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Negative ion mode atmospheric pressure ionization methods in lignin mass spectrometry: A comparative study

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RATIONALE: Mass spectrometry with atmospheric pressure ionization is the most promising method for studying the structure of natural lignin, which is the second most abundant biopolymer in nature. The goal of this study is to compare the efficiency and characteristics of different types of ionization techniques (ESI, APCI, and APPI) in the negative ion mode by the example of softwood lignin.
METHODS: As the subjects of the study, we selected a preparation of spruce dioxane lignin and several phenols, simulating the basic structural fragments of the lignin macromolecule. High-resolution mass spectra were recorded using an Orbitrap mass spectrometer. Acetone was used as a solvent for samples and a dopant in photoionization mode. The ionization conditions were optimized to achieve the maximum intensity of the mass spectra.
RESULTS: The formation of deprotonated lignin molecules is characteristic of all the studied types of ionization; partial fragmentation of the biopolymer occurs in all ionization modes. ESI in the presence of ammonia yields low-intensity signals, leads to a significant decrease in ionization efficiency with increasing molecular weight of lignin oligomers, gives high-intensity impurity peaks in the mass spectra, and demonstrates selectivity for more polar structures. The ionization efficiency increases sharply in the order of ESI < APCI < APPI. The two latter methods are characterized by similar mechanisms of ionization; they ensure detection of approximately 1900 spruce lignin oligomers in the range of molecular weights up to 1.8 kDa. The determination of the elemental composition of oligolignols enabled the four main groups of compounds to be distinguished.
CONCLUSIONS: Photoionization using acetone as a dopant is distinguished by a significantly higher intensity of signals and the lowest sensitivity to contaminants present in the lignin preparation. This ionization method can be considered as preferred for studying the dioxane lignin preparations of woody plants. Copyright © 2016 John Wiley & Sons, Ltd.

Lignin is the second most abundant biopolymer in nature after cellulose; it is a product of enzymatic oxidative polymerization of three monomeric aromatic compounds (monolignols): coniferyl, sinapic, and *p*-coumaric alcohols. Its structure comprises phenylpropanoid units linked together by various bonds. Currently, lignin is viewed as a promising commercial source of a wide range of aromatic compounds, alternative to fossil hydrocarbons. There is a growing interest by researchers in the structure of lignins of various plants, which, in spite of almost 100 years of research, is still a matter of debate.[1] In this regard, mass spectrometric methods, which open up possibilities for sequencing lignin oligomers and for interpreting the plant 'lignome', are the most promising.[2,3]

A substantial amount of information about the mass spectra of lignins of various origins and lignin-derived compounds, the most common of oligomeric structures identified from the mass spectrometric data, and the pathways of their collision-induced dissociation has been accumulated in the literature.[3] Nevertheless, the mass spectra of lignin preparations from the same origin, published in various sources, often differ radically, which, in our opinion, is due to a number of unresolved methodological problems. This is, above all, the selection of ionization conditions of analytes, which is a key factor for obtaining high-quality spectra of natural compounds such as lignins that are extremely difficult to study.

Methods such as matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and atmospheric pressure ionization mass spectrometry, including those in combination with preliminary chromatographic separation, have been used in various studies. Despite the undoubted advantages of MALDI, this method is not currently favored in structural studies of lignin for several reasons, the most important of which is a low ionization efficiency of the biopolymer achieved using conventional matrices.[4,5]

Atmospheric pressure ion sources are used in most studies on the mass spectrometry of lignin, with electrospray ionization (ESI) taking the leading role.[6–17] Given the presence of significant amounts of functional groups in lignin macromolecules, capable of protolytic dissociation (phenolic, hydroxyl, and carboxyl), the negative ion detection mode (ESI(−)) is more frequently used. To

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improve the efficiency of generation of deprotonated molecules, a sample solution was made alkaline by the addition of ammonia (0.25–4%)[6–8,12,16] or sodium hydroxide to pH 11,[13–15] despite the introduction of nonvolatile salts into the mass spectrometer being highly undesirable. Positive ion electrospray ionization (ESI (+)) was also used in several studies devoted mainly to low-molecular-weight degradation products of lignins.[10–12] To obtain high-intensity mass spectra of monolignols and dilignols in this mode, Na^+ cationization was enhanced through the introduction of significant amounts of sodium chloride into test solutions.[13] The disadvantage of this approach is the impossibility of obtaining tandem mass spectra due to the elimination of metal cation during collision-induced dissociation (CID). Another adverse effect of ESI is the high (compared with the analyte) ionization efficiency of a number of impurities contained in both the solvents used and the lignin preparations. This may be a reason for the presence of intense equally spaced peaks in the mass spectra, which were observed by some authors and probably wrongly attributed to lignin.[9–11]

Atmospheric pressure chemical ionization (APCI) is considered as the main alternative to ESI in studies of lignin,[13,18–20] with such advantages over ESI as the ability to ionize weakly polar molecules and the low sensitivity to matrix effects. This method of generating negative ions (APCI(−)) in combination with multistage fragmentation of analytes by CID was used successfully by Morreel *et al*.[18,19] in the development of approaches for sequencing oligolignols. APCI in both negative and positive ion mode was also used in the study of the structure of wheat straw lignin isolated from the plant material with a mixture of formic and acetic acids with water.[20] In the mass spectra obtained, no significant signals were detected at *m/z* above 600; the authors attributed this to the fact that lignin could undergo degradation under relatively stringent APCI conditions. Haupert *et al*. also considered this issue; by the example of model compounds, they showed that upon chemical ionization, the structural fragments of lignin macromolecules easily eliminate water and formaldehyde and are subject to demethylation.[13] It is pointed out in the same publication that APCI has a limited applicability for recording representative mass spectra of lignin due to its extremely low ionization efficiency with respect to the groups with weak acidic properties.

Banoub *et al*.[21] were the first to propose atmospheric pressure photoionization (APPI) for the study of wheat straw lignin; the method had performed well previously in the obtaining of the mass spectra of humic substances and even oxidative degradation products of industrial lignin.[22] Introduction of toluene as a dopant into the ion source enabled the recording of sufficiently intense peaks of both positive ($[M + H]^+$) and negative ($[M–H]^-$) ions in the *m/z* range of 100–1200, corresponding to the total of 57 oligomeric structures. In a recent comprehensive review, the same authors characterized APPI as the most promising method for the soft ionization of lignin, having all the advantages of chemical ionization, including excellent linearity of response and tolerance to matrix effects.[3] However, there are no further details on the application of photoionization in the mass spectrometry of woody plant lignins in publications so far.

The goal of this study is to compare the efficiency and characteristics of different atmospheric pressure ionization techniques by the generation of negative ions as a tool for obtaining high-quality mass spectra of natural lignin, suitable for structural studies of the biopolymer.

EXPERIMENTAL

Reagents and materials

Four monomeric phenols (isoeugenol, coniferyl aldehyde, ferulic acid, and acetovanillone) and four dimeric phenols (guaiacylglycerol-β-guaiacyl ether, secoisolariciresinol, dihydrodehydrodiisoeugenol, and pinoresinol) (Fig. 1) were used to simulate the guaiacyl structural units of softwood lignin macromolecules and the most important types of bonds between them. All the test compounds were purchased from Sigma-Aldrich (Steinheim, Germany) and Fluka (Buchs, Switzerland); the concentration of the main substance in each of them was at least 98%. Dihydrodehydrodiisoeugenol is an exception; it was synthesized from isoeugenol according to a known procedure.[23]

Methanol (J.T. Baker LC/MS grade; Avantor, Gliwice, Poland), acetone (high-purity grade, Komponent-Reaktiv, Saint Petersburg, Russia), and high-purity water obtained from a Milli-Q system (Millipore, Molsheim, France) were used for the preparation of solutions of the above compounds and lignin. An alkaline medium was created by adding ammonium hydroxide (a 30% aqueous solution, ACS reagent, Sigma-Aldrich).

Production and characterization of the lignin preparation

Dioxane lignin (DL), a representative of native (virgin released) woody plant lignins, isolated from spruce *Picea abies* by the Pepper method,[24] was selected for the study. Extractive substances (approximately 2 wt % of oven-dry raw material) were removed from air-dried sawdust by extraction with acetone in a Soxhlet apparatus for 48 h. Then 50 g of the prepared sawdust was placed in a flask with a reflux condenser and poured with 800 mL of a 0.2 M HCl solution in a mixture of 1,4-dioxane and water (9:1). After soaking for 30 min, the mixture was heated in a water bath under nitrogen for 4 h. The resulting extract was neutralized with sodium bicarbonate to pH 7–8 and evaporated under vacuum in a rotary evaporator to a volume of 100 mL. The concentrate was added dropwise into an eightfold excess of water under continuous stirring and nitrogen bubbling. After settling, the precipitated lignin was separated by centrifugation, washed with water, frozen with liquid nitrogen, and freeze-dried. The yield of dioxane lignin was 11% by weight of oven-dry wood.

The molecular weight characteristics of the resulting product were determined by size-exclusion chromatography. The LC-20 chromatographic system (Shimadzu, Kyoto, Japan) consisted of a pump, a vacuum degasser, a column thermostat, an autosampler, and a spectrophotometric detector. Separation was performed in a MCX column (8 × 300 mm; PSS, Mainz, Germany); a 0.1 M aqueous NaOH solution was used as the mobile phase. The test sample and calibration samples were dissolved in the eluent (1 mg mL^{-1}). The injected

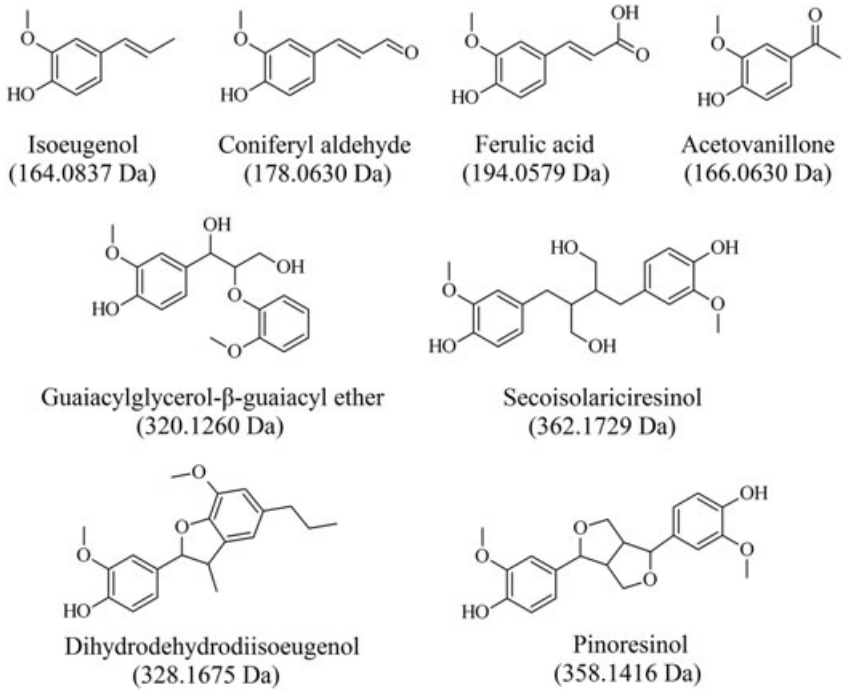

Figure 1. Chemical structures and monoisotopic molecular masses of lignin model compounds.

sample volume was 20 μL. Narrow-dispersed samples of sodium polystyrene sulfonates were used for calibration. Detection was performed at 280 nm. WinGPC software (PSS) was used for data acquisition and processing. The analysis results were: M_n = 1400 Da, M_w = 8200 Da, and a polydispersity index (M_w/M_n) = 5.8.

Elemental analysis of the lignin preparation was performed using a EA3000 CHNS-analyzer (EuroVector, Milan, Italy). The results were (%): H, 6.5; C, 63.4; O, 30.1 (by difference). Recalculated to a guaiacylpropane structural unit, the gross formula of lignin under study can be represented as $C_{10}H_{12.2}O_{3.6}$.

Mass spectrometric analysis

Mass spectra were recorded using a Q Exactive Plus hybrid mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with an orbitrap mass analyzer with a resolution of 70,000 FWHM (for m/z 200) and an Ion Max ion source with interchangeable probes for heated electrospray ionization HESI II, atmospheric pressure chemical ionization, and atmospheric pressure photoionization. In APPI, a krypton discharge lamp with a photon energy of 10.0 eV was used as a radiation source. The mass scale was calibrated using a Calmix mixture (Thermo Scientific). We applied a direct loop injection (5 μL) of the test solution with a concentration of 50 mg L^{-1} into the ion source of the mass spectrometer at a flow rate of 100–300 μL min^{-1} generated by an LC-30 AD chromatographic pump (Shimadzu). Mass spectra were recorded in the m/z range of 50–750 (for model compounds) and 300–3000 (for lignin) with subsequent averaging of the results of at least ten measurements and subtracting the solvent background signal. The peaks were detected using a threshold value of the relative intensity of 0.1%, which corresponded to a signal-to-noise ratio of at least 10.

For each type of ionization, optimal ion source parameters found in preliminary experiments were applied, ensuring the maximum intensity of the mass spectra of the test lignin preparation. The ESI settings were: spray voltage, 3.5 kV; sheath gas (N_2) pressure, 20 psi; Aux and sweep gas (N_2) flow rates, 10 and 2 arbitrary units, respectively; desolvation capillary temperature, 275°C; Aux gas heater temperature, 150°C; S-lens RF level, 55%. The APCI and APPI settings were: discharge current, 25 μA (only for APCI); sheath gas (N_2) pressure, 20 psi; Aux and sweep gas (N_2) flow rates, 5 and 2 arbitrary units, respectively; desolvation capillary temperature, 250°C; vaporizer temperature, 500°C; S-lens RF level, 55%.

Xcalibur software (Thermo Scientific) was used to control the mass spectrometer and to collect and process the data. In determining the elemental composition of the ions corresponding to the peaks in the mass spectra, a value of 3 ppm was taken as a permissible relative deviation of the calculated m/z values from the measured values. To analyze mass spectrometric data and to calculate the Log$P$ values of analytes, we used an ACD Structure Elucidator software package (ACD Labs, Toronto, Canada).

Selection of solvent for the sample

The ionization efficiency of analytes is largely determined by the solvent used, which, in addition to sufficient solvent power with respect to the compounds under study, should ensure the dissociation of the phenolic hydroxyl groups of lignin in solution (for electrospray ionization) or in the gas phase (for chemical ionization or photoionization). Dimethyl sulfoxide and *N*,*N*-dimethylformamide are the best solvents for lignins; however, they cannot be recommended for mass spectrometric studies because of their high boiling points. Experiments on the recording of the mass spectra of lignin,

using methanol, acetonitrile, 1,4-dioxane, and acetone, showed that the latter with the addition of 10% of water (to ensure the high solubility of lignin) yielded the most intense mass spectra. Despite a linear increase in the pK_a values of guaiacol derivatives with increasing mole fraction of the organic solvent in the acetone–water system,[25] the introduction of 0.1% of ammonia into aqueous acetone provides a sufficient degree of dissociation of phenols for ESI and gives results comparable with an ammonia solution in methanol, which was used previously.[6–8] The high basicity of acetone promotes the deprotonation of analyte molecules under APCI and APPI conditions, thus contributing to obtaining high-quality mass spectra of lignin. The advantages of this solvent are the best manifested in photoionization. Because of its low ionization energy in the gas phase (9.7 eV), acetone can act as an effective dopant transmitting UV energy to analyte molecules. Comparing acetone with the most commonly used dopant, toluene,[21] we have found that the former, in addition to the high solubility of lignin therein, is no less effective in generating negative ions from the polymer. The appearance of the obtained mass spectra and the intensity of signals are almost identical for the two dopants. This is consistent with the published data relating the deprotonating action of acetone under APPI conditions with its high proton affinity in the gas phase (812 kJ mol^{-1}) and the formation of ions in the source because of photochemical processes involving highly basic $CH_3COCH_2^-$ ions.[26] When using toluene, the dopant is introduced into the solvent flow just before the ion source, and this did result in an increase in noise and affected the reproducibility of the spectra in our preliminary experiments.

Therefore, all subsequent studies were carried out using an acetone–water mixture (9:1) as a solvent for the samples, and the sample in that solvent was injected into the ion source.

RESULTS AND DISCUSSION

Mass spectra of model compounds

One of the most important factors determining the applicability of different ionization techniques for obtaining the mass spectra of lignin is the possibility of undesirable fragmentation of that high-molecular-weight compound in the ion source. To study the processes of fragmentation of the phenylpropane structural units of macromolecules, we obtained mass spectra of four guaiacyl phenols simulating the most important moieties of softwood lignin macromolecules with different functional groups in the propane chain, such as carbon–carbon double bond (isoeugenol), aldehyde group (coniferyl aldehyde), carboxyl group (ferulic acid), and α-carbonyl group (acetovanillone). The stability of the most common types of bonds between the phenylpropane structural units (ether G(8–O–4)G, carbon–carbon resinol G(8–8)G, phenylcoumaran G(8–5)G, and pinoresinol G(8–8)G) was estimated from the mass spectra of the corresponding dimer models (guaiacylglycerol-β-guaiacyl ether, secoisolariciresinol, dihydrodehydrodiisoeugenol, and pinoresinol). Details of the spectra of the model compounds, recorded under optimal conditions, are presented in Table 1. Note that the mass spectrometer resolution is sufficient to resolve the isoeugenol $[M–CH_3]^-$ fragment ion at *m/z* 149 from the ^{13}C version of the ion at *m/z* 148 (data not shown). In all cases, except for the mass spectra of guaiacylglycerol-β-guaiacyl ether, obtained in APCI and APPI modes, $[M–H]^-$ ions are dominant.

Analysis of the mass spectra shows that none of the ionization methods can prevent the fragmentation of the analytes in the ion source. The main routes of fragmentation are the elimination of methyl radicals and methane from hydrocarbon substituents and methoxyl groups; the loss of water, methanol, or formaldehyde in the case of compounds having aliphatic hydroxyl groups; and, for ferulic acid, the release of CO_2 from the carboxylate anion. A comparison of the intensities of the fragment ions leads to the conclusion that ESI is the softest ionization method, and that the ESI fragmentation routes of initial deprotonated molecules differ markedly from those of APPI and APCI. The latter ionization techniques display both similar fragmentation mechanisms and a similar fraction of fragment ions in the spectra. The effect of the harder APCI and APPI conditions is exhibited particularly in the case of guaiacylglycerol-β-guaiacyl ether, where the spectra have base peaks of $[M–H–H_2O–CH_2O]^-$ rather than the deprotonated molecules.

The presence of fragment ions in the mass spectra of dimers, resulting from the cleavage of the bonds between the monomer structural units, indicates that lignin may undergo depolymerization in the mass spectrometric experiment. As expected, the G(8–O–4)G ether bond, with the lowest dissociation energy, is the most prone to fragmentation during APCI and APPI. Thus, the intensity of the fragment ions formed by the cleavage of the G(8–O–4)G bond in guaiacylglycerol-β-guaiacyl ether is 30 and 40% of the total intensity in the APCI and APPI mass spectra, respectively. The elimination of aromatic units under the same conditions is significantly less pronounced for the pinoresinol structure, while secoisolariciresinol and dihydrodehydrodiisoeugenol generally do not undergo dissociation into monomeric phenols. In this context, an unexpectedly strong fragmentation of dimers under ESI is of great interest; it is not consistent with the data obtained for chemical ionization and photoionization: the stability of bonds between units decreases in a series of guaiacylglycerol-β-guaiacyl ether > dihydrodehydrodiisoeugenol > secoisolariciresinol > pinoresinol. For all the studied dimers, ESI yields quite intense peaks associated with the elimination of guaiacol, which are virtually absent in the APPI and APCI spectra. This is due to a different decay mechanism of dimers under ESI, where thermal dissociation is not a dominant factor. In general, despite it being a mild method and causing less pronounced elimination of functional groups, ESI cannot be considered as a preferred method for the study of intact oligomeric and polymeric lignin molecules.

The comparison of the effectiveness of different ionization methods for model compounds, based on the peak heights of the deprotonated molecules (Table 2), shows that the signal intensity of the $[M–H]^-$ ions and, consequently, the total intensity of the mass spectra increase sharply in the series: ESI < APCI < APPI. The only exception is ferulic acid which yields a more intense peak with ESI than with APCI, because it is present in solution in its fully ionized form (pK_a = 4.52). However, even for this compound, APPI demonstrates a

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Table 1. Mass spectra of model compounds of softwood lignin, obtained using different atmospheric pressure ionization techniques

Compound	Ion	*m/z*	Error, ppm	Relative intensity, %		
				ESI	APCI	APPI
Isoeugenol	$[M–H]^-$	163.0754	–6.4	100	100	100
	$[M–CH_3]^-$	149.0596	–7.9	–	3	6
	$[M–CH_4]^-$	148.0519	–7.6	10	27	20
	$[M–C_3H_7]^-$	121.0283	–10.2	5	–	–
Conyferyl aldehyde	$[M–H]^-$	177.0546	–5.3	100	100	100
	$[M–CH_4]^-$	162.0311	–6.4	4	9	5
Ferulic acid	$[M–H]^-$	193.0497	–4.7	100	100	100
	$[M–CHO_2]^-$	149.0597	–7.7	–	17	12
	$[M–C_2H_4O_2]^-$	134.0360	–9.3	3	9	4
	$[M–C_5H_5O]^-$	113.0231	–12.4	–	16	–
Acetovanillone	$[M–H]^-$	165.0546	–6.5	100	100	100
	$[M–CH_4]^-$	150.0311	–7.7	5	14	8
Guaiacylglycerol-β-guaiacyl ether	$[M–H]^-$	319.1187	–0.13	100	26	16
	$[M–H–H_2O–CH_2O]^-$	271.0976	–0.03	27	100	100
	$[M–C_7H_9O_2]^-$	195.0656	–4.27	5	13	19
	$[M–C_7H_{11}O_3]^-$	177.0548	–5.4	–	10	20
	$[M–C_8H_{12}O_3]^-$	164.0469	–6.3	–	9	9
	$[M–C_9H_{13}O_3]^-$	151.0390	–7.1	–	8	8
	$[M–C_9H_{15}O_3]^-$	149.0234	–7.1	–	4	5
	$[M–C_{10}H_{15}O_4]^-$	121.0283	–10	15	6	9
	$[M–C_{12}H_{15}O_3]^-$	113.0233	–9.6	–	5	10
Secoisolariciresinol	$[M–H]^-$	361.1658	0.4	100	100	100
	$[M–CH_5O]^-$	329.1396	0.5	–	3	9
	$[M–C_{10}H_{14}O_3]^-$	180.0784	–4.5	4	–	–
	$[M–C_{13}H_{19}O_4]^-$	121.0282	–10.4	25	–	–
Dihydrodehydrodiisoeugenol	$[M–H]^-$	327.1602	0.05	100	100	100
	$[M–CH_5]^-$	311.1290	0.7	–	–	6
	$[M–C_{13}H_{19}O_2]^-$	121.0823	–10.1	10	–	–
Pinoresinol	$[M–H]^-$	357.1344	0.15	100	100	100
	$[M–C_8H_{11}O_3]^-$	203.0706	–3.7	–	–	5
	$[M–C_{10}H_{12}O_3]^-$	178.0626	–5.1	17	–	–
	$[M–C_{10}H_{13}O_3]^-$	177.0547	–5.6	–	–	5
	$[M–C_{12}H_{15}O_3]^-$	151.0389	–7.8	–	10	37
	$[M–C_{13}H_{17}O_4]^-$	121.0822	–10.4	24	–	–

threefold improvement in sensitivity. For the other analytes, photoionization yields an order of magnitude gain in efficiency over APCI and up to two orders of magnitude over ESI. This pattern also holds for guaiacylglycerol-β-guaiacyl ether, although it is somewhat less pronounced due to the strong fragmentation of this analyte, resulting in a decrease in the relative intensity of the $[M–H]^-$ ion in the APPI spectrum. Since ESI, unlike the other ionization techniques, is strongly dependent on the presence of analyte ions directly in the test solution, a clear correlation is observed between the peak intensities of the $[M–H]^-$ ions of four selected monomeric phenols and their ionization constants ($r^2 = 0.98$) (Supplementary Fig. S1, Supporting Information). Dimeric compounds, where the pK_a values of the phenolic hydroxyl groups are similar to those of monomers[27] and the intensities of the corresponding peaks an order of magnitude smaller, do not, however, show this dependence. It is interesting that for these analytes, a linear correlation ($r^2 = 0.99$) is observed between the logarithmic peak intensity and a partition constant between octanol and water (Log*P*) rather than the pK_a value (Supplementary Fig. S2, Supporting Information). The reason for this is that the Log*P* value indirectly characterizes the surface activity of molecules and, consequently, their ability to migrate to the surface of the droplets formed during ESI.[28]

Table 2. Comparison of the peak intensities of deprotonated molecules in mass spectra for different types of ionization

Compound	$[M–H]^-$ ion relative intensity, %		
	ESI	APCI	APPI
Isoeugenol	1.83	6.64	35.9
Coniferyl aldehyde	3.21	10.1	100
Ferulic acid	11.9	3.67	36.7
Acetovanillone	3.05	6.51	62.5
Guaiacylglycerol-β-guaiacyl ether	0.37	0.59	2.02
Secoisolariciresinol	0.32	2.97	17.9
Dihydrodehydrodiisoeugenol	0.66	4.24	21.0
Pinoresinol	0.42	2.14	10.5

Mass spectra of spruce dioxane lignin

The main patterns found for the model compounds are also characteristic of the ionization of larger lignin molecules. The general appearance of the mass spectra of a DL preparation, obtained using three types of atmospheric pressure ionization, is shown in Fig. 2.

Noteworthy is the dominance of high-intensity peaks of extraneous compounds in the ESI spectra; most of them can be attributed, based on accurate mass values, to various fatty acids and lipids inevitably present in the preparations of lignin. This is confirmed by MS/MS spectra obtained for the precursor ions with *m/z* 659.5460, 925.8080, and 1192.0676,

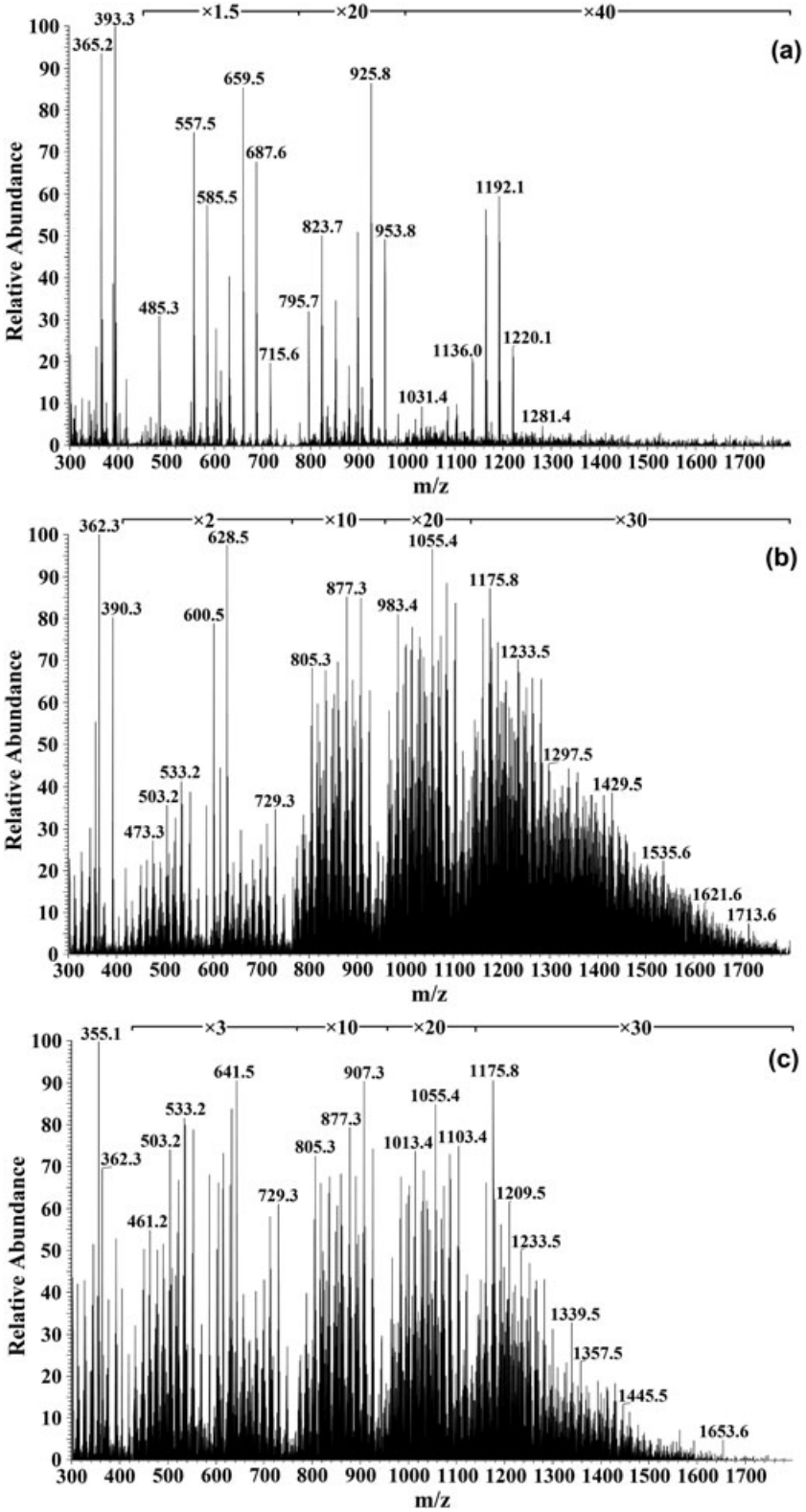

Figure 2. (a) ESI, (b) APCI, and (c) APPI mass spectra of spruce dioxane lignin.

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Table 3. Number-average molecular weight of spruce DL obtained by mass spectrometry and number of peaks of lignin oligomers with a relative intensity above 0.1% ($N_{0.1\%}$) and 1% ($N_{1\%}$), obtained using different atmospheric pressure ionization techniques

	Ionization mode		
Parameter	**ESI**	**APCI**	**APPI**
M_n, Da	490	690	640
$N_{1\%}$	127	987	990
$N_{0.1\%}$	613	1890	1992

in which product ions with m/z 227.2014, 255.2329, 283.2643, and 91.0398 are found – respectively the anions of myristic, palmitic, and stearic acids, and glycerol. Several unidentified peaks with low ring and double bond equivalent (RDBE) values (<4), uncharacteristic of aromatics compounds, appear to belong to different surfactants that have entered the lignin preparations in the processes of their isolation from wood and subsequent purification. Application of chemical ionization facilitates the solution of this problem by means of a sharp decrease in the signal intensity of ionic impurities and surfactants compared with ESI. Photoionization is almost completely devoid of this shortcoming: the peaks of contaminants in the dioxane lignin spectrum either are of a very low intensity or are not found at all.

The low efficiency of ESI, observed for model phenols, is also typical of the lignin preparation. This is reflected in a significant difference in the number of peaks observed in the mass spectra of dioxane lignin, obtained using the three methods of ionization (Table 3). Whereas for APCI and APPI these values are comparable (about 1900 peaks with a relative intensity of more than 0.1%), ESI enables the detection of approximately three times fewer compounds. If we consider only the most intense peaks (greater than 1% relative intensity) in the mass spectrum, the difference becomes even more pronounced: ESI is more than seven times inferior to chemical ionization and photoionization. Attempts to increase the intensity of the ESI mass spectra of lignin by increasing the ammonia concentration in the solution to 4% led to only a slight increase in the number of peaks.

A small number of compounds detected in the ESI mass spectra of lignin cannot be explained by a less pronounced fragmentation of the polymer macromolecules in the ion source, since the greatest improvement of APCI and APPI with respect to ESI is observed in the high-mass region. An illustration of this effect is the difference in the average molecular weight (M_n) values of dioxane lignin, determined from the mass spectrometric data and presented in Table 3. In general, all the methods of ionization demonstrate the problem of discrimination of lignin by mass, and the determined value of M_n is at least twice as low as determined by size-exclusion chromatography (1400 Da). Nevertheless, the observed discrimination against high-mass ions differs significantly for ESI, APCI, and APPI. To study this phenomenon, we selected a number of intense peaks of oligomers in the mass spectra of the DL preparation, containing from two to eight guaiacylpropane structural units and that differed in mass by 196.0736 Da. This value corresponds to a guaiacylglycerol unit, the most common for softwood lignin, with the gross formula of $C_{10}H_{12}O_4$, observed previously in the MALDI-TOF mass spectra of spruce dioxane lignin.[5] The $[C_{20}H_{19}O_6]^-$ ion with m/z 355.1184 which is the base peak in all the mass spectra obtained (excluding the peaks of impurities in ESI spectra) was selected for study. Based on published data,[19] this ion can be attributed to balanopholin (ChemSpider ID 10366582), a compound that has a dimeric structure (Fig. 3) with a phenylcoumaran bond that is resistant to fragmentation in the ion source. The data on the intensities of peaks for selected oligolignols (Fig. 3) show a significant decrease in the efficiency of ESI compared with APCI and APPI with increasing molecular weight of the analytes. This effect is especially pronounced for the tetramer and higher oligomers, and the octamer signal in the ESI mass spectrum is comparable with the noise level. The difference in the intensities of peaks

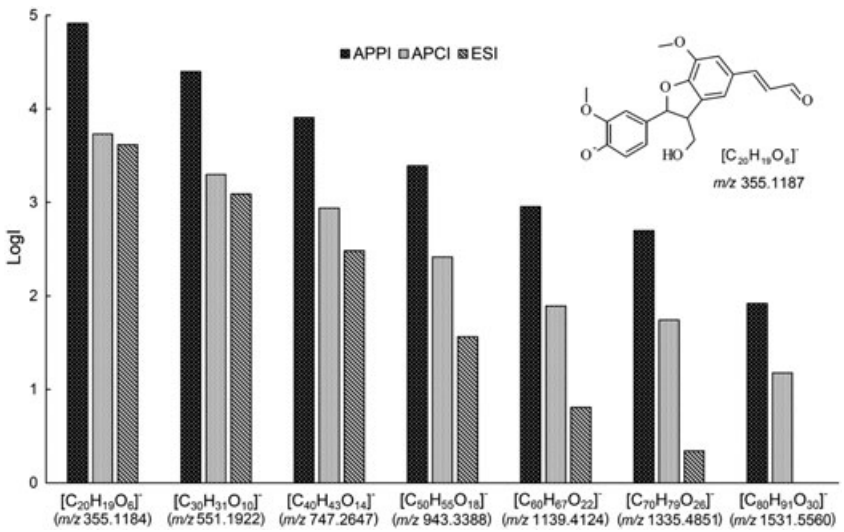

Figure 3. Comparison of the peak intensity (LogI) of lignin oligomers in the mass spectra obtained using ESI, APCI, and APPI.

obtained using chemical ionization and photoionization remains almost constant, at one order of magnitude, across the entire experimental mass range.

The van Krevelen diagrams[29] based on the data on the elemental composition of ions (Fig. 4) illustrate the heterogeneity of dioxane lignin and the selectivity of ionization methods in relation to various fractions of the preparation under study. The ions observed in the dioxane lignin spectra can be grouped into five distinct regions.

The region of H/C ranging from 0.7 to 1.2 and O/C of 0.2 to 0.5 (Region I) contains the maximum number of peaks, including the most intense. Corresponding to an average gross formula of the structural unit of $C_{10}H_{9.5}O_3$, it is characterized by a somewhat lower concentration of oxygen and, particularly, hydrogen than the DL preparation as a whole (based on the elemental analysis data). This is consistent with the results of experiments for model compounds, proving that the partial demethylation and elimination of water from lignin molecules proceed in the ion source. Notably, in the case of ESI, the coordinates of Region I are shifted compared with APCI and APPI along the O/C axis by 0.1. This reflects a certain selectivity of ESI for easily dissociating carboxyl and carbonyl structures.

Region II with an average composition of $C_{10}H_{13}O_5$, situated in the range of H/C and O/C of 1.2–1.5 and 0.4–0.6, respectively, corresponds to guaiacylglycerol structures ($C_{10}H_{14}O_5$), common in softwood lignin, and to syringyl fragments, present in minor quantities. In this region, APCI and APPI give a large number of peaks, some of which have exceptionally high relative intensity (more than 10%). ESI gives almost no possibility of detecting such structures, probably because of their relatively high pK_a values, resulting, among others, from a greater involvement of the phenolic hydroxyl groups in intramolecular hydrogen bonds.

Lignin–carbohydrate complexes, always present in lignin preparations obtained under mild conditions, determine the presence of significant and practically the same number of low-intensity peaks of ions with a high concentration of hydrogen and oxygen (H/C = 1.5–1.9 and O/C = 0.7–0.9) (Region III) in the chemical ionization and photoionization mass spectra. ESI, due to a significantly lower ionization efficiency, was not capable of detecting such minor fractions of the lignin preparation.

Region IV, opposite to Region III, with the highest degree of unsaturation (H/C = 0.5–0.7) and a low oxygen content (O/C = 0–0.1), corresponds to polycondensed aromatic compounds[22] with an average composition of $C_{10}H_{6.5}O_{0.4}$. Such structures are not originally present in lignin and are, probably, formed by the elimination of functional groups and subsequent condensation under harder ionization conditions of APPI and, especially, APCI. Chemical ionization results in high-intensity peaks in this region, with it being inferior to photoionization because of significant degradation of the analyte. ESI, requiring no high temperatures, is also not devoid of this drawback; however, the destruction and condensation of lignin are less significant in this case: the corresponding low-intensity peaks are grouped mainly in the H/C and O/C ranges of 0.6–0.8 and 0.1–0.2, respectively.

As noted above, ESI is characterized by a large number of intense peaks of impurities, grouped in a fairly extensive Region V in the van Krevelen plot, covering the H/C range from 1.2 to 1.8 and characterized by a low oxygen content (many exceptionally intense peaks are observed for the ions with O/C less than 0.1). These ions can be attributed to fatty and resin acids, triterpenoids, or some synthetic surfactants. The spectra obtained using APCI contain a small number of peaks in Region V, while APPI exhibits an extremely low sensitivity to these contaminants (single low-intensity peaks).

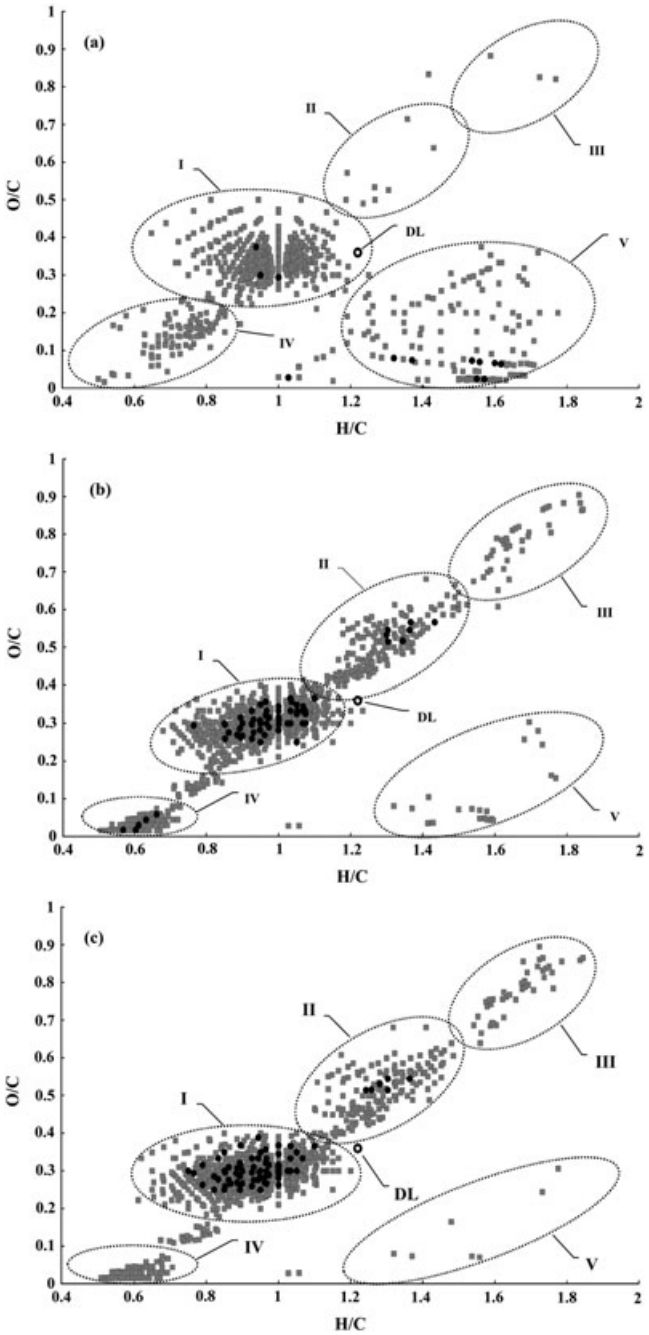

Figure 4. Van Krevelen plots for spruce dioxane lignin, obtained in (a) ESI, (b) APCI, and (c) APPI mode. Grey dots correspond to peaks with relative intensity >1% (>0.1% for ESI); black dots are the most intense peaks (>10%). Elemental composition of the DL preparation is marked with a circle.

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CONCLUSIONS

Because of the high reactivity of natural lignin, none of the studied types of atmospheric pressure ionization in the negative ion mode can prevent its partial fragmentation in the ion source; the routes of fragmentation of macromolecules under electrospray ionization differ significantly from those of chemical ionization and photoionization modes.

ESI is not suitable for obtaining the mass spectra of lignin due to its low ionization efficiency, selectivity for polar structures with low pK_a values, the presence of intense peaks of impurities (mainly lipids) in the mass spectra, and a significant discrimination by mass that narrows the range of molecular weights of the biopolymer available for study.

Atmospheric pressure chemical ionization and atmospheric pressure photoionization using acetone as a dopant yield virtually identical mass spectra of lignin in the molecular weight range up to 1.8 kDa; APPI is distinguished by a significantly higher signal intensity and a lower sensitivity to contaminants present in the lignin preparation. This ionization method can be considered as the preferred means for studying lignin.

The application of orbitrap high-resolution mass spectrometry in combination with acetone-assisted photoionization enables the recording of approximately 2000 peaks of deprotonated oligomer molecules in the mass spectrum of softwood lignin, which, depending on their elemental composition, can be divided into four main groups: typical oligolignols, guaiacylglycerol-based structures, lignin–carbohydrate complexes, and condensed polyaromatic compounds, formed as a result of fragmentation of macromolecules in the ion source.

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