JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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A Stable-Isotope Dilution GC-MS Approach for the Analysis of DFRC (Derivatization Followed by Reductive Cleavage) Monomers from Low-Lignin Plant Materials

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ABSTRACT: The derivatization followed by reductive cleavage (DFRC) method is a well-established tool to characterize the lignin composition of plant materials. However, the application of the original procedure, especially the chromatographic determination of the DFRC monomers, is problematic for low-lignin foods. To overcome these problems a modified sample cleanup and a stable-isotope dilution approach were developed and validated. To quantitate the diacetylated DFRC monomers, their corresponding hexadeuterated analogs were synthesized and used as internal standards. By using the selected-ion monitoring mode, matrix-associated interferences can be minimized resulting in higher selectivity and sensitivity. The modified method was applied to four low-lignin samples. Lignin from carrot fibers was classified as guaiacyl-rich whereas the lignins from radish, pear, and asparagus fibers where classified as balanced lignins (guaiacyl/syringyl ratio=1−2).

KEYWORDS: *DFRC method, plant-based food, dietary fiber, lignin composition, monolignol*

INTRODUCTION

Lignin, an integral component of (secondary) plant cell walls, is a complex polymer that mainly derives from three monomers (monolignols): *p*-coumaryl, coniferyl, and sinapyl alcohols. Building a polymer through oxidative coupling, these monomers form *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units.[1] Radical polymerization of the monomers results in different types of linkages such as β-O-4, β-5, $\beta-\beta$, 5−5, and β-1 linkages, with the β-O-4 linkage being generally dominant.[2] The composition of lignin varies among taxa, tissues, and cell types and depends on the maturation stage of the plants. Lignins from gymnosperms mainly consist of G units, dicotyledonous angiosperms of G and S units, and monocotyledonous angiosperms of H, G, and S units.[3] Also, the amount of S units usually increases during plant growth.[3]

In addition to indigestible polysaccharides and oligosaccharides, lignin is part of the dietary fiber complex. In general, dietary fibers are known to be beneficial to human health with, however, large differences between fiber types.[4] Lignified fibers in particular have been suggested to reduce the colon cancer risk by adsorbing carcinogens such as heterocyclic aromatic amines.[5−7] The extent of in vitro adsorption depends on lignin content as well as lignin composition.[5] Although plant-based foods are a source of lignified dietary fiber, most studies on lignin are limited to wood and forages. The lignin contents and particularly the monomer composition and linkage types of the lignin polymers from plant foods are poorly investigated.

A popular method to characterize the monomer composition of lignin is, next to the thioacidolysis,[8] the derivatization followed by reductive cleavage (DFRC) method, which was first published by Lu and Ralph in 1997.[9,10] Although the different types of linkages in the lignin polymer are resistant to most chemical treatments, the DFRC method can be used to selectively cleave (α- and) β-aryl-ethers to liberate monolignols and to determine their ratio in the polymer. The method is frequently used for the determination of H, G, and S units in lignins from, for example, woods and grasses; its application to plant-based foods is, however, rare.[11] The lignin contents of plant-based human foods are generally lower than in woody samples, resulting in high amounts of matrix (mostly derived from cell-wall polysaccharides), which often contaminates the gas chromatographic system including a potential MS detector. Also, the matrix components can coelute, which complicates the identification and quantification of the DFRC products, especially if a nonselective FID is used for detection.

Another detail of the method that needs to be optimized is the internal standard used for quantification. The protocol of the original method describes tetracosane as internal standard,[9] but 4,4-ethylidenebisphenol is also used.[11] Both compounds do not ideally comply with the requirements of an internal standard because their structures are not similar to the DFRC products. Thus, it is likely that the internal standard does not correctly compensate for sample losses during sample preparation.

Thus, the aim of this study was to develop and validate a stable-isotope dilution GC-MS method for quantification of DFRC products from low-lignin samples. Deuterated DFRC products were synthesized as internal standards. To avoid impurities of the MS system, the sample preparation protocol was modified, in particular the sample cleanup.

MATERIALS AND METHODS

Chemicals. Heat-stable α-amylase Termamyl 120 L (from *Bacillus licheniformis*, 120 KNU/g), the protease Alcalase 2.5 L (from *Bacillus licheniformis*, 2.5 AU/g), and the amyloglucosidase AMG 300 L (from

Received: December 22, 2014
Revised: February 24, 2015
Accepted: February 28, 2015
Published: February 28, 2015

DOI: 10.1021/jf506221p
J. Agric. Food Chem. 2015, 63, 2668−2673

Aspergillus niger, 300 AGU/g) were from Novozymes, Bagsvaerd, Denmark. The complex carbohydrase mixture Driselase (from Basidiomycetes), coniferaldehyde, sinapaldehyde, *p*-coumaric acid, borohydride exchange resin (2.5−5.0 mmol BH_4^-/g resin), acetyl bromide, diisobutylaluminum hydride, and the deuterated solvents were from Sigma-Aldrich, St. Louis, MO. Acetic anhydride, NaOH, NH_4Cl, Na_2HPO_4, NaH_2PO_4, and ethyl acetate were from Roth, Karlsruhe, Germany. Pyridine, glacial acetic acid, $MgSO_4$, HCl, methanol, dichloromethane, petroleum ether, *n*-hexane, 1,4-dioxane, ethanol, and acetone were obtained from VWR, Radnor, PA.

General. NMR analyses were performed on a Bruker (Rheinstetten, Germany) 250 MHz spectrometer using acetone-d_6 as solvent. NMR data of synthesized monolignols and their precursors were compared to those described in the lignin model database.[12] Solid-phase extraction (SPE) columns (Discovery, LC-SI, 500 mg/3 mL) were purchased from Supelco, Bellefonte, PA and Phenomenex, Torrance, CA (STRATA-X, polymeric reversed phase, 200 mg/3 mL). Filter papers were obtained from Macherey & Nagel, Düren, Germany (4−12 μm), and Roth, Karlsruhe, Germany (12−15 μm). GC analyses were carried out on a Shimadzu (Kyoto, Japan) GC-MS system (GC-2010plus, GC-MS-QP2010Ultra) using a 30 m × 0.25 mm, 0.25 μm Rtx-5MS column (Restek, Bellefonte, PA) and on a GC-FID system (GC-2010Plus) using a 30 m × 0.32 mm, 0.25 μm HP-5 column (Agilent, Santa Clara, CA).

Synthesis of Coniferyl and Sinapyl Alcohol. Coniferyl and sinapyl alcohols were synthesized from coniferaldehyde and sinapaldehyde as previously described.[13] In brief, coniferaldehyde/sinapaldehyde (205 mg/200 mg) were reduced in methanol (MeOH) (8 mL) by using a borohydride exchange resin (BER; 920 mg/770 mg), which was washed twice with 6 mL of MeOH before use. The reaction mixture was stirred at room temperature for 1 h/3 h. The resin was filtered off through glass wool, and the filtrate was evaporated. Cleanup was carried out using silica gel SPE columns: The columns were conditioned with 5 mL of ethyl acetate, the reaction mixture was applied in 5 mL of ethyl acetate, and the columns were eluted with 25 mL of ethyl acetate. Crystallization from dichloromethane/petroleum ether yielded pale yellow needles (57−67%) of coniferyl alcohol. Dichloromethane was added to the dried sinapyl alcohol, and the solution was kept in a freezer for several days. Since no crystallization was achieved, sinapyl alcohol was obtained as a yellow oil (67−95%).

Synthesis of *p*-Coumaryl Alcohol. *p*-Coumaryl alcohol was synthesized from *p*-coumaric acid in two steps as previously described.[14] Ethyl *p*-coumarate was prepared by stirring 1 g of *p*-coumaric acid and 5 mL of acetyl chloride in 50 mL of ethanol at room temperature for 17 h. After evaporation of the solvents, the procedure was repeated. The product was crystallized from ethyl acetate/petroleum ether. Ethyl *p*-coumarate was reduced using diisobutylaluminum hydride (DIBAL-H). Ethyl *p*-coumarate (640 mg) in 30 mL of toluene was ice cooled under nitrogen, and 12 mL of DIBAL-H was slowly added. The reaction mixture was stirred for 1 h, and the reaction was quenched by adding 5 mL of ethanol. The solvent was partially removed under reduced pressure, and 50 mL of water was added. The aqueous layer was extracted four times by using ethyl acetate. Pale yellow crystals of *p*-coumaryl alcohol (85−94%) were obtained by crystallization from dichloromethane/petroleum ether.

Synthesis of Diacetylated *p*-Coumaryl, Coniferyl, And Sinapyl Alcohols. Diacetylation of the three *p*-hydroxycinnamyl alcohols was carried out as described in the protocol of the DFRC method.[9] *p*-Hydroxycinnamyl alcohols (*p*-coumaryl alcohol, 162 mg; coniferyl alcohol, 189 mg; sinapyl alcohol, 216 mg), acetic anhydride (3.3 mL), and pyridine (3.2 mL) were stirred for 40 min at room temperature. The solvents were evaporated, and coevaporation was carried out using ethanol. The diacetylated compounds were obtained as yellow oils. For the synthesis of deuterium-labeled (hexadeuterated) *p*-hydroxycinnamyl alcohols acetic anhydride-d_6 was used. ^{1}H NMR (*p*-coumaryl diacetate-d_6), δ [ppm]: δ 7.50 (d, 2H, J = 8.6 Hz, H2/H6), 7.10 (d, 2H, J = 8.7 Hz, H3/H5), 6.71 (d, 1H, J = 16.0 Hz, Hα), 6.35 (dt, 1H, J = 16.0, 6.2 Hz, Hβ), 4.69 (dd, 2H, J = 6.2, 1.4 Hz, Hγ). ^{1}H NMR (coniferyl alcohol diacetate-d_6), δ [ppm]: δ 7.23 (s, 1H, H2), 7.02 (m, 2H, H5/H6), 6.69 (dt, 1H, J = 15.9, 1.3 Hz, Hα), 6.37 (dt, 1H, J = 15.9, 6.2 Hz, Hβ), 4.69 (dd, 2H, J = 6.2, 1.4 Hz, Hγ), 3.85 (s, 3H, OCH_3). ^{1}H NMR (sinapyl alcohol diacetate-d_6), δ [ppm]: δ 6.85 (s, 2H, H2/H6), 6.67 (dt, 1H, J = 15.9, 1.2 Hz, Hα), 6.39 (dt, 1H, J = 15.9, 6.2 Hz, Hβ), 4.69 (dd, 2H, J = 6.2, 1.3 Hz, Hγ), 3.81 (s, 6H, OCH_3).

Plant Material. All fruits and vegetables (pear (*Pyrus communis* L.), carrot (*Daucus carota* L. subsp. *sativus*), asparagus (*Asparagus officinalis* L.), and small radish (*Raphanus sativus* L. var. *sativus*), also known as European radish) were obtained from a local grocery store. Asparagus was peeled whereas pear, carrot, and European radish were used unpeeled; however, the cores of the pears were removed.

Preparation of Insoluble Fibers. All fruits and vegetables were freeze dried and milled to a particle size of <0.5 mm. Dried material (20 g) was suspended in 0.08 M sodium phosphate buffer pH 6.2 (200 mL), and 1.5 mL of α-amylase was added. The suspension was placed in a water bath (92 °C) for 20 min and shaken every 5 min. The samples were cooled to room temperature, and the pH was adjusted to 7.5 with 0.275 M NaOH. After adding 600 μL of protease, the samples were incubated for 30 min at 60 °C with continuous agitation. The samples were cooled down; the pH was adjusted to 4.5 with 0.375 M HCl and, after adding 700 μL of amyloglucosidase, incubated in a water bath for 30 min at 60 °C. The suspensions were filtered through filter paper (12−15 μm), and the residues were washed three times with hot water (60 °C), ethanol, and acetone and finally air dried under the fume hood. To remove small amounts of remaining lignin-like low molecular weight material, the insoluble fibers were further extracted with ethanol (8 h), ethyl acetate (8 h), and *n*-hexane (8 h) using a Soxhlet apparatus. Finally, the insoluble fibers were dried at 40 °C in a vacuum oven.

Enzyme Digestion. Insoluble fibers (1 g) were suspended in 100 mL of water and incubated with 60 mg of Driselase at 37 °C for 48 h. After inactivation of the enzymes (5 min, 100 °C), the samples were centrifuged for 10 min at 2500 rpm and washed twice with water, ethanol, and acetone. The residues were dried in a vacuum oven at 40 °C.

DFRC Method. The DFRC method was performed as described by Bunzel et al.[11] with minor modifications. About 10−25 mg (pear, 24 mg; radish, 11 mg; carrot and asparagus, 15 mg) of enzyme-digested insoluble dietary fiber were mixed with 7.5 mL of acetyl bromide reagent (acetyl bromide/glacial acetic acid, 20/80, v/v), and the mixture was stirred at 50 °C for 3 h. The solvent was removed by rotary evaporation, and 7.5 mL of acidic reduction solvent (dioxane/glacial acetic acid/water, 5/4/1, v/v/v) and about 50 mg of zinc dust were added. The mixture was stirred at room temperature for 40 min. This mixture, 10 mL of dichloromethane, and 10 mL of saturated NH_4Cl were transferred into a separatory funnel, and the internal standards (deuterium-labeled *p*-hydroxycinnamyl alcohol diacetates) were added. The amounts of added standard compounds depend on the dilution after sample cleanup (final internal standard concentration, 30 μM). The organic layer was separated, and extraction of the aqueous phase with 10 mL of dichloromethane was repeated twice. The combined organic layers were washed twice with 10 mL of saturated NH_4Cl and dried over $MgSO_4$. The mixture was filtered through filter paper (4−12 μm), and the solvent was evaporated.

For the acetylation step, 1.5 mL of dichloromethane, 200 μL of pyridine, and 200 μL of acetic anhydride were added to the residue. The mixture was stirred overnight, and the solvents were removed under reduced pressure (coevaporation with ethanol, 3×).

Sample Cleanup. The residue, which was obtained from the DFRC method, was dissolved in 1 mL of MeOH (ultrasonic bath). The mixture was centrifuged (3 min at 13 000 rpm), and the supernatant was removed and evaporated. The residue after evaporation was dissolved in 1 mL of MeOH/H_2O (40/60, v/v). Sample cleanup was carried out using polymeric reversed phase SPE tubes (conditioning with 3 mL of each MeOH and H_2O, application of the sample, washing with 3 mL of each H_2O and MeOH/H_2O (20/80, v/v), elution with 9 mL of MeOH), and the solvent was removed under reduced pressure.

DOI: 10.1021/jf506221p
J. Agric. Food Chem. 2015, 63, 2668−2673

Table 1. GC-MS Fragmentation Patterns of Diacetylated *p*-Coumaryl (HDiAc), Coniferyl (GDiAc), and Sinapyl (SDiAc) Alcohols and Their Hexadeuterated Compounds (d_6)[a]

compound	*m/z* (rel. intensity)
HDiAc	133 (100),[d] 149 (94), 192 (86),[c] 131 (65) 150 (65), 121 (53), 132 (45), 107 (41),[e] 134 (40), 105 (38), 103 (37), 234 (17),[b] 104 (15), 115 (15), 193 (12), 108 (7), 122 (6), 119 (6), 151 (5), 135 (5), 102 (5), 175 (5), 176(5)
HDiAc-d_6	150 (100), 134 (93),[d] 196 (80),[c] 132 (58), 122 (52), 133 (45), 152 (43), 135 (37), 106 (33), 104 (25), 109 (25), 103 (17), 240 (16),[b] 151 (13), 197 (12), 131 (11), 115 (11), 108 (10),[e] 105 (8), 123 (7), 116 (6), 102 (5), 120 (5), 149 (3)
GDiAc	222 (100),[c] 131 (77), 179 (51), 119 (37), 103 (36), 124 (26), 180 (25), 151 (24), 164 (20), 163 (19),[d] 147 (17), 162 (17), 137 (16),[e] 223 (13), 132 (11), 152 (10), 102 (8), 120 (8), 130 (8), 149 (7), 133 (7), 105 (7), 264 (7),[b] 104 (6), 148 (5), 115 (5)
GDiAc-d_6	226 (100),[c] 131 (76), 180 (61), 103 (33), 119 (29), 152 (27), 164 (21),[d] 165 (17), 148 (16), 182 (15), 126 (15), 227 (15), 120 (13), 139 (12), 163 (11), 181 (9), 270 (8),[b] 102 (8), 104 (8), 130 (8), 162 (7), 150 (7), 154 (7), 125 (7), 147 (6), 134 (5), 133 (5), 138 (4)[e]
SDiAc	252 (100),[c] 161 (35), 149 (30), 209 (28), 210 (18), 133 (17), 105 (17), 253 (14), 194 (14), 154 (13), 181 (13), 193 (13),[d] 131 (10), 121 (10), 162 (9), 103 (9), 177 (8), 182 (8), 192 (8), 167 (8), 118 (8), 106 (7), 119 (6), 115 (6), 147 (5), 135 (5), 179 (5), 132 (5), 134 (5), 107 (5), 294 (4),[b] 167 (1)[e]
SDiAc-d_6	256 (100),[c] 161 (32), 210 (31), 149 (27), 133 (15), 105 (15), 195 (15), 257 (15), 194 (13),[d] 182 (12), 212 (12), 162 (9), 131 (9), 103 (9), 121 (8), 156 (8), 150 (7), 118 (6), 193 (6), 115 (6), 184 (6), 132 (6), 119 (5), 106 (5), 211 (5), 178 (5), 164 (5), 300 (3),[b] 168 (3)[e]

[a]Only relative intensities ≥ 3% are shown. The *m/z* values chosen for selected-ion monitoring mode are underlined. [b]Molecular ion. [c]Fragment resulting from ketene loss from the molecular ion. [d]Fragment resulting from McLafferty rearrangement after ketene loss. [e]Benzylic cation fragment.

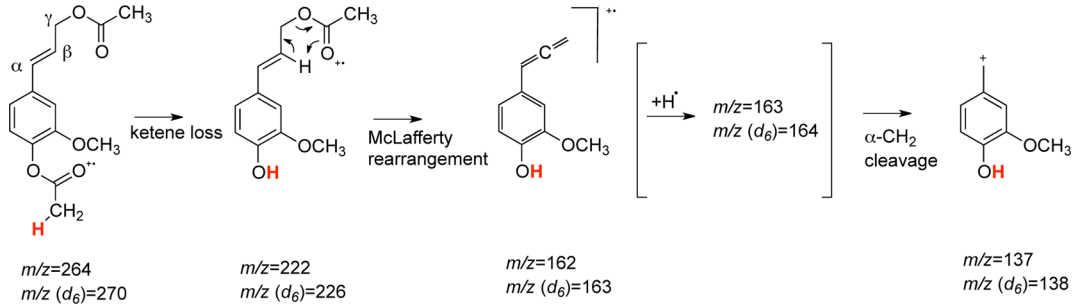

Figure 1. Characteristic fragmentations (EI-MS) of diacetylated coniferyl alcohol. *m/z* values of the products from the nondeuterated and the hexadeuterated (identified by the -d_6 descriptor in the figure) compounds resulting from ketene loss, McLafferty rearrangement, and α-CH_2 cleavage are indicated. Fragmentation reactions of diacetylated *p*-coumaryl and sinapyl alcohols are analogous.

GC-MS and GC-FID Analysis. DFRC monomers were separated and quantitated by GC-MS (EI) using He (1 mL/min) as carrier gas. GC conditions were as follows: initial column temperature 160 °C, ramped at 10 °C/min to 230 °C, ramped at 5 °C/min to 240 °C, ramped at 20 °C/min to 260 °C, ramped at 10 °C/min to 300 °C and held for 10 min; injector temperature 250 °C; split ratio 1/30. MS conditions were as follows: ion source temperature 220 °C, interface temperature 275 °C. Quantification was carried out in the selected-ion monitoring (SIM) mode using ions with the following *m/z*: *m/z* = 192/196 for *p*-coumaryl alcohol diacetate/*p*-coumaryl alcohol diacetate-d_6; *m/z* = 222/226 for coniferyl alcohol diacetate/coniferyl alcohol diacetate-d_6; *m/z* = 252/256 for sinapyl alcohol diacetate/sinapyl alcohol diacetate-d_6. Mass spectrometric detection was carried out only near the retention times of the compounds to avoid unnecessary contamination of the MS system.

GC-FID was used for analyzing low-lignin samples using the original DFRC protocol.[9] GC-FID conditions were as follows: He (1 mL/min) as carrier gas, injector temperature 220 °C, split ratio 1/10, detector temperature 310 °C. GC conditions: initial column temperature 160 °C, ramped at 5 °C/min to 210 °C, ramped at 2 °C/min to 280 °C, ramped at 10 °C/min to 310 °C, and held for 10 min.

Method Validation. Calibration curves were obtained from standard solutions (5–95 μM of the diacetylated hydroxycinnamyl alcohols) in MeOH. The deuterium-labeled internal standard compounds were added to the standard solutions (final concentration of the deuterium-labeled compounds, 30 μM). All solutions were measured in triplicate. The following validation parameters were tested: homogeneity of variances, detection limit (here signal-to-noise ratio of 5:1–10:1; see also Results and Discussion), quantification limit (here lowest calibration point used; see also Results and Discussion), linearity (residual plots, correlation coefficient). The recovery was estimated by comparing the peak area of ions from the hexadeuterated internal standard compounds obtained during sample application to the average peak area obtained from direct injection of the hexadeuterated internal standard compounds (concentration 30 μM) in the GC-SIM-MS sytem.

RESULTS AND DISCUSSION

GC-MS Fragmentation of Standard Compounds. GC-FID is routinely used to determine the ratios of diacetylated DFRC products. However, due to high levels of coeluting matrix compounds, peak identification and integration is often problematic, especially for low-lignin samples. GC-MS analysis in the SIM mode offers both higher selectivity and higher sensitivity. Ideally GC-SIM-MS is combined with using stable-isotope-labeled standard compounds. Here, by acetylation of the *p*-hydroxycinnamyl alcohols using perdeuterated acetic anhydride, six deuterium labels were incorporated into each of the three *p*-hydroxycinnamyl alcohol diacetates. A prerequisite for using the deuterium-labeled diacetates as internal standards is the occurrence of at least one main fragment that is different from the nonlabeled diacetates and thus can be used for quantification. Table 1 summarizes the EI-MS fragmentation patterns (70 eV) of the synthesized compounds. Generally, phenolic acetates lose ketene, which has also been described for lignin dimers.[15] During the loss of ketene one proton of the phenolic acetyl group remains with the parental molecule (Figure 1, using the example of diacetylated coniferyl alcohol). This explains the difference of four mass units between the ketene loss fragment of the nonlabeled (e.g., *m/z* = 222 for diacetylated coniferyl alcohol) and the corresponding hexadeuterated compounds (e.g., *m/z* = 226 for the deuterated

DOI: 10.1021/jf506221p
J. Agric. Food Chem. 2015, 63, 2668–2673

diacetylated coniferyl alcohol). The loss of m/z = 60 can be explained by a McLafferty rearrangement leading to cleavage of γ-acetates. Also, cleavage of α-CH_2 structures, leading to their corresponding benzylic cations, can be observed. In general, the fragments are comparable to those described in the literature,[10] but the intensities are different from literature data.

For coniferyl alcohol diacetate/coniferyl alcohol diacetate-d_6 (GDiAc/GDiAc-d_6) and sinapyl alcohol diacetate/sinapyl alcohol diacetate-d_6 (SDiAc/SDiAc-d_6) the base peak was chosen for GC-SIM-MS quantification. This was not possible for *p*-coumaryl alcohol diacetate (HDiAc) because the base peak of *p*-coumaryl alcohol diacetate-d_6 (HDiAc-d_6) occurs in the spectrum of the nondeuterated compound and vice versa. Thus, m/z = 192 and 196 were chosen to quantitate HDiAc and HDiAc-d_6, respectively. Figure 2 shows the GC-SIM-MS chromatogram of the synthesized standard compounds and their corresponding labeled compounds.

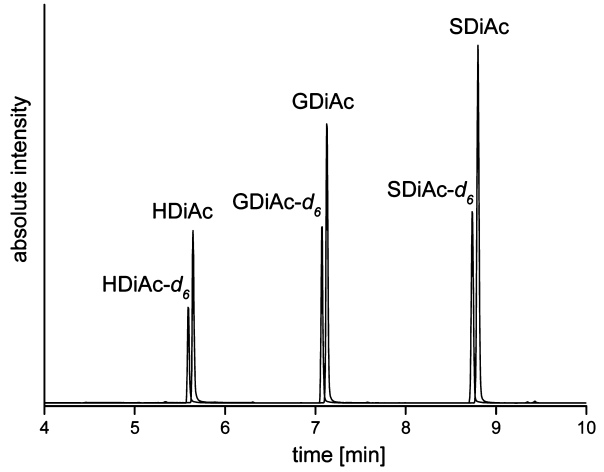

Figure 2. GC-MS chromatogram of the analysis of a standard mixture containing diacetylated *p*-coumaryl (HDiAc), coniferyl (GDiAc), and sinapyl (SDiAc) alcohols and their corresponding labeled compounds (d_6). Detection was carried out in selected-ion monitoring mode with m/z = 192/196 for HDiAc/HDiAc-d_6, m/z = 222/226 for GDiAc/GDiAc-d_6, and m/z = 252/256 for SDiAc/SDiAc-d_6.

Method Validation. The calibration curves of the three standard compounds were repeatedly analyzed in a range of 5−95 μM. Standard deviations of repeated injections were partially ≥20% depending on the concentration and/or day, suggesting less stable fragmentation. By using the corresponding deuterium-labeled compounds as internal standards to calculate the calibration curves, the standard deviations were more acceptable (0.1−2.0%). Thus, regression lines were obtained by plotting the area of analyte/area of internal standard [30 μM, see below] on the *y*-axis vs analyte concentration on the *x*-axis. The fact that less reproducible fragmentation can be corrected for by using the deuterated compounds as internal standards demonstrates the importance of internal standards that are structurally similar to the diacetylated DFRC products. For all calibration points an internal standard concentration of 30 μM was used, roughly representing the middle of the concentration range tested. The use of a fixed internal standard concentration implies a disadvantage that is, however, minor compared to the advantages of the method: The ready-to-analyze sample solutions need to contain the defined concentration of internal standards (30 μM). If absolutely no information about the lignin amount/composition of the sample is known or assessable, a preceding analysis of the sample material has to be performed. This additional performance is necessary to determine if and which final dilution is necessary to get the sample solution into the calibrated concentration range. Then, the concentration of the internal standard compounds added during sample preparation has to be adapted to this dilution.

Visual evaluation of the standard curves of the three acetylated monomers revealed that higher order polynomial regression may fit the data better than a linear regression. Therefore, a polynomial regression (second order) was used and confirmed by the correlation coefficient and residual analysis. Because homogeneity of variances at the highest and lowest concentration was not given, a weighted regression (with reduced chi^2, weighting factor 1/standard deviation^2) was used. The standard deviation of each calibration point was between 0.1% and 2.0% applying the parameters used. The most important validation parameters are shown in Table 2.

Table 2. Validation Parameters for the Calibration of *p*-Coumaryl Alcohol Diacetate (HDiAc), Coniferyl Alcohol Diacetate (GDiAc), and Sinapyl Alcohol Diacetate (SDiAc)[a]

	calibration equation (polynomial model)	correlation coefficient	LOD[b] [μM]	LOQ [μM]	recovery[c] (%)
HDiAc	$y = 0.00654 + 0.04094x + (8.59968 \times 10^{-5})x^2$	1.0000	0.007	5	99−142
GDiAc	$y = 0.00225 + 0.03314x + (1.2895 \times 10^{-4})x^2$	1.0000	0.001	5	68−185
SDiAc	$y = -0.00984 + 0.03872x + (1.553252 \times 10^{-4})x^2$	0.9997	0.05	5	77−179

[a]The corresponding deuterium-labeled compounds (final concentration: 30 μM) were used to correct for less stable fragmentation. The tested range was 5−95 μM for all three compounds. LOD, limit of detection; LOQ, limit of quantification. [b]Rough estimate due to less stable fragmentation (see text). [c]Estimated from the recovered area of the internal standard compounds added during sample preparation (see text).

Due to the less stable fragmentation of the analytes, the limits of detection and quantification (LOD, LOQ) determined via the signal-to-noise ratio are rather rough estimates than definite values. Also, due to the less stable fragmentation it was not possible to define exact concentrations with signal-to-noise ratios of 3:1 (LOD) or 9:1 (LOQ). Therefore, the LOD was defined by injecting standard solutions with decreasing concentrations in triplicate until a signal-to-noise ratio of about 5:1−10:1 was found in all three replicates. The limit of quantification was set as the lowest calibration point due to the mentioned problems in determining this validation parameter.

To test day-to-day reproducibility, the standard solutions were reinjected after 8 days and regression lines were compared. The calibration equations were similar, suggesting that the method was robust if performed as described. Also, the standard solutions were freshly prepared and analyzed. In this case, the regression lines vary in an acceptable way due to minor variations during solution preparation. These data suggest that a complete calibration with each sample set is not necessary. Instead, it is recommended to double check the regression lines by three calibration points before analysis of each sample set.

DOI: 10.1021/jf506221p
J. Agric. Food Chem. 2015, 63, 2668−2673

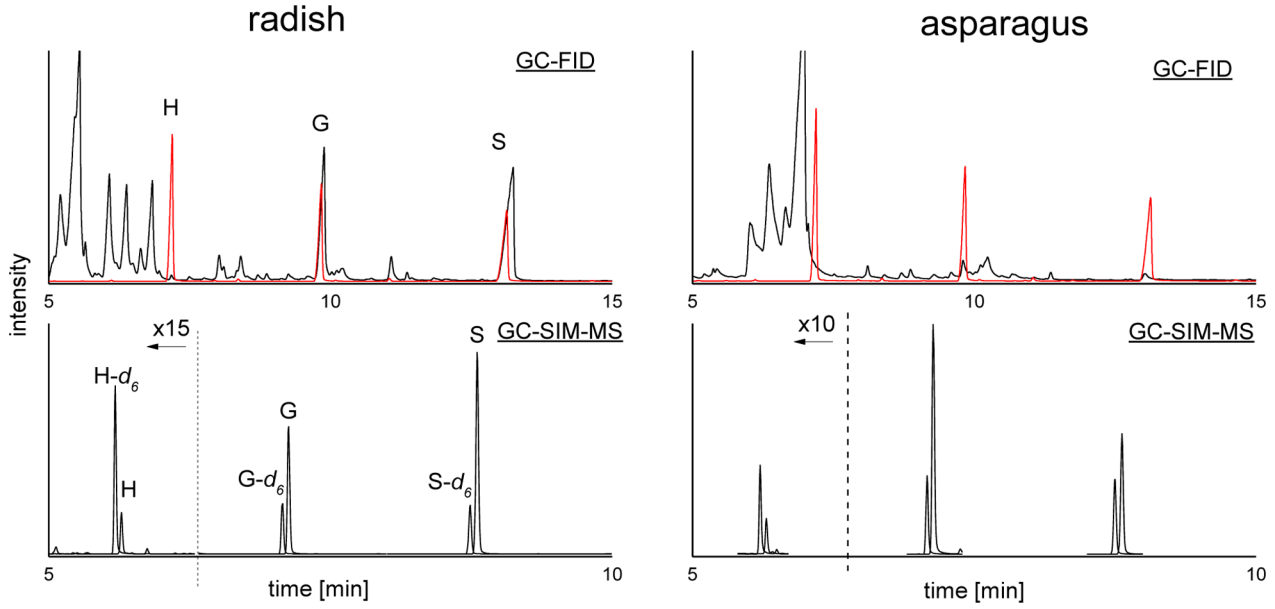

Figure 3. Comparison of chromatograms from derivatization followed by reductive cleavage (DFRC) products from radish (left) and asparagus (right). GC-FID chromatograms were obtained without modified sample cleanup. Colored lines represent the overlaid chromatograms of the standard compounds. GC-SIM-(selected-ion monitoring)-MS chromatograms were obtained by using the modified method. Peaks representing *p*-coumaryl alcohol diacetate and deuterated *p*-coumaryl alcohol diacetate are enlarged 15- (radish) and 10-fold (asparagus). HDiAc, *p*-hydroxyphenyl unit; G, guaiacyl unit; S, syringyl unit; d_6, hexadeuterated internal standard compounds.

Recoveries were estimated by using the analyzed areas of the internal standard compounds added before extraction of the liberated monomers during application of the method to low-lignin[11] samples (pear, radish, carrot, and asparagus, see below). Recovery rates were calculated by comparing these areas to the average areas of the deuterated internal standards obtained from direct injection of the internal standard solution (30 μM). It has to be kept in mind that the recovery rates determined here are only rough estimates due to one-point calibration without correcting this data point for fragmentation instabilities. The recovery rates were between 99% and 142% for HDiAc-d_6, 68−185% for GDiAc-d_6, and 77−179% for SDiAc-d_6. As described above, the standard deviations of repeated analyses of a single calibration point without adjustment for fragmentation instabilities using an internal standard are often ≥20% and can be up to 40%. However, despite large variations of the recovery rates due to noncorrected fragmentation instabilities, the average values demonstrate that major quantities of the liberated DFRC products are indeed analyzed after sample preparation and cleanup (see below). Furthermore, the MS fragmentation and detection does not seem to be suppressed significantly by matrix effects. In addition, the calculation of the actual monomer amount is not as important as the determination of the monolignol ratios. Because the DFRC method only cleaves β-O-4 linkages, the calculated ratios represent the monomers that are involved in these units. Because the β-O-4 linkages usually represent > 50% and up to 80% of the linkages in the lignin polymer the obtained ratios reflect well the composition of the lignin polymer. This has also been demonstrated by comparison of the G/S ratios of lignins from plant-based foods determined by the DFRC method with the G/S ratios of isolated lignins analyzed by 2D-NMR.[16]

Sample Cleanup. Sample cleanup is adapted to low-lignin samples to reduce the high matrix-derived content that can contaminate the GC-MS system. The original protocol describes a dissolution of the sample in dichloromethane after the acetylation step and before GC-MS analysis.[9] Dichloromethane dissolves the entire residue, i.e., DFRC products and (acetylated) matrix-derived components. Because the acetylated DFRC products are soluble in MeOH this solvent was chosen instead of dichloromethane to dissolve the DFRC residue after acetylation. Using MeOH leaves, conveniently, a large amount of matrix-derived components undissolved. Additionally, an SPE step was established for further cleanup. A polymeric reversed phase, which provides strong retention of aromatic compounds due to $\pi-\pi$ bonding interactions next to hydrogen bonding, was chosen as the stationary phase. The loading solution was MeOH/water (40/60, v/v), which was tested to completely dissolve the standard compounds. SPE conditions were tested by using a mixture of all standard compounds. The solution was loaded onto the column, which was washed and eluted in several steps using different volumes of H_2O and MeOH mixtures in different ratios. All eluates were collected separately and analyzed for DFRC products.

The results showed that loading in MeOH/H_2O (40/60, v/v) and washing with H_2O and MeOH/H_2O (20/80, v/v) result in a loss of about 1% of the standard compounds. Also, the recovery rates of the internal standard compounds (68−185% depending on the determined monomer) as determined during application of the sample cleanup on different plant-based food samples reveal applicability of the chosen conditions for complex, high-matrix samples.

Application. The developed method was applied to fibers from four plant-based food samples: pear, radish, carrot, and asparagus. The enzymatic digestion of the extracted fibers by using an enzyme mixture capable of digesting cell wall polysaccharides is recommended because it reduces the sample matrix load, which contaminates the MS system. Figure 3 shows the chromatograms of radish and asparagus DFRC products measured by GC-FID (original DFRC protocol[9]) or GC-SIM-MS (modified sample cleanup). The GC-FID

DOI: 10.1021/jf506221p
J. Agric. Food Chem. 2015, 63, 2668−2673

chromatogram reveals the existence of a large amount of matrix from low-lignified samples, complicating the identification and/or quantification of the acetylated DFRC products. Using the newly developed GC-SIM-MS method these problems are minimized.

The results from the application of the DFRC method to food samples are summarized in Table 3. To characterize the lignin composition H:G:S proportions and G/S ratios were calculated. The insoluble dietary fiber lignin of carrots shows a high G/S ratio (ca. 38) and can be classified as a G-rich lignin. In contrast, lignins from pear, radish, and asparagus are characterized by balanced G/S ratios. These values are generally comparable to literature data.[11] Smaller differences can be explained by different maturation stages of the used samples and methodological differences. Furthermore, by using the GC-SIM-MS method, H units were detected in quantifiable amounts in all samples analyzed. In comparison to G and S units, the amounts were low, with the exception of carrot lignin with an H/S ratio of 0.87. Bunzel et al.[11] could detect quantifiable amounts of H units from lignins of radish only. Although differences in the plant materials are possible, the detection of minor amounts of H units in this study is most likely due to the increased selectivity and sensitivity of the newly developed stable-isotope dilution GC-MS approach.

Table 3. Monolignol Composition of Fibers from Pear, Radish, Carrot, and Asparagus Determined by the Derivatization Followed by Reductive Cleavage (DFRC) Method with Modified Sample Cleanup and GC-Selected-Ion Monitoring (SIM)-MS Analysis[a]

	H:G:S [mol %]	ratio G/S	ratio G/S from literature[11]
pear	0.18:53.28:46.54	1.15 (±0.03)	0.6 (±0.04)
radish	1.29:44.75:53.96	0.80 (±0.14)	1.5 (±0.18)
carrot	2.32:94.97:2.71	35.24 (±2.45)	38.8 (±6.11)
asparagus	0.85:67.04:32.11	2.10 (±0.17)	3.3 (±0.28)

[a]Ratios of H (*p*-hydroxyphenyl unit), G (guaiacyl unit), and S (syringyl unit) were determined as percentages on a molar basis. G/S ratios are calculated on molar basis too. Each sample was measured in duplicate. Uncertainties of measurement are indicated in brackets as range/2. For quantification, the hexadeuterated internal standards were used at a concentration of 30 μM.

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Funding

This work was funded by a fellowship from the Carl-Zeiss Foundation.

Notes

The authors declare no competing financial interest.

ABREVIATIONS USED

BER, borohydride exchange resin; DFRC, derivatization followed by reductive cleavage; DIBAL-H, diisobutylaluminum hydride; H, *p*-hydroxyphenyl unit; HDiAc, *p*-coumaryl alcohol diacetate; G, guaiacyl unit; GDiAc, coniferyl alcohol diacetate; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; S, syringyl unit; SDiAc, sinapyl alcohol diacetate; SIM, selected ion monitoring; SPE, solid-phase extraction

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DOI: 10.1021/jf506221p
J. Agric. Food Chem. 2015, 63, 2668−2673