FTMS of Natural Polymers*

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The ability of Fourier-transform mass spectrometry to produce spectra of some natural polymers – peptides, lignin and humic acid – that include stable, high-mass ions formed by laser desorption/ionization (LDI) and matrix assisted laser desorption/ionization (MALDI) is demonstrated.

Key words: mass spectrometry, Fourier transform, LDI, MALDI, peptides (met-enkephalin, dermorphin, dermorphin-Pro(D_5), dermorphin-Pro(D_7)), spruce lignin, lake sediment humic acid.

INTRODUCTION

Over the past decade, mass spectrometry (MS) has become an important tool for the analysis of natural polymers. Revolutionary developments in ionization techniques and instrumentation have extended the range of masses amenable to study by MS to above a hundred kilodaltons. Ionization sources are capable of producing intact, gas-phase macromolecular ions of such non-volatile and thermolabile molecules. Coupling of these ionization sources to high-performance analyzers can provide high mass resolution, accurate molecular weight measurement and structural characterization of large biomolecules.

The first technological breakthrough widely applicable to the mass spectrometry of non-volatile and thermolabile molecules came with the introduc-

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tion of fast atom bombardment (FAB).[1] Bombarding a sample in a non-volatile liquid matrix (glycerol) with high-energy (keV) neutrals or ions could transfer the analyte into the gas phase; it could then be ionized and mass analyzed with minimal decomposition. FAB permitted the analysis of relatively small biological molecules in the low kilodalton range.[2,3]

A method capable of ionizing large biomolecules was plasma desorption (PD)[4] using ≈ 100 MeV particles from the spontaneous fission of a ^{252}Cf source. Although PDMS methods are now used only in special applications,[5] their pioneering results stimulated the research that produced, in late 1980s, the present promising soft ionization methods for large biomolecules – electrospray ionization (ESI),[6–8] laser desorption/ionization (LDI) and matrix-assisted laser desorption/ionization (MALDI).[9–16]

Electrospray ionization technique forms stable, multiply charged ions of large molecules by evaporation of solvent from small electrostatically charged droplets at atmospheric pressure. The ions remaining after the solvent evaporation are transported into the high vacuum of the mass spectrometer through several stages of pumping. The greatest benefit of multiply charged ions is that higher mass ions can be detected at a low mass-to-charge (m/z) ratio (usually $500 < m/z < 2500$). This shifted scale enables the high resolution detection of high mass ions because the mass-resolving power of analyzers is inversely proportional to m/z.

The other major advance in the analysis of large biomolecules by MS was the application of laser desorption/ionization and matrix-assisted laser desorption.[9–16] LDI makes use of laser irradiation for rapid heating of the sample. While being transferred from the condensed phase into the gas phase, the sample is ionized by processes such as protonation, deprotonation and cationization. The laser is usually used in a pulsed mode (pulse width of the order of 100 ns or less), employing wavelengths from ultraviolet to infrared. In MALDI, the sample is mixed with an excess of matrix, which preferentially absorbs the laser irradiation. The matrix is able to transfer energy more gently to the sample and gas phase ions are formed with significantly less internal energy than those formed by LDI in the absence of matrix. A major benefit of producing only molecular ions with complete absence of molecular fragmentation is that MALDI can easily be applied to direct analysis of mixtures, which is complicated if each species produces multiple signals. Aromatic compounds containing the carboxylic acid functional group – nicotinic acid, sinapinic acid, 2,5-dihydroxybenzoic acid – are often used as matrix for almost all wavelengths, and succinic acid, glycerol and urea for IR desorption.

The success of high-molecular-weight mass spectrometry is also very dependent on the development of new instrumental capabilities, especially

time-of-flight (TOF)[17] mass spectrometer and Fourier transform mass spectrometer (FTMS).[18–19] Besides TOF's ability to analyze all masses from a single ionization event, it has a virtually unlimited mass range, another advantage in its application to large molecules. The TOF instruments are highly sensitive, but their resolving power is still insufficiently high.

The FTMS is ideally suited for measurements on large molecules using LDI, MALDI, and ESI.[20–22] Ultra high resolving power combined with high mass accuracy makes FTMS even more powerful then sector instruments.[16] Another advantage of FTMS is its nondestructive ion detection. Applying RF pulses to eliminate unwanted ions can selectively trap ions inside the FTMS cell. After this isolation step, activation is commonly achieved by collision of ions with neutrals (collision-activated dissociation, CAD or collision-induced dissociation, CID) or by irradiation with photons. These processes can provide detailed structural information. Furthermore, multiple steps of ion isolation and subsequent dissociation can be linked together to permit investigation of several »generations« of fragment ions. This process, referred to as tandem mass spectrometry (MS^n, where n is the number of stages of MS/MS), [23,24] is readily achieved on trapped-ions instruments such as FTMS[25,26] because its implementation needs no additional instrumentation. One of the more prominent areas in which MS/MS is applied is peptide sequencing.[27] The MS/MS spectrum of the selected peptide usually contains a mix of C-terminal containing product ions (X, Y, Z), product ions containing the N-terminus (A, B, C) and internal fragments. Under favorable conditions, there is a complete series of one type of product ion, or overlapping partial series of more than one type of product ion. In such cases, the amino acid sequence can be deduced. However, various factors influence the types of ions formed and thus it is still difficult to interpret the MS/MS spectra of peptides.

In this report, we present an overview of our investigations on the use of LDI and MALDI FTMS for the analysis of different natural polymers – linear molecules such as peptides as well as complex organic structures of lignin and humic acid.

The capabilities of MALDI FTMS were used not only for molecular weight determination of investigated pentapeptide met-enkephalin, **I**, and heptapeptide dermorphin, **II**, but also for stable isotope measurements of deuterated dermorphin **III** (with Phe(D$_5$)) and **IV** (with Pro(D$_7$)), and structure determination of dissociation products and ionized molecules. Peptide fragments could be correlated to the peptide amino acid sequence.

Tyr-Gly-Gly-Phe-Met	**I**
Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser	**II**
Tyr-D-Ala-Phe(D$_5$)-Gly-Tyr-Pro-Ser	**III**
Tyr-D-Ala-Phe-Gly-Tyr-Pro(D$_7$)-Ser	**IV**

Lignin[28] and humic acid[29] are fundamentally different from other bio-polymers, such as peptides, in which the type of linkage and order of units is stipulated. In lignin, which is a major cell wall component of all vascular plants, at least ten types of binding are found to different extents to link the phenylpropane units into a high irregular three-dimensional polymer. Its monomeric precursor in conifers is predominantly coniferyl alcohol, whereas additional precursors are sinapyl alcohol in deciduous trees and *p*-cumaryl alcohol in grasses and herblike dicotyledons. The complex organic structure of humic acid, formed by chemical and biological degradation of plant and animal residues, consists of aromatic rings joined by alkyl chains and substituted by methyl, cyano and oxygen-containing functional groups (COOH, OH).

Using soft ionization methods some encouraging results have been obtained for humic acid[30,31,33] and well reproducible LDI[32,33] and MALDI[34–36] mass spectra of lignins have been reported. In the present paper, the LDI FTMS analyses of humic acid isolated from lake sediments were carried out and the results were compared with those obtained for spruce lignin.

EXPERIMENTAL

All experiments were performed on an FT/MS 2001-DD Fourier transform mass spectrometer (Finnigan FT/MS, Madison, WI, USA) equipped with a 3 T superconducting magnet, and a Nicolet 1280 data station. Both peptides and deuterated analogues were mixed with an excess of 2,5-dihydroxybenzoic acid and irradiated with a pulsed nitrogen laser (VSL-337ND-S, Laser Science, Inc. Franklin, MA, USA) at 337 nm for MALDI experiments. LDI of lignin and humic acid were performed using the fundamental 1064 nm (humic acid and lignins), 532 nm (humic acid) and 355 nm (humic acid) outputs of a pulsed Nd:YAG laser (Quanta Ray DCR–11, Spectra-Physics, Inc. Mountain View, CA, USA). The sample was dried onto the tip of a stainless steel solid probe, which was then inserted into the high vacuum region and positioned adjacent to the source side of the FTMS cell. The laser radiation was focused onto this probe tip and the resulting ions simply drifted into the source compartment of the dual cubic trap. All positive- and negative-ion mass spectra were acquired at $(3–5) \times 10^{-8}$ Torr in the source compartment of the cell, and the trapping voltage was maintained at ± 2 V during the experimental sequence. Following ion formation, 100 ms to 300 s delay was employed for relaxation of the initial cyclotron and axial motion by collisions with background neutrals.

The syntheses of both peptides were described earlier.[37,38] Samples of the milled wood lignin from spruce (*Picea mariana*) were obtained by the standard isolation procedure from wood.[39] Humic acid was isolated from the Butoniga Lake sediments (Istria, Croatia) by alkaline extraction, purified by reprecipitation, dialysis and ion exchange on Chelex-100 followed by freeze-drying.[40]

RESULTS AND DISCUSSION

In the vapour phase under MALDI conditions, both peptides investigated here prefer to form alkali cationized molecular, M, and fragment ions.

Ions $[M+Na]^+$ and $[M+K]^+$ are the base peaks in their mass spectra. The negligible molecular fragmentation especially for the dermorphin could be attributed to irradiation at a wavelength strongly absorbed by the matrix, but not by the peptide. Collisions with background neutrals activate its fragmentation after a protracted time delay.

As a result of rearrangement reactions, the mass spectrum of pentapeptide Tyr-Gly-Gly-Phe-Met, **I** (Figure 1), is dominated by rearrangement ions $[M+Na-Met+OH]^+$, $m/z = 465$; $[M+K-Met+OH]^+$, $m/z = 481$; $[M+Na-Met-Phe+OH]^+$, $m/z = 318$; and $[M+K-Met-Phe+OH]^+$, $m/z = 334$. These ions $[B_{n-1,2..}+Na+OH]^+$ or $[B_{n-1,2..}+K+OH]^+$ can be considered alkali cationized peptides, *i.e.* one, two or more residue shorter than the original n residue peptide. (The initial peptide ions $[M+Na]^+$ and $[M+K]^+$ are equivalent to $[B_{n-1,2..}+Na+OH]^+$ and $[B_{n-1,2..}+K+OH]^+$.) This reaction of alkali cationized peptide, to undergo a rearrangement with the C-terminal hydroxy transfer to the adjacent residue with loss of the C-terminal residue, was observed as a metastable process for FAB generated ions using sector mass spectro-
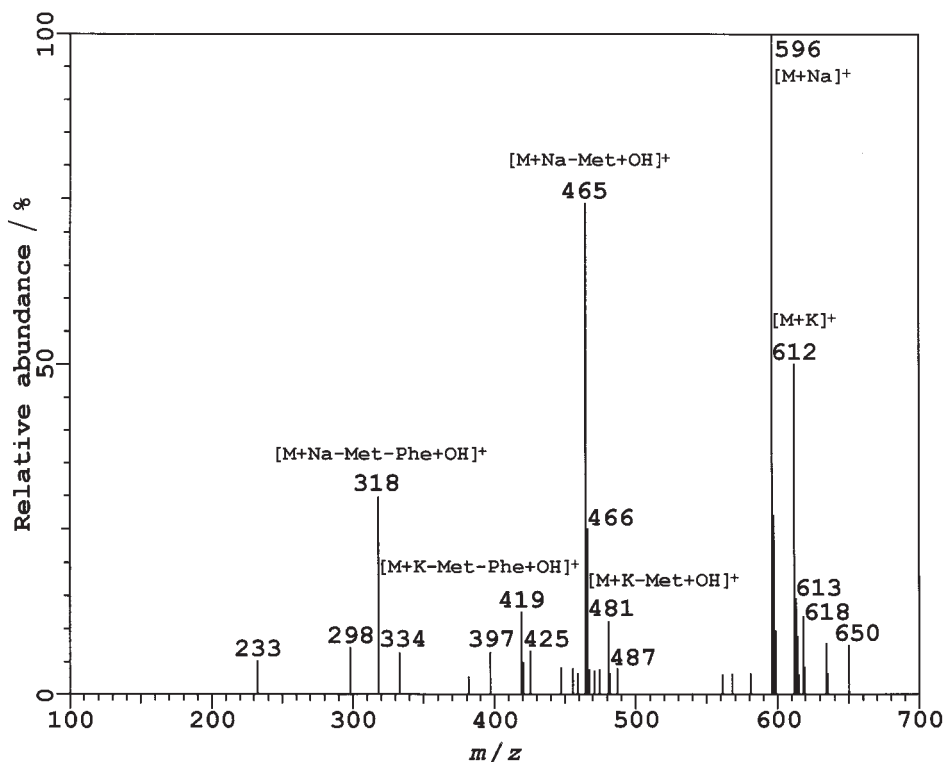

Figure 1. MALDI FTMS spectrum of Tyr-Gly-Gly-Phe-Met (**I**) (time delay 100 ms).

meter[41,42] as well as an intensive process in ion trap instruments.[43] This
suggests that it is a low energy process favoured in instruments in which
collisional activation conditions tend to generate ions from low energy pro-
cesses. The behavior of pentapeptide **I** under conditions of the MALDI
FTMS experiment provides an almost ideal case in which MS^n can be use.

The mass spectrum of heptapeptide Tyr-Ala-Phe-Gly-Tyr-Pro-Ser, **II**, is
dominated by fragment ion at m/z = 596 (Figure 2a). Deuterium labeling on
phenylalanine, Phe(D_5), increases its mass as molecular ion mass by five
units to m/z = 601 (Figure 2b). This is not the case of peptide deuterated on
proline, Pro(D_7), where the base peak is again at m/z = 596 and the molecu-
lar ion is by seven units greater (Figure 2c). These results demonstrate the
presence of Phe in ion m/z = 596 and prove its composition as [M+Na-Ser-
Pro-CO]+. The great abundance of this $[A_{n-2}+Na]^+$ ion (cleavage between
–CR_2–CO–) indicates the possible interactions between phenyl rings in Tyr,
Phe and Tyr which stabilize the formation of this ion (Scheme I).

Lignins produced negative ions up m/z = 3000 in the gas phase under
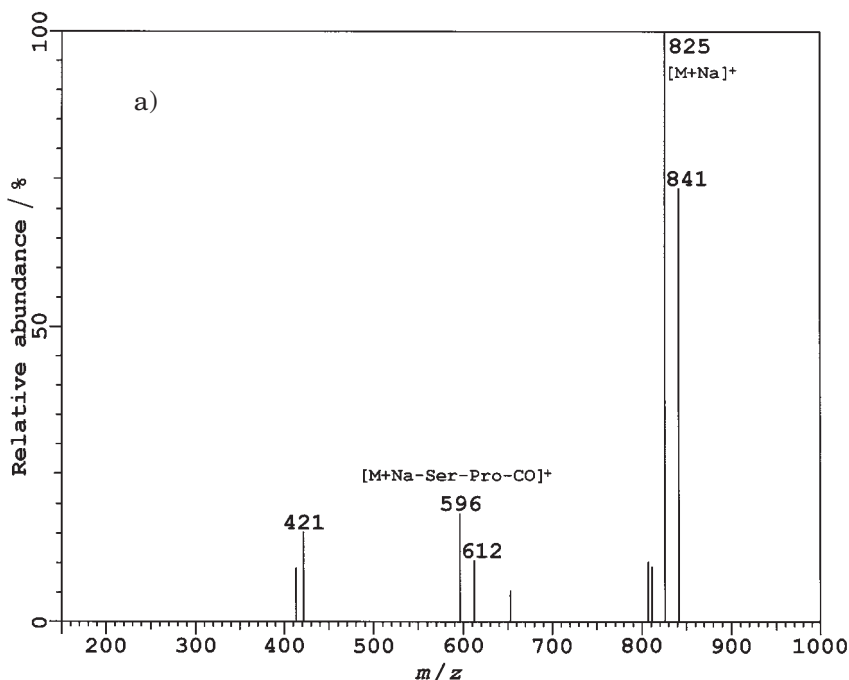LDI conditions (Figure 3). The peaks observed in the mass spectra can be
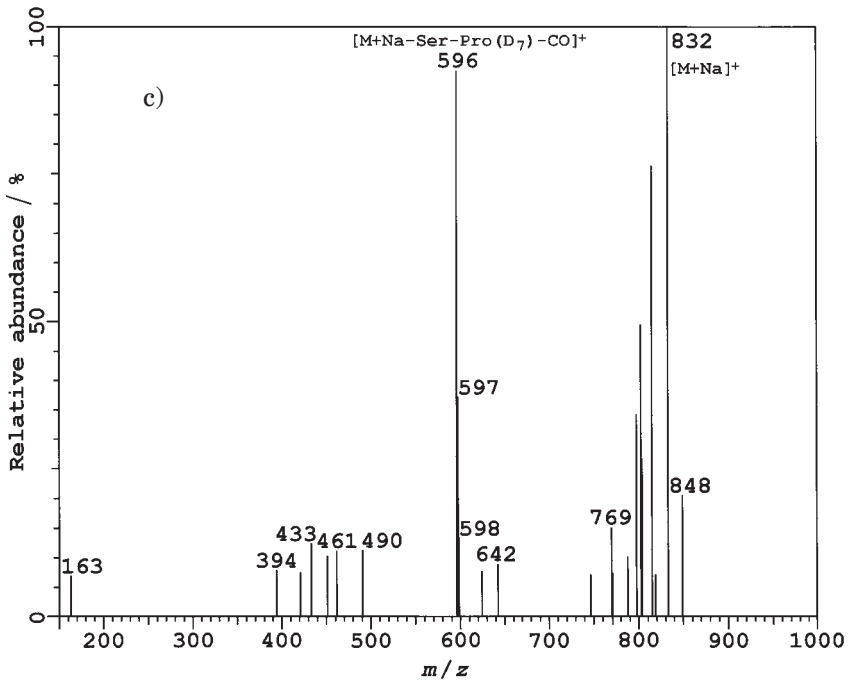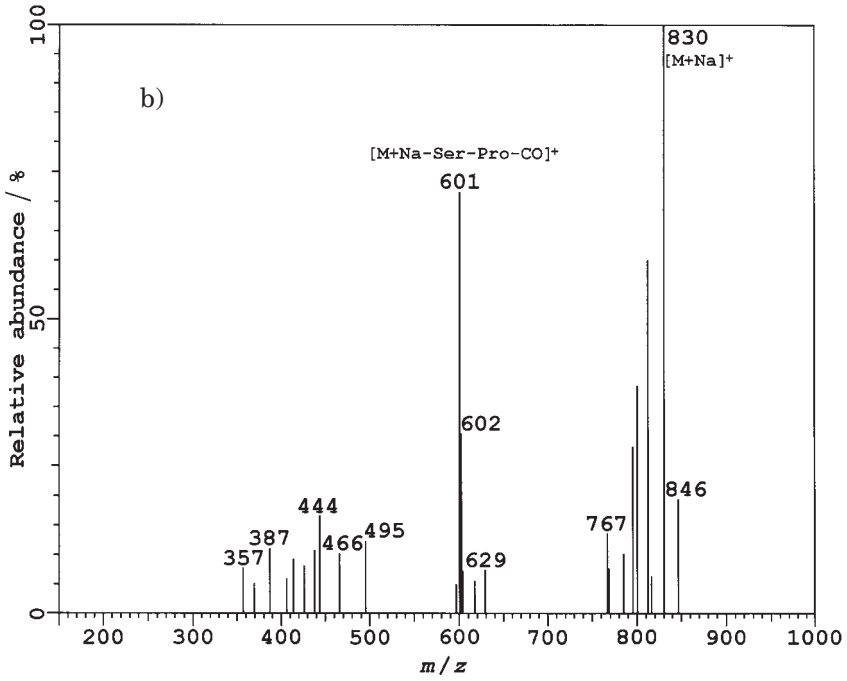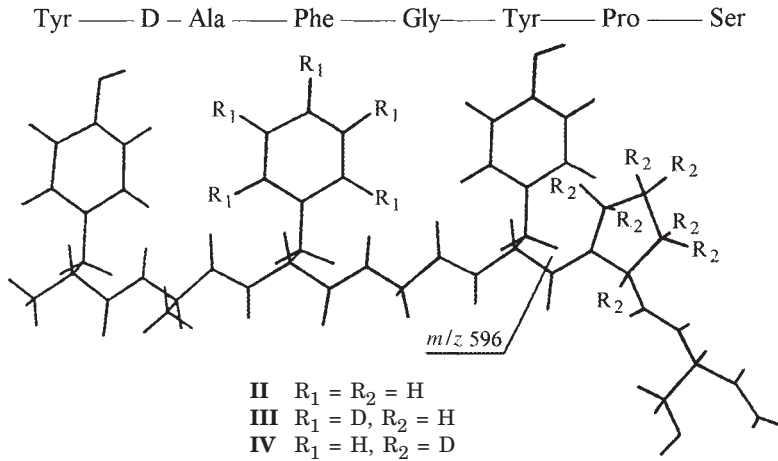

Figure 2. MALDI FTMS spectra of a) Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser (**II**); b) Tyr-D-
Ala-Phe(D_5)-Gly-Tyr-Pro-Ser (**III**); c) Tyr-D-Ala-Phe-Gly-Tyr-Pro(D_7)-Ser (**IV**) (time
delay 5 s for all spectra).   ⇒

Tyr —— D – Ala —— Phe —— Gly—— Tyr—— Pro —— Ser

II $R_1 = R_2 = H$
III $R_1 = D, R_2 = H$
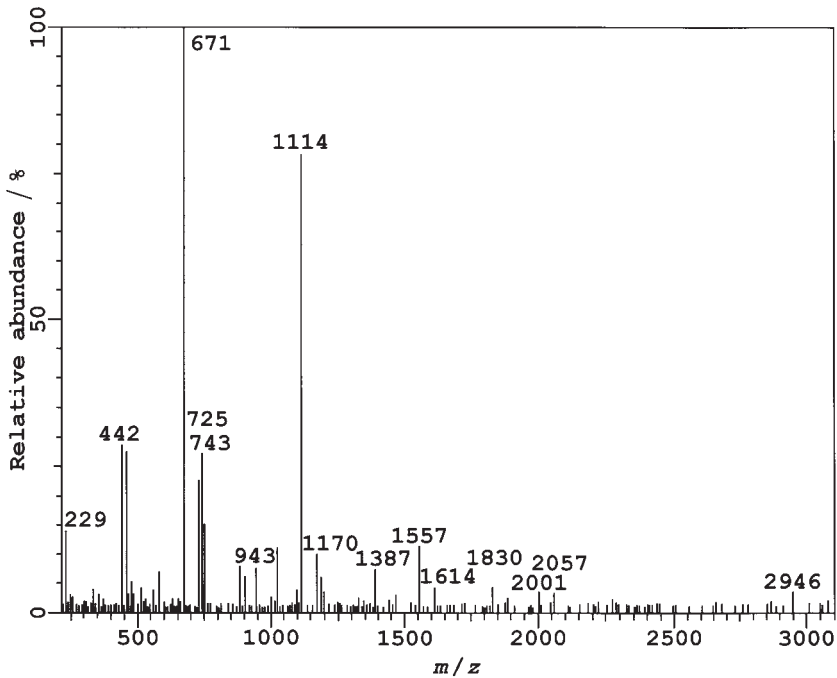IV $R_1 = H, R_2 = D$

Scheme I.

Figure 3. LD FTMS spectrum of spruce lignin (time delay 100 ms).

assigned to individual oligomers at well defined spacings. Three sequences
with $\Delta m \approx 444$

$m/z = 2946 \xrightarrow{-444,-445} m/z = 2057 \xrightarrow{-443} m/z = 1614 \xrightarrow{-444} m/z = 1170 \xrightarrow{-444} m/z = 725$

$m/z = 2001 \xrightarrow{-444} m/z = 1557 \xrightarrow{-443} m/z = 1114 \xrightarrow{-443} m/z = 671 \xrightarrow{-442} m/z = 229$

$m/z = 1830 \xrightarrow{-443} m/z = 1387 \xrightarrow{-444} m/z = 943$

confirm the existence of the stable trimer building blocks (Scheme II) in the composition of the investigated spruce lignin in agreement with coniferyl alcohol as monomeric precursor in conifers.
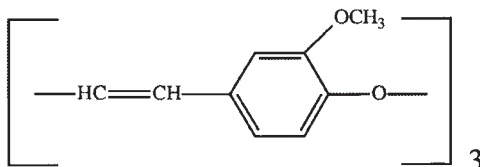
Scheme II.

In contrast to lignin, humic acid produces predominantly positive ions in the vapour phase under LDI conditions. An unusually long time delay (300 s) was needed to obtain satisfactory mass spectra (Figure 4). Increase of the
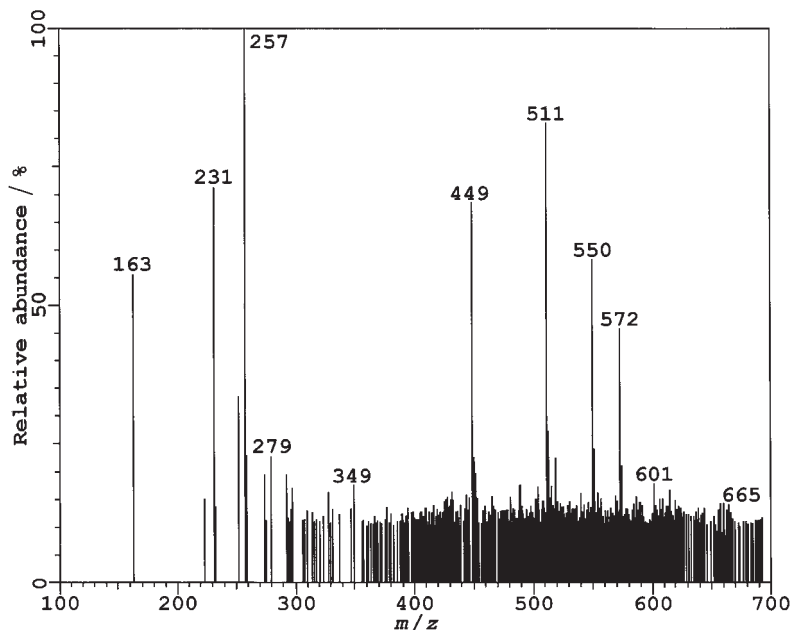

Figure 4. LD FTMS spectrum of humic acid (time delay 300 s).

time delay somewhat simplified the spectrum and extended the mass range. Experiments with the laser working at wave lengths λ = 1064 nm, 532 nm and 355 nm indicated that the spectra were susceptible to beam energy. Measurements with λ = 1064 nm yielded ions up to m/z = 700 with characteristic peaks at m/z = 163, 231, 257 (base peak), 449, 511, 550 and 572. With λ = 532 nm, the ion at m/z = 550 became the base peak followed by m/z = 523, 495 and 298, whereas with λ = 355 nm peaks up to m/z = 700 were observed, with m/z = 317 and 550 being most prominent. However, at this stage, we are unable to propose composition and/or structures for the observed ionic species. Thus, the present spectra show that, indeed, humic acid, which can be assumed to be a degradation product of lignins, has little resemblance to its source structure.

The results presented here show that LDI and MALDI are very promising ionization techniques in the field of research of natural polymers. The detailed structures of these molecules will require many additional MS^n measurements and FTMS experiments are in progress along this line.

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SAŽETAK

FTMS prirodnih polimera

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Dan je prikaz mogućnosti Fourier-transformirane spektrometrije masa u izučavanju nekih prirodnih polimera – peptida, lignina i humusne kiseline. Desorpcija i ionizacija tih termolabilnih uzoraka velikih molekulskih masa provedena je laserom (LDI) bez i uz korištenje matrice (MALDI).