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Rapid analysis of *O*-acetylated neuraminic acids by matrix assisted laser desorption/ionization time-of-flight mass spectrometry

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***N*-Acetylneuraminic acid (a sialic acid) occurs mainly as a terminal substituent of oligosaccharides of glycoconjugates. Derivatives of neuraminic acid occur widely, substituted in the amino and hydroxy side chains, as well in the C-9 carbon skeleton. These derivatives are responsible for specific functions of sialic acids during cell-cell, cell-substrate, or cell-virus interactions. The study of *O*-acetylated neuraminic acids is difficult, because only small amounts are extractable from natural sources and they are generally unstable to acids and bases. We report a new method for the rapid analysis of *O*-acetylated neuraminic acids, using a combination of reversed phase HPLC and MALDI-TOF mass spectrometry. A mixture of neuraminic acids from *bovine submaxillary gland* mucins was analysed, as well as neuraminic acids variously substituted in the amino and hydroxy side chains with acetyl and glycolyl groups, respectively.**

***Keywords*: *O*-acetylated neuraminic acids, MALDI-TOF mass spectrometry**

Introduction

Sialic acids‡ mainly occur in the terminal position of glycoproteins and glycolipids. With a few exceptions, their natural occurrence is restricted to higher invertebrates and vertebrates [1–3]. The report of *N*-acetylneuraminic acid in the embryo of *Drosophila melanogaster* [4] is still the only known case of sialic acids in insects. With the exception of buckwheat [5], plants do not seem to contain sialic acids.

Sialic acids show diverse substitutions of the side chain amino and hydroxy functions of the common C-9 skeleton [1, 3, 6] (see Figure 1). These different structures serve different biological functions, such as modulation of cellular adhesion, aggregation or agglutination processes and receptor functions [7–15], influences on conformation, solubility, viscosity and charge of glycoproteins [16–22], protection of glycoconjugates and cells from degradation [23, 24] and modulation of enzyme activity [9, 25–27].

Therefore highly sensitive methods are needed to detect and identify different sialic acids. To date the detection of small quantities of *O*-acetylated neuraminic acids was difficult, due to the instability of the acetyl groups, *eg* the spontaneous migration of the acetyl group from the 7-position to the 9-position.

The first step in the analysis of sialic acids is their release from sugar residues of N- and O-glycans, either with sialidase (E.C. 3.2.1.18) or by the use of mild acid conditions. However, it should be noted that in most cases much lower amounts of sialic acids are released by sialidase than by acid [28–30], even some derivatives, *eg* 4-*O*-acetyl-*N*-acetylneuraminic acid, are resistant to sialidase [31]. For complete cleavage without loss of *O*-acetyl groups mild acidic conditions are favoured [31]. Further identification of purified sialic acids is difficult and a precise quantification is not possible, particularly when they are present in small amounts or as the components of a mixture. Gas-liquid chromatography/mass spectrometry (GLC/MS) was originally used to characterize many of the O-substituted neuraminic acids [2]. However, this technique is complicated by the need for derivatization, variable recovery from the GLC column and different detector responses to the different sialic acids. Nuclear magnetic resonance spectroscopy (NMR) provides information on the linkage and substitution profile of native neuraminic acids, but suffers from the disadvantage that large amounts of substance

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‡ Sialic acid comprises all types of substituted neuraminic acids with the common structure of 5-amino-3,5-dideoxy-D-glycero-D-galactononulosonic acid.

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are required, and it is difficult to analyse mixtures of two or more compounds [32]. Fast-atom bombardment-mass spectrometry (FAB-MS) was successfully introduced, permitting the analysis of naturally occurring modifications of sialic acids in small amounts from natural sources, with and without further derivatization [33]. Most recently liquid chromatography-electrospray ionization-mass spectrometry was introduced to analyse derivatized *O*-acetylated neuraminic acids. This method lacked the possibility to obtain data from mixtures of sialic acids [34].

We report here a new method which combines sensitive high performance liquid chromatography (HPLC) of fluorescently labelled sialic acids [35] and their detection by matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). As mentioned above, *O*-acetylated sialic acids are not resistant to acid or alkaline conditions, though the derivatization with 1,2-dihydroxy-4,5-methylendioxybenzol (DMB, the reaction products are proposed in Figure 2 as given [36]) in light acidic solution always carries the risk of saponification or migration of the *O*-acetyl groups.

Using this method, we identified unambiguously small amounts of *O*-acetylated neuraminic acids in the picomolar range. We also characterized and quantified various *O*-acetylated neuraminic acids from *bovine submaxillary gland* mucins, and differentiated between two positional isomers.

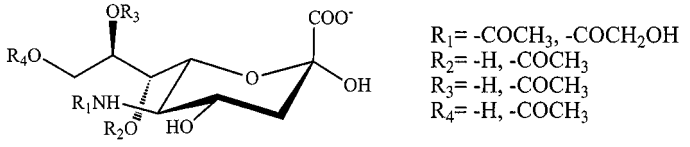

Figure 1. The sialic acids. The molecule is shown in the chair conformation. The various substitutions described (at R_1, R_2, R_3 and R_4) are indicated. Additional diversity is generated by various types of linkage to the glycosidic hydroxyl group at C-2, e.g. *a*2,3-, *a*2,6- or *a*2,8-bound to galactose or sialic acid.

Materials and methods

Materials

Mucins (type I and type I-S) from *bovine submaxillary glands* were purchased from Sigma Chemicals, USA. Standard *O*-acetylated neuraminic acids were from Oxford Glycoscience, England. Solvents for chromatography were of HPLC grade. All solutions were made with double-distilled water. Other compounds were of analytical grade.

Release and purification of sialic acids

To release neuraminic acids from mucins, up to 1 mg of sample was added to 200 µl 2 mol l^{-1} acetic acid in a screw capped glass vial. The suspension was heated for 1 h at 80 °C. The reaction products were dried in a vacuum centrifuge (speed-vac), then resuspended in 200 µl of water and dried again to remove the remaining acetic acid.

Dried products were dissolved in 100 µl water, reacidified to pH 5 and loaded onto a 2 ml gel of AG-50W-X12 cation exchanger (Bio-Rad, USA). Elution was performed with 6 ml water and products were dried in a vacuum centrifuge.

Derivatization with diaminomethylendioxybenzene (DMB)

Dried products were resuspended in 100 µl of double-distilled water, carefully mixed and sonicated to dissolve free neuraminic acids. After centrifugation 20 µl were removed and added to 100 µl of a DMB-solution (7 mmol l^{-1} DMB, 1 mol l^{-1} β-mercaptoethanol, 12 mmol l^{-1} sodium sulfite). Derivatization was carried out at 56 °C for 2.5 h. All labelled compounds were kept in the dark to prevent degradation.

Chromatography

All chromatographic procedures were carried out on a Knauer HPLC-system (Knauer, Germany) equipped with a fluorescence detector (excitation wavelength 373 nm, emission wavelength 448 nm). Eluent A contained water: acetonitrile : methanol (92 : 4.5 : 3.5 by vol) while eluent B contained water : acetonitrile : methanol (84 : 9 : 7 by vol).

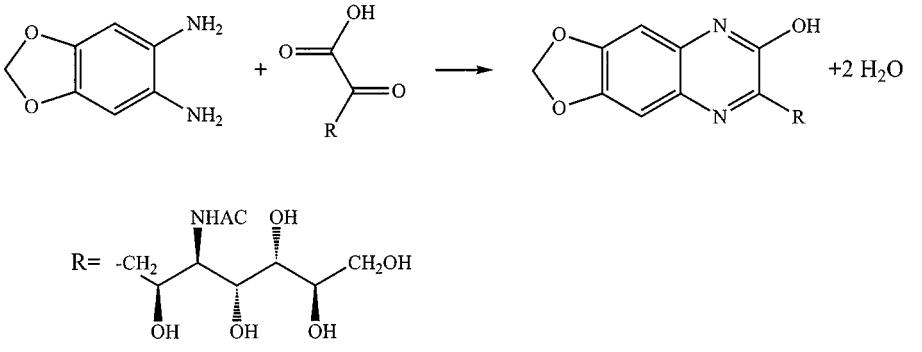

Figure 2. Reaction scheme of *a*-keto carboxylic acids with DMB resulting in quinoxalinones. Substitution R defines the *N*-acetyl neuraminic acid.

All separations were made with an RP18-column (Lichrosorb RP18, 250 × 3.1 mm, 5 µm, Bischoff Analysentechnik, Germany) starting at 20% eluent B increasing to 65% B in 9 min, then for 18 min isocratically, and finally in 18 min to 100% B at a flow rate of 0.5 ml min^{-1}.

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out on a Bruker Biflex instrument (Bruker, Germany). Ionization was accomplished with a 337 nm beam from a nitrogen laser. The mass spectra presented here are the averages of 50–100 laser shots. All mass spectra were recorded in the positive ion mode using the reflector. The ion acceleration potential was set to 10.5 kV and the potential in the reflector was 9.7 kV. 2,5-Dihydroxybenzoic acid (10 mg ml^{-1}) in 60% ethanol was used as matrix. Typically an aqueous solution of the monosaccharide quinoxalinone (2–20 pmol μl^{-1}) was mixed with an equal volume of the matrix and 0.5–1 µl was placed on the target.

Results

Liberation of neuraminic acids

The liberation of neuraminic acids from mucins was performed by mild acid hydrolysis. The loss of acetyl groups attached to the hydroxyl groups at C-7, C-8 or C-9 of the neuraminic acid was determined to be less than 7%. This was calculated by comparing standard *O*-acetylated neuraminic acids with and without acidic treatment (data not shown).

Chromatographic analysis

The sialic acids from two *bovine submaxillary gland* mucins were fluorescently labelled and fully separated on reversed phase chromatography (see Figure 3). For quantification of sialic acids it was assumed that the molar response factor for all sialic acids is identical. This assumption is made as the detection intensity of the substances is only dependent on the fluorescent label, and *O*-acetyl groups have no fluorescence activity. Thus each modification of sialic acid is detected with the same sensitivity.

The analysis showed that the total amount of sialic acids in mucin I-S is five times as high as that in mucin I.

N-Acetylneuraminic acid (Neu5Ac) and 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac_2) were the two dominant compounds in mucin I. The minor compounds are *N*-glycolylneuraminic acid (Neu5Gc), 7-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,7Ac_2), 9-*O*-acetyl-*N*-glycolylneuraminic acid (Neu5Gc9Ac) and 7(8),9-di-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,7(8),9Ac_3). In mucin I-S, the distribution is more balanced so that the amounts of Neu5Ac and Neu5Gc are almost identical. The most abundant sialic acid was Neu5,9Ac_2, while the rare modifications of *O*-acetylated neuraminic acids are also remarkably abundant. For detailed sialic acid patterns in mucins see Table 1.

Mass spectrometry

Liberated and separated sialic acids from *bovine submaxillary gland* mucins were subjected to MALDI-TOF MS. Spectra were obtained for all compounds from the HPLC separation. Each HPLC fraction yielded the expected signal for the sodium cationized pseudomolecular ion in *m/z*.

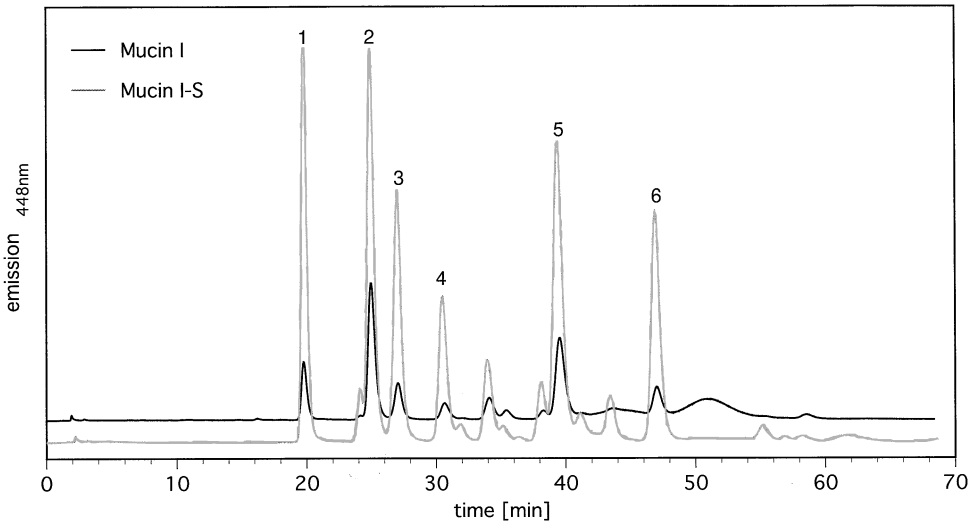

Figure 3. HPLC chromatograms of purified neuraminic acids from *bovine submaxillary gland* mucins. Peaks are assigned as follows: (1) Neu5Gc; (2) Neu5Ac; (3) Neu5,7Ac_2; (4) Neu5Gc9Ac; (5) Neu5,9Ac_2; (6) Neu5,7(8),9Ac_3.

Besides the sodium adduct no potassium cationized pseudomolecular ion could be detected. Generally, as a soft ionization, MALDI-TOF-MS enables the detection of non-fragmentated pseudomolecular ions. Post-source-decay experiments to investigate fragmentation of sialic acid quinoxalinones were not performed. The spectrum presented in Figure 4F showed a second significant pseudomolecular ion at *m/z* 461.0 which probably did not represent a fragment of the assigned sialic acid Neu5,7(8),9Ac_3. It was likely that this compound coeluted on the RP18-HPLC with the fluorescent labelling reagent which led to the impurity observed and the unlikely data in the mass spectrometry. Experimental data were averaged and given as the mean of three different measurements. For exact molecular mass ion signals see Table 2.

Table 1. Distribution of sialic acids in *bovine submaxillary gland* mucins in nmol sialic acid/mg protein.

	Mucin I	*Mucin I-S*
Neu5Gc	26	181
Neu5Ac	70	202
Neu5,7Ac_2	20	150
Neu5Gc9Ac	11	108
Neu5,9Ac_2	61	247
Neu5,7(8),9Ac_3	18	172

Table 2. Pseudo molecular ions for fluorescent labelled sialic acids (theoretical and experimental (mean of three spectra) values).

Sialic acid	**(M+Na^+)**	
	theoretical	*experimental*
Neu5Gc	464,8	464,7
Neu5Ac	448,5	448,7
Neu5,7Ac_2	490,5	490,9
Neu5Gc9Ac	506,8	506,6
Neu5,9Ac_2	490,5	490,6
Neu5,7(8),9Ac_3	532,6	532,7

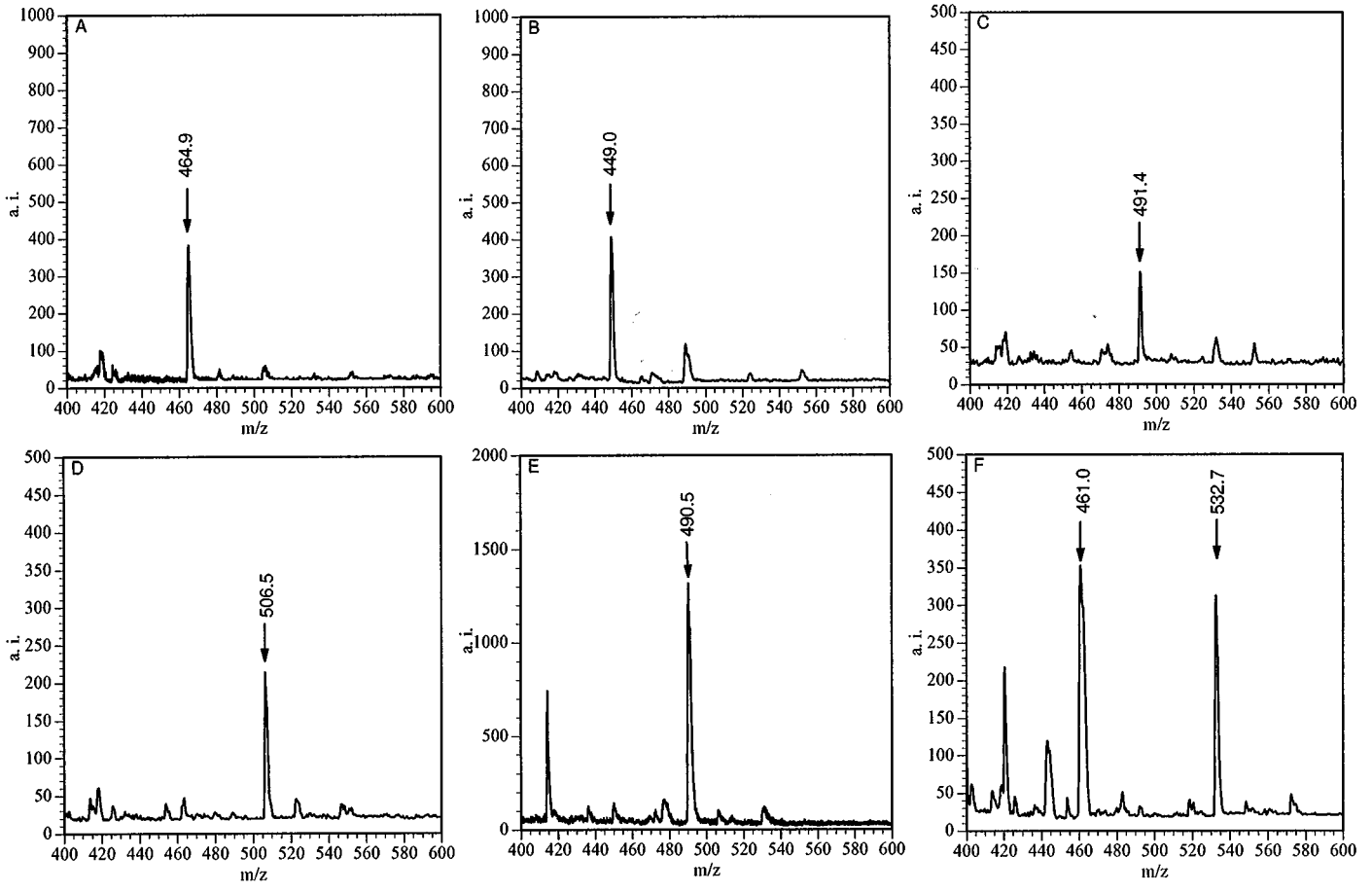

Figure 4. MALDI-TOF mass spectra of neuraminic acids fractionated by RP-18 HPLC. (A) Neu5Gc; (B) Neu5Ac; (C) Neu5,7Ac_2; (D) Neu5Gc9Ac; (E) Neu5,9Ac_2; (F) Neu5,7(8),9Ac_3 at *m/z* 532.7; further signals derive from fragmentation or impurities.

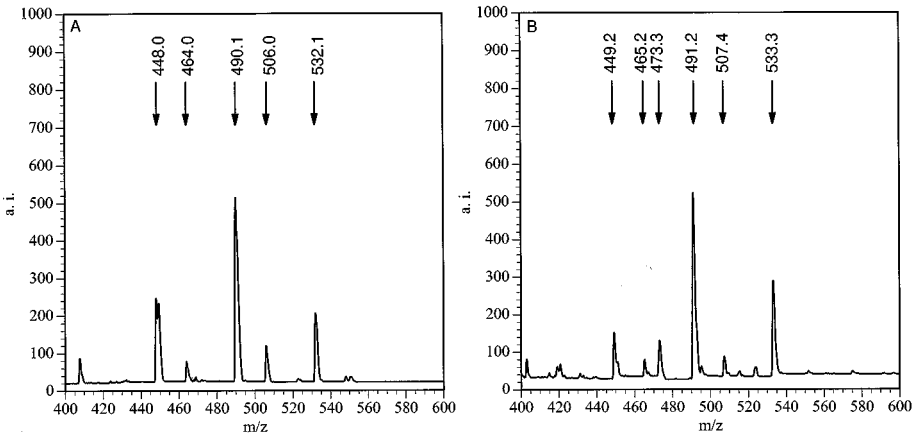

Figure 5. MALDI-TOF MS spectra of total sialic acids from *bovine submaxillary gland* mucins. For details of recording see material and methods. (A) mixture of sialic acids from mucin I; (B) mixture of sialic acids from mucin I-S.

To determine how effective our method is we studied the complex mixtures of purified sialic acids from *bovine submaxillary gland* mucins. These experiments showed that it was possible to recognize the presence of several compounds in a given sample (see Figure 5). All pseudomolecular mass ions reflecting to a specific sialic acid were found. In Figure 5A five mass ions were assigned. The mass at *m/z* 490.1 was consistent with the positional isomers Neu5,7Ac_2 and Neu5,9Ac_2. In the other spectrum (see Figure 5B) one additional significant pseudomolecular mass ion was detected. The mass at *m/z* 473.3 differed from the mass at *m/z* 491.2 by 18 Da. This difference suggested a lactonization between C4 and C8 of the sialic acid to form a thermodynamically favoured 6-membered ring by loss of water as mentioned for ESI-MS spectra of sialic acid quinoxalinones [34].

Discussion

A new fast method is presented for the analysis of complex mixtures of *O*-acetylated sialic acids from natural sources, using HPLC and MALDI-TOF-MS.

This method has two major advantages over NMR-spectroscopy. First, only small amounts of samples are needed for mass spectrometry and second, complex mixtures can be analysed with MALDI-TOF-MS.

Sialic acids from *bovine submaxillary gland* mucins were released under mild acidic conditions to prevent loss of *O*-acetyl groups. After further purification, sialic acids were fluorescently labelled with DMB. We carried out the reaction of sialic acids with the DMB under light acid conditions generated by adding sodium hydrogensulfite to an aqueous solution as stated in the original work [35]. These authors have since refined their method to address the problem of acetyl migration [37]. So that they now use a 1.4 molar solution of acetic acid instead of water. We could not observe migration of *O*-acetyl groups with the original method.

Labelled in this way sialic acids could be detected in the picomolar range. Separation was performed by RP-18 HPLC. Fractionated compounds were dried, redissolved, and directly analysed by MALDI-TOF mass spectrometry. With this method it is possible to obtain molecular ion data without further desalting, derivatization and purification after the chromatography, procedures that can lead to the loss of *O*-acetyl groups.

Ionization of samples by the MALDI-TOF-MS procedure yields data for the pseudomolecular mass ions, and this soft ionization method produces almost no signals of fragmentation. Thus, even mixtures of *O*-acetylated sialic acids can be analysed. Notably, the spectra obtained reflect the relative amounts of each of the different sialic acids present. This conclusion is supported by similar relative amounts for the peak areas from both MALDI-TOF-MS and RP18-HPLC (data not shown) as already compared for HPAEC[‡] and MALDI-TOF-MS [38].

The combination of RP-18 HPLC with MALDI-TOF-MS presented here even offers the possibility of analysing positional isomers such as Neu5,7Ac_2 and Neu5,9Ac_2. As the *O*-acetyl group in Neu5,9Ac_2 is positioned by the terminal hydroxyl function, the difference in polarity compared with Neu5,7Ac_2 is sufficient to separate both the isomers by this procedure. In addition, the derivatization with a hydrophobic fluorescence label changes the physicochemical properties of sialic acids, so that a much improved separation by reversed-phase chromatography becomes possible. Nevertheless, not all positional isomers can be analysed, as the 7-*O*-acetyl group is very unstable

[‡]HPAEC: high performance anion exchange chromatography.

and can easily migrate to the 9-position. Even if the hydroxyl group at C-9 is blocked by another acetyl group, migration from C-7 to C-8 is possible under mild alkaline conditions. This always leads to a mixture of Neu5,7,9Ac_3 and Neu5,8,9Ac_3, preventing an unambiguous analysis by any combination of chromatography and mass spectrometry. This problem may be solved by using NMR-spectroscopy alone.

Our method is very helpful for screening samples containing different sialic acids. It has the advantage over other mass spectrometric techniques like ESI-MS of giving very reliable data for complex mixtures. Although our method does not produce all the information needed for a complete analysis, it is a very effective method for obtaining data from natural sources containing only small amounts of sialic acids.

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