**Electrospray Ionization with High-Resolution Mass Spectrometry as a Tool for
Lignomics: Lignin Mass Spectrum Deconvolution**

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Abstract

Capability to characterize lignin, lignocellulose, and their degradation products is essential for development of new renewable feedstocks. Electrospray ionization high-resolution time-of-flight mass spectrometry (ESI HR TOF MS) method was developed expanding the lignomics toolkit while targeting the simultaneous detection of low and high molecular weight (MW) lignin species. The effect of a broad range of electrolytes and various ionization conditions on ion formation and ionization effectiveness was studied using a suite of mono-, di- and triarene lignin model compounds as well as intact lignin. Contrary to the previous studies, the positive ionization mode was found to be more effective for methoxy-substituted arenes and polyphenols, i.e., species of a broadly varied MW structurally similar to the native lignin. For the first time, we report an effective formation of multiply charged species of lignin with the subsequent mass spectrum deconvolution in the presence of 100 mmol·L⁻¹ formic acid in the positive ESI mode. The developed method enabled the detection of lignin species with an MW between 150 and 9,000 Da or higher, depending on the mass analyzer. The obtained M_n and M_w values of 1,500 and 2,500 Da, respectively, were in good agreement with those determined by gel permeation chromatography. Furthermore, the deconvoluted ESI mass spectrum was similar to that obtained with matrix-assisted laser desorption/ionization (MALDI) TOF MS, yet featuring a higher signal-to-noise ratio. The formation of multiply charged species was confirmed with ESI ion mobility HR Q-TOF MS.

Introduction

Lignin constitutes the structural support of vascular plants' tissues and algae contributing up to 30% of biomass [1-4]. As a result, lignin is one of the most abundant sources of renewable carbon, a potential feedstock for renewable liquid fuel and valuable phenolic chemicals [5]. Various approaches to transform lignin into commercially beneficial substances are being investigated including extraction as well as thermal, catalytic and biological degradation [1, 3, 6-9].

The determination of produced low molecular weight (MW) species (<400 Da) present in the mixture of decomposition products is usually accomplished by one or two dimensional gas chromatography (GC or GC×GC) with either flame ionization (FID) or mass spectrometry (MS) detection, or by liquid chromatography with electrospray ionization (ESI) MS (LC-MS) [9]. This analysis allows for a comprehensive characterization of up to 20% of the product mass extractable by organic solvents, so-called bio-oil [9]. However, a significant non-GC-able portion of degradation products, which consists of phenolic oligomers and a so-called "coke" solid soot-like residue originating from native lignin, is only tentatively characterized [9]. Furthermore, for a comprehensive assessment of the degradation efficiency and understanding of decomposition pathways, a thorough characterization of intact lignin in the form of a native polymer is essential, yet extremely challenging.

The most common method employed for intact lignin characterization is size exclusion chromatography (SEC). Recent studies suggest Agilent PLgel columns to be the most suitable for biomass and lignin analysis in THF-based systems [10, 11]. While this method is an excellent tool for regular polymer MW determination, its application to lignin is limited because of the lack of pertinent calibration standards. As a result, calibration is usually conducted with polystyrene

standards, which do not match the structure of lignin [12]. Additionally, lignin's heteropolymeric nature contributes to secondary interactions of various functional groups with the column stationary phase [12-15].

Steric effects typical for rigid phenolic polymers also tend to skew SEC results [12]. To improve the accuracy of MW determination by SEC, detectors such as low-angle light scattering and multi-angle laser light scattering are frequently used [13, 16-21]. However, the determined MW values may still be skewed by the fluorescence and UV absorptivity of lignin constituents along with possible lignin macromolecule association [22].

While mass spectrometry is often the method of choice, Table 1 demonstrates the limitations of various MS approaches and unresolved issues reported for intact lignin characterization alone [14], or in combination with pyrolysis (Pyr) GC [23-33]. Pyr-GC is traditionally used to determine the type of lignin or lignocellulose, however it neither provides any MW information nor allows for a differentiation of small and large MW species [9]. The application of Pyr-GC-MS typically targets the speciation of syringol and guaiacol within lignin structural units [32].

Matrix-assisted laser desorption/ionization (MALDI) MS is currently considered the only suitable MS approach [34] for determination of MW distribution in synthetic polymers, proteins and polysaccharides [34]. However, MALDI provides only limited structural information [35-37] due to partial fragmentation, which makes it impossible to distinguish small and large molecules [35-37]. This issue could be addressed by coupling MS to LC; however, MALDI cannot be used as an online detector due to the required sample preparation procedure, which includes the analyte co-crystallization in a mixture with a matrix. Moreover, sample co-crystallization leads to undesirable association effects [38]. MALDI ionization also results in the predominant formation of single charged species, thus suppressing the ionization of high MW species and promoting the

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4 formation of ions featuring low m/z ratios [36]. Additionally, the analyte MW is typically limited
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6 to the mass analyzer operation range [39]. These limitations result in a noisy lignin spectrum
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8 lacking resolved peaks, particularly on the higher end of the m/z scale [34, 37, 39, 40].
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11 Intact lignin has also been investigated using MS with ESI and atmospheric pressure
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13 chemical ionization (APCI). However these studies focused only on a limited m/z range (up to
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15 1,000 m/z [41-47] and 1,500 m/z [42, 48-51]) aiming to evaluate lignin degradation products (Table
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17 1) [14, 22]. The significance of these studies was in the successful elucidation of the structural
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19 patterns and common linkages in various types of lignin, yet the MW was left undetermined [41-
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21 51], as well as a potential formation of multiply charged species characteristic for ESI, which is
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23 widely used in proteomics [52], yet has not been reported to our knowledge for lignin. Several
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25 research groups utilized ESI with an extended m/z range (up to 3,500 m/z [53] and 7,000 m/z [38]),
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27 however the reported spectra showed a low signal-to-noise ratio and low intensity above 1,000 m/z ,
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29 possibly due to the use of ionization conditions optimized for low MW lignin model compounds.
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31 To the best of our knowledge, a thorough optimization of ionization conditions and mass analyzer
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33 parameters within an extended m/z range aiming for a greater peak resolution and higher signal-
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35 to-noise ratio has not been reported.
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43 The intermediate products of lignin degradation, i.e., lignin mono- to oligomeric standards,
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45 appear to be suitable model compounds for understanding the ionization mechanism of intact and
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47 degraded lignin [42, 45-47]. An extensive optimization of ESI and APCI conditions toward the
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49 analysis of mono- and several diarene lignin model compounds was reported, to our best
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51 knowledge, only in the study by Hauptert et al. while utilizing a linear quadrupole ion trap MS [54].
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53 Kosyakov et al. assessed ESI, APCI and APPI as ionization techniques focusing on specific
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55 additives for analysis of lignin and its model compounds using a quadrupole-orbitrap MS [47].
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The limitation of APCI and APPI is their applicability only to a relatively low MW range, below 1,800 Da, and the predominant formation of singly-charged ions. In addition, APCI was shown to suffer from excessive fragmentation of lignin model compounds thus making this method inappropriate for both analysis of complex degradation mixtures [43, 54] and determination of intact lignin MW [44]. Thus, APPI and APCI may be preferred for studying oligomeric species and structural patterns in lignin. However, these ionization techniques cannot be applied for detection of high MW species in lignin.

Sodium hydroxide and sodium chloride have been claimed to be the most effective electrolytes while utilizing ESI, as minimal molecular ion fragmentation was observed for acidic lignin model compounds in the negative mode and for non-acidic compounds in the positive mode [54]. The ESI MS with NaOH method was successfully applied in combination with reversed phase LC by Owen et al., enabling a structural elucidation of some low MW components in a mixture of lignin degradation products [55]. By contrast, Kiyota et al. showed that oligomers with a MW up to 852 Da could be successfully ionized in the negative mode in the presence of 20 mmol·L⁻¹ formic acid and created a do-it-yourself oligomeric database, which included mono- to tetrameric phenolic species synthesized in house [45]. Proposed in 2010 the MS-based sequencing of lignin oligomers as an identification tool allowed for coining the term “lignomics” [42, 46]. Oligomeric model compounds and isolated oligolignols (up to 650 *m/z*) were efficiently ionized with APCI and ESI in the negative mode; furthermore, the fragmentation pathways of the major bonding structures in the gas phase were elucidated [42, 46]. All of these approaches laid the foundation for the development of an MS-based sequencing algorithm, yet they varied in ionization approaches and were limited by their focus on relatively low *m/z* values (<900). Thus,

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4 detection of high MW species, which are essential components of lignin, remains challenging and
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6 prevents lignin MW elucidation with the currently available MS toolkit.
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9 In this study, we have developed an MS protocol extending the lignomics toolkit through the
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11 detection of high-MW lignin components as well as low-MW phenolics. Building on previous
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13 studies [42, 45, 49, 50, 54], we investigated the effect of a broader suite of electrolytes on ESI of
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15 two representative lignin-like dimers. This was followed by narrowing the range of electrolytes
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17 while assessing the ionization of eleven mono-, di- and triarene lignin model compounds featuring
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19 different oxygenated functional groups and linkages typical for lignin and then expanded the
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21 method to native lignin. This investigation led to the development of a protocol for successful
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23 ionization of high MW lignin species with a focus on the multiply charged ion formation. We also
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25 postulated and confirmed that higher MS resolution led to an improved detection of high MW
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27 species when an ion mobility (IM) feature was employed with HRMS. The determined average
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29 MW values of lignin were compared to those obtained by LDI MS and GPC.
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Table 1. Comprehensive Overview of the MS Approaches Employed for the Analysis of Intact Lignin and High MW Standards

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
APPI						
–	Q-orbitrap	Dioxane lignin	Acetone–water mixture (9:1)	0.1% NH ₄ OH	300–1800 m/z	[47]
+	HR Q-TOF-MS	Wheat straw lignin	Dioxane (100%) or	NR ^a	Positive mode:	[41]
–	HR Q-TOF-MS	Wheat straw lignin	Dioxane/MeOH/CHCl ₃		300–1,120 m/z	
+/–	HR Q-TOF-MS/MS	Oligomeric species in lignin	(1:1:1)		Negative mode: 200–700 m/z	
APCI						
–	Q-orbitrap	Dioxane lignin	Acetone–water mixture (9:1)	0.1% NH ₄ OH	300–1800 m/z	[47]
–	Ion trap	Synthesized oligomeric standards	H ₂ O/MeOH (50:50);	0.1% acetate	100–1,000 m/z	[42]
–	Single quadrupole	Lignin partially depolymerized under acetic conditions	H ₂ O/MeOH	NR	150–650 m/z	[43]
+/–	Quadrupole– hexapole– quadrupole	Wheat straw intact and acetylated lignin	CHCl ₃ /MeOH (2:1)	A mixture of formic and acetic acid	100–550 m/z	[44]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
			ESI			
–	Q-orbitrap	Dioxane lignin	Acetone–water mixture (9:1)	0.1% NH ₄ OH	300–1800 m/z	[47]
–	Q-TOF	Dried sorghum feedstock, corn stover hydrolysate, xylobiose and xylotetraose	H ₂ O/traces of MeOH	None or traces of H ₂ SO ₄	50–2,000 m/z	[51]
+	Linear trap quadrupole – Fourier transform hybrid linear trap/7- T Fourier transform – ion cyclotron resonance MS	Bagasse lignin (100–100,000 g·mol ^{–1} ¹ with a maximum at 2·10 ³ g·mol ^{–1} according to GPC); steam explosion lignin	DMSO/MeOH (9:1)	NR	150–2,000 m/z	[48]
–	Magnetic sector	Spruce and eucalyptus dioxane lignin, eucalyptus kraft -organosolv and oxygen spruce lignin	MeOH/H ₂ O (1:1)/2.5% NH ₃ or dioxane/H ₂ O (7:3)	2.5% NH ₄ OH ^b	100–7,000 m/z (unresolved at higher m/z)	[38]
–	Q-TOF	Low-molecular-weight fraction of E. globulus dioxane lignin	MeOH/H ₂ O (1:1)/0.25% NH ₃	0.25% NH ₄ OH ^b	50–1,500 m/z	[49]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
+	Triple Q	Oligolignols (3–8 aromatic rings) synthesized from coniferyl alcohol with horseradish peroxidase	ACN/H ₂ O	Cetyltrimethylammonium sulfate	200–1,500 m/z	[50]
–	Magnetic sector- quadrupole	Wood and residual lignin from kraft pulps, black liquor lignin	MeOH/H ₂ O (1:1)	0.25% NH ₄ OH ^b	300–3,500 m/z	[53]
–	Triple-quadrupole	Synthetic lignin and soluble lignin extracted from sugar cane	ACN/H ₂ O	0.1% formic acid	100–850 m/z	[45]
– (nano- mate spray)	Linear trap quadrupole Fourier transform	Isolated oligolignols	ACN/H ₂ O (eluent Collected from RP LC)	0.1% acetic acid	120–1,400 m/z	[42]
MALDI						
–	Quadrupole-ion-trap analyzing system (linear trap quadrupole-XL)	Hand-cut sections of stems of two <i>Eucalyptus</i> species	NA	Matrix was substituted with thin layer chromatography grade silica	100–1,000 m/z	[56]
+	TOF	Organosolv lignin and glyoxalated lignin resin	NA	DHB ^c	200–700 m/z	[57]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
+	TOF	Bagasse lignin (100–100,000 g·mol ⁻¹ ¹ with a maximum at 2·10 ³ g·mol ⁻¹ according to GPC); steam explosion lignin	NA	DHB	Mass distribution with a maximum around m/z 360; no signal above 2000 m/z	[48]
+/-	TOF	Lignin fractions extracted from <i>Miscanthus x giganteus</i> under alkali or acid conditions	NA	CHCA ^d /α-cyclodextrin	100–800 Da	[35]
+/-	TOF	Ssoftwood kraft lignin, mixed hardwood organosolv lignin, acid hydrolysis lignin from bagasse, and steam explosion lignin from aspen (acetylated sample)	NA	DHB or all- <i>trans</i> -retinoic acid	50–14,000 m/z (unresolved at higher m/z); 50– 14,000 Da (singly charged ions are almost exclusively generated)	[39]
+	TOF	Milled wood lignin, alkali lignin and a synthetic lignin (G-type)	NA	DHB, 2-aminobenzoic acid or sinapinic acid	200–1,700 Da	[58]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or <i>m/z</i> Range Detected	Ref
+/-	Quadrupole ion trap TOF (QIT-TOF)	obtained by enzymic		(Sinapinic acid was		
		polymerization of coniferyl alcohol		preferred.)		
		Dioxane lignin	NA	<i>N-tert</i> -butyl- <i>N</i> -isopropyl- <i>N</i> -	50–2,000 Da	[34]
				methylammonium α -cyano-	(unresolved) and	
				4-hydroxycinnamate ionic	2000–6000 Da	
+	TOF			liquid or no matrix. The	(unresolved)	
				latter provided poor spectral		
				data.		
+	TOF	Milled wood lignin and synthetic	NA	DHB	400–2,600 Da	[36]
		lignin (polymerisation of coniferyl alcohol with peroxidase)				
+	TOF	Pyrolytic lignin and its SEC	NA	DHB or no matrix	50–4,000 Da	[40]
		fractions			(unresolved)	
NA	TOF	Soda hardwood lignin (Eucalyptus)	NA	DHB	70–700 Da	[59]
		and softwood kraft lignin				
+	TOF	Native and degraded birch wood	NA	DHB	1,000–2,000 <i>m/z</i>	[37]
		lignin				
Secondary Ion MS (SIMS)						

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
+	TOF	Milled wood lignin, birch kraft lignin, isolated	DCM/EtOH (2:1) or DMSO prior to deposition on silver disk	None	10–1,200 m/z	[60]

^a NR denotes not reported

^b The w/v concentration was provided in respect to NH₃

^c DHB stands for 2,5-dihydroxybenzoic acid

^d CHCA stands for α -cyano-4-hydroxycinnamic acid

Experimental

Materials and reagents

All standards used in this study as lignin model compounds are listed in Table 2, along with their acronyms, while details are provided in Supplementary Table 1. Alkali lignin (CAS Number: 8068-05-1), cesium iodide (99.999%), ammonium acetate ($\geq 99\%$), sodium iodide (99.999%), lithium chloride (99.999%), lithium hydroxide (99.998%) and sodium hydroxide ($\geq 98\%$) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Ammonium hydroxide, trifluoroacetic, acetic and formic acids (puriss p.a., eluent additive for LC-MS) were obtained from Fluka.

MALDI matrices, i.e., 2-(4'-hydroxybenzeneazo)benzoic acid (HABA, $\geq 99.5\%$) and α -cyano-4-hydroxycinnamic acid (CHCA, $\geq 99\%$), were purchased from Sigma-Aldrich. HPLC-MS grade water, acetonitrile (ACN), unstabilized tetrahydrofuran (THF) and methanol (MeOH) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All chemicals were used as received, without further purification. Deionized water was obtained using a Direct-Q® 3 system, Millipore, Billerica, MA, USA.

Table 2. List of Lignin Model Compounds Used in This Study

Compounds	Acronym	Characteristic functionality ^a	MW (g·mol ⁻¹)	Supplier/Synthesized	Purity
Vanillin	V	Carbonyl	152.15	Sigma-Aldrich ^b	99%
Guaiacol	G	—	124.24	Sigma-Aldrich	98%
Eugenol	E	Alkenyl	164.20	Acros Organics ^c	99%
Vanillic acid	VA	Carboxyl	168.15	Fluka ^d	97%
Syringol	S	Methoxy	154.16	Acros Organics	99%
Homovanillyl alcohol	HA	Aliphatic hydroxyl	168.19	Sigma-Aldrich	99%
Veratrole	VER	—	138.16	Sigma-Aldrich	99%
Syringaldehyde	SA	Carbonyl	182.17	Sigma-Aldrich	98%
Pinoresinol	P2	Hydroxyl, methoxy	358.38	Sigma-Aldrich	≥95%
Guaiacylglycerol- β -guaiacyl ether	G- β -2	Aliphatic hydroxyl, methoxy, β -O-4	320.34	In-house synthesis [61].	≥95%
1,2-Dimethoxy-4-[(2-methoxyphenoxy)methyl]benzene	ET2	Methoxy, ether dimer	274.12	In-house synthesis [62].	≥95%
4-(1-Hydroxyethyl)-2-methoxyphenyl benzoate	ALC2	Aliphatic hydroxyl dimer, ester	272.1	In-house synthesis [62].	≥95%
4-Formyl-2-methoxyphenyl benzoate	EST2	Carbonyl, ester dimer	256.07	In-house synthesis [62].	≥95%
(<i>E</i>)-4,4'-(Ethene-1,2-diyl)bis(2-methoxyphenol)	ALK2	Hydroxyl, methoxy, alkene dimer	272.10	In-house synthesis [59].	≥95%

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Dehydrodivanillin	D2V	Carbonyl, hydroxyl, methoxy, 5-5 dimer	302.07	In-house synthesis [63, 64].	≥95%
4-[2-(3,4-Dimethoxybenzyl)-4,5-dimethoxybenzyl]-2-methoxyphenol and 1-(3,4-dimethoxybenzyl)-4,5-dimethoxy-2-[(2-methoxyphenoxy)methyl]benzene	ET3-1	Methoxy, ether trimer	424.19	In-house synthesis [62].	≥95%
	ET3-2				

^a Functional groups and linkages (for oligomers) featured in the studied methoxyphenols compared to guaiacol

^b Sigma-Aldrich (St. Louis, MO, USA)

^c Acros Organics (Morris Plains, NJ, USA)

^d Fluka (Steinheim, Germany)

Several dimeric lignin model compounds, i.e., D2V [63, 65], G- β -2 [61], ET2 [62], EST2 [62], ALC2 [62], ALK2 [62], and ET3 [62] featuring different functional groups and linkages (Table 2) were synthesized according to the procedures published earlier, with an addition of column chromatography and recrystallization purification steps. A detailed description of the syntheses can be found under Supplementary Material. These compounds were characterized by ¹H NMR, GC-MS and direct infusion ESI HR TOF MS.

Stock solutions of lignin mono-, di-, and trimeric standards were prepared in 50% MeOH/water with a final concentration of 100 ppm w/v. For intact lignin analysis, it was essential to dissolve the polymer while avoiding the use of aggressive solvents such as dimethyl sulfoxide or *N,N*-dimethylformamide. We have shown that alkali lignin may be completely dissolved in ACN-water (1:1) or THF-water (1:1) mixtures, with concentrations up to 10,000 ppm and 50,000 ppm (w/v), respectively. It is of note that neither pure organic solvents (ACN, THF) nor water dissolved any amounts of lignin to form a true solution. For direct infusion analysis of lignin, alkali lignin was completely dissolved in either water/ACN (1:1) or water/THF (1:1) at a final concentration of 100 or 1000 ppm, respectively. The solutions were diluted to a final lignin concentration of 80 or 90 ppm prior to the analysis. Neither of the utilized electrolytes caused lignin precipitation. All samples and stock solutions were stored in a refrigerator at 4 °C prior to analysis.

To address the solubility issue in THF, the effect of acetylation was evaluated [66]. Acetylation of lignin samples was performed by a conventional method [67]. In brief, about 50 mg of the sample was completely dissolved in 500 μ L of dry pyridine and reacted with 500 μ L of acetic anhydride. The reaction mixture was stirred for 12 h at room temperature. Then, 200 μ L of methanol was added to the reaction mixture to terminate the reaction. Solvents were evaporated

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4 under a stream of nitrogen and the residue was dried in a vacuum oven at 30 °C overnight.
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6 Acetylated lignin was completely dissolved in 5.5 mL of THF resulting in a 10,000 ppm w/v
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8 solution, which was further diluted with water/ACN (1:1) or water/THF (1:1) mixtures for the ESI
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10 TOF MS analysis.
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15 16 *MS Analysis: Ionization* 17

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19 An Agilent 6210 ESI HR TOF-MS system was used throughout the study for method
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21 development and parameter optimization. An initial optimization of MS conditions included
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23 selection of the ionization polarity, electrospray (e.g., capillary) and collision-induced dissociation
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25 (e.g., fragmentor) potentials, nebulization temperature, nebulizing gas flow rate and nebulization
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27 pressure. Samples were introduced *via* a direct infusion with a syringe pump at a flow of 5 $\mu\text{L}\cdot\text{min}^{-1}$
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29 ¹ for the initial optimization. ESI potentials were optimized between 2000 and 5000 V.
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31 Nebulization pressure, gas flow and vaporizer temperature were varied between 18–40 psi, 4–12
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33 $\text{L}\cdot\text{min}^{-1}$, and 250–400 °C, respectively. The full range of electrolytes specified in the Materials and
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35 Reagents section was evaluated. In the experiments involving THF, all PEEK tubings were
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37 replaced with stainless steel.
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43 To optimize the electrolyte concentration, a flow injection analysis (FIA) was performed
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45 employing an Agilent 1100 Series HPLC. An aliquot (20 μL) of the prepared solution was injected
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47 into a mobile phase consisting of 50% ACN or MeOH in water at a flow rate of 0.2–1.0 $\text{mL}\cdot\text{min}^{-1}$
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49 and delivered directly to the TOF-MS system (no LC column was installed). In this study, we
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51 optimized the electrolyte concentration in the mobile phase by doping it only into the sample as
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53 we did previously in our work [68]. We experimentally confirmed that the final electrolyte
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concentration in the mobile phase after the injection of an electrolyte-doped sample remained the same (Supplementary Table 2).

The TOF-MS system was calibrated with an ESI (50–3500 m/z) tuning mixture purchased from Agilent. For higher m/z measurements (i.e., analysis of intact lignin in a range of 150–10,000 m/z), the calibration was performed in the positive mode while using $[(CsI)_n+Cs]^+$ clusters formed by an introduction of cesium iodide [30 mmol·L⁻¹ solution in ACN/water 1:1 (v/v)] *via* direct infusion at a flow rate of 5 μ L·min⁻¹ (Supplementary Fig. 1). Agilent 6560 IM Q-TOF system equipped with an ESI source was used to acquire IM mass spectra under the optimized conditions determined with 6210 TOF MS system.

A MALDI SYNAPT G2-Si Q-TOF MS System (Waters, Milford, MA, USA) with CryLaS FTS355-Q laser (a repetition rate of 2.5 kHz, wavelength 355 nm) was employed to acquire MALDI MS spectra in the range 50–8000 m/z . The system was manually calibrated with red phosphorus, and the experiments were performed in the positive resolution mode (20,000). The laser energy was set to 350 arbitrary intensity units. Typically, HABA [36, 37, 39, 40, 48, 57-59], CHCA [34, 35], retinoic [39] or sinapinic [58] acids were used as matrices for lignin analysis. Contradicting results were reported on the analysis with no matrix used (LDI); some studies claim successful ionization [40, 56] while the others report it to be ineffective [34]. In this study, we evaluated lignin ionization with CHCA and HABA used as matrices and without any matrix. The best ionization effectiveness was achieved when no matrix was used.

MS Characteristics and Data Processing

The 6210 HR TOF-MS system with a mass resolution of >13,000 (at m/z 2,722) and mass accuracy <2 ppm (m/z 609.2807) was utilized. A 6560 IM Q-TOF MS system used for IM mass

imaging had a resolution of >42,000 (at m/z 2,722) and mass accuracy <2 ppm. The resolving power of SYNAPT G2-Si MS system was 50,000 and the mass accuracy was under 1 ppm.

Mass Hunter software packages, B.02.00 and B.07.00, were used for data processing. The spectra of intact lignin recorded in the positive mode, which included multiply charged ions, were deconvoluted using a built-in tool utilizing an unbiased isotope model with a peak spacing tolerance of 0.0025 m/z . The maximal assigned charge state was not limited. Both hydrogen and sodium were considered as the charge carriers. The peaks selected for deconvolution were filtered based on their absolute height (≥ 100 counts) and the relative height of the largest peak, which was set to $\geq 0.1\%$ of the largest peak unless otherwise stated. The maximum number of peaks was not specified.

Equation 1 was used for the MW (M) calculation of the multiply charged species:

$$\frac{m}{z} \cdot z - [(atomic\ mass\ of\ charge\ carrier\ (^1H\ or\ ^{23}Na) - electron\ rest\ mass) \cdot z] = M, Da \quad (1)$$

An open source alternative software for mass spectrometric data analysis *mMass* Data Miner [69] was used for MALDI data processing.

To qualitatively assess the MW distribution of lignin utilizing the MS data, we applied an approach used in our previous work for MALDI MS data interpretation [11]. To calculate the number average (M_n), weight average (M_w) and z-average (M_z) MW of lignin samples, equations 2–4, where I_i is the absolute abundance of the deconvoluted species of a given MW (M_i), were used.

$$M_n = \frac{\sum I_i M_i}{\sum I_i} \quad (2)$$

$$M_w = \frac{\sum I_i M_i^2}{\sum I_i M_i} \quad (3)$$

$$M_z = \frac{\sum I_i M_i^3}{\sum I_i M_i^2} \quad (4) [70].$$

Results and Discussion

Electrolyte Screening: Effect on the Representative Model Compounds

We optimized the ESI TOF MS ionization targeting a broad range of electrolytes using two representative dimers as model compounds (Fig. 1). Two dimers, G- β -2 and ET2, were selected for this initial screening, the former featuring both aromatic and aliphatic hydroxyl groups, while the latter does not have any hydroxyl groups. Both of these standards exhibited the most efficient ionization while forming sodium adducts in the positive mode in the presence of formic or acetic acids at $\leq 10 \text{ mmol} \cdot \text{L}^{-1}$ concentration (Fig. 1).

A preferable ionization of ET2 in the positive ESI mode was expected, due to the lack of hydroxyl groups in its structure. For the hydroxylated compound G- β -2, contrary to expectations, the ionization efficiency improved in the positive ESI mode resulting in an abundant sodium adduct ion (Fig. 1a). The formation of sodium adduct ions even when sodium was not purposely added to the samples is known to occur because of traces of sodium leaching from glassware [71].

An effective ionization was also observed with no electrolyte present (Fig. 1a), the feature frequently observed in ESI TOF MS [72]. However, these conditions were deemed to be non-optimal as the lack of a buffer could result in pH instability and consequently cause the dependence of ionization on the sample composition and, as a result, irreproducible data [72].

a)

b)

Figure 1. ESI TOF MS response obtained in the presence of different electrolytes *via* a direct infusion for two representative dimers (a) G-β-2; (b) ET2 in the positive and negative ionization modes. For most of the electrolytes used, the response for $[M+Na]^+$ and deprotonated molecular ions is shown, except for LiCl and LiOH whose application resulted in the formation of $[M+Li]^+$. The electrolyte concentration was $1.0 \text{ mmol} \cdot \text{L}^{-1}$ unless specified otherwise

Sodium hydroxide, which was previously claimed as an effective ionization agent for lignin model compounds in both positive and negative ESI modes [54], did not promote the formation of sodium or protonated adducts for G- β -2 as much as the majority of other evaluated electrolytes (Fig. 1a). Moreover, in contrast to the previous work [54], excessive fragmentation was observed in the presence of sodium hydroxide in the positive (Fig. 2c) and negative modes, whereas a “clean” mass spectrum was obtained when either ammonium acetate or acetic acid were used (Fig 2a,b). Perhaps, the reason for a different fragmentation pattern upon ionization of G- β -2 was a varied stability (fragmentation) of ions in different mass analyzers used in previous studies, as Hauptert et al. [54] utilized a linear quadrupole ion trap MS whereas in our study we used TOF.

Figure 2. Positive ESI TOF mass spectra of 5 ppm G- β -2 ($[M+Na]^+$ 343.1152 m/z) in the presence of 1.0 mmol·L⁻¹ of a) ammonium acetate (mass accuracy error 5 ppm); b) acetic acid (mass accuracy error 7 ppm); and c) sodium hydroxide (mass accuracy error 6.7 ppm)

ESI: Impact of Oxygenated Functional Groups on Ionization

Based on the screening experiments (*cf.* Fig. 1), formic and acetic acids appeared to be the most efficient electrolytes. Thus, we compared these acids to frequently used ammonium acetate [73], to evaluate the impact of these electrolytes on the ionization effectiveness of a broader suite of mono-, di- and triarene lignin model compounds featuring different linkages and functional groups (Table 2), and investigated the contribution of various oxygenated functional groups: The results are shown in Table 3. Contrary to the previously preferred negative ionization mode [45, 46], we showed ionization of all considered compounds (with hydroxyl, methoxy and carboxyl groups) in the positive ESI mode with both formic/acetic acids and ammonium acetate, although some selectivity toward specific oxygenated functional groups was observed.

The compounds *without* phenolic hydroxyl groups, with multiple *methoxy* groups, or with *aliphatic* hydroxyl groups, were preferentially ionized in the positive mode (the upper portion of Table 3) corroborating the results obtained earlier for non-acidic lignin model compounds [54, 55]. Thus the positive ESI mode is preferable as similar structural features, i.e., prevailing methoxy over phenolic hydroxyl functional groups, are also characteristic for alkali lignin (4.6 vs. 3.6 moles/1,000 g as claimed by the supplier).

While both electrolyte systems seemed to show satisfactory performance, acids were more effective for the majority of species with no hydroxyl groups and prevailing methoxy groups (Table 3). It is of note that some of the standards showed low response or could not be detected in the negative ESI mode at all, e.g., VER, EST2, ET2, ET3-1, SA, ET3-2, P2, G- β -2). This could perhaps be explained by the absence of hydroxyl groups or their steric hindrance (the structure motifs occurring in these molecules are shown in Table 2). For example, in case of syringaldehyde (SA) two MeO groups in the *ortho*-position to the hydroxyl moiety made the deprotonation of these compounds difficult.

Highly hydroxylated monomeric phenolic standards with no more than one methoxy group (the bottom portion of Table 3), those containing *carboxyl* groups as well as other compounds of high *acidity* such as VA, V and D2V, showed a higher ionization efficiency in the negative mode, as expected (Table 3). Nevertheless, as mentioned above, such a high ratio of hydroxylation/methoxylation is not characteristic for intact lignin and thus does not seem to be suitable for selection of ionization conditions.

Table 3. ESI TOF MS Response with Acids (Either Formic or Acetic) and Ammonium Acetate as ESI Electrolytes for Representative Lignin Mono- to Trimeric Structure Model Compounds in both Positive and Negative Ionization Modes^a

Model Compounds	Numbers of Oxygenated Functional Groups			pK _a	Intensity of the Target Ion Response			
	-OH	-OCH ₃	-COOH		Acid (Formic/Acetic)		Ammonium Acetate	
					Positive	Negative	Positive	Negative
VER	0	2	0	-	++++ ^{b, c}	ND ^d	+++	ND
EST2	0	1	0	-	++++	ND	++++	ND
ET2	0	3	0	-	++++	ND	+++	ND
ET3-1	0	5	0	-	++++	ND	+++	ND
ET3-2	1	5	0	-	++++	+	+++	+
P2	2	2	0	9.76	++++	+	+++	+
G-β-2	1 +2 aliphatic	2	0	9.88	++++	+	+++	+
HA	1 +1 aliphatic	1	0	10.19	+++	+	++++	++
ALC2	1 aliphatic	1	0		+++	+	++++	++
S	1	2	0	9.98	++	+++	++++	+
SA	1	2	0	7.8	+++	+	++++	++

Numbers of Oxygenated Functional					Intensity of the Target Ion Response			
Model Compounds	Groups			pK _a	Acid (Formic/Acetic)		Ammonium Acetate	
	-OH	-OCH ₃	-COOH		Positive	Negative	Positive	Negative
VA	1	1	1	4.45	++	+++	+	++++
V	1	1	0	7.38	+	+++	++	++++
D2V	2	2	0	7.04	++	+++	+	++++
G	1	1	0	9.93	++	+++	+	++++
E	1	1	0	10.19	+	++++	+	++++

^a For the majority of analytes, the response was monitored for $[M+Na]^+$ and $[M-H]^-$ ions in the positive and negative ionization modes, respectively. The electrolyte concentration upon direct infusion was $2.5 \text{ mmol} \cdot \text{L}^{-1}$

^b “++++” indicates the system resulting in the most efficient ionization; “+” indicates the system with the least efficient ionization

^c The mass spectra recorded under the optimal conditions indicated as “++++” are shown in Supplementary Fig. 2

^d “ND” denotes no molecular ions or adducts were detected

Lignin ESI: Electrolyte Selection

Similarly to lignin model compounds, electrolyte screening was performed for efficient ionization of lignin itself over a broad range of commonly used salts and acids in both the positive and negative modes. The urgent goal was to ensure an effective ionization of higher MW species forming high m/z ions (Fig. 3). Formic acid at its highest concentrations of 100 and $200 \text{ mmol} \cdot \text{L}^{-1}$ in the positive ESI mode enhanced the formation of multiply charged species. Following the deconvolution (as explained in the next section), this protocol allowed for detecting the masses of up to $9,000 \text{ Da}$ (Fig. 3b).

Under these conditions, the ionization of lower MW lignin model compounds was somewhat suppressed, yet sufficient for their effective detection (*cf.* Fig. 1), thus providing more balanced mass spectra.

Since acetylation is used in a number of studies to enhance lignin's solubility, we investigated its ESI ionization as well. Similar to intact lignin, the positive mode was preferred for derivatized lignin when the hydroxyl groups were substituted with acetyloxy (CH_3COO^-) groups (Fig 3c). Lignin acetylation prevented a facile deprotonation in the negative mode (Fig. 3c). The acetylated lignin spectrum also featured multiply charged species allowing for subsequent deconvolution (Fig. 3d).

a) Intact alkali lignin. The highest observed m/z prior to deconvolution.

b) Intact alkali lignin. The highest MW (Da) after deconvolution of the mass spectra.

c) Acetylated alkali lignin. The highest m/z prior to deconvolution.

d) Acetylated alkali lignin. The highest MW (Da) after deconvolution of the mass spectra.

Figure 3. The highest mass-to-charge (m/z) values of the ions detected in the mass spectrum of (a) 15–90 ppm intact alkali lignin and (c) 15 ppm acetylated alkali lignin dissolved in ACN-water 1:1 in the positive and negative ionization modes while using different electrolytes; MW (Da) obtained after spectrum deconvolution of (b) 15–90 ppm intact alkali lignin and (d) 15 ppm acetylated alkali lignin. The electrolyte concentration was $1.0 \text{ mmol} \cdot \text{L}^{-1}$ unless specified otherwise

Lignin ESI: Mass Spectrum Deconvolution

ESI of lignin in the positive mode in the presence of $100 \text{ mmol} \cdot \text{L}^{-1}$ formic acid allowed for minimizing the ionization discrimination. Thus, lower MW species were mainly observed as singly charged ions, whereas high MW lignin constituents carried multiple charges. For example, vanillin was detected as a singly charged ion of $153.0570 \text{ } m/z$ (protonated adduct $[\text{C}_8\text{H}_8\text{O}_3 + \text{H}]^+$, 16 ppm mass accuracy error) (Fig. 4a). The corresponding deconvoluted species possessed a mass of 152.05 Da (Fig. 4b) calculated based on equations 1 and 5, which was equal to vanillin's MW.

$$153.0570 \cdot 1 - [(1.007825 - 0.000549) \cdot 1] = 152.05 \text{ Da} \quad (5)$$

High MW species were predominantly observed as multiply charged ions, for example the $597.2073 \text{ } m/z$ ion carried a charge of +7 (the spectrum is shown in Fig. 4c). The mass spectrum

deconvolution resulted in a calculated MW of 4173.40 for the corresponding species (equation 6), the spectrum is shown in Fig. 4d.

$$597.2073 \cdot 7 - [(1.007825 - 0.000549) \cdot 7] = 4173.040 \text{ Da} \quad (6)$$

a)

b)

c)

d)

Figure 4. Positive ESI HR TOF mass spectra of an 80 ppm solution of intact lignin in ACN-water (1:1) with 100 mmol·L⁻¹ formic acid: a), c) and e) zoomed and full original mass spectra; b), d) and f) zoomed and full mass spectra after deconvolution

Thus, analysis of lignin under the suggested ESI conditions allowed for a simultaneous determination of lignin constituents varying in size and structure *via* direct infusion analysis with minimal sample preparation. An introduced feature of the multiply charged ion formation allowed for an efficient ionization of high MW lignin species, which, to our knowledge, was not previously reported. This approach is an essential contribution to the lignomics toolkit allowing for analysis of higher MW species, as shown in the next section.

Lignin MW Determination by ESI HR TOF MS

To assess lignin MW, we previously adapted an approach from NIST [74] employing equations 2–4 to the deconvoluted spectral data [11]. This calculation is typically used for evaluating GPC data rather than MS, yet, the obtained values, 1,480 Da, 2,520 Da and 3,790 Da

for M_n , M_w and M_z , respectively, were in good agreement with the MW values determined earlier by GPC (Table 4) [11]. Interestingly, similar MW values were determined for acetylated lignin, i.e., 1,570 Da, 2,440 Da and 3,530 Da for M_n , M_w and M_z , respectively. This could be explained either by fragmentation with the loss leaving the most abundant molecular ions as ions $[M-CH_3CO^+]$ or incomplete acetylation of lignin due to sterical hindrance, thus still detecting mostly intact lignin. In either case, it appears that the acetylated lignin MS data could also serve for the MW determination of the original, native lignin without a typical mass correction on acetylation.

Table 4. Number-Average, Weight-Average and z-Average Molecular Weight of Lignin Determined by ESI HR TOF MS, GPC and MALDI HR TOF MS

	ESI HR TOF MS	GPC [11]	MALDI HR TOF MS
M_n	1,480	1,630	830
M_w	2,520	2,740	1,250
M_z	3,790	3,720	2,230

Ionization: MALDI HR TOF MS vs. ESI HR TOF MS

Similar mass spectra of intact lignin were recorded with MALDI and ESI as ionization sources (Fig. 5 compared to Fig. 4f). MALDI MS was selected as a reference technique frequently used for polymer MW determination [14, 34, 39, 40, 75-77]. To achieve the optimal lignin ESI, we evaluated the ionization efficiency with CHCA and HABA matrices and also without a matrix (Supplementary Fig. 3). The mass spectrum recorded with no matrix was of higher clarity, showing well resolved peaks of higher intensity above 2500 m/z . Apparently, the matrix structurally similar to lignin increased the background and complexity of spectra, perhaps, due to the undesirable association effects occurring during the sample co-crystallization with a matrix [38]. The recorded

LDI mass spectrum (i.e., without a matrix) demonstrated several local maxima at 500, 1000, 2900, 4500 and 6200 m/z (Fig. 5) with the decreasing signal at m/z values of 7000. The trends in the observed MALDI (LDI) and ESI HR TOF spectra were similar; however, ESI HR TOF MS allowed for obtaining a cleaner deconvoluted spectra for the high MW species.

We evaluated the LDI MS data using the calculations shown in equations 2–4. The determined MW values agreed with the results obtained with GPC and ESI MS (Table 4). The MW elucidated while employing MALDI was shifted toward lower values, perhaps, due to the suppression of high MW species ionization because their detection was limited by the predominant formation of singly charged ions.

Figure 5. LDI (no matrix) HR TOF mass spectrum of alkali lignin. The insert shows a zoomed in part of the spectrum in the range 2800–8000 m/z

Ion Mobility ESI MS: Confirmation of the Multiply Charged Species Formation

To confirm the formation of multiply charged species, intact lignin was analyzed with IM ESI Q-TOF MS (Fig. 6a). Five distinguishable regions observed in the two-dimensional spectrum (drift time (ms) vs. m/z) suggest the occurrence of species carrying either varied charges or structural conformations (Fig. 6a). The deconvoluted positive IM ESI Q-TOF mass spectrum was similar to that recorded with the TOF MS (Fig. 6b compared to Fig. 4f). Species with a higher MW featured a higher abundance when analyzed by IM MS, as expected due to a better ion focusing typical for this technique. Notably, both deconvoluted spectra featured the same ion species observed after deconvolution.

a)

b)

Fig. 6. a) Ion mobility image of a 100 ppm solution of intact lignin in ACN-water (1:1) with 100 mmol·L⁻¹ formic acid, recorded in the positive ESI mode. b) Original and deconvoluted mass spectra of a 100 ppm solution of intact lignin in ACN-water (1:1) with 100 mmol·L⁻¹ formic acid recorded in the positive ESI mode. The blank spectrum was subtracted before deconvolution. An accurate deconvolution algorithm is described in equation 1.

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4 With IM ESI HR Q-TOF MS, high MW species with masses up to 20,000 Da were observed,
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6 while ESI TOF MS allowed for the detection of species only up to 10,000 Da. This difference
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8 (compared to TOF MS) may be due to a higher sensitivity of the newer IM ESI Q-TOF instrument,
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10 possibly due to the IM feature allowing for a higher resolution and ion focusing.
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15 16 **Conclusions** 17

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19 An ESI TOF MS method for intact lignin analysis has been developed allowing for a
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21 simultaneous detection of both low and high MW species *via* direct infusion with minimal sample
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23 preparation. The most efficient ionization conditions were achieved in the positive ESI mode with
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25 100 mmol·L⁻¹ formic acid as an electrolyte. For the first time, the formation of multiply charged
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27 ions promoting the ionization of high MW lignin species was shown. Determination of multiply
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29 charged ions was possible due to an inherently high resolving power of an applied HR TOF mass
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31 analyzer. To elucidate MW, the mass spectrum was deconvoluted. The obtained M_n, M_w, and M_z
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33 values of 1,480 Da, 2,520 Da and 3,790 Da, respectively, were in good agreement with those
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35 determined previously for similar samples by gel permeation chromatography. The presence of
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37 multiply charged lignin ions was confirmed by IM MS using ESI IM HR Q-TOF MS. The
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39 developed method may extend the lignomics toolkit while targeting higher-MW species.
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Tables

Table 1. Comprehensive Overview of the MS Approaches Employed for the Analysis of Intact Lignin and High MW Standards

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
APPI						
–	Q-orbitrap	Dioxane lignin	Acetone–water mixture (9:1)	0.1% NH ₄ OH	300–1800 m/z	[47]
+	HR Q-TOF-MS	Wheat straw lignin	Dioxane (100%) or	NR ^a	Positive mode:	[41]
–	HR Q-TOF-MS	Wheat straw lignin	Dioxane/MeOH/CHCl ₃		300–1,120 m/z	
+/-	HR Q-TOF-MS/MS	Oligomeric species in lignin	(1:1:1)		Negative mode: 200–700 m/z	
APCI						
–	Q-orbitrap	Dioxane lignin	Acetone–water mixture (9:1)	0.1% NH ₄ OH	300–1800 m/z	[47]
–	Ion trap	Synthesized oligomeric standards	H ₂ O/MeOH (50:50);	0.1% acetate	100–1,000 m/z	[42]
–	Single quadrupole	Lignin partially depolymerized under acetic conditions	H ₂ O/MeOH	NR	150–650 m/z	[43]
+/-	Quadrupole– hexapole– quadrupole	Wheat straw intact and acetylated lignin	CHCl ₃ /MeOH (2:1)	A mixture of formic and acetic acid	100–550 m/z	[44]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
			ESI			
–	Q-orbitrap	Dioxane lignin	Acetone–water mixture (9:1)	0.1% NH ₄ OH	300–1800 m/z	[47]
–	Q-TOF	Dried sorghum feedstock, corn stover hydrolysate, xylobiose and xylotetraose	H ₂ O/traces of MeOH	None or traces of H ₂ SO ₄	50–2,000 m/z	[51]
+	Linear trap quadrupole – Fourier transform hybrid linear trap/7- T Fourier transform – ion cyclotron resonance MS	Bagasse lignin (100–100,000 g·mol ^{–1} ¹ with a maximum at 2·10 ³ g·mol ^{–1} according to GPC); steam explosion lignin	DMSO/MeOH (9:1)	NR	150–2,000 m/z	[48]
–	Magnetic sector	Spruce and eucalyptus dioxane lignin, eucalyptus kraft -organosolv and oxygen spruce lignin	MeOH/H ₂ O (1:1)/2.5% NH ₃ or dioxane/H ₂ O (7:3)	2.5% NH ₄ OH ^b	100–7,000 m/z (unresolved at higher m/z)	[38]
–	Q-TOF	Low-molecular-weight fraction of E. globulus dioxane lignin	MeOH/H ₂ O (1:1)/0.25% NH ₃	0.25% NH ₄ OH ^b	50–1,500 m/z	[49]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
+	Triple Q	Oligolignols (3–8 aromatic rings) synthesized from coniferyl alcohol with horseradish peroxidase	ACN/H ₂ O	Cetyltrimethylammonium sulfate	200–1,500 m/z	[50]
–	Magnetic sector- quadrupole	Wood and residual lignin from kraft pulps, black liquor lignin	MeOH/H ₂ O (1:1)	0.25% NH ₄ OH ^b	300–3,500 m/z	[53]
–	Triple-quadrupole	Synthetic lignin and soluble lignin extracted from sugar cane	ACN/H ₂ O	0.1% formic acid	100–850 m/z	[45]
– (nano- mate spray)	Linear trap quadrupole Fourier transform	Isolated oligolignols	ACN/H ₂ O (eluent Collected from RP LC)	0.1% acetic acid	120–1,400 m/z	[42]
MALDI						
–	Quadrupole-ion-trap analyzing system (linear trap quadrupole-XL)	Hand-cut sections of stems of two <i>Eucalyptus</i> species	NA	Matrix was substituted with thin layer chromatography grade silica	100–1,000 m/z	[56]
+	TOF	Organosolv lignin and glyoxalated lignin resin	NA	DHB ^c	200–700 m/z	[57]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
+	TOF	Bagasse lignin (100–100,000 g·mol ⁻¹ ¹ with a maximum at 2·10 ³ g·mol ⁻¹ according to GPC); steam explosion lignin	NA	DHB	Mass distribution with a maximum around m/z 360; no signal above 2000 m/z	[48]
+/-	TOF	Lignin fractions extracted from <i>Miscanthus x giganteus</i> under alkali or acid conditions	NA	CHCA ^d /α-cyclodextrin	100–800 Da	[35]
+/-	TOF	Ssoftwood kraft lignin, mixed hardwood organosolv lignin, acid hydrolysis lignin from bagasse, and steam explosion lignin from aspen (acetylated sample)	NA	DHB or all- <i>trans</i> -retinoic acid	50–14,000 m/z (unresolved at higher m/z); 50– 14,000 Da (singly charged ions are almost exclusively generated)	[39]
+	TOF	Milled wood lignin, alkali lignin and a synthetic lignin (G-type)	NA	DHB, 2-aminobenzoic acid or sinapinic acid	200–1,700 Da	[58]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
+/-	Quadrupole ion trap TOF (QIT-TOF)	obtained by enzymic		(Sinapinic acid was		
		polymerization of coniferyl alcohol		preferred.)		
		Dioxane lignin	NA	<i>N-tert</i> -butyl- <i>N</i> -isopropyl- <i>N</i> -	50–2,000 Da	[34]
				methylammonium α -cyano-	(unresolved) and	
				4-hydroxycinnamate ionic	2000–6000 Da	
+	TOF	Milled wood lignin and synthetic lignin (polymerisation of coniferyl alcohol with peroxidase)	NA		liquid or no matrix. The (unresolved)	
					latter provided poor spectral	
					data.	
+	TOF	Pyrolytic lignin and its SEC fractions	NA	DHB or no matrix	400–2,600 Da	[36]
					(unresolved)	
NA	TOF	Soda hardwood lignin (Eucalyptus and softwood kraft lignin)	NA	DHB	70–700 Da	[59]
+	TOF	Native and degraded birch wood lignin	NA	DHB	1,000–2,000 m/z	[37]
Secondary Ion MS (SIMS)						

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
+	TOF	Milled wood lignin, birch kraft lignin, isolated	DCM/EtOH (2:1) or DMSO prior to deposition on silver disk	None	10–1,200 m/z	[60]

^a NR denotes not reported

^b The w/v concentration was provided in respect to NH₃

^c DHB stands for 2,5-dihydroxybenzoic acid

^d CHCA stands for α -cyano-4-hydroxycinnamic acid

Table 2. List of Lignin Model Compounds Used in this Study

Compounds	Acronym	Characteristic functionality ^a	MW (g·mol ⁻¹)	Supplier/Synthesized	Purity
Vanillin	V	Carbonyl	152.15	Sigma-Aldrich ^b	99%
Guaiacol	G	—	124.24	Sigma-Aldrich	98%
Eugenol	E	Alkenyl	164.20	Acros Organics ^c	99%
Vanillic acid	VA	Carboxyl	168.15	Fluka ^d	97%
Syringol	S	Methoxy	154.16	Acros Organics	99%
Homovanillyl alcohol	HA	Aliphatic hydroxyl	168.19	Sigma-Aldrich	99%
Veratrole	VER	—	138.16	Sigma-Aldrich	99%
Syringaldehyde	SA	Carbonyl	182.17	Sigma-Aldrich	98%
Pinoresinol	P2	Hydroxyl, methoxy	358.38	Sigma-Aldrich	≥95%
Guaiacylglycerol-β-guaiacyl ether	G-β-2	Aliphatic hydroxyl, methoxy, β-O-4	320.34	In-house synthesis [61].	≥95%
1,2-Dimethoxy-4-[(2-methoxyphenoxy)methyl]benzene	ET2	Methoxy, ether dimer	274.12	In-house synthesis [62].	≥95%
4-(1-Hydroxyethyl)-2-methoxyphenyl benzoate	ALC2	Aliphatic hydroxyl dimer, ester	272.1	In-house synthesis [62].	≥95%
4-Formyl-2-methoxyphenyl benzoate	EST2	Carbonyl, ester dimer	256.07	In-house synthesis [62].	≥95%
(E)-4,4'-(Ethene-1,2-diyl)bis(2-methoxyphenol)	ALK2	Hydroxyl, methoxy, alkene dimer	272.10	In-house synthesis [59].	≥95%

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Dehydrodivanillin	D2V	Carbonyl, hydroxyl, methoxy, 5-5 dimer	302.07	In-house synthesis [63, 64].	≥95%
4-[2-(3,4-Dimethoxybenzyl)-4,5-dimethoxybenzyl]-2-methoxyphenol and 1-(3,4-dimethoxybenzyl)-4,5-dimethoxy-2-[(2-methoxyphenoxy)methyl]benzene	ET3-1 ET3-2	Methoxy, ether trimer	424.19	In-house synthesis [62].	≥95%

^a Functional groups and linkages (for oligomers) featured in the studied methoxyphenols compared to guaiacol

^b Sigma-Aldrich (St. Louis, MO, USA)

^c Acros Organics (Morris Plains, NJ, USA)

^d Fluka (Steinheim, Germany)

Table 3. ESI TOF MS Response with Acids (Either Formic or Acetic) and Ammonium Acetate as ESI Electrolytes for Representative Lignin Mono- to Trimeric Structure Model Compounds in Both Positive and Negative Ionization Modes^a

Model Compounds	Numbers of Oxygenated Functional Groups			pK _a	Intensity of the Target Ion Response			
	-OH	-OCH ₃	-COOH		Acid (Formic/Acetic)		Ammonium Acetate	
					Positive	Negative	Positive	Negative
VER	0	2	0	-	++++ ^b	ND ^c	+++	ND
EST2	0	1	0	-	++++	ND	++++	ND
ET2	0	3	0	-	++++	ND	+++	ND
ET3-1	0	5	0	-	++++	ND	+++	ND
ET3-2	1	5	0	-	++++	+	+++	+
P2	2	2	0	9.76	++++	+	+++	+
G-β-2	1	2	0	9.88	++++	+	+++	+
	+2 aliphatic							
HA	1	1	0	10.19	+++	+	++++	++
	+1 aliphatic							
ALC2	1 aliphatic	1	0		+++	+	++++	++
S	1	2	0	9.98	++	+++	++++	+
SA	1	2	0	7.8	+++	+	++++	++
VA	1	1	1	4.45	++	+++	+	++++
V	1	1	0	7.38	+	+++	++	++++
D2V	2	2	0	7.04	++	+++	+	++++
G	1	1	0	9.93	++	+++	+	++++
E	1	1	0	10.19	+	++++	+	++++

^a For the majority of analytes, the response was monitored for [M+Na]⁺ and [M-H]⁻ ions in the positive and negative ionization modes, respectively. The electrolyte concentration upon direct infusion was 2.5 mmol·L⁻¹

^b “++++” indicates the system resulting in the most efficient ionization; “+” indicates the system with the least efficient ionization

^c “ND” denotes no molecular ions or adducts were detected

Table 4. Number-Average, Weight-Average and z-Average Molecular Weight of Lignin
Determined by ESI HR TOF MS, GPC and MALDI HR TOF MS

	ESI HR TOF MS	GPC [11]	MALDI HR TOF MS
M_n	1,480	1,630	830
M_w	2,520	2,740	1,250
M_z	3,790	3,720	2,230

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Figure 1. ESI TOF MS response obtained in the presence of different electrolytes *via* a direct infusion for two representative dimers (a) G- β -2; (b) ET2 in the positive and negative ionization modes. For most of the electrolytes used, the response for $[M+Na]^+$ and deprotonated molecular ions is shown, except for LiCl and LiOH whose application resulted in the formation of $[M+Li]^+$. The electrolyte concentration was $1.0 \text{ mmol}\cdot\text{L}^{-1}$ unless specified otherwise

Figure 2. Positive ESI TOF mass spectra of 5 ppm G- β -2 ($[M+Na]^+$ 343.1152 m/z) in the presence of $1.0 \text{ mmol}\cdot\text{L}^{-1}$ of a) ammonium acetate (mass accuracy error 5 ppm); b) acetic acid (mass accuracy error 7 ppm); and c) sodium hydroxide (mass accuracy error 6.7 ppm)

Figure 3. The highest mass-to-charge (m/z) values of the ions detected in the mass spectrum of (a) 15–90 ppm intact alkali lignin and (c) 15 ppm acetylated alkali lignin dissolved in ACN-water 1:1 in the positive and negative ionization modes while using different electrolytes; MW (Da) obtained after spectrum deconvolution of (b) 15–90 ppm intact alkali lignin and (d) 15 ppm acetylated alkali lignin. The electrolyte concentration was $1.0 \text{ mmol}\cdot\text{L}^{-1}$ unless specified otherwise

Figure 4. Positive ESI HR TOF mass spectra of an 80 ppm solution of intact lignin in ACN-water (1:1) with $100 \text{ mmol}\cdot\text{L}^{-1}$ formic acid: a), c) and e) zoomed and full original mass spectra; b), d) and f) zoomed and full mass spectra after deconvolution

Figure 5. LDI (no matrix) HR TOF mass spectrum of alkali lignin. The insert shows a zoomed in part of the spectrum in the range 2800–8000 m/z

Fig. 6. a) Ion mobility image of a 100 ppm solution of intact lignin in ACN-water (1:1) with $100 \text{ mmol}\cdot\text{L}^{-1}$ formic acid, recorded in the positive ESI mode. b) Original and deconvoluted mass

spectra of a 100 ppm solution of intact lignin in ACN-water (1:1) with 100 mmol·L⁻¹ formic acid recorded in the positive ESI mode. The blank spectrum was subtracted before deconvolution. An accurate deconvolution algorithm is described in equation 1.

Figure 1

Figure 2

Figure 4

Figure 5

Figure 6

b)

Table 1

Table 1. Comprehensive Overview of the MS Approaches Employed for the Analysis of Intact Lignin and High MW Standards

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or <i>m/z</i> Range Detected	Ref
APPI						
–	Q-orbitrap	Dioxane lignin	Acetone–water mixture (9:1)	0.1% NH ₄ OH	300–1800 <i>m/z</i>	[47]
+	HR Q-TOF-MS	Wheat straw lignin	Dioxane (100%) or	NR ^a	Positive mode:	[41]
–	HR Q-TOF-MS	Wheat straw lignin	Dioxane/MeOH/CHCl ₃		300–1,120 <i>m/z</i>	
+/–	HR Q-TOF-MS/MS	Oligomeric species in lignin	(1:1:1)		Negative mode: 200–700 <i>m/z</i>	
APCI						
–	Q-orbitrap	Dioxane lignin	Acetone–water mixture (9:1)	0.1% NH ₄ OH	300–1800 <i>m/z</i>	[47]
–	Ion trap	Synthesized oligomeric standards	H ₂ O/MeOH (50:50);	0.1% acetate	100–1,000 <i>m/z</i>	[42]
–	Single quadrupole	Lignin partially depolymerized under acetic conditions	H ₂ O/MeOH	NR	150–650 <i>m/z</i>	[43]
+/–	Quadrupole– hexapole– quadrupole	Wheat straw intact and acetylated lignin	CHCl ₃ /MeOH (2:1)	A mixture of formic and acetic acid	100–550 <i>m/z</i>	[44]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
ESI						
–	Q-orbitrap	Dioxane lignin	Acetone–water mixture (9:1)	0.1% NH ₄ OH	300–1800 m/z	[47]
–	Q-TOF	Dried sorghum feedstock, corn stover hydrolysate, xylobiose and xylotetraose	H ₂ O/traces of MeOH	None or traces of H ₂ SO ₄	50–2,000 m/z	[51]
+	Linear trap quadrupole – Fourier transform hybrid linear trap/7- T Fourier transform – ion cyclotron resonance MS	Bagasse lignin (100–100,000 g·mol ^{–1} ¹ with a maximum at 2·10 ³ g·mol ^{–1} according to GPC); steam explosion lignin	DMSO/MeOH (9:1)	NR	150–2,000 m/z	[48]
–	Magnetic sector	Spruce and eucalyptus dioxane lignin, eucalyptus kraft -organosolv and oxygen spruce lignin	MeOH/H ₂ O (1:1)/2.5% NH ₃ or dioxane/H ₂ O (7:3)	2.5% NH ₄ OH ^b	100–7,000 m/z (unresolved at higher m/z)	[38]
–	Q-TOF	Low-molecular-weight fraction of E. globulus dioxane lignin	MeOH/H ₂ O (1:1)/0.25% NH ₃	0.25% NH ₄ OH ^b	50–1,500 m/z	[49]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
+	Triple Q	Oligolignols (3–8 aromatic rings) synthesized from coniferyl alcohol with horseradish peroxidase	ACN/H ₂ O	Cetyltrimethylammonium sulfate	200–1,500 m/z	[50]
–	Magnetic sector- quadrupole	Wood and residual lignin from kraft pulps, black liquor lignin	MeOH/H ₂ O (1:1)	0.25% NH ₄ OH ^b	300–3,500 m/z	[53]
–	Triple-quadrupole	Synthetic lignin and soluble lignin extracted from sugar cane	ACN/H ₂ O	0.1% formic acid	100–850 m/z	[45]
– (nano- mate spray)	Linear trap quadrupole Fourier transform	Isolated oligolignols	ACN/H ₂ O (eluent Collected from RP LC)	0.1% acetic acid	120–1,400 m/z	[42]
MALDI						
–	Quadrupole-ion-trap analyzing system (linear trap quadrupole-XL)	Hand-cut sections of stems of two <i>Eucalyptus</i> species	NA	Matrix was substituted with thin layer chromatography grade silica	100–1,000 m/z	[56]
+	TOF	Organosolv lignin and glyoxalated lignin resin	NA	DHB ^c	200–700 m/z	[57]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or m/z Range Detected	Ref
+	TOF	Bagasse lignin (100–100,000 g·mol ⁻¹ ¹ with a maximum at 2·10 ³ g·mol ⁻¹ according to GPC); steam explosion lignin	NA	DHB	Mass distribution with a maximum around m/z 360; no signal above 2000 m/z	[48]
+/-	TOF	Lignin fractions extracted from <i>Miscanthus x giganteus</i> under alkali or acid conditions	NA	CHCA ^d /α-cyclodextrin	100–800 Da	[35]
+/-	TOF	Ssoftwood kraft lignin, mixed hardwood organosolv lignin, acid hydrolysis lignin from bagasse, and steam explosion lignin from aspen (acetylated sample)	NA	DHB or all- <i>trans</i> -retinoic acid	50–14,000 m/z (unresolved at higher m/z); 50– 14,000 Da (singly charged ions are almost exclusively generated)	[39]
+	TOF	Milled wood lignin, alkali lignin and a synthetic lignin (G-type)	NA	DHB, 2-aminobenzoic acid or sinapinic acid	200–1,700 Da	[58]

[illegible]

Ionization Mode	Mass Analyzer	Analyte	Solvent	Electrolyte/ MALDI Matrix	MW or <i>m/z</i> Range Detected	Ref
+	TOF	Milled wood lignin, birch kraft lignin, isolated	DCM/EtOH (2:1) or DMSO prior to deposition on silver disk	None	10–1,200 <i>m/z</i>	[60]

^a NR denotes not reported

^b The w/v concentration was provided in respect to NH₃

^c DHB stands for 2,5-dihydroxybenzoic acid

^d CHCA stands for α -cyano-4-hydroxycinnamic acid

Table 2. List of Lignin Model Compounds Used in this Study

Compounds	Acronym	Characteristic functionality ^a	MW (g·mol ⁻¹)	Supplier/Synthesized	Purity
Vanillin	V	Carbonyl	152.15	Sigma-Aldrich ^b	99%
Guaiacol	G	—	124.24	Sigma-Aldrich	98%
Eugenol	E	Alkenyl	164.20	Acros Organics ^c	99%
Vanillic acid	VA	Carboxyl	168.15	Fluka ^d	97%
Syringol	S	Methoxy	154.16	Acros Organics	99%
Homovanillyl alcohol	HA	Aliphatic hydroxyl	168.19	Sigma-Aldrich	99%
Veratrole	VER	—	138.16	Sigma-Aldrich	99%
Syringaldehyde	SA	Carbonyl	182.17	Sigma-Aldrich	98%
Pinoresinol	P2	Hydroxyl, methoxy	358.38	Sigma-Aldrich	≥95%
Guaiacylglycerol-β-guaiacyl ether	G-β-2	Aliphatic hydroxyl, methoxy, β-O-4	320.34	In-house synthesis [61].	≥95%
1,2-Dimethoxy-4-[(2-methoxyphenoxy)methyl]benzene	ET2	Methoxy, ether dimer	274.12	In-house synthesis [62].	≥95%
4-(1-Hydroxyethyl)-2-methoxyphenyl benzoate	ALC2	Aliphatic hydroxyl dimer, ester	272.1	In-house synthesis [62].	≥95%
4-Formyl-2-methoxyphenyl benzoate	EST2	Carbonyl, ester dimer	256.07	In-house synthesis [62].	≥95%
(E)-4,4'-(Ethene-1,2-diyl)bis(2-methoxyphenol)	ALK2	Hydroxyl, methoxy, alkene dimer	272.10	In-house synthesis [59].	≥95%

Dehydrodivanillin	D2V	Carbonyl, hydroxyl, methoxy, 5-5 dimer	302.07	In-house synthesis [63, 64].	≥95%
4-[2-(3,4-Dimethoxybenzyl)-4,5-dimethoxybenzyl]-2-methoxyphenol and 1-(3,4-dimethoxybenzyl)-4,5-dimethoxy-2-[(2-methoxyphenoxy)methyl]benzene	ET3-1 ET3-2	Methoxy, ether trimer	424.19	In-house synthesis [62].	≥95%

^a Functional groups and linkages (for oligomers) featured in the studied methoxyphenols compared to guaiacol

^b Sigma-Aldrich (St. Louis, MO, USA)

^c Acros Organics (Morris Plains, NJ, USA)

^d Fluka (Steinheim, Germany)

Table 3. ESI TOF MS Response with Acids (Either Formic or Acetic) and Ammonium Acetate as ESI Electrolytes for Representative Lignin Mono- to Trimeric Structure Model Compounds in Both Positive and Negative Ionization Modes^a

Model Compounds	Numbers of Oxygenated Functional Groups			pK _a	Intensity of the Target Ion Response			
	-OH	-OCH ₃	-COOH		Acid (Formic/Acetic)		Ammonium Acetate	
					Positive	Negative	Positive	Negative
VER	0	2	0	-	++++ ^b	ND ^c	+++	ND
EST2	0	1	0	-	++++	ND	++++	ND
ET2	0	3	0	-	++++	ND	+++	ND
ET3-1	0	5	0	-	++++	ND	+++	ND
ET3-2	1	5	0	-	++++	+	+++	+
P2	2	2	0	9.76	++++	+	+++	+
G-β-2	1 +2 aliphatic	2	0	9.88	++++	+	+++	+
HA	1 +1 aliphatic	1	0	10.19	+++	+	++++	++
ALC2	1 aliphatic	1	0		+++	+	++++	++
S	1	2	0	9.98	++	+++	++++	+
SA	1	2	0	7.8	+++	+	++++	++
VA	1	1	1	4.45	++	+++	+	++++
V	1	1	0	7.38	+	+++	++	++++
D2V	2	2	0	7.04	++	+++	+	++++
G	1	1	0	9.93	++	+++	+	++++
E	1	1	0	10.19	+	++++	+	++++

^a For the majority of analytes, the response was monitored for [M+Na]⁺ and [M-H]⁻ ions in the positive and negative ionization modes, respectively. The electrolyte concentration upon direct infusion was 2.5 mmol·L⁻¹

^b “++++” indicates the system resulting in the most efficient ionization; “+” indicates the system with the least efficient ionization

^c “ND” denotes no molecular ions or adducts were detected

Table 4. Number-Average, Weight-Average and z-Average Molecular Weight of Lignin
Determined by ESI HR TOF MS, GPC and MALDI HR TOF MS

	ESI HR TOF MS	GPC [11]	MALDI HR TOF MS
M _n	1,480	1,630	830
M _w	2,520	2,740	1,250
M _z	3,790	3,720	2,230

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Supplemental Information

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