

# Oligosaccharides and Glycan Separation via Capillary Electrophoresis Coupled with Mass Spectroscopy

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**Abstract:** After 2014, capillary electrophoresis (Fig. 1) has become an efficient method for carbohydrate evaluation. The methodology features a large resolution capable of distinguishing carbohydrates by the ratio of charge to scale. These primary features heavily focus on N-glycans, which seem to be extremely relevant for biological therapeutics and biomarker researches. Innovations in tools used during molecular or structural recognition of N-glycan involve time-indexing of migration and lectin profiling, also exoglycosidase along with mass spectrometry. Techniques of capillary electrophoresis have been formed that are able to separate glycans with the same sequence of simple sugars similar to other instruments but it can detect different positional isomers, and also specify whether alpha or beta is linked to monosaccharides comprising glycans. Based on the significant accomplishments of capillary electrophoresis to glycoscience from 2014 to the present, as addressed in this study, groundbreaking, evolving techniques in the field are highlighted and the potential direction of the technique is predicted.

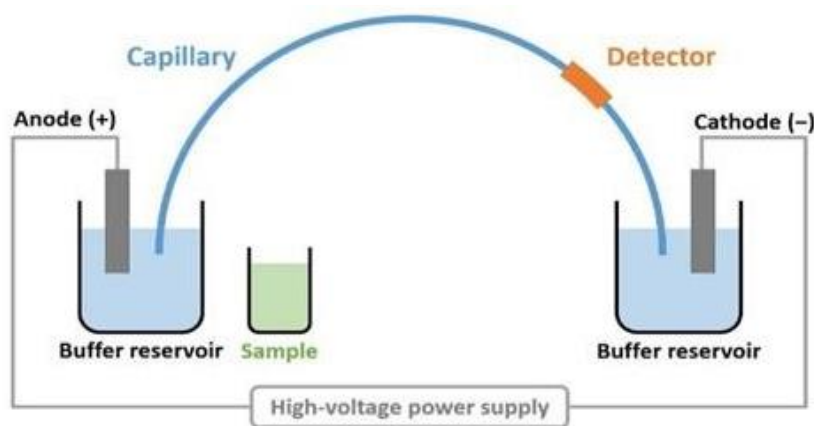


Fig. 1 Capillary electrophoresis working simple visual representation.

**Key words:** N-glycan, exoglycosides, migration time index, APTS, CE-MS systems.

## 1. Introduction

Carbohydrates also known as sugars are playing the main role in structuring, signaling, and providing energy. Their molecules are part of diseases, renewable energy, therapeutics, and food. Glycans' nature to participate in signaling helps the diagnosis and prognosis of different diseases and necessary therapeutic intervention. Science surrounds a wide

range of usage in chemistry such as operating glycoproteins in protein substrates or explaining the importance of glycosylation in processes like ligand-receptor binding and signaling in eukaryotic cells. Glycosylation regulates biological actions and intensively impacts the antibody effector functions. There is the probability to drastically ameliorate drug activity and performance but demands the availability of technologies, such as capillary electrophoresis (CE) in order to observe microheterogeneity throughout production.

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The attentiveness for CE in glycoscience usage is present by the latest great interest in written reviews in 2016 and 2017. This chemical review concentrates on the examination of fled oligosaccharides to emphasize the accelerated evolution in CE instrumentation and application. These achievements are beneficial increasing antibody pharmaceutical market, which assumed to top an annual global value of approximately \$125 billion in 2020.

Analytical methodologies are continuously adapting to coincide with the evolving challenges present in carbohydrate analysis because glycoscience researches require the presence of CE. This chemical review is a comprehensive explanation of the latest advances in capillary electrophoresis applications, highlighting findings from 2014 till 2017. Mainly, three primary suggestions are considered in this review to properly recognize glycan peaks with help of migration time indexing, mass spectrometry (MS), and enzyme or lectin profiling. There is a review of utilization of glycans in biomarker development, applications to the food industry, pathogen analyses, and characterization of therapeutics. The article winds up fields of emphasizing CE technologies that may have a great impact on the area of glycosciences with future improvement.

## 2. Fundamentals of Saccharides and CE

A short explanation is represented in this review focusing on important elements of CE. Several sources can supply supplementary details regarding the description of glycan structure and nomenclature, also an overview of different types of fragmentation patterns using methods of MS. For example, the location of the hydroxyl substituents is the difference between hexose saccharides: glucose, galactose, and mannose. Another important carbohydrate as an example of substituted hexose saccharides can be N-acetylneuraminic acid, N-acetyl glucosamine containing different hydroxyl substituents [11]. Deoxy-hexose saccharide can be fucose. In addition, the bonding orientation can differ

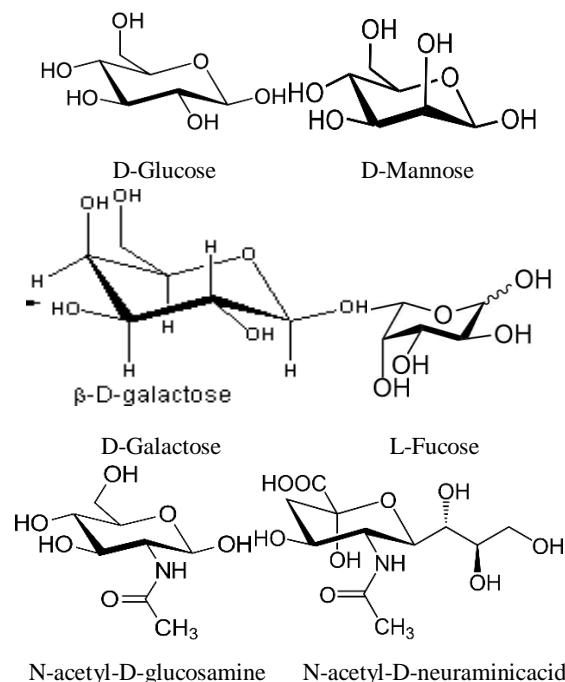


Fig. 2 Six different types of hexose.

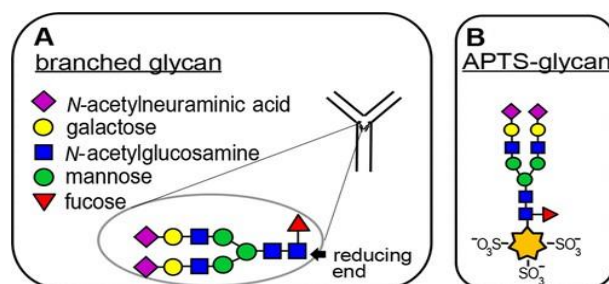


Fig. 3 Representation of linear glycan and its linkage to APTS.

alpha or beta, the situation of linkages is also different as shown in the trisaccharides portrayed in Fig. 2. Even though the familiar biosynthetic pathways for oligosaccharides restrict the diversity of structures that are physiologically pertinent. This results in an arrangement of various carbohydrate structures that are the reason that molecules play a weighty role in cell-signaling [13]. This encompasses linear polysaccharides produced of periodic repeating disaccharides which are glycosaminoglycans also known as mucopolysaccharides. This type of polysugars helps taking a big part in living organism processes like motor function, anticoagulation, and rapid cell growth. They are created with famous molecular categories such as heparin, hyaluronic acid,

chondroitin, and keratin sulfate [14]. N-glycans range by the existence of galactose, extra branching, or bisecting N-acetyl glucosamine, fucose, sialic acid capping, and other modifications: poly-lactosamine capping (Fig. 3). Despite N-glycans being part of one class of carbohydrates, glycoscience focuses on their structural representation while CE analysis, the reason is that they pose post-translational modifications with strong suggestions in functioning physiology. All these help N-glycans to be described for biomarker discovery and utilization of biological therapeutics. First great example, deeply discussed a charged tag which is very compatible with CE on the N-glycan structure.

Capillary zone electrophoresis separations are the perfect method for biomolecular separations, the method is dependent on conveying in an electric field. The two fundamental mechanisms play an important role in operating CE: Electroosmotic flow (EOF) and electrophoretic mobility. EOF keeps the bulk flow of the electrolytes. There is bare fused silica in which capillary EOF transfers from the specific anode to cathode, which happens because of the surface charge of the capillary (negative) and the appearance of the electric field. The first part—electrophoretic transferring is a function of charge/size ratio, in addition, the analyte is attached to the anode or cathode, the size of the molecule defines the rapidness of the conveying process [14]. The methods of separations of carbohydrates by Capillary Electrophoresis are distinctive because most of saccharides that are being analyzed are uncharged, excluding glycans. The main reason stated in the research was that their components contain acidic sugars e.g. N-acetylneuraminic acid or iduronic acid. Furthermore, sugar molecules have a deficiency of a chromophore and are unable to perceive with absorbance in the represented UV range. These astonishing features are highlighted by labeling the carbohydrate molecules with a fluorophore. The strategy says that large amount of fluorophores are

firstly charged in advance of the CE experiment, which is because it guarantees the migration of the labeled carbohydrate towards the electric field. The most known fluorescent label is abbreviated as APTS meaning 8-aminopyrene-1,3,6-trisulfonate, one of the first dyes used for oligosaccharides during CE analysis. While tagged glycans become negatively charged, the segregation of the mixture is altered to count on electrophoretic transport in order to quicken up and underline differences in charge/size ratio. For instance, the experiment proved that sialylated glycans tagged with APTS become much more negatively charged, creating a consequence—accelerated migration. Mathematically, the easiest representation of the relationship between molecular weight and molecular size of the certain compound gives the formula for electrophoretic mobility being the function of molecular weight:

$$\mu_{eph} = \frac{q}{6\pi\eta r}$$

where  $q$  is the representation of charge,  $\eta$ —viscosity of the electrolyte, and eventually,  $r$  is the hydrodynamic radius of the molecule. In addition, viscosity can take part in frictional drag that is proved with the equation provided above and it is an efficient methodology to reach the high resolution to separate oligosaccharide isomers, accordingly. There are additives to the background electrolyte to enhance the resolution of the CE performance.

Many types of analytical instrumentation are modified and used for the separation of glycans, some of them are chromatography, MS, lectin arrays, ion mobility, and lastly CE discovering new approach distinguishes glycans and their isomers. In 2014, the scientists Huffman et al. stated a comprehensive judgment of CE application but in relation to (1) reversed-phase chromatography coupled along with fluorescence detection matrix, (2) laser desorption ionization—also known as time-of-flight MS vs. liquid chromatography coupled with electrospray ionization-MS. All these methods were checked by

counting N-glycans according to IgG molecules but specifically in plasma samples, approximately 1,201 samples were tested. These trials managed by the authors proved the unique advantages of CE.

First trials provided the proof of the high resolution that permitted isomer separation and bonding analysis. The second method contained quantification with CE, the liquid chromatography method coupled with fluorescence detection. The third experiment utilized equipment with 16-capillary arrays, the scientists taking part in these trials achieved priceless methodology with the usage of both liquid chromatography and CE. The authors presented the cost of the experiments and benefits of CE, also stated minor differences between this instrumentation in precision, and separation performance, and error accuracy. The information about detailed separation-based methods of N-glycan in the large sample was assessed resulting in the glycosylation process to be present on IgG antibodies.

### 3. Carbohydrate Derivatization

Carbohydrate derivatization is the first step to start the analysis, so glycan tagging with APTS was maintained as the method for CE separations. The preferred strategy is glycan labeling with APTS with the help of amination which also consists of Michael addition or otherwise, hydrazide or oxime formation. In general, taking advantage of catalysts is necessary in order to conduct automated parallel processing because it decreases the hydrogen cyanide generation using sodium cyanoborohydride in the acid [15]. Even though there are many other methods, the usage of APTS is a common strategy for glycan separation analysis by Capillary electrophoresis because of few beneficial characteristics. First, glycans with APTS are 40-fold higher than unconjugated APTS [15]. Furthermore, the background intrusion from biomatrices must be decreased, the reason is that excitation wavelength of labeling-APTS falls the visible range ( $\lambda_{\text{ex}} = 488 \text{ nm}$ ). But the other dyes used in the

experiments can be excited in the ultraviolet range, for instance 2-amino benzoic acid at  $\lambda_{\text{ex}} = 325 \text{ nm}$ .

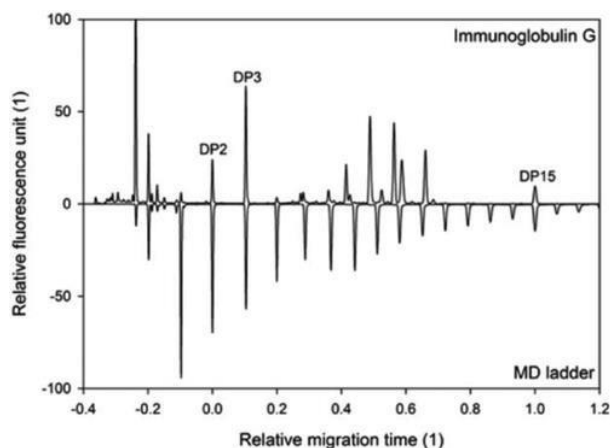
### 4. Methods to Identify Glycans and Their Isomers

The variety of methodology gives scientists several strategies such as calibration of migration time with ladders and standards, MS analysis, exoglycosidase, and lectin reactions in Capillary electrophoresis. CE uses a migration time index in order to define different types of glycans. In short, analyte migration must be described as a size ladder created by a homologous series of linear glucose polymer [1]. Analyte possessing mobility is represented as the total amount of electrophoretic mobility plus bulk EOF. Additionally, there are factors that assist the operation of EOF: (1) adsorption of biomolecules to the surface, (2) changes in the pH (because of electrolysis in the anodic and cathodic reservoir), (3) a change in the ionic strength of the background electrolyte.

Usually, a glycan ladder is important for assuming the relative size of the sample utilizing the migration time of the standard oligomer and the result showed that the analytes referred to experimentally analyzed changes in ladder migration connecting to helical turn specifically happening in malto-oligosaccharides [3]. This procedure may occur when the polymerization degree of the structures reaches 7.85 and beyond that value. Nevertheless, as shown in Fig. 4 this strategy is simplified with co-injected size standards in order to precisely guide proper migration time without any problems and to prevent comigration of the sample. The procedure has straightforward representation as an equation, known as GU value:

$$GU_x = G_N + \frac{t_x - t_N}{t_{N+1} - t_N}$$

In this equation,  $G_N$  is the number of maltose molecules in the neighboring malto-oligosaccharide ladder peak, but  $t_x$ ,  $t_N$ , and  $t_{N+1}$  are different migration times of the target glycan. Thus,  $N$  and  $N + 1$  represent number of malto-oligomers [1].

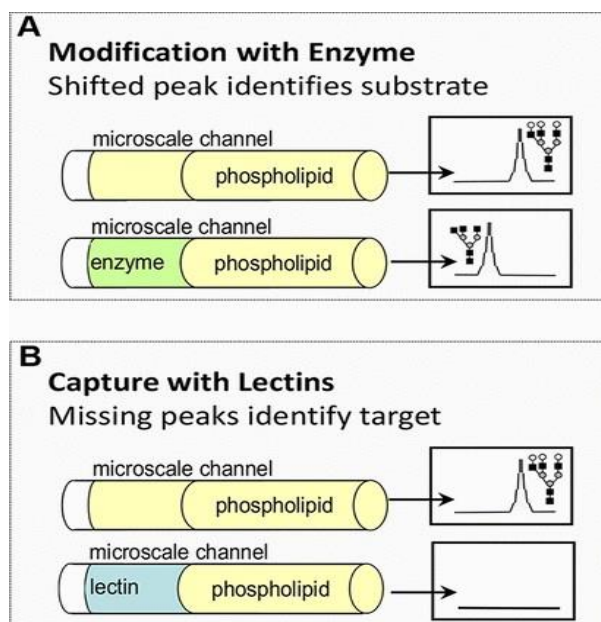


**Fig. 4** Electropherogram of standards maltose, maltotriose, and maltopentadecaose, accordingly plus APTS labeled N-linked glycan freed specifically from human immunoglobulin G.

GU values are used for assessing the effect of temperature on the hydrodynamic size of N-glycans (branched and linear) utilizing CE. The observation results proved that GU values drastically impacted on function because of the temperature change between 20-50 °C utilized background electrolytes saturated with the presence of linear polymer additives or without the presence of them. Another method consists of identification with enzymes called Exoglycosidases. They tend to cleave residue of an oligosaccharide but specifically attract the nonreducing end of an analyzed glycan, or oligosaccharide (see Fig. 4). As it is proved, those enzymes have the characteristic of being highly selective according to the shape and chemical nature of the substrate or the molecule, therefore they are searching for specific oligosaccharide monomers [6]. Furthermore, enzymes can have selectivity corresponding to the linkage orientation such as ortho position of the carbon-carbon linkage (e.g.  $\alpha_{2-3}$  vs.  $\alpha_{2-6}$  sialic acid as shown in Fig. 4). There are examples of galactosidases—the enzymes that cleave the very last galactose residues from an N-glycan resulting in the possibility of elucidation of Michaelis-Menten constant helped authors to develop the strategy of identification for in-line enzymatic sequences in N-glycan isomers. These experiments were achieved

the particular sequence and bonding of oligosaccharides [25]. Traditionally, the above-described reactions are conducted overnight with intense usage of fluorescent detection feature of APTS that tagged N-glycans; this made the approach very appropriate for nanomolar N-glycan samples [1]. During the analysis, the substrate goes through the nanogel, and most importantly the incubation period was managed electrophoretically, this occurs when voltage goes in forward and then goes to reverse polarity to let the analyte rotate and cycle through the column containing the specific enzyme. The study also discovered the relevant amount of concentration of the enzyme that plays a big role in an experiment with CE.

Another protein can be used as a method to identify certain glycans—Lectins are proteins that tend to spot structural differences like neuraminic acids or mannose structural appearance. Lectins' specificity ranges between several types of them: for instance, sambucus nigra lectin can attach itself to  $\alpha_{2-6}$  neuraminic acids when another one maackia amurensis lectin has the possibility to attract  $\alpha_{2-3}$  neuraminic acids [10]. Another available process can fill up the whole capillary column with protein-lectin then it encloses



**Fig. 5** Pictuere A portrays a galactosylated glycan.

the entire to a zone within the capillary utilizing reversible nanogel. The method enabled the identification and separation of Gal  $\beta$ 1-3GlcNAc from Gal $\beta$ 1-4GlcNAc linkages in N-glycans [1]. The benefit of this method can be defined as an amalgamation of lectins and enzymes that can generate unique specificity. In the article written by Holland et al., there were examples of an enzyme connecting to only  $\beta$ 1-4 galactose coupled with erythrina cristagalli lectin. All these were proved by MS analysis (Fig. 5).

## 5. Application of Glycans

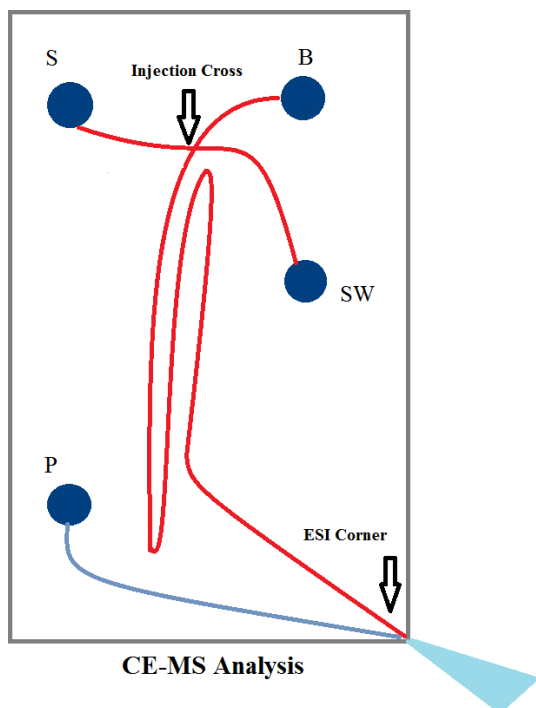
The chemical review states that capillary electrophoresis is a separation-based assay for proper identification and quantification of carbohydrates. Usually, the strategy was developed for addressing obstacles technologies to precisely measure specific molecules in the mixture [8]. Afterwards, it was transferred into a permanent methodology for detecting important industrial or clinical processes [4]. Some specific areas about activation of glycan separation were described in this review, especially from 2014 to 2017. These studies can be ascribed to distinguish power of CE and the capability to solve complicated mixtures' compositions. For example, how to segregate positional isomers according to differences in hydrodynamic volume. Glycan separations are commonly utilized in biomarker discoveries, autoimmune disorders, cancer, biomarkers for cell-signaling, application to biological therapeutics, glycosaminoglycans separation and etc.

## 6. Technique Necessity and Future Applications

The development of Capillary Electrophoresis provided scientists with many types of applications because of its importance in glycosciences, engineers started to work on this equipment too. The rapid discoveries in glycosylation and high demand in CE conditioned a prominent role in this area of science.

There are several aspects impacting the foundation of new methodology in the separation of glycans: 1st—area drastically affected the development of a tool to complement characterization and quantification of position linkages of certain isomers to detonate biological therapeutics as a demanding field [14]. Noteworthy progression was a result of migration time databases with help of oligosaccharides, polysaccharides, or DNA ladders that created unparalleled accuracy in time-based peak identification during CE separations. This large bunch of samples contributes to manufacturers of biologics to have complete power to easily detect changes in glycosylation happening while changing the cell culture conditions [7]. In this report, there is the demonstration of other methods as well, which utilizes the main differences in mobilities in order to differentiate linkage positions in the isomers or tack the MS to recognize peaks that can migrate thought CE. “These include the means to decouple the separation current from the electrospray process while avoiding dilution associated with the transfer of the nanoliter flow rates in the electrophoresis capillary” [1]. Developments in the compatibility of two beneficial instruments CE and MS, coupled with innovative ionization strategies and more pliability in merging monitor modalities, will grow the popularity of CE in biomarker invention.

The latest highlights on separations of glycopeptides and glycoproteins with the utilization of coupled instrumentation—CE-MS systems changed glycosylation process's detection and made it easier to know the location of glycosylation helping to conduct powerful analysis in biomarkers and test their sensitivity. The unity of CE characteristics—a migration database and most importantly structural determination managed to lead to a high level of precision in identification methods obtaining better results [27]. In Fig. 6, it is evident that a microfluidic electrophoresis device is specifically produced for MS analysis. This device focuses on glycoprotein and glycopeptide separations



**Fig. 6** Schematic representation of microfluidic chip for capillary electrophoresis-electrospray coupled with ionization-mass spectrometry. It contains sample reservoir indicated as S, electrolyte reservoir B, electrospray ionization pump P, and sample waste SW.

and their peaks' analyses. MS relevant buffers were chosen for intense segregation of glycoproteins, glycopeptides, oligosaccharides, and monosaccharides. The sugars experimented with tandem of mass labeling, clearly signified the structural recognition and quantification discerned with advanced throughput and automated benchtop instrumentations.

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