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Chemical Characterization of Klason Lignin Preparations from Plant-Based Foods

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ABSTRACT: To analyze the accuracy of the Klason lignin method as applied for the determination of lignin contents in plant based-food products, Klason lignin preparations from curly kale, pears, whole wheat grains, and corn bran were chemically characterized. Characterization included routine ash and protein determinations and the extraction of fat/waxes as well as cutin/suberin depolymerization and extraction of the liberated monomers. Fat/wax and cutin/suberin amounts in the Klason lignin preparations were determined gravimetrically, and their compositions were analyzed by using GC-MS. Typical fat, wax, and cutin (and suberin) constituents such as saturated and unsaturated fatty acids, hydroxy and/or epoxy fatty acids, and phenolic acids were identified in all samples, whereas the detection of long-chain hydrocarbons, alcohols, and ketones, sterols, stanols, and dioic acids was dependent on the sample analyzed. Estimation of the contribution of non-lignin compounds to the Klason lignin contents reduced the noncorrected Klason lignin contents of the insoluble fibers from 28.7% (kale), 22.8% (pear), 14.8% (wheat), and 9.9% (corn) to maximum lignin contents of 6.5% (kale), 16.4% (pear), 4.9% (wheat), and 2.3% (corn). These data demonstrate that certain commonly used statements such as "cereal brans are highly lignified" need to be revised.

KEYWORDS: lignin, waxes, cutin, suberin, dietary fiber

INTRODUCTION

Plant cell walls are complex entities composed of polysaccharides, structural proteins, and, depending on the cell type, other polymers such as lignin, suberin, and cutin. Whereas structural proteins do not contribute to the dietary fiber complex, cell wall polysaccharides are the main source of dietary fiber in the human diet. The 2001 dietary fiber definition of the American Association of Cereal Chemists International explicitly mentions lignin as a dietary fiber component, too, and compounds such as waxes, suberin, and cutin are covered by the term dietary fiber "associated plant substances".[1]

Lignins are complex phenolic polymers that are generated through radical polymerization of phenolic compounds, especially the monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. However, other compounds such as phenolic acids can be partially incorporated into the lignin polymer, too.[2,3] The most important reactions in lignification are coupling reactions between lignin monomers and the growing polymer, predominantly resulting in two types of linkages, β-O-4-linkages and, to a lesser degree, β-5-linkages. The β-O-4-ether linkage, which usually represents >50% and often >80% of the linkages, and other common linkage types in natural lignins are stable against most chemical treatments, explaining the high resistance of lignin against chemical degradation. Although stable against most chemical treatments, the β-O-4-ether linkage can be cleaved by certain treatments such as those used in thioacidolysis and the derivatization followed by reductive cleavage (DFRC) methodologies.

Lignin as dietary fiber constituent has an impact on our diet's physiological effects. In in vitro assays it was demonstrated that lignins effectively adsorb heterocyclic aromatic amines,[4−6] thus preventing their being absorbed in the human small intestine and consequently from being activated by phase I/II enzymes in the liver. Although it was also discussed that lignins contribute to the adsorption of bile acids to dietary fiber, this hypothesis was recently disproved in an in vitro experiment.[7] Incorporated into cell walls, lignins can reduce in vitro fermentability of cell wall polysaccharides by human gut bacteria and prolong fermentation times.[8,9] As expected for phenolic polymers, lignins are also able to scavenge radicals, as was demonstrated in several studies.[10,11] Finally, it was shown in an animal model that, despite the chemical resistance of lignin, mammalian lignans can be formed from lignins, most likely by the action of gut bacteria on the lignin polymer.[12]

By using the Klason methodology, average daily lignin intakes of 1.6−2.0 g/day were estimated.[13] However, the Klason lignin methodology, which was originally developed to measure lignin contents in wood,[14] is known to overestimate lignin contents in other plant materials such as forage grasses.[15] There are also strong hints that the Klason lignin method does not accurately measure lignin contents in food products such as cereal grains,

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fruits, and vegetables. Although, for example, wheat bran was frequently described as being highly lignified, we were not able to extract substantial amounts of lignin from wheat bran insoluble fiber.[16] We consequently reasoned that lignin contents for wheat bran and most likely for cereal grains in general are highly overestimated without knowing, though, which plant constituents contribute to the Klason lignin besides "true lignin". Therefore, the aim of this study was to analyze the components that are determined as Klason lignin from plant-based food products and to estimate how closely the Klason lignin contents describe the actual lignin concentrations in these samples.

MATERIALS AND METHODS

General. If not otherwise specified, chemicals were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). All chemicals were of appropriate purity for analytical applications. The enzymes Alcalase 2.4 L (≥2.4 AU/mL), a protease, and AMG 300 (≥300 AGU/mL), an amyloglucosidase, were from Novozymes (Franklinton, NC). Amylex 4T (≥24540 LU/g), a heat-stable α-amylase, was from Danisco (Copenhagen, Denmark). All analyses were performed in duplicate, and concentrations are reported on a dry matter basis. Individual compounds were only reported as present in the samples if they were detected in the GC-MS chromatograms of both replicates.

Materials. Wheat (*Triticum aestivum* L. 'BacUp') was grown and harvested in Minnesota in 2007; whole grains were used for this study. Corn bran had its origin from corn (*Zea mays* L.) that was grown and harvested in Indiana in 2008. Curly kale (*Brassica oleracea* var. *sabellica* L. 'Darkibor') was grown in California in 2010, and pears (*Pyrus communis* L. 'Green Anjou') were harvested in 2010 in the state of Washington. The kale was used as-is, whereas pear cores were removed before dietary fiber preparation.

Preparation of Insoluble Dietary Fibers. Whole wheat kernels and corn bran were ground to pass a 0.25 mm sieve. Kale and pears were cut into small pieces and freeze-dried. The kale was ground to a particle size of <0.18 mm, whereas, due to the presence of stone cells, the lyophilized pears were ground to pass a 0.85 mm sieve. Isolation of dietary fiber was performed by using a scaled up enzymatic procedure as described previously.[17] In brief, samples (20 g) were destarched in a phosphate buffer (300 mL) by applying α-amylase (1.5 mL, 100 °C, pH 6.0, 30 min) and amyloglucosidase (700 μL, 60 °C, pH 4.5, 30 min). Deproteinization was achieved by using the protease Alcalase (600 μL, 60 °C, pH 7.5, 30 min). After centrifugation, the residues were washed with water (twice, 50 mL each, 70 °C), 95% (v/v) ethanol (twice, 50 mL each), and acetone (twice, 50 mL each), dried, and weighed. This material is referred to as uncorrected insoluble dietary fiber and was used for the isolation of Klason lignin. Also, ash and residual protein contents were determined to calculate insoluble dietary fiber contents.

Analysis of Klason Lignin Contents and Preparation of Klason Lignin Samples for Chemical Characterization. Insoluble dietary fiber (0.5 g (analytical procedure)/2.0 g (preparative procedure)) was added to ice-cold H_2SO_4 (12 M, 10 mL (analytical procedure)/40 mL (preparative procedure)) in Erlenmeyer flasks (please note that some Klason lignin procedures use 72% H_2SO_4 instead of 12 M H_2SO_4). The flasks were closed, and the suspension was stirred for 30 min while being ice-cooled and for an additional 2 h at room temperature. The acid was diluted with water (65 mL (analytical procedure)/260 mL (preparative procedure)), and the suspension was heated in a boiling water bath for 2 h. Samples were filtered through previously dried and weighed glass fiber filters (2.5 cm diameter, 1.6 μm particle retention, 1820-025 GF/A, Whatman, Buckinghamshire, U.K.) and washed acid free by using water (ca. 500 mL). The residues were dried, weighed, and scraped off the filters for ash correction (analytical approach) and Klason lignin characterization (preparative approach).

Ash Determination. Ash contents of the uncorrected insoluble dietary fibers and Klason lignins from the analytical approach were analyzed by incineration at 550 °C.

Protein Determination. Residual protein contents in the uncorrected insoluble fibers and protein contents in the Klason lignin preparations were determined by the nitrogen combustion method in tin capsules using a LECO TruSpec Nitrogen Determinator (Leco, St. Joseph, MI). Protein contents were calculated from the analyzed nitrogen contents by using the unspecific factor 6.25.

Gravimetric Determination and Identification of Fat and Wax Constituents. Klason lignin samples (200 mg) were weighed into Erlenmeyer flasks with ground joints. A mixture of *tert*-butyl methyl ether (TBME) and methanol (MeOH) (9:1, v/v, 20 mL) was added, and the suspensions were stirred for 24 h at room temperature. After filtration through previously weighed and dried 1820-025 GF/A glass fiber filters, the filtrates were reduced in volume (rotary evaporator, maximum 25 °C), whereas the residues were dried, weighed, and kept for further analysis (cutin, suberin constituents) as described below. The filtrates were quantitatively transferred into weighed vials, and the solvents were evaporated under a stream of nitrogen. After the residues had dried for 8 h under high vacuum, the TBME/MeOH-soluble compounds, which are referred to as fat and wax constituents, were gravimetrically determined. For identification purposes, the residues were dissolved in hexane (4 mL) and transferred into a round-bottom flask. Potential hexane-insoluble residues were dissolved in small volumes of TBME/MeOH (9:1, v/v) and added to the flask. BF_3 (14%) in MeOH (10 mL) was added, and the solutions were refluxed for 3 h under nitrogen. After evaporation under reduced pressure, water (50 mL) was added to the residues. The suspensions were extracted with chloroform (three times, 30 mL each), and the combined organic extracts were evaporated under reduced pressure, transferred into vials, and dried under high vacuum. The residues were silylated by using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; TCI America, Portland, OR)/pyridine (2:1, v/v), 600 μL) and heating at 70 °C for 1 h in reaction vials. The derivatized fat and wax constituents were analyzed by GC-MS (5890 GC, 5970 mass selective detector, Hewlett-Packard, Palo Alto, CA) on an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (Agilent, Santa Clara, CA). Chromatographic conditions were as follows: initial column temperature, 40 °C, ramped at 9 °C/min to 130 °C, ramped at 2 °C/min to 280 °C, held for 10 min; split (split ratio, 1/20) or splitless injection; injector temperature, 280 °C; carrier gas, helium, 1 mL/min. MS parameters were as follows: transfer line, 275 °C; ion source, 200 °C; ionization energy, 70 eV; mass range, m/z 50−650. Compound identification was achieved by using a database (PAL 600) and comparison with literature data,[18−23] interpretation of mass spectra, and, for some simple fatty acids, comparison with standard compounds. A mixture of the following fatty acid methyl esters was used (Grain FAME Mix; Supelco, Bellefonte, PA): C8:0, C10:0, C12:0, C13:0, C14:0, C14:1Δ9, C15:0, C16:0, C16:1Δ9, C17:0, C18:0, C18:1Δ, C18:1trΔ9, C18:2Δ9, C18:3Δ9, C20:0, C20:1Δ11, C22:0, and C22:1Δ13.

Gravimetric Determination and Identification of Cutin and Suberin Constituents. An aliquot (60 mg) of the residues after extraction of the fat and wax constituents was weighed into round-bottom flasks. BF_3 (14%) in MeOH (20 mL) was added, and the suspensions were refluxed for 16 h under nitrogen.[24] After cooling to room temperature, the samples were filtered through previously dried 1820-025 GF/A glass fiber filters. The filtrates were evaporated at room temperature under reduced pressure, and water (50 mL) was added to the residue. The mixture was extracted with chloroform (three times, 30 mL each), and the combined organic layers were evaporated at room temperature under reduced pressure. The residues were quantitatively

transferred into vials, solvents were removed under a stream of nitrogen, and the residues were dried for 8 h under high vacuum. After correction for methyl esterification as described under Results and Discussion, the gravimetrically determined residues were referred to as cutin and suberin constituents. For identification purposes, the samples were silylated for 1 h at 70 °C by using BSTFA/pyridine (2:1, v/v, 600 μL) and analyzed by GC-MS using the conditions described for the wax and fat constituents.

RESULTS AND DISCUSSION

Determination of Klason Lignin Contents of Insoluble Dietary Fibers. Insoluble dietary fibers from wheat (whole kernels), corn (bran), pears, and curly kale were isolated by using a scaled up enzymatic procedure following the basic steps of the dietary fiber analysis. The uncorrected fiber contents of the dried samples were 42.6 ± 0.2% (kale), 17.2 ± 0.2% (pear), 12.4 ± 0.2% (whole grain wheat), and 61.3 ± 0.6% (corn bran). Following correction for ash and residual protein contents, the insoluble dietary fiber concentrations of the dried samples were 31.9 ± 1.4% (kale), 16.2 ± 0.4% (pear), 10.8 ± 0.3% (whole grain wheat), and 57.8 ± 0.6% (corn bran). Klason lignin contents were determined using a two-step acid hydrolysis to remove the bulk of non-lignin compounds. After correction for ash contents, the Klason lignin contents of the corrected insoluble dietary fibers were 28.7 ± 0.5% (kale), 22.8 ± 0.7% (pear), 14.8 ± 0.2% (whole grain wheat), and 9.9 ± 1.2% (corn bran). Although ash correction is a requirement of the Klason lignin method, ash contents in the Klason lignin preparations were usually <3%, thus having a minor impact on the Klason lignin concentrations of the corrected insoluble dietary fibers. To further characterize the Klason lignin preparations, the Klason lignin method was performed on a preparative scale and the resulting preparations were chemically analyzed.

Nitrogen/Protein Content of the Klason Lignin Preparations. Nitrogen contents of the Klason lignin preparations were analyzed by the combustion method. By calculating protein as N × 6.25, protein contents of 23.0 ± 0.8% (kale), 5.8 ± 1.6% (pear), 21.3 ± 2.6% (whole grain wheat), and 20.1 ± 2.0% (corn bran) were determined. These data partially reflect the residual protein contents that were found in the dietary fibers, for example, 16.0% in kale but only 4.4% in pear. However, the protein contents in the cereal Klason lignin preparations do not follow residual protein trends of the insoluble dietary fibers (wheat, 12.3%; corn bran, 4.3%). This means that proteins of the dietary fiber preparations from corn remain in higher proportions in the acid-hydrolyzed residue than those of wheat dietary fiber preparations. However, the analyzed nitrogen may not completely stem from proteins but can also represent, for example, water-insoluble, potentially high molecular weight Maillard reaction products that can be especially formed during the heating steps in the dietary fiber and Klason lignin procedures.

Fat and Wax Constituents of the Klason Lignin Preparations. The enzymatic−gravimetric analysis of dietary fiber requires only samples with fat contents >10% to be defatted by using petroleum ether.[25,26] Therefore, none of our samples had to be defatted before fiber preparation. Some lipids are partially removed during ethanol and acetone washing steps of the fiber. Heat treatment with strong acid during the Klason lignin preparation should cleave, at least partially, ester linkages between the fatty acids and glycerol. Glycerol and most of the amphiphilic fatty acids should then be removed while the residues are washed until acid free. However, whether or not these processes are complete and remove all fat from the Klason lignin preparations is not known.

Waxes can be found as epicuticular (exterior to cutin) and intracuticular (residing "within" the cutin) waxes, which help the plant in water loss reduction, but are also discussed to be involved in protection against plant pathogens and repulsion or attraction of insects. Additionally, waxes can be associated with suberin. Waxes are soluble in organic solvents, and their compositions are extremely complex: long-chain (>20 carbons) hydrocarbons, long-chain primary and secondary alcohols, and long-chain aldehydes and ketones, as well as free and esterified fatty acids. The chains often contain up to 40 carbons for the monomers, with the fatty acid esters (esters of $C_{16}-C_{34}$ fatty acids and $C_{20}-C_{36}$ primary alcohols)[27] containing more than 40 carbons. Besides the aliphatic compounds, waxes also include pentacyclic triterpenoids. Mixtures of these compounds form the intra- and epicuticular waxes, and their compositions are very different between plant species and also depend on environmental conditions. Due to their lipophilic nature most waxes need to be removed during the washing steps of the dietary fiber. However, because waxes tightly interact with the cuticle (especially the intracuticular waxes), a major portion potentially remains with the isolated fiber. Consequently, waxes are also described as dietary fiber-associated substances in the dietary fiber definition. Again due to their lipophilic properties, a substantial proportion of the waxes may also stay in the Klason lignin preparations.

To analyze the Klason lignin preparations for residual fat and wax constituents, the lignin samples were extracted for 24 h with TBME/MeOH (9:1, v/v), which was suggested by Bauer et al.[28] as a less toxic alternative than chloroform extraction. Following a first 24 h extraction, a second 24 h extraction was performed using the same solvent. Because the second extraction did not remove additional lipids, the standard procedure used a 24 h extraction only. The gravimetric determination of the high vacuum-dried residues revealed the following amounts of fat and wax constituents in the Klason lignin preparations: 42.4 ± 0.2% (kale), 5.3 ± 0.6% (pear), 7.8 ± 0.3% (whole grain wheat), and 23.8 ± 4.1% (corn bran).

The fat and wax constituents were then identified as their methyl esters and trimethylsilyl ethers (Tables 1 and 2). Saturated and unsaturated fatty acids, hydroxy and/or epoxy fatty acids, and ferulic acid were identified in all samples, whereas long-chain hydrocarbons, alcohols, and ketones (15-nonacosanone) as well as sterols and stanols were not identified across all samples (Tables 1 and 2). As a leafy vegetable, curly kale has a large wax-covered surface, which easily explains the high percentage of fat and wax constituents in the Klason lignin preparations. Although we did not aim to quantify the different constituents, it should be noted that the GC-MS chromatogram was dominated by signals which were identified to represent hexadecanoic acid, unsaturated C_{18} fatty acids, nonacosane, and 15-nonacosanone. Although not unambiguously identified, another large peak at 82.3 min, eluting just before 15-nonacosanone, was probably due to coeluting 14- and 15-nonacosanol as described previously.[27] Nonacosane, 15-nonacosanone, 14/15-nonacosanol, and related C_{29} compounds are typical wax constituents found in *B. oleracea* cultivars,[29−31] but C_{29} secondary alcohols were, for example, also found in waxes from *Pisum sativum* leaves.[32] These compounds, together with long-chain alcohols (hexacosanol, heptacosanol) and fatty acids, which are typically found in waxes such as nonadecanoic (likely branched) and tetracosanoic acid, prove

Table 1. Tentatively Identified Acids That Were Extracted (either Free or Esterified) with a *tert*-Butyl Methyl Ether/Methanol (9:1, v/v) Mixture from Klason Lignin Preparations[a]

	kale	pear	wheat	corn bran
saturated acids				
hexadecanoic acid (palmitic acid)	+	+	+	+
heptadecanoic acid[b]	+	−	−	−
octadecanoic acid (stearic acid)	+	+	+	+
nonadecanoic acid[b]	+	−	−	−
eicosanoic acid (arachidic acid)	−	+	−	+
docosanoic acid (behenic acid)	−	+	+	−
tetracosanoic acid (lignoceric acid)	+	−	−	+
hexacosanoic acid (cerotic acid)	−	−	+	−
octacosanoic acid	−	+	−	−
unsaturated acids				
octadecenoic acid (oleic acid)	+	+	+	+
octadecadienoic acid (linoleic acid)	+	+	+	+
octadecatrienoic acid (α-linolenic acid)	+	+	−	−
nonadecadienoic acid[b]	+	−	−	−
nonadecatrienoic acid[b]	+	−	−	−
eicosenoic acid	−	−	+	+
hydroxy and epoxy acids				
10,16-dihydroxyhexadecanoic acid	−	+	−	−
(9,10-epoxy-18-hydroxyoctadecanoic acid)[c]	−	+	+	−
9,10,18-trihydroxyoctadecanoic acid	−	+	+	−
18-hydroxyoctadecenoic acid	−	−	+	+
2-hydroxytetracosanoic acid	+	+	+	+
phenolic acids				
ferulic acid	+	+	+	+

[a] Analyzed in the form of their methyl esters, trimethylsilyl ethers. [b] Possibly branched. [c] See text.

that a substantial amount of kale waxes will be isolated as Klason lignin. Other fatty acids such as C_{16} and C_{18} fatty acids can stem from wax esters or from triglycerides or their (partial) degradation products after acidic hydrolysis used during the Klason lignin preparation. Even though less noticeable in the kale sample when compared to the other samples, partial degradation of the cutin polyester network into extractable monomers or oligomers during Klason lignin preparation may also contribute to the "fat and wax" fraction. Although, for example, 2-hydroxytetracosanoic acid was found in waxes,[33] it was also described as a monomer of the cutin polyester and/or the polyaliphatic domain of the suberin polymer.[34−36] Partial degradation of these polymers potentially also contributed to the large hexadecanoic acid signal in the kale fat/wax fraction.

Only about 5.3% of the pear Klason lignin was extractable by using TBME/MeOH. The GC-MS chromatograms of these fractions were dominated by signals, representing, again, hexadecanoic acid and nonacosane. However, contrary to kale, typical cutin monomers, that is, 9,10,18-trihydroxyoctadecanoic acid and 10,16-dihydroxyhexadecanoic acid, were other dominant signals in the fat/wax fraction, demonstrating again partial degradation of cutin during the preparation of the Klason lignin. Because the cutin monomers are mostly bound through ester linkages, a partial degradation under the strong acidic conditions of the Klason lignin method is not unexpected. However, it is still

Table 2. Tentatively Identified Alkanes, Alcohols, Ketones, and Sterols That Were Extracted with a *tert*-Butyl Methyl Ether/Methanol (9:1, v/v) Mixture from Klason Lignin Preparations[a]

	kale	pear	wheat	corn bran
alkanes				
nonacosane	+	+	−	−
hentriacontane	+	−	−	−
alcohols				
docosanol	−	+	−	−
hexacosanol	+	+	−	−
heptacosanol	+	−	−	−
ketones				
15-nonacosanone	+	−	−	−
sterols and stanols				
campesterol	−	−	−	+
stigmasterol	−	−	−	+
β-sitosterol	+	+	−	+
stigmastanol	−	−	−	+

[a] Analyzed in the form of their trimethylsilyl ethers (hydroxyl group containing compounds).

surprising that the extensive washing of the Klason lignin with water does not remove more of the amphilic free acids. It is possible, though, that the free acids were not analyzed in this approach but that less polar but TBME/MeOH extractable oligomers were extracted from which the acids were liberated by MeOH/BF_3 treatment before GC-MS analysis.

In the TBME/MeOH fractions from both whole wheat kernels and corn bran C_{16} and C_{18} fatty acids dominated the GC-MS spectra. Contrary to wheat, corn bran Klason lignin also contained sterols and stanols with β-sitosterols as one of the dominant signals in the chromatogram. Phytosterols are frequently found in wax preparations from plants. However, it is discussed whether they are wax constituents or coextracted from plasma membranes.[27] In our corn sample, phytosterols could be wax compounds, but they can easily stem from internal lipids, too. However, long-chain fatty acids such as tetracosanoic acid are more likely to be wax constituents[37] than from internal lipids. Again, the occurrence of 18-hydroxyoctadecenoic acid suggests that some polyester degradation products were extracted by using TBME/MeOH. Different from corn, substantial amounts of 9,10,18-trihydroxyoctadecanoic acid were found as cutin degradation products in the fat/wax fraction from wheat. Surprisingly, a signal at 68.8 min, which was also detected in the pear samples, showed a mass spectrum that matches the spectrum of 9,10-epoxy-18-hydroxyoctadecanoic acid.[19] Whereas this epoxy acid is a common cutin monomer, it was not expected to be detected due to both the harsh acidic conditions during the Klason lignin preparation and the (trans)esterification by using MeOH/BF_3. During both steps the epoxy group is supposed to be the subject of an opening, resulting in either diol or methoxyhydrin derivatives. Thus, the mass spectral identification of this potential epoxy acid remains somewhat ambiguous.

Cutin (and Suberin) Constituents of the Klason Lignin Preparations. The surface of the epidermal cells of aerial organs of land plants, for example, fruits, leaves, and primary stems, is sealed by the cuticle, which is composed of a lipophilic polymer matrix, the cutin, and the already mentioned waxes. Suberin is

deposited in both external and internal tissues and is found, for example, in the periderm, the hypodermis, and the endodermis and in the Casparian band. It is also an important factor in wound healing of plant organs, best demonstrated for potatoes.[38−40] Cutin is mostly composed of C_{16} and C_{18} hydroxy fatty acids, for example, 16-hydroxyhexadecanoic acid and 18-hydroxyoctadec-9-enoic acid, and especially hydroxy fatty acids with additional hydroxyl or epoxyl groups, for example, 10,16-dihydroxyhexadecanoic acid or 9,10-epoxy-18-hydroxyoctadecanoic acid. Small amounts of phenolic acids can be found, too. Suberin is composed of a polyaliphatic and a polyphenolic or polyaromatic domain. The polyaromatic domain consists of hydroxycinnamic acids and their derivatives such as ferulic acid and feruloyl tyramine.[41,42] The polyaliphatic domain is a polyester with hydroxy fatty acids, α,ω-dicarboxylic acids, and glycerol as dominant constituents and alcohols and unsubstituted fatty acids being less abundant.[43] In the past, α,ω-dicarboxylic acids were described to be characteristic for suberin, but *Arabidopsis* cutin was demonstrated to have high proportions of these acids, too.[35] Whereas the dietary fiber preparation does not contain steps suitable to remove either suberin or cutin, the strong acidic conditions during the Klason lignin preparation should cleave the ester linkages, at least partially.

The potential polyesters in the Klason lignin preparations were depolymerized by MeOH/BF_3 treatment, and the liberated monomers were extracted, dried under high vacuum, and weighed as cutin and suberin constituents. To test the recovery of fatty acid methyl esters under the high vacuum conditions applied, we used 20 mg of our fatty acid methyl ester standard mixture and dried it under the same conditions. Because we did not find a significant reduction in weight after the drying step, we assumed that no systematic error seriously conflicts with the results presented here. As the acids are liberated and weighed in the form of their methyl esters the results need to be corrected for the added methyl group. Because we did not quantitate the individual cutin and suberin monomers in the residues, we corrected the gravimetric results by taking the weighed residue as being completely methyl octadecanoate. This approach results in a correction factor of 0.953 for the determined residues and approximate cutin/suberin contents of 11.7 ± 4.4% (kale), 16.4 ± 2.0% (pear), 35.9 ± 2.0% (whole grain wheat), and 32.5 ± 1.5% (corn bran) in the Klason lignin preparations. However, due to the indirect determination of cutin and suberin monomers used here, these data should be classified as semiquantitative data at best.

After silylation of potential hydroxyl groups, the cutin/suberin constituents were identified by using GC-MS (Table 3). The composition of this fraction clearly demonstrates that cutin is determined as Klason lignin in all samples analyzed. Although no quantification of the liberated monomers was attempted, the signal representing 18-hydroxyoctadecenoic acid clearly dominates the wheat chromatogram. This cutin monomer was also analyzed as one of the two dominating cutin monomers in wheat bran samples by Matzke and Riederer.[36] In addition to 18-hydroxyoctadecenoic acid, Matzke and Riederer determined 9,10-epoxy-18-hydroxyoctadecanoic acid as a major cutin monomer from wheat bran.[36] This monomer was not found in our wheat cutin/suberin fraction. However, 9,10,18-trihydroxyoctadecanoic acid was another dominant signal in the wheat chromatogram. As discussed earlier, this monomer can be potentially formed from 9,10-epoxy-18-hydroxyoctadecanoic acid during the acid hydrolysis required for the Klason lignin preparation. Among the phenolic compounds ferulic acid dominates the

Table 3. Tentatively Identified Acids That Were Extracted with Chloroform from Klason Lignin Preparations after *tert*-Butyl Methyl Ether/Methanol Extraction and Methanol/BF_3 Treatment[a]

	kale	pear	wheat	corn bran
saturated acids				
hexadecanoic acid (palmitic acid)	+	+	+	+
heptadecanoic acid[b]	+	−	−	−
octadecanoic acid (stearic acid)	+	+	+	+
unsaturated acids				
octadecenoic acid (oleic acid)	−	−	+	+
octadecadienoic acid (linoleic acid)	+	+	+	+
octadecatrienoic acid (α-linolenic acid)	+	−	−	−
nonadecadienoic acid[b]	+	−	−	−
nonadecatrienoic acid[b]	+	−	−	−
hydroxy acids				
16-hydroxyhexadecanoic acid	+	+	+	+
10,16-dihydroxyhexadecanoic acid	+	+	(+)[c]	−
18-hydroxyoctadecanoic acid	−	−	+	+
9,10,18-trihydroxyoctadecanoic acid	−	+	+	−
18-hydroxyoctadecenoic acid	+	+	+	+
22-hydroxydocosanoic acid	−	−	+	+
2-hydroxytetracosanoic acid	+	+	+	+
2-hydroxytetracosenoic acid	+	−	−	−
24-hydroxytetracosanoic acid	−	−	+	+
26-hydroxyhexacosanoic acid	−	−	+	−
α,ω-dioic acids				
hexadecane-1,16-dioic acid	+	−	−	−
octadecene-1,18-dioic acid	+	−	+	−
tetracosane-1,24-dioic acid	−	−	(+)	−
phenolic acids				
vanillic acid	+	+	+	+
syringic acid	−	+	+	+
p-coumaric acid	(+)[c]	+	(+)[c]	+
ferulic acid	+	−	+	+
dehydrodiferulic acids[d]	−	−	−	+

[a] Analyzed in the form of their methyl esters, trimethylsilyl ethers. [b] Possibly branched. [c] Coeluting compound complicates identification. [d] See text.

wheat chromatogram. In accordance with results reported by Beaugrand and co-workers[44] we detected dioic acids, that is, small amounts of octadecene-1,18-dioic acid and trace amounts of tetracosane-1,24-dioic acid, in the wheat sample. Dioic acids are discussed as markers for suberin,[45] but were recently also found in the cuticle from *Arabidopsis* leaves.[35,46]

The corn chromatogram was dominated by phenolic acids, especially *p*-coumaric acid, but also ferulic acid. Other large signals were identified as 18-hydroxyoctadecenoic acid and unsaturated C_{18} fatty acids (Table 3). Whereas the involvement of small amounts of phenolic compounds in the formation of cutin is well-known, it was rather unexpected to find the phenolic acids among the dominant signals in the chromatogram of this particular "cutin fraction" isolated from a Klason lignin preparation. Because *p*-coumaric acid can be ester-linked to the lignin monomer sinapyl alcohol in corn,[2,47] some *p*-coumaric acid could have its origin from potential lignin in this fraction. The overall composition of this fraction, however, suggests that lignin is not

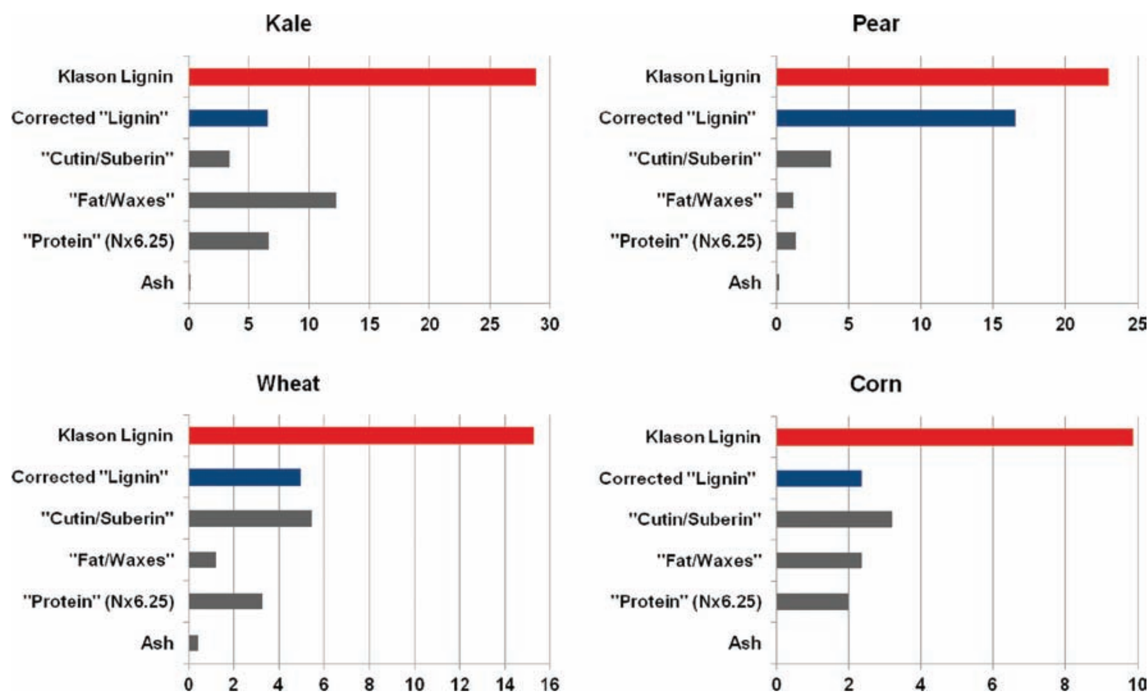

Figure 1. Uncorrected Klason lignin contents (red bars) and corrected "lignin" contents (blue bars) in insoluble dietary fibers from kale, pear, wheat (whole grains), and corn (bran). The corrected "lignin" contents may represent true lignin but possibly also other plant cell components which are not acid-extractable and which were not analyzed as non-lignin components of the Klason lignin preparations in this study, for example, cutan. The gray bars indicate an estimation of the amounts of different plant cell wall compounds that are isolated from the insoluble dietary fibers by using the Klason lignin methodology. Please note that Klason lignin data are usually corrected for the ash contents.

the major source for *p*-coumaric acid in this fraction. For example, ferulic acid is not attached to the lignin polymer through ester linkages, which is the only linkage type to be cleaved by the MeOH/BF_3 treatment. The attachment of ferulic acid to lignin monomers (and consequently to the polymer) was demonstrated to be through either C−C or ether linkages,[48−50] neither of which is cleaved under the conditions used here. Finally, two signals with m/z 558 and 486, respectively, indicate the presence of ferulic acid dehydrodimers in this fraction.[51] Whereas m/z 558 is indicative for a dimer that does not involve a hydroxyl group in the linkage between the two ferulate moieties, m/z 486 represents those diferulates with the phenolic group involved in the linkage between the monomers such as 8-*O*-4-dehydrodiferulic acid.

The cutin/suberin fraction of kale Klason lignin contained 10,16-dihydroxyhexadecanoic acid, a typical cutin monomer. Other dominating signals in the GC-MS chromatogram were identified as hexadecanoic acid, unsaturated C_{18} fatty acids, and, tentatively, nonadecatrieonic acid. The pear GC-MS chromatogram was dominated by 10,16-dihydroxyhexadecanoic acid. Whereas the identification of this cutin monomer was not unexpected, the phenolic composition was more surprising. Due to the analogy of its mass spectrum with vanillic acid (characteristic fragments + m/z 30) syringic acid was identified as one of the dominant phenolic compounds in this fraction in addition to vanillic acid. Vanillic acid and syringic acid were identified as suberin monomers,[52] but their origin in these pear Klason lignin samples remains to be elucidated in more detail.

Corrected "Lignin" Contents. Klason lignin data are routinely corrected for their ash contents. Correction of the ash-free Klason lignin data for "proteins", residual fat, and waxes as well as cutin (and suberin) results in maximal lignin contents of 6.5% in kale insoluble fiber (instead of 28.7%), 16.4% in pear insoluble fiber (instead of 22.8%), 4.9% in wheat insoluble fiber (instead of 14.8%), and 2.3% in corn insoluble fiber (instead of 9.9%) (Figure 1). Although our semiquantitative approach used here still contains potential sources of errors (e.g., protein equal to N × 6.25, methyl esterification of cutin/suberin monomers), these data clearly demonstrate that the Klason lignin methodology may produce fair data (although far from being perfect) for samples such as pears, which are indeed known to contain higher amounts of lignin and from which clean lignin preparations can be isolated and characterized by NMR.[16] However, these data also demonstrate that the Klason lignin methodology is not suitable for samples such as cereal grains. Although small amounts of lignin or lignin-like compounds are indeed cereal grain polymers/oligomers,[44,50,51] there is no justification to describe cereal grains/bran as highly lignified. The "high" lignin content of cereal brans is often used to explain some physiological effects of cereal dietary fibers such as low fermentability. We demonstrated earlier that only small amounts of considerably impure lignin can be isolated from wheat.[16] This led us to conclude that lignin contents of cereal grains were probably highly overestimated in the past. The data presented here clearly support this conclusion. Although one major uncertainty of this study is whether the correction for protein is correct as other nitrogen sources such as Maillard reaction products can potentially contribute to the nitrogen content of the Klason lignin preparations, it also has to be kept in mind that even more of the Klason lignin is potentially represented by non-lignin polymers. For example, in our approach we cannot differentiate between lignin and cutan in the residue after cutin/suberin extraction.

Finally, it should be noted that partial solubilization of lignin during the acid treatment is another potential problem of the

Klason lignin methodology. This aspect, which we investigated only to some extent, can lead to (slight) underestimation of the lignin contents. In our studies we neutralized the acid fraction after hydroylsis and extracted it with ethyl acetate. UV scans of these fractions indicated the presence of lignin fragments. This was confirmed by applying the DFRC methodology on, for example, the pear extract. The detection of low amounts of the DFRC products of sinapyl alcohol and coniferyl alcohol hints that some lignin is not measured by using the Klason lignin method. More in-depth studies are needed, however, to quantify these losses during the acid hydrolysis of the Klason lignin procedure.

Whereas the Klason lignin method is clearly not suitable to analyze lignin in plant-based food products, especially in dietary fiber, the bigger challenge will be now to develop methods to analyze the lignin contents in these complex, often low-lignin, samples more accurately. Pre-extraction steps, which sometimes precede the actual Klason lignin isolation, may help to reduce the amounts of waxes and lipids in the Klason lignin. However, this would not reduce the impact of suberin and cutin on the Klason lignin contents. Also, whereas the combination of different proteases may slightly reduce the concentrations of residual proteins in the dietary fiber and potentially Klason lignin preparations, it is unlikely that this problem can be solved by just using enzymatic protein removal, especially because certain structural proteins are known to be inefficiently degraded by (commercially available) proteases.

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ABBREVIATIONS USED

BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; MeOH, methanol; TBME, *tert*-butyl methyl ether; DFRC, derivatization followed by reductive cleavage.

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