analytical chemistry

Quantification of Lignin and Its Structural Features in Plant Biomass Using ^{13}C Lignin as Internal Standard for Pyrolysis-GC-SIM-MS

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S *Supporting Information*

ABSTRACT: Understanding the mechanisms underlying plant biomass recalcitrance at the molecular level can only be achieved by accurate analyses of both the content and structural features of the molecules involved. Current quantification of lignin is, however, majorly based on unspecific gravimetric analysis after sulfuric acid hydrolysis. Hence, our research aimed at specific lignin quantification with concurrent characterization of its structural features. Hereto, for the first time, a polymeric ^{13}C lignin was used as internal standard (IS) for lignin quantification via analytical pyrolysis coupled to gas chromatography with mass-spectrometric detection in selected ion monitoring mode (py-GC-SIM-MS). In addition, relative response factors (RRFs) for the various pyrolysis products obtained were determined and applied. First, ^{12}C and ^{13}C lignin were isolated from nonlabeled and uniformly ^{13}C labeled wheat straw, respectively, and characterized by heteronuclear single quantum coherence (HSQC), nuclear magnetic resonance (NMR), and py-GC/MS. The two lignin isolates were found to have identical structures. Second, ^{13}C-IS based lignin quantification by py-GC-SIM-MS was validated in reconstituted biomass model systems with known contents of the ^{12}C lignin analogue and was shown to be extremely accurate (>99.9%, $R^2 > 0.999$) and precise (RSD < 1.5%). Third, ^{13}C-IS based lignin quantification was applied to four common poaceous biomass sources (wheat straw, barley straw, corn stover, and sugar cane bagasse), and lignin contents were in good agreement with the total gravimetrically determined lignin contents. Our robust method proves to be a promising alternative for the high-throughput quantification of lignin in milled biomass samples directly and simultaneously provides a direct insight into the structural features of lignin.
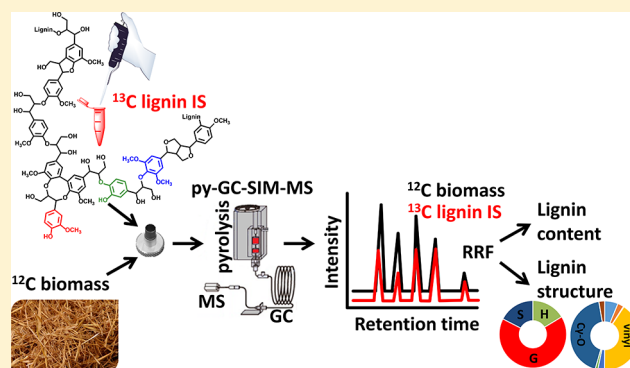
Understanding and improving the conversion of plant biomass heavily depends on the characterization and quantification of its constituents. The major constituents of poaceous plant biomasses are cellulose (30−50% (w/w)), hemicellulose (mainly xylan) (20−40% (w/w)), and lignin (5−25% (w/w)).[1] Cellulose and xylan are polysaccharides and lignin is a cross-linked phenolic macromolecule, which is composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) subunits. These subunits are linked through multiple carbon−carbon and aryl−ether linkages, resulting in a complex structure.[2] While characterization and quantification of the plant carbohydrates are considered routine analyses, the quantification of lignin and its subunit composition is not well established.[3−5]

Common quantification of lignin, still, mainly relies on gravimetric analysis after sulfuric acid hydrolysis known as Klason-lignin analysis or a variant hereof.[6,7] The outcome of such gravimetric analysis is highly disturbed by the presence of nonlignin acid-insoluble material, e.g. proteins and chitin of fungal origin. In addition, this analysis does not distinguish

different lignin structures.[6] Therefore, one of the main challenges for biomass analysis is the development of an analytical tool for the specific quantification of lignin in absolute amounts that is able to simultaneously characterize lignin structural features in a robust and high-throughput manner.

Solving this analytical challenge has been attempted with vibrational spectroscopy and nuclear magnetic resonance (NMR), both proven powerful techniques for the structural elucidation of lignin.[5,8−11] For quantification of lignin, however, serious drawbacks remained: both techniques suffered from poor accuracy, dependency on structurally similar calibration standards, and/or minimal structural information that was obtained.[8−11] Additionally, for NMR throughput was low.[12]

As an alternative, our research aimed at the development of a rapid and specific lignin quantification method via analytical pyrolysis coupled to gas chromatography with mass-spectrometric detection (py-GC/MS). This technique requires minimal sample amounts (10−100 μg) and only milling of the sample is needed prior to analysis. Furthermore, py-GC/MS has been shown to enable distinguishing lignins' subunits, i.e. H, G, and S substructures.[13−15] In contrast, thus far, py-GC/MS has been found inaccurate for the absolute quantification of lignin in plant biomass.[16−20] That inaccuracy has been proposed to relate to two main aspects. The first relates to the need for a proper internal standard (IS), that during pyrolysis behaves like (native) lignin in biomass, together with the large number of pyrolysis products formed.[13,21] Second, different ratios of pyrolysis products ask for the determination and use of (relative) response factors.[16,21,22]

Previously, absolute lignin quantification by py-GC/MS and py-GC/FID (flame ionization detection) was attempted by relating the relative peak area of lignin-derived compounds, so-called "py-lignin", to lignin content as determined by gravimetric methods. The models that were obtained showed low and/or unstable accuracy.[17−20] This was a consequence of the absence of response factors for the obtained pyrolysis products. Furthermore, the extents to which CO_2 and other low molecular weight gases and char were formed from both lignin and nonlignin components of the cell-wall upon pyrolysis were not accounted for. Matrix-effects during pyrolysis may have further influenced the outcomes.[22−24] Alternatively, Bocchini et al. approached lignin quantification by the measurement of lignin-derived pyrolysis products via monomeric internal standards and the application of response factors, which resulted in severe underestimation.[16] Again, the formation of nonmonomeric pyrolysis products was excluded and matrix-effects were expected to affect the monomeric internal standard differently than lignin macromolecules in biomass.

To specifically correct for the formation of nonmonomeric pyrolysis products from lignin and monitor matrix-effects properly, the use of polymeric lignin as internal standard can be hypothesized for lignin quantification by py-GC/MS, a currently unexplored field of research.

Thereto, this research aimed to apply the ideal standard, a ^{13}C-labeled polymeric lignin isolate, for the absolute lignin quantification by py-GC/MS. Relative response factors for lignin-derived products were determined and applied to allow quantification, via the ^{13}C-lignin as internal standard, of structurally diverse lignins in four poaceous biomasses.

■ EXPERIMENTAL SECTION

Materials. All chemicals were obtained from commercial suppliers and used without further purification. Water used in all experiments was purified via a Milli-Q water system (Millipore, Billerica, MA, USA). For lignin content determination experiments, wheat straw (WS) and corn stover (CS) were kindly provided by CNC (Milsbeek, The Netherlands). Sugar cane bagasse (SCB) and barley straw (BS) were supplied by Sime Darby (Kuala Lumpur, Malaysia) and Unifarm Wageningen (Wageningen, The Netherlands), respectively. Compositional analysis was performed as described in Supporting Information.

Preparation of nonlabeled and ^{13}C-labeled wheat straw. Nonlabeled ("^{12}C", 98.9 atom % ^{12}C) and uniformly ^{13}C-labeled ("^{13}C", 97.7 atom % ^{13}C) spring wheat plants (*Triticum aestivum* L. cv. "Baldus") were produced under identical growth conditions in modified, custom designed, airtight, high-irradiance labeling chambers of the facility ESPAS (Experimental Soil Plant Atmosphere System, IsoLife, Wageningen, The Netherlands).[25] Details of wheat straw preparation are provided in Supporting Information.

Isolation of lignin from ^{12}C and ^{13}C wheat straw. The isolation of lignin was performed according to a modified method reported by Björkman.[26] Freeze-dried straw (3 g), either ^{12}C or ^{13}C, was cut to a size of 1−3 mm and acetone-extracted for 3 h at 30 °C under magnetic stirring (750 rpm) to remove extractives. Insoluble material was removed by filtration, dried under a stream of nitrogen and ball milled in a PM100 planetary ball mill (Retsch, Haan, Germany) in a 50 mL zirconium dioxide jar containing 17 φ10 mm zirconium dioxide balls at a frequency of 600 rpm with a net milling time of 4 h. After every 15 min of milling a pause of 10 min was set to prevent overheating. Ball milled material was subsequently water-extracted in a concentration of 5% (w/w) at 50 °C for 15 h under magnetic stirring (750 rpm). Insoluble material (water unextractable solids, WUS) was removed by centrifugation (60,000xg, 10 min, 20 °C) and washed 3 times with 30 mL water. The wet residue was suspended in dioxane and adjusted to 80% aqueous dioxane (v/v) at a material loading of 5% (w/w) and extracted twice (2 × 24 h) at room temperature with magnetic stirring (500 rpm) under nitrogen atmosphere. Supernatants were recovered by centrifugation (30,000xg, 5 min, 20 °C), combined and freeze-dried to obtain crude lignin isolates (ISOcrude). The obtained crude isolates were purified by enzymatic carbohydrate removal. ISOcrude was thereto suspended in 50 mM sodium acetate buffer at pH 4.8 at 5% (w/w) material loading, charged with 0.075% (w/w) Viscostar 150L (Dyadic, Jupiter, FL, USA) (protein content:[27] 40 mg·mL$^{−1}$) and incubated under rotary shaking (20 rpm) at 50 °C for 4 h. Insoluble material was removed by centrifugation (8000xg, 5 min, 20 °C) and washed 3 times with water before freeze-drying to obtain pure lignin isolates (LIGpure).

Compositional analysis of total biomass and lignin isolates. Carbohydrate content and composition, protein content, ash content and lignin content were determined by modifications of previously published procedures.[3,15,28] Detailed procedures are provided in Supporting Information.

Characterization total biomass and lignin isolates. *NMR spectroscopy.* NMR of the pure lignin isolates (^{12}C-LIGpure and ^{13}C-LIGpure) was performed according to Del Río et al.[29] For NMR experiments 12 mg ^{12}C-LIGpure was dissolved in 450 μL DMSO-d_6, while for ^{13}C-LIGpure 1.3 mg was dissolved in 450 μL DMSO-d_6. The HSQC (heteronuclear single quantum coherence) NMR-experiments were recorded with a hsqcetgpsisp2.2 pulse sequence on a Bruker AVANCE III 600 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm cryo-probe. The internal temperature of the probe was set at 298 K. The spectral widths were 6000 Hz (10−0 ppm) for the ^1H-dimension and 25000 Hz (165−0 ppm) for the ^{13}C-dimension. The number of complex points was 2048 in the ^1H-dimension, 32 scans were collected with a relaxation time of 1.5 s. In the ^{13}C-dimension, 256 time increments were recorded. The $^1J_{CH}$ was set at 145 Hz. The data was processed with Bruker TopSpin 3.2 software. For the Fourier transformation in the ^1H-dimension, Gaussian apodization was used. For the ^{13}C-dimension, zero-filling up to 1024 was applied prior to Fourier transformation with a squared cosine window function. Zero-order phase correction

Table 1. Pyrolysis Products and Their Signature Fragments Used for py-GC-SIM-MS Analyses

#	Compound	CAS	Retention time (min)	Structural feature	RRF (-)	M_w ^{12}C (g·mol^{-1})	Fragments ^{12}C [a]	M_w ^{13}C (g·mol^{-1})	Fragments ^{13}C [a]	Segment (min)
1	phenol*	108952	9.98	H, unsub.	0.54	94	94, 66	100	100, 71	9.70–10.30
2	guaiacol*	90051	10.12	G, unsub.	0.67	124	124, 109	131	131, 115	
3	2-methylphenol*	95487	11.10	H, methyl	0.81	108	108, 107	115	114, 115	
4	4-methylphenol*	106445	12.14	H, methyl	0.76	108	107, 108	115	114, 115	10.30–13.00
5	4-methylguaiacol*	93516	12.77	G, methyl	0.70	138	138, 123	146	146, 130	
6	2,4-dimethylphenol	105679	13.26	H, methyl	0.79[g]	122	107, 122	130	114, 130	
7	4-ethylphenol*	123079	14.32	H, ethyl	1.00	122	107, 122	130	114, 130	13.00–15.40
8	4-ethylguaiacol*	2785899	14.91	G, ethyl	0.95	152	137, 152	161	145, 161	
9	4-vinylguaiacol*	7786610	16.39	G, vinyl	0.22	150	150, 135	159	159, 143	
10	4-vinylphenol*	2628173	16.57	H, vinyl	0.33	120	120, 91	128	128, 98	15.40–16.80
11	eugenol*	97530	16.98	G, misc.[b]	0.40	164	164, 149	174	174, 158	
12	4-propylguaiacol	2785877	17.05	G, misc.	0.37[h]	166	137, 166	175	145, 175	16.80–17.30
13	syringol*	91101	17.73	S, unsub.	0.48	154	154, 139	162	162, 146	
14	cis-isoeugenol*	97541	18.33	G, misc.	0.34	164	164, 149	174	174, 158	17.30–18.50
15	3-methoxy-5-methylphenol[f]	3209130	18.92	G, methyl	-	138	138, 109	146	146, 116	
16	4-propenylphenol	539128	19.31	H, misc.	0.27[i]	134	134, 133	143	143, 142	18.50–19.45
17	trans-isoeugenol*	97541	19.60	G, misc.	0.34	164	164, 149	174	174, 158	19.45–19.80
18	4-methylsyringol*	6638057	19.95	S, methyl	0.55	168	168, 153	177	177, 161	
19	vanillin*	121335	20.08	G, C_α-O[c]	0.66	152	151, 152	160	159, 160	19.80–20.20
20	4-propyneguaiacol	-	20.33	G, misc.	0.40[j]	162	162, 147	172	172, 156	
21	4-alleneguaiacol	-	20.59	G, misc.	0.40[j]	162	162, 147	172	172, 156	20.20–21.00
22	hydroquinone[f]	123319	20.61	H, misc.	-	110	110, 81	116	116, 85	
23	homovanillin	5603242	21.53	G, C_β-O[d]	0.59[k]	166	137, 166	175	145, 175	21.00–21.85
24	4-ethylsyringol	14059928	21.69	S, ethyl	0.98[l]	182	167, 182	192	176, 192	
25	acetovanillone*	498022	22.00	G, C_α-O	0.51	166	151, 166	175	159, 175	21.85–22.90
26	4-hydroxybenzaldehyde	123080	22.81	H, C_α-O	0.53[m]	122	122, 121	129	129, 128	
27	4-vinylsyringol	28343228	23.01	S, vinyl	0.28	180	180, 165	190	190, 174	22.90–23.30
28	guaiacylacetone*	2503460	23.19	G, C_β-O	0.53	180	137, 180	190	145, 190	
29	4-allylsyringol*	6627889	23.41	S, misc.	0.27	194	194, 167	205	205, 176	23.30–24.40
30	guaiacyl vinyl ketone	-	24.16	G, C_α-O	0.53[n]	178	151, 178	188	188, 159	
31	cis-4-propenylsyringol	26624135	24.55	S, misc.	0.27[o]	194	194, 179	205	205, 189	
32	4-propynesyringol	-	25.19	S, misc.	0.27[o]	192	192, 131	203	203, 140	24.40–26.10
33	4-allenesyringol	-	25.39	S, misc.	0.27[o]	192	192, 131	203	203, 140	
34	trans-4-propenylsyringol	26624135	25.84	S, misc.	0.27[o]	194	194, 179	205	205, 189	
35	syringaldehyde*	134963	26.45	S, C_α-O	0.30	182	182, 181	191	191, 190	26.10–27.20
36	cis-coniferyl-alcohol	458355	26.53	G, C_γ-O[e]	0.097[p]	180	137, 180	190	145, 190	
37	homosyringaldehyde	-	27.42	S, C_β-O	0.30[q]	196	167, 196	206	176, 206	27.20–28.05
38	acetosyringone*	2478388	27.88	S, C_α-O	0.44	196	181, 196	206	190, 206	
39	trans-coniferyl-alcohol	458355	28.23	G, C_γ-O	0.097[p]	180	137, 180	190	145, 190	28.05–28.70
40	trans-coniferaldehyde*	458366	28.60	G, C_γ-O	0.097	178	178, 135	188	143, 188	
41	syringylacetone	19037582	28.79	S, C_β-O	0.30[q]	210	167, 210	221	176, 221	28.70–29.85
42	syringyl vinyl ketone	-	29.67	S, C_α-O	0.30[q]	208	208, 181	219	219, 190	
43	coniferylacetate[f]	-	29.88	S, C_γ-O	-	222	131, 222	234	140, 234	29.85–32.60
44	cis-sinapyl-alcohol	537337	31.74	S, C_γ-O	0.068[r]	210	167, 210	221	176, 221	
45	trans-sinapyl-alcohol	537337	33.45	S, C_γ-O	0.068[r]	210	210, 167	221	176, 221	32.60–34.00
46	trans-sinapaldehyde*	4206580	33.65	S, C_γ-O	0.068	208	208, 165	219	219, 174	

*Relative response factor (RRF) for compound determined (RSD < 5%). [a]Most abundant fragment first. [b]Miscellaneous. [c]C_α-oxygen. [d]C_β-oxygen. [e]C_γ-oxygen. [f]Not included in quantitative analyses due to background interference. [g]Average #3,#4. [h]Average #11,#17. [i]#17/#2 × #1. [j]#11. [k]Average #19,#25. [l]Average #7,#8. [m]#7/#2 × #1. [n]#28. [o]#29. [p]#40. [q]#35. [r]#46.

was performed manually. The DMSO solvent-peak was set as internal reference at δ_C 39.5; δ_H 2.49 ppm. HSQC correlation peaks were assigned by comparison with literature.[29−31] Semiquantitative analysis of the volume integrals was performed according to Del Río et al.[29]

py-GC/MS. Pyrolysis was performed with an EGA/PY-3030D Multishot pyrolyzer (Frontier Laboratories, New Ulm, MN, USA) equipped with an AS-1020E Autoshot autosampler. The pyrolyzer was coupled to GC-MS using a Trace GC equipped with a DB-1701 fused-silica caplillary column (30 m x 0.25 mm i.d. 0.25 μm film thickness) coupled to a DSQ-II mass

spectrometer (both Thermo Scientific, Waltham, MA, USA). Pyrolysis, GC and MS settings were based on previously described research.[15] Samples were weighed using a XP6 excellence-plus microbalance (Mettler Toledo, Columbus, OH, USA). Pyrolysis of total biomass (80−90 μg) and lignin isolates (20−30 μg) was performed at 500 °C for 1 min with an interface temperature of 300 °C. Pyrolysis products were injected on the column via split/splitless injection (at 250 °C) with a splitratio of 1:133 and helium was used as carrier gas with constant flow at 1.5 mL·min^{-1}. The GC oven was programmed from 70 °C (2 min) to 270 °C at 5 °C·min^{-1} and held at 270 °C for 15 min. MS detection was used with EI at 70 eV, a source temperature of 250 °C, a scan range of m/z 50−550 and a scan rate of 4.0 scans/sec. Compounds were identified by comparing retention time and mass spectrum with standards, the NIST library and data published by Ralph and Hatfield.[13] Results were combined in a (^{12}C) target library. A unique ^{13}C target library was built on the basis of retention time and expected fragmentation from ^{12}C mass spectra and carbon number.

For qualitative analysis, pyrograms were processed by AMDIS software (version 2.71, NIST, USA). For identification and deconvolution the following software settings were used: minimum match factor at 60 with multiple identifications per compound, component width at 20, adjacent peak subtraction at two, resolution at high, sensitivity at very high and shape requirements at low. Compounds identified on the basis of reference standards were annotated by evaluation of retention time (± 0.1 min), reverse search (≥ 80) and simple search (≥ 45). Peak molar area was calculated as defined by Del Río et al.[32] All samples were analyzed in triplicate.

Absolute lignin quantification using py-GC/MS. *Relative response factor determination of pyrolysis products.* Relative response factors of 21 (of a total of 46) lignin-derived pyrolysis products, indicated in Table 1 with an asterisk, were determined by injecting an equimolar mixture of authentic standards and 9-fluorenone as internal standard into the py-GC/MS system.[21] Standards and 9-fluorenone were dissolved in 50:50 (v/v) Ethanol (EtOH):Chloroform (CHCl$_3$) in a concentration of 10 mM, taking the declared purity of the standard into account, and mixed to give an equimolar solution of 0.45 mM. All solutions were kept in amber vials with minimal exposure to air and kept at −20 °C. Five microliter of the equimolar mixture was injected into a pyrolysis cup and directly measured by py-GC/MS as described in section 'Characterization total biomass and lignin isolates−py-GC/MS' in triplicate. Different injection volumes were tested and showed similar relative response factors, confirming linearity of the response over the used concentration range. Stability of compounds during pyrolysis, i.e. the formation of a single compound peak only, was confirmed by injecting all compounds individually at 0.4 mM mixed with 9-fluorenone at the same concentration. Xcalibur 2.2 was used for data analysis. The two most abundant fragments per compound were used for quantification (Table 1). Peak areas (A_i) were manually integrated to avoid erroneous integration by method settings and normalized for molarity (M_i). Areas for *cis*-isoeugenol and *trans*-isoeugenol were summed as they were present as a mixture. Relative response factors (RRFs) were calculated versus the highest peak area (4-ethylphenol, A_{4EP}) normalized for molarity (M_{4EP}) according to eq 1.

$$RRF_i = \frac{A_i/M_i}{A_{4EP}/M_{4EP}} \quad (1)$$

where i refers to compound number (Table 1). *cis*- and *trans*-isomers were assumed to have similar RRFs. RRFs for pyrolysis products for which no commercial standard was available were estimated from structurally closest molecules (Table 1). In addition, RRFs of *cis*-/*trans*-coniferyl alcohol and *cis*-/*trans*-sinapyl alcohol were estimated from their aldehyde analogues, as they were shown to be insufficiently stable to analyze via "liquid injection". Molar RRFs (^{12}C) were assumed to be similar for ^{13}C analogues.

^{13}C lignin internal standard based lignin quantification in reconstituted biomass model systems. ^{12}C-LIGpure was dissolved in 50:50 (v/v) EtOH:CHCl$_3$ in a concentration of 1.0 mg·mL^{-1}. 10−35 μL of this solution was added to 40−65 μg cellulose to obtain 75 μg "reconstituted" biomass (m_{sample}) with six different lignin contents (12−43% (w/w)) and dried at 30 °C for 1 h. ^{13}C-LIGpure (=internal standard, IS) was dissolved in a similar manner. Ten microliter IS solution of 1.0 mg·mL^{-1} (m_{IS}) was added to the reconstituted biomass and dried overnight at room temperature before analysis. All samples were prepared and analyzed by py-GC/MS in triplicate.

Py-GC/MS settings and data processing for quantitative analyses. The pyrolysis and GC setup used was similar to the qualitative analysis. MS detection was applied in selected ion monitoring (SIM) mode. The two most abundant fragments per compound were monitored, with a maximum of 8 fragments (4 ^{12}C + 4 ^{13}C) per segment (Table 1). Dwell time was set at 25 ms to ensure at least 25 data points per peak. Data was processed using Xcalibur 2.2. Peaks were integrated using ICIS peak integration where smoothing points was set at 5, area noise factor was set at 5, peak noise factor was set at 10 and baseline window optimized per compound (range 12−55). A manual correction was only applied when irregular peak shapes lead to erroneous peak integration with method settings. Areas were normalized by dividing by RRFs and summed (A_i/RRF_i, where i refers to the compound number in Table 1). To correct for the higher molecular weight and concomitant lower response per weight of ^{13}C pyrolysis products, a correction factor for detected ^{13}C total area was included. This correction factor was determined by calculating total molar area of ^{13}C-LIGpure on the basis of ^{12}C and ^{13}C pyrolysis product molecular weights (Table 1) as described by Del Río et al.[32] in >25 samples. The calculated correction factor was equal to 1.057 with a standard deviation below 0.05%. Lignin content was quantified following eq 2.

$$Lignin\ content\ (w/w)\% = \frac{\sum_{i=1}^{46} \frac{A_i^{12C}}{RRF_i} \cdot m_{IS} \cdot P_{IS}}{\sum_{i=1}^{46} \frac{A_i^{13C}}{RRF_i} \cdot m_{sample} \cdot 1.057} \cdot 100 \quad (2)$$

where i refers to compound number (Table 1), A is area, RRF is relative response factor (Table 1), m_{IS} is the amount of IS (μg; ^{13}C-LIGpure), m_{sample} is the amount of sample (μg), and P_{IS} is a correction factor for the purity of the IS (0.895). In i, compounds 15, 22, and 23 were not included.

Application of ^{13}C lignin IS method for lignin quantification. Lignin in water-unextractable solids (WUS) of WS, CS, BS, and SCB, obtained as described in the Supporting Information, was quantified using the novel method as

Table 2. Composition and Yield of ^{12}C and ^{13}C Lignin Isolates % (w/w) Determined in Duplicate

	^{12}C-WS	^{13}C-WS	^{12}C-LIGpure	^{13}C-LIGpure
carbohydrate	68.5 ± 0.5	67.3 ± 0.3	6.8 ± 0.2	7.1 ± 0.1
protein	0.9 ± 0.04	0.7 ± 0.1	4.3 ± 0.1	3.0 ± 0.1
ash	1.5 ± 0.2	1.4 ± 0.1	0.8 ± 0.4	0.4 ± 0.1
lignin	20.6 ± 0.8[a]	19.3 ± 1.3[a]	88.1 ± 0.5[b]	89.5 ± 0.2[b]
Isolation yield (%)			19.0 ± 0.7	21.5 ± 1.4

[a]Estimated according to Jurak et al.[15] [b]Remaining content of dry matter after subtraction of carbohydrates, protein, and ash.

described in the section "Py-GC/MS settings and data processing for quantitative analysis". Approximately 75 μg of material was mixed with 10 μL of ^{13}C-IS solution (1.0 mg· mL^{-1}) and dried at 30 °C for 3 h. All samples were prepared and analyzed by py-GC/MS in triplicate and compared to Klason lignin content (AIL corrected for ash and protein + ASL).

■ RESULTS AND DISCUSSION

The quantification potential of our new approach could best be evaluated using reconstituted biomass model systems, on the basis of structurally similar ^{12}C and ^{13}C lignins to which cellulose was added, to mimic a plant biomass matrix. This allowed us to evaluate the performance of the novel method on samples with a known, "true" lignin content, without relying on inaccurate and unselective procedures. Thereto, first lignin was isolated from nonlabeled ("^{12}C") and uniformly ^{13}C-labeled ("^{13}C") wheat straw that was produced under identical growth conditions.

Compositional analysis of lignin isolates. The abundance of the main constituents of the lignin isolates is indicated in Table 2. Lignin content was calculated as the remaining content of dry matter after subtraction of carbohydrates, protein, and ash, since acetone and water extractives were removed prior to dioxane extraction. High purity (∼90%) isolates were obtained, with carbohydrates as the most abundant impurity (∼7%). The carbohydrate contents of the isolates were approximately two times lower than previously reported for unpurified dioxane/milled wood lignins, achieved by water extraction prior to and enzymatic carbohydrate removal after dioxane extraction.[33] Enzymatic purification is considered a milder and less laborious alternative compared to the commonly used chemical workup procedure, maintaining efficiency.[26,34] The significant amount of protein (nitrogen) found in the isolates was likely the result of protein adsorption, although coextraction has been reported as well.[35,36] Estimated lignin contents of ^{12}C and ^{13}C wheat straw (total biomass) were 20.6 ± 0.8% (w/w) and 19.3 ± 1.3% (w/w), respectively, and used for calculation of isolation yields.[15] Final lignin isolation yields of approximately 20% were achieved for both isolates, which is in line with literature on lignin isolates obtained from wheat straw with a similar extraction procedure.[29]

Characterization of lignin isolates. The lignin isolates were characterized by 2D-NMR to provide information on the interunit linkages present within the lignins. Chemical shift assignments were based on previous studies.[29−31] The aliphatic (δ_C/δ_H 50−90/2.5−6.0) and aromatic/unsaturated (δ_C/δ_H 90−160/6.0−8.0) regions of the recorded HSQC spectra along with the structures of the assigned correlation peaks are shown in Supporting Information Figures S-1 and S-2. Clear lignin signals could be observed in the aromatic region (S$_{2,6}$, G$_2$, H$_{2,6}$, and several tricin, p-coumarate, and ferulate related

signals). The aliphatic region was dominated by methoxyl group- and β-O-4′ substructure (A, A′, and A$_{ox}$) related correlation peaks. Other interunit linkages such as phenyl-coumaran (B) and resinol (C), and to a lesser extent spirodienone (F) and dibenzodioxocins (D) substructures, were also observed in the HSQC spectra. α,β-Diaryl ether (E) substructures, the presence of which was confirmed by del Río et al., albeit in minor amounts, could not be detected in the isolated lignins.[29] A summary of the relative abundance of the aromatic units and interunit linkages of the ^{12}C and ^{13}C lignin isolates, determined by semiquantitative analysis, is presented in Table 3.

Table 3. Relative Abundance of Lignin Interunit Linkages and Aromatic Units of ^{12}C/^{13}C Lignin Isolates by Semiquantitative HSQC NMR Analysis

	^{12}C-LIGpure	^{13}C-LIGpure
Lignin interunit linkages (%)[a]		
β-O-4′ aryl ethers (A/A′)	80	81
α-oxidized β-O-4′ aryl ethers (Aox)	3	3
phenylcoumarans (B)	8	8
resinols (C)	5	4
dibenzodioxocins (D)	1	1
spirodienones (F)	4	4
α,β-diaryl ether (E)	n.d.	n.d.
total	100	100
Lignin aromatic units[b]		
H (%)	6 (14)	3 (13)
G (%)	58 (54)	60 (56)
S (%)	36 (30)	37 (31)
S/G	0.62 (0.56)	0.62 (0.56)

[a]percentage of total lignin interunit linkage (A−F; see Supporting Information for details), n.d.: not detected [b]Molar percentages (H + G + S = 100) excluding or including (in brackets) p-coumaric acid (H) and ferulic acid (G).

Besides lignin, multiple carbohydrate-related signals (δ_C/δ_H 65−80/2.8−3.6) were readily detectable in both isolates, despite their relatively low abundance (Table 2), which could provide additional information on the structural features of carbohydrates and lignin involved in lignin−carbohydrate complexes (LCC). This is not further discussed in this paper.

The distribution of aromatic units and lignin interunit linkages closely matched with earlier reports on HSQC NMR analyses of wheat straw lignin.[29,31,33] Hence, it was concluded that the isolated lignins were representative of lignin in wheat straw. In addition, a highly similar relative abundance of lignin interunit linkages and aromatic units was found for both isolates. This good similarity of the ^{12}C and ^{13}C lignins was further used for py-GC/MS analysis to identify and annotate for the first time pyrolysis products formed from ^{13}C lignin.

Pyrograms of the two lignin isolates can be found in Supporting Information Figure S-3. Due to the lack of authentic ^{13}C standards, ^{13}C pyrolysis products were identified on the basis of expected retention time ($R_{t,12C} = R_{t,13C}$) and molecular ion ($M^{+\bullet}_{13C} = M^{+\bullet}_{12C}$ + carbon number) and fragmentation spectra of their ^{12}C counterparts. The ^{12}C and ^{13}C MS fragmentation spectra of two abundant lignin-related pyrolysis products are shown in Figure 1. Identical fragmentation
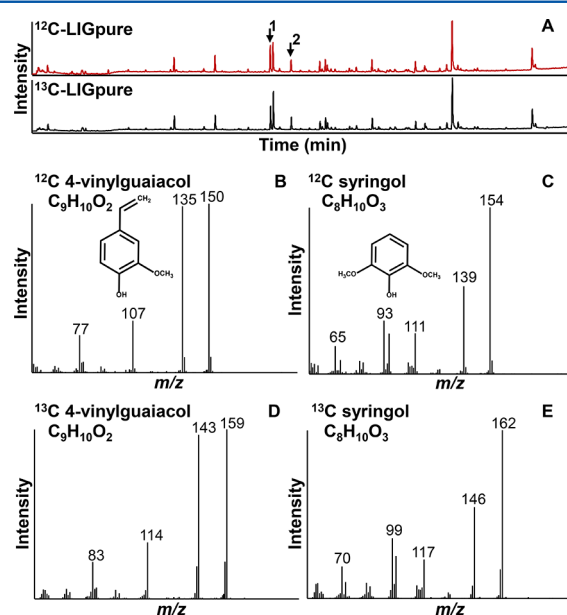
Figure 1. Pyrograms (A) of ^{12}C-LIGpure and ^{13}C-LIGpure where 4-vinylguaiacol (1) and syringol are indicated (2) and EI-MS (70 eV) spectra of [12]C 4-vinylguaiacol (B), ^{12}C syringol (C), [13]C 4-vinylguaiacol (D), and ^{13}C syringol (E). Most abundant fragments are indicated. Full size pyrograms with peak annotation can be found in Supporting Information Figure S-3.

behavior was observed for ^{12}C and ^{13}C analogues. MS fragmentation spectra of all other identified ^{13}C lignin-related pyrolysis products are presented in Supporting Information Figure S-4.

Like NMR, py-GC/MS analysis revealed that the chemical composition of the isolated ^{12}C and ^{13}C lignins was highly similar. Furthermore, all lignin-related pyrolysis products found for the isolates were also present in "native" lignin in total biomass, although their relative distribution differed (Table 4). Note that in Table 4 the data were not corrected for relative response factors (see text below) to allow comparison with literature. Most profound were the lower relative amounts of unsubstituted and vinyl substituted compounds in the isolates and higher amounts of methyl substituted and oxygen containing products, which is in line with findings by Del Río et al.[29]

The lower amount of vinyl substituted products in the isolates can be explained by the fact that a significant part of the detected vinyl compounds arise from structures that cross-link lignin to arabinoxylan, like ferulic acid, that could remain in the residue after dioxane extraction.[37] Furthermore, changes in the relative distribution of structural features might be the result of modification and/or selective extraction of a specific lignin population.

It should be noted that in py-GC/MS lignin interunit linkages as well as p-coumarate (pCA) and ferulate (FA) lead to

Table 4. Py-GC/MS Relative Abundance of Structural Features within ^{12}C/[13]C Wheat Straw (WS) and ^{12}C/[13]C Lignin Isolates (LIGpure) on the Basis of Molar Peak Area[a]

	^{12}C-WS	^{13}C-WS	^{12}C-LIGpure	^{13}C-LIGpure
H	36.3 ± 2.0	41.1 ± 1.7	22.4 ± 2.1	25.5 ± 3.3
G	47.9 ± 2.1	45.1 ± 1.5	52.4 ± 1.5	49.7 ± 3.6
S	15.7 ± 0.7	13.9 ± 0.4	25.2 ± 0.7	24.8 ± 0.9
S/G	0.3	0.3	0.5	0.5
unsub.	21.6 ± 1.6	18.5 ± 0.5	13.9 ± 0.5	13.5 ± 0.6
methyl	4.1 ± 0.2	3.8 ± 0.2	13.3 ± 1.0	11.9 ± 0.4
ethyl	2.0 ± 0.2	1.6 ± 0.1	2.7 ± 0.2	2.5 ± 0.3
vinyl	49.1 ± 2.4	52.4 ± 2.0	28.1 ± 2.1	30.2 ± 3.4
C_α-O[b]	4.4 ± 0.2	5.0 ± 0.5	9.5 ± 0.2	10.4 ± 0.3
C_β-O[c]	2.9 ± 0.3	2.2 ± 0.1	4.6 ± 0.1	4.7 ± 0.3
C_γ-O[d]	9.9 ± 0.5	10.9 ± 0.3	20.3 ± 1.2	19.8 ± 3.6
misc.[e]	6.0 ± 0.2	5.5 ± 0.4	8.9 ± 0.4	8.0 ± 0.3

[a]Structural classification is shown in Table 1. Average and standard deviation of triplicates. Not corrected for relative response factors. [b]C_α-oxygen. [c]C_β-oxygen. [d]C_γ-oxygen. [e]miscellaneous.

the formation of the same pyrolysis products, namely 4-vinylphenol and 4-vinylguaiacol, respectively, and can therefore not be independently quantified.[38,39] Interestingly, our findings showed that when relative response factors for pyrolysis products, discussed in the next section, were taken into account, the relative distribution of aromatic units by py-GC/MS (H:G:S = 15:58:26) and NMR (including pCA and FA) (H:G:S = 13:56:31) were in good agreement. This showed that the commonly found discrepancy between py-GC/MS and NMR partially originates from the analytical approach, and heavily depends on the used definition of lignin.[37]

Lignin quantification in a reconstituted biomass model system. After establishing a complete pyrolysis product library for ^{12}C and ^{13}C products, as explained in the previous section, a py-GC-SIM-MS method was set up for quantification purposes. By applying ^{13}C lignin as internal standard, the necessity of monitoring the formation of nonmonomeric pyrolysis products from lignin, such as CO_2 or char, is bypassed. Furthermore, matrix effects during pyrolysis and system performance are corrected for properly via the use of polymeric ^{13}C lignin as internal standard. In our opinion, this can be seen as huge improvement compared to previous lignin quantification attempts by py-GC/MS.[16−20,22]

Selected ion monitoring (SIM) was used for detection as this MS-mode benefits from a significant reduction of background noise and therefore lowers the limit of quantification. Previously it was shown that the lignin-derived pyrolysis products of various grasses, soft- and hardwoods are similar, although their distribution is different.[17,20,29,32] Monitoring all identified lignin-derived pyrolysis products (43, Table 1) thereto ensured unbiased and complete quantification, also when the method is ultimately applied on samples with different product ratios.

Quantification with ^{13}C lignin as internal standard (IS) requires the simultaneous measurement of ^{12}C-sample derived and ^{13}C-IS derived products. Therefore, the number of quantified products is equal to 86. Pyrograms were thereto divided in segments to ensure sufficient data points per peak.

To prove the principle of ^{13}C lignin internal standard based quantification via py-GC/MS, the developed method was applied on ^{12}C lignin with close to identical structure (Tables 3 and 4) in reconstituted biomass systems, with known lignin

content. The pyrolysis products derived from cellulose in these mixtures were similar as previously reported, of which levoglucosan was the most abundant, and did not interfere with lignin analysis.[13,23] In the latter setup similar relative abundance of lignin-derived pyrolysis products (molar based) in the used ^{12}C lignin and ^{13}C lignin internal standard circumvented the need for response factor corrections and allowed the use of total peak area for quantification directly. However, as a consequence of weight-based quantification, a correction factor was required to compensate for the molecular weight differences between ^{12}C and ^{13}C pyrolysis products. This correction factor was calculated from the difference in ^{12}C and ^{13}C molecular weight based total molar peak area calculated for the same IS sample. As a result, it was found that ^{13}C total areas should be multiplied with a correction factor of 1.057. Resulting correlations for ^{12}C lignin input and ^{13}C lignin IS-based quantified ^{12}C lignin amount are shown in Figure 2. It can be observed from the slope of the equation
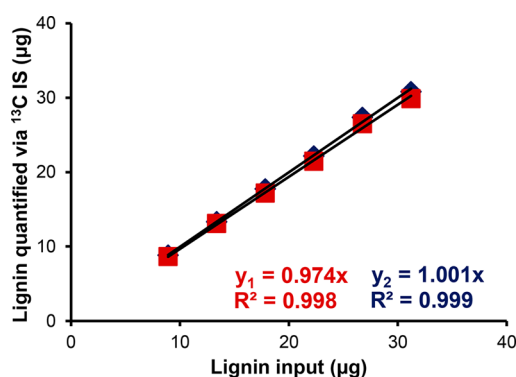
Figure 2. Lignin input and quantified ^{13}C-IS based (total area SIM-MS) output in a reconstituted biomass model system. Square, y_1: uncorrected, Diamond, y_2: corrected for RRF. Average and standard deviation on the basis of triplicates (RSD < 1.5%).

(0.974) and excellent linearity ($R^2 > 0.998$) that ^{12}C lignin can be correctly quantified on the basis of the ^{13}C lignin internal standard in the applied lignin content range. The high reproducibility (RSD < 1.5%) was achieved by solubilizing the lignin isolates in 50:50 (v/v) EtOH:CHCl$_3$ and pipetting specified volumes, opposed to the use of a microbalance. Besides improving reproducibility, this approach facilitated high-throughput sample preparation.

To allow the ultimate use of the internal standard for the quantification of lignins with dissimilar relative distribution of pyrolysis products as well, relative molar response factors (RRFs) were determined and applied. RRFs are of utmost importance since they include compound stability during pyrolysis at 500 °C, transfer efficiency through injector and column and sensitivity of the detector as a result of ionization efficiency and ion coverage by the monitored fragments in SIM. An overview of the relative response factors of all lignin-derived pyrolysis products can be found in Table 1. The relative response factors that were obtained were different from RRFs calculated from calibration curves published by Groenewold et al.[21] Underlying might be different ion coverages as a result of a different number of fragments that were followed per compound. Furthermore, the lability of compounds during pyrolysis at 500 °C was taken into account in our approach, while Groenewold et al. determined RRFs directly with MS without pyrolysis.[21] By applying the RRFs, correcting for small

differences in the relative composition of the pyrolysis products between the ^{12}C and ^{13}C isolates (Table 4), the obtained slope was similar to the theoretical slope of 1 (1.001) and resulted in excellent accuracy (>99.9%) across the measured lignin content range (Figure 2). Surprisingly, highly similar outcomes were obtained when RRFs of Groenewold et al. were applied (data not shown).[21]

Considerations regarding the selectivity of ^{13}C-IS lignin quantification. In grasses, relatively high amounts of *p*-coumaric acid and ferulic acid are present, that form similar products as "core lignin" upon pyrolysis, and can thus not be distinguished.[13] Both coumarylation and feruloylation of arabinoxylan and lignin have been described in the literature and potentially could be a source of error when quantifying lignin on the basis of the formed pyrolysis products. Nevertheless, the major part of the hydroxycinammic acids found in grasses is thought to be attached to lignin and is considered an integral part of it, rather than being present as "free decorations" of arabinoxylan.[37,40] Hence, all vinylic pyrolysis products are assumed to be fully lignin-derived.

Traces of selected masses for ^{12}C pyrolysis products at given retention times were detected in the ^{13}C lignin isolate, and vice versa, but did not form a significant interference (<1% of total peak area) and were therefore neglected. Further, the pyrolysis products phenol, 2-methylphenol, and 4-methylphenol can originate from both lignin and nonlignin components of the cell wall (like aromatic amino acids in protein) (data not shown). These products were fully included in our lignin quantification as they comprised only a minor part (<1% of total peak area) of lignin-derived products. Nevertheless, to avoid significant interference in samples that are high in protein and low in lignin, it is suggested to monitor the formation of indole as protein marker pyrolysis product (R_t 17.81 min, *m/z* 117) and correct detected areas for phenol, 2-methylphenol, and 4-methylphenol accordingly.[41]

Extensive changes in the relative distribution of pyrolysis products over the applied full range (10 to 35 μg) were not observed, indicating that besides content, valuable information about the lignin structure can be obtained concurrently. To further explore the potential of the novel quantitative method, ^{13}C IS based lignin quantification was applied on four different common poaceous biomass sources and compared to the classical Klason method. Compositional data of these four grasses is presented in Supporting Information Tables S-1 and S-2.

Lignin quantification in poaceous biomass. In Figure 3, a comparison is shown between lignin content as quantified by the classical Klason lignin method and the novel ^{13}C-IS based py-GC-SIM-MS method. The results of Klason lignin analysis are presented as acid-insoluble lignin (AIL) corrected for ash and protein and acid-soluble lignin (ASL). AIL determined by this method is often not corrected or contaminated with chitin from fungal origin that cannot be corrected for.[15,17,20] The importance of ash correction was clearly demonstrated as ash contents of hydrolysis residues (AI ash) up to 25% (w/w) were found. Even though the amount of acid-insoluble protein was rather limited (~4% of hydrolysis residue), it might be important to be taken into account for more protein-rich samples. The spectrophotometric analysis of ASL depends highly on the used wavelength and corresponding extinction coefficients. As a result of different lignin compositions per biomass, extinction coefficients vary per biomass. Since the determination of this extinction coefficient is laborious, all types
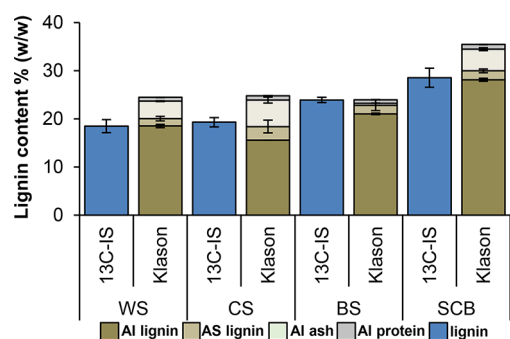
Figure 3. Lignin content determination via ^{13}C-IS based py-GC-SIM-MS (triplicate) and Klason (duplicate). WS: wheat straw, CS: corn stover, BS: barley straw, SCB: sugar cane bagasse, AI: acid-insoluble, AS: acid-soluble.

of biomass were analyzed at the same wavelength (205 nm) with an averaged extinction coefficient (110 g·L^{-1}·cm^{-1}), based on the literature.[7,42,43] For that reason the ASL-content based on the common Klason protocol does not result in an accurate lignin content. Lignin contents determined according to Klason (AIL and ASL) agreed well with previous studies on these biomasses and were therefore considered valid for the evaluation of the performance of the novel method.[29,44,45]

The lignin contents of the four biomasses quantified via the ^{13}C-IS py-GC/MS approach were in good accordance with the classical Klason method (AIL + ASL) with an insignificant relative deviation on the order of 5%. High reproducibility was obtained for the lignin content of all biomasses (RSD < 7%). Compared to previous py-GC/MS attempts on lignin quantification, our novel method thus showed greatly enhanced accuracy and reproducibility, with acceptable costs and time-investments.[16−20,22] The latter is concluded from the fact that from 1 g of ^{13}C labeled wheat straw 45 mg of 90% pure ^{13}C lignin internal standard can be isolated in 1 week, which is sufficient to perform 45 000 pyrolysis runs. Compared to external ^{12}C lignin based quantification, significant enhancements in terms of accuracy were achieved by applying ^{13}C lignin as internal standard (Supporting Information Figure S-5), indicating the importance of matrix-effects during pyrolysis.

The use of extractive-free biomasses ensured compounds potentially interfering with lignin-analysis by py-GC/MS analysis to be removed. Furthermore, the presence of ash and/or chitin (in fungal treated biomass) does not interfere with py-GC/MS analysis, as potential matrix-effects are corrected for by the internal standard approach and all chitin-derived pyrolysis products can be distinguished from lignin-derived products. Thereto, the sum of the lignin derived pyrolysis products was a reliable representation of lignin content and lignin content could thus be similarly determined as shown for reconstituted biomass samples. Due to the fact that the internal standard was added as a liquid and subsequently dried, the internal standard was well dispersed throughout the sample. The use of a microfurnace pyrolyzer combined with low standardized particle size (<250 μm) and standardized low sample amount (∼75 μg) furthermore ensured rapid pyrolysis with heating rates expected to be >2000 °C·s^{-1}.[23,24] The mechanisms underlying pyrolysis were thereto expected not to differ between sample lignin and internal standard lignin.

The application of relative response factors (Table 1) enabled us to accurately determine the contents of lignins that were structurally distinct and dissimilar from the structure of the internal standard as well (Table 5 and Figure S-5). This flexibility is a clear benefit compared to existing alternatives for the quantification of lignin content in plant biomass, that depend on structurally similar calibration standards.[8−11] Future analysis of a larger variety of biomasses, including softwoods, hardwoods, and chemically and/or enzymatically modified ones, will further improve understanding of the performance of our ^{13}C-IS based novel method for lignin quantification.

As previously discussed in the section "Characterization of lignin isolates", the relative composition of the lignin subunits as found by py-GC/MS when RRFs were applied was comparable to 2D-NMR. Besides content, valuable information on the subunit composition of the biomasses could thus be obtained concurrently. A more accurate view on the composition of the pyrolysates was obtained and demonstrated the importance of oxygen-containing pyrolysis products (C$_\alpha$-O, C$_\beta$-O, and C$_\gamma$-O) for describing lignins structural features (Table 5).

Table 5. Py-GC-SIM-MS Relative Abundance of Structural Features within Biomasses and ^{13}C Internal Standard (^{13}C -IS) on the Basis of RRF Corrected Molar Peak Area[a]

	WS	CS	BS	SCB	^{13}C-IS[b]
Lignin % (w/w)[c]	18.5 ± 1.3	19.3 ± 1.0	23.9 ± 0.6	28.5 ± 2.0	–
H	16.3 ± 0.8	42.8 ± 0.8	15.4 ± 0.7	49.0 ± 4.2	17.1 ± 5.0
G	66.2 ± 1.4	47.7 ± 1.1	59.9 ± 1.4	35.8 ± 1.8	60.2 ± 3.9
S	17.5 ± 0.4	9.5 ± 0.2	24.7 ± 0.7	15.3 ± 0.7	22.7 ± 1.2
S/G	0.3	0.2	0.4	0.4	0.4
unsub.	6.2 ± 0.2	5.1 ± 0.1	5.4 ± 0.05	6.8 ± 0.4	4.5 ± 0.3
methyl	2.8 ± 0.1	3.0 ± 0.1	2.5 ± 0.1	4.6 ± 0.5	2.1 ± 0.5
ethyl	0.3 ± 0.01	0.3 ± 0.1	0.2 ± 0.01	0.4 ± 0.05	0.2 ± 0.04
vinyl	40.8 ± 0.9	68.3 ± 1.3	34.8 ± 1.3	59.5 ± 4.2	25.3 ± 5.1
C$_\alpha$-O[d]	3.1 ± 0.1	4.0 ± 0.1	3.5 ± 0.06	4.1 ± 0.2	4.3 ± 0.3
C$_\beta$-O[e]	1.2 ± 0.03	0.8 ± 0.05	1.4 ± 0.05	1.5 ± 0.1	1.4 ± 0.2
C$_\gamma$-O[f]	42.9 ± 1.4	15.9 ± 0.3	49.4 ± 1.2	17.7 ± 1.7	58.8 ± 3.0
misc.[g]	2.7 ± 0.04	2.6 ± 0.08	2.7 ± 0.06	5.1 ± 0.2	3.3 ± 0.4

[a]Structural classification is shown in Table 1. Average and standard deviation of triplicates. WS: wheat straw, CS: corn stover, BS: barley straw, SCB: sugarcane bagasse. [b]Average and standard deviation of ^{13}C-IS added to all biomasses in triplicate. [c]Determined by ^{13}C lignin IS based py-GC-SIM-MS. [d]C$_\alpha$-oxygen. [e]C$_\beta$-oxygen. [f]C$_\gamma$-oxygen. [g]Miscellaneous.

CONCLUSIONS

Here, we describe a novel method for the concurrent quantification of absolute lignin content and subunit composition in plant biomass. By employing a ^{13}C-labeled polymeric lignin isolate as internal standard (IS) for the first time and correcting for relative response factors of the formed lignin-derived pyrolysis products, py-GC/MS could be applied for this purpose. Our ^{13}C lignin IS based approach was validated in biomass model systems containing a structurally similar ^{12}C lignin analogue with known lignin contents. Structurally distinct lignins in four common poaceous biomass sources were quantified with an insignificant relative deviation in the order of 5% compared to classic gravimetric analysis. The simultaneous acquirement of structural information with high reproducibility in a more high-throughput fashion makes our method a huge improvement compared to current alternative methods for lignin quantification. We reckon that our new lignin quantification method opens up possibilities for more accurate analysis of lignin content and subunit composition in various research fields dealing with lignin containing plant biomass.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.7b02632.

Detailed experimental procedures for preparation of nonlabeled and ^{13}C-labeled wheat straw, compositional analysis of total biomass and lignin isolates, HSQC-NMR spectra with annotated structures, py-GC/MS pyrograms of ^{12}C-LIGpure and ^{13}C-LIGpure, ^{13}C lignin EI-MS library, comparison of ^{12}C external standard and ^{13}C internal standard based lignin quantification via py-GC-SIM-MS, impact of RRF application on ^{13}C-IS lignin quantification in poaceous biomasses, composition of poaceous biomasses (PDF)

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Author Contributions

GvE, HG, and MAK designed research; GE performed research and analyzed data; RdV prepared the ^{12}C and ^{13}C wheat straw; WS performed compositional analysis of biomasses; DM performed NMR analyses; PdG provided technical support for py-GC/MS; GE and MAK wrote the paper. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Buranov, A. U.; Mazza, G. Ind. Crops Prod. 2008, 28, 237−259.
(2) Vanholme, R.; Demedts, B.; Morreel, K.; Ralph, J.; Boerjan, W. Plant Physiol. 2010, 153, 895−905.
(3) Englyst, H. N.; Cummings, J. H. Analyst 1984, 109, 937−942.
(4) Ruiz, R.; Date, T. E. Laboratory Analytical Procedure No. 002; National Renewable Research Laboratory, 1996.
(5) Lupoi, J. S.; Singh, S.; Parthasarathi, R.; Simmons, B. A.; Henry, R. J. Renewable Sustainable Energy Rev. 2015, 49, 871−906.
(6) Hatfield, R.; Fukushima, R. S. Crop Sci. 2005, 45, 832−839.
(7) Sluiter, J. B.; Ruiz, R. O.; Scarlata, C. J.; Sluiter, A. D.; Templeton, D. W. J. Agric. Food Chem. 2010, 58, 9043−9053.
(8) Rodrigues, J.; Faix, O.; Pereira, H. Holzforschung 1998, 52, 46−50.
(9) Wolfrum, E. J.; Sluiter, A. D. Cellulose 2009, 16, 567−576.
(10) Jiang, N.; Pu, Y.; Ragauskas, A. J. ChemSusChem 2010, 3, 1285−1289.
(11) Fu, L.; McCallum, S. A.; Miao, J.; Hart, C.; Tudryn, G. J.; Zhang, F.; Linhardt, R. J. Fuel 2015, 141, 39−45.
(12) Gao, X.; Laskar, D. D.; Zeng, J.; Helms, G. L.; Chen, S. ACS Sustainable Chem. Eng. 2015, 3, 153−162.
(13) Ralph, J.; Hatfield, R. D. J. Agric. Food Chem. 1991, 39, 1426−1437.
(14) Murciano Martínez, P.; Punt, A. M.; Kabel, M. A.; Gruppen, H. Bioresour. Technol. 2016, 216, 44−51.
(15) Jurak, E.; Punt, A. M.; Arts, W.; Kabel, M. A.; Gruppen, H. PLoS One 2015, 10, e0138909.
(16) Bocchini, P.; Galletti, G.; Camarero, S.; Martinez, A. J. Chromatogr. A 1997, 773, 227−232.
(17) Alves, A.; Schwanninger, M.; Pereira, H.; Rodrigues, J. J. Anal. Appl. Pyrolysis 2006, 76, 209−213.
(18) Alves, A.; Rodrigues, J.; Wimmer, R.; Schwanninger, M. J. Anal. Appl. Pyrolysis 2008, 81, 167−172.
(19) Ross, K.; Mazza, G. World Journal of Agricultural Sciences 2011, 7, 763−776.
(20) Lourenço, A.; Gominho, J.; Marques, A. V.; Pereira, H. BioResources 2013, 8, 2967−2980.
(21) Groenewold, G. S.; Johnson, K. M.; Fox, S. C.; Rae, C.; Zarzana, C. A.; Kersten, B. R.; Rowe, S. M.; Westover, T. L.; Gresham, G. L.; Emerson, R. M. Energy Fuels 2017, 31, 1620.
(22) Torri, C.; Adamiano, A.; Fabbri, D.; Lindfors, C.; Monti, A.; Oasmaa, A. J. Anal. Appl. Pyrolysis 2010, 88, 175−180.
(23) Patwardhan, P. R.; Satrio, J. A.; Brown, R. C.; Shanks, B. H. Bioresour. Technol. 2010, 101, 4646−4655.
(24) Patwardhan, P. R.; Brown, R. C.; Shanks, B. H. ChemSusChem 2011, 4, 1629−1636.
(25) Gorissen, A.; Kraut, N. U.; de Visser, R.; de Vries, M.; Roelofsen, H.; Vonk, R. J. Food Chem. 2011, 127, 192−196.
(26) Björkman, A. Sven. Papperstidn. 1956, 59, 477−485.
(27) Kabel, M. A.; Van der Maarel, M. J.; Klip, G.; Voragen, A. G.; Schols, H. A. Biotechnol. Bioeng. 2006, 93, 56−63.
(28) Jones, D. B. US Agric. Circ 1931, 1−16.
(29) Del Río, J. C.; Rencoret, J.; Prinsen, P.; Martínez, A. n. T.; Ralph, J.; Gutiérrez, A. J. Agric. Food Chem. 2012, 60, 5922−5935.
(30) Rencoret, J.; Marques, G.; Gutiérrez, A.; Nieto, L.; Santos, J. I.; Jiménez-Barbero, J.; Martínez, Á. T.; del Río, J. C. Holzforschung 2009, 63, 691−698.
(31) Zeng, J.; Helms, G. L.; Gao, X.; Chen, S. J. Agric. Food Chem. 2013, 61, 10848−10857.
(32) Del Río, J. C.; Gutiérrez, A.; Rodríguez, I. M.; Ibarra, D.; Martínez, Á. T. J. Anal. Appl. Pyrolysis 2007, 79, 39−46.
(33) Zikeli, F.; Ters, T.; Fackler, K.; Srebotnik, E.; Li, J. Ind. Crops Prod. 2014, 61, 249−257.
(34) Chang, H.-m.; Cowling, E. B.; Brown, W. Holzforschung 1975, 29, 153−159.
(35) Rencoret, J.; Prinsen, P.; Gutiérrez, A.; Martínez, A. n. T.; del Río, J. C. J. Agric. Food Chem. 2015, 63, 603−613.
(36) Zeng, J.; Singh, D.; Gao, D.; Chen, S. Biotechnol. Biofuels 2014, 7, 161.
(37) Ralph, J. Phytochem. Rev. 2010, 9, 65−83.
(38) Akazawa, M.; Kojima, Y.; Kato, Y. J. Anal. Appl. Pyrolysis 2016, 118, 164−174.
(39) Akazawa, M.; Kato, Y.; Kojima, Y. J. Anal. Appl. Pyrolysis 2016, 122, 355−364.
(40) Koshijima, T.; Watanabe, T. Association between lignin and carbohydrates in wood and other plant tissues; Springer-Verlag: Berlin, 2003.
(41) Biller, P.; Ross, A. Algal Res. 2014, 6, 91−97.

(42) Nicholson, D.; Aaron, T.; Francis, R. C. *Cellul. Chem. Technol.* **2013**, *48*, 53−59.

(43) Dence, C. W. In *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer Berlin Heidelberg: Berlin, Heidelberg, 1992; pp 33−61.

(44) Murciano Martínez, P.; Bakker, R.; Harmsen, P.; Gruppen, H.; Kabel, M. *Ind. Crops Prod.* **2015**, *64*, 88−96.

(45) Kim, T. H.; Kim, J. S.; Sunwoo, C.; Lee, Y. *Bioresour. Technol.* **2003**, *90*, 39−47.