Profiling of diferulates (plant cell wall cross-linkers) using ultrahigh-performance liquid chromatography-tandem mass spectrometry

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Recalcitrance of grasses to enzymatic digestion arises to a significant degree from a complex array of phenolic crosslinks between cell wall glycopolymer chains that inhibit their conversion to biofuels and lower their nutritive value for animal feed applications. Polysaccharide esters of ferulic acid are abundant in plant cell walls. Crosslinks between polysaccharides are formed through oxidative dehydrodimerization of ferulates, producing dehydrodiferulates (henceforth termed diferulates). Such ferulates and diferulates further crosslink plant cell walls by radical coupling cross-reactions during lignification. Although cell wall digestibility can be improved by cell wall metabolic engineering, or post-harvest by various pretreatment processes, a more comprehensive understanding of the role and impact of ferulate crosslinking on polysaccharide hydrolysis would be accelerated by availability of analytical methods that can distinguish the various diferulates released during biomass pretreatments, many of which are isomers. In this report, we present an ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) strategy for comprehensive separation and identification of diferulate isomers. Collision-induced dissociation (CID) mass spectra of [M+H]+ ions distinguished various isomers without requiring derivatization. Characteristic product ions for 8-0-4-, 8-8-non-cyclic, 8-8-cyclic, 8-5-cyclic, 8-5-non-cyclic, and 5-5-linked isomers were identified. All diferulates were identified either as di-acids in extracts of NaOH-hydrolyzed corn stover, or as a diverse group of diferulate mono- and di-amides in extracts of Ammonia Fiber Expansion (AFEX™)-treated corn stover. This approach allows for direct analysis of released diferulates with minimal sample preparation, and can serve as the foundation for high-throughput profiling and correlating pretreatment conditions with biomass digestibility in biorefineries producing biofuels and biochemicals.

Introduction

Grasses serve society in myriad ways, providing food in the form of grain (e.g., rice, wheat, maize, and oats), and nutrition for livestock as grain and/or silage. Society has turned increasing attention to developing sustainable resources to provide biomass for conversion to renewable liquid fuels. In light of increasing worldwide demand for food, and the escalating costs and environmental impacts of liquid fossil fuels, improvement of grasses for biomass production, pest resistance, and conversion to bioenergy products will rely on better understanding the chemistry of grass cell walls and the genes that regulate this chemistry. The exciting developments in plant engineering, DNA sequencing, and metabolic profiling,1 offer prospects for substantial improvements in the production of sustainable renewable plant resources that provide high-value products. The chemistry of cell walls in grasses governs important functions including the regulation of plant growth, mechanical strength, resistance to pathogens and insects, and cell wall degradation.2 These cell walls consist of about one-third hemicelluloses (total cell wall mass fraction basis) including various (glucuronoro-arabino)xylans, glucans, and xyloglucans, roughly one-half cellulose, with the balance coming from other biopolymers (notably lignin), polyphenol derivatives, ash, and extractives.3 Recalcitrance of cell walls to enzymatic digestion presents the major hurdle to economic development of grass-derived biomass in applications ranging from animal nutrition to biofuels.4 A substantial part of this recalcitrance arises from crosslinking of cell wall arabinoxylans via dehydrodimerization of ferulate esters that produces stable ether and carbon-carbon bonds.5,6 Although this crosslinking presents a substantial obstacle to enzymatic digestion,8,9 thermochemical pretreatments (including AFEX™) cleave these ester linkages, releasing diferulates (or derivatives) and arabinoxylans, allowing for increased sugar conversion.11,12 As diferulates act as cell wall cross-linkers, identification and quantitative profiling of diferulates released by chemical or enzymatic pretreatment provides a revealing and important measure of the role of cell wall crosslinking on wall deconstruction efficiency. This quantitative information serves as a key metric for guiding process optimization. Cross-linked
Fig. 1 A schematic of grass cell wall hemicelluloses, showing crosslinking of arabinoxylans by diferulates. This is a schematic model showing key features of grass cell walls—there are many more arabinosyl (Ara) units in arabinoxylans without ferulate/diferulate substitution; there are also other substitutions such as with glucuronate units not shown. Note that Ara substitution has been shown at the 3-position of xylosyl (Xyl) residues (where it is most frequently found)\textsuperscript{13}, and that ferulate is invariably on the primary (C5) OH of Ara units. The model shows cross-linking of the arabinoxylan chains by 5–5-, 8–5-, 8–O–4-, and two forms of 8–8-diferulates. Acetyl substitution on the 2-, 3-, and 2,3-positions of xylan units is also shown. Finally, note that Ara units branching off the xylan chain may themselves have xylosyl substitution (usually/invariably at C2)—hence the R = H, Xyl designation here.\textsuperscript{14–18} arabinoxylans are a major polysaccharide constituent of grass hemicelluloses,\textsuperscript{19,20} as illustrated in Fig. 1. Their structure is based on β-D-(1,4)-linked xylose monomers that are substituted with arabinosyl groups as side-chains on every 2-3 xylose units.\textsuperscript{13} The side-chain arabinosyl C-5 hydroxyl groups are often acylated by the hydroxycinnamates ferulate and $p$-coumarate.\textsuperscript{13, 21} Feruloylated oligosaccharides have been isolated and identified from cell walls of maize,\textsuperscript{22,23} barley straw,\textsuperscript{24} sugar cane bagasse,\textsuperscript{25} and wheat bran.\textsuperscript{26} Oxidative dehydrodimerization (simply termed dimerization from here on) of arabinoxylan ferulate esters forms covalent crosslinks between arabinoxylan chains, unambiguously documented with the isolation of a diferulate from bamboo shoots in which each ferulate moiety acylated an arabinoxylan fragment.\textsuperscript{27} A more recent investigation detected pentose-diferulate linkages in corn fibers.\textsuperscript{28} Diferulate ester crosslinks are distributed among diverse chemical forms because they are formed by free-radical coupling at the O–4-, 5-, and 8-positions to form dimeric structures annotated as 5–5-, 8–5-, and 8–8– (formed through C–C bonds) and 8–O–4– and 4–O–5– (formed through ether bonds) coupled diferulates as a total of 7 topological isomers plus additional chemical forms.\textsuperscript{3, 29, 30} It has been suggested that different isomers of diferulate crosslinks may have distinct physiological functions,\textsuperscript{3} but control over chemical radical coupling reactions makes this an unlikely plant strategy.\textsuperscript{5} Regardless, the scarcity of accurate quantitative information about abundances of specific diferulate links in various cells and tissues presents limits on our fundamental understanding of cell wall structure and its deconstruction by biomass pretreatments. Recognition and quantitation of diferulate isomers released from cell walls has been non-routine because of limited availability of authentic standards,\textsuperscript{31} even though syntheses have been reported.\textsuperscript{6, 32} To date, high-performance liquid chromatography with ultraviolet (HPLC-UV) spectrophotometric detection and gas chromatography-mass spectrometry (GC-MS) have been the main analytical tools for identification and quantification of diferulate isomers from hydrolyzed cell walls.\textsuperscript{31, 32} HPLC with UV detection lacks selectivity, as phenolic compounds may co-elute. GC/MS provides an alternative approach, but requires derivatization to confer sufficient volatility needed for GC separations. The requirement that solvents be removed before derivatization may result in irreproducible and/or poor derivatization yields, and serves as a barrier to high-throughput sample analyses needed for process optimization.
Fig. 2 Structures of anticipated diacid (Di-Ac, black), acid-amide (Ac-Am, blue), and diamide (Di-Am, red) products of ammonolysis and hydrolysis of 8-O-4, 8-8NC, 8-C, 8-S, and 4-O-5-isomers of plant cell wall diferulates during ammonia-based biomass pretreatment using AFEX™ process. Note that only a single 8-5-diferulate (the cyclic phenylcoumaran structure) is found in the wall, but that various noncyclic isomers arise following hydrolysis or ammonolysis.

In addition, the 70 eV electron ionization (EI) mass spectra of diferulate trimethylsilyl derivatives are characterized by molecular ions that are frequently of low abundance, and these spectra present few abundant fragment ions that distinguish isomers. Avoidance of derivatization provides important practical advantages for diferulate analyses, and is compatible...
with LC/MS/MS. However, collision-induced dissociation MS² spectra have yet to be reported for the entire suite of diferulate isomers. Tandem mass spectrometric methods have found success in distinguishing a variety of polyphenolic metabolites, with one of the more prominent reports by Clifford and co-workers who reported LC-MS⁸ identification of chlorogenic acid analogs including isomers including caffeoylquinic, p-coumaroylquinic and feruloylquinic acids.³⁵,³⁶ In a similar report, Kulher et al. reported the use of tandem mass spectrometry for distinguishing feruloyl and isoferruloyl quinic acids. In another recent related paper, Morreel and co-workers published negative-mode collision-induced dissociation (CID) spectra of dilignols, a class of compounds with close similarity to diferulates, but lacking carboxylic acid groups.³⁸,³⁹ Although the behaviors of some linkage types among monolignols were investigated, the chemical structures of dilignols are sufficiently different from diferulates that information in the monolignol CID spectra cannot be extrapolated to distinguish diferulate isomers. Four diferulates were also detected in extracts of alkali-treated maize grain using LC/atmospheric pressure chemical ionization MS in positive-ion mode. This approach distinguished the four isomers largely based on abundances of in-source fragments arising from losses of 1-2 water molecules, but did not present MS/MS spectra.⁴⁰ If profiling of the multitude of isomers from biomass hydrolysates was not already sufficiently challenging, pretreatments such as ammonia fiber expansion (AFEXTM),⁴¹,⁴² which can cleave diferulate bridges through combinations of hydrolysis and ammonolysis,⁴³ (Fig. 2) make the task yet more formidable. In ammonia-based biomass treatments, the two diferulate ester groups can be converted to either their amide or carboxylic acid forms, and this multiplies the complexity of released product isomers. In the current report, we have assembled a library of tandem mass spectra of protonated diferulic acid (Di-Ac), diamide (Di-Am), and bifunctional acid-amide (Ac-Am) dimers, with the intent that the MS² spectra will be helpful for distinguishing released dimers when authentic standards are not available for comparisons. When isomer-specific fragment ions are formed, these can provide the foundation for rapid LC/multiple reaction monitoring profiling of released diferulates.

In addition, this report also describes an ultrahigh-performance liquid chromatography (UHPLC) tandem mass spectrometry approach for separation of modified and unmodified diferulates that does not require chemical derivatization. Extracts from corn stover treated with aqueous NaOH or the AFEXTM process were characterized to demonstrate the application of this methodology.

Experimental
AFEX⁰™ pretreatment

Corn stover (biomass) was pretreated in a stainless steel high pressure reactor (Parr Instrument Company, Moline, IL) with liquid ammonia at 130 °C, 0.6:1 water to biomass loading and 1:1 NH₃ to biomass loading (w/w) for 15 min total residence time. Detailed protocols can be found in our previous publication.¹²

NaOH pretreatment of corn stover

One gram of untreated corn stover was pretreated with 2 mL of 2 M NaOH at 25 °C for 20 h as previously suggested.³⁴ Afterwards, the reaction mixture was brought to pH 7 with formic acid, and aliquots of the mixture were analyzed without further processing and dilution.

Synthesis of diferulic acid ester standards

Diferulate esters (8–O–4, 8–8NC, 8–8C, 8–5NC, 8–5C, and 5–5 isomers) were synthesized from ethyl ferulate via oxidative coupling catalyzed by copper(II)-tetramethylethylenediamine complex in acetonitrile as recently reported.³²

Synthesis of Ac-Am and Di-Am diferulates

To generate various Di-Am and Ac-Am derivatives, 200 μL of 15 M NH₂OH was added to 1 mg of each of the ethyl diferulate esters in 200 μL of dichloromethane solution, and each mixture was stirred at 25 °C for 16 h. Reactions were quenched by addition of 1 mL water and 1 mL dichloromethane, and the organic layer was collected. Aliquots were diluted 100-fold in acetonitrile before UHPLC/MS analysis.

Separation and analysis of synthetic diferulates and pretreatment byproducts using UHPLC/MS and UHPLC/MS/MS

Diferulates were profiled using separation on an Ascentis Express C18 column with either UHPLC/TOF MS and multiplexed collision-induced dissociation on a Waters LCT Premier or MS/MS on an AB/Scieq QTRAP 3200 mass spectrometer, using N₂ as collision gas in the latter, at 3.3 x 10⁻² Torr. Additional experimental details regarding these analyses and profiling of byproducts of NaOH- and AFEX™-pretreatments of corn stover are provided in Electronic Supplemental Information (ESI).

Results and Discussion

Complexity of treated biomass extract

UHPLC/TOF MS profiling of constituents in an aqueous extract of AFEX™-treated corn stover reveals a rich mixture composed largely of cell wall-derived aromatic substances (Fig. 3a). Automated peak extraction and integration yielded more than 2000 features after deisotoping, including numerous phenolic compounds,¹² as expected from a plant extract. In anticipation of observing diferulate derivatives, extracted-ion chromatograms were generated for ions corresponding to protonated Di-Ac, Ac-Am and Di-Am diferulates, yielding more than 30 chromatographic peaks with these nominal masses. Of these, 15 compounds gave masses within 5 ppm of theoretical values for diferulate, Di-Ac, Ac-Am or Di-Am forms of diferulates (SI-Table S3).

Diferulate isomers can be distinguished from their CID MS² spectra

Discrimination of metabolites based on their CID mass spectra remains one of the great challenges for metabolite discovery efforts that rely on tandem mass spectrometry. Unlike the case for the dilignols which often exist as heterodimers,³⁵ diferulates crosslinks in plant cell walls exist as homodimers but in multiple isomeric forms. To determine whether isomeric diferulates can be distinguished by their CID-generated fragment ions, MS² spectra of product ions derived from [M+H]+ of synthetic diferulic acid isomers were generated using collision energy of 20 eV (Fig. 3b-3g) on the QTRAP mass spectrometer. MS² spectra of the
diferulate synthetic standards in negative-ion mode (on [M-H]+) are also provided in SI-Fig. S6, but this discussion will focus on positive ion behavior. MS² spectra differentiated all isomers in both positive- and negative-ion modes, often displaying unique fragments from specific isomers. In other cases, relative abundances of fragment ions distinguished isomers. These observations set the basis for isomer differentiation using CID spectra. Although phenolic compounds ionize more efficiently in electrospray negative-ion mode owing to their acidity, fragmentation in positive-ion mode yielded more extensive cleavage of bonds that aids structure elucidation.

**Fragment ions derived from side-chain neutral losses**

Ferulic acid and other 4-hydroxycinnamic acids undergo rapid decarboxylation upon heating to 200 °C, and such losses of portions of the groups attached to the aromatic rings were anticipated during CID. For the purpose of further discussion, side-chains on diferulates are defined as all substituents attached to rings, such as carboxylic acid groups, methoxyl groups, and oxidized propyl moieties. Positive-mode CID mass spectra of all diferulic acid (Di-Ac) isomers exhibited fragment ions (Fig. 3 and SI-Table S1) corresponding to losses of 1 or 2 neutral water molecules (m/z 369 and 351), as anticipated following protonation at the OH portion of the carboxylic acid groups. Most isomers underwent subsequent losses of CO that are presumed to be charge-directed losses from the acylium ions that result from prior losses of neutral H₂O. However, the 8–O–4-isomer stands out as an exception, exhibiting negligible amounts of such fragment ions. In contrast, an even-mass fragment (m/z 326) is abundant in this region of the CID spectrum, corresponding to neutral losses of CO and a CH₃ radical from [M+H-H₂O]+. This was the only isomer to yield significant amounts of radical fragment ions. Significant abundance of the fragment corresponding to neutral loss of CO₂ (-44 Da; m/z 343) from [M+H]+ was only observed for the 8–5C isomer, but combined losses of CO₂ plus other side-chain-derived neutrals were common for all isomers except the 5–5-isomer. CID mass spectra of this phenyl-phenyl linked isomer yielded few fragments beyond those corresponding to losses of H₂O, but showed fragments corresponding to neutral losses of two H₂O and both one and two molecules of CH₃OH (m/z 319 and 287). Although losses of CH₃OH from aromatic methoxyl groups are unusual in 70 eV EI mass spectra, they have been observed in positive-ion mode CID spectra of specific isomers of methyl ethers of the flavonoid myricetin.

**Characteristic fragment ions that distinguish diferulic acid isomers**

Each diferulic acid isomer standard yielded fragment ions upon CID that allowed for isomer discrimination, and these are highlighted with bold italic labels in the CID mass spectra (Fig. 3b-g). In one case, the 8–O–4-isomer, the fragment at m/z 193 corresponds to cleaving the dimer in half, i.e., from cleavage of the ether link between monomeric units and elimination of neutral ferulic acid. In contrast, the 5–5-isomer yielded a characteristic fragment at m/z 319, derived from losses of two water molecules from the carboxylic acid groups plus CH₃OH from the aromatic methoxyl group. This isomer did not yield significant fragments below m/z 250, demonstrating a resistance to dissociation of its core structure. The two 8–8-isomers were distinguished by two prominent fragments. For isomer 8–8NC, the fragment at m/z 245 is attributed to an unusual elimination of one water and methoxyphenol (guaiacol) that can be explained by rearrangement and cleavage of a bond attached to a aromatic ring. In comparison, the 8–8C isomer yielded a dominant fragment at m/z 297 (losses of H₂O, CO, and CO₂), which was less abundant from isomers 8–8NC and 8–5C. The two 8–5-linked dimers also yielded distinguishing fragments, with m/z 293 (loss of H₂O, CO₂, and CH₃OH) and 307 (2H₂O and CO₂) at 40-50% of the base peak in 8–5C, whereas m/z 309 (loss of H₂O, CO, and CH₃OH) was prominent in 8–5NC. Assignments of structural features to fragment ions can be challenging, but supporting evidence can be derived from MS² spectra and from accurate measurements of fragment masses using multiplexed and nonselective CID, which subjects all ions to collisional activation without prior mass filtering. All of the fragment ions described above were detected as m/z values measured by UHPLC/TOF MS consistent with the proposed elemental formulas (± 10 ppm). Further evidence about structures of these fragments came from observations of granddaughter (secondary product) ion masses. A summary of fragment ion annotations is presented in Fig. 4, along with proposed pathways of fragment ion formation.

**Comparison of CID spectra of diferulates with mono and di-amide functionalities**

When cell wall diferulate esters are cleaved by a combination of ammonolysis and hydrolysis, Di-Am or two isomers of each asymmetrical Ac-Am product (except from the 8–8NC-diferulate that has a center of symmetry) may form (Fig. 2). The CID spectra of synthetic Ac-Am and Di-Am standards bear great similarity to the corresponding Di-Ac spectra, differing primarily in loss of NH₃ instead of H₂O, and in relative abundances of analogous fragments. In addition, CID spectra of 8–O–4 Ac-Am (the later-eluting isomer) and Di-Am (Fig. 3h-j) exhibited even-mass fragments at m/z 192 corresponding to cleavage of the central ether with charge retention almost exclusively on the amide portion. The two 8–O–4 Ac-Am isomers, differing in the position of the amide, were readily distinguished from one another by their CID spectra (SI-Fig. S1a and S1b) in that the loss of water (m/z 368) is more prominent in the earlier eluting isomer (isomer 1) as is the m/z 308 fragment. This fragment ion is consistent with losses of two water molecules plus CO and methyl radical from [M+H]+. Furthermore, isomer 2 yields higher relative abundance of fragments at m/z 326 (loss of one water plus CO and CH₂•) but minimal amounts of m/z 308. In other cases, the two Ac-Am isomers were not distinguished by their CID spectra, as illustrated by 8–5C isomers (SI-Fig. S1d and S1e) and other means such as chromatographic retention time are necessary to distinguish the isomers. In the absence of standards of the individual isomers, their structural assignments (Fig. 2) should be regarded as tentative.
Fig. 3 (a) UHPLC/TOF-MS summed extracted ion chromatogram for ions with m/z 383, 384 and 385, the same nominal masses of [M+H]+ ions of the three forms of released diferulates (Ac-Am, Di-Ac or Di-Am) of aqueous extract of AFEX®-treated corn stover using electrospray ionization in positive ion mode.; (b-g) CID MS² spectra of diferulic acid standards and (h-j) CID MS² spectra of Di-Ac, Ac-Am and Di-Am synthetic standards for 8–O–4-diferulate. Precursor ions were [M+H]+ at m/z 387, 386, and 385 for Di-Ac, Ac-Am, and Di-Am, and all CID mass spectra were generated on a QTRAP 3200 mass spectrometer using a collision energy of 20 eV. Isomer-distinguishing fragment ions are highlighted on each spectrum in bold italic font.
Fig. 4 Summary of fragment ion annotation for CID MS² spectra of diferulic acid isomers. Assignments and proposed fragmentation pathways are based on accurate fragment masses measured using UHPLC/TOF MS with non-selective CID and from MS³ spectra generated on a QTRAP mass spectrometer. Each isomer-distinguishing fragment ion is highlighted inside an ellipse.
annotated as the 4–O–5-isomer, the only other anticipated isomer with a long retention time similar to 8–O–4-isomers, this product is suggestive of an ether linkage. Based on this observation and the retention times of the identified peaks, the products of NaOH pretreatment. Released 8–8NC-diferululate is consistent with observation of the corresponding Di-Ac forms in previous publications.

Identification of diferulates released from corn stover cell walls upon NaOH catalyzed hydrolysis

To demonstrate the potential utility of selective fragment ions for characterizing diferulates released from biomass, diferulates derived from pretreatment of corn stover with NaOH were profiled using UHPLC/MS. Released Di-Ac diferulates were identified by generating an extracted-ion chromatogram for [M+H]⁺ (m/z 387, Fig. 5a). At least twelve chromatographic peaks were identified as diferulates based on accurate mass measurements and MS² spectra, which are provided in SI-Fig. S3 for comparison with the MS² spectra of synthetic standards. Di-Ac 8–O–4-, 8–5C-, 8–5NC-, 8–8NC-, 8–8C- and 5–5-isomers were confirmed based on coelution with standards and equivalent MS² spectra. Peaks 1, 7 and 11 gave molecular masses consistent with diferulates and shared some side-chain loss fragments with other diferulates, but lacked unique product ions necessary to distinguish them from other Di-Ac isomers. Peak number 1 remains unauthenticated but it exhibits a fragment at m/z 193 suggestive of an ether linkage. Based on this observation and the long retention time similar to 8–O–4-isomers, this product is annotated as the 4–O–5-isomer, the only other anticipated isomer capable of forming m/z 193 via cleavage of the ether linkage. It has been reported before that the 4–O–5-isomer is not common in grasses and cereal fibers, and its low relative abundance is consistent with these earlier findings. The possibility of a configurational isomer (e.g., with one or both of the double bonds as cis geometrical isomers) of 8–O–4-diferulate is discounted because this compound was not formed upon photoirradiation of 8–O–4-diferulate, whereas Peak 2 increased during these experiments. Additional peaks are annotated as diferulates but did not match retention times with any standards. It is expected that some of the observed Di-Ac products are cis-isomers. For example, peak numbers 5 and 8 are both assigned as 8–5NC because of indistinguishable MS² spectra. The UHPLC retention time of compound 8 matched the synthetic standard of 8–5NC-diferulate. Information regarding all diferulate products including accurate masses is represented in SI-Fig. S2. Elution order of diferulates in a reversed-phase separation, and occurrence of identified diferulate isomers in corn stover presented here, are consistent with those in previous publications.

Identification of diferulate isomers in corn stover presented here, are consistent with those in previous publications. Compound annotations are provided in Supplemental Information Figs. S2 and S3.

Identification of diferulates released from corn stover cell walls upon AFEX™ pretreatment

Hydrolysis and ammonolysis of diferulate esters are the two primary reactions that take place during AFEX™ pretreatment. As suggested in Fig. 2, it is expected that AFEX™ treatment cleaves diferulates via a combination of ammonolysis and hydrolysis, removing the hemicellulose cross-links, and releasing Di-Ac, Ac-Am, and Di-Am diferulates. After biomass pretreatment, the amounts of the individual diferulates released from the cell wall depend upon AFEX™ process conditions, abundances of various diferulate esters in the cell wall, and relative rates of hydrolysis and ammonolysis. Yields of these products reflect yields of cross-link removal, and quantification of these compounds provides measures of process efficiency in generating more digestible cell wall glycopolymers. Profiles of the three diferulate forms released upon AFEX™ treatment of corn stover are evident from extracted-ion chromatograms for m/z 385, 386, and 387 ([(M+H)⁺] for Di-Am, Ac-Am, and Di-Ac combined; Fig. 5b). At least 12 of the 15 diferulate peaks have been identified in extracts of AFEX™-treated corn stover. Retention times of the identified peaks were based in part on retention time and MS² matches (SI-Fig. S5) with synthesized Di-Ac, Ac-Am and Di-Am standard isomers. Information regarding all diferulate products including accurate masses is represented in SI-Fig. S4. In both NaOH and AFEX™ pretreatment, products derived from 8–O–4-diferulate were the most abundant. Ac-Am and Di-Amides of 8–O–4-, 8–8C-, 8–5NC- and 5–5-diferulates were present in the AFEX™-treated corn stover extract, and are consistent with observation of the corresponding Di-Ac forms in the products of NaOH pretreatment. Released 8–8NC-diferulate products were observed as Di-Ac products from NaOH pretreatment, but were only observed in AFEX™ products in the Ac-Am form. Although the 8–5C Di-Ac isomer was the second most abundant product from NaOH hydrolysis, this compound or its other cyclic analogs were not observed in any form following AFEX™ pretreatment. AFEX™ conditions likely converted it to 8–5NC variants that may undergo decarboxylation, but such products were not detected in nontargeted UHPLC/TOF MS analyses of AFEX™ products. Based on integrated peak areas,
over 90% of AFEX™-released diferulates contained at least one amide group (i.e., nearly all released identified diferulates are either Ac-Am or Di-Am, with 8–O–4-Di-Ac accounting for about 9% of the total diferulate products). Under these AFEX™ treatment conditions, rates of ammonolysis were therefore apparently greater than those of hydrolysis. Several of the observed Ac-Am and Di-Am diferulates are identical to products detected in reaction products of diferulate esters with liquid ammonia and water under AFEX™-like conditions.  

Similar CID fragmentation using different mass spectrometers

To explore whether the findings of CID behavior can be extended to other kinds of mass spectrometers, CID spectra were generated for all diferulate forms on a hybrid linear ion trap (QTRAP 3200) using enhanced product-ion scanning, a triple quadrupole (Quattro Premier XE) using product-ion scans, and a quadrupole/time-of-flight hybrid (QToF Ultima API). In addition, nonselective CID spectra were generated on an orthogonal TOF instrument (LCT Premier). A comparison of CID spectra for 8–O–4-Di-Ac is presented in SI-Fig. S7. Despite differences in the time frame between ion-molecule collision and ion detection, and collision gases (N2 in QTrap, Ar in others), all CID spectra share common major fragment ions, suggesting that the identifications and analysis here will be universally useful.

To provide for quantitative analysis of individual Di-Ac forms of diferulates, a multiple reaction monitoring (MRM) method was developed using transitions from [M+H]+ to a characteristic product ion for five isomeric forms. Relative response factors (RRFs) were determined for each isomer (Fig. S8). Although it is recognized that response factors will vary across different instrument platforms and with various instrumental conditions, the differences between isomer responses were consistent with a combination of two factors: (1) increasing ionization efficiency as the methanol content of the mobile phase increased, and (2) differences in yields of the detected product ions, as evident from the product ion spectra (Fig. 3).

Conclusions

Comprehensive determination of the chemical diversity of diferulate cross-linkers in plant cell walls provides a rich source of information useful for guiding the breeding of grasses for improved digestibility and resistance to pests. Future efforts to engineer plant cell walls for desirable traits will depend on analytical tools for rapidly profiling diferulates released from plant tissues. The discoveries presented above demonstrate that diferulate structural isomers are distinguished by their MS2 product-ion spectra when combined with UHPLC retention times, though discrimination of cis- and trans- isomers may require their chromatographic resolution. In many cases, isomer-specific fragment ions offer the prospect for rapid UHPLC/MS/MS analyses using MRM for quantitative analyses. This approach avoids the need for derivatization and related sample processing steps, allowing direct analysis of crude biomass extracts, and promises to address the need for more robust quantitative methods for ferulate oligomers. The primary barrier to adopting such strategies lies in the limited availability of authentic standards, though most of the diferulate esters themselves are now readily available. Cell walls also accumulate larger ferulate oligomers and lignin cross-coupling products that will present a yet more complex array of isomeric products. Our findings also demonstrate the diversity of diferulates from NaOH and AFEX™ pretreatment of cell walls, showing most of the anticipated diferulate isomers but revealing additional complexity. It is our hope that the CID spectra can be used in MS/MS spectrum libraries to aid diferulate characterization, and that the ion fragmentation chemistry demonstrated in this work will guide future efforts to characterize higher ferulate oligomers and establish the levels and roles of diferulates in cell wall structures of wild and engineered plant materials.

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Notes and references
