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Analysis of sialyl oligosaccharides by high-performance liquid chromatography-electrospray ionisation-mass spectrometry with differentiation of α2-3 and α2-6 sialyl linkages

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Abstract

A method for analysing sialyl oligosaccharides from bovine colostrum using high-performance liquid chromatography-electrospray ionisation-mass spectrometry (HPLC-ESI-MS) is described. Under positive ionisation mode, mass spectra of α2-3 and α2-6 linkages were different, and the former produced a prominent B_2 (or B_3 in disialyl lactose) mass fragment. This fragment was absent from mass spectra with α2-6 linkages. Two sialyl oligosaccharides, which have not been reported previously, were tentatively identified. One comprises a *N*-acetyl neuraminic acid (Neu5Ac), two hexoses (Hex), and one *N*-acetyl hexosamine (HexNAc) residue ($(Neu5Ac)_1(Hex)_2(HexNAc)_1$), and the other comprises one Neu5Ac and one Hex residue ($(Neu5Ac)_1(Hex)_1$).
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Keywords: Sialyl oligosaccharides; Bovine colostrum; HPLC-ESI-MS; Aminopropyl column; Sialyl linkage; Mass spectrum

1. Introduction

Sialyl oligosaccharides (Fig. 1) containing sialic acids (*N*-acetyl neuraminic acid (Neu5Ac) or *N*-glycoyl neuraminic acid (Neu5Gc)) occur in several body fluids and they are also important components of glycoproteins [1]. Mammalian milk contains a highly complex mixture of sialyl oligosaccharides [2]. They have been shown to act as cell surface receptors for pathogens [3].

Bovine colostrum (the yellowish fluid secreted by the mammary glands immediately after birth) contains a higher content of sialyl oligosaccharides than mature bovine milk does. There are at least eight sialyl oligosaccharides present in the bovine colostrum [4,5]. HPLC is the popular choice to analyse sialyl oligosaccharides. High-performance anion exchange chromatography (HPAEC) using pellicular resins with a pulsed amperometric detector has many advantages including good resolution and a very low detection limit [6,7]. However, a limitation of the HPAEC method is that it cannot give structural information.

Aminopropyl columns are widely used for carbohydrate analysis [8]. Application of these columns in sialyl oligosaccharides analysis is also common [9–11]. The mobile phase used is almost exclusively a mixture of acetonitrile and water. Unlike HPAEC, this mobile phase is compatible with an electrospray ionisation (ESI)-mass spectrometry (MS) detector.

The complexity of oligosaccharides makes the MS detector a superior tool for analysis. The advent of ESI-MS made the HPLC and MS a satisfactory combination, and in recent years, a few papers have been published to characterise the sialylated sugars using HPLC-ESI-MS [12–16]. The columns used include C18 [12–14] and graphitised carbon [15,16]. The C18 column is not a good choice because of its poor resolution for carbohydrates, and in most of the cases, either the samples were derivatized [12] or an ion-suppression technique had to be used [13]. Graphitised carbon is a relatively

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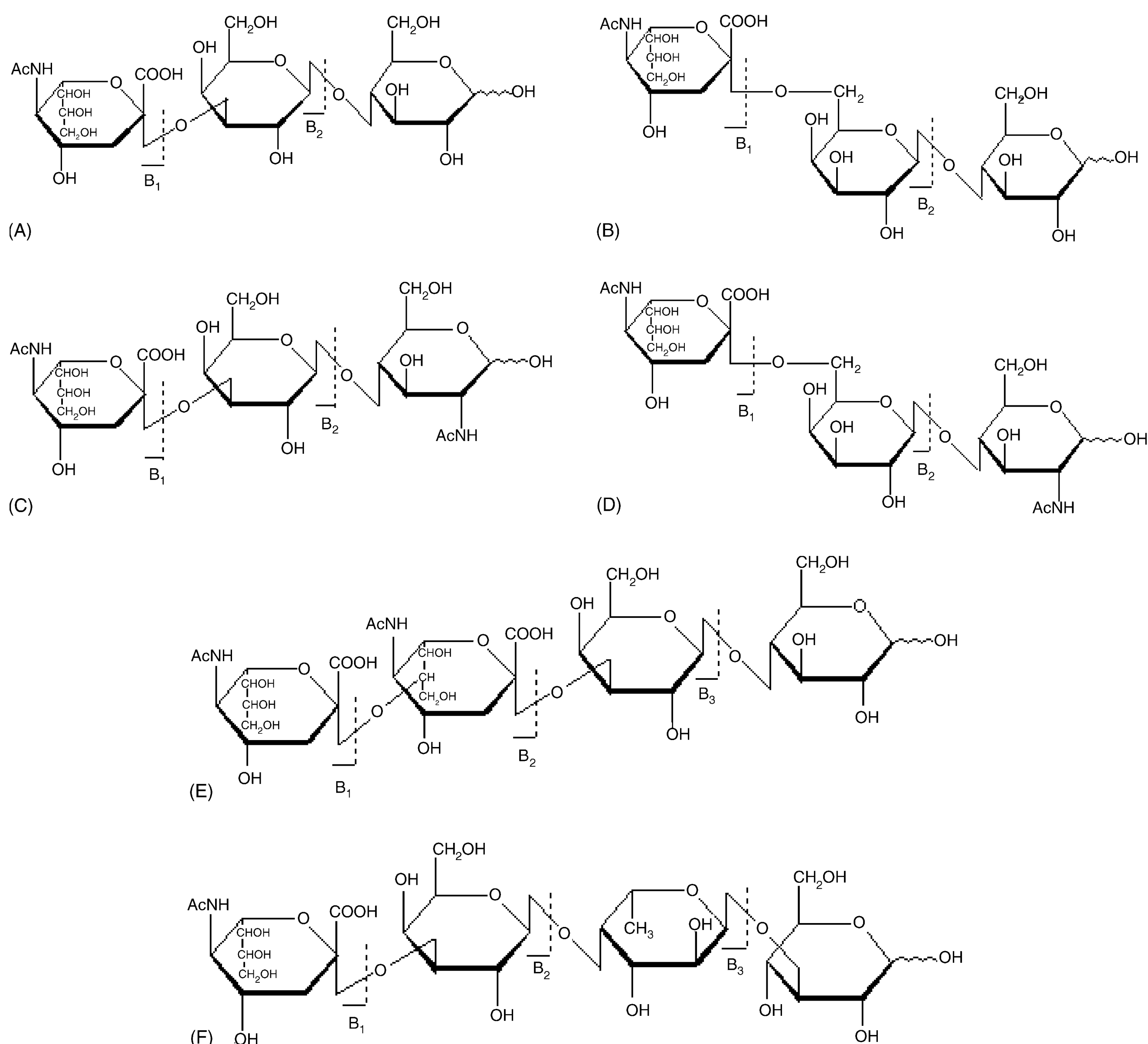

Fig. 1. Structures and mass spectra fragmentation patterns of sialyl oligosaccharides (for the nomenclature of fragmentation, refer to [31]). (A) α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-D-Glc (3′-SL); (B) α-Neu5Ac-(2 → 6)-β-D-Gal-(1 → 4)-D-Glc (6′-SL); (C) α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-D-GlcNAc (3′-SLN); (D) α-Neu5Ac-(2 → 6)-β-D-Gal-(1 → 4)-D-GlcNAc (6′-SLN); (E) α-Neu5Ac-(2 → 8)-α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-D-Glc (DSL); (F) α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-α-L-Fuc-(1 → 3)-D-Glc (SFL).

new kind of column. For the analysis of sulfated oligosaccharides from mucin glycoproteins using HPLC-ESI-MS [17], an aminopropyl column and a graphitised carbon column appeared to give comparable resolutions. Sialic acids are most commonly linked α2-3 or α2-6 to the Gal residues of glycoproteins [18] or gangliosides [19]. Determination of the linkage position traditionally involves the selective cleavage of α2-3 or α2-6 linkage by enzymes, followed by HPLC or matrix-assisted-laser desorption ionisation (MALDI) mass spectrometry for the identification of the residual glycans [20]. In recent years, several attempts were made to differentiate the two kinds of linkages using MS with "soft ionisation" methods such as fast atom bombardment (FAB) [21], MALDI [20,22,23], and ESI [19,20,24]. Most of the studies were carried out in negative ionisation mode because the negatively charged sialic acids give stronger signals. However, in this work positive ionisation was used because it allowed the two sialyl linkages to be readily distinguished from each other.

In this paper, we report on the development of a method, which used an aminopropyl column HPLC-ESI-MS. The method was applied to a mixture of sialyl oligosaccharides extracted from bovine colostrum. We believe that is the first time HPLC with an aminopropyl column and MS detection has been used to analyse sialyl oligosaccharides. Two new sialyl oligosaccharides in bovine colostrum have been tentatively identified using this method. We found that under ex-

perimental conditions, the α2-3 or α2-6 sialyl linkage could produce different mass spectra in the ESI-MS, which can be utilised to differentiate the two linkages.

2. Experimental

2.1. Materials

Bovine colostrum was obtained at the first milking of Friesian cows at a local dairy farm. Acetonitrile (HPLC grade) was obtained from Scharlau (Barcelona, Spain), Pyridine, glacial acetic acid, and ammonium bicarbonate were from BDH (Poole, UK). Water was purified by a Milli-Q system (Millipore, Milford, MA, USA). α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-D-Glc (3′-SL), α-Neu5Ac-(2 → 6)-β-D-Gal-(1 → 4)-D-Glc (6′-SL), α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-D-GlcNAc (3′-SLN), α-Neu5Ac-(2 → 6)-β-D-Gal-(1 → 4)-D-GlcNAc (6′-SLN), and α-Neu5Ac-(2 → 8)-α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-D-Glc (disialyl lactose or DSL) (all as sodium salts), were purchased from Dextra Laboratories (Reading, UK). Neu5Ac and α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-α-L-Fuc-(1 → 3)-D-Glc (sialyl fucosyllactose or SFL, sodium salt) standards, as well as GF_{254} TLC plates were purchased from Sigma (Steinheim, Germany). Dowex resin (1-X4, 200-400 mesh, Cl^- form) was purchased from Supelco (Bellefonte, PA, USA). Sephadex G-25 (fine) resin was from Pharmacia (Uppsala, Sweden).

2.2. Sample preparation

The mixture of sialyl oligosaccharides was extracted from bovine colostrum using a modification of a published method [5]. In brief, the colostrum sample (500 ml) was skimmed at 4 °C to remove the fat, then an equal volume of methanol was added and the solution was centrifuged at 4 °C. The supernatant was applied to a column of Dowex 1-X4 resin (2.8 cm × 40 cm), which was pre-conditioned with 1 M NaOH. The column was washed with water to remove lactose, and then eluted with a mixture of equimolar pyridine/acetic acid solution (0.1 M). Fractions (5 ml each) were collected and 5 μl of each fraction were spotted on a GF_{254} TLC plate and visualized with the orcinol–H_2SO_4 spray reagent [5]. Appropriate sialyl oligosaccharide containing fractions were combined, reduced to 10 ml by rotary evaporation at 40 °C, and then loaded to a column (2 cm × 100 cm) of Sephadex G-25 resin (fine) for desalting. The appropriate sialyl oligosaccharide containing fractions from the Sephadex column were collected, combined and lyophilised.

2.3. HPLC-ESI-MS

The HPLC analyses were performed using a HP1100 (Agilent Technologies, Wilmington, DE, USA) with autosampler and photodiode array detector, along with a Phenosphere NH_2 column (250 mm × 4.60 mm, 5 μm; Phenomenex, Torrance, CA, USA). The sialyl oligosaccharide mixture (10 mg) was dissolved in 10 ml of water. Solutions of the sialyl oligosaccharide standards were made to 1 mg/ml each. An isocratic mobile phase of acetonitrile and 5 mM ammonium bicarbonate aqueous solution (80/20, v/v) was used. The flow rate was 1 ml/min and the chromatograms were recorded at 195 nm. The injection volume was 20 μl. The outlet from the diode array detector was connected to an on-line splitter, subsequently to a Mariner Mass Spectrometer (PerSeptive, Biosystems, Framingham, MA, USA), using an electrospray source in positive ionization mode with time-of-flight mass analyzer. The splitting ratio was 20:1. The operating parameters were: spray tip potential 3804 V; spray chamber temperature 0 °C; acceleration potential 4000 V; scan range *m/z* 99–1200; seconds per spectrum 1.00.

3. Results and discussion

3.1. Results

HPLC chromatograms of sialyl oligosaccharides extracted from bovine colostrum, based on selected ion monitoring (SIM) results, are shown in Fig. 2. Neu5Ac (retention time 8.7 min) was not detected in the sample. The retention times of two other sialyl oligosaccharide standards, 3′-SLN and SFL (which are not found in bovine colostrum), were 11.9 and 25.9 min respectively. Standards of 3′-SL, 6′-SL, 3′-SLN, 6′-SLN, DSL and SFL were also analysed by HPLC-ESI-MS and the mass spectra are shown in Fig. 3. The mass spectra of the sialyl oligosaccharides from colostrum were virtually the same as that of the standards. Mass spectra of three minor sialyl oligosaccharides, present in the bovine colostrum sample (Fig. 2D–F), are shown in Fig. 4.

3.2. HPLC of sialyl oligosaccharides from bovine colostrum

The four major sialyl oligosaccharides in the bovine colostrum, 3′-SL, 6′-SLN, 6′-SL and DSL, can be well separated using aminopropyl column HPLC-ESI-MS (Fig. 2A–C). It is accepted that the retention of carbohydrates on aminopropyl columns is due to hydrogen bonding between the hydroxyl groups of the sugars and the amine groups of the stationary phase [25]. The 6′-SLN was eluted earlier than 6′-SL, which can be explained by the presence of an acetamido group instead of a hydroxyl group at the C-2 position of 6′-SLN. Previous studies showed that such substitution would reduce the retention time because less hydroxyl groups available for the binding with the stationary phase [26]. The elution of 3′-SLN was immediately before that of 3′-SL, which supports the above hypothesis.

Sialyl oligosaccharides with α2-6 linkages were eluted later than their α2-3 linked isomers, presumably due to the greater flexibility of the α2-6 linkages, which allows an increased interaction of hydroxyl groups with the matrix of the stationary phase [26,27].

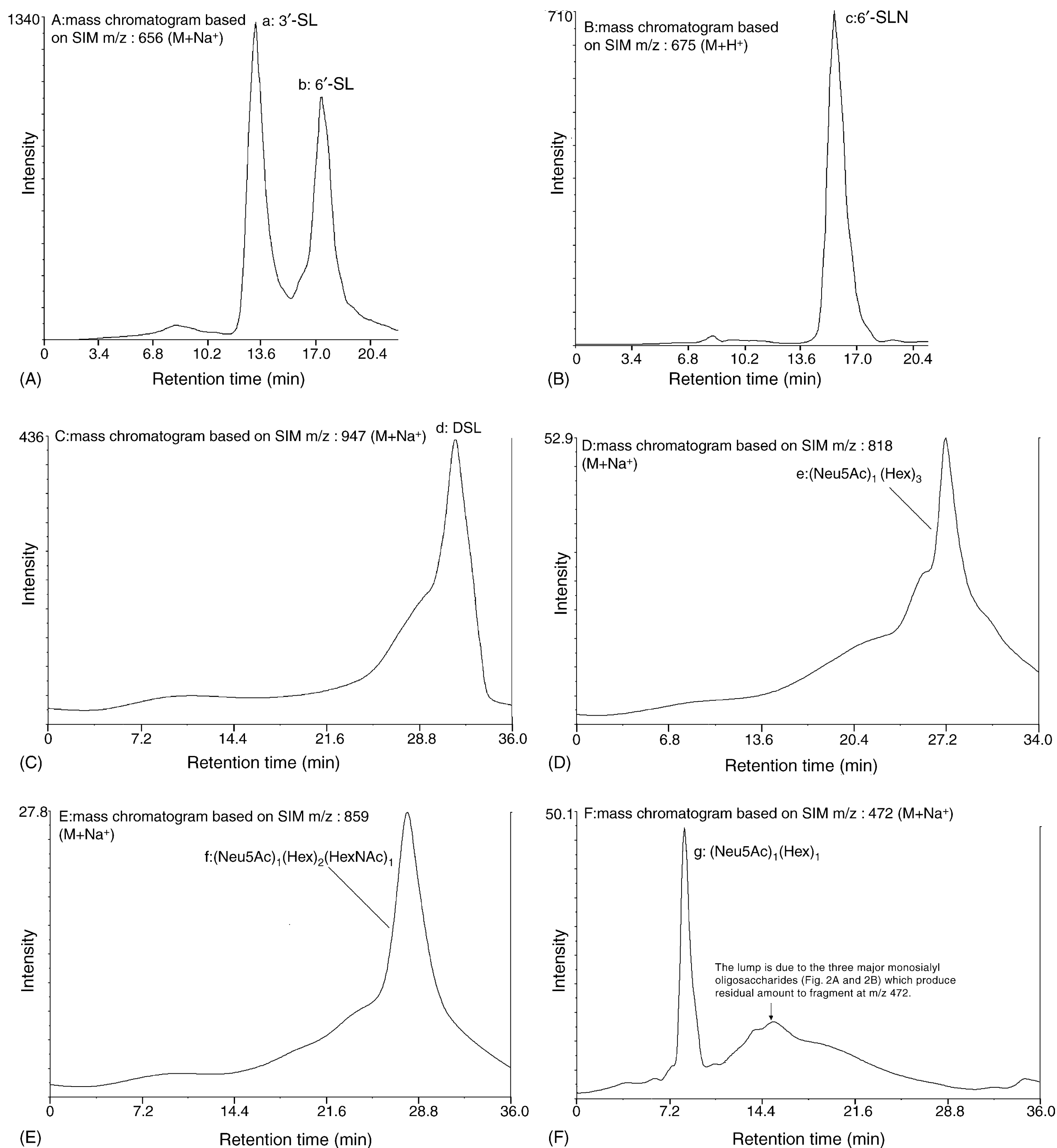

Fig. 2. Mass chromatograms of sialyl oligosaccharides extracted from bovine colostrum. The chromatograms (from the same experiment) were made with selected ion monitoring (SIM) which corresponds to the pseudomolecular mass ($M + Na^+$ or $M + H^+$) of a particular sialyl oligosaccharide. For chromatographic conditions, refer to Section 2.3. Peak identification: (a) 3′-SL; (b) 6′-SL; (c) 6′-SLN; (d) DSL; (e) $(Neu5Ac)_1(Hex)_3$; (f) $(Neu5Ac)_1(Hex)_2(HexNAc)_1$; (g) $(Neu5Ac)_1(Hex)_1$.

Peaks e, f and g (Fig. 2D–F) in the chromatograms of the sample could not be identified by the use of standards. Their intensities in the HPLC chromatograms were roughly two orders of magnitude lower than that of the three major monosialyl oligosaccharides (Fig. 2A and B), which suggests that their concentrations in the bovine colostrum are relatively low. Based on the pseudomolecular mass of peak e (*m/z* 796, Fig. 4A), the structure is postulated to be: $(Neu5Ac)_1(Hex)_3$. A monosialyl oligosaccharide with the same mass as peak e has previously been reported

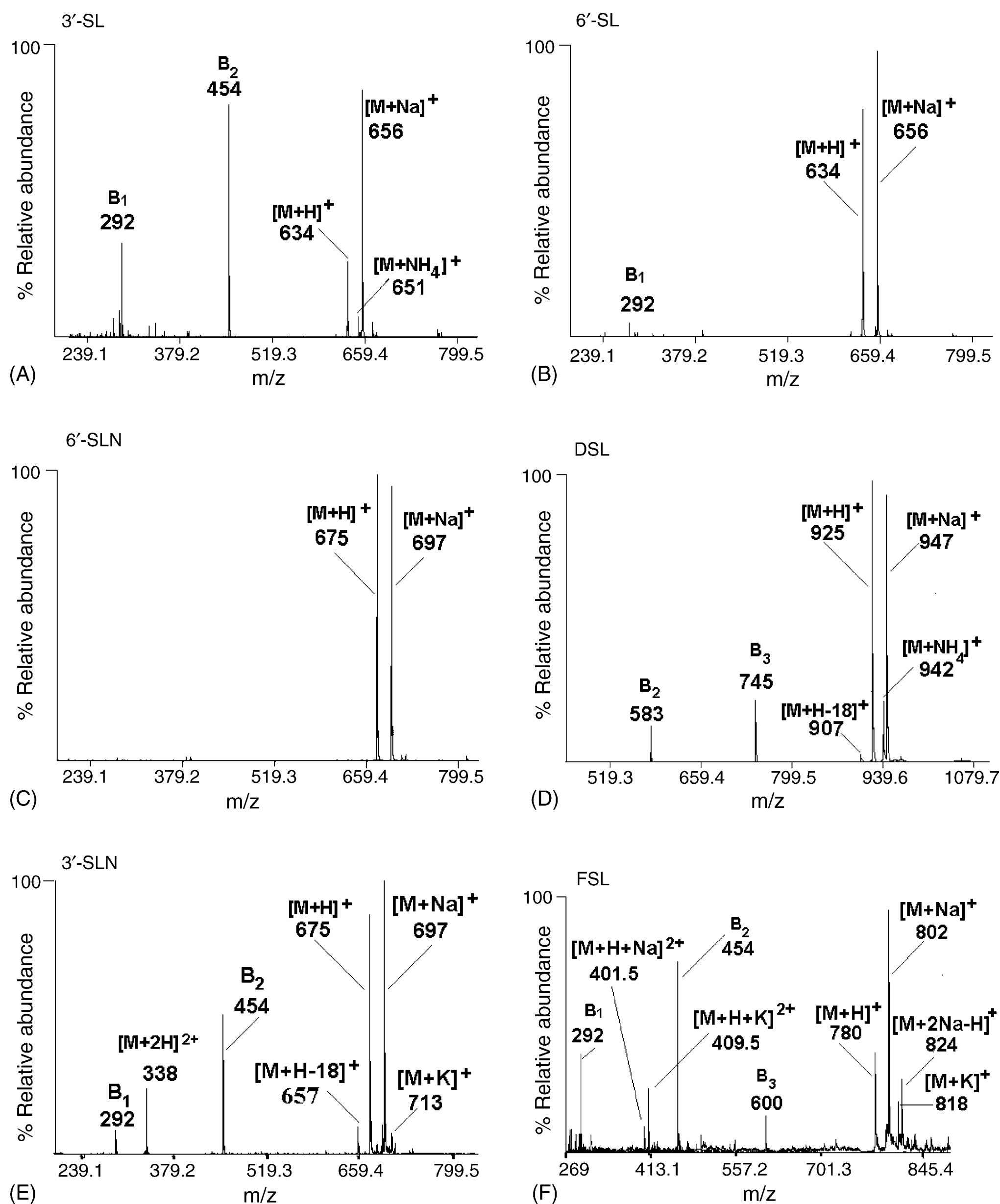

Fig. 3. Mass spectra of sialyl oligosaccharides. For nomenclature of fragmentation, refer to Fig. 1. (A) 3′-SL; (B) 6′-SL; (C) 6′-SLN; (D) DSL; (E) 3′-SLN; (F) SFL.

from bovine colostrum [4]: α-Neu*p*5Ac-(2 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc. It is possible that peak e corresponds to the same compound. The retention time of SFL is 25.9 min, which is slightly shorter than that of peak e. The molecular structure of SFL is very similar to that postulated for peak e, which may explain the close retention times of the two compounds. The longer retention time of peak e can be attributed to its additional hydroxyl group, as discussed earlier.

Peak f with a retention time of 27.9 min is identified as: $(Neu5Ac)_1(Hex)_2(HexNAc)_1$, based on its mass spectrum (Fig. 4B). From the mass spectrum (Fig. 4C), peak g comprises Neu5Ac and Hex units and it is likely to be: $(Neu5Ac)_1(Gal)_1$. In aminopropyl columns, the elution times of oligosaccharides are primarily based on their molecular sizes [9]. The retention time of peak g is 8.9 min, which is between the retention time of Neu5Ac (8.7 min) and 3′-SL (13.0 min). Likewise the retention times of peaks e and f are 27.2 and 27.9 min respectively, which are between that of 3′-SL and DSL (31.9 min). So it appears that the retention times of peak e, f, and g are within the expected range.

The presence of $(Neu5Ac)_1(Hex)_2(HexNAc)_1$ and $(Neu5Ac)_1(Gal)_1$ in bovine colostrum have not been reported before, probably due to their low concentrations.

Peaks e, f and g in the sample could not be detected by the UV detector. Although sialyl oligosaccharides absorb UV more strongly than neutral sugars, they are still quite weak UV absorbing compounds. However, the three peaks

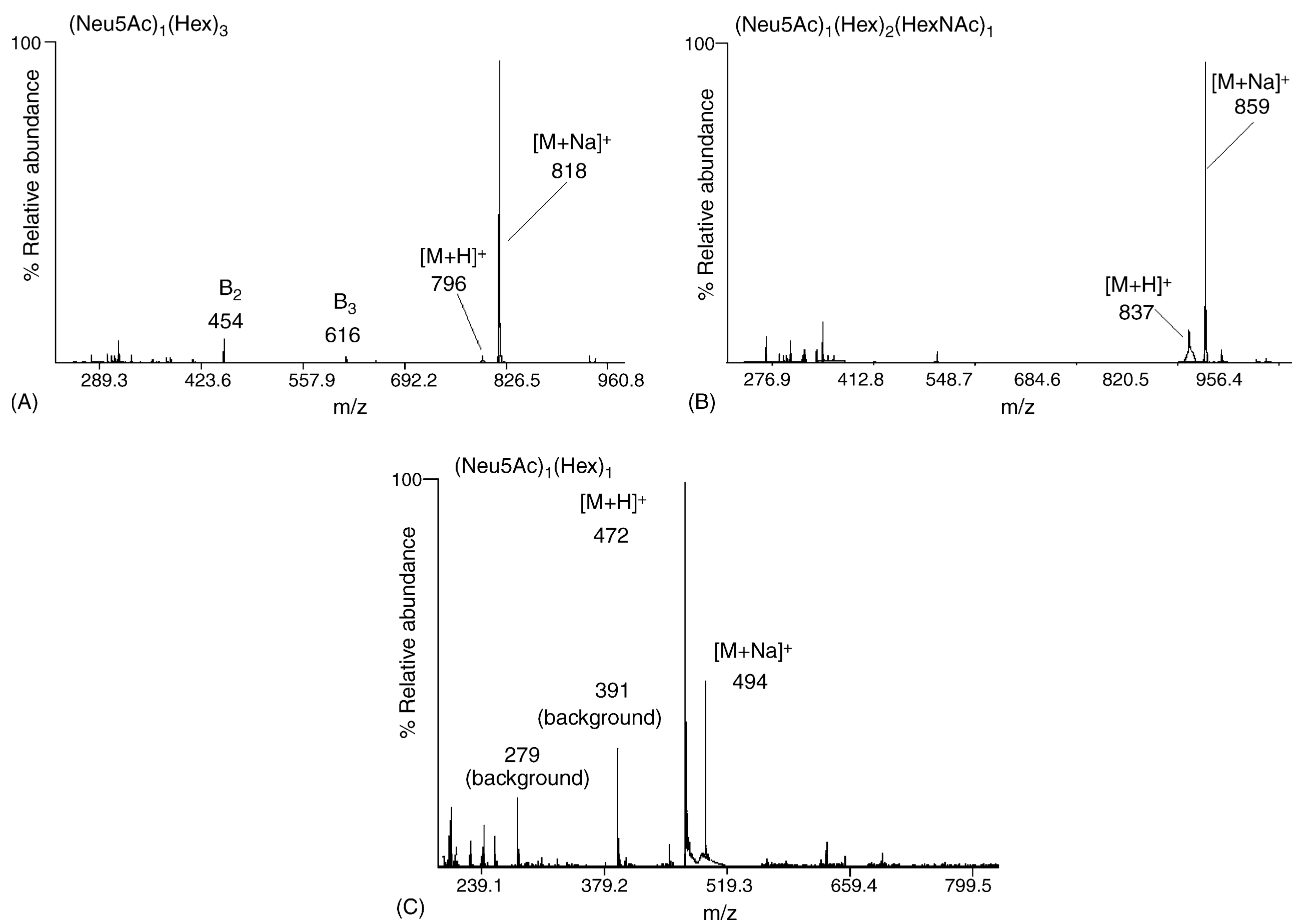

Fig. 4. Mass spectra of three minor sialyl oligosaccharides present in the bovine colostrum sample. Refer to Fig. 2 for the respective retention times. For nomenclature of fragmentation, refer to Fig. 1.

can be readily detected by a MS detector using the SIM method.

3.3. ESI mass spectra of sialyl oligosaccharides

Fig. 3 shows that under the experimental conditions, the base peak for each standard is the pseudomolecular peak of $[M+H]^+$ or $[M+Na]^+$, and in some cases $[M+K]^+$ or $[M+NH_4]^+$ also occurs.

There is a major difference between the mass spectra of α2-3 and α2-6 linked sialyl oligosaccharides (Fig. 3). For 3′-SL (or 3′-SLN), a major fragment is B_2 (see Fig. 1 for fragmentation nomenclature), which is produced by the loss of a Glc (or GlcNAc) residue. But the B_2 fragment is absent from the mass spectrum of 6′-SL (or 6′-SLN). 3′-SL (or 3′-SLN) also produced a much higher proportion of B_1 fragment, compared with 6′-SL (or 6′-SLN). Clearly the linkage difference caused the different fragmentation patterns. The same trend also applies to the spectra of other sialyl oligosaccharides. In DSL, the Neu5Ac residue links to the Gal residue at C-3 position and the fragments B_2 and B_3 are present in the mass spectrum (Fig. 3D). In the case of SFL, the linkage between Neu5Ac and Gal is also α2-3 and two major fragments (B_1 and B_2) can also be found (Fig. 3F). An explanation is that the steric hindrance effect of the α2-3 linkage is quite large because the two sugar units linking to Gal: Neu5Ac and the other sugar (Glc, GlcNAc, or Fuc), are on the same side of the pyranose ring of Gal. In the high voltage field, this steric hindrance causes the removal of either sugar unit on the Gal residue. In contrast, the steric hindrance effect of α2-6 linked sugars is much more reduced because the C-6 is not in the pyranose ring of Gal and has larger freedom of rotation. A previous study [29] utilising circular dichroism suggested that the Gal residue in 6′-SL has essentially the same aglycone behaviour as, say, an ethyl group, whereas 3′-SL presents much more steric interaction which could inhibit eclipsing of the glycosidic oxygen and hence favour the form in which the ring oxygen is eclipsed.

MALDI [22] and ESI [24] studies under negative ionisation mode also found that α2-3 sialyl linkages cleave more easily than α2-6 sialyl linkages, and the B_1 fragment, not the B_2 fragment was observed for α2-3 linked sialyl oligosaccharides. In the present study, although B_1 fragment (or B_2 fragment in DSL) is present in all the spectra of α2-3 linked sialyl oligosaccharides (as well as 6′-SL), the abundance of B_2 fragment (or B_3 in DSL) is significantly higher. The existence of COOH groups makes the glycosidic linkages between the Neu5Ac and other monosaccharide residues re-

markably labile [30], and such linkages are easily cleaved in the mass spectrometric analysis [19,28]. Stability will be even more problematic if the COOH group is negatively charged as in the negative ionisation mode, because of the increased electron withdrawing ability. The present study was conducted under positive mode; this might explain its different fragmentation patterns, as compared to other studies [22,24].

This mass spectrum pattern will be useful for identifying the linkage for unknown sialyl sugars or sugar chains in glycoproteins. Compared with similar studies [22,24], our method has a distinct advantage because the fragment used for identification is present in the α2-3 linkage and clearly absent in the α2-6 linkage sialyl oligosaccharides.

B_2 and B_3 fragments are present in the mass spectrum of peak e (Fig. 4A), but are absent in the spectrum of peak f (Fig. 4B). As discussed earlier, in peak e, Neu5Ac may link to the C-3 position of the Gal residue and in peak f, the linkage could be α2-6, which may also explain that although containing an additional acetamido group, peak f still elutes later than peak e in the HPLC.

4. Conclusions

Aminopropyl column HPLC-ESI-MS is suitable for analysing sialyl oligosaccharides. The sialyl oligosaccharides in bovine colostrum were separated by this system, which has the potential to analyse higher molecular weight sialyl oligosaccharides, which are present in many biological sources. The mass spectra of α2-3 and α2-6 linked sialyl oligosaccharides are different, and can be used for linkage identification.

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