Separation and Detection of Cell Wall-Bound Ferulic Acid Dehydrodimers and Dehydrotrimers in Cereals and Other Plant Materials by Reversed Phase High-Performance Liquid Chromatography With Ultraviolet Detection

DIANE DOBBERSTEIN[†] AND MIRKO BUNZEL[*,‡]

[†]Institute of Biochemistry and Food Chemistry, Department of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany, and [‡]Department of Food Science and Nutrition-CFANS, University of Minnesota, 1334 Eckles Avenue, St. Paul, Minnesota 55108

Ferulate dehydrodimers and the more recently discovered dehydrotrimers play an important role in the cell wall architecture of plant-based foods and forages. High-performance liquid chromatography methods to determine ferulate dimers often lack specificity; methods for trimers did not exist yet. A method for the determination of 11 cell wall-bound ferulate dehydrodimers and -trimers was developed, including the crucial separation of the di/trimers from the often dominating phenolic monomers. Validation parameters for the basic calibration of the dimers and trimers met our acceptance criteria. However, the matrix calibration revealed that lignin-rich matrices lead to problems with precision and accuracy that likely can be addressed by using a more specific detection, that is, mass spectrometric detection, next to improved sample preparation procedures. The method was used to analyze low-lignin fibers from corn, wheat, and rye grains, wild rice, asparagus, and sugar beet. With the exception of wild rice, the 5-5/8-*O*-4-, 8-*O*-4/8-*O*-4-, and 8-8(aryltetralin)/8-*O*-4-dehydrotrimers were detected in all analyzed samples, however, often in amounts below the limit of quantitation.

INTRODUCTION

Hydroxycinnamates are minor constituents of the plant cell walls of two major groups of angiosperms, the commelinid monocotyledons, which include the grasses, and the core Caryophyllales (*1*). While *p*-coumarate is mainly bound to lignin (*2, 3*) with only smaller amounts attached to polysaccharides (*4, 5*), ferulate is mostly ester-linked to plant cell wall polysaccharides such as arabinoxylans or pectins (*6*). Small amounts of sinapate are supposed to be linked to polysaccharides as well (*7*); however, this has not yet been unambiguously proven. Hydroxycinnamates, especially ferulate, form dimers and oligomers (*8, 9*), thus cross-linking plant cell wall polymers with important implications for plant physiology, plant pathology, food science, and human and ruminant nutrition (*8*). For example, diferulate cross-links have been discussed and are involved in genotypic resistance of corn to Gibberella stalk rot and Giberella ear rot (*10, 11*), in maintaining the crispness of fruits and vegetables (*12−14*), in reducing forage digestibility (*15, 16*), in influencing the physicochemical properties of dietary fiber (*17*), and in explaining dough and baked good properties (*18*). Currently, after alkaline hydrolysis of the ester linkages, hydroxycinnamic acid monomers and ferulic acid dehydrodimers are analyzed by both high-performance liquid chromatography (HPLC) and gas chromatography (GC) after

derivatization (*17, 19, 20*). Whereas GC shows better resolution for the ferulic acid dimers and can separate "blocks" of monomers from "blocks" of dimers, it also requires a derivatization step and is not suitable for separating higher oligomers such as ferulic acid trimers. On the other hand, HPLC methods often lack separation of phenolic monomers from ferulic acid dehydrodimers. As a consequence, the absence of some ferulic acid dimers, especially the early eluting 8-8-coupled dimers, was falsely claimed for some plant materials. Also, a general problem for the development of analytical methodologies that separate ferulate dimers and trimers is the availability of pure standard compounds, which often prevents researchers from validating the methodologies used. While syntheses for ferulic acid dimers have been described (*19, 21*), these procedures are often time-consuming and require advanced synthetic skills (*19*). More recently, we described the isolation of ferulic acid dimers as standard compounds from corn bran, a rich source of ferulates in general (*22*). Synthetic procedures for higher oligomers than dimers are not available yet; however, isolation procedures have been described recently (*23−25*).

The aim of this paper is to describe an improved method for the separation of ferulic acid dimers while incorporating ferulic acid trimers for the first time. Also, the application of the whole set of validation parameters to our method provides insight into how accurately ferulate oligomers can be quantified by using reversed phase (RP)-HPLC/ultraviolet (UV).

*To whom correspondence should be addressed. Tel: +1-612-624-1764. Fax: +1-612-625-5272. E-mail: mbunzel@umn.edu.

MATERIALS AND METHODS

Materials and General Experimental Procedures. Monomeric phenolic compounds with the exception of *cis-p*-coumaric and *cis*-ferulic acid were obtained from Merck (Darmstadt, Germany), Sigma (St. Louis, MO), or Acros Organics (Geel, Belgium). The *cis*-isomers of ferulic and *p*-coumaric acid were obtained from UV irradiation (14 h) of the *trans*-isomers in methanol (MeOH) (for ferulic acid) or 0.5 M aqueous ammonia (for *p*-coumaric acid). This treatment resulted in the formation of 46% *cis*-ferulic acid and 83% *cis-p*-coumaric acid, respectively (*26*). The diacetylated, dimethylated precursor of the 8-8(tetrahydrofuran)-dehydrodiferulic acid (*27*) and the 5-5(methylated)-dehydrodiferulic acid [monomethylated 5-5-dehydrodiferulic acid (*9*)], which was used as an internal standard, were kindly donated by Prof. John Ralph (Department of Biochemistry, University of Wisconsin, Madison, WI). Cellulose was obtained from Acros Organics (Geel, Belgium); xylan and Organosolv lignin were from Sigma (St. Louis, MO). To remove low molecular weight byproducts of the commercial lignin preparation, the lignin was extracted with diethyl ether (three times, 8 h each) in a Soxhlet extractor. Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from *Bacillus licheniformis*, 120 KNU/g), the protease Alcalase 2.4 L (EC 3.4.21.62, from *B. licheniformis*, 2.4 AU/g), and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were kindly donated by Novo Nordisk (Bagsvaerd, Denmark). Sephadex LH-20 was from Amersham Pharmacia Biotech (Freiburg, Germany).

Sephadex LH-20 chromatography instrumentation (L-6000 pump and L-7400 UV-detector) was from Merck/Hitachi (Darmstadt, Germany). Semipreparative C-18 HPLC columns were purchased from Macherey-Nagel (Düren, Germany). Analytical phenyl-hexyl HPLC columns were purchased from Phenomenex (Aschaffenburg, Germany). RP-HPLC was carried out using either of the following instrumentations: (1) isolation of standard compounds: L-6200 intelligent pump, T-6300 column thermostat, and L-7400 UV detector (all Merck/Hitachi); (2) analysis of phenolic monomers: L-6200 intelligent pump (Merck/Hitachi), 2155 column oven (LKB, Bromma, Sweden), L7400 UV-detector (Merck/Hitachi), and Spectra series AS 100 autosampler (Thermo Separation Products, Darmstadt, Germany) (analysis of phenolic monomers); and (3) analysis of ferulic acid dimers and trimers: L-7150 intelligent pump, L-7300 column oven, L-7455 photodiode array detector (PDA), and L-7200 autosampler (Merck/Hitachi, Darmstadt, Germany). NMR experiments were performed on a Bruker DRX-500 (Rheinstetten, Germany) instrument. Solvents [MeOH and acetonitrile (ACN)] were HPLC grade. Water was distilled and deionized; diethyl ether was purified and stored over potassium hydroxide to avoid peroxide formation.

Plant Materials. Corn bran was kindly provided by Hammermühle Maismühle GmbH (Kirrweiler, Germany). Corn stover was kindly provided by Prof. Frieder Schwarz and Dr. Friederike Zeller (Technical University of Munich, Department of Animal Nutrition). Whole grains of corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), wild rice (*Zizania aquatica* L.), and fresh white asparagus (*Asparagus officinalis* L.) were obtained from a local German supplier. Extracted sugar beet (*Beta vulgaris* L. ssp. vulgaris var. altissima DOELL) chips were provided by a local German sugarplant. Insoluble fibers (not corrected for ash or residual protein) were obtained from the plant materials as described previously (*17, 28*).

Isolation of Standard Compounds. With the exception of the 4-*O*-5-dehydrodiferulic acid and the 8-8(tetrahydrofuran)-dimer, all known ferulate dehydrodimers (*19, 27*) as well as the three major dehydrotriferulic acids [5-5/8-*O*-4-, 8-*O*-4/8-*O*-4-, and 8-8(aryltetralin)/8-*O*-4-dehydrotriferulic acids] (*23, 24*) were isolated from the alkaline hydrolyzate of corn bran following different, previously described chromatographic fractionations (*22*). The recently described 8-8(tetrahydrofuran)-dehydrodiferulic acid (*27*) was obtained by saponification of its esterified precursor.

Alkaline Hydrolysis of Insoluble Corn Fiber and Extraction of Phenolic Acids. Insoluble corn fiber was prepared from acetone-extracted corn bran by using an enzymatic procedure (heat-stable α-amylase, protease, and amyloglucosidase) described previously (*22*). Saponification (2 M NaOH) was carried out under nitrogen, protected from light, and under continuous stirring for 18 h. Following acidification (pH < 2), the liberated phenolic acids were extracted with diethyl ether. Ether extracts were extracted with NaHCO₃ solution (5%). This step extracts phenolic acids

Table 1. Amounts and Purities of ferulic acid dehydrodimers (DFA) and dehydrotrimers (TriFA) Isolated as Standard Compounds for the Method Development

	amount (mg)	purity[a] (%)
8-8(aryltetralin)-DFA	18.2	>96
8-8-DFA	4.3	>99
8-8(tetrahydrofuran)-DFA	12.8	>97
5-5-DFA	121.2	>98
8-*O*-4-DFA	52.4	>95
8-5(benzofuran)-DFA	22.6	>94
8-5-DFA	16.6	>99
8-5(decarboxylated)-DFA	25.5	>99
5-5(methylated)-DFA	10.8	>98
5-5/8-*O*-4-TriFA	19.6	>98
8-8(aryltetralin)/8-*O*-4-TriFA	3.8	>93
8-*O*-4/8-*O*-4-TriFA	2.2	>94

[a] Purities were estimated based on ¹H-NMR data.

into the aqueous layer; other phenolic compounds mainly remain in the organic layer and are discarded. The aqueous layers were acidified (pH < 2), and phenolic acids were re-extracted into diethyl ether. Ether extracts were dried over Na₂SO₄, evaporated to dryness, and redissolved in MeOH/water 50/50 (v/v) (ultrasonic bath, addition of a few drops of acetone to improve solubility).

Sephadex LH-20 Chromatography. Sephadex LH-20 chromatography was performed as described previously (*22*) with some minor modifications. In brief, the sample was applied to the column (gel bed: 2.5 cm × 85 cm) preconditioned with 0.5 mM aqueous trifluoroacetic acid (TFA)/MeOH 95/5 (v/v). Elution was carried out as follows: (1) elution with 0.5 mM TFA/MeOH 95/5 (v/v) for 72 h, flow rate: 1.5 mL/min; (2) elution with 0.5 mM TFA/MeOH 50/50 (v/v) for 72 h, flow rate: 1.0 mL/min; (3) elution with 0.5 mM TFA/MeOH 40/60 (v/v) for 65 h, flow rate: 1.0 mL/min; and (4) rinsing step with 0.5 mM TFA/MeOH 10/90 (v/v). Detection was carried out at 280 and 325 nm. Fractions were collected over 12 min periods, combined according to the chromatograms (data not shown), and evaporated. Fractions were further separated/purified by using semipreparative RP-HPLC or recrystallization.

Further Purification of Dehydrodi- and Triferulic Acids. Further separation of Sephadex LH-20 fractions was achieved by semipreparative RP-HPLC (*22*) using a Nucleosil 100-5 C18-HD column (250 mm × 10 mm i.d., 5 μm particle size) and binary gradient systems made up of 1 mM aqueous TFA and MeOH, used at a flow rate of 2.7 mL/min. The injection volume was 60 μL, and the separation was performed at either 35 or 45 °C. Chromatograms were monitored at 280 and 325 nm. Used were the following gradients [eluent A, 1 mM aqueous TFA; eluent B, MeOH/1 mM aqueous TFA 90/10 (v/v)]: purification of 8-8(aryltetralin)-, 8-*O*-4-, 8-5(benzofuran)-dehydrodiferulic, 5-5/8-*O*-4-, and 8-*O*-4/8-*O*-4-dehydrotriferulic acid: initially 90% A, 10% B held for 10 min, linear over 5 min to 60% A, 40% B, held for 5 min, linear over 5 min to 50% A, 50% B, held for 5 min, linear over 5 min to 20% A, 80% B, held for 5 min, following a rinsing and an equilibration step. Purification of 8-8-, 8-5-dehydrodiferulic, and 8-8(aryltetralin)/8-*O*-4-dehydrotriferulic acid: initially 75% A, 25% B held for 20 min, linear over 5 min to 40% A, 60% B, held for 5 min, linear over 5 min to 10% A, 90% B, held for 5 min, following a rinsing and an equilibration step. The 8-8(tetrahydrofuran) dimer was deacetylated and demethylated according to a previously described two-step saponification (*29*). Following saponification, the isolated dimer was also purified by semiprepative RP-HPLC using the following binary gradient: initially 70% A, 30% B, held for 5 min, linear over 5 min to 60% A, 40% B, linear over 10 min to 50% A, 50% B, linear over 5 min to 20% A, 80% B, held for 5 min, following an equilibration step. Identity and purity of all isolated standard compounds were confirmed by using phenyl-hexyl RP-HPLC-PDA (chromatographic conditions as described below) and ¹H-NMR. ¹H-NMR experiments were performed in acetone-*d₆*. Amounts and purities of the isolated standard compounds are listed in **Table 1**.

Method Development and Validation. The following phenolic monomers, dimers, and trimers were included into the methods: monomers (in order of elution on the phenyl hexyl column): 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, caffeic acid, vanillic acid, syringic acid, 4-hydroxybenzaldehyde, 4-hydroxyphenylpropionic acid, *trans-p*-coumaric

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acid, vanillin, *cis-p*-coumaric acid, syringaldehyde, *trans*-ferulic acid, sinapic acid, and *cis*-ferulic acid; dehydrodiferulic acids (in order of elution on the phenyl hexyl column): 8-8(aryltetralin)-, 8-8-, 8-8(tetrahydrofuran)-, 8-5-, 5-5-, 8-O-4-, 8-5(benzofuran)-, 5-5(methylated)-, and 8-5(decarboxylated)-dehydrodiferulic acid; and dehydrotriferulic acids (in order of elution on the phenyl hexyl column): 8-8(aryltetralin)/8-O-4-, 5-5/8-O-4-, and 8-O-4/8-O-4-dehydrotriferulic acid.

Both the phenolic monomers and the phenolic oligomers are analyzed from the same alkaline plant hydrolysates. As the amounts of monomers, dimers, and trimers are supposedly very different in most plant materials, different concentration ranges were used to validate the method for the different phenolic acids/aldehydes. Because of the prevalence of *trans*-ferulic acid and *trans-p*-coumaric acid among the monomers, the concentration range 25−125 µg/mL with 25, 50, 75, 100, and 125 µg/mL steps was used for these monomers. All other monomers were used in a range between 2.5 and 12.5 µg/mL (steps 2.5, 5, 7.5, 10, and 12.5 µg/mL). The dimers and trimers were, mostly depending on their approximate abundance in corn, used either in a range between 2.5 and 12.5 µg/mL, too, or in a range between 6 and 30 µg/mL (steps 6, 12, 18, 24, and 30 µg/mL) (**Table 4**). For both basic and matrix calibration, all concentrations were measured three times. To determine the homogeneity of variances throughout the tested concentration range, the lowest and the highest concentrations were measured six times, and an *F* test was performed.

The basic calibration was performed by dissolving appropriate amounts of the standard compounds in MeOH/water 50/50 and diluting the stock solution to the required concentrations. Parameters tested for the basic calibration were selectivity (baseline separation, less than 10% peak overlap), homogeneity of variances, detection limit (signal-to-noise ratio of 3:1), quantitation limit (signal-to-noise ratio of 9:1), linearity (visual inspection of residual plots, correlation coefficient), sensitivity (slope), and precision (residual standard deviation). In addition, the standard deviation of the procedure and the variation coefficient of the procedure were calculated. The standard deviation of the procedure was calculated from the quotient of the residual standard deviation and the sensitivity (slope). The variation coefficient of the procedure in % was calculated by dividing the standard deviation of the procedure by the center of the concentration range and multiplying times 100. In addition to the basic calibration, a matrix calibration was performed, mimicking the plant cell wall matrix by using a cellulose/xylan/lignin mixture [50/45/5 (w/w/w)]. This mixture was saponified as described below for the plant materials. The alkaline hydrolysates were spiked with standard mixtures of known concentrations before the hydrolysates were acidified, extracted with diethyl ether, evaporated, redissolved in tetrahydrofuran (THF)/water (50/50, v/v), and analyzed by HPLC as described for the analysis of the plant materials. As described for the basic calibration, the variation coefficient of the procedure was calculated from the residual standard deviation, the slope of the regression line, and the concentration representing the center of the tested concentration range. In addition, the recovery rate was calculated from plotting the spiked concentrations versus the analyzed concentrations and determining the slope of the regression line. Correction factors (matrix calibration) were calculated against caffeic acid and 5-5-(methylated)-dehydrodiferulic acid as potential internal standards for the determination of the phenolic monomers or dehydrodiferulic and dehydrotriferulic acids, respectively. Correction factors were determined from the slope of the regression line resulting from plotting concentration (analyte)×area (internal standard) on the *y*-axis vs concentration (internal standard)× area (analyte) on the *x*-axis.

Alkaline Hydrolysis of Plant Materials and Extraction of Phenolic Acids. Saponification was performed as described for the isolation of standard compounds; however, plant materials and reagents were downscaled to 75 mg of plant fibers (destarched) and 5 mL of 2 M NaOH. Following acidification (ca. 0.95 mL of concentrated HCl), caffeic acid (1 mg) and 5-5(methylated)-dehydrodiferulic acid [10 or 50 µg (depending on the plant material)] were added as internal standards for the quantification of phenolic acid monomers and ferulic acid oligomers, respectively. Extraction was carried out with diethyl ether (three times, 4 mL of diethylether) without additional cleanup steps. Diethyl ether extracts were dried under a stream of nitrogen and redissolved in THF/water [50/50 (v/v); 500 µL]. Whereas these extracts were directly used for the determination of ferulate dimers and trimers, they were diluted 1 to 10 with THF/water [50/50 (v/v)] for the determination of phenolic acid monomers.

Phenyl-hexyl RP-HPLC-PDA of Phenolic Acids. Monomeric phenolic acids as well as ferulic acid dimers and trimers were analyzed using a Luna phenyl hexyl column (250 mm × 4.6 mm i.d., 5 µm particle size, plus 3 mm × 4.6 mm i.d. guard column) and ternary gradient systems made up of 1 mM aqueous TFA, ACN, and MeOH at a flow rate of 1.0 mL/min. The injection volume was 20 µL, and the separation was performed at 45 °C. Chromatograms were monitored at 280 nm. The following gradients were used eluent A, 1 mM aqueous TFA; eluent B, ACN/1 mM aqueous TFA [90/10 (v/v)]; and eluent C, MeOH/1 mM aqueous TFA [90/10 (v/v)]. Separation of phenolic monomers: initially 87% A, 13% B, and 0% C held for 10 min, linear over 10 min to 77% A, 20% B, and 3% C, linear over 5 min to 70% A, 25% B, and 5% C, linear over 5 min to 25% A, 50% B, and 25% C, following a 10 min equilibration step. Separation of ferulate dimers and trimers: initially 85% A, 15% B, and 0% C, linear over 15 min to 82% A, 18% B, and 0% C, linear over 5 min to 80% A, 20% B, and 0% C, linear over 5 min to 72% A, 25% B, and 3% C, linear over 5 min to 70% A, 25% B, and 5% C, linear over 10 min to 65% A, 30% B, and 5% C held for 5 min, linear over 10 min to 55% A, 40% B, and 5% C, following a rinsing and an equilibration step.

RESULTS AND DISCUSSION

HPLC procedures described for the determination of ferulic acid dehydrodimers often lack specificity. A major problem is the separation of the early eluting ferulate dimers (especially the 8-8-dehydrodimers) from late-eluting monomers such as *trans/cis*-ferulic and *trans/cis-p*-coumaric acid. Also, the vast differences in abundance of the dominant ferulic and *p*-coumaric acids as compared to the minor oligomeric compounds often lead to additional problems in the chromatography. As a result, the 8-8-dimers were often reported to be absent in plant samples that should contain these dimers. The recent discovery of an additional 8-8-coupled dimer, the 8-8(tetrahydrofuran)-dehydrodiferulic acid (*27*), and of several dehydrotrimers (*8*) adds to the complexity of the analysis of ferulate dimers. A preseparation of phenolic monomers from dimers/trimers by using Sephadex LH-20 was developed earlier by our group and applied to several materials (*30*); however, this procedure is very time-consuming, and recovery rates were decreased by the additional separation step (*31*). Two different HPLC gradients were developed to separate (a) the phenolic monomers and (b) the ferulate dehydrodimers and the three likely dominating ferulate dehydrotrimers. This approach requires two HPLC runs to determine all phenolic compounds in the samples; however, the same alkaline extract can be used for the HPLC separation (after 1/10 dilution of the extract for the determination of the monomers). Different from most studies describing the separation of phenolic acids, a pheny-hexyl-HPLC column was used as stationary phase instead of the more often used C18 or C8 columns. The phenyl-hexyl stationary phase is able to interact with phenolic metabolites via $\pi-\pi$ linkages, resulting in a higher selectivity for phenolic compounds. This effect is described to be more distinct by applying MeOH as an organic modifier as compared to using ACN. In our system, a tertiary gradient made up of 1 mM TFA, MeOH, and ACN was applied.

Separation and Detection of Phenolic Monomers. Although the focus of this study was to develop a suitable methodology to separate ferulate dimers and trimers, a separation of the most common monomers in cereal grain alkaline hydrolysates was developed as well. The phenolic monomers cover phenolic acids that were found in cereals in the past: *trans/cis*-ferulic acid, *trans/cis-p*-coumaric acid, *trans*-sinapic acid, *trans*-caffeic acid, 4-hydroxyphenylpropionic acid, 4-hydroxyphenylacetic acid, syringic acid, vanillic acid, and 4-hydroxybenzoic acid. Oxidative degradation of hydroxycinnamic acids under alkaline conditions involves the decomposition of the propenyl side chain resulting in the formation of benzaldehyde derivatives. Thus, the degradation products of the
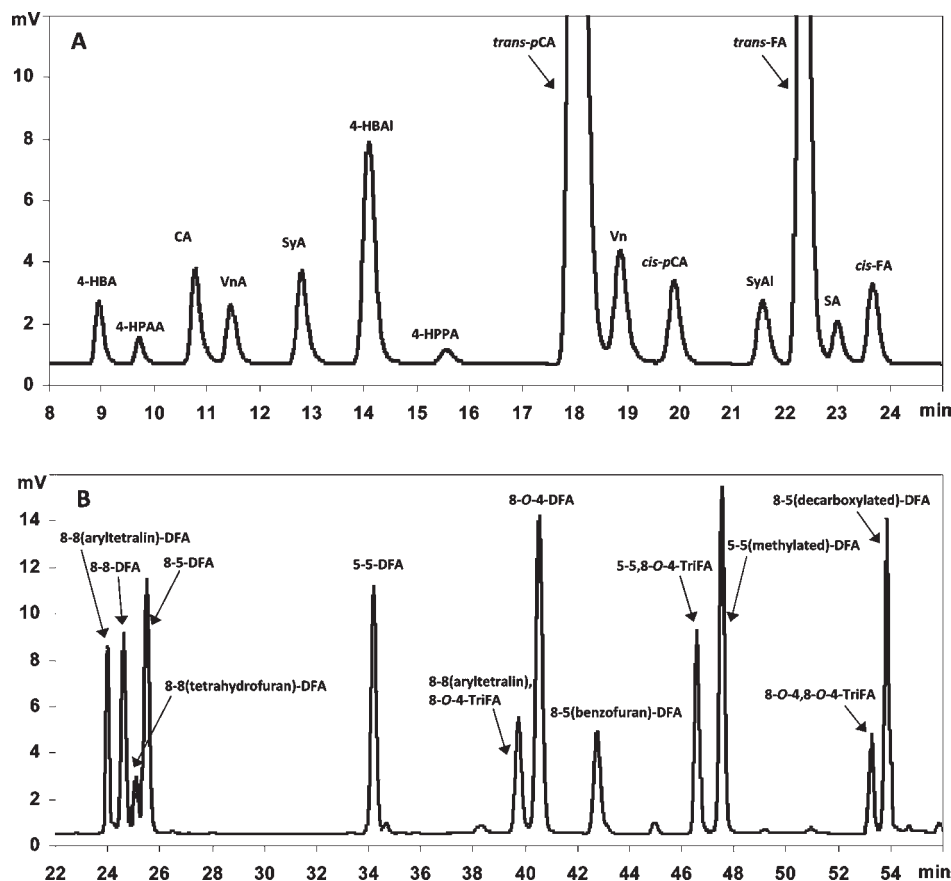
Figure 1. Separation of A) phenolic monomers and B) ferulate dehydrodimers (DFA) and -trimers (TriFA) by using RP-HPLC/UV (detection at 280 nm). 4-HBA: 4-hydroxybenzoic acid, 4-HPAA: 4-hydroxyphenylacetic acid, CA: caffeic acid, VnA: vanillic acid, SyA: syringic acid, 4-HBAl: 4-hydroxybenzaldehyde, 4-HPPA: 4-hydroxyphenylpropionic acid, *trans*-pCA: *trans-p*-coumaric acid, Vn: vanillin, *cis*-pCA: *cis-p*-coumaric acid, SyAl: syringaldehyde, *trans*-FA: *trans*-ferulic acid, SA: sinapic acid, *cis*-FA: *cis*-ferulic acid.

Table 2. Relative Retention Times and Correction Factors of Phenolic Monomers (for Abbreviations See **Figure 1**) in Relation to Caffeic Acid as Well as of Ferulic Acid Dehydrodimers (DFA) and Ferulic Acid Dehydrotrimers (TriFA) in Relation to 5-5(Methylated)-dehydrodiferulic Acid[a]

phenolic monomers	relative retention time	correction factor	DFAs and TriFAs	relative retention time	correction factor
4-HBA	0.83	1.672	8-8(aryltetralin)-DFA	0.54	4.350
4-HPAA	0.89	5.458	8-8-DFA	0.58	1.936
CA	1.00	1.000	8-8(tetrahydrofuran)-DFA	0.59	6.040
VnA	1.06	1.316	8-5-DFA	0.60	1.509
SyA	1.18	0.799	5-5-DFA	0.74	1.514
4-HBAl	1.32	0.320	8-8(aryltetralin),8-*O*-4-TriFA	0.85	3.591
4-HPPA	1.46	5.255	8-*O*-4-DFA	0.86	0.845
trans-pCA	1.70	0.444	8-5(benzofuran)-DFA	0.91	4.597
Vn	1.76	0.565	5-5,8-*O*-4-TriFA	0.98	2.089
cis-pCA	1.86	0.774	5-5(methylated)-DFA	1.00	1.000
SyAl	2.00	1.145	8-*O*-4,8-*O*-4-TriFA	1.12	-[b]
trans-FA	2.09	0.754	8-5(decarboxylated)-DFA	1.14	1.341
SA	2.14	1.845			
cis-FA	2.21	1.111			

[a] As described in the text, different gradient systems were used for the separation of the monomers and dimers/trimers, respectively. [b] Matrix calibration was not performed; see the text.

dominant hydroxycinnamic acids vanillin (formed from ferulic acid), 4-hydroxybenzaldehyde (from *p*-coumaric acid), and syringaldehyde (from sinapic acid) were integrated into the methodology. Baseline separation (less than 10% peak overlap) of all 14 monomers within 24 min (**Figure 1A**) was achieved by using only small amounts of MeOH in the gradient. While higher amounts of MeOH in the eluent significantly increased the separation of the dimers and trimers, higher MeOH portions were actually detrimental for the separation of the monomers. Monomer detection was performed at 280 nm as only those phenolic

monomers with a propenylic side chain show adsorption at 325 nm, too. Depending on the plant sample, one of the monomeric phenolic compounds included in this methodology can be used as an internal standard (if wished), or other compounds such as 3-hydroxy-5-methoxybenzaldehyde or *ortho*-coumaric acid can be integrated into the method. In our studies analyzing corn stover, caffeic acid was not naturally occurring in any detectable levels in our samples, making caffeic acid a convenient internal standard for the determination of phenolic monomers. The relative retention times in relation to caffeic acid are given in **Table 2**.
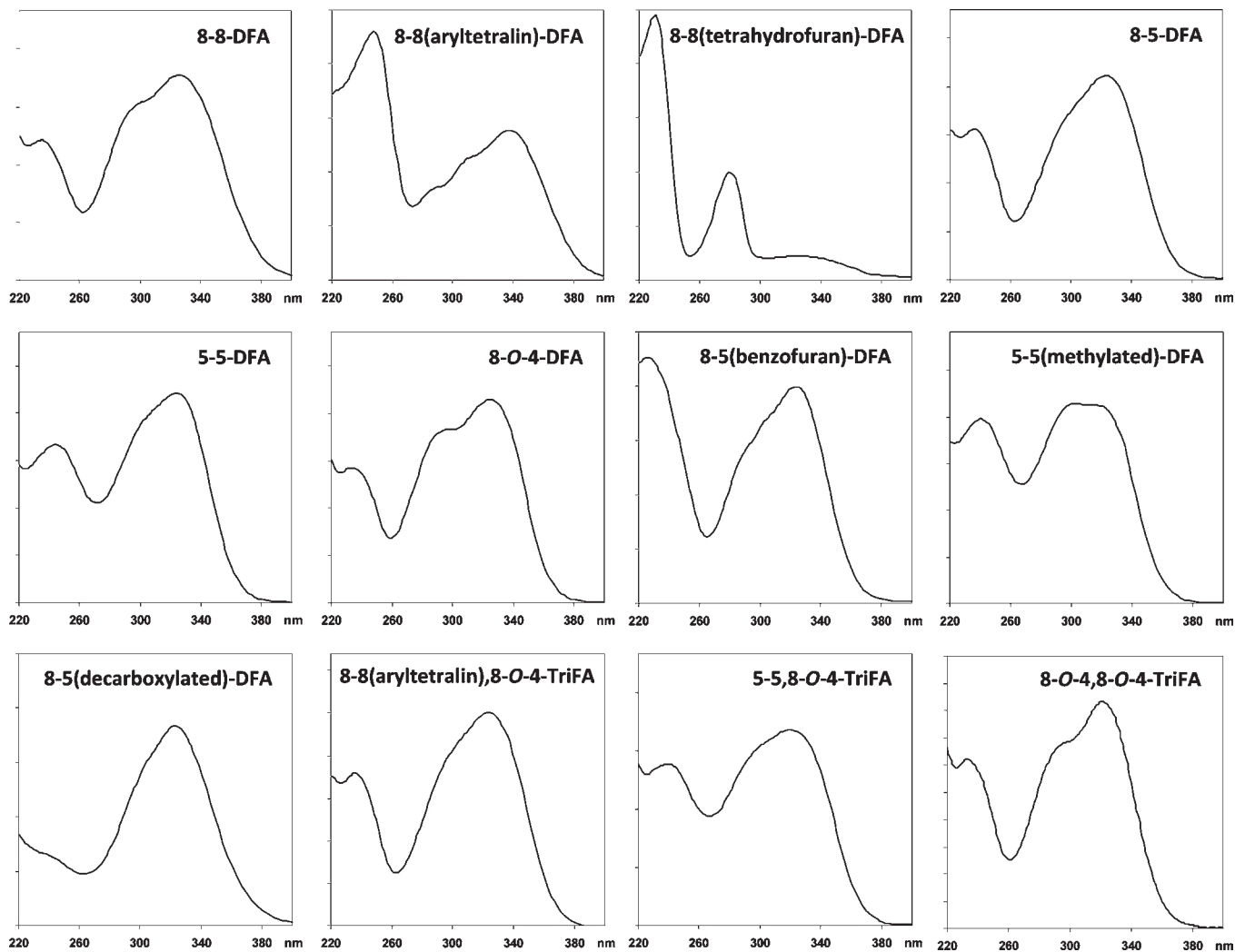
Figure 2. UV-spectra of ferulate dehydrodimers (DFA) and -trimers (TriFA).

Separation and Detection of Ferulate Dehydrodimers and Dehydrotrimers. With the exception of the 4-*O*-5-dehydrodiferulic acid and the 8-8(tetrahydrofuran)-dehydrodiferulic acid, all other dehydrodiferulic acids were semipreparatively isolated from corn bran. While the 8-8(tetrahydrofuran)-dehydrodiferulic was prepared from a synthesized precursor, the 4-*O*-5-dehydrodiferulic acid, which generally only occurs in traces in cereal samples (*17*,*32*), was not integrated into the methodology. Three major trimers, 5-5/8-*O*-4-, 8-8(aryltetralin)/8-*O*-4-, and 8-*O*-4/8-*O*-4-dehydrotriferulic acids, were isolated from corn bran as well.

The UV spectra of all dimers and trimers are shown in **Figure 2**. The detection of the dimers and trimers was performed at 280 nm, respectively. As discussed later, the choice of 280 versus 325 nm (another often used wavelength for hydroxycinnamate derivatives) has some implications on the selectivity of the method when applied to certain plant materials; however, choosing 325 nm would exclude the detection of the recently discovered 8-8-(tetrahydrofuran)-diferulic acid. Of course, these problems can be avoided by using a PDA or a dual wavelengths detector (if available) instead of a single wavelength UV detector. For the separation of the nine dimers and three trimers, MeOH was only used in the eluent after 20 min to achieve the separation from the phenolic monomers. Introducing MeOH was, however, crucial to achieve a good separation of the dimers and trimers in an acceptable time (55 min) (**Figure 1B**). The relative retention times in relation to 5-5(methylated)-dehydrodiferulic acid are given in **Table 2**.

Method Validation, Phenolic Monomers. Homogeneity of variances was given for all monomers in the concentration ranges tested in the basic calibration. The most important validation parameters for the phenolic monomers are shown in **Table 3**. The limits of detection and quantitation can be largely decreased by measuring the absorption maxima of the individual compounds as opposed to measuring at a single wavelength (280 nm). The coefficients of variation of the procedure, calculated from the residual standard deviation, the sensitivity (slope), and the center of the tested concentration range, was between 2.1 and 4.8% for the basic calibration and 1.2 and 5.4% for the matrix calibration. With the exception of the 4-hydroxyphenylacetic acid, the recoveries for the phenolic monomers were between 97 and 109%, which may indicate some coelution from matrix compounds, especially lignin degradation products derived from the saponification process. As these compounds are, however, unknown and may change from sample to sample depending on factors such as lignin composition and degree of polymerization, it is not possible to adequately address this problem by using a rather unspecific detection at 280 nm or other suitable UV wavelengths. Overall, the method described is a good procedure for the determination of the phenolic monomers without any coelution from dimers and higher ferulate oligomers resulting in reliable results. However, depending on the sample, the influence of the matrix, especially of ligninlike compounds, has to be kept in mind and critically evaluated.

Table 3. Calibration Equations, Correlation Coefficients, Limits of Detection (LOD) and Limits of Quantitation (LOQ), and Coefficients of Variation of the Procedure (CV) for the Basic Calibration (BC) of the Phenolic Monomers (for Abbreviations See **Figure 1**) in the Concentration Ranges Tested as Indicated[a]

	range tested (μg/mL)	calibration equation (linear model)	correlation coefficients	LOD (μg/mL)	LOQ (μg/mL)	CV (BC) (%)	CV (MC) (%)	recovery (%)
4-HBA	2.5–12.5	$y = 3894x - 335$	0.9992	0.05	0.15	2.1	3.2	103
4-HPAA	2.5–12.5	$y = 1231x - 444$	0.9982	0.34	1.03	3.0	4.2	80
CA	2.5–12.5	$y = 6307x - 2375$	0.9956	0.04	0.11	4.8	3.9	106
VnA	2.5–12.5	$y = 4807x - 976$	0.9989	0.05	0.15	2.3	2.2	106
SyA	2.5–12.5	$y = 7853x - 1233$	0.9982	0.04	0.12	3.0	2.5	102
4-HBAl	2.5–12.5	$y = 20004x - 3481$	0.9985	0.02	0.06	2.7	2.0	105
4-HPPA	2.5–12.5	$y = 1203x - 115$	0.9975	0.43	1.29	3.6	4.6	108
trans-pCA	25–125	$y = 13936x - 26284$	0.9985	0.05	0.15	2.5	5.3	105
Vn	2.5–12.5	$y = 11721x - 3498$	0.9985	0.08	0.24	2.8	2.0	104
cis-pCA	2.5–12.5	$y = 8148x - 2119$	0.9985	0.04	0.11	2.8	5.4	102
SyAl	2.5–12.5	$y = 5432x - 1394$	0.9989	0.05	0.16	2.4	3.0	107
trans-FA	25–125	$y = 8295x - 18779$	0.9984	0.07	0.21	2.7	2.1	100
SA	2.5–12.5	$y = 3272x + 746$	0.9978	0.12	0.34	3.3	3.3	109
cis-FA	2.5–12.5	$y = 5593x - 837$	0.9987	0.04	0.11	2.6	1.2	97

[a] The recovery was determined from the matrix calibration as described in the text. Also, the coefficients of variation of the procedure for the matrix calibration (MC) are shown.

Table 4. Calibration Equations, Correlation Coefficients, Limits of Detection (LOD) and Limits of Quantitation (LOQ), and Coefficients of Variation of the Procedure (CV) for the Basic Calibration (BC) of the Ferulic Acid Dehydrodimers (DFA) and Dehydrotrimers (TriFA) in the Concentration Ranges Tested as Indicated[a]

|	range tested (μg/mL)	calibration equation (linear model)	correlation coefficients	LOD (μg/mL)	LOQ (μg/mL)	CV (BC) (%)	CV (MC) (%)	recovery (%)
8-8(aryltetralin)-DFA	2.5–12.5	$y = 0.344x - 0.059$	0.9975	0.30	0.89	3.6	7.6	90
8-8-DFA	2.5–12.5	$y = 0.604x - 0.576$	0.9893	0.21	0.63	7.5	47.5	81
8-8(tetrahydrofuran)-DFA	2.5–12.5	$y = 0.198x - 0.312$	0.9931	0.43	1.28	6.9[b]	45.5	76
8-5-DFA	6.0–30.0	$y = 1.005x - 1.052$	0.9984	0.25	0.76	2.9	18.2	81
5-5-DFA	6.0–30.0	$y = 0.936x - 1.153$	0.9980	0.21	0.63	3.2	24.8	86
8-8(aryltetralin),8-*O*-4-TriFA	6.0–30.0	$y = 0.397x - 2.205$	0.9864	1.01	3.04	8.4	80.1	34
8-*O*-4-DFA	6.0–30.0	$y = 1.553x - 1.522$	0.9997	0.20	0.62	1.1	24.4	89
8-5(benzofuran)-DFA	6.0–30.0	$y = 0.537x - 0.440$	0.9997	0.38	1.13	1.3	13.6	53
5-5,8-*O*-4-TriFA	2.5–12.5	$y = 0.695x - 1.318$	0.9839	0.44	1.33	9.2	65.5	45
5-5(methylated)-DFA	6.0–30.0	$y = 1.462x - 0.893$	0.9981	0.18	0.54	3.1	11.5	86
8-*O*-4,8-*O*-4-TriFA	1.0–5.0	$y = 0.340x - 0.015$	0.9979	0.13	0.40	3.3	-	-
8-5(decarboxylated)-DFA	2.5–12.5	$y = 1.093x - 0.333$	0.9992	0.15	0.46	2.1	57.4	62

[a] The recovery was determined from the matrix calibration as described in the text. Also, the coefficients of variation of the procedure for the matrix calibration (MC) are shown.
[b] The concentration 10 μg/mL was eliminated as an outlier within the calibration function.

Method Validation, Ferulic Acid Dehydrodimers and Dehydrotrimers. Homogeneity of variances was given for the dimers and trimers in the concentration ranges tested in the basic calibration. The results for the basic calibration are generally acceptable with coefficients of variation of the procedure ranging from 1.1 to 7.5% for the ferulic acid dehydrodimers and 3.3 to 9.2% for the ferulic acid dehydrotrimers (**Table 4**). However, variation coefficients of the procedure for the matrix calibration are severely impaired by the influence of the matrix and/or the extraction procedure, resulting in coefficients ranging between 7.6 and 57.4% for the dimers and 65.5 and 80.1% for the trimers (due to the limited amount of the standard compound the matrix calibration for 8-*O*-4/8-*O*-4-dehydrotriferulic acid was not performed). Also, the recoveries for some dimers [8-8(tetrahydrofuran)-, 8-5(benzofuran)-, and 8-5(decarboxylated)-dehydrodiferulic acids] and the trimers do not meet the general acceptance criterion (80–110%). Factors, which probably most heavily influence the variation coefficients and/or the recovery, are matrix lignin, the liquid/liquid extraction step, and redissolving the extracted phenolics after evaporating the diethyl ether. Alkaline degradation products from the lignin preparation can coelute with the analytes, thus influencing the precision (and the accuracy, although the recoveries are generally too low for the dimers/trimers). While this effect can have an effect on samples such as grass, stems, etc., which are more heavily lignified, this effect should be minor for the analysis of cereal grains, which are, if at all, only slightly lignified (*33–35*). The precision can also be impacted by the reproducibility of redissolving the dimers/trimers in THF/water after evaporation. Redissolving some ferulate dimers/trimers in general can be an issue. From our preparative experiences, a THF/water mixture was preferred over the generally used MeOH/water mixture as THF/water was a better solvent and only slightly influenced the subsequent chromatography. However, the results of the matrix calibration clearly indicate that analytical data for the ferulic acid dehydrodimers and trimers should be used very carefully; in certain cases, it might be more appropriate to talk about a semiquantitative determination, reflecting the limited precision and accuracy for some of the dimers and trimers.

Application to Plant Samples. The developed methodology was applied to a range of plant fibers from corn stover (leaves and stems), whole corn, wheat and rye grains, wild rice, sugar beet, and asparagus. The comparison of the chromatograms of corn stover (**Figure 3A**) and corn grains (**Figure 3B**) reflects the susceptibility of the HPLC method to interference by other UV-active compounds released from the plant cell wall, for example, lignin degradation products. Corn grain cell walls are only slightly lignified, and because of the protection of the grains by the husk, photochemically formed cyclobutandimers of ferulic and/or p-coumaric acid (*36*) are only minor. Accordingly, analysis of the chromatogram of the alkaline corn grain hydrolysate is straightforward. On the other hand, corn stover cell walls can be heavily lignified (stem), and because of the influence of light (i.e., true for the leaves), cyclobutane dimers are very abundant (*28*).
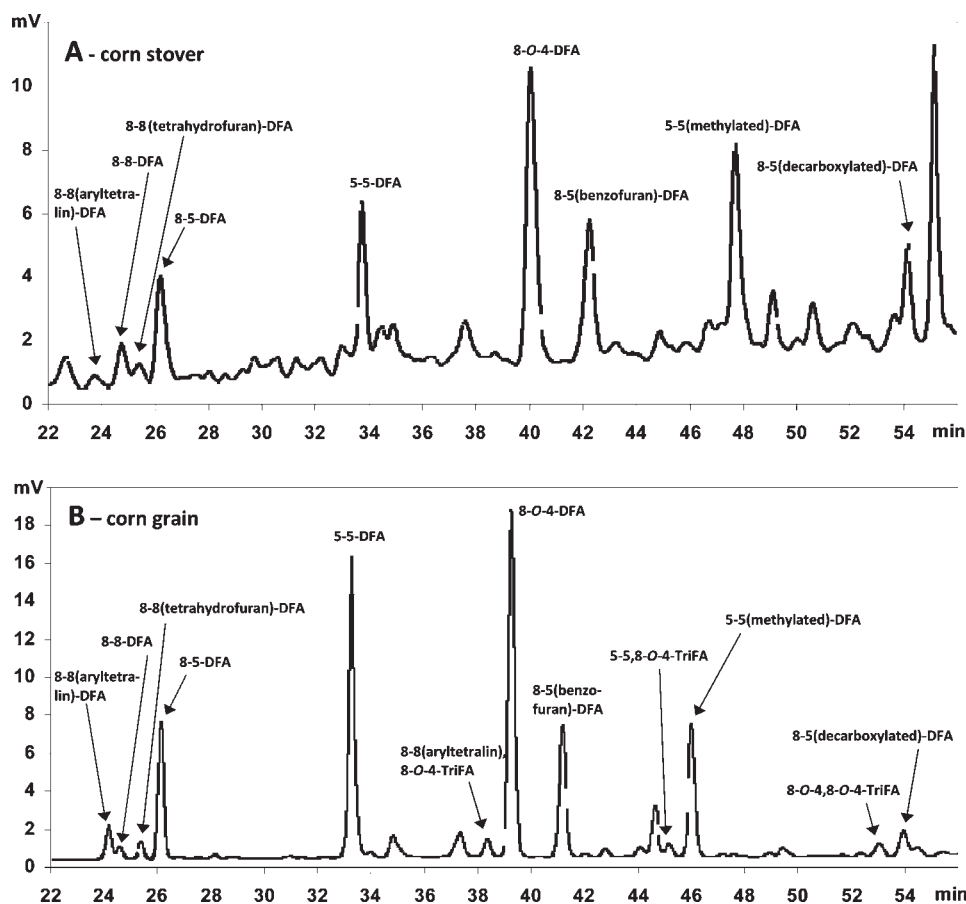
Figure 3. Separation of ferulate dehydrodimers (DFA) and -trimers (TriFA) in (**A**) corn stover and (**B**) insoluble fibers from whole corn grains (detection at 280 nm).

Table 5. Concentrations of ferulic acid dehydrodimers (DFA) and dehydrotrimers (TriFA) in Insoluble Fibers from Different Plant Materials

	corn stover	corn grain	wheat grain	rye grain	sugar beet	asparagus
$\sum$DFA (μg/g)	2091	13965	1956	1925	1586	545
$\sum$8-8 (% DFA)	24	21	25	26	26	44
$\sum$8-5 (% DFA)	57	42	44	45	43	43
5-5 (% DFA)	8	19	16	16	15	4
8-*O*-4 (% DFA)	11	17	16	14	16	9
$\sum$TriFA (μg/g)		916				
5-5,8-*O*-4 (% TriFa)	$-^a$	86	$+^b$	+	+	+
8-8(aryltetralin),8-*O*-4 (% TriFA)	$-$	6	+	+	+	+
8-*O*-4,8-*O*-4 (% TriFA)	$-$	8	+	+	+	+

[a] Not detected. [b] Detected but the amounts were below the quantitation limits.

The interference of the cyclobutane dimers can be reduced by measuring at 325 nm where cyclobutane dimers do not absorb. If a two-wavelength UV detector is available, the use of both wavelengths is recommended, with the use of a PDA ideal. However, measuring at 325 nm does not eliminate all problems with coeluting compounds: Although we isolated and identified the ferulic acid trimers integrated in the methodology formerly from the same stover material (*28*), we were not able to unambiguously identify or even quantitate them by using the developed methodology. Whereas the identification and quantitation of the ferulic acid dehydrodimers and -trimers from rye and wheat grain fiber and from sugar beet fiber were straightforward (**Table 5**), problems with coelution occurred for asparagus and especially for wild rice. In both cases, coelution was apparent for the early eluting dimers. It is known that wild rice contains considerable amounts of 8-8-linked dehydrodisinapic dimers (*29*),

which might be the coeluting compounds. Because of the severe coeluting in the wild rice chromatograms, no data were given for wild rice in **Table 5**.

All three trimers were identified in the analyzed samples with the exception of wild rice in which the 5-5/8-*O*-4- and 8-*O*-4/8-*O*-4-dehydrotriferulic acids were detected only. Although identifiable, the amounts of dehydrotrimers were below the quantitation limit in most of the samples. Up to now, only the 5-5/8-*O*-4-trimer was identified in other plant materials than corn bran, for example, in rye and in wheat (*37−41*). The existence of ferulic acid trimers was also suggested for asparagus; however, because of a lack of standard compounds, the trimers were not identified (*42*).

In conclusion, a methodology for the separation of 11 dehydrodimers and dehydrotrimers of ferulic acid without coelution of the most common monomeric phenolic acids and their degradation products was developed. Whereas data for the basic

calibration met acceptance criteria, the matrix calibration revealed that lignin-rich matrices lead to both unsatisfactory precision and accuracy of the method. Precision and accuracy of the method can be impaired by matrix components but also by the liquid/liquid extraction step and inadequate redissolution of the dimers and trimers after liquid—liquid extraction and evaporation. While it is likely that sample preparation problems can be addressed in further studies by testing more solvent combinations, the problem of coeluting lignin degradation products requires a mass spectrometric detection to be properly addressed. Unvalidated HPLC/UV methodologies were used in the past to determine ferulic acid dimers and trimers (no validation data were published to the best of our knowledge), and often because of the lack of standard compounds, the identification of the ferulic acid oligomers was often performed by means of retention time and (rather unspecific) UV spectra. The validation data of the method presented should be taken as a note of caution not to over-interpret data from nonvalidated methodologies. In addition, data obtained from nonvalidated methods should be labeled as semiquantitative data as compared to quantitative data.

ABBREVIATIONS USED

ACN, acetonitrile; MeOH, methanol; PDA, photodiode array detector; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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