JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Dietary Fiber Lignins from Fruits and Vegetables Using the DFRC Method

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Insoluble fiber fractions from 11 fruits and vegetables were investigated for their lignin composition using the derivatization followed by reductive cleavage (DFRC) methodology. To enrich lignin contents and to minimize polysaccharide excess that led to nonanalyzable DFRC chromatograms, the insoluble fibers were degraded by a carbohydrolases mixture. The residues that were found to be representative for the insoluble fiber lignins were analyzed. The investigated fibers differ considerably in their lignin contents and also in their lignin compositions. With the exception of radish fiber, only trace amounts (or none) of the products resulting from *p*-hydroxyphenyl units were detected. Lignins noticeably differed in the ratio of the DFRC products resulting from syringyl units (S) and guaiacyl (G) units (G/S ratios ranged from ~39 to 0.2). The insoluble fiber lignins were classified as G-rich lignins (G/S ratio > 3; carrot, spinach, kiwi, curly kale, radish, and asparagus), S-rich lignins (S/G ratio > 3; rhubarb), or balanced lignins (0.3 < G/S ratio < 3; pear, apple, small radish, and kohlrabi). Information about further structural characteristics, for example, cinnamyl endgroups, was obtained from the analysis of DFRC minor products.

KEYWORDS: Dietary fiber; fruit; vegetable; lignin composition; DFRC method; acetyl bromide soluble lignin; monolignol

INTRODUCTION

In 2001 the American Association of Cereal Chemists (AACC) adopted the following definition of dietary fiber (*1*):

"Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation."

Next to polysaccharides and oligosaccharides, lignin is emphasized in this definition, whereas other dietary fiber components such as waxes, tannins, or suberin are combined as "associated plant substances". Despite its accentuated position in this definition and the fact that dietary fiber lignins are discussed as being protective against colon cancer by binding carcinogens such as heterocyclic aromatic amines (*2*), knowledge about lignins in food is rather limited. Lignins represent a class of polymers that can be defined as polymeric natural products arising from an enzyme-initiated dehydrogenative polymerization of three primary precursors (*p*-coumaryl, coniferyl, and sinapyl alcohols). However, this definition is very general and does not consider that other phenolics in addition to the three mentioned hydroxycinnamyl alcohols are present in lignins and should be regarded as lignin monomers (*3*). 5-Hydroxyconiferyl alcohol, hydroxycinnamaldehydes, hydroxycinnamyl *p*-hydroxybenzoates, and hydroxycinnamyl *p*-coumarates are all examples of phenolics that may be incorporated into lignins. Although there has been substantial progress in lignin structural analysis, and as a result also a deeper insight into lignin biosynthetic pathways, most lignin studies are limited to wood and forages. Lignins from foods are poorly investigated. Usually, these investigations are limited to the determination of Klason lignin contents, a method that is also integrated into some dietary fiber determination methodologies. However, the determination of Klason lignin does not prove that real lignin is present because plant material residues such as structural proteins or waxes may contribute to acid-insoluble residues. As a consequence, quantitative determinations are imprecise, and published lignin contents differ over a wide range (*4*). Knowledge of the composition of food lignins is cursory at best. Recently, the composition of cereal grain lignins was independently investigated by two groups using thioacidolysis or the derivatization followed by reductive cleavage (DFRC) method (*5*−*7*). This study aims to expand such analysis to dietary fiber lignins from fruits and vegetables. Furthermore, we tested the acetyl bromide soluble lignin (ABSL) methodology for the quantitative determination of lignin in fruit and vegetable fibers.

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10.1021/jf0520037 CCC: $30.25
Published on Web 11/08/2005

MATERIALS AND METHODS

General. 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 *Bacillus licheniformis*, 2.4 AU/g), and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were from Novo Nordisk, Bagsvaerd, Denmark. The carbohydrase mixture "Driselase" (from Basidiomycetes) was from Sigma, St. Louis, MO. Amberlite XAD-2 was obtained from Serva (Heidelberg, Germany), and Bond Elut SI 3 mL SPE-columns were from Varian, Darmstadt, Germany. GC was carried out on a Thermo Electron Focus GC-FID (Dreieich, Germany) using a 30 m × 0.25 mm i.d., 0.25 μm, HP-5MS capillary column (Hewlett-Packard, Waldbronn, Germany) or, for structural identification purposes, on a Hewlett-Packard 5890 series II GC and 5972 series mass selective detector using a 30 m × 0.25 mm, 0.25 μm, DB-5MS capillary column (J&W Scientific, Folsom, CA).

Plant Material. All fruits and vegetables were obtained from a local German supplier (January−March 2005). Cultivars and origins indicated here were confirmed by the supplier: radishes (*Raphanus sativus* L. var. *niger* cv. Minowase summer cross, harvested in 2005 in the Lazio region, Italy); small, red radishes (*Raphanus sativus* var. *sativus* cv. Topsi, harvested in 2005 in the Lazio region, Italy); carrots (*Daucus carota* L. cv. Rotin, harvested in 2004 in the Vierlande region, Germany) (carrots were stored between sand plies in the soil until they were sold in early 2005); kohlrabi (turnip cabbage) [*Brassica oleracea* L. ssp. *oleracea* convar. *caulorapa* (DC.) Alef. var. *gongylodes* L. cv. Primulux, harvested in 2005 in the Lazio region, Italy]; curly kale (*Brassica oleracea* L. ssp. *oleracea* var. *sabellica* L. cv. Arsis, harvested in 2005 in the Vierlande region, Germany); spinach (*Spinacia oleracea* L. cv. Monnopa, harvested in 2005 in the Pfalz region, Germany); asparagus (*Asparagus officinalis* L. cv. Gijnlim, harvested in 2005 in Spain); rhubarb (*Rheum rhabarbarum* L. cv. Esta, harvested in 2005 in the Rheinland region, Germany); kiwi fruits (Chinese gooseberries) (*Actinidia chinensis* Planch. cv. Hayward, harvested in 2005 in Italy); apples (*Malus domestica* Borkh. ssp. *domestica* cv. Cox's orange, harvested in 2004 in the region of Altes Land, Germany) (apples were stored under controlled atmosphere conditions until they were sold in early 2005); and pears (*Pyrus communis* L. cv. Conference, harvested in 2005 in Belgium). Only edible parts of the fruits and vegetables were used. All fruits and vegetables were prepared for consumption as typically done in the household kitchen. In detail, the radish and carrot main roots and asparagus were washed, and the outer layers were thinly pared using a potato peeler; small radishes were washed, and the thickened hypocotyl was used as a whole. Kohlrabi leaves were removed, and the tubers were thinly pared. Curly kale and spinach leaves were washed and used without further preparations. The rhubarb leaves were cut, and the petioles were washed and thinly pared. The kiwi fruits were pared, whereas unpared apples and pears were used. However, the apple and pear cores were removed.

Preparation of Insoluble Fibers (IF). All fruits and vegetables were roughly crushed, frozen, and lyophilized for 5−7 days. The dried material was further milled using a mortar and pestle to a particle size of <0.5 mm. Two insoluble dietary fiber samples were prepared from each fruit and vegetable. Dried plant materials (15 g) were suspended in 0.08 M sodium phosphate buffer, pH 6.0 (300 mL), and 1125 μL of α-amylase was added. Beakers were placed in a boiling water bath for 20 min and shaken gently every 5 min. The pH was adjusted to 7.5 with ∼60 mL of 0.275 M NaOH, and samples were incubated with 450 μL of protease at 60 °C for 30 min with continuous agitation. After the pH had been adjusted to 4.5 with ∼60 mL of 0.325 M HCl, 525 μL of amyloglucosidase was added, and the mixture was incubated at 60 °C for 30 min with continuous agitation. Following centrifugation, the residue was washed three times with hot (70 °C) water and once with 95% (v/v) ethanol and acetone and finally dried at 40 °C overnight in a vacuum oven. To remove possibly remaining small amounts of low molecular weight material, the insoluble fibers were further extracted with ethanol (8 h), dichloromethane (8 h), and *n*-hexane (8 h) using a Soxhlet apparatus. Finally, the extracted insoluble fibers (IF) were dried at 40 °C overnight in a vacuum oven.

Enzymatic Polysaccharide Degradation of Insoluble Fibers. IF (1 g) was suspended in 100 mL of H_2O and incubated with 60 mg of Driselase at 37 °C for 48 h. After heat inactivation of the enzymes (10 min, 100 °C) and centrifugation, the residues ["enriched insoluble fiber" (EIF)] were collected in glass filter crucibles (porosity 3, pore size = 16−40 μm) and washed twice with 10 mL of water. The filtrate and the washing water were combined, lyophilized, redissolved in 10 mL of water ["enzymatic polysaccharide hydrolysate" (EPH)], and further treated as described below. The EIF was further washed with ethanol (2 × 10 mL) and acetone (2 × 10 mL) and dried overnight in a vacuum oven. The EPH solution was applied to a column (18 × 0.8 cm) of Amberlite XAD-2 beforehand conditioned with water. Elution was carried out with 40 mL of H_2O, 50 mL of MeOH/H_2O 50:50 (v/v), and 50 mL of MeOH. The MeOH/H_2O and MeOH fractions were combined and evaporated ["phenolics-containing enzymatic polysaccharide hydrolysate" (PEPH)].

Determination of Acetyl Bromide Soluble Lignin (ABSL). The acetyl bromide lignin assay was performed as detailed by Fukushima and Hatfield (*8*). Briefly, in a screw-cap tube 20−100 mg of IF or EIF (∼100 mg of curly kale, spinach, asparagus, carrot, radish, kohlrabi, small radish, and apple IF, 50 mg of kiwi and rhubarb IF; 20 mg of pear IF; 50 mg of curly kale, spinach, asparagus, carrot, radish, kohlrabi, small radish, and apple EIF; 40 mg of kiwi EIF; 20 mg of pear EIF; and 10 mg of rhubarb EIF) was digested with 4 mL of a 25% acetyl bromide in acetic acid solution at 50 °C for 2 h. The tube was cooled, and 12 mL of acetic acid was added. After centrifugation, 1 or 0.5 mL (pear IF, pear and kiwi EIF) of this solution was added to a tube containing 2.5 mL of acetic acid and 1.5 mL of 0.3 M NaOH. After mixing, 0.5 mL of 0.5 M hydroxylamine hydrochloride solution was added, and the volume was made up to 10 mL with acetic acid. The absorbance of the mixture was read at 280 nm. In the same way a sample blank was run. The concentration was determined using the following equation:

$$c = \frac{A(\text{sample}) - A(\text{blank})}{\epsilon \times L}$$

c = ABSL concentration in the final solution (mg/mL), L = cell thickness (cm), and ϵ = absorptivity of lignin standard (cm^{-1} mg^{-1} mL). For these calculations an absorption coefficient of 20 cm^{-1} mg^{-1} mL was used. This absorption coefficient was suggested as the standard value for herbaceous plant materials by Iiyama and Wallis (*9*).

In addition, UV scans of the acetyl bromide soluble solutions were recorded over the range of 200−400 nm. They showed the characteristic absorption profile with a maximum (or shoulder) at 280 nm.

DFRC Method. The DFRC method was performed as described by Lu and Ralph (*10*) with some minor modifications. In brief, ∼100 mg [rhubarb (I), curly kale, spinach, radish, asparagus, red radish, carrot, apple], 40 mg [kohlrabi, rhubarb (II), kiwi], or 20 mg (pear) of EIF was weighed into a Pyrex tube or the PEPH fraction was completely used (amounts varied between 6.1 and 122.7 mg depending on the sample). Acetyl bromide reagent (acetyl bromide/acetic acid, 20:80, v/v; 7.5 mL) was added. The mixture was stirred at 50 °C for 3 h. The solvent was removed by rotary evaporation below 50 °C. Acidic reduction solvent (dioxane/acetic acid/water, 5:4:1, v/v/v; 7.5 mL) was added to the evaporation residue. Following the addition of zinc dust (∼50 mg), the mixture was stirred for 40 min. This mixture, CH_2Cl_2 (10 mL), and saturated NH_4Cl (10 mL) were transferred into a separatory funnel. The internal standard 4,4′-ethylidenebisphenol (0.05 mg in CH_2Cl_2) was added, and the pH of the aqueous phase was adjusted to below 3. After vigorous mixing, the organic layer was separated, and the extraction was repeated twice with 5 mL of CH_2Cl_2. The combined organic fractions were transferred into a separatory funnel, and the separation step between CH_2Cl_2 and saturated NH_4Cl was repeated twice. Following the last separation step the combined organic layers were dried over $MgSO_4$, evaporated, and redissolved in 1.5 mL of CH_2Cl_2 and acetylated with 0.2 mL of acetic anhydride and 0.2 mL of pyridine overnight. Following the acetylation, the residue was obtained by 3-fold coevaporation with ethanol under reduced pressure. The acetylation product was redissolved in 500 μL of CH_2Cl_2 and transferred to a silica SPE column previously conditioned with 2 mL of CH_2Cl_2. The products were eluted using 9 mL of $CHCl_3$/ethyl acetate, 10:1. For testing purposes the SPE column was eluted with

another 9 mL of $CHCl_3$/ethyl acetate, 10:1. Because this repeated elution step does not elute further DFRC products, the original elution with 9 mL of eluent was maintained. After evaporation without heating, the samples were dissolved in CH_2Cl_2 (200 μL) and analyzed by GC-MS and GC-FID.

GC-MS and GC-FID of DFRC Monomers. (*E*)-*p*-Coumaryl diacetate, (*E*)-coniferyl diacetate, and (*E*)-sinapyl diacetate liberated from plant materials were authenticated by comparison of their relative retention times and mass spectra with authentic standards using a GC-MS system. Rarely found DFRC monomers were identified by their mass spectra and retention behavior, both compared with data from the literature (*11*−*13*). DFRC monomers were separated by GC-MS. He (1 mL/min) was used as carrier gas. GC conditions were as follows: initial column temperature, 160 °C, ramped at 5 °C/min to 300 °C, and held for 10 min; injector temperature, 220 °C; split, 1/30. Quantification was carried out by GC-FID: carrier gas, He (1 mL/min); injector temperature, 220 °C; split, 1/10; detector temperature, 300 °C. GC conditions were as follows: 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. Monomer determination was carried out using response factors [*p*-coumaryl diacetate (P),1.42; coniferyl diacetate (G), 1.39; sinapyl diacetate (S), 1.44] derived from monomer standards against the internal standard. Generally, both samples from one fruit or vegetable source were determined by GC-FID, whereas only one sample was injected into the GC-MS system to identify DFRC monomers and to estimate peak purities.

RESULTS AND DISCUSSION

Apples and kiwis were chosen for lignin investigations because they are widely consumed, popular fruits, whereas the presence of stone cells renders pears interesting for our studies. The vegetables were selected to represent a cross section of commonly consumed plant organs. Carrot and radish represent root vegetables with the difference that carrot is a phloem or bast root vegetable, whereas radish is a xylem or wood root vegetable. Although carrot and radish also integrate small parts of the hypocotyl, they differ from the small red radish, which represents a hypocotyl tuber. The edible part of kohlrabi is a rounded enlarged stem section without hypocotyl sections. Asparagus represents a monocotyledonous stem vegetable that is not capable of secondary growth. Spinach and curly kale represent leaf vegetables. The edible part of rhubarb is the petiole (leaf stalk).

Insoluble fibers (IF) of the investigated fruits and vegetables were prepared by lyophilization and enzymatic degradation (α-amylase, protease, amyloglucosidase) of the plant material followed by exhaustive extraction with organic solvents. Fiber contents (on a dry matter basis) achieved by this methodology are given in **Table 1**.

Acetyl Bromide Lignin Methodology. Lignin contents were determined to estimate the lignin enrichment by enzymatic treatment of IF and to standardize liberated DFRC products to lignin contents of the samples. All methods for lignin determinations in different plant materials are not fully satisfactory, leading to lignin under- or overestimations (*14*, *15*). The most prominent lignin methodology used for food is the Klason lignin method. This gravimetric method determining the acid-insoluble residue as lignin may lead to imprecise results due partly to solubilization of lignin in the acid and often more importantly due to the codetermination of structural proteins, waxes, etc. In our studies, the need for a method to determine lignin in small sample amounts (e.g., in our enriched insoluble fibers) excluded the Klason lignin methodology. The only method sensitive enough for our sample amounts is the acetyl bromide soluble lignin (ABSL) method (*16*), which has undergone several changes and improvements over the years. However, this spectrophotometric method based on lignin solubilization in 25% acetyl bromide in glacial acetic acid and measurement of the absorbance at 280 nm was found to be less suitable for carrot and celery fibers due to a less distinct absorption maximum at 280 nm (*17*). UV scans of our samples (**Figure 1**) indicate that most samples show a maximum/shoulder at 280 nm that is at least comparable to those published for forages for which the ABSL method is routinely used. However, some samples (especially small radish and spinach) showed the described less distinct absorption maximum, possibly due to poor solubility of these lignins in 25% acetyl bromide or simply due to minor lignin contents. Another drawback of this methodology used for fruits and vegetables is an unsatisfactory day-to-day reproducibility (10% variations are possible), which is annoying from an analytical point of view but does not affect the conclusions from our experiments. With knowledge of the existing shortcomings of other methodologies, and due to the lack of better applicable methods, we used the acetyl bromide procedure, keeping its limitations in mind.

Table 1. Insoluble Fiber, Enriched Insoluble Fiber, and Acetyl Bromide Soluble Lignin (ABSL) Contents of the Investigated Fruits and Vegetables

			ABSL[a]	
	insoluble fiber[b] (%)	residue after Driselase digestion[c] (%)	insoluble fiber (%)	digestion residue (%)
apple	9.8	46.2	1.7	3.1
kiwi	11.9	53.0	8.3	19.5
pear	12.9	85.7	23.5	25.1
asparagus	18.0	25.6	5.6	12.1
carrot	10.3	34.6	3.2	3.4
curly kale	33.4	24.8	2.5	5.6
kohlrabi	6.2	19.6	3.8	11.2
radish	12.6	31.0	3.4	7.0
small radish	18.3	30.6	3.4	7.9
rhubarb	26.7	54.9	9.0	14.2
spinach	28.5	43.9	3.3	2.9

[a] Lignin content determined by using the ABSL methodology. [b] Residue after treatment with α-amylase, protease, and amyloglucosidase and exhaustive solvent extraction; calculated on dry matter basis. [c] One gram of insoluble fiber was treated with 60 mg of the carbohydrolase mixture Driselase; the residue is designated "enriched insoluble fiber" (EIF).

DFRC Methodology. The most frequent type of interunit linkage in lignins is *β*-*O*-4, arylglycerol-*β*-aryl ethers formed by endwise coupling of monolignols with the growing lignin polymer. The DFRC method cleaves these ethers, resulting in the diagnostic formation of the peracetates of the monolignols from which lignins are biosynthesized (*10*, *12*, *18*). Other types of linkages between lignin units such as *β*-5, *β*-*β*, 5-5, 5-*O*-4, and *β*-1 are more resistant to chemical degradation and are not cleaved using the DFRC method, but can be detected in releasable dimers (*19*). Application of the DFRC method to IF led to complex chromatograms that were nonanalyzable by GC-FID. Interfering peaks presumably have their origin in polysaccharide degradation products (*10*). The samples had an almost oily character, also preventing their routine analysis by GC-MS. Introduction of a second silica SPE step in the cleanup process did not improve the purity of the DFRC samples. Therefore, IFs were degraded with a carbohydrolases mixture to reduce the amount of polysaccharides in the sample and also to increase the lignin contents in the residues (EIF). Further variations in the temperature program led to GC-FID chromatograms that were fully analyzable for the main DFRC products

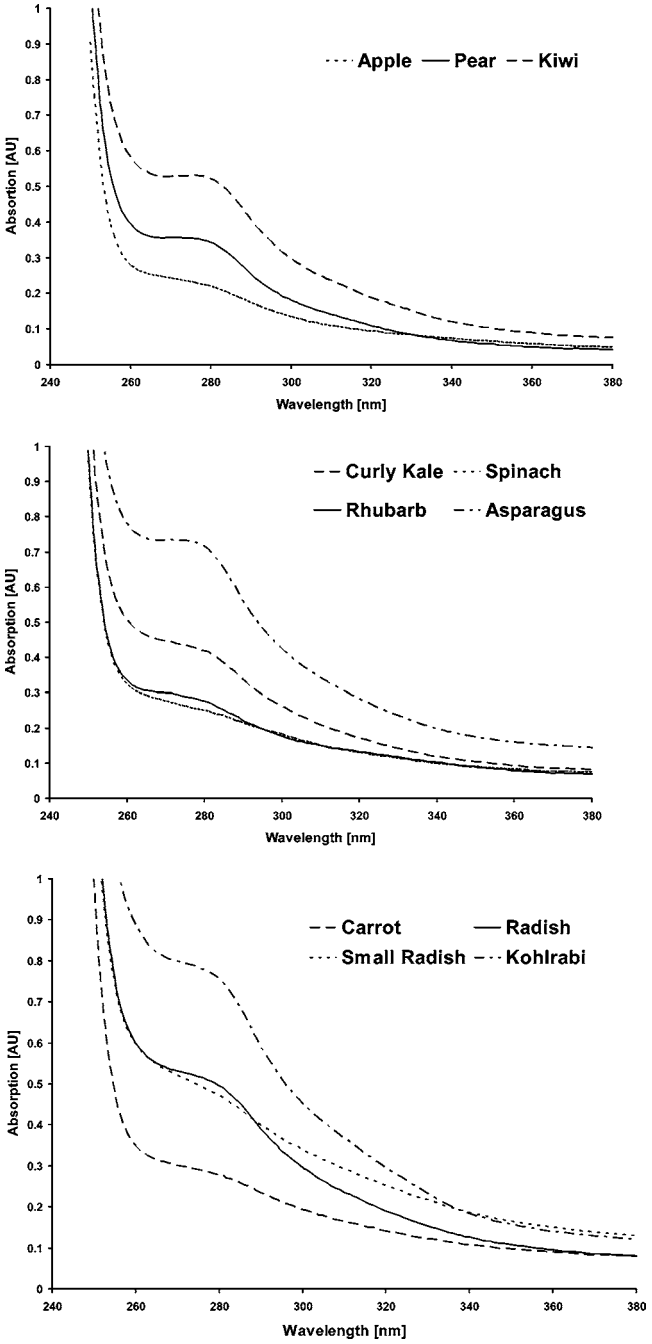

Figure 1. UV spectra of acetyl bromide-treated enriched insoluble fibers of different fruits and vegetables.

coniferyl diacetate (G; derived from guaiacyl units, originally produced by coupling of the monolignol coniferyl alcohol) and sinapyl diacetate (S; derived from syringyl units from the monolignol sinapyl alcohol) (**Figure 2**). In some samples, complete separation of *p*-coumaryl diacetate (P; from *p*-coumaryl units from the monolignol *p*-coumaryl alcohol) from early-eluting interfering peaks, which possibly have their origin in degradation products from residual, undegraded polysaccharides, was difficult to achieve. However, in the only sample in which *p*-coumaryl diacetate was present in substantial amounts (red radish), no problems with interfering peaks occurred.

Enzymatic degradation of IF with carbohydrases resulted in a water soluble hydrolysate (EPH) in addition to the EIF. This hydrolysate should predominantly contain polysaccharide degradation products. However, it is also possible that lignin or lignin-like material is released during this enzymatic procedure, for example, by solubilization of complexes consisting of oligosaccharides and lignin-like oligomers. To make sure that the carbohydrate degradation step did not solubilize lignin and alter the properties of the original IF lignins, the hydrolysates were investigated for possible lignin contents. For this, the EPH was fractionated using Amberlite XAD-2. Whereas polysaccharide degradation products that would make the application of the DFRC method difficult are eluted with water, phenolic compounds and phenolic−oligosaccharide complexes dominated by the phenolic moieties are retained and eluted with MeOH/H_2O and MeOH as eluents. The combined evaporated MeOH/H_2O and MeOH fractions (PEPH) account for 1.6−13.5% of the EPH and were analyzed using the DFRC method as described. Although the chromatograms from these samples generally show more interfering peaks, in most cases it was possible to analyze the chromatograms. However, the very low amounts of liberated acetylated monolignols in combination with more interfering peaks make an exact quantification difficult, so that the amounts given here for the hydrolysates should be regarded as rough estimations.

ABSL Contents. Lignin contents of the insoluble and enriched insoluble fibers are shown in **Table 1**. The high lignin content of pear insoluble fiber (23.5%) is striking. Kiwi and rhubarb insoluble fibers (8.3 and 9.0%) also show comparably high lignin contents. Polysaccharides digestion of the insoluble fibers led in most cases to a considerable increase in the lignin contents (**Table 1**) with the exceptions of pear, carrot, and spinach fibers. However, the reason for the nonenrichment may be different. Pear insoluble fiber was the only fiber that was only minimally hydrolyzed by carbohydrolases, resulting in ∼85% digestion residue (**Table 1**). In contrast, spinach and carrot insoluble fibers were well degraded by carbohydrolases (about 44 and 35% residues), but the lignin contents remained nearly the same. The easiest explanation would be that the ABSL methodology is not suitable for accurately measuring the low lignin contents of carrots and spinach. A likely explanation is that other phenolic compounds that are measured at 280 nm and that are solubilized during carbohydrate degradation are involved in the determination of the insoluble fiber lignin content. For spinach, for example, it is well-known that ferulate is bound to pectins that are degradable using the Driselase enzyme preparation (*20*, *21*).

Detection of Lignin Monomers from Cleaving *β*-Ether Units: Lignin Compositions. Lignin structures were detected in all investigated EIF using the DFRC method. G and S monomers were easily detected and quantified in all investigated enriched insoluble fibers. Monomer P was not detected or detected in only trace amounts, except in small radish, in which a substantial amount was found (**Table 2**). The G/S ratio differed considerably between the investigated enriched insoluble fibers. This is of interest in relation to recent results from our analysis of the adsorption of carcinogenic heterocyclic aromatic amines to lignins with different G/S ratios in model cell wall studies (unpublished results). These studies indicate different adsorption abilities of the lignified cell walls that are dependent not only on the lignin contents but also on the lignin compositions. It is possible to classify the lignins from the investigated fibers as G-rich lignins (G/S ratio > 3; carrot, spinach, kiwi, curly kale, radish, and asparagus), S-rich lignins (S/G ratio >3; rhubarb), or balanced lignins (0.3 < G/S ratio < 3; pear, apple, small radish, and kohlrabi) (**Table 2**). The very high G/S ratios of carrot (38.8), spinach (29.6), and kiwi (18.5) and the high S/G ratio of rhubarb (6.2) are notable. The S/G ratio of rhubarb may even be underestimated because the GC-MS chromatogram of

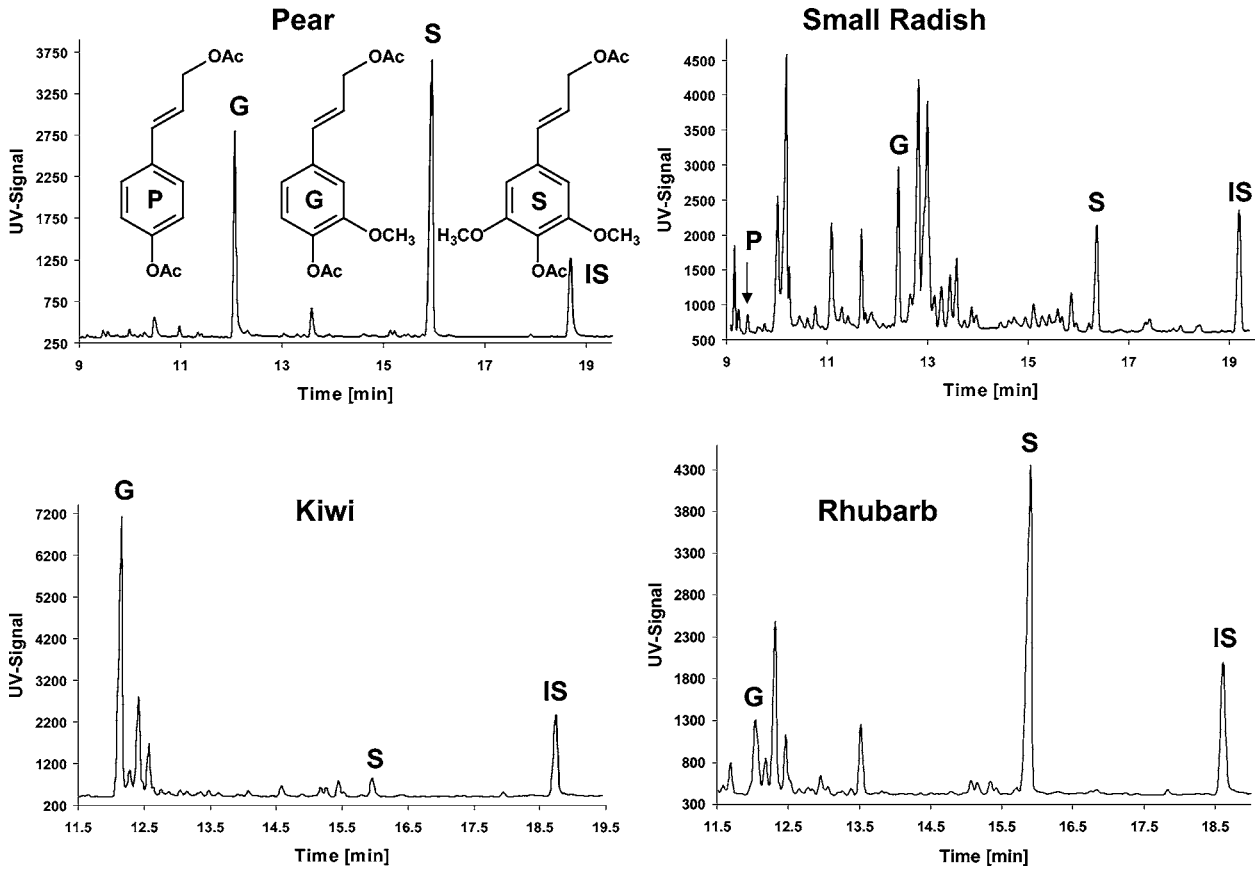

Figure 2. GC-FID chromatograms of DFRC products from pear, small radish, kiwi, and rhubarb enriched insoluble fibers. The main DFRC products P (*p*-coumaryl diacetate, derived from *p*-coumaryl units from the monolignol *p*-coumaryl alcohol), G (coniferyl diacetate, derived from guaiacyl units from the monolignol coniferyl alcohol), and S (sinapyl diacetate, derived from syringyl units from the monolignol sinapyl alcohol) are illustrated in the area of the pear chromatogram. IS, internal standard.

Table 2. Molar Amounts of Released Lignin Monomers from Cleaving β-Ether Units via DFRC Based on Enriched Insoluble Fiber (EIF) and on Acetyl Bromide Soluble Lignin (ABSL) Contents

	ΣG+S (μmol/g of EIF)	ΣG+S[a] (μmol/g of ABSL)	P[a] (μmol/g of ABSL)	ratio G/S[a,b]	ratio S/G[b]
apple	5	158 (±49.2)	nd[c]	1.2 (±0.03)	0.8
kiwi	38	199 (±72.0)	nd	18.5 (±6.17)	0.06
pear	130	514 (±24.3)	nd	0.6 (±0.04)	1.6
asparagus	33	274 (±31.8)	nd	3.3 (±0.28)	0.3
carrot	5	161 (±60.4)	nd	38.8 (±6.11)	0.03
curly kale	13	232 (±27.0)	tr[d]	4.9 (±1.15)	0.2
kohlrabi	23	195 (±26.2)	tr	2.4 (±0.05)	0.4
radish	1	20 (±5.0)	tr	3.7 (±0. 81)	0.3
small radish	8	98 (±1.2)	6 (±0.3)	1.5 (0.18)	0.7
rhubarb	23	165 (±8.7)	nd	0.17[e] (±0.005)	6.2[e]
spinach	4	52 (±9.8)	nd	29.6 (±0.25)	0.03

[a] Standard deviations are valid for the whole procedure (preparation of insoluble fiber, solvent extraction, enzymatic polysaccharide degradation, DFRC method) and do not reflect just the reproducibility of the DFRC methodology. [b] G/S and S/G ratios are calculated on a molar basis. [c] Not detected using the experimental conditions described under Materials and Methods; not detected is here defined as a signal-to-noise ratio of <3 in the extracted ion chromatogram mode (*m*/*z* 192) from the total ion GC-MS chromatograms. [d] Traces; signal-to-noise ratio of >3, but amounts too small to be quantified using GC-FID. [e] See also text.

the DFRC products from this fiber revealed some coelution of unknown compounds with G. Lignins this rich in S are rarely found; kenaf bast fiber lignins are among the highest, with an S/G ratio of ~6 (*22*).

As with the ABSL contents, the amounts of liberated G and S vary among the investigated enriched fibers and, as expected, fiber sources with higher lignin contents also released more G and S units (**Table 2**). However, the lignin content is not the only parameter influencing the monomer levels. Lignin composition and solubilization of the lignin in the reaction medium that may be hindered by the fiber matrix also influence the G/S liberation. For example, lignins that are mainly composed of G units should be less degraded using the DFRC method because the β-ether content is lower; the C5 positions are available for coupling reactions, leading to chemically resistant linkages (5-5, β-5, 5-*O*-4). This is apparent with regard to the yields of liberated G and S units on an ABSL basis. The molar sum of liberated G and S ranged between about 20 and 514 μmol/g of lignin (**Table 2**). Extremely low yields were found for radish (20 μmol/g of lignin) and spinach (52 μmol/g), which had a very high G/S ratio. However, kiwi and carrot also show a high G/S ratio but have considerably higher liberation yields. The highest yield was achieved from pear EIF (514 μmol/g of lignin), a yield that is higher than yields achieved from cereal fibers (50−250 μmol/g of lignin) (*7*) and grass stems (~350 μmol/g of lignin) but lower than yields from woods (800 μmol/g of lignin or more). The yields achieved from all other vegetable and fruit lignins are in the range of cereal grains. However, it must be emphasized that lignin contents, liberation yields, and G/S ratios in these studies represent fruits and vegetables at the stage of maturity at which they are sold to consumers. Usually, the lignin contents rise with increasing maturity. Kohlrabi and other stem and root vegetables may turn to a

Table 3. Released Lignin Monomers from Cleavage of β-Ether Units by DFRC from the Phenolics-Containing Enzymatic Polysaccharide Hydrolysate (PEPH) That Was Achieved from 1 g of Insoluble Fiber.

	G (μmol/ PEPH)	S (μmol/ PEPH)	P (μmol/ PEPH)	ratio G/S	ratio S/G
apple	0.01	0.01	nd[a]	1.4	0.7
kiwi	0.36	0.10	nd	3.6	0.3
pear	0.48	0.21	nd	2.3	0.4
asparagus	0.06	0.05	nd	1.2	0.9
carrot	tr[b]	nd	nd		
curly kale	tr	tr	tr		
kohlrabi	tr	tr	nd		
radish	0.02	0.01	tr	1.3	0.8
small radish	tr	tr	nd		
rhubarb	0.03	0.03	nd	0.9	1.1
spinach	0.13	0.05	nd	2.8	0.4

[a] Not detected using the experimental conditions described under Materials and Methods; not detected is here defined as a signal-to-noise ratio of <3 in the extracted ion chromatogram mode [*m*/*z* 192 (P), 222 (G), 252 (S)] from the total ion GC-MS chromatograms. [b] Traces; signal-to-noise ratio of >3, but amounts too small to be quantified using GC-FID.

woody, nonpalatable plant organ. Also, the G/S ratio usually decreases in mature tissues. The other important point is the preparation of the fruits and vegetables. We tried to simulate a preparation for consumption typical in the kitchen. For example, we thinly pared the asparagus, thus partly removing outer vascular bundles and in the case of later stages of maturity also the outer sclerenchyma sheath.

The analysis of the PEPH showed that the results from the application of the DFRC method to the EIF are representative for the lignins from the IF. Although the G/S ratios in the PEPH fractions may differ from those of the EIF (**Tables 2** and **3**), it is important to note that the lignin or lignin-like material in the PEPH fraction generally only constituted a small fraction of the IF lignin without a significant influence on the overall lignin composition. Generally, the amounts of liberated G and S units from the PEPH only account for up to 2% for the total liberated G and S units (sum of liberated units EIF and PEPH). Exceptions to this are in radish and spinach, for which the G/S units from the PEPH contribute 6 and 8.4% to the total liberated G and S sum, respectively.

Detection of Minor Monomeric Components Originating from End Groups and Keto Units. Minor DFRC monomers were identified by analyzing MS chromatograms and spectra for DFRC monomers described in various papers of Lu and Ralph (*12*, *13*, *18*, *23*). In our investigations only peaks resulting from DFRC monomers that elute after P were detected. This procedure was necessary due to early-eluting impurities that would excessively contaminate the MS. *p*-Coumaroylated units were not detected in all samples investigated. However, with the exception of asparagus, a monocot plant in which alkali-releasable, wall-bound *p*-coumaric acid was detected (*24*), this peculiar structural feature of grasses was not expected to be found. Small amounts of 3,4-diacetoxycinnamyl acetate and 5-acetoxyconiferyl acetate were found in nearly all samples investigated. These products are likely demethylation products of coniferyl diacetate and sinapyl diacetate and not DFRC products from naturally occurring caffeyl alcohol or 5-hydroxyconiferyl alcohol units.

α-Keto units that that are only partially liberated using the DFRC method and that result in several DFRC products were tentatively identified in some samples. For example, the 1-acetoxy-1-phenylpropan-2-ones G−CHOAc−CO−CH_3 and S−CHOAc−CO−CH_3 (note that here and in the following G and S represent only the aromatic part of coniferyl and sinapyl diacetate), the major DFRC products from coniferyl and sinapyl α-keto β-ethers, were found in pear, kiwi, and, surprisingly, radish EIF. From rhubarb EIF only the S product was detected, and from kohlrabi only the G product was found.

Cinnamyl end groups are more characteristic of "bulk" lignins, in which a fast polymerization occurs. Lignins that are formed by slow and continuous addition of lignin monomers contain lower amounts of cinnamyl end groups because dimerization of lignin monomers is minor. From the analysis of the GC-MS chromatograms for cinnamyl end groups in all investigated EIF with the exception of rhubarb, carrot, apple, and radish, G−$CHOAcCH_2CH_2Br$ was identified by its retention behavior and mass spectrometric data. This compound is one compound that was formed from a coniferyl alcohol end-group model (*13*). However, the main compound formed by application of the DFRC method to this model, G−$CH_2CH_2CH_2Br$, was much harder to find and was detected in only trace amounts, often seen in only the selected ion mode (*m*/*z* 153, 244, 246). Syringyl analogues were not detected, but are always less prevalent because sinapyl alcohol heavily favors β-β-dimerization, which does not produce a cinnamyl alcohol end group. In addition to cinnamyl alcohol end groups, the existence of cinnamaldehyde end groups is implied by the mass spectrometric identification of some compounds that are formed from such groups following DFRC (*13*). In pear EIF both isomers of the G−cyclopropyl−OAc compound were detected. Sinapaldehyde end groups were also identified in pear EIF by detection of one isomer of the S−cyclopropyl−OAc compound and the identification of S−CHOAc−CH_2CH_2OAc, a minor product from the application of the DFRC method to the mentioned model compound. Although the identification of the DFRC minor products is already an indication for the existence of the described structures within fruit and vegetable lignins, future NMR studies will be needed to prove these structural characteristics, for example, the incorporation of cinnamaldehydes into these lignins, and to obtain deeper insight into the different structures of these important food components.

ABBREVIATIONS USED

ABSL, acetyl bromide soluble lignin; DFRC, derivatization followed by reductive cleavage; EIF, enriched insoluble fiber; EPH, enzymatic polysaccharide hydrolysate; G, coniferyl diacetate; IF, insoluble fiber; IS, internal standard; P, *p*-coumaryl diacetate; PEPH, phenolics-containing enzymatic polysaccharide hydrolysate; S, sinapyl diacetate.

ACKNOWLEDGMENT

A standard mixture of the DFRC monomers *p*-coumaryl, coniferyl, and sinapyl diacetates was a kind gift from Fachuang Lu and John Ralph, Dairy Forage Research Center, USDA-ARS, Madison, WI. We thank Andreas Heinze for experimental support.

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Received for review August 15, 2005. Revised manuscript received September 20, 2005. Accepted September 21, 2005.

JF0520037