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Positive and negative electrospray ionisation travelling wave ion mobility mass spectrometry and low-energy collision-induced dissociation of sialic acid derivatives

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Mono- or oligosaccharide-containing samples, whether they are derived from biological sources or products of chemical synthesis, are often mixtures of spatial or constitutional isomers. The possibility of characterising or performing quality control on such samples by mass spectrometry is hampered because these isomers cannot be separated by their mass-to-charge ratio alone. Therefore, the use of techniques to separate the isobaric sample compounds prior to mass spectrometric characterisation is mandatory. Travelling wave ion mobility separation offers the possibility of separating mixtures based on their compound's collisional cross-sections in the gas phase and can easily be combined with mass spectrometry for further characterisation. Here, we use 5-*N*-acetylneuraminic acid and several derivatives as model compounds to evaluate the separation power of travelling wave ion mobility spectrometry and present an approach to clearly identify constitutional isomers in mixtures in combination with low-energy collision-induced dissociation (CID) in the negative ion mode even if they cannot be completely separated by ion mobility.

For several decades, ion mobility spectrometry was mainly used to analyse small volatile molecules most notably for detection of explosives or chemical warfare agents.[1,2] By combining ion mobility separation and mass spectrometry, analytical instruments were developed, which allowed the characterisation of analyte ions based on their three-dimensional structure or more exactly the collisional cross-section as well as their mass/charge ratio. These instruments – many of them being home-built or otherwise customised – were of very heterogeneous designs as they combined different types of ion sources, ion mobility spectrometers and mass analysers.[2–4] Ion mobility mass spectrometry has now been successfully applied to study structural or conformational isomers as well as complex samples, where it allows the partitioning of compounds by substance class or charge state.[5] The samples that were studied covered a broad range of molecular weights from small, even chiral, molecules,[6] complex peptide mixtures,[7,8] synthetic polymers,[9] and proteins up to intact protein assemblies.[10,11] The characterisation of sugars, oligosaccharides or glycans is especially challenging for mass spectrometry alone, due to the sheer number of isobaric species arising from constitutional and spatial isomerism, which often exist simultaneously and in equilibrium. When characterising such samples, the option to additionally separate the compounds based on their collisional cross-section is quite valuable. Consequently, many studies utilising different types of ion mobility separators were performed on mono- and oligosaccharides or glycoconjugates. Using an instrument equipped with a drift-tube-type ion mobility spectrometer operated at ambient pressure, the separation of differently linked di- and trisaccharides[12,13] was shown, as well as the separation of anomeric species of monosaccharides.[14] Using a high-field asymmetric waveform (FAIMS)-type ion mobility separator, the possibility of separating linkage and position isomers of disaccharides was shown.[15] Since the introduction of a travelling wave (T-wave) type ion mobility mass spectrometer by Waters,[16] several studies using this instrument to analyse carbohydrates have been presented. The possibility of distinguishing protein-derived glycan isomers,[17] and differently linked tri- and hexasaccharides,[18] and of significantly reducing the complexity of mass spectra when analysing glycoconjugate mixtures of extreme heterogeneity[19] was shown.

This study focuses on the use of ion mobility mass spectrometry (IM-MS) to analyse mixtures of constitutional isomers of sialic acids, which are a large family of derivatives of neuraminic acid.[20] Most of these compounds are found as components of glycoproteins,[21] glycolipids,[22] or as part of bacterial cell walls.[23,24] A common modification of sialic acids is *O*-acetylation at carbons 4, 7, 8 or 9.[25] A number of bacterial pathogens use sialic acids. While *E. coli* K1, *N. meningitides* and *C. jejuni* obtain sialic acids by *de novo* synthesis, others, like *H. influenzae*, *N. gonorrhoeae* and *E. coli* K12, are able to take up free sialic acids from the environment by specific transporters.[26] To study the catabolism of *O*-acetylated sialic acids in bacteria, pure preparations of sialic acid derivatives are required. Using such preparations, Steenbergen *et al*. recently identified the YihS gene product of *E. coli* required for growth on 9-*O*-acetylated sialic acid.[27]

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There are several other chromatographic or spectroscopic methods available, which can be used to separate such mixtures and characterise the components. Nuclear magnetic resonance (NMR) is widely used for structural elucidation or confirmation of synthesis products and would allow the determination of the absolute configuration. However, compared with MS-based methods, NMR is slow, needs high amounts of sample and is not generally applicable to even simple mixtures.[28] Chromatography-based methods like high pH anion-exchange chromatography[29] or gas chromatography[30] have already been used to separate sialic acids. These methods either require sample derivatisation or suffer from the need to use volatile solvent compounds when directly coupled to mass spectrometers.

EXPERIMENTAL

Materials

All chemicals were of analytical grade (p.a.). *N,N*-Dimethyl-4-pyridinamine (DMAP) (>98%), 2,2-dimethoxypropane (98%), *p*-toluenesulfonic acid monohydrate (98,5%) and acetic anhydride (98%) were purchased from Sigma Aldrich (Deisenhofen, Germany). The cation-exchange (50W-X8 resin, 20–50 mesh, hydrogen form) and the anion-exchange material (1-X8, 100–200 mesh, formate form) were from Bio-Rad Laboratories (Hercules, CA, USA). Silica gel N (without binder, for thin layer chromatography) was purchased from Machery-Nagel (Düren, Germany). Sodium hydroxide, acetone, methanol, 2-propanol, methylene chloride, formic acid and acetic acid were from Fluka (Buchs, Switzerland). Neu5Ac was obtained from Codexis (Jülich, Germany). 8,9-*O*-Isopropylidene-5-*N*-acetylneuraminic acid (Neu5Ac-8,9-protected) was an intermediate product of the Neu4,5Ac$_2$ synthesis. 5-*N*-Acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac$_2$) was obtained from Applied Biotech (Salzburg, Austria).

Synthesis of 5-*N*-acetyl-4-*O*-acetylneuraminic acid and 5-*N*-acetyl-8,9-*O*-isopropylideneneuraminic acid

The synthesis of 5-*N*-acetyl-4-*O*-acetylneuraminic acid was performed according to Ogura *et al.*[31] with a modification in the acetylation step, where DMAP was used as catalyst instead of pyridine. Briefly, Neu5Ac (1.00 g, 3.23 mmol) and 1.0 g Dowex 50W X-8 (H^+) cation-exchange resin were stirred in 30 mL methanol for 16 h at room temperature. The ion-exchange resin was removed by filtration and the solution evaporated in vacuum to obtain Neu5Ac1Me (780 mg, 75 %). Neu5Ac1Me (780 mg, 2.41 mmol) was dissolved in 15 mL acetone. After addition of 2,2-dimethoxypropane (1.20 g, 11.5 mmol) and 1.5 mg *p*-toluenesulfonic acid the suspension was stirred for 1 h at room temperature. Then, 0.2 g of Dowex 1-X8 (formate) anion-exchange resin was added and stirred for 15 min at room temperature. The ion-exchange resin was removed by filtration and the solution was evaporated in vacuum to obtain 8,9-*O*-isopropylidene-Neu5Ac1Me (850 mg, 97%). 8,9-*O*-Isopropylidene-Neu5Ac1Me (730 mg, 2.00 mmol) was suspended in 10.0 mL 0.2 M NaOH (2.00 mmol) and stirred for 4 h at room temperature. The solution was neutralised with 0.5 g of Dowex 50 W X-8 (H^+) cation-exchange resin. The ion-exchange resin was filtered off and the filtrate evaporated in vacuum to obtain 8,9-*O*-isopropylidene-Neu5Ac (620 mg, 89%, used for MS analysis). 8,9-*O*-Isopropylidene-Neu5Ac (200 mg, 0.57 mmol) was suspended in 8 mL methylene chloride. DMAP (167 mg, 1.37 mmol) and acetic anhydride (500 mg, 4.9 mmol) were added and the suspension stirred at room temperature for 2 h. The reaction mixture was extracted with H_2O (4 × 1 mL) and the aqueous phase re-extracted once with methylene chloride (1 mL). The combined H_2O phases containing 8,9-*O*-isopropylidene-Neu4,5Ac$_2$ were used in the next step without isolation. To the aqueous solution of 8,9-*O*-isopropylidene-Neu4,5Ac$_2$ 4 mL of concentrated acetic acid were added and stirred for 3 h at 60 °C. After evaporation of the solvent in vacuum the resulting crude Neu4,5Ac$_2$ was dissolved in 1 mL of 1-propanol/H_2O (3:1, v/v), loaded onto a short silica gel column and chromatographed by dry-column flash chromatography. Elution was started with pure 1-propanol followed by 90%, 80% and 75% 1-propanol/H_2O (v/v) as eluents. The column was sucked to dryness after each fraction of the solvent. Fractions containing Neu4,5Ac$_2$ were pooled and evaporated to obtain Neu4,5Ac$_2$ (71 mg, 35% over 2 steps).

MS and MS/MS measurements

All mass spectrometric and ion mobility mass spectrometric measurements were carried out using a SYNAPT HDMS instrument of IM/QRTOF geometry (Waters, Manchester, UK) equipped with a LockSpray dual electrospray source. The source conditions were as follows: source temperature: 80 °C; desolvation gas: nitrogen (flow rate: 600 L/h, temperature: 250 °C); cone gas: nitrogen (flow rate: 50 L/h). The acquisition parameters were: electrospray voltage: 2.0–2.5 kV; extraction cone voltage: 2.5 V, Ar flow rate: 1.5 mL/min (resulting in a pressure of 0.01 mbar in the trap/transfer cell); trap and transfer CE (collision energy) adjusted as needed to avoid unwanted fragmentation in MS mode (typically 1–2 eV) and induce fragmentation in MS/MS mode (typically 4–12 eV). The samples were dissolved at 0.0025 mg/mL in methanol/water, 1: 1 (v/v) (negative mode) and 0.01 mg/mL in methanol/water, 1: 1 (v/v) containing 0.1% formic acid (positive mode) and infused via a syringe pump at a flow rate of 5 µL/min. The reflectron TOF mass analyser (allowing multiple ion reflections) was operated in V-geometry for the acquisition of positive and negative ion mode mass spectra, and calibration for both polarities in the *m/z* range of 100–1000 was performed using sodium formate solution (generated by mixing equal volumes of aqueous solutions of sodium hydroxide (0.1 mol/L) and formic acid (0.2 mol/L) and diluting 1:500 using 2-propanol/water, 8: 2 (v/v) at a flow rate of 5 µL/min).

All MS and MS/MS spectra were recorded as accurate mass data with Neu5Ac for lockmass correction in positive and negative ion mode (positive mode: 0.01 mg/mL in methanol/water, 1:1 (v/v) containing 0.1% formic acid, negative mode: 0.001 mg/mL in methanol/water, 1:1 (v/v), flow rate 5 µL/min for both modes) using the protonated and deprotonated molecule, respectively. In cases where the signal intensity of the analyte or lockmass exceeded the recommended value for accurate mass measurement of 200 counts per second, the transmission rate was reduced by setting the sample cone voltage to sufficiently low values. Accurate mass values were derived from centroid mode mass

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spectra and every assignment of an ion species to a peak was verified by calculation of the elemental composition from accurate mass data using the Elemental Composition module (version 4.0) included in MassLynx (version 4.1 SCN 704, Waters).

Ion mobility-MS and MS/MS measurements

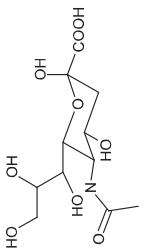
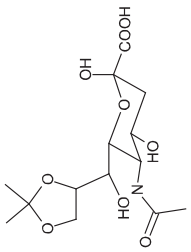
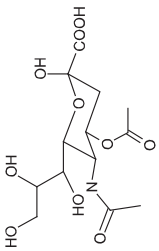
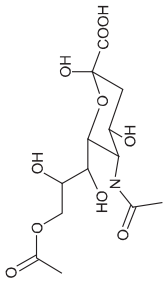
All the T-wave ion mobility measurements were actually carried out as MS/MS mobility experiments, i.e. the quadrupole was operated in resolving mode to ensure that only the ion species of interest (deprotonated, protonated or sodiated molecule) was transferred into the ion mobility cell. In case of mixtures of non-isobaric analytes, the LM (low-mass) resolution parameter controlling the width of the quadrupole isolation window was changed to allow transmission of all species of interest. The acquisition parameters were: electrospray voltage: 2.5 kV; sample cone voltage: 15 V; extraction cone voltage: 3 V; Ar flow rate: 3 mL/min (resulting in a pressure of 0.016 mbar in the trap/transfer cell); trap DC BIAS: 14 V; trap and transfer CE: 6 eV (negative mode) and 5 eV (positive mode); ion mobility gas flow rate: 32 mL/min nitrogen (resulting in a pressure of 0.79 mbar in the ion mobility cell); IMS wave height: 8 V (negative mode) and 7.5 V (positive mode); IMS wave velocity: 300 m/s; scan time: 3 s. The samples were dissolved at 0.001 mg/mL in methanol/water, 1:1 (v/v) (negative ion mode) or 0.005 mg/mL in methanol/water, 1:1 (v/v) containing 0.1% formic acid (positive ion mode) and infused via syringe pump at flow rates of 10 µL/min. The ion mobility data were loaded into Driftscope software (version 2.1, Waters) and exported to MassLynx for display and further processing. To determine the centroids of the arrival time peaks, the data were imported into Origin (version 8.0, Originlabs, Northampton, MA, USA) to fit Gaussian functions.

Actual T-wave IM-MS/MS experiments (with fragmentation taking place in the transfer cell, i.e. after mobility separation of the precursor ions) were carried out by applying a transfer CE of 20 eV.

RESULTS

To evaluate the resolving capability of the ion mobility separation on isobaric and non-isobaric derivatives of Neu5Ac, a set of four substances was chosen: Neu5Ac, the constitutional isomers Neu4,5Ac$_2$ and Neu5,9Ac$_2$ and Neu5Ac-8,9protected, an intermediate product obtained during the synthesis of Neu4,5Ac$_2$ (see Table 1 for detailed information on these substances). The purities and elemental compositions of all synthesis products were assured by accurate mass measurement in positive and negative ion mode (data not shown). Figure 1 shows the arrival time distributions of the deprotonated (left column), the protonated (center column) and sodiated (right column) molecules of each individual substance. The arrival time for each ion was determined and is listed in Table 1. For consecutive measurements of the same sample, variations of ±0.01 ms in arrival time were typically observed (data not shown). Not surprisingly, the arrival times for Neu5Ac in positive and negative ion mode are clearly lower than those determined for its derivatives, which exhibit 40 Da and 42 Da higher molecular weights. These three Neu5Ac derivatives clearly show different arrival times in negative ion mode, but

Table 1. Confirmation of elemental composition via accurate mass measurement, summary of arrival time measurements (shown in Fig. 1) of Neu5Ac and derivatives

	Neu5Ac	Neu5Ac-8,9protected	Neu4,5Ac$_2$	Neu5,9Ac$_2$
Structure				
Systematic name[33]	5-*N*-acetylneuraminic acid	8,9-*O*-isopropylidene-5-*N*-acetylneuraminic acid	4-*O*-acetyl-5-*N*-acetylneuraminic acid	5-*N*-acetyl-9-*O*-acetylneuraminic acid
Formula	$C_{11}H_{19}NO_9$	$C_{14}H_{23}NO_9$	$C_{13}H_{21}NO_{10}$	$C_{13}H_{21}NO_{10}$
$[M–H]^-$ calculated*	308.0982	348.1295	350.1087	350.1087
$[M–H]^-$ measured	not determined	348.1291 (−1.1 ppm)	350.1090 (+0.9 ppm)	350.1089 (+0.6 ppm)
Arrival time** $[M–H]^-$	4.29 ms, FWHM = 0.87	5.62 ms, FWHM = 1.00	4.92 ms, FWHM = 0.87	5.29 ms, FWHM = 0.96
$[M+H]^+$	4.43 ms, FWHM = 0.71	5.13 ms, FWHM = 0.80	5.14 ms, FWHM = 0.76	5.10 ms, FWHM = 0.76
$[M+Na]^+$	4.59 ms, FWHM = 0.66	5.21 ms, FWHM = 0.76	5.37 ms, FWHM = 0.77	5.31 ms, FWHM = 0.77

*Exact *m/z* values of $[M–H]^-$ ions and the observed relative *m/z* errors were calculated using "Elemental Composition" module included in MassLynx software suite.
**The centroid and the full width at half maximum (FWHM) of each molecule's arrival time peak are listed.

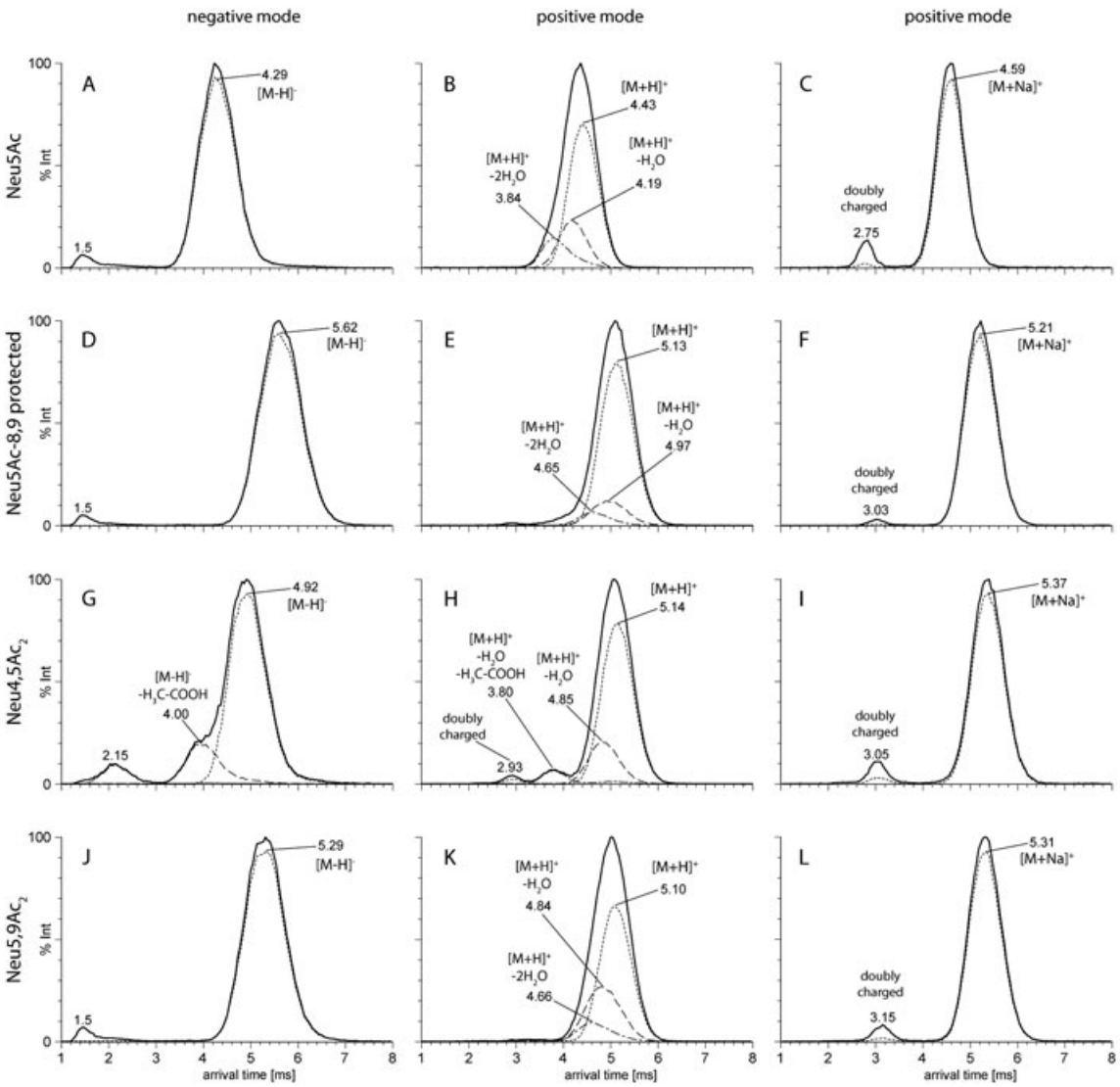


Figure 1. Arrival time distributions for Neu5Ac (A–C) and derivatives (Neu5Ac-8,9protected: D–F, Neu4,5Ac$_2$: G–I, Neu5,9Ac$_2$: J–L) in positive (B, C, E, F, H, I, K, L) and negative (A, D, G, J) ion mode. The bold solid lines show the total arrival time distribution for all ions across the whole *m/z* range, the dotted lines represent the arrival time of the respective intact deprotonated, protonated or sodiated molecule. Arrival time labels represent the centroid of the peak. Additional peaks are caused by product ions generated prior to ion mobility separation and are labelled with their arrival time and plotted using dashed and dashed-dotted lines, in case their peaks overlap the peak of the intact precursor ion. Peaks labelled "doubly charged" arise from doubly charged species.

the arrival times found for the protonated and sodiated molecules are nearly identical. The observed peak widths at half height are also different in positive and negative ion mode, ranging from 0.87–1.00 ms for the deprotonated molecules to 0.71–0.80 and 0.66–0.77 ms for the protonated and sodiated molecules. Although the acquisition conditions have been optimised to avoid fragmentation before and after ion mobility separation, some arrival time distributions show the presence of product ions, which have been generated between the quadrupole and the ion mobility separation cell. In particular, the protonated molecules of all species (Fig. 1, middle column) are susceptible to fragmentation by neutral loss of water and/or acetic acid. The arrival time distributions obtained from sodiated molecules show the presence of considerable amounts of doubly charged ions, with *m/z* values very close to those of the sodiated molecules, which therefore could pass the quadrupole.

The findings described above clearly show that an IM-based separation of these substances can only be successful when carried out in negative ion mode, as the substances show inadequate arrival time differences and significant post-source fragmentation in positive ion mode. To evaluate the applicability of IM-MS on more complex samples, binary mixtures of Neu5Ac and its derivatives were analysed. Figure 2(A) shows the arrival time distribution of a mixture of equal amounts of Neu5Ac and Neu5Ac-8,9protected. This mixture contains the two compounds which exhibit the highest possible difference in ion mobility and can clearly be separated into two major components with arrival times of 4.27 and 5.58 ms, which perfectly match those values, that have been determined for Neu5Ac (4.29 ms) and Neu5Ac-8,9protected (5.62 ms) when analysed individually. Figure 2(B) shows the arrival time distribution obtained for the mixture of

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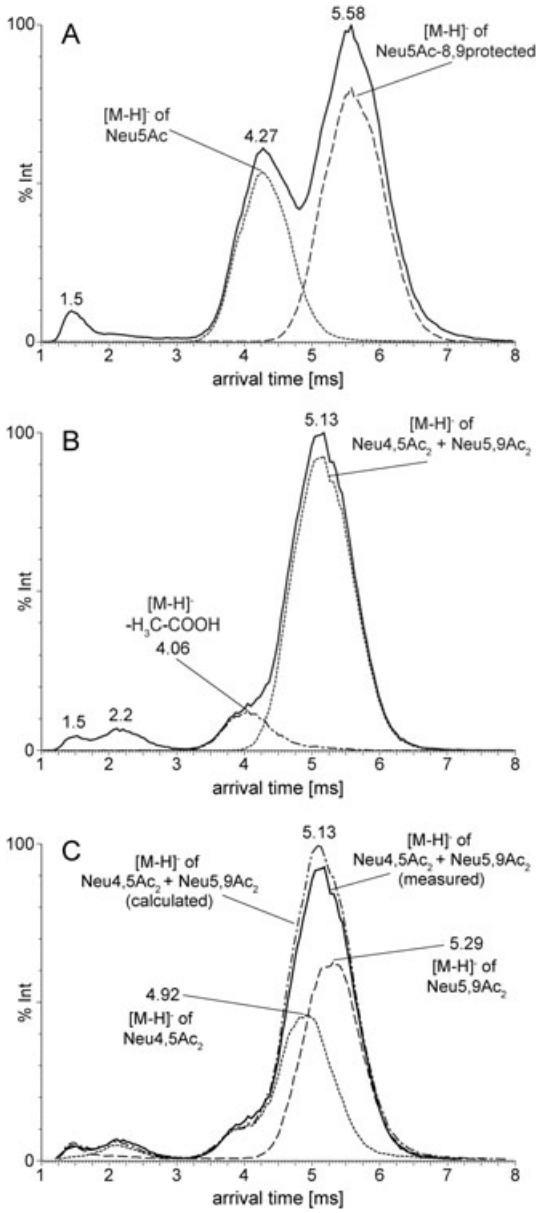

Figure 2. Arrival time distributions for binary mixtures. (A) Mixture of Neu5Ac and Neu5Ac-8,9-protected (0.001 mg/mL each). The bold solid line shows the total arrival time distribution for all ions across the whole *m/z* range, the dotted and dashed line represent each deprotonated molecule. (B) Mixture of $Neu4,5Ac_2$ and $Neu5,9Ac_2$ (0.001 mg/mL each). The bold solid line shows the total arrival time distribution for all ions, the dotted line represents the total intensity for both deprotonated molecules, the dashed-dotted line represents a product ion overlapping the peak of the precursor ion. (C) The bold solid line shows the total arrival time distribution for all ions of the same $Neu4,5Ac_2$/$Neu5,9Ac_2$ – mixture as used in (B); the dotted and dashed lines represent arrival time distributions of $Neu4,5Ac_2$ and $Neu5,9Ac_2$ when recorded individually (shown in Figs. 1(G) and 1(J)). The dashed-dotted line is the sum of both.

the isobaric compounds $Neu4,5Ac_2$ and $Neu5,9Ac_2$. Unfortunately, the arrival time difference and resolution are not sufficient to achieve a level of separation comparable with that of the other mixture. The arrival time distribution shows only a single peak with a centroid at an arrival time of 5.13 ms. It is not distorted or irregularly shaped and does not exhibit a significantly higher width at half maximum height than the compounds when analysed individually (1.06 ms vs. 0.87 and 0.96 ms). Figure 2(C) shows that the curve obtained by summing up the arrival time distributions of $Neu4,5Ac_2$ and $Neu5,9Ac_2$ when recorded individually is nearly identical in terms of centroid, intensity and shape to the peak obtained by analysing the corresponding mixture.

The results achieved so far suggest that the resolving power of the ion mobility separator used in these experiments is not sufficient to achieve even partially separated peaks in the arrival time distributions if the compounds' arrival times do not differ by more than about 1 ms. Therefore, a mixture of isobaric compounds like $Neu4,5Ac_2$ and $Neu5,9Ac_2$ cannot be identified by recording mass and ion mobility spectra alone. As a consequence, MS/MS spectra of $Neu4,5Ac_2$ and $Neu5,9Ac_2$ were recorded in positive and negative ion mode to identify product ions, which allowed the unambiguous assignment of the position of the *O*-acetylation. Figure 3 shows MS/MS spectra of protonated and deprotonated $Neu4,5Ac_2$ and $Neu5,9Ac_2$. The product ions that are generated from the protonated molecules (Figs. 3(A) and 3(B)) are nearly exclusively formed by neutral losses of acetic acid (from *O*-acetylation), ketene (from *N*-acetylation) and water (from hydroxy groups). Naturally, there are differences in the positive ion MS/MS spectra that would allow us to discriminate between $Neu4,5Ac_2$ and $Neu5,9Ac_2$, but these are mainly differences in relative peak intensities rather than in the types of product ions that are observed. This makes the decision as to whether a sample contains a mixture of $Neu4,5Ac_2$ and $Neu5,9Ac_2$ or only a single compound very difficult. The MS/MS spectra generated from sodiated $Neu4,5Ac_2$ and $Neu5,9Ac_2$ (data not shown) are equally unsuitable for discriminating between $Neu4,5Ac_2$ and $Neu5,9Ac_2$, as they show a similar amount of intense peaks corresponding to neutral losses alongside some minor peaks from cross-ring cleavages. The picture is completely different for the negative ion mode MS/MS spectra (Figs. 3(C) and 3(D)) of deprotonated $Neu4,5Ac_2$ and $Neu5,9Ac_2$, which are less complex and mainly show peaks corresponding to product ions from cross-ring cleavages. In addition, the deprotonated $Neu4,5Ac_2$ and $Neu5,9Ac_2$ give rise to different sets of product ions, which easily allow discrimination of the two species or their presence in a mixture. Figure 4 shows the putative fragmentation pathway. For the deprotonated $Neu4,5Ac_2$, the neutral loss of acetic acid seems to be the preferred fragmentation. The resulting product ion (*m/z* 290.0877) can further fragment by 0,4-cross-ring cleavage to form a product ion at *m/z* 170.0447; the formation of the expected second product ion at *m/z* 119.0344 could not be confirmed experimentally. A similar fragmentation process has already been observed for unsaturated glycosides in negative mode fast atom bombardment spectra with high-energy collisions and proposed to be result of a retro-Diels-Alder (RDA) reaction.[32] The product ion at *m/z* 170.0447 gives rise to ions at *m/z* 126.0556 and 98.0607 by neutral loss of CO_2, and of CO_2 followed by CO, respectively. The deprotonated $Neu5,9Ac_2$ can fragment using the same

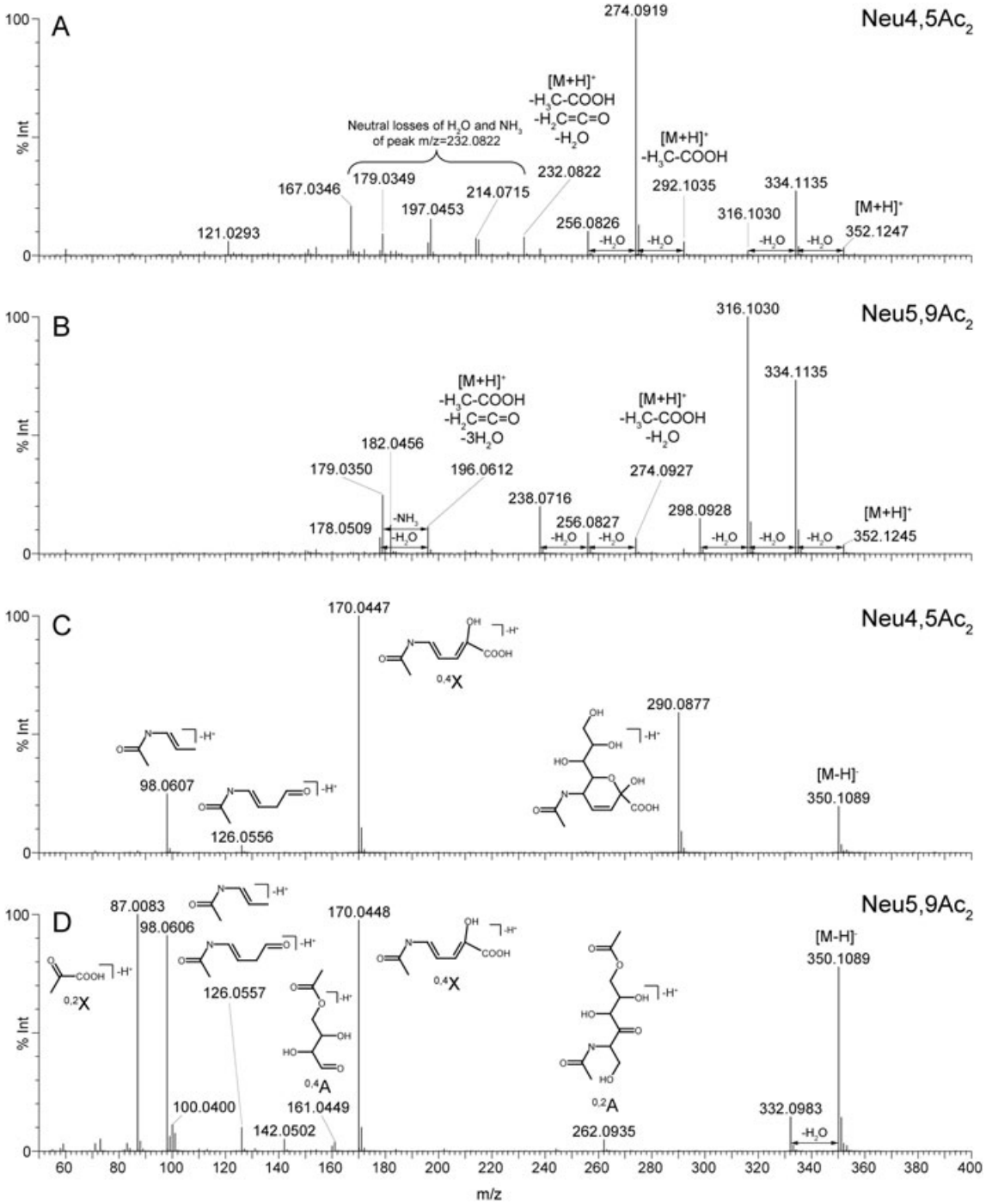

Figure 3. Centroid low-energy CID spectra of Neu4,5Ac$_2$ in positive (A) and negative (C) mode and Neu5,9Ac$_2$ in positive (B) and negative (D) mode. Nomenclature of cross-ring cleavage fragments is according to Domon and Costello.[34] The elemental composition of the suggested ion structures was confirmed by accurate mass measurement.

pathway: neutral loss of water from carbon #4 (according to nomenclature[33]) generating the ion at *m/z* 332.0983 followed by cleavage of bonds 0 and 4 (according to nomenclature[34]) by a RDA reaction to form product ions at *m/z* 170.0448 and 161.0449. The product ions derived from *m/z* 170.0448 are the same as those described for Neu4,5Ac$_2$. The elimination of the substituent at carbon #4 seems to be less favourable for deprotonated Neu5,9Ac$_2$; therefore, product ions can be observed that are generated by a 0,2-cross-ring cleavage (*m/z* 262.0935 and 87.0083), which could not have taken place if bond #2 is a double bond.

With these results it is possible to decide whether a sample contains Neu4,5Ac$_2$, Neu5,9Ac$_2$ or a mixture of both by the absence or presence of peaks from characteristic product ions for each isomer. Of course, this approach is only possible if pure samples of all compounds are available at the beginning of investigations, which is often not the case. In addition, the acquisition and thorough analysis of MS/MS spectra can be

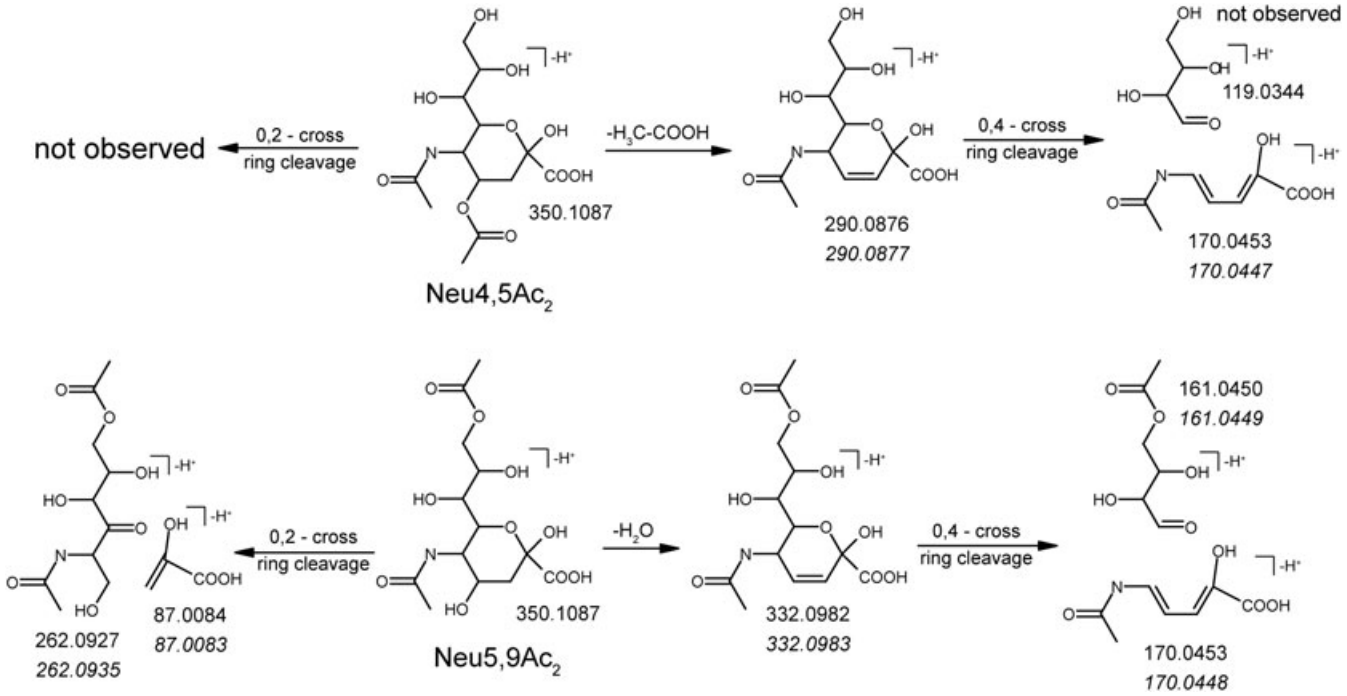

Figure 4. Fragmentation pathways of deprotonated $Neu4,5Ac_2$ and $Neu5,9Ac_2$. Nomenclature of cross-ring cleavage fragments is according to Domon and Costello.[34] For each product ion, the exact mass (calculated using the molecular mass calculator application included in MassLynx) and the observed *m/z* (set in italic) are provided.

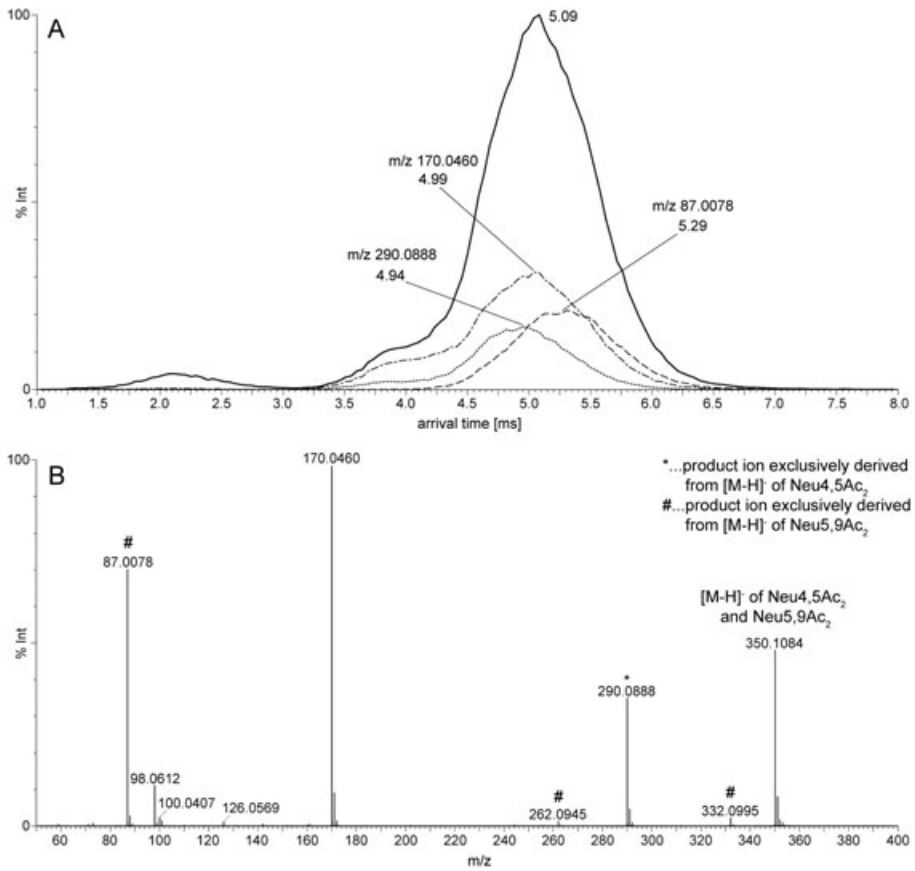

Figure 5. (A) Arrival time distribution of a mobility MS/MS experiment on equimolar $Neu4,5Ac_2/Neu5,9Ac_2$ – mixture. The bold line represents the total arrival time for all ion species; dotted, dashed and dashed-dotted lines represent arrival time distributions for product ions at *m/z* 290.0888, 87.0078 and 170.0460. Labels are arrival time peak centroids. (B) Negative ion MS/MS spectrum of $Neu4,5Ac_2/Neu5,9Ac_2$ – mixture generated by combining across whole arrival time range.

rather time-consuming. Therefore, an IM-MS/MS experiment was set up where the deprotonated molecules of Neu4,5Ac_2 and Neu5,9Ac_2 were first separated as well as possible by ion mobility and subsequently fragmented by low-energy CID. Figures 5(A) and 5(B) show the results of such an IM-MS/MS experiment for an equimolar mixture of Neu4,5Ac_2 and Neu5,9Ac_2. The MS/MS spectrum (Fig. 5(B)) contains all those peaks corresponding to product ions that are generated from each precursor ion – indicating that the applied conditions do not selectively fragment only one compound. The arrival time distribution (Fig. 5(A)) shows the traces for selected product ions: *m/z* 87.0078 (exclusively formed from Neu5,9Ac_2), 290.0888 (exclusively formed from Neu4,5Ac_2), and 170.0460 (formed from both Neu4,5Ac_2 and Neu5,9Ac_2). Those product ions, that are formed exclusively from one compound, are detected at different arrival times (4.94 ms for *m/z* 290.0888 and 5.29 ms for *m/z* 87.0078), indicating that they were derived from partially mobility separated precursors. The arrival times of these product ions are in perfect agreement with those previously determined for their respective precursor ions (Neu4,5Ac_2: 4.92 ms; Neu5,9Ac_2: 5.29 ms). The centroid of the arrival time peak of the product ion *m/z* 170.0460 (5.03 ms), which is derived from both precursors, is located between the arrival times of its precursors. These results clearly show that IM-MS/MS experiments allow the detection of mixtures of isobaric compounds if they give rise to unique product ions.

DISCUSSION

In this study, we investigated the applicability of T-wave ion mobility mass spectrometry for the separation of a mixture of 5-*N*-acetylneuraminic acids *O*-acetylated at different positions and for the unambiguous identification of the compounds. Although the constitutional isomers showed arrival time differences of nearly 0.4 ms, not even partially separated peaks could be observed in the arrival time distributions of equimolar mixtures. This can either be caused by the lower resolving power of the travelling wave type ion mobility spectrometer as it is used in the SYNAPT instrument in comparison with other ion mobility spectrometer types[17] or peak broadening as a consequence of the superposition of different gas-phase structures, conformers and anomers. Unfortunately, there are no data available on the occupancy rates of the possible conformers and anomers for neuraminic acid or derivatives in the used solvent mixture. As the separation of monosaccharide anomers by ion mobility mass spectrometry has already been shown with another instrument type,[14] it has to be assumed that the contribution of different gas-phase structures or conformers to peak broadening is rather marginal. An unexpected finding was that the arrival times of protonated and sodiated molecules were nearly identical for the compounds with similar or identical molecular weights, whereas the arrival times of the deprotonated molecules in negative mode showed far higher differences. However, not only the substances' arrival time differences changed, but also their order: Neu5Ac-8,9protected showed the highest arrival time as a deprotonated and the lowest as a sodiated molecule. Unfortunately, there are no data available on the gas-phase structure of cationised Neu5Ac or derivatives. Calculations for hexoses have shown that sodium adducts are multidentate complexes and that the interaction with sodium can promote a certain conformation of the pyranose ring.[35] Experimental data and calculations for linear polymers and oligosaccharides have shown that the linear molecules adapt cyclic structures upon interaction with cations.[36,37] Therefore, it has to be assumed that Neu5Ac and derivatives form very similar gas-phase structures upon cationisation, whereas the abstraction of a proton does not induce the generation of structures with similar collisional cross-sections.

As a result of the low resolution in ion mobility mode, species with arrival time differences of about 0.4 ms are detected as single peaks in the arrival time distribution. Analysing a mixture of compounds with 1.3 ms arrival time difference revealed two distinct peaks in the arrival time distribution, but these compounds differed by 40 Da in their molecular weight. Probably such arrival time differences cannot be achieved for most small isobaric molecules but, by using CID to fragment the mobility-separated precursors, their arrival time differences are retained by their product ions and can be detected. This approach could be successfully applied to a mixture of Neu5Ac carrying *O*-acetylations at different positions, but only because of the existence of product ions, that allowed the discrimination of the constitutional isomers. Unfortunately, it is very unlikely to find such product ions that would allow the discrimination of standard monosaccharides (e.g. glucose or mannose), but linkage and sugar composition-specific fragmentation has been shown for oligosaccharides.[38]

CONCLUSIONS

The approach presented in this paper of using ion mobility mass spectrometry to detect and separate conformational isomers of acetylated sialic acids has not been completely successful. The resolution (ion mobility resolution Ω (collision cross-sections)/$\Delta\Omega$ (full width of mobility peak at half height) of the T-wave ion mobility spectrometer as implemented in the applied instrument was not sufficient ($\Omega/\Delta\Omega = 10$) to clearly (base-line) separate the isobaric species by their different ion mobilities. Nevertheless, the absence or presence of each of the conformational isomers could be confirmed when ion mobility and negative ion mode MS/MS were used in combination. This experimental setup could also be used to analyse any mixture of isobaric species where pure compounds are not available to analyse them individually. Undoubtedly, this procedure would enormously benefit from instrumental improvement with regard to the performance of the mobility separation (e.g. the recently released SYNAPT G2 or G2S with an improved ion mobility resolving power ($\Omega/\Delta\Omega = 45$)[39]). In addition, independence from liquid-phase separation methods to separate conformational isomers by using ion mobility separation could dramatically reduce analysis time and sample consumption as well as add an additional degree of peak capacity to the commonly applied HPLC/ESI-MS approach. Our approach, despite there being room for improvement, is helpful in monitoring and quality control in chemical synthesis of sialic

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acid derivatives and might be applied in the characterisation of complex hydrolysates of glycans moieties derived from glycoproteins as well as in metabolomics of e.g. bacterial pathogens.

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