Resolution of structural isomers of sialylated oligosaccharides by capillary electrophoresis

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Abstract

The resolution of structural isomers in mixtures of oligosaccharides is often challenging. Capillary electrophoresis was employed to separate three sets of structural isomers of sialylated oligosaccharides found in human milk and bovine colostrum. Different running buffers were necessary to achieve optimal baseline resolution. To resolve 3′- and 6′-sialyllactoses, 0.2 M aqueous sodium phosphate containing 40% methanol as an organic modifier was used as a running buffer. To resolve 3′- and 6′-sialyllactosamines, 0.4 M aqueous sodium phosphate without organic modifier was used. Baseline resolution of sialyllacto-N-tetraose-a and -b and sialyllacto-N-*neo*tetraose-c was achieved with a 0.4 M Tris–HCl buffer containing 250 mM sodium dodecyl sulfate and 10% methanol as the organic modifier. Thus, each of these sets of structural isomers of sialylated oligosaccharides required a unique running buffer with respect to buffer type, concentration, pH, presence of organic modifiers, and surfactants. Similar electrophoresis conditions may be useful for resolving and analyzing other structural isomers of acidic oligosaccharides by capillary electrophoresis. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Oligosaccharides containing *N*-acetyl (or *N*-glycolyl) neuraminic acid, i.e., sialylated oligosaccharides, are important components of glycoproteins and glycolipids [1,2]. In nature, sialylated oligosaccharides often occur as homologous series, with incremental differences in composition, and as structural isomers with subtle differences in monosaccharide sequence and glycosyl linkage and, possibly, with presence or absence of molecular branching. A large number of such sialylated oligosaccharides occur in human milk and bovine colostrum, where they may have important biological functions [3–5]. Major structural isomers of these acidic oligosaccharides in human milk are 3′- and 6′-sialyllactoses and the sialyllacto-*N*-tetraoses; appreciable amounts of 3′- and 6′-sialyllactosamines are found in human urine and in bovine colostrum. Studies of their biological activities, a growing field of interest, require methods for the efficient separation and quantitation of structural isomers. In the past this has usually been achieved by high-performance liquid

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chromatography [6,7] or high-performance anion-exchange chromatography [8–10].

Analysis by capillary electrophoresis (CE) can be more sensitive, reproducible, and facile than those techniques. We previously showed that CE, with direct detection at 205 nm, offered a convenient, rapid, sensitive method for the resolution and detection of the 11 predominant sialylated oligosaccharides of human milk [11], including structural isomers. However, in order to obtain these results in a single run time of less than 60 min, it was necessary to choose conditions that balanced the ability to resolve isomers with the ability to separate molecules of diverse structures and sizes. Thus, baseline resolution of each of the groups of isomers was not achieved. Because related structural isomers can have quite different biological activities, we wished to devise methods for optimum resolution within three groups of structural isomers. Two of these groups are significant components of the 11 predominant human milk acidic oligosaccharides: the sialyllactoses (3′- and 6′-sialyllactose) and the group of three pentasaccharides consisting of two sialyllacto-*N*-tetraoses (-a and -b) plus sialyllacto-*N-neo*tetraose-c. The third group is the sialyllactosamines (3′- and 6′-sialyllactosamine) present in bovine colostrum.

Our goal was to optimize the separations of sialyllactoses, sialyllactosamines, and sialyllacto-*N*-tetraoses while exploring relationships between molecular structure and electrophoretic migration. The rate of migration of these compounds in CE is the net result of two opposing effects – electrophoresis and electroosmotic flow. Optimal conditions for separations were obtained by systematically varying running buffer components that influence these two effects, including the buffer composition, concentration, pH, and organic modifiers.

2. Experimental

2.1. Apparatus

CE was performed with a Hewlett-Packard ^{3D}CE apparatus. The capillary dimensions were: 56 cm effective length×50 μm I.D. with extended light path geometry. Detection was by absorbance at 205 nm, and voltage was 30 kV positive, i.e., sample was loaded at the anode and detected at the cathode. Temperature was maintained at 25°C.

2.2. Materials

Authentic standards of 3′- and 6′-sialyllactose and sialyllacto-*N*-tetraose-a were obtained from Sigma (St. Louis, MO, USA), 3′- and 6′-sialyllactosamine and sialyllacto-*N-neo*tetraose-c from Oxford GlycoSystems (Bedford, MA, USA), and sialyllacto-*N*-tetraose-b from Dextra (Reading, UK). Oligosaccharides from human milk were prepared as described by Shen et al. [11].

2.3. CE analyses

Sample was loaded into the capillary at 50 mbar for 3.5 s. The concentrations of the oligosaccharide solution were in the range of 200 mg/l for each of the sialyllactoses and sialyllactosamines, and 70 mg/ml for each of the sialyllactotetraoses. Initial studies tested the efficacy of a wide variety of buffer systems with varying pH, ionic strength, and organic modifiers (including both alcohols and surfactants); the most promising systems were determined to be the following: CE of 3′-sialyllactose and 6′-sialyllactose was performed with a running buffer consisting of 0.2 *M* aqueous sodium phosphate (pH 7.2) containing up to 40% (v/v) methanol or up to 30% (v/v) 2-propanol as an organic modifier. CE of 3′-sialyllactosamine and 6′-sialyllactosamine was performed with a running buffer containing 0.05–0.4 *M* sodium phosphate (pH 7.2), or 0.4 *M* Tris–HCl (pH 7.6 or 8.1). For the CE of sialyllacto-*N*-tetraose-a and -b and sialyllacto-*N-neo*tetraose-c, four different running buffers were systematically investigated: (a) 0.2–0.6 *M* sodium phosphate (pH 7.2–7.6) without or with methanol (up to 40%, v/v) as an organic modifier, (b) 0.4 *M* Tris–HCl (pH 7.6) without or with methanol (10%, v/v) as an organic modifier, (c) 0.4 *M* sodium phosphate (pH 7.2) with varying amounts of sodium dodecyl sulfate (SDS) as a surfactant, without or with methanol (10%, v/v) as an organic modifier, and (d) 0.4 *M* Tris–HCl (pH 7.6 or 8.1) containing 250 m*M* SDS as a surfactant, without or with methanol (10%, v/v) as an organic modifier.

3. Results

Three sets of conditions were found necessary for the baseline resolution of these sialyllactoses, sialyllactosamines, and sialyllacto-*N*-tetraoses.

For sialyllactoses, variation of buffer components or pH did not improve resolution beyond that obtained with 0.2 *M* sodium phosphate (pH 7.2); only the addition of an organic modifier further improved the separation. Of the many modifiers tested, only methanol and 2-propanol were effective at resolving the two sialyllactoses. As shown in Fig. 1A, the sialyllactoses comigrated when run in 0.2 *M* sodium phosphate, pH 7.2, but were clearly separated by the addition of 20% methanol (Fig. 1B). Resolution of 3′- and 6′-sialyllactose was optimal at 40% methanol (Fig. 1D), and these conditions allowed good sensitivity. When 2-propanol was used, sensitivity deteriorated as the concentration was increased to the level (30%) necessary for optimal resolution (Fig. 1E).

The sialyllactosamines did not resolve when run in 0.1 *M* sodium phosphate buffer (Fig. 2A), and only partially resolved when run in 0.2 *M* sodium phosphate buffer (Fig. 2B). Resolution was improved by further increasing the buffer strength (Fig. 2C), which at 0.4 *M* fully resolved 3′- and 6′-sialyllactosamine while allowing adequate sensitivity and an acceptably short run time (Fig. 2D).

The three sialylated pentasaccharides (sialyllacto-*N*-tetraose-a, -b, and sialyllacto-*N-neo*tetraose-c), when combined and run in 0.2 *M* sodium phosphate buffer, resolved into only two peaks: one contained the branched b isomer, while the other contained the linear a and c isomers. The partial separation of these two peaks was improved by increasing the strength of the sodium phosphate from 0.2 to 0.6 *M* and (as in the case of the two sialyllactoses) by adding methanol (10% to 40%) as an organic modifier. However, this modification produced only partial resolution between the b isomer and the other two, which still comigrated as one peak (not shown). In a second approach, addition of the surfactant SDS (150 m*M*) to the running buffer also resulted in separation of sialyllacto-*N*-tetraose-b from the other two isomers, but again, no combination of conditions was found that would resolve sialyllacto-*N*-tetraose-a from sialyllacto-*N-neo*tetraose-c (not shown).
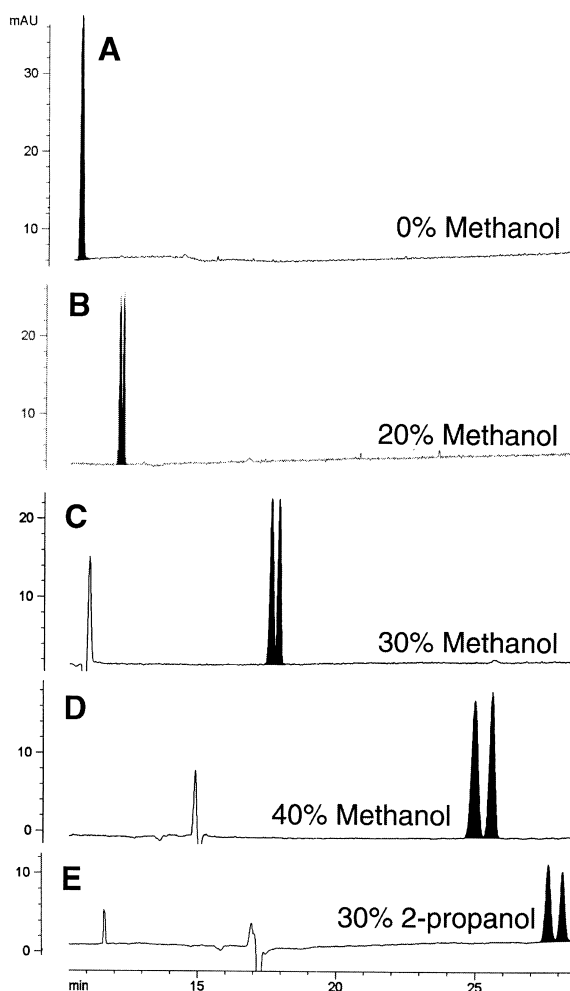

Fig. 1. Resolution of 3′-sialyllactose and 6′-sialyllactose with increasing concentrations of organic modifier. The running buffer consisted of 0.2 *M* sodium phosphate (pH 7.2) containing up to 40% (v/v) methanol (A–D), or up to 30% (v/v) 2-propanol (E) as an organic modifier. For each run, the capillary is loaded with 2 nl of water containing 400 pg of 3′-sialyllactose and 400 pg of 6′-sialyllactose.

Changing to a running buffer system of 0.4 *M* Tris–HCl, pH 7.6, resulted in slight resolution of the linear sialyllacto-*N*-tetraose-a and sialyllacto-*N-neo*tetraose-c (Fig. 3A). No additional resolution resulted from addition of methanol as organic modifier (Fig. 3B). However, a dramatic improvement was achieved by a combination of methanol (10%) and SDS (150 m*M*) (Fig. 3C), or by a higher concentration of SDS alone (250 m*M*) (Fig. 3D).
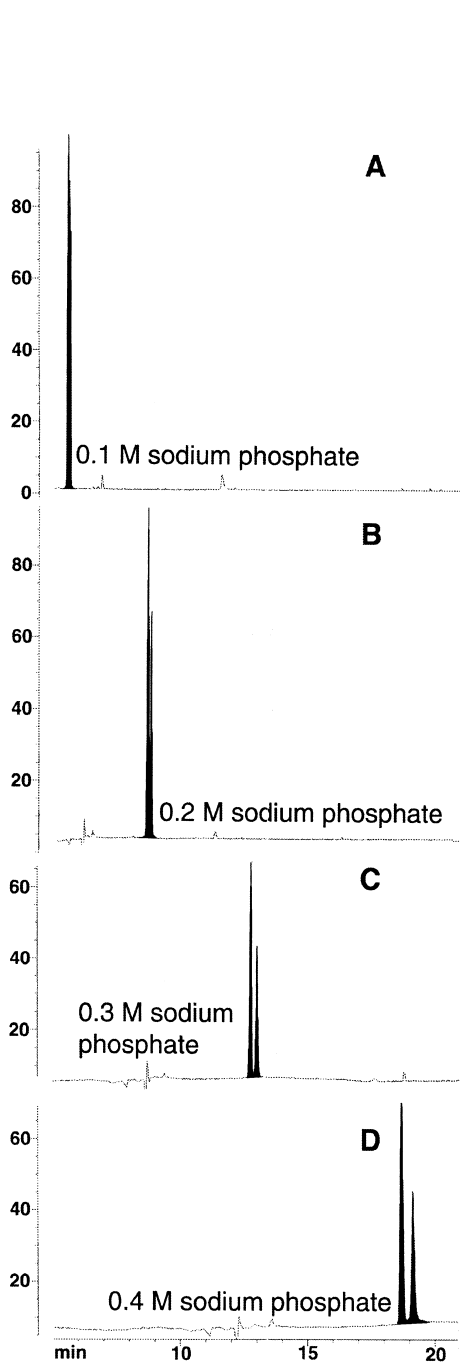
Fig. 2. (A–D) Resolution of 3′-sialyllactosamine and 6′-sialyllactosamine with increasing concentrations of sodium phosphate, pH 7.2, in running buffer. Running buffer contained 0.05–0.4 *M* sodium phosphate. For each run, the capillary is loaded with 2 nl of water containing 400 pg each of 3′-sialyllactosamine and 6′-sialyllactosamine.
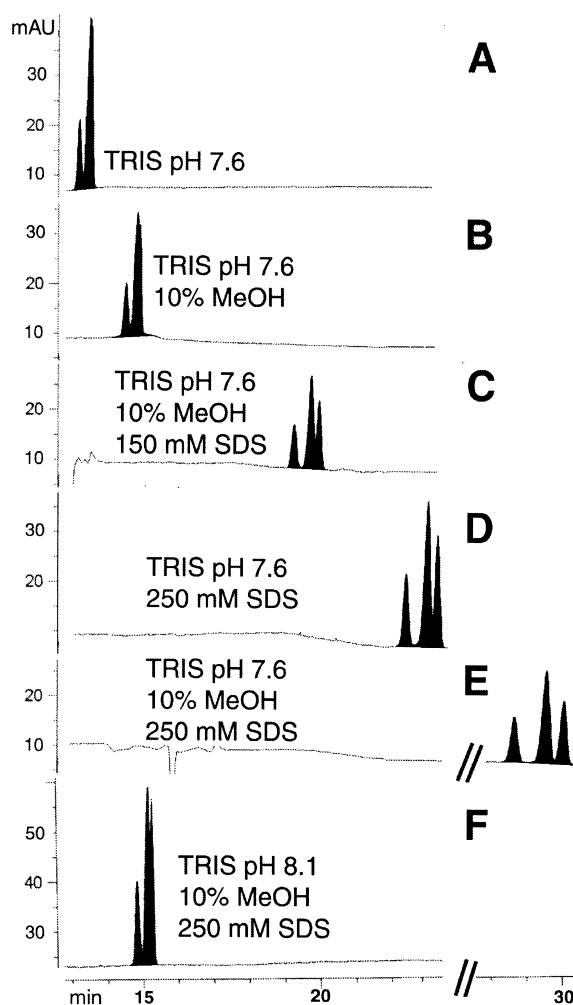

Fig. 3. Resolution of sialyllacto-*N*-tetraose-a, sialyllacto-*N*-tetraose-b, and sialyllacto-*N-neo*tetraose-c using a running buffer containing Tris–HCl, showing effect of modifying composition or pH of the running buffer. (A) The Tris–HCl buffer separates the b isomer and (unlike phosphate buffer) shows a slight separation of a and c isomers. (B) Addition of 10% methanol; (C) addition of 150 m*M* SDS plus methanol; (D) addition of 250 m*M* SDS without methanol; (E) addition of 250 m*M* SDS with 10% methanol; (F) pH changed from 7.6 to 8.1. For each run, the capillary is loaded with 2 nl of water containing 140 pg each of sialyllacto-*N*-tetraose-a, sialyllacto-*N*-tetraose-b, and sialyllacto-*N-neo*tetraose-c.

Ultimately, baseline resolution of the three sialylated pentasaccharides was achieved with 250 m*M* SDS and 10% methanol; under these conditions both running time and sensitivity were satisfactory (Fig. 3E). Interestingly, an increase in pH from 7.6 to 8.1

decreased resolution, especially of the a and c isomers (Fig. 3F). Thus optimal resolution of these three isomers required extensive evaluation of experimental parameters: the running buffer composition and concentration, the buffer pH, the presence, identity and concentration of an organic modifier, the presence of a surfactant and its concentration, and the appropriate combination of organic modifier and surfactant.

Clearly, the difficulty of resolving sialyllacto-*N*-tetraose-a and sialyllacto-*N-neo*tetraose-c, compared with the readily achieved resolution of sialyllacto-*N*-tetraose-b, results from isomer b having a branched structure, unlike the linear structures of isomers a and c. The two linear pentasaccharides, differing only in the terminal sialic acid (NANAα2,3 vs. NANAα2,6) and galactose (Galβ1,3 vs. Galβ1,4) linkages, comigrate under most conditions. Only under the specific conditions defined by the experimental parameters described above were we able to separate these two compounds.

The Tris–HCl system, so successful for separating the pentasaccharides, proved inferior to the phosphate running buffers for the two pairs of sialylated trisaccharides. As the running buffer for the sialyllactoses, 0.4 *M* Tris–HCl at pH 7.6 did not yield any separation and provided only minimal resolution in the presence of methanol. Although baseline resolution of the sialyllactosamines did occur using Tris–HCl under the same conditions, both the degree of separation and the sensitivity were inferior to the results obtained with 0.4 *M* sodium phosphate as running buffer.

When applied to oligosaccharides isolated from a human milk sample, a running buffer of 0.2 *M* sodium phosphate, pH 7.2, with 40% methanol resulted in good resolution and excellent separation of the 3′- and 6′-sialyllactose from the other milk components (Fig. 4). This figure also illustrates that running this sample in 0.4 *M* Tris–HCl, pH 7.6, with 250 m*M* SDS and 10% methanol resulted in a different order of elution of the milk oligosaccharides, with resolution of the three sialylated pentasaccharide isomers. Although complete baseline resolution of the three isomers was not seen in this sample, it is clear that the concentration of the sialyllacto-*N*-tetraose-a isomer was quite low relative to the other two. We have observed a large amount of
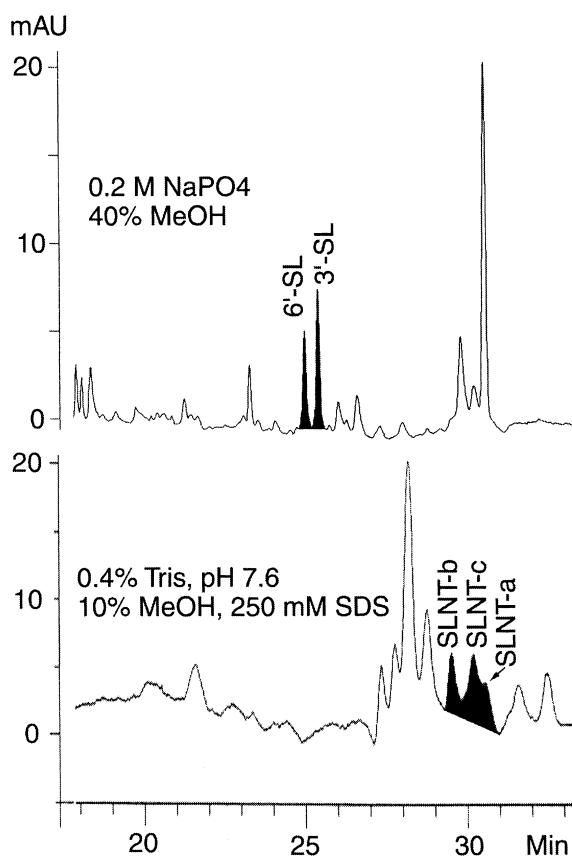

Fig. 4. Resolution of the sialyllactoses (SLs) and sialyllacto-*N*-tetraoses (SLNTs) of human milk. The use of 0.2 *M* sodium phosphate with 40% methanol as the running buffer allowed resolution of the sialyllactoses, while 0.4% Tris, pH 7.6, with 10% methanol and 250 m*M* SDS allowed sialyllacto-*N*-tetraose-a, -b, and sialyllacto-*N-neo*tetraose-c to be measured.

variation in the relative proportions and amounts of these sialylated pentasaccharide isomers in milk samples from different individuals (data not shown).

4. Discussion

Chromatographic resolution of structurally similar oligosaccharides, especially those containing chemically labile sialic acid residues, is a challenging problem [6–10]. CE combines some of the separation principles of column chromatography with those of other forms of electrophoresis; with this technique, we were able to efficiently resolve and

quantify 11 sialylated oligosaccharides, including structural isomers, in human milk [11]. However, those CE conditions were designed for the simultaneous analysis of all 11 oligosaccharides in milk samples from multiple populations, and the running conditions were selected as the best compromise between resolution and running time. The present study developed conditions for baseline resolution of specific sets of isomers within a 35-min run. Unique conditions were needed for each set of isomers. Because no general rule exists for predicting a priori how different species will be separated by electrophoresis, the investigation of the effects of various electrophoretic conditions on CE separations is a critical empirical task. Our selection of buffers was guided by the following general principles.

The pH of the running buffer is the most important parameter for changing the selectivity of CE. Separation by electrophoresis depends upon differing mobilities of the analytes, which are directly related to their size and net charge. The size of an ion depends upon its molecular mass and its degree of hydration; the degree of hydration varies with ionic strength and polarity of the solution. The net charge of an ion is a measure of its degree of ionization, which depends upon the pK value of its acidic or basic functional group and the pH of the running buffer [12].

Increases in salt concentration increase the polarity of the running buffer, and should therefore increase both electroosmotic flow and the electrophoretic mobility, as the same principles underlie both of these phenomena [12]. Increasing the ionic strength can improve efficiency, resolution, and sensitivity of the separation, because in many systems the analyte would spend more time in the capillary column. The separation of sialyllactosamines increased dramatically as the concentration of sodium phosphate in the running buffer increased from 0.1 to 0.4 M (Fig. 2).

Even when the concentration of two buffers is identical, however, different ionic strengths can produce different field strengths, which result in significantly different currents. For this reason, Tris–HCl buffer was tested as well as sodium phosphate and other buffers. Moreover, the concentration and ionic strength of the buffer are not the only critical variables that affect separation at high voltages: the mobility of the buffer constituents, and therefore the

buffer's specific conductance, plays a role as well [12]. The interaction of analytes with buffer components can influence these parameters, and thus buffer systems differ in their ability to resolve different sets of analytes. Therefore, for each analyte, particular concentrations of buffer and particular buffer systems may be required. Our best separation of the sialyllactoses was achieved with 0.2 M sodium phosphate buffer but required organic modifiers to achieve baseline resolution (Fig. 1).

Organic modifiers added to the mobile phase decrease the polarity and alter the viscosity of the buffer electrolyte, thus affecting both the electroosmotic flow and the electrophoretic mobility of the analytes. A number of organic modifiers can be used in CE [12]. The separation of sialyllactoses increased with the addition of increasing amounts of alcohols. Addition of methanol at 40% or 2-propanol at 30% to 0.2 M sodium phosphate buffer resulted in baseline resolution of the sialyllactoses within 30 min of running time, but the methanol allowed shorter runs than did propanol. Also, with methanol there was greater sensitivity than with propanol, probably due to less background absorbance at 205 nm.

The resolution of the three sialylated pentasaccharide isomers proved to be more complex than the previous two separations, requiring surfactant as well as the proper combination of buffer system (Tris), buffer concentration, pH, and organic modifier. Surfactants can be highly effective buffer additives in CE, and are among the most widely used [13]. Below the critical micelle concentration (CMC), ionic surfactant molecule monomers can solubilize hydrophobic solutes, act as ion-pairing reagents, or modify the capillary wall. The monomer surfactant can interact with the solute via ionic interactions with the charged end of the surfactant and/or via hydrophobic interactions between the surfactant alkyl chains and the hydrophobic moieties of the solute. Surfactants may also adsorb to the capillary wall, modifying electroosmotic flow and also limiting potential solute adsorption. Surfactant concentrations above the CMC radically alter the mechanism of separation, creating a different mode of CE usually referred to as micellar electrokinetic chromatography (MEKC) [14]. MEKC allowed baseline resolution, not only of the linear from the branched (b) isomers

of the sialylated pentasaccharides of milk, but also of the linear a and c isomers from one another. Even with MEKC, the pH was still a critical factor, as evident by the loss of resolution when the pH was changed from 7.6 to 8.1 (Fig. 3F).

These methods allow three groups of isomeric sialylated oligosaccharides that occur in human milk, bovine colostrum, and other biological fluids and tissues to be baseline resolved by CE within a convenient running time by using three different running buffers. To achieve the separations it was necessary to test a wide variety of electrophoretic conditions, focusing especially on the composition, concentration and pH of the running buffer, and the presence of organic modifiers and/or surfactants.

Sialyllactoses and sialylated pentaoses are major components of human milk, and sialyllactosamines are found in both human urine and bovine colostrum. The routine resolution and detection of individual structural isomers by CE will facilitate studies of their occurrence and biological roles. Furthermore, the approaches described herein will allow resolution and quantitation of acidic oligosaccharide isomers derived from other sources, such as glycans released from glycoconjugates containing homologous series and structural isomers.

Acknowledgements

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