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# Capillary electrophoresis and off-line capillary electrophoresis–electrospray ionization quadrupole time-of-flight tandem mass spectrometry of carbohydrates

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**Abstract**

The use of off-line high-performance capillary electrophoresis in connection with nanospray electrospray ionization quadrupole time-of-flight tandem mass spectrometry for identification of complex carbohydrates of biological origin is presented. The method was applied to the identification of *O*-glycosylated amino acids and -glycopeptides from the urine of patients suffering from a hereditary disease – *N*-acetylhexosaminidase deficiency. Structural elements typical for *O*-glycosylation of proteins, like expression of core 1 and 2 type *O*-glycans with different numbers of *N*-acetyllactosaminyl repeats and different degrees of sialylation, can be directly detected. 

*Keywords:* Capillary electrophoresis–mass spectrometry; Carbohydrates; Peptides

## 1. Introduction

Capillary electrophoresis (CE) is a sensitive separation method for ions, hence ionic carbohydrates as sulfated and sialylated oligosaccharides can be separated directly based on their electric charge/molecular size ratio [1]. For neutral carbohydrate mixtures CE separation often requires the conversion into charged species via complex formation with other ions such as borate [2] or metal cations [2,3]. Another option for ionization of hydroxy groups of the sugars is to raise the pH, where highly alkaline pH electrolyte solutions provide fair conditions for electrophoretic separation in their non-derivatized state [4,5]. Under proper conditions, CE separation may either off-line or directly precede the mass spectrometric analysis resulting in a powerful and sensitive tool of carbohydrate analysis.

The aim of this work was to develop and optimize an appropriate method for CE separation and off-line CE–electrospray ionization quadrupole time-of-flight (ESI-QTOF) MS coupling, in order to assess the feasibility of a direct CE–ESI-QTOF-MS coupling for carbohydrate characterization.

Complex carbohydrates occur ubiquitously in nature, either as oligosaccharides or as glycoconjugates, where the oligosaccharide moiety is linked to a protein and/or lipid. Carbohydrates are involved in many important biological functions such as cell adhesion, cell recognition, cellular differentiation,

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fertilization and embryonic development, tumor development and inflammation. The great discrepancy between the extreme complexity of the structures found in nature and the minute amounts of material available gave rise to massive efforts in development of sensitive and specific methods for their structure elucidation. The complete structural characterization of a complex carbohydrate includes the determination of (a) molecular mass, (b) type and number of sugar components, (c) sequence and patterns of branching, (d) the sites of glycosidic linkages, (e) anomeric configuration of glycosidic linkages, (f) type and conformation of sugar rings and (g) the secondary structure.

Potentials of MS for high-sensitive analysis of non-derivatized complex carbohydrates increased dramatically after introduction of ESI and matrix-assisted laser desorption/ionization (MALDI) desorption methods from one side and possibility to perform tandem MS experiments for sequencing on the other [6]. In particular, for analysis of native glycopeptides and glycolipids, nanospray ESI (nanoESI)-QTOF-MS–MS in the positive as well as in the negative ion mode was recently shown to be capable in sequencing subpicomolar amounts of biological material, and providing direct information on carbohydrate structure determinants (a)–(d) [7–9]. A major feature in setting up the CE–MS coupling is the compatibility of the CE buffer to ESI-MS, which has to ensure both an optimal electrophoretic separation and an appropriate ionization and desolvatation. The best CE separations of carbohydrates were given, so far, by CE electrolytes based on sodium borate and sodium phosphate, both buffers which are incompatible with ESI-MS ionization requirements.

In order to optimize a direct coupling between CE and MS for carbohydrates identification, Deuteil et al. [10] have shown that aqueous ammonium acetate buffer is CE electrolyte consistent with the requirements of the coupling. Using 40 m*M* ammonium acetate (pH 3.5 and 9.2) as a CE carrier, Deuteil et al. optimized a direct coupling between CE and ESI-MS which was tested on a standard mixture of heparin disaccharides. CE sensitivity reached in these experiments was 0.25 mg/ml ammonium acetate electrolyte (original solution) but no specification related to the injected amount was given.

## 2. Experimental

### 2.1. Method and instrumentation

CE experiments were carried out on a P/ACE™ 5000 series instrument (Beckman, Fullerton, CA, USA) equipped with a UV detector (deuterium lamp, 2 nm wavelength accuracy, 190–380 nm wavelength range with filter selection). The electrophoresis capillaries used in these experiments were polyamide coated fused-silica tubing (BGB Analytik Vertrieb, Germany) with 50 μm I.D.x 375 μm O.D. and an overall length of 57 cm. Each CE capillary was conditioned daily prior to experimental by rinsing it for 20 min with 19 *M* aqueous ammonium hydroxide, 20 min with methanol and then for 30 min with the running buffer. The running buffer used was 5 m*M* aqueous citric acid with ammonium pH adjustment (pH 10.4) which provided a very good CE separation and a stable signal in all CE experiments performed on carbohydrates. All solutions were filtered through 0.2-μm membranes and degassed before use.

Before the injection of each sample, the capillary was rinsed for at least 5 min with the CE electrolyte. Samples were hydrodynamically injected by applying a constant nitrogen high pressure of 0.5 p.s.i. for 3, 4 and 6 s, respectively. The separation voltages of +15 kV (for $BP_2$-$1Py_2$ in CE separation experiment), +20 kV and +30 kV ($BP_2$-$1Py_2$ separation in off-line CE–MS coupling experiments) and +20 kV (β-galactopyranosyl-D-arabinose in both CE separation and separation in off-line CE–MS coupling experiments) respectively were applied to the inlet of the capillary together with the pressure of 0.5 p.s.i. UV absorption at 214 nm (in all cases) was recorded, the detection window being located 7 cm from the outlet end of the CE capillary. The temperature of the capillary cartridge was set to 20°C for all experiments.

### 2.2. CE electrolyte

CE separation experiments were performed on the standard β-galactopyranosyl-D-arabinose disaccharide in the following conditions: 40 m*M* ammonium acetate (pH 10.0) as the CE carrier, 1 mg/ml buffer original concentration, injection time 3 s, $3 \cdot 10^{-5}$

mg/ml buffer concentration of the injected solution did not emphasize, so far, the electrophoretic separation. For this reason, in this work, the CE separation and off-line CE–ESI-QTOF-MS experiments were carried out using aqueous citric acid as the CE electrolyte. Previously, pH conditions were established for sodium borate buffer and they turned out to be appropriate with the new CE buffer. The concentration of the citric acid solution was then gradually decreased during the CE experiments from 20 m*M* down to 5 m*M* in order to establish the lowest possible concentration limit the CE experiment can be performed with. It was found that a 5 m*M* citric acid concentration was fit for both CE separation and ESI-MS analysis.

### *2.3. Mass spectrometry*

MS was carried out on a hybrid QTOF (Micromass, Manchester, UK) instrument detecting negative ions. NanoESI experiments were carried out using self-pulled omega glass capillaries produced on a vertical pipette puller, model 720 (David Kopf Instruments, Tujanga, CA, USA) by an 'omega' shape filament. MS–MS was performed at 30–35 eV and 10 p.s.i. argon (1 p.s.i.=6894.76 Pa).

### *2.4. Carbohydrate samples*

β-galactopyranosyl-D-arabinose was purchased from Sigma (St. Louis, MO, USA). $BP_2$-$1Py_2$ mixture of *O*-glycosylated peptides was obtained from the urine of patients suffering from *N*-acetylhexosaminidase deficiency as described earlier [11,12].

## 3. Results

### *3.1. CE experiments on β-galactopyranosyl-D-arabinose*

β-Galactopyranosyl-D-arabinose sample was dissolved in the CE running buffer to a concentration of 1 mg/ml original solution and $4 \cdot 10^{-5}$ mg/ml injected solution and then injected into the CE capillary by applying the operating pressure for 4 s to the inlet of the capillary. The applied voltage was 20 kV generating a constant electric current of 15.6 μA. UV absorption at 214 nm was recorded and the separation time was 20 min. Under the described conditions, the retention time of β-galactopyranosyl-D-arabinose was 6.66 min. In addition, the electropherogram displays one peak that corresponds to an unknown compound having the retention time of 14.08 min (Fig. 1), which was not identified by off-line CE–ESI-QTOF-MS experiments mainly because of the low sensitivity obtained under off-line conditions.

### *3.2. Off-line CE–ESI-QTOF-MS experiments on β-galactopyranosyl-D-arabinose*

For β-galactopyranosyl-D-arabinose samples an off-line CE–ESI-QTOF-MS experiment was performed. For this purpose, β-galactopyranosyl-D-arabinose ($2 \cdot 10^{-3}$ mg/ml buffer injected, for an injection time of 4 s, 50 mg/ml buffer – the original solution) was injected into the inlet end of the CE capillary and successively collected in ten CE fractions as follows: 3.6 min–I, 6.8 min–II, 9 min–III, 12.2 min–IV, 15.2 min–V, 18.0 min–VI, 22.3 min–VII, 25.8 min–VIII, 27.5 min–IX, 30 min–X. All fractions were analyzed by ESI-QTOF-MS and MS–MS to identify the fraction containing the sugar analyte. The analyte was detected in fraction II corresponding to 6.8 min collection time.

In the off-line CE–ESI-QTOF-MS experiments performed on 2.5 and 5 mg/ml under injection time 4 s, no sugar ions were detectable in the ESI-QTOF-MS.

### *3.3. CE experiments on $BP_2$-$1Py_2$*

The $BP_2$-$1Py_2$ sample was submitted to the first CE experiment in 5 m*M* (pH 10.4) aqueous citric acid as a CE electrolyte in a concentration of 1 mg/ml CE buffer by applying the operating pressure for 3 s and a separation voltage of 15 kV, which generated a constant current of 10.4 μA. The separation time was 30 min. Nine significant peaks were recorded under the above conditions (Fig. 2).

### *3.4. Off-line CE–ESI-QTOF-MS experiments on $BP_2$-$1Py_2$ sample*

At a concentration of 25 mg/ml of original

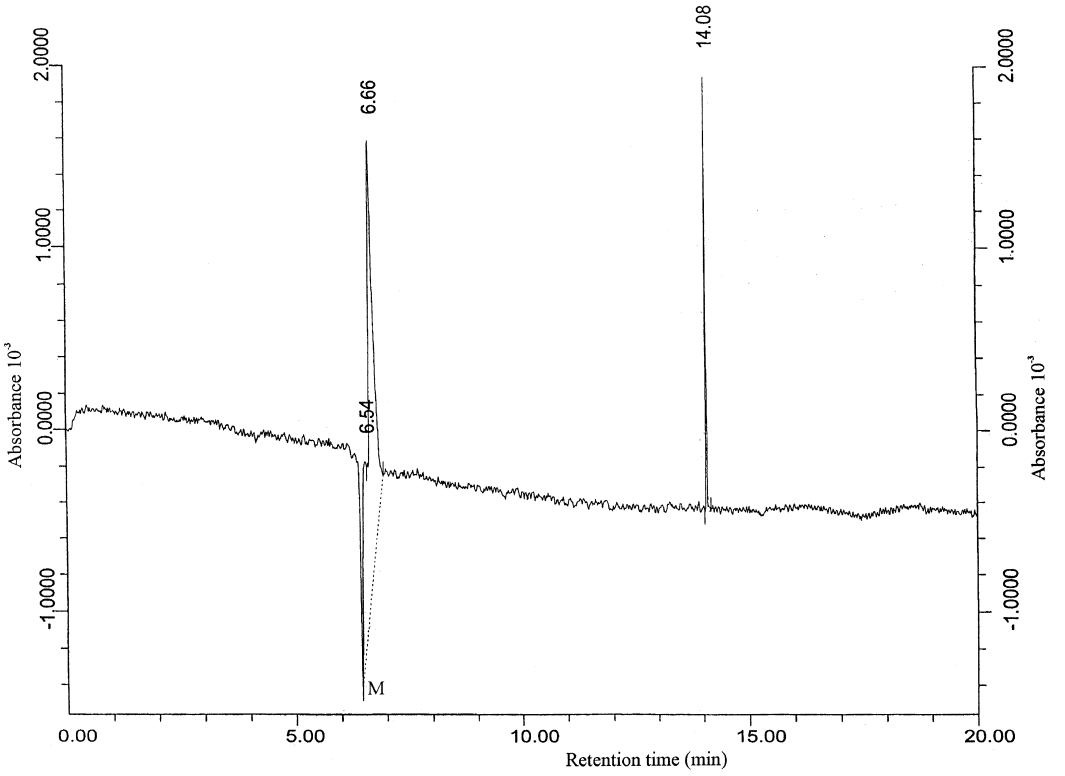


Fig. 1. CE profile of galactopyranosyl-D-arabinose disaccharide (1 mg/ml in 5 m*M* aqueous citric acid). Capillary coated fused-silica (57 cm×50 μm I.D.×375 μm O.D.). Applied potential +20 kV; carrier=5 m*M* aqueous citric acid (pH 10.4); detection UV=214 nm; I=15.6 A; M=methanol.

solution, $1.00 \cdot 10^{-3}$ mg/ml injected solution by applying the injection pressure for 4 s, in the CE running buffer, 5 m*M* aqueous citric acid (pH 10.4), $BP_2$-1 $Py_2$ components were not detectable by ESI-QTOF-MS.

At a concentration of 35 mg/ml original solution, $1.05 \cdot 10^{-3}$ mg/ml injected solution and operating pressure for 4 s, $BP_2$-1 $Py_2$ was detectable only by the single diagnostic ion at *m/z* 308–290 (NeuAc) by ESI-QTOF-MS presence in the third fraction corresponding to a collection time of 6.5 min.

Under the same buffer conditions, $BP_2$-1 $Py_2$ in 80 mg/ml original solution was injected by applying the injection pressure for 3 s, a voltage of 20 kV to the inlet end of the CE capillary and collected in five fractions: 6 min–I, 9.2 min–II, 15 min–III, 21 min–IV, 30 min–V. In the first fraction, three molecular ions from *O*-glycopeptides were detected by ESI-QTOF-MS indicating incomplete CE separation. Gradually improved fractionation in the next experiment yielded five samples: 3 min–I, 6 min–II, 9 min–III, 12 min–IV, 30 min–V. ESI-QTOF-MS and tandem MS–MS experiments carried out on these fractions emphasized $BP_2$-1$Py_2$ separation in the first corresponding to 3 min and the second corresponding to 6 min collection time. Finer fractionation yielded seven samples: 1 min–I, 2 min–II, 3.5 min–III, 5 min–IV, 8 min–V, 15 min–VI, 30 min–VII, where $BP_2$-1$Py_2$ related structures were detected by ESI-QTOF-MS and tandem MS–MS in the third (3.5 min) and the fifth (8 min). Further optimizing the separation by increasing the voltage to 30 kV and the injection time to 6 s gave seven different fractions: 1 min–I, 2 min–II, 3.5 min–III, 5 min–IV, 8 min–V, 15 min–VI, 30 min–VII. $BP_2$-1$Py_2$ related ions were now detected by ESI-QTOF-MS and

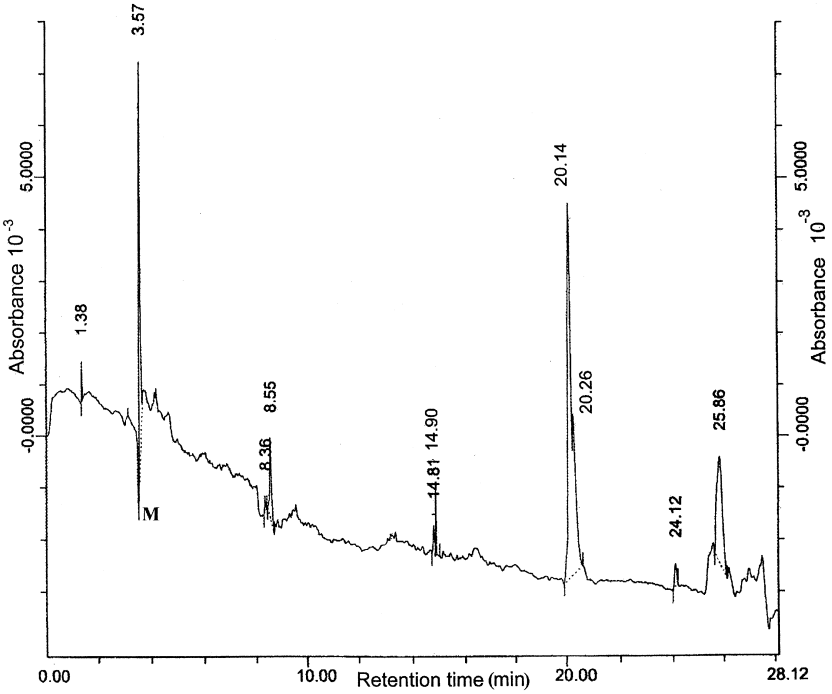


Fig. 2. CE profile of $BP_2$-$1Py_2$ mixture of glycosylated amino acids (1 mg/ml in 5 m*M* aqueous citric acid). Capillary coated fused-silica (57 cm×50 μm I.D.×375 μm O.D.). Applied potential=+15 kV; carrier=5 m*M* aqueous citric acid (pH 10.4); detection UV=214 nm; I=10.4 A; M=methanol.

tandem MS–MS in the second (2 min) and the third (3.5 min), however, with higher sensitivity.

Negative ion mode ESI-QTOF-MS of the third fraction (3.5 min) revealed a number of ions related to glycosylated sialylated peptides (Fig. 3). The most prominent ions are assigned to molecular species originating from the tetrasaccharide $NeuAc_2GalGalNAc$, which is *O*-linked to either Ser, Thr or Thr–Pro. The assignment of the singly and doubly charged ions depicted in Fig. 3 and derived from the tetrasaccharide species is listed in Table 1. Furthermore, low-energy collision-induced dissociation (CID) experiments were carried out using these molecular ions as precursors to detect such fragment ions, which will provide further structural information.

The efficiency of fragmentation can be a crucial parameter for structural assignment in such CE fractions, in which only a low amount of material is present. This parameter was probed by testing the singly and doubly charged ions as precursors for MS–MS fragmentation analysis. In Fig. 4 the tetrasaccharide component $NeuAc_2GalGalNAc$–Ser showing CID decay of $[M\text{-}H]^-$ at $m/z$ 1051 (top

Table 1
Peak assignment for negative ion MS and MS–MS of *O*-glycosylated tetrasaccharide peptides from mixture $BP_2$-$1Py_2$

| $m/z$ | Assignment | Negative charge |
|---|---|---|
| 290 | NeuAc–$H_20$ | 1 |
| 308 | NeuAc | 1 |
| 469 | GalGalNAc–Ser | 1 |
| 470 | NeuAcGal | 1 |
| 483 | GalGalNAc–Thr | 1 |
| 673 | NeuAcGalGalNAc | 1 |
| 760 | NeuAcGalGalNAc–Ser | 1 |
| 774 | NeuAcGalGalNAc–Thr | 1 |
| 1051 | NeuAcGalGalNAc–Ser | 1 |
| 1065 | NeuAcGalGalNAc–Thr | 1 |
| 1163 | NeuAcGalGalNAc–Thr–Pro | 1 |
| 525 | NeuAcGalGalNAc–Ser | 2 |
| 532 | NeuAcGalGalNAc–Thr | 2 |
| 582 | NeuAcGalGalNAc–Thr–Pro | 2 |

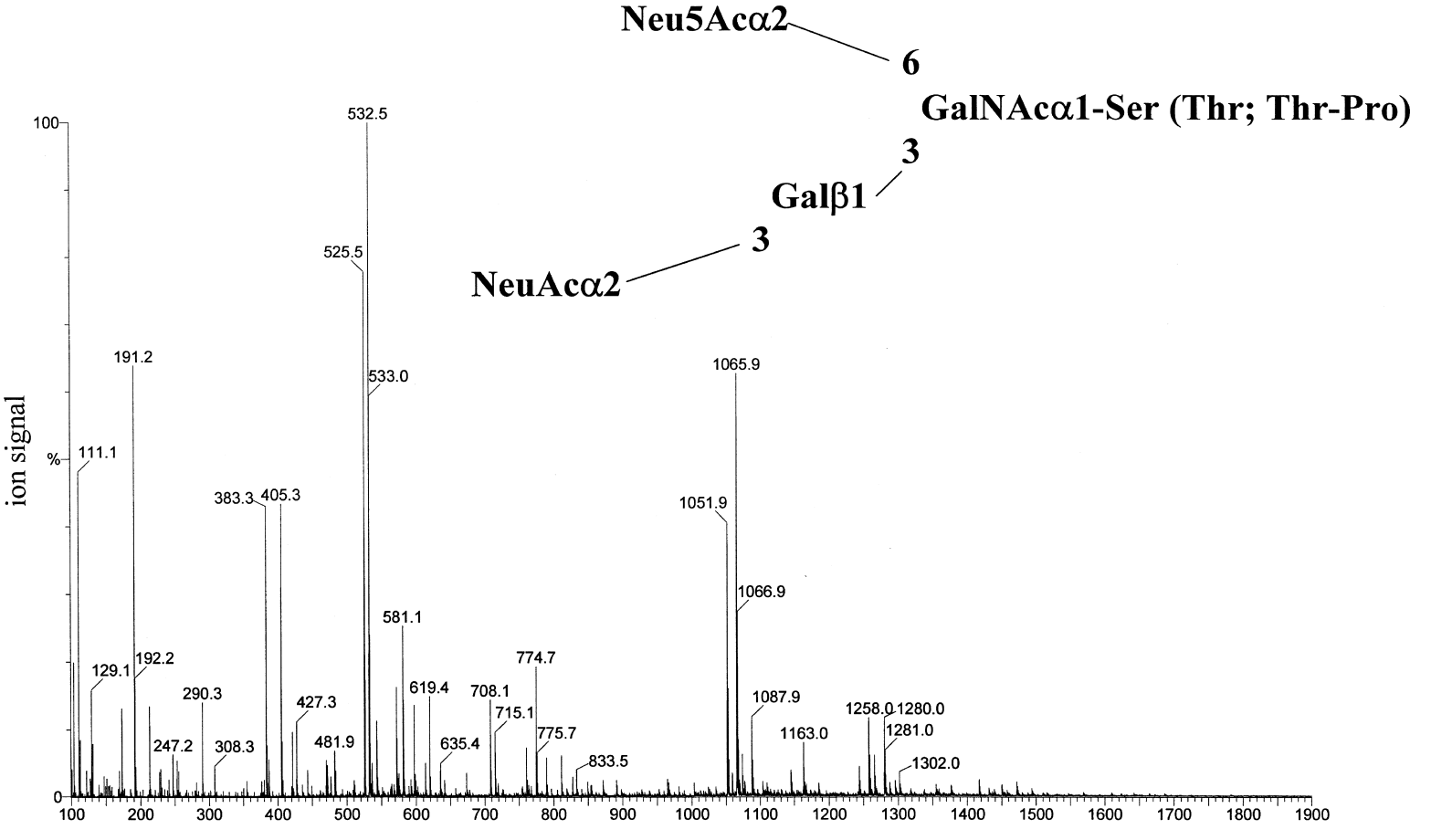


Fig. 3. Negative ion off-line CE–nanoESI-QTOF-MS of the third fraction at 3.5 min retention time. The list of $m/z$ values for abundant ions is given in Table 1.

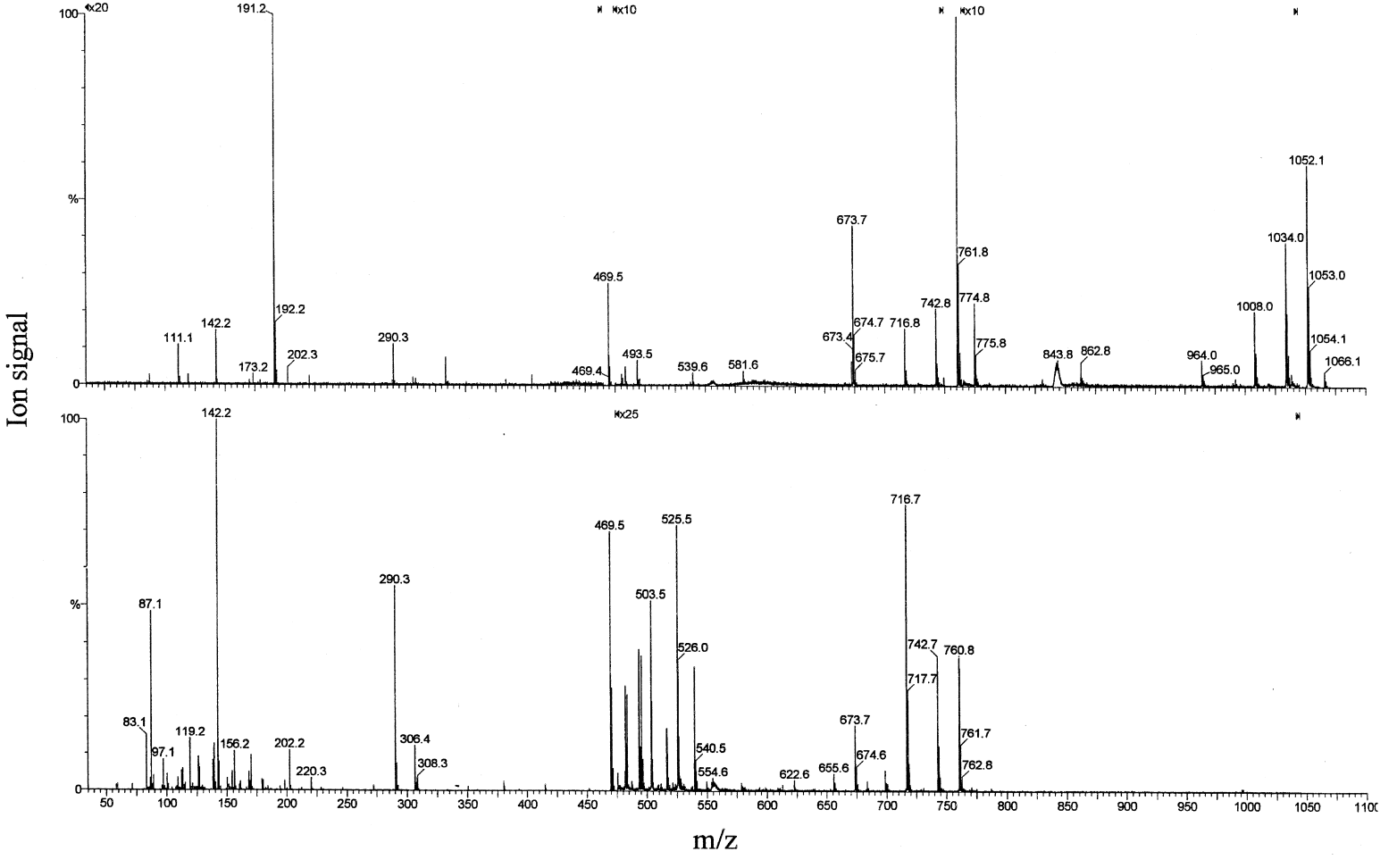


Fig. 4. Negative ion off-line CE–nanoESI-QTOF-MS–MS of $[M\text{-}2H]^{-2}$ (top trace) and $[M\text{-}H]^{-2}$ (bottom trace) arising from the $NeuAc_2GalGalNAc$–Ser at $m/z$ 1051 and 525, respectively. The $m/z$ values for abundant ions are listed in Table 1.

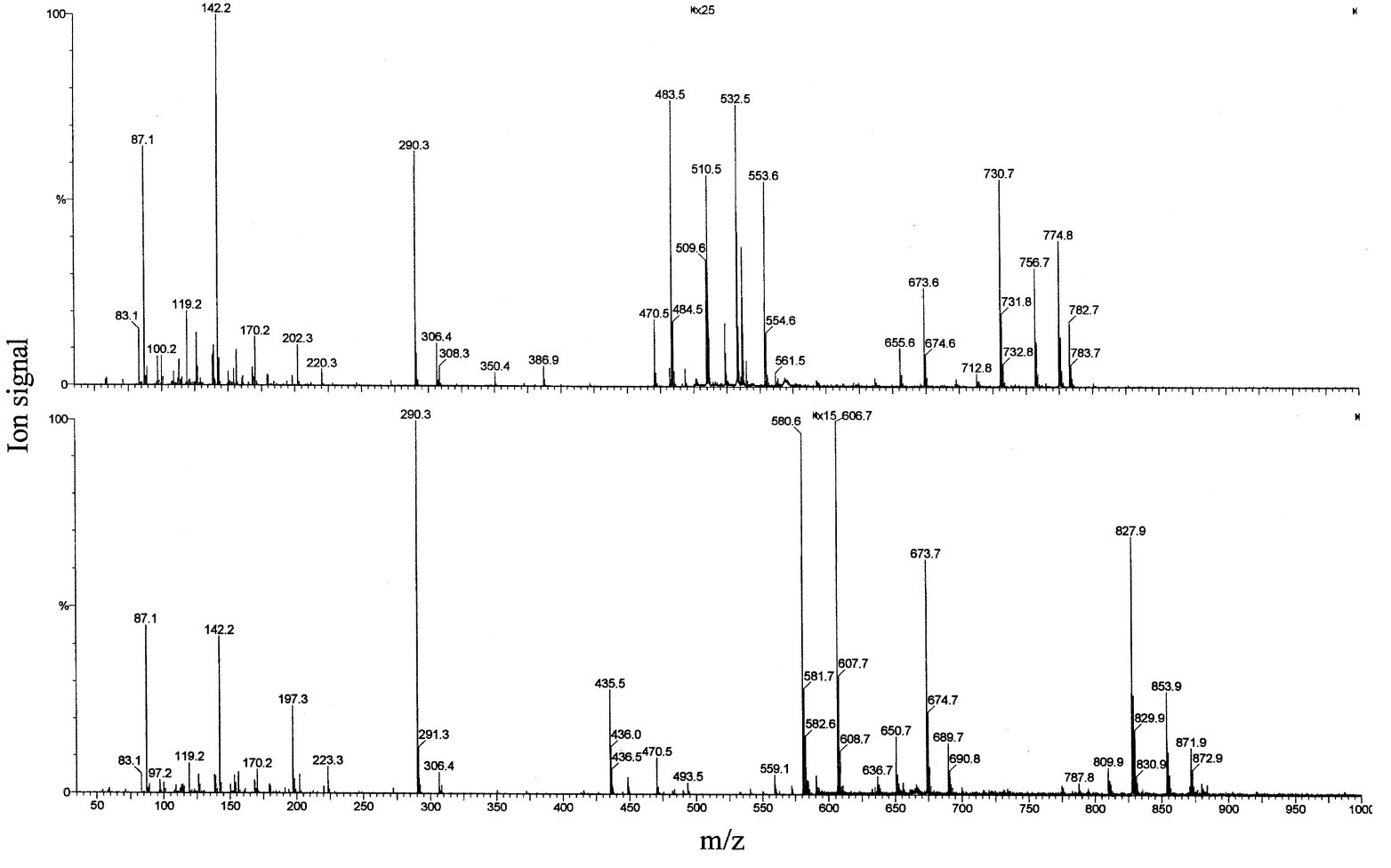


Fig. 5. Negative ion off-line CE–nanoESI-QTOF-MS–MS of $[M\text{-}2H]^{-2}$ arising from the $NeuAc_2GalGalNAc$–Thr at $m/z$ 532 (top trace) and $NeuAc_2GalGalNAc$–Thr–Pro at $m/z$ 582 (bottom trace). $m/z$ values for abundant ions are listed in Table 1.

trace) and $[M\text{-}2H]^{-2}$ at $m/z$ 525 (bottom trace) is presented, which in both cases correlate to formation of the Y-type ions arising from the sequential loss of NeuAc entities at $m/z$ 760 and 469. The 3-linked branch is documented by the C-ion $NeuAcGal^{-}$ at $m/z$ 470. Besides, double cleavages yield characteristic core ions, like the C-ion at $m/z$ 673 ($NeuAcGalGalNAc^{-}$), which can be used diagnostically.

Fragmentation patterns of the $[M\text{-}2H]^{-2}$ arising from the $NeuAc_2GalGalNAc$–Thr at $m/z$ 532 (top trace) and $NeuAc_2GalGalNAc$–Thr–Pro at $m/z$ 582 (bottom trace), respectively, are presented in Fig. 5. The shift of 97 u between the two sets of Y-ions and the identical C-ions between these two species provide data for a validity of this concept.

## 4. Conclusions

Current status of the off-line CE–ESI-MS experiments can be positively summarized concerning the compatibility of the citric acid as a CE buffer and injection conditions, related to identification of complex carbohydrates like the neutral disaccharide β-galactopyranosyl-D-arabinose and the acidic *O*-glycosylated aminoacids and peptides from the $BP_2$-$1Py_2$ fraction. In this contribution, it is clearly illustrated that for carbohydrate samples containing impurities, in particular salts, a citric acid buffer is a superior and compatible ESI spraying agent in comparison to the neutral methanol–water mixtures.

The limitation by the off-line approach by which both the voltage and the operating pressure need to be turned off between two consecutive collections, causing the diffusion of the sample and compromising the separation process, can be handled in relation to the options of ESI-QTOF-MS–MS for analysis of mixtures. The advantage of sequencing single components from the mixtures using their molecular ions as precursors and applying low-energy CID to obtain structure-relevant fragment ions can offer significant perspectives for samples arising from incomplete separation.

The choice of the negative ion mode for analysis of complex carbohydrates was already demonstrated to be advantageous for both neutral and acidic substrates [6]. According to the data that is presented here, we have demonstrated the feasibility of this approach toward a direct CE–ESI-QTOF-MS coupling and indicate the factors influencing the separation and identification of complex carbohydrates. Furthermore, the on-line approach promises to show a significant extention of the sensitivity limit for detection of minor components in complex carbohydrate mixtures.

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