Coupling Capillary Electrochromatography with Electrospray Fourier Transform Mass Spectrometry for Characterizing Complex Oligosaccharide Pools

Amy H. Que, Yehia Mechref, Yunping Huang, John A. Taraszka, David E. Clemmer,* and Milos V. Novotny*

Department of Chemistry, Indiana University, Bloomington, Indiana 47405

To deal with the complexity of the glycan mixtures released from glycoproteins, an efficient form of microcolumn separations, capillary electrochromatography, has been combined with high-performance mass spectrometry (Fourier transform ion cyclotron resonance). Contour plots have been generated from the mixtures of O-linked oligosaccharides originated from bovine mucin and bile salt-stimulated lipase, a large glycoprotein enzyme.

Glycosylation of proteins in eukaryotic cells can represent a highly complex series of events that are all necessary for vital cellular functions such as morphogenesis, proliferation, cellular differentiation, cell−cell recognition, cellular adhesion, and programmed cell death.[1] The biochemical complexity of glycosylation events is invariably paralleled by the enormous complexity of glycan mixtures resulting from the isomerism of oligosaccharides and their propensity to branch, to add modified sugar structures, and to attach more or less selectively at certain amino acid residues within a polypeptide backbone. Glycoproteins are generally viewed as molecules with a precisely determined amino acid sequence, but highly variable glycan structures.

The analytical challenges of glycoprotein structural work typically demand a controlled degradation of these biomolecules to either glycopeptide entities or gently stripping the oligosaccharide chains from the polypeptide (either enzymatically or chemically).[2] There is often a different degree of complexity associated with asparagine-linked (N-linked) and threonine/serine-linked (O-linked) pools of glycans. Unlike *N*-glycans featuring a common core region, O-linked oligosaccharides may exhibit more complex (and less easily predictable) structures. Despite their functional significance,[3,4] much less has been done to characterize *O*-glycan pools compared with the N-linked glycans. The glycan pools originating from either source appears to represent some of the most complex mixtures of hard-to-resolve components. Thus, it is important to combine the best available separation tools with the best means of identification for their analysis.[2]

Modern glycoanalysis is a multimethodological task[2] in which mass spectrometry (MS) is playing an increasingly important role. Recent results on interfacing high-pressure capillary liquid chromatography (CLC) to Fourier transform (FT) MS in high-throughput proteomics[5−7] has encouraged us to consider coupling capillary electrochromatography (CEC) to this type of high-performance MS. The demonstrated success by the Smith group in peptide separation and identification is a task of comparable complexity to the investigation of complex glycan pools. A highly efficient separation of isomeric glycans[8] seemed an appropriate prelude to structural investigations by a tool with utmost in mass-to-charge (m/z) resolution and accuracy.

FTICR MS has now been amply demonstrated to work with either the matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) ion sources in the analysis of biomolecules. In the analysis of relatively uncomplicated mixtures of glycans, MALDI TOF-MS with postsource decay or in conjunction with enzymatic (exoglycosidase) procedures[2,9−12] has been generally preferred over ESI-based methodologies in mass profiling and sequencing due to its sensitivity and the capability of assigning linkages. The power of MALDI FTMS in its tandem mode has been demonstrated for a detailed structural characterization of glycans,[13,14] and also in conjunction with chemical degradation[15] and exoglycosidase digestion[16] by Lebrilla and co-

* Corresponding authors. E-mail: novotny@indiana.edu; clemmer@indiana.edu. Phone: (812)-855-4532. (812)-855-8259. Fax: (812)-855−8300.

(1) Rudd, P. M.; Elliott, T.; Cresswell, P.; Wilson, I. A.; Dwek, R. A. *Science* **2001**, *291*, 2370−2376.
(2) Mechref, Y.; Novotny, M. V. *Chem. Rev.* **2002**, *102*, 321−370.
(3) Varki, A. *Glycobiology* **1993**, *3*, 97−130.
(4) Wells, L.; Vosseller, K.; Hart, G. W. *Science* **2001**, *291*, 2376−2378.
(5) Shen, Y.; Zhao, R.; Belov, M. E.; Conrads, T. P.; Anderson, G. A.; Tang, K.; Pasa-Tolic, L.; Veenstra, T. D.; Lipton, M. S.; Udseth, H. R.; Smith, R. D. *Anal. Chem.* **2002**, *73*, 1766−1775.
(6) Shen, Y.; Tolic, N.; Zhao, R.; Pasa-Tolic, L.; Li, L.; Berger, S. J.; Harkewicsz, R.; Anderson, G. A.; Belov, M. E.; Smith, R. D. *Anal. Chem.* **2002**, *73*, 3011−3021.
(7) Li, L.; Masselon, C. D.; Anderson, G. A.; Pasa-Tolic, L.; Lee, S.-W, Shen, Y.; Zhao, R.; Lipton, M. S.; Conrads, T. P.; Tolic, N.; Smith, R. D. *Anal. Chem.* **2002**, *73*, 3312−3322.
(8) Que, A. H.; Novotny, M. V. *Anal. Chem.*, in press.
(9) Harvey, D. J. *Mass Spectrom. Rev.* **1999**, *18*, 349−450.
(10) Harvey, D. J. *Proteomics* **2001**, *1*, 311−28.
(11) Mechref, Y.; Novotny, M. V. *Anal. Chem.* **1998**, *70*, 455−463.
(12) Mechref, Y.; Baker, A. G.; Novotny, M. V. *Carbohydr. Res.* **1998**, *313*, 145−155.
(13) Cancilla, M. T.; Penn, S. G.; Lebrilla, C. B. *Anal. Chem.* **1998**, *70*, 663−672.
(14) Penn, S. G.; Cancilla, M. T.; Lebrilla, C. B. *Anal. Chem.* **1996**, *68*, 2331.
(15) Cancilla, M. T.; Penn, S. G.; Lebrilla, C. B. *Anal. Chem.* **1998**, *70*, 663−672.
(16) Xie, Y.; Tseng, K.; Lebrilla, C. B. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 877−884.

10.1021/ac025985c CCC: $25.00
Published on Web 00/00/0000

workers. In one additional study, ESI has been employed in the structural analysis of permethylated oligosaccharides through FTMS.[17]

Although considerable attention has been paid to various modes of chromatography in carbohydrate analysis,[2] most previously developed methodologies are neither highly efficient nor easily adaptable for coupling with MS. In our recent investigations, we have developed a hydrophilic-phase mode of CEC,[8] with the mobile-phase conditions amenable to its coupling with electrospray ion trap MS.[18] While this form of MS was suitable as a mass-sensitive detector for CEC because of its sensitivity and high acquisition speed, it was limited by relatively low mass accuracy and resolution.

CEC is a relatively new separation technique, previously unused in carbohydrate research, that combines some of the best features of capillary LC and capillary zone electrophoresis (CZE), such as a much higher separation efficiency than LC, shorter analysis times, and chromatographic selectivity not seen in CZE. New column technologies are fundamental to the success of CEC, as we have recently demonstrated with the development of monolithic (polymer-based) capillaries[8,16−20] for diverse complex mixtures. We have recently developed a polar version of the monolithic column matrix for highly efficient separations of carbohydrates, featuring the separation efficiencies in excess of 250 000 theoretical plates/m.[8]

In this paper, we report coupling of these highly efficient columns to FTMS for high-accuracy mass measurements of the individually separated glycans. Using external calibration, we demonstrate mass accuracy of <4 ppm in the m/z range of 200−2000 for a number of oligosaccharides. In initial studies, we assessed ion formation mechanisms for this class of compounds by examining a series of dextran oligomers. The analytical merit of this combination has been subsequently demonstrated with complex mixtures of O-linked oligosaccharides isolated from bovine mucin and bile salt-stimulated lipase.

MATERIALS AND METHODS

Materials. Acrylamide and *N*,*N*′-methylenebisacrylamide were purchased from Bio-Rad Laboratories (Hercules, CA). Ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), 3-methacryloxypropyltrimethoxysilane (Bind-Silane), poly(ethylene glycol) (PEG, MW 10 000), bovine submaxillary mucin and neuraminidase from *A. ureafaciens* were obtained from Sigma (St. Louis, MO). Vinylsulfonic acid (sodium salt, 25% (v/v)), 2-cyanoethyl acrylate (CEA), and formamide were purchased from Aldrich (Milwaukee, WI). Dextran 1000 was received from Fluka (Buchs, Switzerland). Bile salt-stimulated lipase (BSSL) from human breast milk was isolated as described elsewhere.[21]

Column Preparation. Columns were prepared according to the previously described procedures.[8,19,20] Briefly, fused-silica capillary (Polymicro Technologies, Phoenix, AZ) with 100-μm i.d. and 160-μm o.d. was used to make CEC columns. The stationary phase for the cyano columns consisted of 5% T, 60% C (Hjertén's designation[22] for T and C), 3% PEG, 40% CEA, and 10% vinylsulfonic acid. The reaction mixture was prepared by dissolving 10.0 mg of acrylamide, 30 mg of *N*,*N*′-methylenebisacrylamide, 16.0 μL of CEA, 12.4 μL of vinylsulfonic acid, and 30 mg of PEG in 0.5 mL of formamide and 0.5 mL of 100 mM Tris/150 mM boric acid (pH 8.2). Polymerization was initiated by using 4 μL of 20% (v/v) TEMED and 4 μL of 40% ammonium persulfate added to 0.5 mL of the above monomer solution (heated to 50 °C). The polymerization was allowed to proceed overnight at room temperature. Finally, columns were flushed and conditioned using a solution consisting of 50:50 (v/v) acetonitrile/5 mM phosphate buffer at pH 3.0.

Release of O-Linked Oligosaccharides from Glycoproteins. O-Linked oligosaccharides were cleaved from the glycoproteins using a modified β-elimination procedure under reducing conditions according to our previously published procedure.[23] Briefly, a 0.4-mg sample of each glycoprotein studied here was dissolved in 1 mL of 1 M borane ammonia complex, prepared in 28% ammonia aqueous solution. The solution was incubated at 45 °C for 24 h, dried under a stream of nitrogen, and then reconstituted in 100 μL of water. The solution was then passed through a 100-mg C18 cartridge (Waters, Milford, MA), on top of which 1.5 mL of strong cation-exchange resin in H^+ form (Dowex-50Wx8−400, Sigma) had been placed. Glycans were eluted in a 10-mL volume of water and lyophilized. Next, methanol was repeatedly added to the lyophilized sample and evaporated under a stream of nitrogen to remove the formed borates. The lyophilized glycan sample was then reconstituted in acetonitrile/water solution prior to its analysis. *O*-Glycans derived from BSSL were desialylated using neuraminidase (an exoglycosidase that selectively cleaves sialic acid from glycan structures) prior to their CEC/FTMS analysis.

CEC/ESI-FTMS. The ESI coupling procedure was similar to the one used in a CEC/ion trap setup, described previously.[8,18,20] Briefly, the instrument consisted of a 60-kV power supply (Spellman, Plainview, NY) connected to a platinum electrode in a vial containing the mobile phase. A nanospray needle was prepared from fused silica (250-μm i.d., 360-μm o.d.) using a laser-based micropipet puller (Sutter, Novato, CA) that produces tapered needles with ~10-μm orifice. The separation capillary was inserted into the nanospray needle so that the column outlet was placed well inside the tapered portion of the needle. A sheath liquid was used to complete the circuit between the column outlet and the needle, which was grounded. The nanospray was generated by applying a negative voltage (~1.4 kV) on the capillary electrode. The sheath liquid, containing 1.0% formic acid and 1 mM sodium acetate in an acetonitrile/water (50:50, v/v) mixture, was sustained utilizing a syringe pump at a flow rate of 0.5 μL/min through a microflow syringe pump (model μLC-50, Isco, Inc., Lincoln, NE). This sheath liquid was needed to maintain an efficient electrospray ionization of saccharides in the positive-ion detection mode. A mobile phase consisting of 2.4 mM ammonium acetate buffer (pH 3) and 0.2 mM sodium acetate prepared in 50:50 (v/v) acetonitrile/water mixture was employed isocratically.

(17) Solouki, T.; Reinhold: B. B.; Costello, C. E.; O'Malley, M.; Guan, S.; Marshall, A. *Anal. Chem.* **1998**, *70*, 857−864.
(18) Que, A. H.; Novotny, M. V. *Anal. Chem.* **2002**, *74*, 5184−5191.
(19) Palm, A.; Novotny, M. V. *Anal. Chem.* **1997**, *69*, 4499−4507.
(20) Que, A. H.; Palm, A.; Baker, A. G.; Novotny, M. V. *J. Chromatogr., A* **2000**, *877*, 379−391.
(21) Thurl, S.; Henker, J.; Siegel, M.; Tovar, K.; Sawatzki, G. *Glycoconjugate J.* **1997**, *14*, 795−799.
(22) Hjertén, S. *Arch. Biochem. Biophys.* **1962**, (Suppl 1), 147−151.
(23) Huang, P.; Konse, T.; Mechref, Y.; Novotny, M. V. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1199−1204.

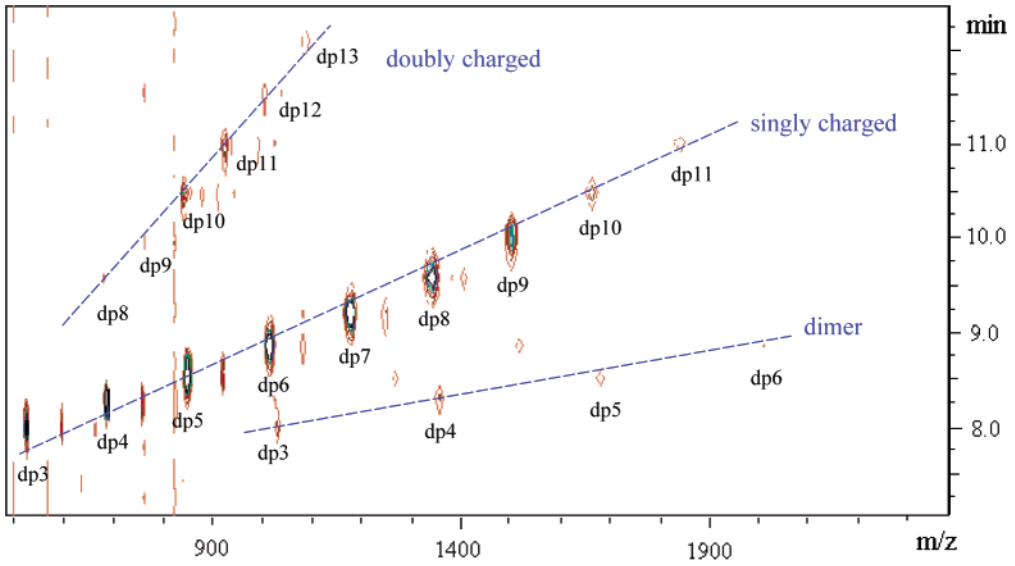

Figure 1. 2-D contour plot of Dextran 1000 ladder (dp3−dp13). Experimental conditions: cyano column, 26 cm; mobile phase, 2.4 mM ammonium formate buffer (240 mM, pH 3) and 0.2 mM sodium acetate in the 60/40 mixture of acetonitrile/water; field strength, 600 V/cm; injection, 12 kV, 30 s; sample, Dextrin 1000 (1.5 mg/mL).

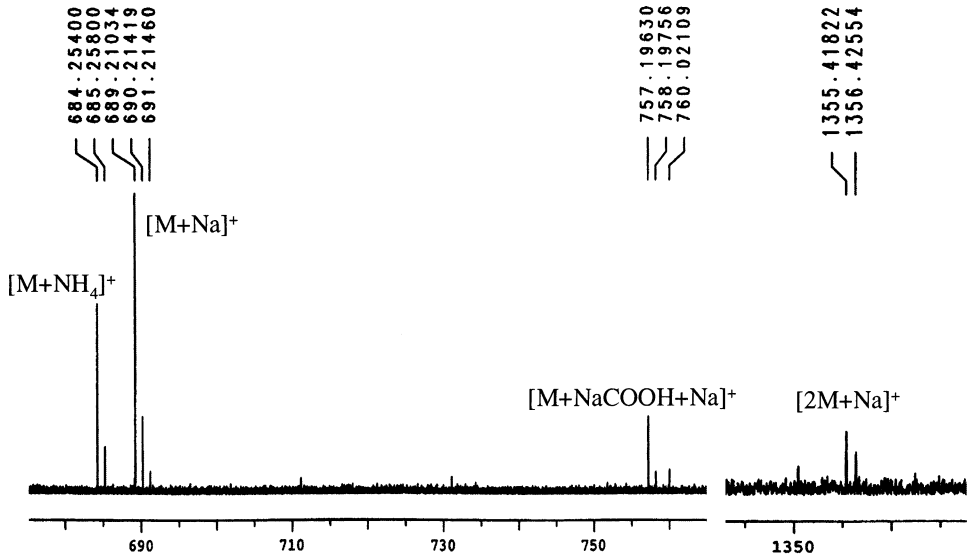

Figure 2. Spectrum of an oligosaccharide (dp4) at a retention time of 8.2 min, extracted from Figure 1.

An Apex III (Billerica, MA) Fourier transform mass spectrometer (Bruker Daltonics Inc., Billerica, MA), was equipped with a 7.0-T shielded superconducting magnet, provided with a patented Infinity Cell; a computer-controlled ESI source was used. Four stages of differential pumping (turbomolecular pumps) were used to evacuate the system providing a base pressure of 1.0×10^{-10} mbar in the analyzer region. Ions originating from the ESI source were first externally accumulated in a hexapole and then transferred into the analyzer stage using a series of electrostatic lenses. Here, the ions were trapped, excited, and detected using the rf-shimmed Infinity Cell. Isolation was performed using the correlated swept excitation.[24] During the sustained off-resonance irradiation collision-induced dissociation (SORI-CID) event, a pulsed valve introduced argon at the pressure of 1.0×10^{-7} mbar. The control software of separation FTMS allowed a display of two-dimensional contour plots in time and mass scales.

The data acquisition speed of FTMS was 1.6−1.8 s/scan in the MS mode. Each data set was collected by averaging eight scans; therefore, a total MS acquisition time of 13−15 s was required. The scans could be saved first and averaged later; however, we averaged first to simplify data interpretation. The number of acquired data points was 256K. The *m*/*z* dimension of the data was calibrated externally using the ions generated from the direct infusion of Dextran 1000 (1.5 mg/mL) prepared in 50/50 (v/v) water/acetonitrile solution containing 1.0% formic acid and 1 mM sodium acetate.

RESULTS AND DISCUSSION

The slow data acquisition speed (1−2 s/scan) of the FTMS instrument limits its suitability to be used directly as an on-line detector for a chromatographic technique such as CEC where an average base peak amounts to 15−25 s. The limitation is caused by an insufficient number of data sets collected across the elution time to produce a peak in a chromatogram. However, a two-

(24) De Konig, L. J.; Nibbering, N. M. M.; Van Orden, S. L.; Laukien, F. H. *Int. J. Mass Spectrom. Ion Processes* **1997**, *165/166*, 209−219.

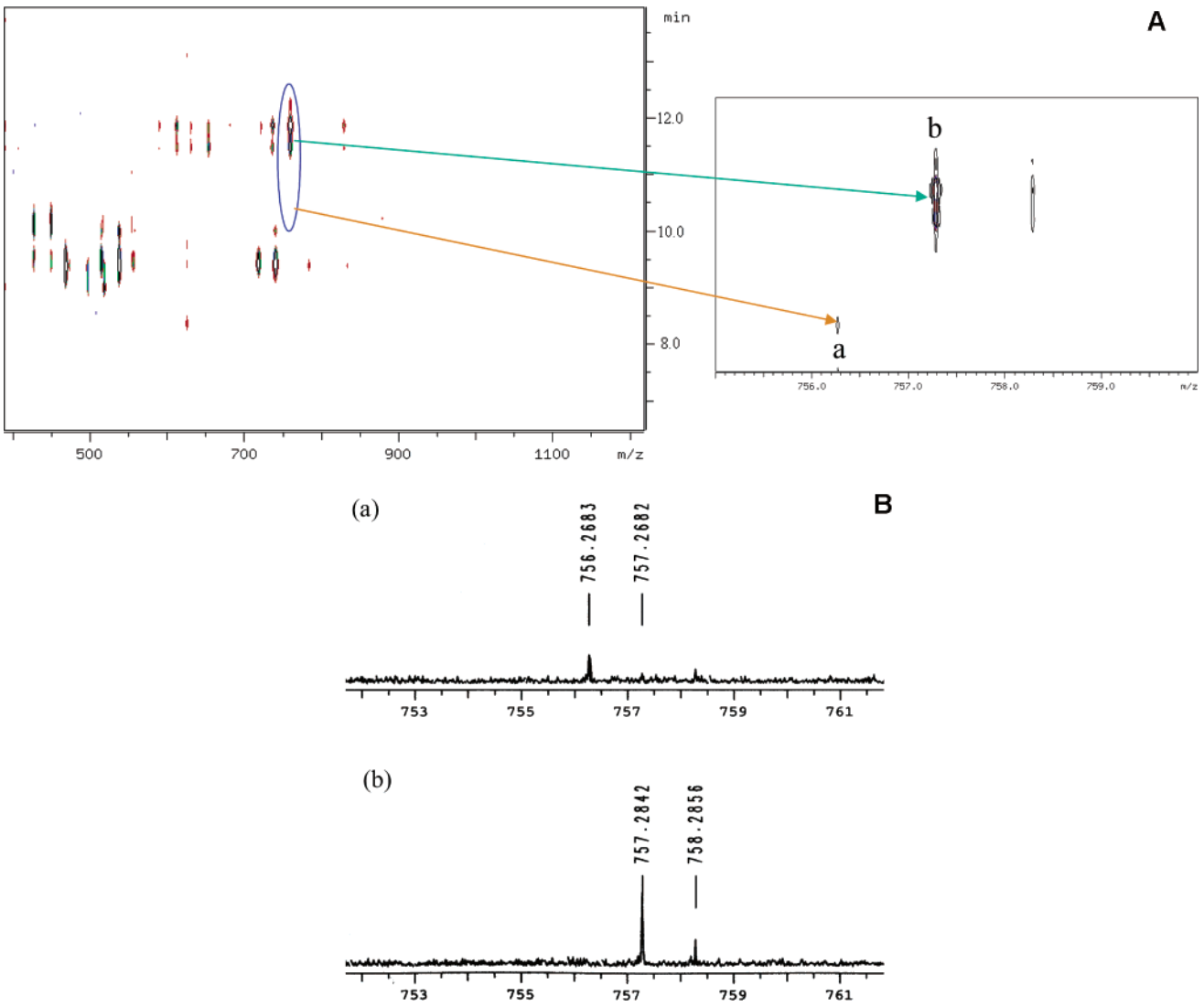

Figure 3. (A) 2-D contour plot of a mixture of *O*-glycans cleaved from mucin; (B) spectra of glycan a with *m*/*z* = 756 and glycan b with *m*/*z* = 757. An average resolution of ~30 000 is demonstrated. Experimental conditions: cyano column, 26 cm; mobile phase, 2.4 mM ammonium formate buffer (240 mM, pH 3) and 0.2 mM sodium acetate in the mixture of acetonitrile/water (71:29, v/v); field strength, 600 V/cm; injection, 12 kV, 30 s.

dimensional (2-D) contour plot of only one data set across one peak has been needed to produce a dot in a 2-D contour plot, providing rationale for a CEC/FTMS coupling. Moreover, sensitivity can be improved by averaging the scans across the peak into one data set.

CEC/FTMS of Dextran 1000. Initially, the suitability of the described system for the analysis of oligosaccharides was tested by utilizing a known mixture of linear oligosaccharides that consists of 3−13 glucose residues (α1−6 linked). As shown in Figure 1, the retention times of oligosaccharides increase linearly with the molecular size or degree of polymerization (dp), as anticipated for a hydrophilic-phase separation on a cyano phase.[6] These oligosaccharides appeared in different forms and charge states in the gas phase: the relatively small sugars (dp3−dp6) formed singly charged monomers $[M + Na]^+$ and dimers $[2M + Na]^+$, while the sugars with intermediate dp numbers (dp8−dp11) formed singly charged $[M + Na]^+$ and doubly charged $[M + 2Na]^{2+}$ ions. Larger oligosaccharides (>dp11, ~1900 MW) formed exclusively doubly charged $[M + 2Na]^+$ ions. Interpretation of the results illustrated in Figure 1 was simplified through clustering the different ions formed along the lines depicting each ion state. Accordingly, ions with same charge state can be interpreted without considering all charge states of an ion.

We also observed that some sugars acquired ammonium ions from the mobile phase to form ammoniated species, while others acquired sodium acetate to form $[M + NaCOOH + Na]^+$ ions, as illustrated in Figure 2, which depicts the spectrum of dp4 extracted from Figure 1. Doubly charged ions [17,25,26] and salt adducts[25] were observed previously in MS of sugars. One-dimensional (1-D) analysis resolves solutes only in the time scale, while a 2-D contour plot provides an extra resolving power by separating the analytes with different mass values in the mass scale. In addition, a 2-D contour plot allows a simple assignment of the charge states (monomers or dimers) and different forms of adduct ions ($[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$, or $[M + NaCOOH + Na]^+$) formed for the individual structures.

(25) Bahr, U.; Pfenninger, A.; Karas, M.; Stahl, B. *Anal. Chem.* **1997**, *69*, 4530−4535.
(26) Harvey, D. J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 900−915.

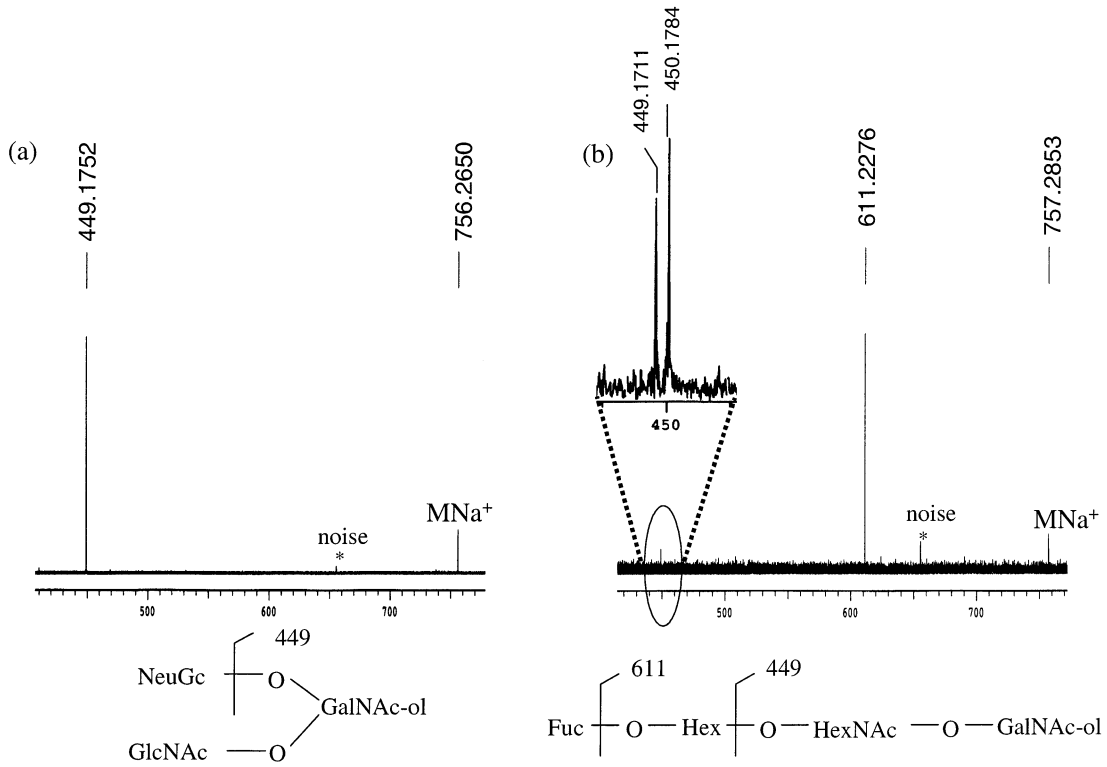

Figure 4. CEC/FTMS/MS spectra of (a) an acidic glycan GlcNAc(NeuGc)GalNAc-ol, $m/z = 756$ and (b) a neutral glycan FucHexHexNAcGalNAc-ol, $m/z = 757$.

Analysis of *O*-Glycans from Bovine Mucin. Proteins are often posttranslationally modified with carbohydrates at Ser or Thr residues through *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), mannose (Man), fucose (Fuc), or glucose (Glc) core monosaccharides.[4] Mucin is a good example of glycoprotein to be used in this study, since it is a heavily glycosylated protein, with predominantly a GalNAc core. There are two classes of relatively small oligosaccharides (400−1200 m/z) known to be formed in bovine mucin: neutral and acidic oligosaccharides. The acidic oligosaccharides contain *N*-acetylneuraminic acid (sialic acid) or *N*-glycolylneuraminic acid residues that are commonly positioned at the nonreducing end of glycans from glycoproteins and glycolipids.[27]

The mixture of *O*-glycans chemically released from mucin was resolved by the CEC/FTMS system as shown in Figure 3A. Each glycan detected in Figure 3A was characterized as listed in Table 1. The average mass measurement accuracy for this analysis with external calibration was 3.9 ppm. The mass resolution and accuracy of FTMS for saccharides was demonstrated in this study by providing characterization of two glycan structures that differ from each other by 1 m/z unit. These were an acidic glycan with $m/z = 756$ (dot a in Figure 3A) and a neutral glycan with $m/z = 757$ (dot b in Figure 3A). The average resolution of the spectrum illustrated in Figure 3A is 30 000. Previously, these structures could not be distinguished by our MALDI-TOF-MS system (unpublished results), because of its inability to isolate either ion for further postsource decay studies. In contrast, these two oligosaccharides could be electrochromatographically separated here from each other, and the high mass accuracy of FTMS allows their accurate mass determination (756.2683 m/z and 757.2851 m/z) (see Figure 3B). Typically, the metastable fragmentation of the highly labile sialic residues hinders the determination of sialylated structures in MALDI-MS. However, both sialylated and asialylated structures were simultaneously detected in the positive ion mode as a result of using acidic mobile phase, which results in protonation of the sialic acid residue. Recently, the metastable fragmentation of sialic acid residues in MALDI-MS were decreased

Table 1. Composition of O-Linked Glycans Released from Bovine Mucin As Observed through CEC/ESI-FTMS

$[M + Na]^+$ calculated	$[M + Na]^+$ measured	error (ppm)	composition[a]
449.1747	449.1706	−9.13	HexNAcGalNAc-ol
537.1907	537.1878	−5.40	NeuAcGalNAc-ol
553.1856	553.1835	−3.80	NeuGcGalNAc-ol
611.2275	611.2232	−7.04	HexHexNAcGalNAc-ol
652.2541	652.2521	−3.07	$HexNAc_2$GalNAc-ol
740.2701	740.2696	−0.68	GlcNAc(NeuAc)GalNAc-ol
756.2650	756.2683	4.36	GlcNAc(NeuGc)GalNAc-ol
757.2854	757.2851	−0.40	FucHexHexNAcGalNAc-ol
773.2803	773.2809	0.78	Hex_2HexNAcGalNAc-ol
814.3069	814.3069	0.00	$HexHexNAc_2$GalNAc-ol
919.3382	919.3371	−1.20	$FucHex_2$HexNAcGalNAc-ol
960.3648	960.3707	6.14	$FucHexHexNAc_2$GalNAc-ol
1065.3961	1065.4055	8.82	Fuc_2Hex_2HexNAcGalNAc-ol
average		3.91	

[a] Abbreviations: Hex, hexose; Fuc, fucose; HexNAc, *N*-acetylhexosamine; GalNAc, *N*-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolyneuraminic acid.

(27) Rosenberg, A. *Biology of the Sialic Acids*; Plenum: New York, 1995.

Table 2. Composition of O-linked Glycans Released from BSSL as Alditols

calculated		measured		error (ppm)		
$[M + Na]^+$	$[M + 2Na]^{2+}$	$[M + Na]^+$	$[M + 2Na]^{2+}$			composition
773.2803		773.2834		4.01		$Hex_2HexNAcHexNAc$-ol
919.3382		919.3418		3.92		$FucHex_2HexNAcHexNAc$-ol
1065.3961		1065.4049		8.26		$Fuc_2Hex_2HexNAcHexNAc$-ol
1138.4125		1138.4128		0.26		$Hex_3HexNAc_2HexNAc$-ol
1284.4704		1284.4741		2.88	3.37	$FucHex_3HexNAc_2HexNAc$-ol
1430.5283		1430.5322		2.73	3.92	$Fuc_2Hex_3HexNAc_2HexNAc$-ol
1503.5447	763.2673	1503.5480	763.2688	2.19	2.03	$Hex_4HexNAc_3HexNAc$-ol
1576.5862	799.7880		799.7874		−0.75	$Fuc_3Hex_3HexNAc_2HexNAc$-ol
1649.6026	836.2962	1649.6128	836.2998		4.30	$FucHex_4HexNAc_3HexNAc$-ol
1795.6605	909.3252		909.3264		1.37	$Fuc_2Hex_4HexNAc_3HexNAc$-ol
1941.7184	982.3541		982.3513		−2.85	$Fuc_3Hex_4HexNAc_3HexNAc$-ol
2014.7348	1018.8623		1018.8638		1.47	$FucHex_5HexNAc_4HexNAc$-ol
2160.7927	1091.8913		1091.8911		−0.14	$Fuc_2Hex_5HexNAc_4HexNAc$-ol
2233.8342	1128.4121		1128.4020		1.00	$Fuc_5Hex_4HexNAc_3HexNAc$-ol
average				2.67		

by transiently elevating pressure in the MALDI-FTMS source into the 1−10 mbar range during ionization.[28]

FTMS/MS of Glycans. The aforementioned structures were further characterized by utilizing the high-resolution MS/MS. Upon collisional activation, the acidic glycan ($m/z = 756$ with the composition of GlcNAc(NeuGc)GalNAc-ol) easily lost its acidic residue at the nonreducing end to form the fragment at $m/z = 449$ (Figure 4a). The glycosidic bonds of sialic acids are readily cleaved from the remainder of glycan structures. Conversely, the neutral glycan ($m/z = 757$ with the composition of FucHexHexNAcGalNAc-ol) produced two product ions with m/z values of 449 and 611. These fragments result from the loss of Fuc and Fuc plus Hex from the nonreducing end of the structures (Figure 4b). The MS/MS data illustrated in Figure 4 were those of CEC-separated glycans.

The above-discussed MS/MS data exemplify the need for separation to unambiguously interpret MS/MS data. This is obvious especially in the case of the glycan with 757 m/z value. The MS/MS data of this structure, acquired under no monoisotopic isolation, exhibited fragment ions corresponding to a loss of $m/z = 308.0938$ and 307.0865, as seen in Figure 4b. The former corresponds to a loss of Hex and Fuc (theoretically 308.1107 m/z), while the later could be mistaken for a loss of NeuGc (theoretically 307.0903 m/z). The second m/z value is actually an isotope with more abundance than expected. Accordingly, it could be erroneously assumed that $m/z = 757$ represents a mixture of two glycan structures. CEC separation of the glycan mixture prior to tandem mass spectrometry analysis eliminates such an ambiguous interpretation of data.

Due to its low acquisition speed, the SORI-CID technique (3−5 s/scan) is not particularly suitable for the fast separations in coupling with FTMS/MS. SORI-CID requires the use of a collision gas pulse, which requires several additional seconds to evacuate the ICR cell to its base pressure. High data acquisition rates are critical for maintaining good chromatographic resolution and high FTMS/MS sensitivity (averaging scans to improve S/N). In this study, the separation was performed at a relatively low column efficiency in order to allow long elution times of the studied peaks and, consequently, to allow for the MS/MS experiments. Recently, electron capture dissociation (ECD) was employed in determining the O-glycosylation sites in peptides.[29] In another study, both ECD and the infrared multiphoton dissociation (IRMPD) have been used in studying N-glycosylated tryptic peptides.[30] In that study, it was demonstrated that IRMPD provided abundant fragment ions of the glycan moiety rather than the peptide backbone, as was the case with ECD. Moreover, IRMPD has been used recently in coupling capillary LC to FTMS.[7] With this technique, a very short irradiation time is needed to obtain effective dissociation (100 ms), permitting MS/MS data to be acquired faster (∼1 s/scan). Accordingly, IRMPD appears more suitable for fast chromatographic separations.

Analysis of *O*-Glycans from Bile Salt-Stimulated Lipase. BSSL is an enzyme with a broad substrate specificity toward mono-, di-, and triacylglycerols, cholesterol esters, fat-soluble vitamins, and lipoamides.[31] It is a glycoprotein that amounts to approximately 0.5−1.0% of the milk protein. The biological roles of BSSL glycans are speculative at present, since glycosylation does not seem to affect the main catalytic activity of this enzyme. It is a relatively large glycoprotein consisting of 722 amino acid residues, with numerous O-glycosylation sites near the C-terminus. The O-linked glycans of this glycoprotein are highly complex, as seen in the profile generated with amino-phase CEC coupled with an ion trap mass spectrometry.[18] Moreover, we have extensively characterized these glycans through MALDI-MS in conjunction with exoglycosidases,[32] so that it became of interest to test this complex sample further through CEC/FTMS.

In this study, the described coupling provided a two-dimensional map of the *O*-glycan fraction released from BSSL (Figure 5). Each glycan in BSSL was identified and listed in Table 2 with an average mass accuracy of ∼2.7 ppm. As expected, the glycans eluted in the order of their size. The smaller glycans formed singly charged molecular ions, while larger oligosaccharides formed doubly charged molecular ions with a relatively long

(28) O'Connor P. B.; Mirgorodskaya, E.; Costello, C. E. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 402−407.

(29) Mirgorodskaya, E.; Roepstroff, P.; Zubarev, R. A. *Anal. Chem.* **1999**, *71*, 4431−4436.

(30) Hakansson, K.; Cooper, H. J.; Emmett, M. R.; Costello, C. E.; Marshall, A. G.; Nilsson, C. L. *Anal. Chem.* **2001**, *73*, 4530−4536.

(31) Mechref, Y.; Chen, P.; Novotny, M. V. Glycobiology **1999**, 9, 277−234.

(32) Mechref, Y.; Huang, Y.; Novotny, M. V., to be submitted.

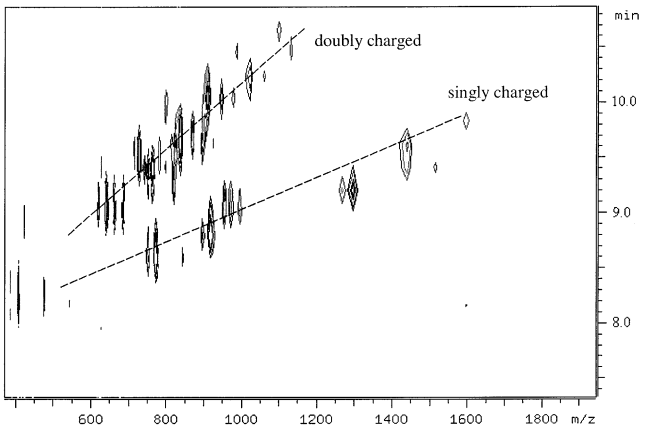

Figure 5. 2-D contour plot of a mixture of *O*-glycans cleaved from BSSL. An average resolution of ~30 000 is demonstrated. Experimental conditions: cyano column, 26 cm; mobile phase, 2.4 mM ammonium formate buffer (240 mM, pH 3) and 0.2 mM sodium acetate in the mixture of acetonitrile/water (60:40, v/v); field strength, 600 V/cm; injection, 12 kV, 30 s.

elution time. Isomeric glycans were not resolved in this study, which could be attributed to the low efficiency at which the experiment had to be run. The broader bands might contain isomers; however, the closely eluted isomers could not be resolved in time because of the low data sample acquisition speed of FTMS (13−15 s/sample). Two components would have to be at least 13−15 s apart in order to be resolved in our experiments. Sodium acetate was additionally used in mobile phase to form mainly sodium adducts. This higher salt concentration is expected to affect adversely CEC resolution.

CONCLUSIONS

Two-dimensional contour plots obtained through CEC/FTMS provide very high overall resolving power for complex glycan mixtures through combining a high spatial resolution of CEC and a high mass resolution of FTMS. Glycans can be identified and structurally determined by FTMS, with high mass measurement accuracies for both MS and MS^n stages. The slow data acquisition of FTMS with SORI-CID limited the capability of multistage MS/MS experiments performed on-line for fast chromatographic separations. The high resolution in mass scale can be particularly effective for coeluting components of a mixture.

ACKNOWLEDGMENT

This work was supported by Grants 24349 (M.V.N.) and 59145 (D.E.C.) from the National Institute of General Medical Sciences, U.S. Department of Health and Human Services. We also gratefully acknowledge a partial support from the 21st Century Fund of the State of Indiana.

Received for review July 25, 2002. Accepted January 10, 2003.

AC025985C