent advance in glycomic analysis. CE is suitable for both derivatized and underivatized carbohydrates and it is established as a powerful and versatile separation tool thanks to the brief analysis time, the ability to handle very complex glycan mixtures with high sensitivity, the minute quantities of samples and reagent required, the capacity to resolve isomeric glycans and the possibility to acquire high resolution obtained under physiological conditions [5]. In this review, our attention is focused on the recent development in CE techniques for carbohydrate analysis.

2 Structure and functions of carbohydrates

Carbohydrates play a variety of extensive roles in all forms of life. Living organisms use carbohydrates as accessible energy to fuel cellular reactions and for energy storage [6].

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Small carbohydrate molecules act as metabolic intermediates improving cell identification, cell signaling and complex immune system responses. The carbohydrate monomers deoxy-ribose and ribose are involved in the structural framework of DNA and RNA molecules. They are crucial in cell support and act as structural elements in plants (cellulose), cell walls of bacteria (peptidoglycan or murein) and animals (chitin). Cells also link carbohydrate molecules to different proteins and lipids, modifying structures to enhance functionality. These linkages are important in cell-cell communication and in interactions between cells or other elements in the cellular environment.

The general empirical structure for carbohydrates is $(CH_2O)_n$ and, in their basic form, carbohydrates are simple monosaccharides (Figure 1). These basic units can combine with one another to form more complex carbohydrates. The combination of two monosaccharides forms a disaccharide. Carbohydrates consisting of two to ten simple sugars are generally called oligosaccharides and the longer chains are known as polysaccharides.

Monosaccharides are often required as energy sources and they consist of a carbon chain of three or more carbon atoms containing a hydroxyl group attached to every carbon atom except one. They may be subcategorized in aldose or ketose sugars. Because the arrangements of individual atoms within a sugar molecule vary, many monosaccharides exist in a variety of isomer forms [7, 8]. Saccharides with identical functional groups but with different spatial configurations have different chemical and biological properties. Sugars may be modified by enzymatic reactions into compounds that retain the basic configuration of saccharides but have different functional groups. Important modified sugars are sugar alcohols (also known as polyols or poly-alcohols, which are the hydrogenated forms of the aldoses or ketoses), amino sugars (modified by the addition of a new functional group) and uronic acids (which have a carboxyl group on the carbon that is not part of the ring) [9].

Carbohydrate chains can be extended by additional dehydration synthesis reactions, adding one monomer at a time to a growing chain. Short carbohydrate chains, the oligosaccharides, can be found free (milk oligosaccharides are an example of free functional oligosaccharides) or attached to lipids and proteins through a process named glycosylation [10]. They are covalently attached to proteins by either *N*-glycosidic or *O*-glycosidic bonds. These molecules support immune system functions, participate in cell communication and help attach cells to extracellular surfaces and other cells.

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Longer carbohydrate chains, with hundreds or more of monosaccharide units, the polysaccharides, can be further classified into homo- and hetero-polysaccharides based on the composition of individual monosaccharides. Differences in the structure and function of these macromolecules are often due to the nature of glycosidic bonds. Moreover, branched polysaccharides can be formed by glycosidic bonds on different carbons of the monosaccharide units. Polysaccharides play structural roles but also constitute a vital energy storage as they can be easily built and broken down by enzymes (glycogen and starch). Other polysaccharides form strong fibers that provide protection and structural support in both plants and animals (cellulose and chitin) [7, 8].

Complex heteropolysaccarides contain different kinds of monosaccharides and they usually provide extracellular support for organisms. Glycosaminogycans (GAGs) are heteropolysaccarides formed by the repetition of a disaccharide unit of an amino-sugar and an acid sugar; moreover several sulfate groups can be linked to certain monosaccharides. Hyaluronic acid, chondroitin sulfates, dermatan sulfate, heparan sulfate and heparin are important GAGs and prominent components of the extracellular matrix. These heteropolysaccharides are degraded by lysosomal hydrolases. A deficiency of one of these enzymes results in a lysosomal storage disease called mucopolysaccharidosis. GAGs are often found associated with proteins forming proteoglycans. These molecules play important roles in structural support, water metabolism regulation, biological lubrication and signaling, among other functions [11, 12].

3 Analytical techniques for carbohydrates study

A number of different analytical techniques have traditionally been employed in carbohydrate analysis (Table 1). High pressure liquid chromatography (HPLC) has been largely applied to separate and quantify carbohydrates with no previous sample derivatization. HPLC-UV detection relies on simple UV absorbance but it is challenging for carbohydrates generally lacking in chemical groups able to absorb UV radiation [13]. Different detection modes have been reported for HPLC carbohydrate analysis such as refractive index (RI), evaporative light-scattering detection (ELSD), a universal detector also having the power to detect interfering compounds with similar elution times, charged aerosol

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detection (CAD) and pulsed amperometric detection (PAD), which is one of the most useful analytical methods [14-16].

Due to the hydrophilic nature of glycans, separation systems based on hydrophilic interaction-liquid chromatography (HILIC) [17] are being increasingly used. Furthermore, porous graphitized carbon (PGC) offers high chemical inertness, good performance at elevated temperatures and stability at different pH ranges. Reversed phase liquid chromatography (RP)-LC [18] and high-performance anion exchange chromatography (HPEAC) [16] are other separation techniques able to separate carbohydrate isomers to a certain extent. Size exclusion chromatography (SEC) is commonly used to separate carbohydrates depending on their hydrodynamic volume but a difference in the molecular weight is required. Moreover, it has relatively long run times also generally showing difficulty in separating glucose from fructose or other isomers [19]. Another common technique for carbohydrate analysis is gas chromatography (GC) which, however, requires several derivatization steps to volatilize the carbohydrate samples [20].

Mass spectrometry (MS), in particular electron spray ionization (ESI)-MS and matrix-assisted laser desorption/ionization (MALDI)-MS, has evolved as one of the most powerful tools for glycan analysis [21]. Usually, MS-based measurements are not suitable for carbohydrate analysis due to their inherent hydrophilicity which results in an inefficient desolvation during electrospray ionization and a consequential signal loss. Therefore, to amplify sensitive detection, carbohydrates can be derivatized to include an appropriate label, but the poor sensitivity of MS towards glycan isomers reduces the reliability of MS analysis. MS-related technologies can overcame this critical issue by supplementing it with other techniques in the context of a larger, integrated approach. Many MS based on-line methods have been developed, such as GC-MS, LC-MS, PGC-LC-ESI-MS (an important tool able to distinguish isomeric glycans with a minimum sample amount) [22], ion-mobility spectrometry (IMS), CE-MS of native and derivatized glycans [21], MALDI-time-of flight MS (MALDI-TOF MS) [23].

Another important technique in carbohydrate study is nuclear magnetic resonance (NMR). ^{1}H and ^{13}C chemical shifts of carbohydrate samples have been used to determine different structural aspects of these molecules with the advantage of being a non-destructive

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method also offering the possibility of quantification with no further derivatization. However, this technique requires expensive instrumentation, significant expertise and often a large amount of pure sample (Table 1) [5, 24].

Finally, the use of lectins for affinity-based chromatographic and electrophoretic methods has proven useful due to their potential for high throughput, analysis of glycans in their physiological context, selectivity and resolution in separation/fractionation studies. Unfortunately, this method is limited by the availability and specificity of characterized lectins [5, 25].

4 CE for carbohydrate analysis

CE is a powerful analytical technique for the separation of polar molecules. In CE, analytes migrate into the capillary column filled with a conducting buffer solution under the influence of an applied electrical field. The capillary inner-surface is negatively ionized by an electrolyte solution contact and an electrical double layer is formed when cations bind on the negatively charged capillary wall. Cations move to the cathode when voltage is applied, which results in a solution flow, the electroendoosmotic flow (EOF). The rate of movement of individual molecules through the capillary and detector depends on several factors including the analyte charge, the charge to hydrodynamic volume ratio, buffer system (pH and ionic strength), voltage applied, temperature, ~~the~~ length and diameter of the capillary and the nature of the capillary wall [26]. CE has high resolving power, requiring a relatively short time, short post-run washes, since undesirable sample components can be flushed out after analysis, and equipment that can be applied for high-throughput automation [27]. On the other hand, apart from carbohydrates possessing negatively charged and easily ionizable acidic groups, a large number of sugars lack ~~of~~ molecular charge, ionizable molecules and chromophore or fluorophore groups. As a consequence, carbohydrates display several limitations for CE technology. However, in the recent year different strategies have been developed to overcome these issues, making CE an election technique for carbohydrate analysis.

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Both direct and indirect CE detection have been performed for carbohydrate analysis. Direct CE-UV is one of the first detection methods for oligosaccharide analysis. The disadvantage of this approach is generally related to the low sensitivity, generally mM range, at the absorbance wavelengths of 191-195 nm. Pre-column derivatization with chromophores or fluorophores and detection by absorption or fluorescence have been developed to enhance sensitivity in the direct CE method.

Reductive amination of the aldehyde group at the reducing end of sugars is one of the most frequent methods for the derivatization of carbohydrates. Labeling reagents include 2-aminopyridine, 2-aminoacridone (AMAC), biotin aminopyridine, *p*-aminobenzoic acid, 2-aminobenzamide, 4-aminobenzonitrile, 8-aminonaphthalene-1,3,6-trisulfonic acid, 1-phenyl-3-methyl-5- pyrazolone, 1-(4-methoxy)phenyl-3-methyl-5-pyrazolone, 6-aminoquino-line, 1-maltohepaosyl-1,5-diaminonaphthalene, 8-aminopyrene-1,3,6-trisulfonate and *p*-hydrazine-benzenesulfonic acid (Figure 2). UV, conventional fluorescence and/or laser induced fluorescence (LIF) may be used for detection depending on the properties of the derivatives obtained [28-31]. However, the pre-column derivatization method can be expensive and time consuming. On the contrary, in the indirect detection mode, a chromophore or fluorophore is added directly to the background electrolyte and a negative peak shows a signal when molecules are detected in the background absorption [32, 33].

For underivatized sugars, CE analysis is possible using an electrolyte with a pH above the pKa of the sugars (pH >12) useful to ionize carbohydrates lacking in acidic functional groups. In a strong alkaline solution, the hydroxyl groups of carbohydrates are ionizable and they can migrate in capillary zone electrophoresis (CZE) as anions. Different detection modes can be applied in this procedure, such as electrochemical detection [34, 35], PAD [36], ESI-MS [37], indirect UV/LIF detection [38] and contactless conductivity detection (C4D) [39]. Recently, a direct UV method without pre-column derivatization has been able to separate several neutral carbohydrates. This procedure uses a high pH buffer and direct UV detection via the formation of an absorbing intermediate by photo-oxidation in the detection window at 270 nm [40-42].

The use of a borate buffer is another method to analyze both derivatized and underivatized carbohydrates since sugars are able to complex with borate and can be

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converted into anions. This complex shows an increased UV sensitivity at 195 nm [43-45]. Furthermore, complexation with copper has been used. However, copper may induce the formation of supramacromolecular structures with other compounds [46].

Micellar electrokinetic chromatography (MECK) is a modification of CZE which includes the introduction of surfactant micelles in the running buffer able to provide a two-phase chromatographic system, mimic reverse phase-LC. This technique can be easily used for the separation of carbohydrates at pH 7.5. However, the addition of sodium dodecyl sulfate (SDS) as surfactant can lead to interaction with proteins and lipids present in complex mixtures [47]. In addition, capillary affinity electrophoresis (CAE) using lectins is based on the high-resolution separation of fluorescently labeled glycans by CE-LIF in the presence of lectins, allowing simultaneous determination of glycan structures in a complex mixture of glycans [48, 49]. Finally, CE chip-based systems have recently been developed [50-52], enabling significant reduction in analysis times, possible high-throughput analysis and more reproducible separations.

5 Recent developments in the CE separation of monosaccharides

Indirect UV detection has been widely applied to monosaccharide analysis. Cebolla-Cornejo et al. [28] developed a routine analysis system for the determination of different compounds in fruit and vegetable crops. Sugars such as fructose, glucose and sucrose along with organic acid and the amino acid glutamate were determined using a running buffer with 20 mM 2,6-pyridine dicarboxylic acid pH 12.1 and 0.1% hexadimethrine bromide (HDMB) under optimal conditions, enabling a large number of injections (200) without disturbances in the same capillary and reducing time analysis down to 12 min [33]. Indirect CE-UV has also been used by Rizelio et al. for the determination of fructose, glucose and sucrose in seven multifloral honey samples. The detection limits were in the range from 22 and 29 mg/L and a rapid separation (less than 2 min) was achieved [32]. Analysis of 13 varieties of stingless bee honey using indirect detection was also developed by Biluca at al. analyzing fructose, sucrose, glucose and 5-hydroxymethylfurfural (5-HMF) content [53].

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Direct UV/LIF detection with pre-column derivatization has been used in several fields for monosaccharide analysis. Abo et al. derivatized reducing monosaccharides with 2-aminobenzoic acid (2-AA) [54] and Sun et al. developed a CE method for the separation of ribose enantiomers using 7-aminonaphthalene-1,3-disulfonic acid as derivatization reagent [55]. In 2013, 7-amino-4-methylcoumarin was used to label monosaccharides and oligosaccharides from glycoproteins [56]. Hu et al. developed a rapid analytical method for the simultaneous separation and determination of 10 monosaccharides labeled with 1-phenyl-3-methyl-5-pyrazolone (PMP) (Figure 3) [35, 36, 57] and Sarazin et al. [42] proposed a direct UV strategy to analyze neutral carbohydrates, fructose, glucose, lactose and sucrose. This method is based on the use of 98 mM sodium hydroxide and 120 mM sodium chloride and direct UV detection via the formation of an absorbing intermediate by photo-oxidation in the detection window. The photochemical reaction of saccharides during direct ultraviolet absorbance detection has been recently investigated by Schmid et al. [60, 61]. A similar method has also been applied by Oliver et al. in a quantitative comparison between CE and HPLC [62, 63] and by Toutounji et al. for the analysis of sucrose, maltose, glucose and fructose in breakfast cereals [64].

C4D has also been used as a detection mode for the analysis of simple carbohydrates by CE. Tuma et al. developed a CE-C4D method for monitoring low-molecular saccharides such as glucose, fructose, galactose, mannose, ribose, sucrose and lactose in food and drinks in a less than 3 min separation with LODs lower than 1 µM (Figure 4) [39]. Furthermore, Vochyanova et al. applied C4D for the rapid (less than 1 min) determination of sucrose, glucose and fructose in high-energy drinks [65].

Recently, Kubo et al. reported an effective CE separation and detection procedure for sugars using a complexation between quinolineboronic acid and multiple hydroxyl structure of sugar alcohol. Six sugar alcohols were successfully separated and the limits of detection were estimated at 15 and 27 µM [66].

6 Recent application of CE for the analysis of oligosaccharides, polysaccharides and glycan-derivatives

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One of the most important fields for oligosaccharide analysis is human milk oligosaccharide (HMOs) determination. HMOs are unique due to their highly complex nature and important emerging biological and protective functions during early life. CE has been widely applied for the separation of underivatized or labeled acidic (sialylated) and neutral HMOs. Recently, Galeotti et al. [67] performed an analytical CE technology based on derivatization with AMAC and simple UV detection at 254 nm to characterize the main HMOs along with lactose. MECK, direct CE-UV and CE coupled with MS have also been performed in HMOs analysis [68]. In a recent study, Zhong et al. performed a CE-ESI-MS for the quantitative analysis of glycans labeled with multiplex carbonyl-reactive tandem mass tags (TMT). The authors characterized the ESI and CID behavior of the aminoxy-TMT-labeled neutral and sialylated glycans [69]. Also, a high-throughput method for the characterization of HMOs using multiplexed CGE-LIF [70] and a CAE method were developed for bovine milk oligosaccharide evaluation [51].

Complex heteropolysaccharides, GAGs and GAG-derived oligosaccharides and disaccharides, are other important subjects in carbohydrate analysis. GAGs are important structural and functional macromolecules often involved in several diseases and GAG-derived disaccharides can be important diagnosis markers. A quantitative and highly sensitive CE method for the analysis of GAG-derived disaccharides, with AMAC derivatization and LIF detection has been presented by Chang et al. [71]. This method enables a complete separation of 17 GAG-derived disaccharides in a single run with a limit of detection at the attomole level and approximately 100-fold more sensitive than traditional CE-ultraviolet detection (Figure 5). Lin et al. [72] demonstrated a new method for the analysis of heparin oligosaccharides, including disaccharides, ultra-low molecular weight heparin and a low-molecular weight heparin, using a novel electrokinetic pump-based CE-MS coupling electrospray ion source. Recently, Maccari et al. separated hyaluronic acid, chondroitin sulfate and heparan sulfate disaccharides fluorotagged with AMAC using CE-LIF at 488 nm [73]. A similar protocol has been applied by Casado et al. for the analysis of the urine oligosaccharide pattern derivatizated with 8-aminopyrene-1,3,6-trisulfonate (Figure 6). They developed a screening method for oligosaccharidoses and related diseases [74]. Whereas enzymatic degradation is time-consuming and expensive, there are also reports about the separation of intact GAGs. Zhao et al. [75] developed a CE method to separate

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hyaluronic acid, chondroitin sulfate, dermatan sulfate and heparin in a single run establishing an economic and labor-saving method for the determination of intact GAGs in plasma. They reported the use of diethylenetriamine as background electrolyte using an uncoated fused silica capillary at 37°C with a run time of 23 min.

CE has also been applied for the separation of chito-oligosaccharides (COS) from other saccharides in aqueous solution. COS are hydrolysates of chitosan, exhibit better water solubility and major physiological activities involving antitumor, free radical scavenging and antimicrobial activities, immune modulatory and wound healing effects, maintaining the chemical structure of chitosan intact. Hattori et al. described a simple CE of COS in an acidic solution using a positively charged capillary coated with N-trimethoxypropyl-N,N,N-trimethylammonium chloride and indirect photometric detection with crystal violet nitrate background solution (Figure 7) [76].

CE can also be coupled to MS to analyze different carbohydrate derivatives. To this aim, Ito et al. developed a high-sensitivity CE-electrospray ionization quadrupole ion trap TOF-MS to structurally characterize four kinds of pyridylaminated (PA) oligosaccharides derived from neutral glycosphingolipids [77]. Sialic acids are *N*- or *O*-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone. *N*-acetylneuraminic acid and its hydroxylated form, *N*-glycolylneuraminic acid (Figure 1), are the two major sialic acids found in mammals. Sialic acid-rich oligosaccharides are important biological molecules often involved in pathological disease and present on many carbohydrate derivatives. The identification of sialo-glycoconjugate presents several difficulties since many methods used in the structural analysis of glycans can degrade sialic acid, such as (??) the application of strong acid catalyst in labeling reductive amination. The most popular agent for sialic acid oligosaccharides derivatization and analysis is AMAC. For example, Szabo et al. developed an AMAC derivatization CE-LIF method for rapid sialic acid speciation on glycoproteins [78].

Recently, a novel method for the separation of underivatized cellodextrin oligosaccharides by CE with direct photochemically induced UV-detection has been proposed by Alinat et al [79]. They reported the simultaneous analysis of 15 carbohydrates in a 20 min total runtime, carried out at 25°C with a background electrolyte (BGE) composed

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of 77.4 mM NaOH and 183 mM NaCl to adjust the conductivity at the optimum value (Figure 8).

A novel carbohydrate labeling method for CE-LIF analysis, utilizing transfer hydrogenation-mediated reductive amination, has been developed by Kovács et al. [80]. They propose a catalytic hydrogen transfer from formic acid catalyzed by water-soluble iridium(III)- and ruthenium(II)-phosphine complexes as a novel alternative to hydrogenation. This procedure is environmentally friendly and reduces the health risks for the industry. Moreover, Szigeti et al. observed that the resolution between some glycan structures was temperature dependent and introduced a temperature gradient CE to enhance separation selectivity for branched glycans of biotherapeutic interest [81].

Microfluidic CE systems are recent developments in technology improving sensitivity and small sample quantities due to their compact scale. Khatri et al. developed a microfluidics-based CE-MS system for the analysis of released glycans, glycopeptides and monosaccharides. They used glycan TMT-labeling to improve electrophoretic migration enabling multiplexed quantitation by tandem MS [82].

Another important application of CE in carbohydrate analysis is glycosylation. Glycans expressed on cell surfaces play a dominant role in various biological processes. A structural change in glycans is often related to metabolic disorders and pathological conditions. Moreover, new biological glycoprotein-based therapies have been discovered in ~~the~~ recent years and rapid and reproducible analytical methods are required for the analysis of these biopharmaceutical products. CE provides a directly orthogonal approach to LC and MS. After releasing the glycans from the protein surface, both enzymatic and chemical methods are available [83] and different CE technologies can be applied for glycosylation analysis (Table 2). Typical CE-LIF, micellar electrokinetic capillary chromatography (MEKC) and CAE are the most used but also microchip-based systems have been developed. For example, CE-LIF has been widely used for the identification of monoclonal antibody glycosylation [84] but also for diagnostic and research purposes such as ovarian cancer *N*-glycome (Table 2) [85].

7 Conclusions

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Carbohydrates are widespread molecules involved in several aspects of life. Over the past years, there has been a notable transformation in understanding the structure, biology and functions of complex glycans thanks to the development of synthesis, analytical and informatic tools and technologies to study glycan structure-function relationships. As a consequence, today, different research fields require a fast and sensitive method for carbohydrate analysis, namely the food industry, plant and wood analysis, pathological and diagnostic research, pharmaceutical development and glycosylation analysis. CE has proven to be a fast and reliable technology for carbohydrate analysis due to its high resolving power and short time, short post-run washes, since undesirable sample components can be flushed out after analysis, and equipment that can be converted for high- throughput automation analysis. Moreover, the CE separation mode of analytes can be easily changed without difficult procedures by replacing the BGE. The major CE disadvantages in carbohydrate analysis are the lack of molecular charge for non-acidic sugars, easily ionizable or chromophore groups and the difficulty involved in its coupling with MS technology. However, in the last years, most of these issues have been overcome thanks to the development of new detection methods (photo-oxidation) or derivatization procedures. Moreover, different on-line, orthogonal or microfluidic methods regarding CE have been achieved. CE technology has surely high potential to become one of the election methods for carbohydrate analysis and further research should be carried forward to consolidate and spread its capacities and applications.

Acknowledgments

This review partially derives from the PRIN 2012 National Research Program project funded by MIUR, Ministero dell'Istruzione, dell'Università e della Ricerca, Prot. 20122EK9SZ_002, entitled "Comprehensive approach to mucopolysaccharidoses: application of highly specific methods for neonatal diagnosis and assessment of therapeutic efficacy in patients and in experimental animals".

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LEGEND TO FIGURES

Figure 1. Structure of the most common monosaccharides commonly found in more complex carbohydrates such as oligosaccharides and polysaccharides. IUPAC name: GlcNAc, β-D-(acetylamino)-2-deoxy-glucopyranose; GalNAc, 2-(acetylamino)-2-deoxy-D-galactose.

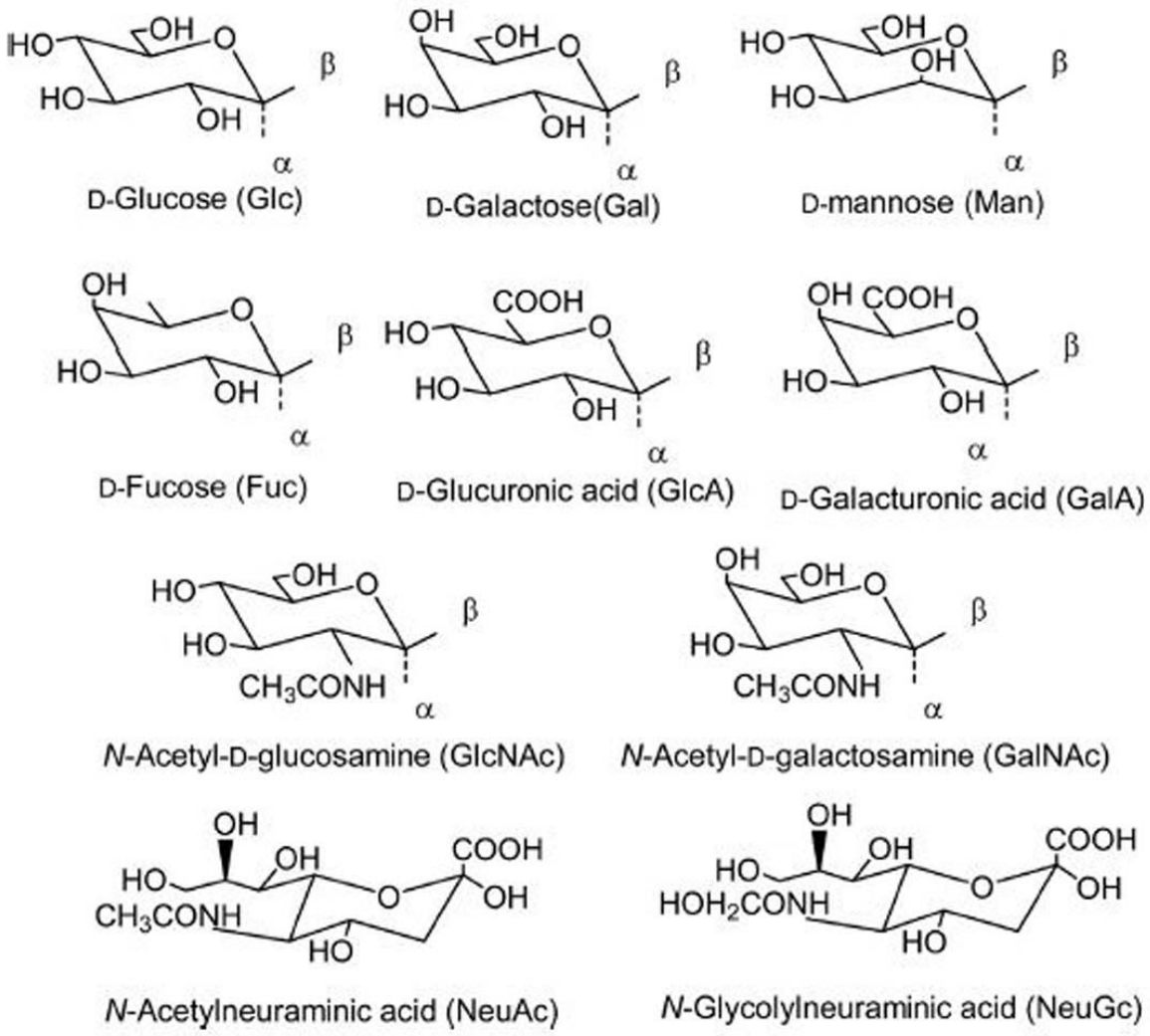

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Figure 2. Common labeling reagents for reductive amination of carbohydrates. AP, 2-aminopyridine; 2-ABA, 2-aminobenzamide; 3-ABA, 3-aminobenzamide; AA, 2-aminobenzoic acid; APTS, 8-aminopyrene-1,3,6-trisulfonic acid; ANTS, 8-aminonaphtalene-1,3,6-trisulfonic acid; PMP, 3-methyl-1-phenyl-5-pyrazolone; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; ABEE, 4-aminobenzoic acid ethyl ester; AMC, 7-amino-4-methylcoumarin; AMAC, 2-aminoacridone; ABN, aminobenzonitrile; ANDS, 7-aminonaphthalene-1,3-disulfonic acid; 6-AQ, 6-aminoquinoline.

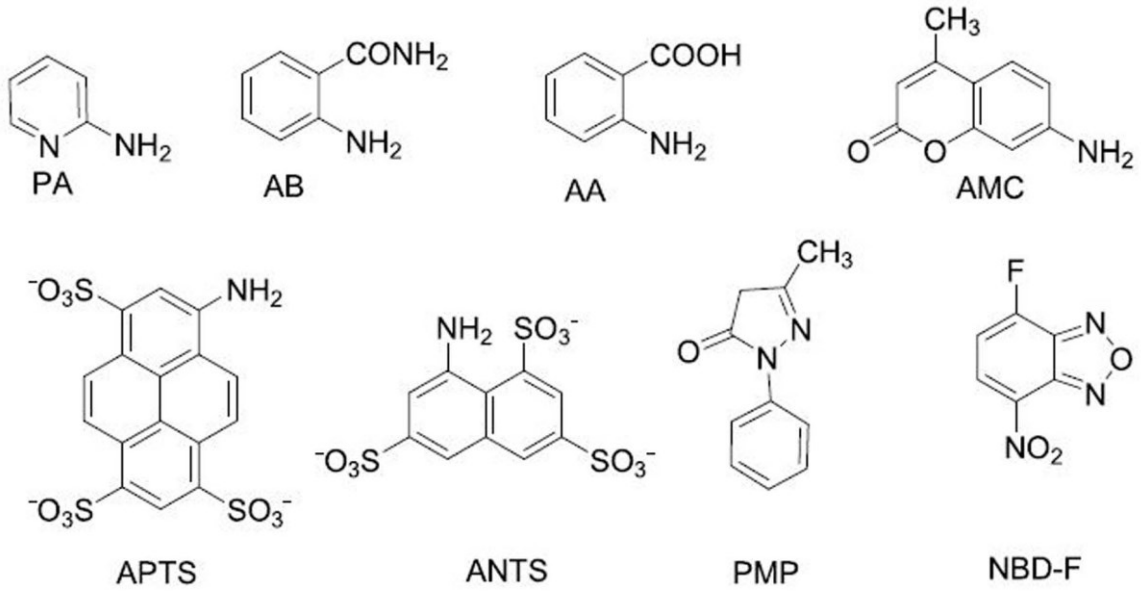

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Figure 3. Separation of ten PMP-labeled standard monosaccharides by CZE. Analytical conditions are: fused-capillary 58.5 cm (48.5 cm to the detector) × 50 μm i.d., 175 mM borate buffer pH 11.0, applied voltage 20 kV, capillary temperature 25°C and UV detection at 245 nm. Peaks: 1, xylose; 2, arabinose; 3, glucose; 4, ribose; 5, rhamnose; 6, fucose; 7, galactose; 8, mannose; 9, glucuronic acid; 10, galacturonic acid. Reprinted with permission from Reference [57].

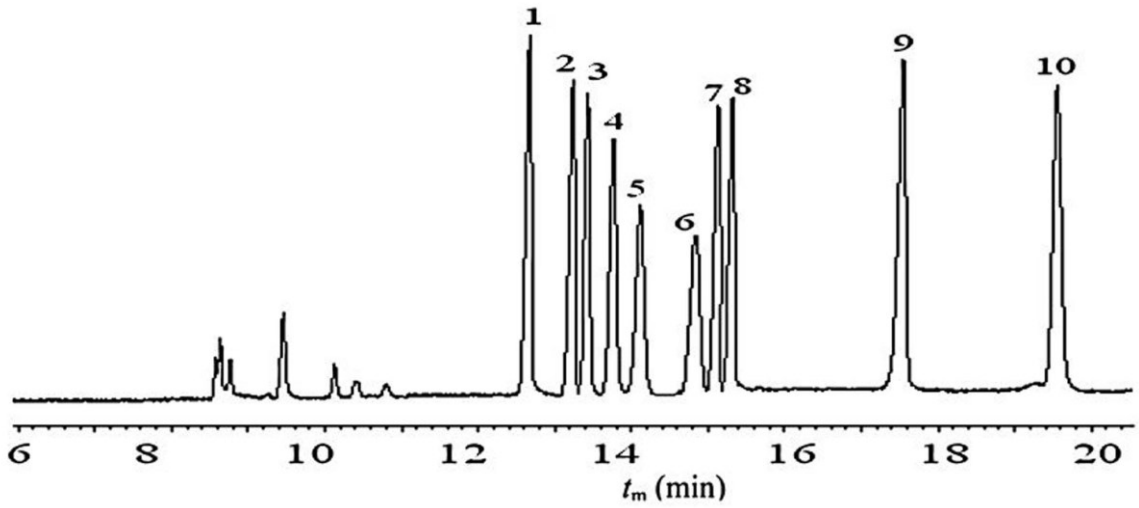

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Figure 4. CE-C4D separation of a mixture of saccharides. Optimized conditions are: BGE 75 mM NaOH pH 12.81, capillary i.d. 5 µm, total length 32.6 cm, length to the detector 18.3 cm. Hydrodynamic injection at a pressure of 50 mbar for 80 s. Voltage, +15 kV, current 1.6 µA. Sample concentration, 100 mg/L in 50% v/v acetonitrile. Peak identification: 1, sucrose; 2, lactose; 3, galactose; 4, glucose; 5, mannose; 6, fructose and 7, ribose. Reprinted with permission from Reference [39].

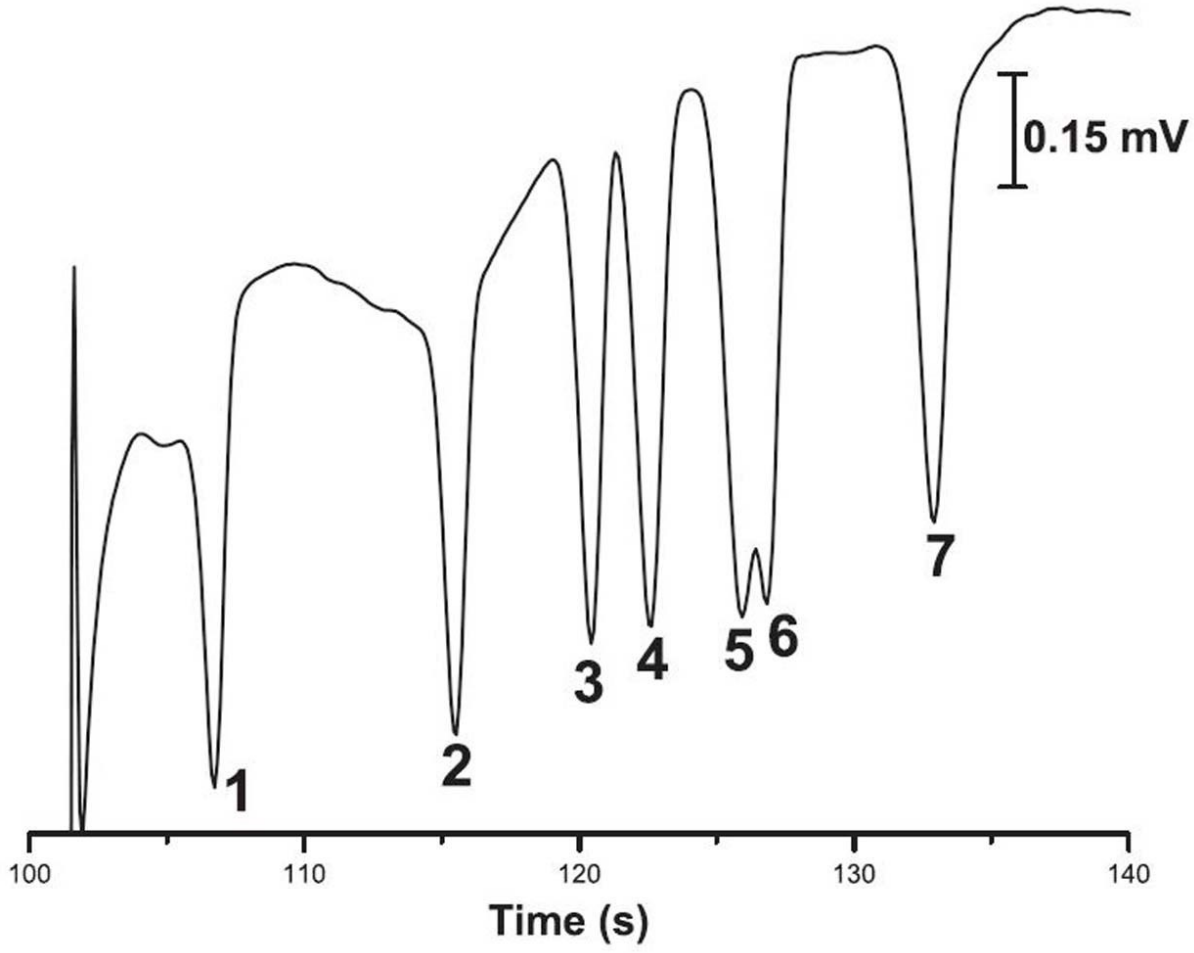

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Figure 5. CE separation of AMAC-labeled ΔUA-disaccharides from different GAGs. Optimized conditions are: 25°C, pressure injection of 50 mbar x 5 s at reversed polarity, 20 kV separation voltage using 50 mM phosphate buffer pH 3.5. Injection volume is 5 nL, and concentration of solution is 5 ng/L. The 17 standard disaccharides from GAGs are as follows: 1, TriSCS; 2, TriSHS; 3, SDCS; 4, SBCS; 5, SECS; 6, 2S6SHS; 7, NS2SHS; 8, NS6SHS; 9, 2SCS; 10, 6SCS; 11, 4SCS; 12, 2SHS; 13, 6SHS; 14, NSHS; 15, 0SHS; 16, 0SCS; 17, 0SHA (for structure see the original publication). IS, Internal Standard. RFU, relative fluorescence units. Reprinted with permission from Reference [71].

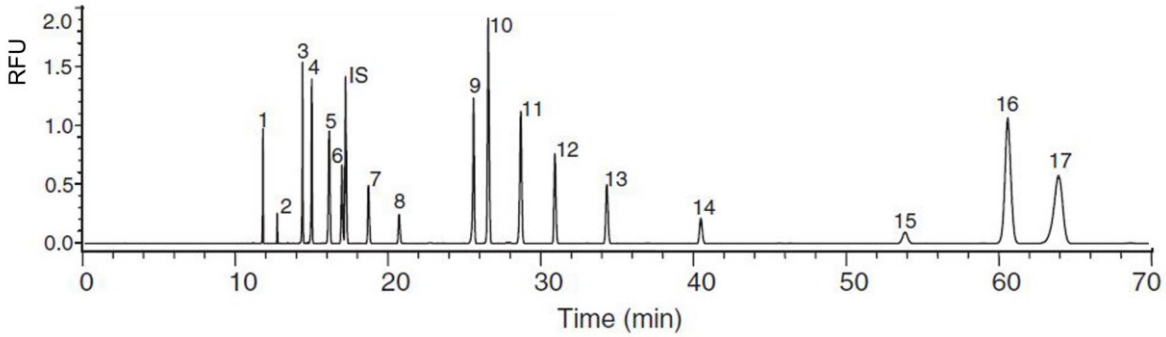

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Figure 6. Electropherograms showing the separation of the APTS-labelled malto-oligosaccharide ladder standard. The G numbers indicate the glucose residues of the ladder oligosaccharide (G2, G3, …, Gn corresponding to disaccharide, trisaccharide, …, n-oligosaccharide, respectively). CE was performed using a Beckman P/ACE MDQ system equipped with a 488-nm argon ion laser module. The compounds were separated in neutrally coated NCHO capillaries with a 50 µm i.d., a 60.2 cm total length and a 50 cm distance to the detector. The capillary cartridge was maintained at 20 °C using liquid coolant. All of the new capillaries were conditioned with deionized water for 10 minutes and running buffer for 2 minutes. Samples were injected at a pressure of 0.5 psi for 3 seconds. A separation voltage of 30 kV was applied with reverse polarity, which resulted in an electrophoretic current of 14 µA. Modified with permission from Reference [74].

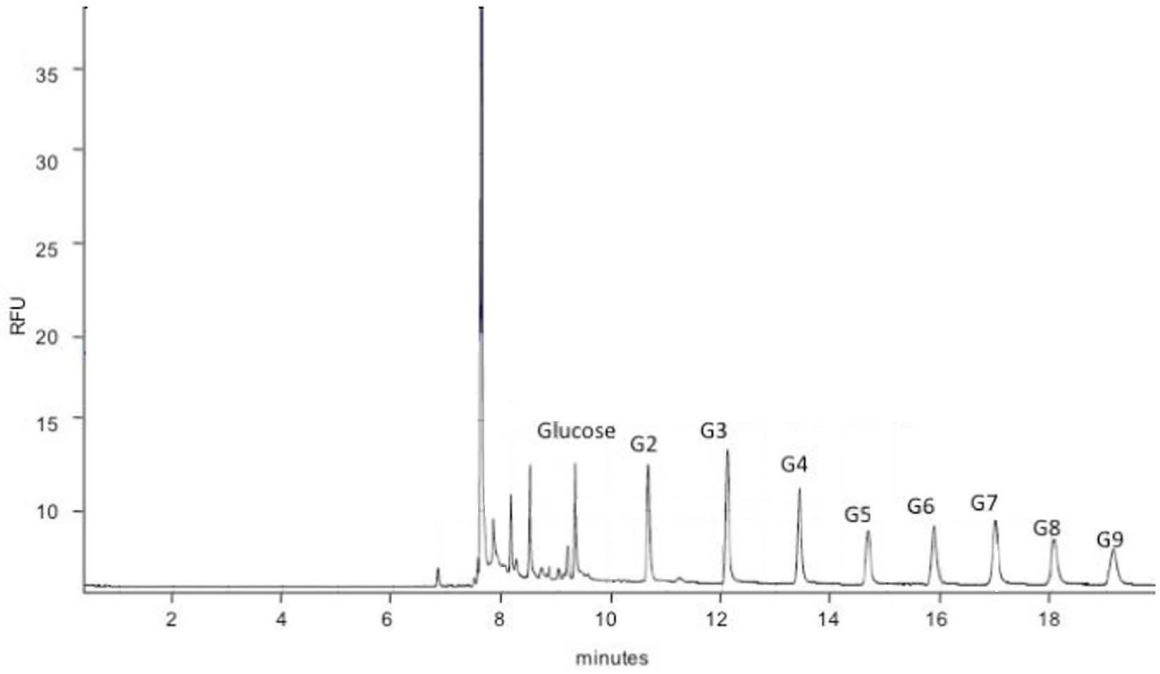

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Figure 7. CE separation of chito-oligosaccharides having a different degree of polymerization (labeled 1-6) performed using a high voltage supplier, a coated capillary column of 80 cm × 50 μm i.d. (55 cm effective length) and a UV-VIS detector. The coated capillary was conditioned with the crystal violet acidic solution for 5 min at high pressure. Sample injection was performed at a low pressure of 4 kPa for 3 s. The separation voltage and detection wavelength were adjusted to −15 kV and 540 nm, respectively. Reprinted with permission from Reference [76].

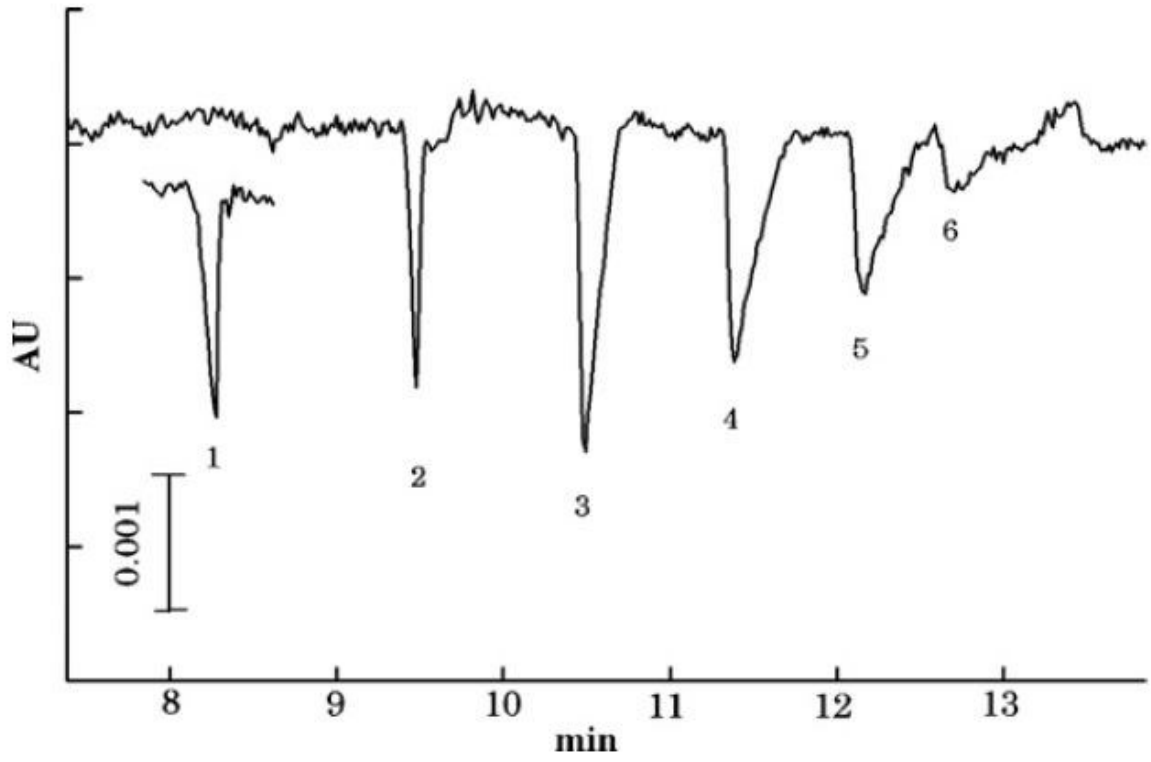

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Figure 8. Electropherogram of a standard mixture of 15 carbohydrates. Bare-fused silica capillary of 50 µm (id) × 60 cm (detection at 51.5 cm) was used. BGE was 95 mM NaOH (calculated pH, 12.98), 123 mM NaCl containing 0.1 g/L HDMB (hexadimethrine bromide). Temperature was set at 25°C, applied voltage was -13 kV, hydrodynamic injection was performed at 50 mbars for 5 s, UV detection was at 270 nm. 1, ribose; 2, xylose; 3, fructose; 4, mannose; 5, glucose; 6, galactose; 7, maltose; 8, cellobiose; 9, lactose; 10, cellotriose; 11, cellotetraose; 12, cellopentaose; 13, cellohexaose; 14, celloheptaose; 15, sucrose. Reprinted with permission from Reference [79].