ISSN 1061-9348, Journal of Analytical Chemistry, 2019, Vol. 74, No. 14, pp. 1412–1420. © Pleiades Publishing, Ltd., 2019.
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ARTICLES

Study of Nettle (*Urtica dióica*) Lignin by Atmospheric Pressure Photoionization Orbitrap Mass Spectrometry

I. I. Pikovskoi[a], D. S. Kosyakov[a, *], I. S. Shavrina[a], and N. V. Ul'yanovskii[a]

[a]*Core Facility Center "Arktika," M.V. Lomonosov Northern (Arctic) Federal University, Arkhangelsk, 163002 Russia*

**e-mail: d.kosyakov@narfu.ru*

Received December 14, 2018; revised January 18, 2019; accepted January 18, 2019

Abstract—Orbital ion trap high-resolution mass spectrometry with acetone doped atmospheric pressure photoionization is first used to characterize the structure of grass lignins using an example of nettle (Urtíca dióica) dioxane lignin. The obtained mass spectrum contains about 3,000 peaks of deprotonated molecules of lignin oligomers in the molecular mass range up to 1.6 kDa. The study of tandem mass spectra and of a composition of monomers formed in the collision-induced dissociation of lignin macromolecules showed the special role of *p*-hydroxycinnamic acids in the formation of nettle lignin. Based on the results of tandem mass spectrometry, possible structures of nettle lignin oligomers formed by the addition of guaiacyl- and syringylpropane units followed by etherification by *p*-coumaric, ferulic, and dihydroferulic acids are proposed.

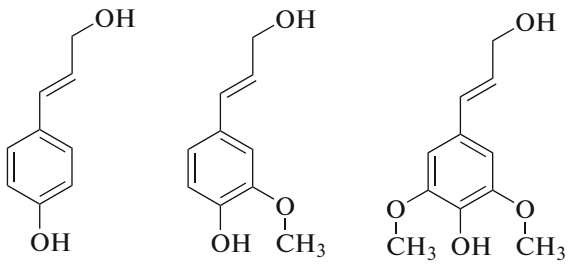
Keywords: high-resolution mass spectrometry, atmospheric pressure photoionization, APPI, grass lignin, nettle

DOI: 10.1134/S1061934819140090

INTRODUCTION

Lignins belong to the least studied class of natural high-molecular compounds because of the extreme complexity and lability of their structures. They form in the enzymatic dehydrogenative polymerization of three monolignols: *p*-coumaric, coniferyl, and sinapic alcohols (Fig. 1), which are precursors of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monomeric structural units of the macromolecule bound by various bonds [1, 2]. The prevailing are ether bonds with the participation of phenol hydroxyl groups and the β-carbon atom of the propane chain (β–O-4), involving more than one half of phenolic structures of wood lignins. Alkyl–alkyl β–β, alkyl–aryl β–5 and diaryl (generally 5–5) carbon–carbon bonds and also alkyl–aryl α–O-4, aryl–aryl 4–O-5 and alkyl–alkyl (α–O-γ and γ–O-γ) ether bonds are also met [3]. The functional composition, ratio of structural units (S : G : H), and also the prevalence of various dimeric structures strongly depend on the lignin nature and even on the method of its isolation. Today the greatest amount of structural information is accumulated for lignins of the most widespread coniferous and deciduous wood, whereas grass lignins are studied very poorly. In particular, it is known that grass lignins differ by an increased concentration of H-type structures and also by the presence of significant amounts of hydroxycinnamic (ferulic and *p*-coumaric) acid residues [4]. The last ones may be attached to the lignin macromolecule by α–O-4 and β–O-4 bonds and are involved in the formation of esters with carbohydrates [5].

The chemical composition of lignins is most often characterized using degradation methods (pyrolysis, nitrobenzene oxidation) followed by the chromatographic analysis of the obtained monomers and also using methods of 2D NMR spectrometry, which are highly laborious, time-consuming, and require substantial amounts of test materials [6]. Recently, an increasing attention in lignin chemistry has been given to possibilities of mass spectrometry (MS), opening prospects for the rapid characterization of lignins and the sequencing of individual oligomers [7–9].



p-Coumaric alcohol | Coniferyl alcohol | Sinapic alcohol

Fig. 1. Monomeric precursors of a lignin (monolignols).

Despite the success achieved recently [10], methods of mass spectrometry with matrix-assisted laser desorption/ionization have still not found wide application to the study of lignin structure because of the complexity of the material. The most elaborated methods are atmospheric-pressure chemical (APCI) and photochemical (APPI) ionization mass spectrometry [11–14]. Their application to the study of wheat straw lignin [13, 14] suggested that oligomers in the mass range available for the study (<1 kDa) have linear structures and consist mainly of phenyl coumaran dimeric units. Despite this, the data on the mass-spectrometric sequencing of lignins accumulated in the literature remain sketchy.

Taking into account the presence of a large number of isobaric compounds with equal nominal masses in lignins, the use of high- and ultrahigh-resolution mass analyzers is of special importance for their study [15, 16]. In addition to instruments of ion cyclotron resonance, high prospects in solving lignomics problems are demonstrated by orbital ion trap (Orbitrap) mass spectrometers, the resolution and accuracy of mass determination by which ensure the reliable determination of the elemental compositions of lignin oligomers even with molecular weights higher than 1 kDa. Orbitrap MS in combination with acetone-doped atmospheric pressure photoionization is one of the most effective methods for the study of lignins [17], ensuring the recording of signals of thousands of deprotonated molecules of lignin oligomers. It has been successfully applied recently to the study of oligomeric products of the depolymerization of hydrolysis lignin and allowed the researchers to reveal a number of specific features in their structures [18]. The further development of this method for the characterization of low-studied grass lignins on an example of a preparation of native nettle lignin is the aim of this work. The choice of the test sample was determined by the prevalence and availability of the plant, and also by its value as a medicinal raw material and a foodstuff.

EXPERIMENTAL

Reagents and materials. For the isolation and purification of the lignin preparation, we used acetone, 1,4-dioxane, and diethyl ether of analytical grade and hydrochloric acid and sodium hydrocarbonate of chemically pure grade (Komponent-Reaktiv, Russia). Solutions for mass-spectrometry analysis were prepared using acetone of high-purity grade (Komponent-Reaktiv, Russia) and ultra-pure water obtained with a Simplicity UV system (Millipore, France).

Lignin preparation and its characterization. The initial raw material of nettle (*Urtíca dióica*) was collected in the Primorskii district of the Arkhangelsk region (Russia). Fresh plants were dried in air and then leaves were separated from the culm. Dry culms were ground to uniform powder in a cutting mill and subjected to extraction with acetone for 48 h in a Soxhlet apparatus for the complete removal of extractive substances.

The preparation of native nettle dioxane lignin was isolated by the Pepper method [19] with small modifications. Eight hundred milliliters of a 0.2 M HCl solution in aqueous dioxane was added to a weighed portion of the prepared plant raw material (100 g) in a round-bottom flask, so that the ratio of 1,4-dioxane to water was 9 : 1 (v/v); the mixture was allowed to stand for 30 min at room temperature in an inert atmosphere for impregnation. Then the mixture was heated on a boiling water bath for 4 h in a nitrogen (99.99%) flow and the undissolved residue was filtered on a Bühner funnel and two times washed with 40-mL portions of 1,4-dioxane; the solution obtained was united with the filtrate. The liquid was neutralized by $NaHCO_3$ (20–25 g) to the termination of gas liberation; nitrogen was blown within 5 min; and the flask was hermetically closed and allowed to stand for a night. After replicate filtration, the solution was evaporated on a rotor evaporator under vacuum to a volume of 100 mL. The obtained concentrate was added dropwise to an 8-fold excess of water. The precipitated lignin was separated from the solution by centrifugation and dried under vacuum. Residual amounts of impurities were removed by the reprecipitation of lignin from 1,4-dioxane in diethyl ether. The yield of the obtained preparation was 4% of the mass of the initial oven dried raw material.

Number-average (M_n) and mass-average (M_w) molecular weights of the obtained lignin preparation were determined by exclusion chromatography [20] using an LC-20 Prominence HPLC system (Shimadzu, Japan) with an SPD-20A spectrophotometric detector. Separation was performed at 40°C on an MCX column for the analysis of water-soluble polymers, 300 × 8 mm with particle size of 1000 Å (PSS, Germany). Detection was performed at the wavelength 275 nm. The mobile phase and the sample solvent was a 0.1 M sodium hydroxide solution. The system was calibrated on standard monodisperse samples of sodium polystyrene sulfonate (PSS, Germany). The found M_n and M_w values were 1400 and 1700 Da, respectively.

To determine the S : G : H ratio, we used pyrolitic gas chromatography/mass spectrometry [6] on an GCMS-QP2010 Plus system (Shimadzu, Japan) equipped with an EGA/PY-3030D pyrolizer (Frontier Lab, Japan) with a cryotrap cooled by liquid nitrogen. The thermal decomposition of the sample (~ 150 μg) was performed in a helium atmosphere at the temperature 450°C within 30 s. The products formed after cryofocusing were separated on an HP-5MS column (Agilent, United States), 30 m × 0.25 mm, stationary phase film thickness 0.25 μm. Carrier gas was helium (1 mL/min). Injector temperature was 270°C. The temperature program of the thermostat was as follows: linear increase from 40 to 340°C within 30 min.

Detection was performed in the scanning mode in the m/z range 15–400. The ratio of syringyl, guaiacyl, and *p*-hydroxyphenyl structural fragments was determined as the ratio of the total areas of chromatographic peaks of monomeric phenols of the specified types identified in the chromatogram. The obtained S : G : H ratio was 17 : 75 : 8.

The elemental composition of the sample was determined by catalytic combustion on a EuroEA-3000 CHNS-analyzer (EuroVector, Italy). The results of analysis were as follows, %: C, 60.3; H, 6.3; O, 33.4 (by the difference) and correspond to the gross formula of the preparation in terms of the quaiacylpropane structural unit $C_{10}H_{12.5}O_{4.2}$.

Analysis by mass spectrometry. Mass spectra were recorded on a Q Exactive Plus hybrid mass spectrometer (Thermo Scientific, USA) with an orbital ion trap mass analyzer at a resolution of 70000 (FWHM, for m/z 200). An Ion Max ion source equipped with an APPI system with a krypton lamp with a quantum energy of 10.0 eV was used. The mass scale was calibrated according to the recommendations of the manufacturer, using a solution of a mixture of Pierce standards (Thermo Scientific, United States). A lignin solution (5 μL) with the concentration 50 mg/L in an acetone : water (9 : 1) mixture was injected in the ion source with a solvent flow (200 μL/min) using a chromatographic LC-30 system (Shimadzu, Japan), which consisted of a pump, a degasser, and an autosampler. Mass spectra were recorded in the negative ion mode in the m/z ranges 90–300 and 300–2000 by averaging of the results of not less than 10 measurements and subtracting the background signal of the solvent. We used the target value of filling the C-Trap (AGC) ion trap 5×10^5. The peaks were picked using the threshold value of relative intensity (I_{rel}) 0.1%.

We used the following optimum parameters of the ion source, ensuring the maximum intensity of mass spectra of the studied lignin preparation and found in preliminary experiments: drying gas pressure 20 psi; flow rates of the nebulizing and curtain gases 5 and 2 arbitrary units, respectively; desolvation line temperature 250°C; ion source temperature 500°C; and RF voltage on the S-lens 55 arbitrary units.

Tandem mass spectra were recorded using collision-induced dissociation (CID) with nitrogen in the HCD cell of the mass spectrometer.

The operation of the mass spectrometer and data collection and processing were controlled using the Xcalibur software (Thermo Scientific, USA).

The elemental compositions of the lignin oligomers were determined on the basis of their exact molecular masses using the acceptable deviation of the experimental m/z value from the calculated one at a level of 3 ppm and the following restrictions on the number of element atoms: C, 4–100; H, 0–200; O, 0–50.

RESULTS AND DISCUSSION

Mass spectrum of nettle lignin. The mass spectrum of the obtained lignin preparation contained as many as about three thousand peaks of deprotonated oligomer molecules $[M-H]^-$ with relative intensities higher than 0.1% (Fig. 2).

As in the case of woody plant lignins [17, 18], peaks in the mass spectrum were united into groups corresponding to oligomers with different degrees of polymerization. The largest oligomers detected with the good signal-to-noise ratio belonged to the octamer and had masses up to 1600 Da. The distance between the peak groups varied in the range from 190 to 210 Da. The average mass of a structural monomer unit was close to 200 Da and corresponded to the predominance of quaiacylpropane structures, which agrees with the results of determination of the S : G : H ratio. By this indicator, nettle dioxane lignin is similar to native coniferous lignin, for which the prevailing value is 196 Da, which corresponds to the most widespread quaiacylglycerol structural unit [21]. The spectrum in the region of low masses (Fig. 2b) includes mainly peaks of monomeric aromatic acids and phenols, formed in the course of the partial degradation of lignin macromolecules in an ion source. Our attention was attracted by the prevalence of cinnamic acids – *p*-coumaric and ferulic (m/z 163.0401 and 193.0506, respectively) and also, probably, of 3,4-dimetoxycinnamic (m/z 207.0663) and 3,4-dihydroxydihydrocinnamic (dihydrocaffeic) with m/z 181.0506 among these compounds.

Calculations of the elemental compositions of all oligomers detected in the range m/z 300–1000 (dimer–pentamer) on the basis of their exact masses and the representation of the results on van Krevelen coordinates [22] allowed us to obtain an "image" of the studied preparation of nettle dioxane lignin (Fig. 3). As in the case of coniferous wood lignin [17], in which G-type units also prevailed, the majority of oligomers are characterized by ranges of elemental compositions H/C = 0.6–1.2 and O/C = 0.2–0.6. A distinguishing feature of nettle lignin is in the presence of a significant amount of oligomers with increased oxygen content (O/C = 0.4–0.6), which is characteristic for aromatic acids, and also in the presence of a certain amount of strongly unsaturated oxygen-containing structures with H/C = 0.3–0.6 at O/C = 0.2–0.6. This points to an increased percentage of oxidized aromatic structures containing carbonyl and carboxyl groups. This conclusion agrees with the results of peak assignment in the region m/z 90–200 and also with the data obtained by tandem mass spectrometry.

Application of tandem mass spectrometry to the study of the structure of nettle lignin. For the detailed characterization of monomeric structural units of nettle lignin, we used the approach [18] proposed recently based on recording a mass spectrum (MS/MS) of CID products of a set of precursor ions in a wide range of

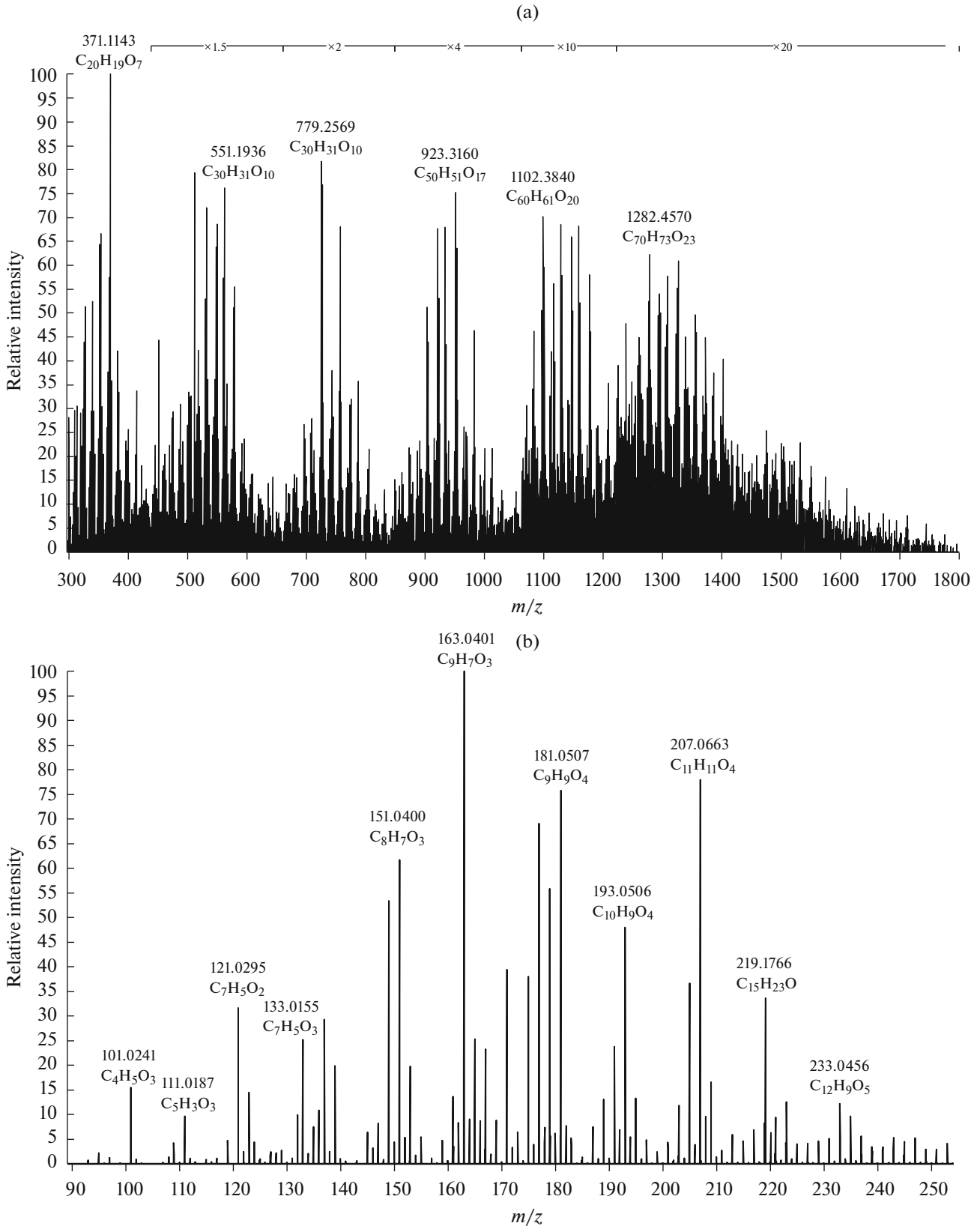


Fig. 2. Mass spectra of nettle dioxane lignin recorded in the acetone-doped atmospheric pressure photoionization mode (a) in the range m/z 300–1800 and (b) in the range m/z 90–300.

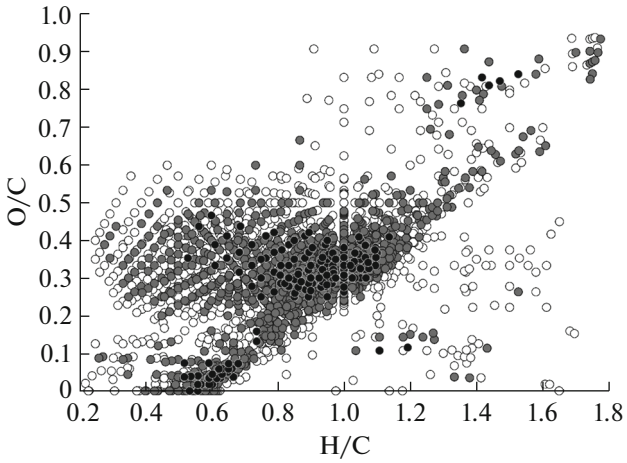


Fig. 3. Van Krevelen diagram for nettle dioxane lignin (point colors correspond to relative peak intensities in the mass spectrum: white, 0.1–1%; gray, 1–10%; black, >10%).

m/z extracted by a quadrupole mass filter. To do this, we selected the region of trimers (*m/z* 450–650) and collision energy of 30 eV (Table 1), which allowed us to ensure the maximum yield of product ions with molecular masses 100–200 Da. As in the corresponding region of the MS^1 spectrum, the main peaks in the obtained tandem mass spectrum corresponded to hydroxycinnamic, *p*-coumaric (the maximum signal intensity), ferulic and dihydroferulic acids, the amount of which was more than one third of the total peak intensity of all monomers. The presence of such amount of phenylpropane acids among the CID products is a highly specific feature, which drastically distinguishes the studied preparation from coniferous wood lignin [18]. An exclusively high intensity of the peak of *p*-coumaric acid (about 25% of the total intensity) indicates that it corresponds at least to the majority of H-type structures in the studied lignin preparation. The second in prevalence group of monomers after acids were aromatic products of polymer degradation bearing no side propane chains. First of all, this was a compound with the gross formula C_8H_8O, which is most likely 4-vinylphenol, but may be also acetophenone or coumaran, because the corresponding structures are quite typical for lignins. Guaiacyl structures, hydroxyanisole, vanillin, and vanillyl alcohol, were also found in large amounts. In the region of lower masses (<100 Da), the CID products contained residues of three acids, glyoxalic ($C_2H_2O_3$), glycolic ($C_2H_4O_3$), and pyruvic ($C_3H_4O_3$), formed on the cleavage of side chains of phenylpropane structures.

The most valuable information on the structure of the lignin oligomer can be obtained in the study of tandem mass spectra of individual precursor ions. Solving this problem is complicated, on one hand, by the presence of a large number of isomeric and isobaric structures that cannot be isolated individually with a quadrupole mass filter for the subsequent CID and, on the other hand, by the unreality of their preliminary chromatographic separation. In this regard, for the further experiments with CID, we selected two ions with *m/z* 371.1143 (dimer $[C_{20}H_{19}O_7]^-$) and *m/z* 581.2024 (trimer $[C_{31}H_{33}O_{11}]^-$) from the lignin mass spectrum, which are characterized by the highest peak intensity and the minimum interferences with isobaric structures. The MS/MS spectra (Fig. 4) demonstrate the abstraction of methyl groups and formaldehyde, characteristic for lignins, and also the break of bonds between structural units with the release of monomeric fragments. Their most intense peaks with *m/z* 163.0401 and 193.0506 corresponded to *p*-coumaric and ferulic acids. The spectrum of the trimer also exhibited an intense peak with *m/z* 195.0662, which can be assigned to dihydroferulic acid. Based on the data of tandem mass spectrometry, we proposed possible structural formulae of the studied oligomers (Fig. 4). The dimer structure possibly includes dihydroferulic and *p*-coumaric acids, forming a β–O-4 bond. One of carboxyl groups may be esterified by methanol, which is attested by the easy loss of a methyl group with the formation of an ion giving an intense signal at *m/z* 356.0902. The anticipated trimer structure includes a syringylpropane moiety bound to phenolic hydroxyl groups of dihydroferulic and *p*-coumaric acids by α–O-4 and β–O-4 bonds. The growth of the macromolecular chain can proceed by the further esterification of the phenolic hydroxyl group of the syringylpropane or quaiacylpropane unit and the attachment of a new acid residue with the formation of additional α–O-4 and β–O-4 bonds. This allows us to explain the formation of a sequence of intense peaks of deprotonated molecules of tri-, tetra-, and pentamers,

Table 1. Main fragments formed in the CID of deprotonated trimer molecules of nettle dioxane lignin (precursor ions *m/z* 450–650, collision energy 30 eV)

Anticipated structures	Ion	*m/z* (exp)	*m/z* (theor)	Δ, ppm	I_{rel}, %
Dihydroxybenzene	$[C_6H_4O_2]^-$	108.0216	108.0217	–0.83	19
2-Furancarboxylic acid	$[C_5H_3O_3]^-$	111.0087	111.0088	–0.33	19
Hydroxymethylfuran	$[C_5H_5O_3]^-$	113.0244	113.0244	–0.06	37
Acetophenone, 4-vinylphenol, coumaran	$[C_8H_7O]^-$	119.0502	119.0502	–0.29	47
Hydroxybenzoquinone	$[C_6H_3O_3]^-$	123.0088	123.0088	0.35	21
Guaiacol	$[C_7H_7O_2]^-$	123.0452	123.0451	0.55	11
Methoxyfuraldehyde	$[C_6H_5O_3]^-$	125.0244	125.0244	0.10	11
3,4-Dihydroxybenzaldehyde 4-hydroxybenzoic acid	$[C_7H_5O_3]^-$	137.0244	137.0244	–0.18	10
Vanillin	$[C_8H_7O_3]^-$	151.0401	151.0401	0.07	20
Syringol, vanillyl alcohol	$[C_8H_9O_3]^-$	153.0558	153.0557	0.54	7
p-Coumaric acid	$[C_9H_7O_3]^-$	163.0401	163.0401	0.08	100
4-Methoxycinnamic acid	$[C_{10}H_9O_3]^-$	177.0557	177.0557	0.38	17
Acetosyringol, caffeic acid	$[C_9H_7O_4]^-$	179.0349	179.0350	–0.35	10
Syringic acid, dihydrocaffeic acid	$[C_9H_9O_4]^-$	181.0506	181.0506	–0.13	17
Ferulic acid	$[C_{10}H_9O_4]^-$	193.0506	193.0506	–0.26	19
Dihydroferulic acid	$[C_{10}H_{11}O_4]^-$	195.0662	195.0663	–0.25	24
3,4-Dimethoxycinnamic acid	$[C_{11}H_{11}O_4]^-$	207.0663	207.0662	1.08	6

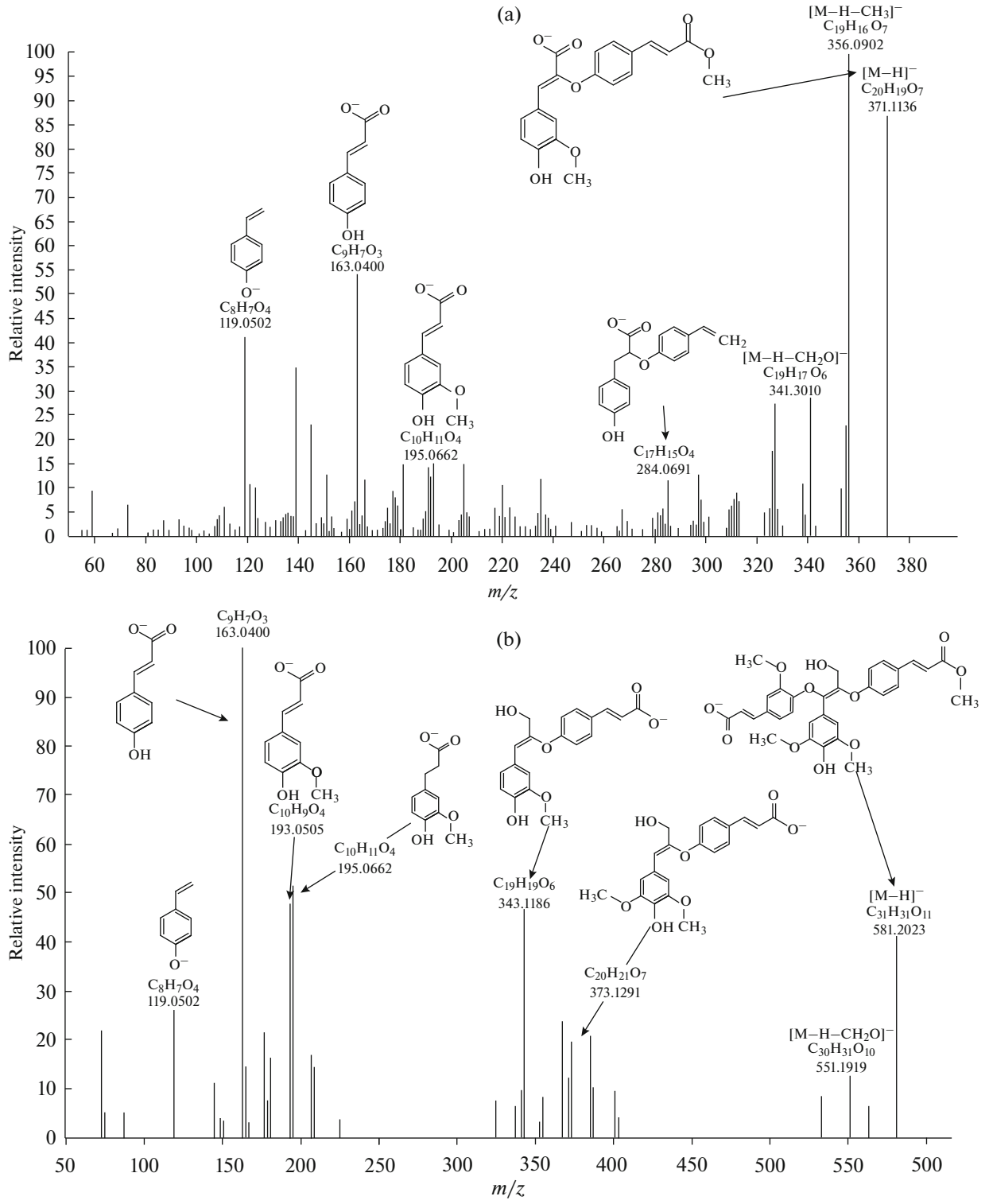


Fig. 4. Mass spectra of the products of collision-induced dissociation of a dimer with *m/z* 371 and a trimer with *m/z* 581 (collision energy 30 eV).

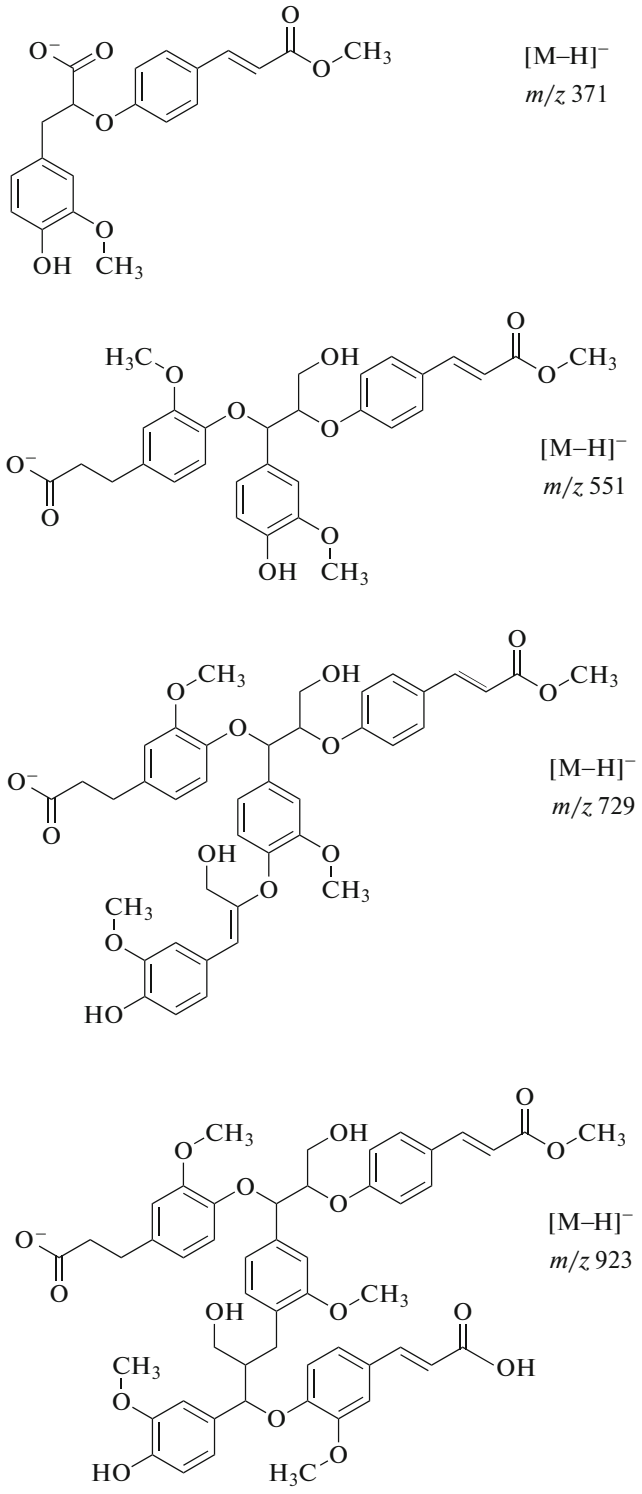


Fig. 5. Anticipated structural formulas of deprotonated molecules of di-, tri-, tetra-, and pentamer with *m/z* 371, 551, 729, 923 in a nettle dioxane lignin preparation.

observed in the MS^1 spectrum at *m/z* 551.1936, 729.2569, 923.3160, respectively (Fig. 5). An increase in the degree of polymerization by the esterification of hydroxycinnamic acid carboxyl groups with the formation of linear macromolecules may be an alternative.

CONCLUSIONS

The application of high-resolution orbitrap mass spectrometry and acetone-doped atmospheric pressure photoionization allows obtaining valuable structural information on grass lignins and their difference from much better studied wood lignins. The study of the mass spectra of monomeric fragments obtained in the CID of deprotonated molecules in the wide *m/z* range and also of tandem mass spectra of individual oligomers allows us to draw a conclusion about the key role *p*-hydroxycinnamic acids (ferulic and *p*-coumaric) in the growth of nettle lignin macromolecules.

ACKNOWLEDGMENTS

This work was performed using instrumentation of the "Arktika" Core Facility Center of the Northern (Arctic) Federal University. The development of the infrastructure the "Arktika" Core Facility Center was supported by the Ministry of Education and Science of the Russian Federation (unique identifier RFMEFI59417X0013).

FUNDING

This work was supported by Russian Foundation for Basic Research, project no. 18-33-00705-mol-a.

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Translated by E. Rykova