Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Research 341 (2006) 1065–1070
Carbohydrate RESEARCH

Note

The signal-to-noise ratio as a measure of HA oligomer concentration: a MALDI-TOF MS study

Katja Busse,[a] Marco Averbeck,[b] Ulf Anderegg,[b] Klaus Arnold,[a] Jan C. Simon[b] and Jürgen Schiller[a,*]

[a]*University of Leipzig, Faculty of Medicine, Institute of Medical Physics and Biophysics, Härtelstr. 16-18, D-04107 Leipzig, Germany*
[b]*University of Leipzig, Faculty of Medicine, Department of Dermatology, Venerology and Allergology, Philipp-Rosenthal-Str. 23-25, D-04103 Leipzig, Germany*

Received 29 November 2005; received in revised form 1 March 2006; accepted 6 March 2006
Available online 11 April 2006

Abstract—MALDI-TOF MS (matrix-assisted laser desorption and ionization time-of-flight mass spectrometry) was used to determine ng amounts of defined hyaluronan (HA) oligomers obtained by enzymatic digestion of high molecular weight HA with testicular hyaluronate lyase. The signal-to-noise (S/N) ratio of the positive and negative ion spectra represents a reliable concentration measure: Amounts of HA down to about 40 fmol could be determined and there is a linear correlation between the S/N ratio and the HA amount between about 0.8 pmol and 40 fmol. However, the detection limits depend considerably on the size of the HA oligomer with larger oligomers being less sensitively detectable. The advantages and drawbacks of the S/N ratio as concentration measure are discussed.

Keywords: Testicular hyaluronate lyase; Hyaluronic acid fragments (HAF); MALDI-TOF mass spectrometry; Signal-to-noise (S/N) ratio

Hyaluronan (HA) is an ubiquitous animal polysaccharide[1] that occurs in high amounts in the extracellular matrix,[2] for example, cartilage, skin, etc.[3] In addition to high molecular weight (MW) HA, smaller hyaluronan fragments (HAF) merit also huge interest.[4] For instance, it was shown that certain HAFs are able to enhance the activities of enzymes released from fibroblasts[5] and contribute to wound repair.[6]

Many details of the occurrence of HA oligomers are so far unknown because their quantitative determination is difficult. The 8-aminonaphthalene 1,3,6-trisulfonic acid (ANTS)-labelling technique,[7] followed by electrophoretic separation[8] is often used although this method has several drawbacks and leads to sample alteration. Despite its limitations in accuracy and selectivity, the traditional carbazole assay by Bitter and Muir is still in use.[9]

More recently, mass spectrometric (MS) methods became also available for the analysis of HA oligosaccharides. Electrospray (ESI)[10] MS was so far most frequently used and it was shown that individual HAFs can be quantified by this approach using maltose as internal standard.[11] However, previous separation and MS/MS is required. In contrast to ESI MS, MALDI-TOF (matrix-assisted laser desorption and ionization time-of-flight) MS was so far less frequently applied. This is primarily due to the rather low extent of analyte desorption induced by laser irradiation of acidic polysaccharides samples.[12,13]

Although HA with a high MW cannot be detected, individual HAFs obtained by digestion of HA with hyaluronate lyase [EC 3.2.1.35] can be easily differentiated in the positive and negative ion spectra.[14] MALDI-TOF MS is especially useful because it can be performed easily

* Corresponding author. Tel.: +49 341 9715733; fax: +49 341 9715709; e-mail: schij@medizin.uni-leipzig.de

0008-6215/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2006.03.005

and has a higher tolerance in comparison to ESI towards salts and buffer contaminations.[15]

Unfortunately, quantitative MALDI-TOF MS data on HA are so far barely available. In the present investigation, we will show that the quantitative determination of different HAFs by MALDI-TOF MS is possible by using the signal-to-noise (S/N) ratio. The special advantage of this approach is that there are no alterations of the sample because no internal standard is required. Although the S/N ratio was already used for the quantitative determination of acidic lipids[16] and lysophospholipids,[17] this is to our knowledge its first application to HA.

Figure 1 shows the positive (left) and negative (right) ion MALDI-TOF mass spectra of selected, chromatographically purified HA oligomers. The mass spectra of HA-6 (**1a**,**d**), HA-12 (**1b**,**e**) and HA-18 (**1c**,**f**) are shown as representative examples in order to monitor the influence of increasing MW on the spectral quality.

Due to the strongly varying molecular weights of the individual samples, it was not possible to use the same *x*-axis scaling in all cases. However, the same mass range (200 Da) is shown in all cases. Along the *y*-axis, all spectra were scaled in such a way that the most intense peak possesses the same intensity in all spectra.

It is obvious that with an increasing MW the spectral resolution and the signal-to-noise ratio decrease. The MW of (neutral) HA-6 is 1155 g/mol. Accordingly, the positive ion spectrum (**1a**) exhibits one intense peak at $m/z = 1178$ that corresponds to cationization by one

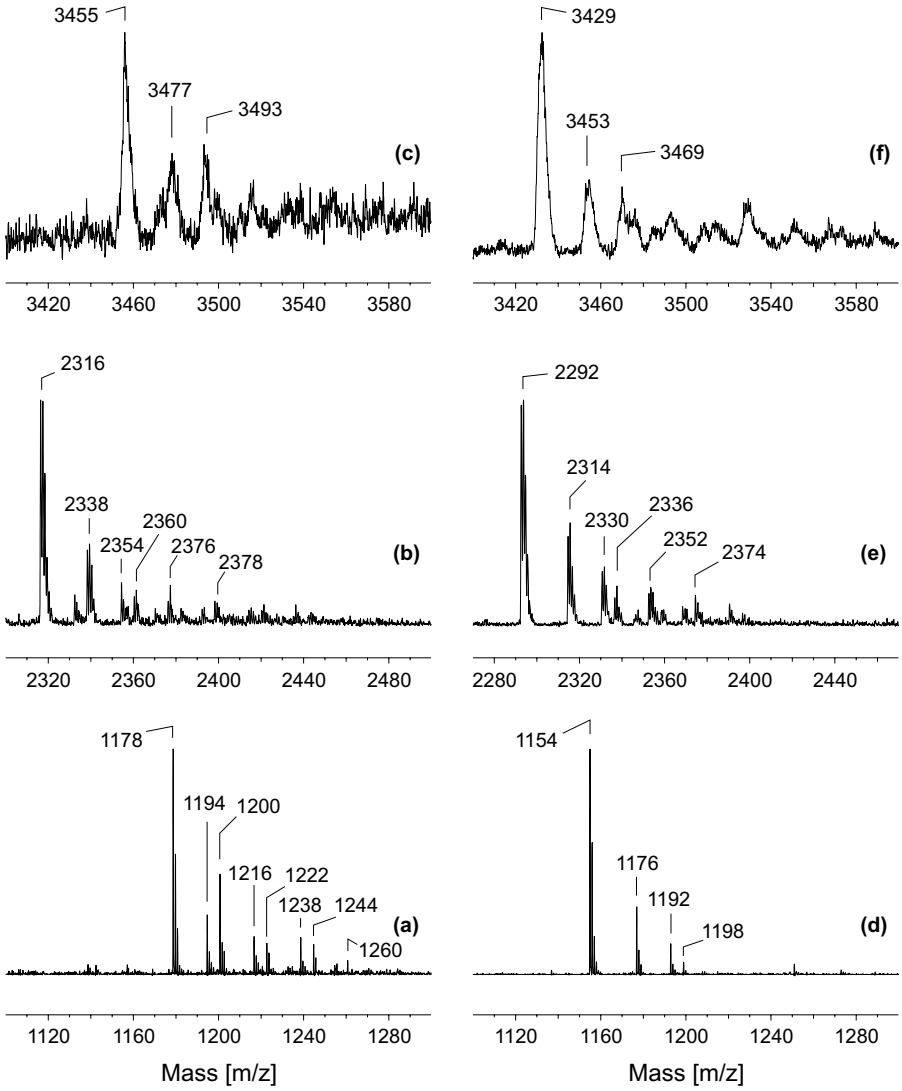

Figure 1. Positive (left) and negative ion (right) MALDI-TOF mass spectra of aqueous solutions of hyaluronan with different molecular weights: HA-6 (a,d), HA-12 (b,e) and HA-16 (c,f). A 50 μg/mL solution of HA was used in all cases. This solution was diluted 1:1 (v/v) with the matrix and 1 μL of this mixture was transferred to the MALDI target. Accordingly, the amount of applied HA was just about 10 ng. All spectra were recorded in the reflector mode. The relative scaling along the *x*-axis is comparable in all spectra. Along the *y*-axis, spectra were scaled according to the intensity of the most intense peak. All peaks are marked according to their individual *m*/*z* ratios.

sodium ion, whereas $m/z = 1194$ represents the potassium adduct. All further peaks correspond to the gradual exchange of one H^+ by Na^+ or K^+. The ratio between H^+, Na^+ and K^+ adducts can be altered by changing the buffer composition.[18]

In the corresponding negative ion spectrum (**1d**), one intense peak at $m/z = 1154$ is detectable that corresponds to the abstraction of one H^+ from the neutral HA-6 molecule. The cation exchange pattern is less complex than in the case of the positive ion spectrum (**1a**). As characteristic of acidic compounds, the negative ion spectrum is characterized by a lower noise level than the positive ion spectrum.

The spectra of HA-12 (**1b**,**e**) can be explained in an analogous way. However, it is obvious that the spectral resolution is poor in comparison to HA-6 although exactly the same experimental parameters were used. Therefore, the increased MW is responsible for that difference. The influence of the MW is still more pronounced when HA-18 is investigated (**1c**,**f**): in this case isotopic resolution is completely lost and the peaks are considerably broadened. It is also evident that the negative ion spectrum can be recorded with an improved signal-to-noise ratio in comparison to the positive ion spectrum.[14]

The achievable mass resolution is about 9000 in the case of HA-6, 4000 with HA-12 and only about 900 in the case of HA-18. This indicates that the m/z ratios of HA-18 can be determined with an accuracy of about ± 4 Da, HA-12 with ± 0.6 Da and HA-6 with about ± 0.15 Da. This is the reason why no decimals are given in this paper. Since the spectral resolution is anyway lost in the cases of larger HAFs, spectra can also be recorded in the so-called 'linear' mode:[15] under these conditions HA can be detected more sensitively but with reduced resolution. Since the linear mode is even available on older MALDI devices, this technique is more generally applicable.[15]

Our next aim was to investigate to what extent the MALDI-TOF mass spectra are altered when the HA concentration is gradually reduced. Some selected spectra of HA-6 recorded with different concentrations are shown in Figure 2. Spectra on the left hand correspond to the positive ion spectra, whereas spectra on the right hand represent the negative ion spectra. Spectra (**2a**) and (**2d**) were recorded with a 6.25 μg/mL, (**2b**) and (**2e**) with 0.81 μg/mL and (**2c**) and (**2f**) with a 92.4 ng/mL HA-6 solution. Considering the dilution with matrix (1:1, v/v), the fact that only 1 μL of the matrix/analyte mixture was deposited onto the sample plate and the MW of HA-6 (1155 g/mol), these concentrations correspond to absolute amounts of 2.71, 0.35 and 0.04 pmol on the sample plate, respectively. It is obvious that the negative ion detection mode is much more sensitive than the positive ion mode: When comparing the spectra obtained with the lowest concentration of HA-6 (trace **2c** and **2f**) the positive ion (**2c**) spectrum is characterized by a much poorer S/N ratio that is close to the detection limit: A S/N ratio of 3 was arbitrarily defined as the detection limit. The reader should also note that the peaks detectable in **2a** (marked with asterisks) at $m/z = 1233$ and 1245 are not caused by HA but are stemming from the DHB matrix. It is well-known that DHB gives under conditions of laser irradiation some charged oligomers.[19] Accordingly, the peak at $m/z = 1233$ can be assigned to $[7M-6H^+ + 7Na^+]$ and at

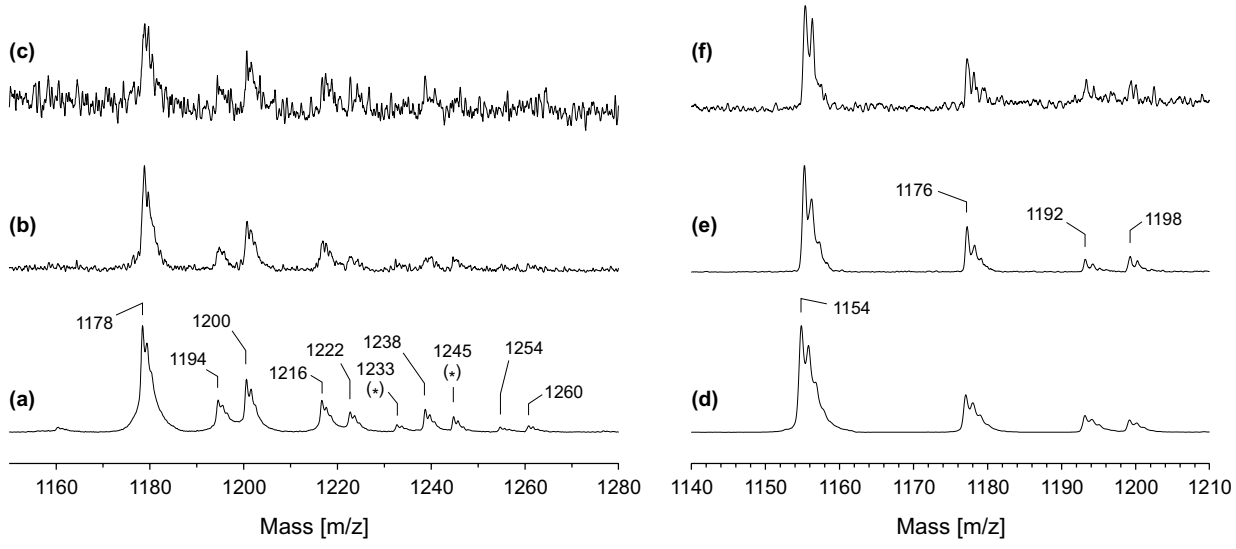

Figure 2. Positive (left) and negative ion (right) MALDI-TOF mass spectra of aqueous solutions of HA-6 in dependence on the amount of HA on the MALDI target. 3.13 ng (a,d), 0.41 ng (b,e) and 0.05 ng (c,f) were deposited onto the sample plate. All spectra were recorded in the linear detection mode for optimum sensitivity. All peaks are marked according to their individual m/z ratios. Peaks arising from the DHB matrix are marked with asterisks.

$m/z = 1255$ to $[7M-7H^{+}+8Na^{+}]$, where 'M' denotes the molecular weight of DHB (154 g/mol). These characteristic matrix peaks can be used as internal calibrants.

It must be emphasized that detection limits of HA may vary between individual MALDI-TOF devices and the used parameters. Therefore, absolute peak intensities are no suitable concentration measures.

For MALDI-TOF mass spectra to be quantitatively analyzed, different approaches were used so far, for instance the addition of a known reference compound, the comparison of the signal of interest with a defined matrix peak and the signal-to-noise ratio.[16] The addition of a known reference compound with a chemical structure similar to the analyte seems the most appropriate method. However, some prior knowledge of the sample is needed to avoid the addition of an excess of the standard. Additionally, peak overlap between the analyte and the standard may occur in complex mixtures. Since the intensity of the matrix signals depends on the ion concentration,[19] obtaining quantitative data by using matrix peaks is difficult.

We had already had good experiences with the S/N ratio for the analysis of phosphoinositides that contain a carbohydrate moiety.[16] The achievable S/N ratio derived from the HA-6 spectra is shown in Figure 3. The most intense signals were used in all cases, that is, the peak at $m/z = 1154$ was used in the negative ion mode (**3a**) and the peak at $m/z = 1178$ in the positive ion mode (**3b**). Nearly the same results, but slightly reduced S/N ratios were obtained when the experiments were performed in the presence of elevated salt concentrations (data not shown).

It is obvious that there is a nearly linear correlation between the S/N ratio and the absolute amount of HA-6 on the MALDI target when the positive ion spectra (**3b**) are analyzed. However this detection mode is less sensitive than the negative ion mode, although there is a more complex dependence of the S/N ratio on the amount of sample (**3a**). Interestingly, the S/N ratio remains nearly constant when more concentrated samples than shown in Figure 3 are investigated (data not shown). In the range between about 3 and 43 pmol HA on the MALDI target, a 'saturation' of the detector occurs leading to a constant S/N ratio. This holds at least when distilled water is used for sample dilution. More relevant, complex buffer systems were not yet tested.

It is our future aim to investigate the effects of common buffer systems on the detectability of HAFs by MALDI-TOF MS in more detail. In these experiments it is also planned to use an internal standard and to compare the peak intensity ratios with the S/N ratio. However, since peak intensities depend also on the molecular weight (as well as the charge state), we expect that standards with different MWs will be necessary in order to determine HA oligomers with varying MWs. Since the addition of several standards might lead to peak overlap between the standard and the analyte, we have favoured the S/N ratio so far.

Finally, in Figure 4, the detection limits in dependence on the size (number of monosaccharide units) of the corresponding HA oligomers are shown. Since the individual HA oligomers are characterized by strongly varying MWs, the amount of HA on the MALDI target is used. It is obvious that the detectability decreases when the MW of the HA of interest increases, that is, larger amounts of HA are required when HAFs with a higher MW are investigated. It is also clear that this effect is more pronounced when the positive ion spectra are analyzed: since each polymer repeat unit (composed of one glucuronic acid and one *N*-acetylglucosamine unit) adds one negative charge, the sensitivity difference between the positive and negative detection mode increases when the MW of HA increases.

We conclude that MALDI-TOF MS offers the possibility to detect different HA oligomers and to determine their amounts by the S/N ratio. The main advantages of the MALDI procedure described in this paper is that

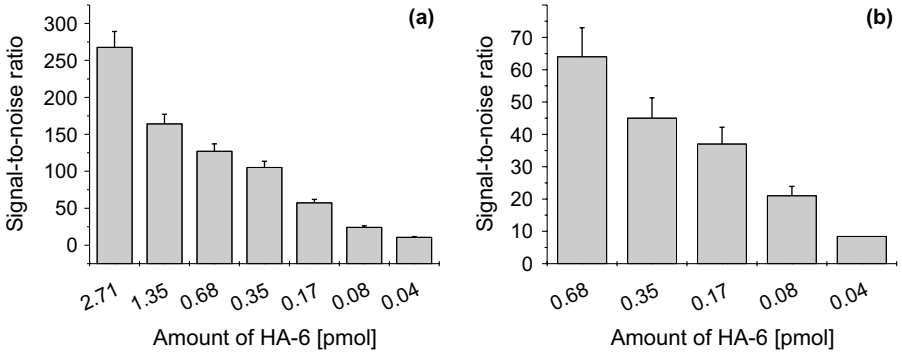

Figure 3. Signal-to-noise ratios determined from the most prominent peaks in the individual spectra in dependence on the amount of HA-6 on the MALDI target. In (a, negative ion spectrum) the intensity of the peak at $m/z = 1154$ was analyzed, whereas in (b, positive ion spectrum) the peak at $m/z = 1178$ was evaluated. Standard deviations from three independent measurements are shown. A signal-to-noise ratio of 3:1 was regarded as the detection limit. Please note the reduced sensitivity when the positive ions spectra are analyzed, and the different scales along the *y*-axis. HA amounts lower than about 0.02 pmol gave signal intensities below the detection limit.

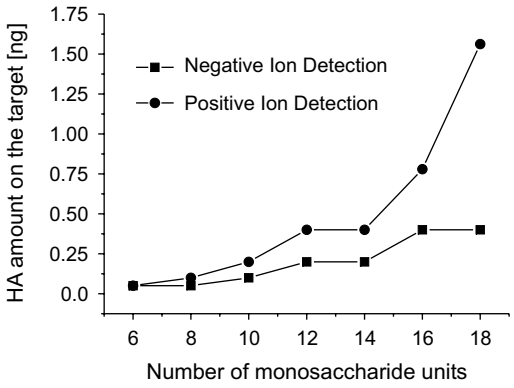

Figure 4. Detection thresholds (amount of HA on the sample plate) in dependence on the size (number of monosaccharide units) of the individual investigated HAF. The deviations were estimated to be of the order of ±5%. Signal-to-noise ratios were determined as described in Figure 3 and a signal-to-noise ratio of 3:1 was regarded as the detection limit. Please note the considerable deviations between positive and negative detection mode when HA fractions with a higher molecular weight were investigated.

neither previous derivatization (e.g., with semicarbazide)[20] nor internal standards are needed. Detection limits in the ng range clearly indicate the sensitivity of this method.

Nevertheless, the so far performed experiments are somewhat simplified because distilled water was used as solvent. Since MALDI-TOF mass spectra are influenced by the ion composition of the solvent (especially when ions with several charges, e.g., phosphate are present), detection limits may be higher under these conditions. Therefore, it is our next aim to clarify the sensitivity changes when pure water is replaced by commonly used buffers.

1. Experimental

1.1. Chemicals

All chemicals and solvents were obtained in highest commercially available purity from Fluka Feinchemikalien GmbH (Deisenhofen, Germany). All chemicals were used without further purification.

1.2. Preparation of HA fragments

High molecular weight hyaluronan (HEALON™) for clinical application with an endotoxin content <0.1 ng/mg was provided by Amersham Pharmacia Biotech. Small HA-fragmentation products were generated by enzymatic digestion as described previously.[21] After separation of the mixture into individual fractions, the sample was lyophilized and re-dissolved in water. The final concentration of all HAF was 100 μg/mL in water. This concentration was determined by the carbazole assay according to Bitter and Muir.[9] The molecular weights of HAFs were calculated assuming that all negative charges are exclusively compensated by protons, that is, only free hyaluronic acid, but no hyaluronate was considered.

1.3. MALDI-TOF mass spectrometry

All MALDI-TOF mass spectra were acquired on a Bruker Autoflex™ mass spectrometer (Bruker Daltonics, Leipzig, Germany). The system utilizes a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage was 20 kV and gated matrix suppression was applied to prevent the saturation of the detector by matrix ions.[15] 128 single laser shots were averaged for each mass spectrum. The laser strength was kept about 5% above threshold to obtain optimum signal to noise ratio. In order to enhance the spectral resolution, some spectra were acquired in the reflector mode using delayed extraction. However, the majority of spectra was recorded in the linear mode. Both, positive and negative ion spectra were recorded.

For all samples, a 10 mg/mL 2,5-dihydroxybenzoic acid (DHB) solution in water containing 0.1% trifluoroacetic acid (as an additional cationizing agent in order to improve the quality of the positive ion spectra) was used as matrix. All HAF solutions were mixed prior to their deposition onto the MALDI target 1:1 (v/v) with the matrix. Droplets (1 μL) were spotted onto the sample plate and afterwards dried rapidly under a moderate, warm stream of air. The signal-to-noise (S/N) ratio was determined by using the program FlexAnalysis™ provided by Bruker Daltonics.

The S/N ratio is defined as the height of the mass peak above its baseline relative to the standard deviation of the noise, that is, peak height/$(3 \times \sigma)$, where σ represents the standard deviation of the noise.

Acknowledgements

This work was supported by the Interdisziplinäre Zentrum für Klinische Forschung (IZKF) Leipzig at the Faculty of Medicine of the Universität Leipzig (Project A 17).

References

1. Garg, H.; Hales, C. *Chemistry and Biology of Hyaluronan*; Elsevier: Amsterdam, ISBN 0-08-044382-6, 2004.
2. Schiller, J.; Fuchs, B.; Arnhold, J.; Arnold, K. *Curr. Med. Chem.* **2003**, *10*, 2123–2145.
3. Fraser, J. R.; Laurent, T. C.; Laurent, U. B. *J. Intern. Med.* **1997**, *242*, 27–33.
4. Termeer, C.; Sleeman, J. P.; Simon, J. C. *Trends Immunol.* **2003**, *24*, 112–114.

5. Fieber, C.; Baumann, P.; Vallon, R.; Termeer, C.; Simon, J. C.; Hofmann, M.; Angel, P.; Herrlich, P.; Sleeman, J. P. *J. Cell Sci.* **2004**, *117*, 359–367.
6. Brown, J. A. *J. Wound Care* **2004**, *13*, 48–51.
7. Oonuki, Y.; Yoshida, Y.; Uchiyama, Y.; Asari, A. *Anal. Biochem.* **2005**, *343*, 212–222.
8. Lee, H. G.; Cowman, M. K. *Anal. Biochem.* **1994**, *219*, 278–287.
9. Bitter, T.; Muir, H. M. *Anal. Biochem.* **1962**, *4*, 330–334.
10. Price, K. N.; Tuinman, A.; Baker, D. C.; Chisena, C.; Cysyk, R. L. *Carbohydr. Res.* **1997**, *303*, 303–311.
11. Kuhn, A. V.; Raith, K.; Sauerland, V.; Neubert, R. H. *J. Pharm. Biomed. Anal.* **2003**, *30*, 1531–1537.
12. Harvey, D. J. *Mass Spectrom. Rev.* **2000**, *18*, 349–450.
13. Chi, L.; Amster, J.; Linhardt, R. J. *Curr. Anal. Chem.* **2005**, *1*, 223–240.
14. Schiller, J.; Arnhold, J.; Benard, S.; Reichl, S.; Arnold, K. *Carbohydr. Res.* **1999**, *318*, 116–122.
15. Schiller, J.; Arnold, K. Mass Spectrometry in Structural Biology. In *Encyclopedia of Analytical Chemistry*; Meyers, R. A., Ed.; John Wiley & Sons: Chichester, 2000; pp 559–585.
16. Müller, M.; Schiller, J.; Petković, M.; Oehrl, W.; Heinze, R.; Wetzker, R.; Arnold, K.; Arnhold, J. *Chem. Phys. Lipids* **2001**, *110*, 151–164.
17. Petković, M.; Schiller, J.; Müller, J.; Müller, M.; Arnold, K.; Arnhold, J. *Analyst* **2001**, *126*, 1042–1050.
18. Schiller, J.; Arnhold, J.; Benard, S.; Müller, M.; Reichl, S.; Arnold, K. *Anal. Biochem.* **1999**, *267*, 46–56.
19. Petković, M.; Schiller, J.; Müller, M.; Benard, S.; Reichl, S.; Arnold, K.; Arnhold, J. *Anal. Biochem.* **2001**, *289*, 202–216.
20. Rhomberg, A. J.; Shriver, Z.; Biemann, K.; Sasisekharan, R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12232–12237.
21. Termeer, C. C.; Hennies, J.; Voith, U.; Ahrens, T.; Weiss, J. M.; Prehm, P.; Simon, J. C. *J. Immunol.* **2000**, *165*, 1863–1870.