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Fast and Robust Method To Determine Phenoyl and Acetyl Esters of Polysaccharides by Quantitative ^{1}H NMR

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ABSTRACT: The acetyl (AcE), feruloyl (FE), and *p*-coumaroyl (pCE) ester contents of different cereal and grass polysaccharides were determined by a quantitative ^{1}H NMR-based method. The repeatability and the robustness of the method were demonstrated by analyzing different plant polysaccharide preparations. Good sensitivity and selectivity for AcE, FE, and pCE were observed. Moreover, an optimized and easy sample preparation allowed for simultaneous quantification of AcE, FE, and pCE. The method is suitable for high-throughput analysis, and it is a good alternative for currently used analytical procedures. A comparison of the method presented to a conventional HPLC-based method showed that the results obtained are in good agreement, whereas the combination of the optimized sample preparation and analysis by the ^{1}H NMR-based methodology results in significantly reduced analysis time.

KEYWORDS: *acetyl ester, phenoyl ester, polysaccharides, NMR*

INTRODUCTION

Analysis of plant cell wall polysaccharides from various feedstocks is necessary to characterize biomass conversion processes, to assist in the selection of suitable feedstocks, and for the development of optimized hydrolysis procedures.[1] Studies have shown that acetyl (AcE), feruloyl (FE), and *p*-coumaroyl (pCE) esters are xylan substituents present in cereal cell walls with their respective contents being dependent on the source of xylan.[2] Of particular relevance is the presence of AcE, FE, pCE, and other substituents in oligosaccharides recalcitrant to enzymatic hydrolysis.[3] A previous study demonstrated the presence of AcE, FE, and pCE in recalcitrant polysaccharides at different levels, and enzymatic substrate conversion showed a dependency on the degree of xylan backbone substitution.[4] To fully hydrolyze these polysaccharides, accessory enzymes, such as acetyl xylan esterases and feruloyl esterases, are required. Their required dosage for efficient hydrolysis depends on the source of the feedstock, as feedstocks show significant differences in their composition.[5] Although the presence of AcE, FE, and pCE in plant cell wall polysaccharides has been known for a long time for various plant species,[6,7] little information is available for bioenergy feedstocks. Furthermore, different stages of maturation and growing conditions also have an influence on the composition of xylan substituents.[8,9] Therefore, knowledge of the levels of substituents is important to assess the quality of a feedstock and its susceptibility to hydrolysis.

Analysis of AcE, FE, and pCE contents by analyzing the acetic acid (AcA), feruloylic acid (FA), and *p*-coumaric acid (pCA) released upon alkaline treatment can be time-consuming due to the required sample preparation and chromatographic analysis. A variety of methods for HPLC analysis of phenolic acids have been reported,[10] showing variations in solvent systems, elution, column choice, column temperature, flow rates, and detection systems. No *single* standardized procedure for quantification has been generally adopted, indicating that the field is still in need of a robust technique for phenoyl and acetyl ester analysis. The use of different analytical procedures may lead to variations in the data and may cause difficulties in reproducing the data. Specific sample preparation and analysis procedures have been reported for the determination of AcA, FA, and pCA separately by chromatographic analysis.[11] Separate analysis procedures may increase error rates compared to measurements by a single analysis procedure and may be labor-intensive. Moreover, HPLC-based methods have rather long running times of 30−150 min,[10] and they are dependent on the availability and inclusion of standards. A reduced sample analysis time (mobile phase sequence of 9.5 min and equilibration of 2.5 min) using an UPLC-MS/MS method has been reported.[12] In comparison, NMR allows for even shorter analysis times and has the advantage of being fully quantitative without the need of reference compounds for calibration curves.[13] This makes it a good alternative to chromatographic measurements.[14] Several recent studies describe NMR as a useful technique regarding the characterization of plant extracts and polysaccharides.[15−18]

In the present study, a method allowing fast and robust determination of AcE, FE, and pCE has been developed using quantitative ^{1}H NMR as an analytical tool. The method allows quantification of AcA, FA, and pCA by a single sample preparation procedure and a single recorded ^{1}H NMR spectrum. The results obtained are compared to results obtained by HPLC-based methods.

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Table 1. AcE, FE, and pCE Contents (% w/w) Determined by an NMR-Based Method of (i) Corn Silage (cs) with Different Saponification/Sonication Conditions and (ii) Corn Silage−Starch Mixtures (cs:starch)

sample	pCE (SD)	FE (SD)	AcE (SD)	saponification/sonication	MA addition[a]
cs 1−5	0.46 (0.01)	0.44 (0.03)	1.41 (0.03)	1 h	bh
cs 6−7	0.45 (0.01)	0.39 (0.02)	1.39 (0.02)	1 h	ah
cs 8−9	0.44 (0.03)	0.43 (0.03)	1.38 (0.01)	on[b]	bh
cs:starch (1:10)	0.07	0.05	0.23	1 h	bh
cs:starch (1:100)	0.0	0.0	0.02	1 h	bh

[a]bh, before hydrolysis; ah, after hydrolysis. [b]200 min sonication, incubation of 16 h.

Table 2. Contents (% w/w) of pCE, FE, and AcE in Various Corn Samples (Corn Silage, Corn Cob WUS, Corn Fiber AIS, and Corn Stover WUS) following Sample Preparation with Different Saponification/Sonication Times

	corn silage			corn cobs			corn fiber			corn stover		
saponification/sonication (h)	pCE	FE	AcE	pCE	FE	AcE	pCE	FE	AcE	pCE	FE	AcE
0.5	0.49	0.36	1.40									
1	0.58	0.41	1.47	1.75	1.17	2.36	0.13	2.26	2.45	1.09	0.54	1.72
2	0.58	0.41	1.40									
16[a]	0.56	0.43	1.45	2.33	1.09	2.36	0.19	2.06	2.47	1.87	0.46	1.85

[a]200 min sonication, incubation of 16 h.

MATERIALS AND METHODS

Feedstock Materials. Dried, milled corn and grass silage were provided by DSM (Heerlen, The Netherlands). Water-unextractable solids (WUS) of corn cobs and corn stover were prepared as described elsewhere.[19] Corn fiber alcohol-insoluble solids (AIS) were prepared as described by Kabel et al.[20] Commercially available potato starch (101252 starch) and maleic acid were from Merck (Darmstadt, Germany). Deuterated solvents were purchased from Cambridge Isotopes Laboratories (Andover, MA, USA).

Sample Preparation and Analysis. *Sample Preparation for NMR Analysis.* Samples (10−30 mg) were accurately weighed in glass tubes. Hydrolysis was done in 1 mL of 0.5 M NaOD in D_2O, under nitrogen atmosphere in the dark at room temperature with sonication with a Branson 5510 ultrasonic cleaner (Branson Ultrasonics, Danbury, CT, USA). Maleic acid (MA; 0.1 mL of a 5.277 g/L stock solution in D_2O) was added as internal standard.

The hydrolysis procedure involved sonication for 100 min followed by overnight incubation at room temperature and subsequent sonication for 100 min. Furthermore, shorter hydrolysis procedures with sonication times ranging from 0.5 to 2 h and different time points of MA addition (before or after hydrolysis with NaOD) were applied (Tables 1 and 2). D_2O containing EDTA (0.1 g/L; 2.5 mL) was added to the samples before analysis by NMR.

Sample Analysis by NMR. A 700 MHz Bruker Avance III spectrometer, equipped with a 5 mm TCI cryoprobe, and a SampleJet autosampler (Bruker, Billerica, MA, USA) suitable for 3 × 103.5 mm tubes were used for NMR analysis.

^1H NMR spectra were recorded at 300 K. For quantitative measurements a pulse program with water suppression was used (zgcppr) and water suppression power corresponding to 10 Hz suppression. A 90° pulse length was applied with a relaxation delay of 30 s. The number of scans was 8; 65536 data points were collected, and a spectrum width of 12 ppm was used. The spectra were analyzed with TopSpin 3.1 (Bruker).

Quantification by NMR. The AcA, FA, and pCA concentrations were calculated from the ^1H NMR spectrum by applying eq 1. For pCA, the integrals corresponding to the aromatic proton peak at 7.44 ppm (doublet) and the integral area of the internal standard maleic acid proton resonance at 6.03 ppm (singlet) were used for this purpose. FA and AcA amounts were calculated using the peaks at 7.17 and 1.95 ppm, respectively, and the internal standard as mentioned above. The molecular weights used for pCA, FA, and AcA were 164.16, 194.18, and 60.05, respectively.

$$P_x = \frac{A_x}{A_{st}} \times \frac{n_{st}}{n_x} \times \frac{MW_x}{MW_{st}} \times \frac{W_{st}}{W_x} \times P_{st} \quad (1)$$

A_x = area peak of product, A_{st} = area peak of internal standard, N_{st} = number of protons corresponding to the internal standard peak, n_x = number of protons corresponding to the product peak, MW_x = molecular weight of product, MW_{st} = molecular weight of the internal standard, W_{st} = weight of the internal standard (g), W_x = weight sample (g), and P_{st} = purity internal standard (%).

Sample Preparation for HPLC Analysis of FA, pCA, and AcA. FA and pCA were analyzed according to the procedure described by Appeldoorn et al.[3] with some minor modifications: Each sample (10−20 mg) was dissolved in 200 μL of methanol. Five milliliters of 0.5 M KOH (flushed with N_2) was added, and samples were put under a N_2 atmosphere. The samples were kept in the dark for 16 h and sonicated two times for 100 min (5510 Branson ultrasonic cleaner). After 16 h, the pH was adjusted to 2 by the addition of 0.75 mL of HCl (6 M). Extraction of FA and pCA was done by 4 mL of ethyl acetate. The extraction was repeated once. The ethyl acetate fraction was dried under N_2 at room temperature, and the residue was dissolved in 1 mL of methanol.

For AcA determination, each sample (20−40 mg) was saponified with 1 mL of 0.4 N NaOH in isopropanol/H_2O (1:1 mixture) for 1 h on ice and for 2 h at room temperature under regular mixing.

HPLC/UHPLC Analysis of FA, pCA, and AcA. For phenolic acid analysis an Acella UHPLC system (Thermo Scientific, Rockford, IL, USA) equipped with a PDA detector and coupled to an LTQ XL mass detector equipped with an ESI source (Thermo Scientific) was used. Separation was performed on a Hypersyl GOLD column (1.9 × 150 mm; 1.9 μm; Thermo Scientific).

For the determination of FA and pCA the mobile phase was composed of (A) H_2O + 1% (v/v) acetonitrile + 0.2% (v/v) acetic acid and (B) acetonitrile + 0.2% (v/v) acetic acid. Elution was done at 0.4 mL/min by the following profile: 5 min, isocratic 0% B; 5−23 min, linear from 0 to 50% B; 23−24 min, linear from 50 to 100% B; 24−27 min, isocratic at 100% B; 27−28 min, linear from 100 to 0% B, followed by reconditioning of the column for 7 min. Spectral data collection was done from 200 to 600 nm. Quantification was done at 320 nm on the basis of standards.

MS data were collected in the negative mode with an ion spray voltage of 3.5 kV, a capillary voltage of −20 V, and a capillary temperature of 350 °C. Full MS scans were made within the range m/z 150−1500, and MS^2 data of the most intense ions were obtained.

For acetic acid analysis, an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a Shodex RI detector (Showa

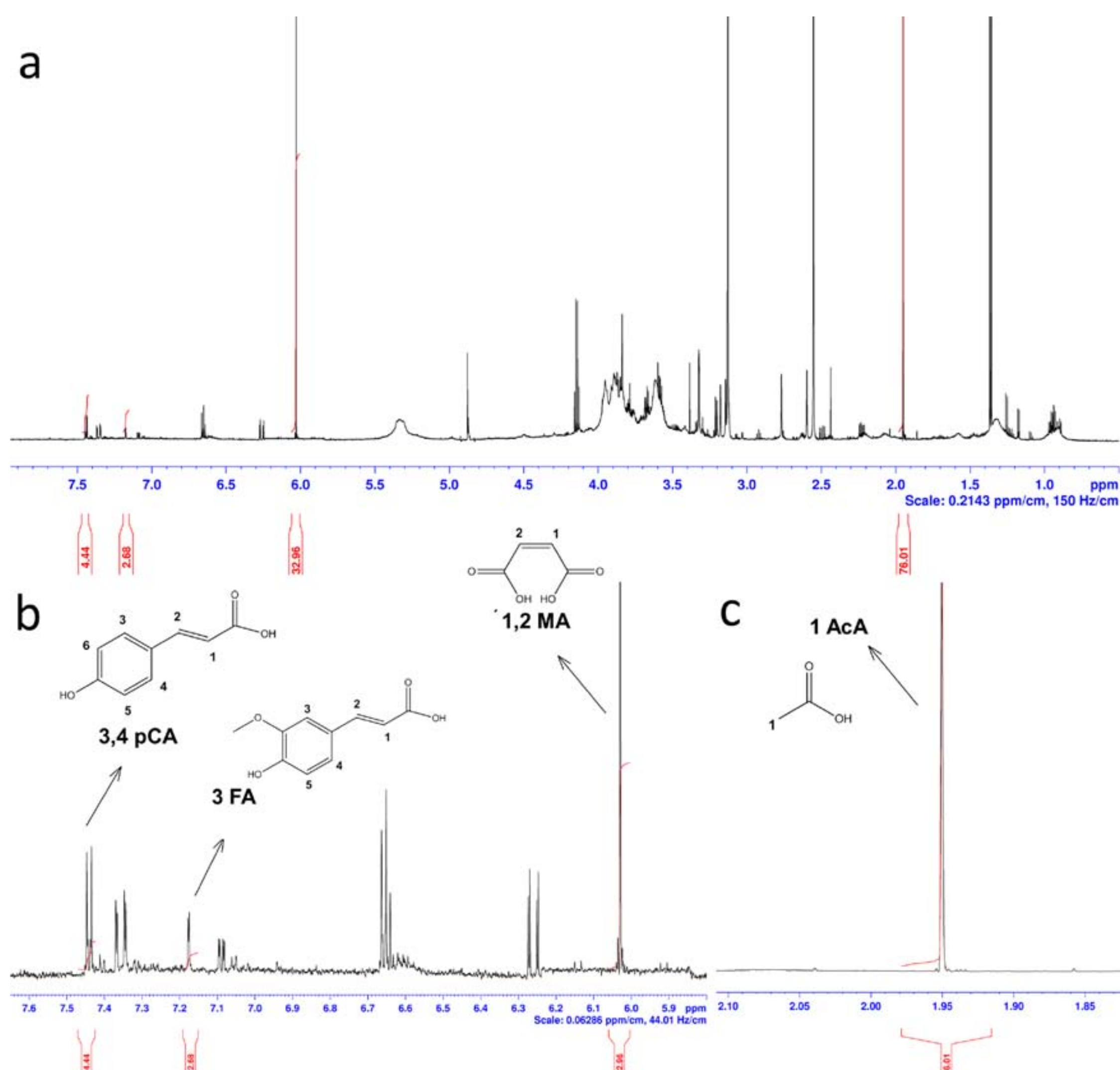

Figure 1. ^{1}H NMR spectrum of saponified corn silage sample: (a) region from 8.00 to 0.5 ppm; (b) region from 8.00 to 6 0.00 ppm; (c) region from 2.3 to 1.7 ppm.

Denko Europe GmbH, Munich, Germany) and an Aminex HPX87H column (300 mm × 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA) were used for AcA analysis. For the determination of AcA by HPLC, elution was performed with H_2SO_4 (5 mM) at a flow rate of 0.6 mL/min and a column oven temperature of 40 °C.

■ RESULTS AND DISCUSSION

Determination of AcE, FE, and pCE Contents. *Optimization of Experimental Parameters.* FA-, pCA-, and AcA-containing standards were analyzed by NMR. The quantified data obtained by NMR analysis were in agreement with the amounts weighed [R^2 values > 0.999; 0.2−0.02 mg/mL (FA, pCA); 20−0.0002 mg/mL (AcA)]. The signal-to-noise ratio was clearly above the quantification limit of 10:1 [International Conference on Harmonization (ICH), Chicago, 2005].[21] Linearity of the results was observed for the standard concentrations with signal-to-noise ratios >10:1 [0.2−0.02 mg/mL (FA, pCA); 20−0.0002 mg/mL (AcA)]. This range covers the expected range of concentrations for AcA, FA, and pCA in plant cell wall polysaccharides, using the sample preparation applied in this study. Whereas 0.02 mg/mL (FA, pCA) could be reliably quantified [signal-to-noise ratios of 19:1 (FA) and 32:1 (pCA)], quantification was not possible for FA and pCA below a concentration of 0.002 mg/mL and for AcA below a concentration of 0.0002 mg/mL with the measurement conditions applied (8 scans, analysis time = 4−5 min).

For the optimization of experimental parameters, plant polysaccharides were used. Figure 1 shows the recorded ^{1}H NMR spectrum after sample preparation of corn silage with the proton signals used for AcA, FA, and pCA quantification (1.95, 7.17, and 7.44 ppm). The variance of the method (sample preparation and quantification) was assessed. The AcE, FE, and pCE contents of corn silage by repeated measurements under the same or modified conditions (different saponification/sonication times, different amounts of sample, and addition of MA before and after hydrolysis) were calculated (% w/w) and are listed in Table 1. Standard deviations (SD) within a series of repeated measurements were between 0.01 and 0.04 (Table 1) and ≤0.02 between the average values of three measurement series. Signal-to-noise ratios of >2000:1 for AcA, 45:1 for FA, and 68:1 for pCA were observed for corn silage, being significantly above the detection and quantification limits. To investigate the suitability of the method for the determination of very low amounts of AcA, FA, and pCA in polysaccharide-containing samples, corn silage/starch mixtures were analyzed [starch, no FE and pCE present, and only minor amounts of

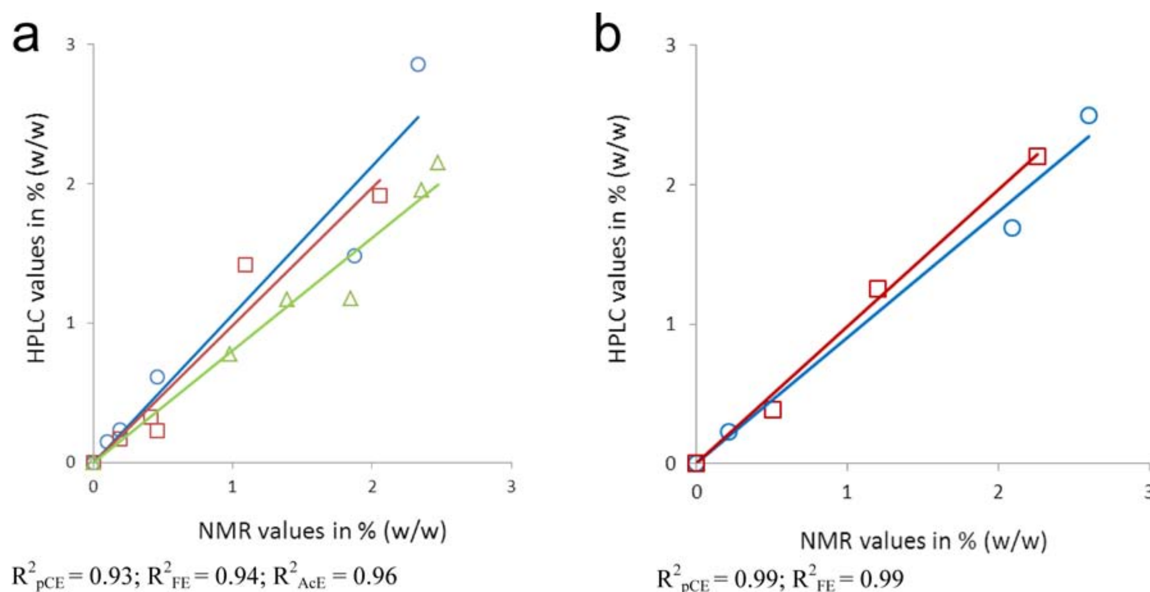

Figure 2. Correlations observed between the results obtained from independent NMR- and HPLC-based analysis of different feedstocks: (a) analysis according to the NMR method presented and HPLC-based methods (feedstocks analyzed: grass silage, corn silage, corn cob WUS, corn stover WUS, corn fiber AIS); (b) FE and pCE levels of the same hydrolysate (feedstocks analyzed: corn cob WUS, corn stover WUS, corn fiber AIS); (red, squares) FE; (blue, circles) pCE; (green, triangle) AcE. Each data point represents the analysis of one sample.

acetic acid (0.015% w/w)]. Signal-to-noise ratios of 780:1, 45:1, and 50:1 for AcA, FA, and pCA, respectively, allowed good quantification in a 1:10 corn silage/starch mixture. In a 1:100 corn silage/starch mixture, AcE could still be reliably quantified (Table 1), whereas reliable quantification of pCE and FE was not possible (signal-to-noise ratios < 10:1). The quantification and detection limits for each sample can be easily assessed from each individually recorded NMR spectrum on the basis of the signal-to-noise ratios.

Optimization of Pretreatment. Saponification of the samples was done with NaOD (0.5 M) at room temperature. It has been reported that relatively mild NaOH extraction conditions (1 M or lower) at room temperature specifically release phenolic acid residues of nonlignin origin,[22,23] as the release of phenolic acids from lignin requires strongly oxidizing conditions (cupric oxide−NaOH, 175 °C).[24]

The saponification times ranged between 30 min and 16 h (Table 2), in agreement with reported saponification procedures.[10] The differences observed when the saponification conditions were altered are shown in Table 2 for four different corn-derived feedstocks (corn silage, corn cob WUS, corn stover WUS, corn fiber AIS). In the case of corn silage, all saponification and sonication times tested resulted in comparable AcE levels, but short saponification/sonication times (0.5 h) gave lower pCE and slightly lower FE levels. Therefore, a saponification/sonication time of 1 h seems to be required to obtain full release of FE and pCE from corn silage. Prolonged saponification/sonication did not release additional FE or pCE.

For corn cobs and corn stover, an increase of pCE levels was observed with prolonged saponification/sonication time (2 × 100 min sonication, overnight saponification). This indicates that the suitability of sample pretreatment conditions depends on the feedstock to be analyzed and its previous processing conditions. On the other hand, it is possible to optimize saponification/sonication times easily for routine analysis of a specific feedstock (as shown for corn silage), thereby reducing the sample preparation time. As pCE levels were higher with prolonged saponification/sonication for corn cobs, corn fiber, and corn stover, overnight saponification was chosen as the standard sample pretreatment.

High-Throughput Determination of AcE, FE, and pCE by NMR. The analysis time of AcE, FE, and pCE in plant feedstocks is significantly reduced by the method presented compared to conventional HPLC methods. NMR analysis of a hydrolyzed sample took 4.52 min (equilibration time was set to 1 min). Reported HPLC methods for phenolic acid analysis vary significantly and range from 30 to 150 min, with some methods including equilibration and others including it as a separate step.[10] Analysis times by UPLC analysis are shorter (12 min).[12] Therefore, significant analysis time reductions of >80 and >50% were achieved by the NMR method compared to HPLC and UPLC methods, respectively.

Methods that include separate specific sample preparation and analysis procedures for AcE,[25] FE, and pCE[10] are frequently used for compositional analysis of feedstocks.[3,19] However, separate analysis procedures may increase error rates and analysis time. Determination of AcE, FE, and pCE from a single hydrolysate and a single recorded ^{1}H NMR spectrum significantly reduces labor input (a single sample preparation) and analysis time (analysis by one recorded NMR spectrum) and may also lower error rates. The use of an autosampler for NMR analysis in this study allowed automated measurement of multiple samples, providing a platform for high-throughput analysis with high precision by NMR quantification.

Comparison of NMR- and HPLC-Based Methods. AcE, FE, and pCE contents of five different substrates (grass and corn silage, corn cob WUS, corn stover WUS, and corn fiber AIS) were determined by NMR, and the results were compared to those obtained by HPLC methods (Figure 2). A regression analysis of the AcE, pCE, and FE contents independently determined by the method presented here and by a conventional HPLC-based method[3] showed high correlations of 0.96, 0.94, and 0.93, respectively (Figure 2a). Therefore, the

measured AcE, pCE, and FE values are in good agreement and confirm the validity of the method presented. A comparison of the same hydrolysate prepared by a single sample preparation procedure (see Sample Preparation for NMR Analysis) of corn cob WUS, corn stover WUS, and corn fiber AIS by NMR and HPLC analysis gave even higher correlations (R^2 = 0.99 for FA and pCA; Figure 2b). This observation indicates that small differences between the results obtained by the two methods are not due to the analytical technique used, but to errors that originate from the sample preparation procedure. Therefore, simplifying the sample preparation as well as analyzing a single hydrolysate, as described for the method presented here, lowers the error rate.

The results obtained by both the NMR- and HPLC-based methods are in agreement with published data for corn cobs, corn stover, and corn fiber.[11] Small differences between the values obtained and the published values are to be expected due to the preparation of the WUS (cobs, stover) fraction and the inhomogeneity of natural plant feedstocks with regard to their chemical composition in general.[26,27]

In conclusion, our data show that the NMR-based method presented is suitable for the determination of AcE, FE, and pCE occurring in plant cell wall polysaccharides. The method presented is a fast, robust, and reliable alternative to conventional methods, and it is therefore of relevance for the characterization of these substituents in cereal and grass cell wall polysaccharides.

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Notes

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■ ABBREVIATIONS USED

AcA, acetic acid; AcE, acetyl ester; AIS, alcohol-insoluble solids; EDTA, ethylenediaminetetraacetic acid; FA, ferulic acid; FE, feruloyl ester; HPLC, high-performance liquid chromatography; MA, maleic acid; MS, mass spectrometry; NMR spectroscopy, nuclear magnetic resonance spectroscopy; pCA, *p*-coumaric acid; pCE, *p*-coumaroyl ester; UPLC, ultra-performance liquid chromatography; WUS, water-unextractable solids

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