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Influence of Lignification and Feruloylation of Maize Cell Walls on the Adsorption of Heterocyclic Aromatic Amines

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Both epidemiological and experimental data indicate that a diet rich in fiber may reduce cancer risk. One possible mechanism is by adsorbing carcinogens and transporting them out of the body without metabolic activation. We investigated the role of fiber lignification and feruloylation on the adsorption of four of the most relevant heterocyclic aromatic amines in food: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC). Adsorption experiments, under conditions mimicking the small intestine, were carried out using nonlignified and artificially lignified primary maize walls with defined lignin and ferulate/diferulate concentrations and defined lignin compositions. Lignin concentration and composition both influenced the adsorption of heterocyclic aromatic amines, especially the more hydrophobic types. Heterocyclic aromatic amine adsorption increased with lignin concentration. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and 2-amino-9*H*-pyrido[2,3-*b*]indole were better adsorbed by guaiacyl-rich lignins, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline by syringyl-rich lignins, whereas the adsorption of 2-amino-3-methylimidazo-[4,5-*f*]quinoline was not clearly influenced by lignin composition. Nonlignified cell walls adsorbed lesser amounts of heterocyclic aromatic amines. Variations in cell wall feruloylation had no effect on heterocyclic aromatic amine adsorption.

KEYWORDS: Heterocyclic aromatic amines; carcinogens; adsorption studies; *Zea mays*; dehydrogenation polymer−cell wall complexes; lignin; monolignols; ferulates; diferulates

INTRODUCTION

Dietary fiber is generally thought to protect against colorectal and possibly other forms of cancer. Although some studies indicate no association between dietary fiber intake and colorectal cancer (*1*−*3*), large studies such as the European prospective investigation into cancer and nutrition (EPIC) and the U.S.-based prostate, lung, colorectal, and ovarian (PLCO) cancer screening trial showed a cancer-preventive role of dietary fiber (*4*, *5*). Several direct and indirect mechanisms are proposed by which dietary fibers may decrease cancer risk. The indirect mechanisms are mainly based on effects induced by dietary fiber degradation products, especially short-chain fatty acids such as butyrate, formed by colonic bacteria. Direct mechanisms such as increase of fecal bulk and shortening of transit time by undegradable dietary fibers may reduce exposure of colonic mucosal cells to carcinogens. The ability of some dietary fibers to adsorb and transport carcinogens out of the body is also proposed as a direct mechanism for preventing cancer (*6*).

A widely discussed group of carcinogens are heterocyclic aromatic amines (**Figure 1**), which are mostly found in heated protein-rich foods such as meat or fish. Nonmetabolized heterocyclic aromatic amines are considered as not being carcinogenic, but their activated metabolites show mutagenic and possibly carcinogenic properties. One of the main mechanisms of metabolic activation occurs when heterocyclic aromatic amines absorbed by the small intestine are transported to the liver where oxidation of the exocyclic amine group by cytochrome P450s leads to *N*-hydroxy-amines. These metabolites are then acetylated or sulfonated by phase II enzymes. The unstable esters readily undergo heterolytic cleavage to produce reactive nitrenium ions, which are able to form promutagenic DNA adducts (*7*). Another important metabolic pathway is based on glucuronidation of *N*-hydroxy metabolites and cleavage by intestinal flora, which is then followed by the already described acetylation or sulfonation and cleavage to reactive nitrenium ions (*8*). Adsorption of heterocyclic aromatic amines by fiber may prevent their absorption by the small intestine and bioactivation in the liver.

With the use of in vitro conditions simulating the small intestine, it has been shown that dietary fibers, particularly those

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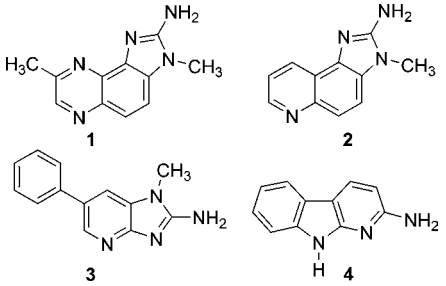

Figure 1. Structures of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) **1**, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) **2**, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) **3**, and 2-amino-9*H*-pyrido-[2,3-*b*]indole (AαC) **4**.

containing lignin or suberin, readily adsorb heterocyclic aromatic amines (*9*, *10*). Lignin increases fiber hydrophobicity, and this may enhance adsorption of hydrophobic carcinogens (*10*). However, the interpretation of these experiments is not easy, because the model dietary fibers differed in lignin composition and concentration but also other dietary fiber characteristics. As a result, it is not clear whether lignin or some other component was responsible for enhanced adsorption of heterocyclic aromatic amines by some types of fiber.

The two primary precursors (i.e., monolignols) of lignin are coniferyl alcohol and sinapyl alcohol, but other 4-hydroxyphenylpropanoids and conjugates may also be incorporated into the macromolecule. In the presence of peroxidase and H_2O_2, monolignols form electron-delocalized radicals, which couple at various sites with other monolignol radicals or predominantly the growing lignin molecule, to form a complex polymer with a variety of interunit linkages (*11*). Ferulate monomers (**7**), ester-linked to polysaccharides in cereal dietary fiber, also undergo radical coupling to form various dimers (**8**) and trimers (**Figure 2**) (*12*−*15*). Ferulates also oxidatively couple with the growing lignin polymer (*16*, *17*). Oxidative coupling of ferulate with itself and with lignin leads to extensive cross-linking of the fiber matrix, thus affecting the physicochemical properties of dietary fibers (*18*, *19*).

The aim of our study was to clearly delineate how various types and concentrations of lignin affect the adsorption of several heterocyclic aromatic amines (**Figure 1**) by fiber. In these studies, we artificially lignified maize cell walls to form dehydrogenation polymer−cell wall complexes (DHP−cell walls) with defined lignin and polysaccharide properties. In other studies, feruloylated nonlignified maize walls were used to investigate the adsorption of heterocyclic aromatic amines by ferulates and diferulates.

MATERIALS AND METHODS

General. Coniferyl alcohol, sinapyl alcohol, 5-hydroxyconiferyl alcohol, dihydroconiferyl alcohol, and γ-acetylated sinapyl alcohol were kindly provided by Hoon Kim and Fachuang Lu (U.S. Dairy Forage Research Center, Wisconsin). Coniferyl aldehyde was obtained from Aldrich, and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (amine **1**), 2-amino-3-methylimidazo[4,5-*f*]quinoline (amine **2**), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (amine **3**), and 2-amino-9*H*-pyrido[2,3-*b*]indole (amine **4**) (**Figure 1**) were from Toronto Research Chemicals, North York, Canada. Triethylamine phosphate buffer (10 mM, pH 6.5) was freshly prepared before each HPLC analysis.

Synthesis of AIP−Cell Walls. Nonlignified cell walls (**Table 1**) were prepared according to Grabber et al. (*20*). In brief, 350 mL of suspension cultures of maize (*Zea mays* L. cv. Black Mexican) were grown in 1 L flasks (media consisting of 4.5 g/L Murashige and Skoog basal medium, 30.0 g/L sucrose, and 2 mg/L 2,4-dichlorophenoxyacetic acid, pH 5.6−6.8). During cell growth 0, 0.4, or 10.6 mg of 2-aminoindan-2-phosphonic acid (AIP)/L of suspension culture was added to vary the degree of cell wall feruloylation (*20*). After 14 days of culture, cells were collected on a nylon mesh (20 μm pore size) and washed with cold 1,4-piperazinediethanesulfonic acid (PIPES) buffer (10 mM, pH 7.0). Cells suspended in PIPES buffer were ruptured by two passages through a Parr nitrogen bomb maintained at 1500 psi. After each passage, cell fragments were collected on a nylon mesh and washed with PIPES buffer. After washing with $CaCl_2$ solution (50 mM) and water cell walls were resuspended in water, and a dilute aqueous solution of H_2O_2 (0.3 mmol of H_2O_2 per g dry weight of cell walls) was added over a 1 h period to one-half of the cell walls of each AIP treatment. Cell walls were then collected on glass microfiber filters (3.1 μm retention), washed thoroughly with water and acetone, and dried at 55 °C.

Synthesis of DHP−Cell Walls. Formation of DHP−cell walls (**Table 2**) was carried out according to Grabber et al. (*21*). Maize cultures were grown without AIP, and cell walls were isolated as described above. Cell walls were resuspended in homopiperazine-1,4-bis(2-ethane-sulfonic acid) (HOMOPIPES) buffer (25 mM, pH 5.5) and dilute aqueous H_2O_2 (0.2 mmol of H_2O_2 per gram dry weight of cell walls) was added over a 1.5 h period. After 30 min of additional stirring, separate solutions of monolignols (0.3−1.0 mmol of monolignol per gram dry weight of cell walls, on ice) dissolved in water/acetone (80/20, v/v) and dilute aqueous H_2O_2 (0.5−1.5 mmol of H_2O_2 per gram dry weight of cell walls) were added dropwise over a period of 5, 10, or 15 h to form DHP−cell walls with about 5%, 10%, and 14% lignin. The following monolignols were used as lignin precursors in different ratios: coniferyl alcohol (**1**), sinapyl alcohol (**2**), 5-hydroxy coniferyl alcohol (**3**), γ-acetylated sinapyl alcohol (**4**), dihydroconiferyl alcohol (**5**), and coniferyl aldehyde (**6**) (**Figure 2**). Following monolignol additions, cell walls were stirred for an additional 24 h, and then cell walls were collected on glass microfiber filters, washed extensively with water and acetone, and dried at 55 °C.

Characterization of Cell Walls. The lignin content of DHP−cell walls was determined by a modified Klason lignin method (*22*). Ferulic acid and diferulic acids were determined by GC-FID following alkaline hydrolysis of cell walls, ether extraction, and silylation as described by Ralph et al. (*12*).

Incubation of Cell Walls with Heterocyclic Aromatic Amines. Minor impurities were removed by mixing DHP−cell walls (60 mg) with water (10 mL) for 30 min at 37 °C on a rotating shaker. After centrifugation of cell wall suspensions, supernatants were discarded, and the washing procedure was repeated three times with water (at 37 °C) and three times with acetone (at room temperature). After washing, cell walls were dried under a stream of nitrogen and stored at 55 °C.

Incubations of heterocyclic aromatic amines with cell walls were done in triplicate, using a modification of the method described by Roberton et al. (*23*). Cell walls (15 mg) were weighed into conical glass centrifuge tubes and swelled overnight in 1128 μL of phosphate-buffered saline (10 mM sodium phosphate buffer, pH 6.5, containing 130 mM sodium chloride). After adding 72 μL of an amine **1**, **2**, **3**, or **4** solution (2.5 μg/mL in methanol/water (50/50, v/v)), samples were incubated on a rotating shaker (35 rpm) at 37 °C for 1 h. Following centrifugation (3200 rpm), 200 μL of each supernatant was taken for HPLC analysis.

HPLC Analysis of Nonadsorbed Heterocyclic Aromatic Amines. Adsorption of each heterocyclic aromatic amine to cell walls was determined indirectly by quantifying the amount of nonadsorbed heterocyclic aromatic amines in the supernatant after incubation. Quantification was performed by HPLC−UV (D-6000A interface, AS-4000A intelligent autosampler, L-6200 intelligent pump, L-4250 UV−vis detector) (Merck, Darmstadt, Germany) with a column oven with control unit (Knauer, Berlin, Germany) using a 250 mm × 4.6 mm i.d., 5 μm Luna Phenyl-Hexyl 100A column (Phenomenex, Aschaffenburg, Germany). The injection volume was 40 μL. The column temperature was 45 °C, and the flow rate was maintained at 1.2 mL/min. Detection wavelengths were 275 nm (amine **1**), 260 nm (amine **2**), 315 nm (amine **3**), and 338 nm (amine **4**). Elution was carried out using 10 mM triethylamine phosphate buffer, pH 6.5 (A) and acetonitrile (B). For AIP−cell walls, the following elution systems were used: amine **1**, A 80%, B 20%, held isocratically for 15 min; amine **2**, A

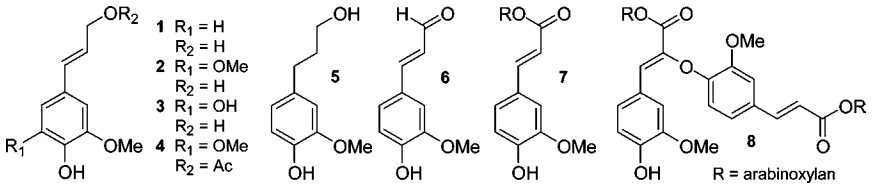

Figure 2. Structures of monolignols and ferulates: coniferyl alcohol **1**, sinapyl alcohol **2**, 5-hydroxyconiferyl alcohol **3**, γ-acetylated sinapyl alcohol **4**, dihydroconiferyl alcohol **5**, coniferyl aldehyde **6**, *trans*-ferulic acid ester **7**, 8-*O*-4-dehydrodiferulic acid ester **8**, one of several possible diferulate regioisomers.

Table 1. Ferulate Concentrations of Investigated Cell Walls Treated with 2-Aminoindan-2-phosphonic Acid (AIP–CWs)[a]

cell walls	AIP addition [mg/L suspension culture]	H_2O_2 addition	ferulates [mg/g CW] monomers	dimers	total
AIP–CW 1	–	–	12.84	1.79	14.63
AIP–CW 2	0.4	–	5.24	1.26	6.50
AIP–CW 3	10.6	–	2.14	0.45	2.59
AIP–CW 4	–	+	9.97	4.03	14.00
AIP–CW 5	0.4	+	3.83	2.01	5.84
AIP–CW 6	10.6	+	1.43	0.70	2.14

[a] Ferulate monomers were partly dimerized by H_2O_2 addition.

Table 2. Precursors and Klason Lignin Concentrations of Investigated Dehydrogenation Polymer–Cell Walls (DHP–CWs)

cell walls	precursors for DHP–cell wall complexes	Klason lignin [%]
DHP–CW 1	coniferyl alcohol	7.56 13.86 19.02
DHP–CW 2	coniferyl alcohol + sinapyl alcohol 2:1	7.51 13.92 16.21
DHP–CW 3	coniferyl alcohol + sinapyl alcohol 1:2	6.88 11.88 12.42
DHP–CW 4	sinapyl alcohol	4.80 9.48 11.34
DHP–CW 5	coniferyl alcohol + sinapyl alcohol + 5-hydroxyconiferyl alcohol 1:1:1	6.95 11.02 16.23
DHP–CW 6	coniferyl alcohol + sinapyl alcohol + dihydroconiferyl alcohol 1:1:1	7.12 13.06 18.82
DHP–CW 7	coniferyl alcohol + sinapyl alcohol + coniferyl aldehyde 1:1:1	7.44 13.70 20.44
DHP–CW 8	coniferyl alcohol + sinapyl alcohol + γ-acetylated sinapyl alcohol 1:1:1	6.76 11.52 13.17
control	none	0.4 (*20*)

86%, B 14%, held isocratically for 15 min; amine **3**, initially A 67%, B 33%, linear over 6 min to A 85%, B 15%, held isocratically for 1 min, following an equilibration step; amine **4**, initially A 57%, B 43%, linear over 3 min to A 45%, B 55%, held isocratically for 2 min, following an equilibration step. Elution systems used for analysis of DHP−cell walls were the same for amines **1** and **3**. Amine **2** was analyzed using A 84%, B 16%, held isocratically for 15 min. The elution system for amine **4** was for DHP−cell walls 1−4 (**Table 2**) the same as described above. DHP−cell walls 5−8 were analyzed with the following elution system: A 58%, B 42%, held isocratically for 15 min.

Heterocyclic aromatic amine concentrations of HPLC samples were calculated from peak areas using external calibration curves of five standard solutions, generally concentrated between 35 and 175 ng/mL. External calibration was carried out by incubating and centrifuging heterocyclic aromatic amine solutions in a manner analogous to that used for the investigated cell walls and subsequent HPLC analysis. Thus, heterocyclic aromatic amine losses due to possible unspecific adsorption to glassware or other losses during procedure were considered. However, recovery rates were >97%. Quantitation limits (calculated from a signal-to-noise ratio of 10) were 10 ng/mL for amine **2**, 15 ng/mL for amines **1** and **4**, and 35 ng/mL for amine **3**.

RESULTS AND DISCUSSION

Cell Wall Characteristics. In our studies, cell walls with varying degrees of lignin content, lignin composition, cell wall feruloylation, and ferulate dehydrodimerization were produced for heterocyclic aromatic amine adsorption studies. Cell walls from maize cell suspensions contain only trace amounts of lignin (0.4% Klason lignin), and their polysaccharide composition is typical of primary walls in cereals (*20*).

Artificially lignified cell walls (DHP−cell wall complexes) were formed by slowly adding monolignols and H_2O_2 to cell walls isolated from maize suspensions (**Table 2**). The artificial lignins formed by polymerization of monolignols by in situ wall-bound peroxidases were shown in previous work to be structurally similar to natural grass lignins (*21*). DHP−cell walls with about 5% lignin were prepared to mimic quantities reported for insoluble dietary fibers of rye bran (4.5%) and maize bran (5.7%) (*24*). To further evaluate the effects of lignin concentration on heterocyclic aromatic amine adsorption, additional DHP−cell walls with 10% and 14% lignin were also prepared. Recently, comparable lignin concentrations were detected in kiwi (8.3%) and rhubarb (9.0%) insoluble fiber (*25*). Actual levels of Klason lignin in DHP−cell walls varied somewhat depending on the monolignol mixture used to lignify cell walls (**Table 2).** As in previous work, very high proportions of sinapyl alcohol occasionally reduce the yields of generated polymeric complexes (*26*). This reflects the results from studies on peroxidases showing that most peroxidases are less efficient in oxidizing sinapyl alcohol than in oxidizing coniferyl alcohol (*11*). However, as also shown in previous work, DHP complexes of mixed monolignols were readily generated (*21*). For example, determination of nonbound monolignols in the reaction media by UV scans showed that DHP−cell wall complexes containing guaiacyl and guaiacyl−syringyl (1:2) units from cell walls with about 10% lignin both incorporated about 94% of added monolignols. These results confirm that lignins of the desired monolignol ratios were formed.

DHP−cell walls were formed with coniferyl alcohol and sinapyl alcohol in ratios of 1:2 and 2:1 to form lignins representing the range of syringyl−guaiacyl composition observed in cereal fiber (e.g., ratios of 0.4 in insoluble rye bran dietary fiber and 1.6 in insoluble maize bran dietary fiber (*24*)). In cereal grain lignins, guaiacyl and syringyl units predominate, whereas *p*-hydroxyphenyl units are only detected in trace amounts (*24*). To further probe the effect of lignin composition on heterocyclic aromatic amine adsorption, additional DHP−

cell wall complexes were formed with only coniferyl alcohol or sinapyl alcohol and with other 4-hydroxyphenylpropanoids that have been detected in natural lignins and particularly in mutant and transgenic plants. Extremely low syringyl−guaiacyl ratios are characteristic for carrots (0.03) and kiwis (0.06), whereas rhubarb has a very high syringyl−guaiacyl ratio (6.2) (*25*). Dihydroconiferyl alcohol and γ-acetylated sinapyl alcohol are both found incorporated into natural plant lignins (e.g., γ-acetylated sinapyl alcohol in kenaf and dihydroconiferyl alcohol in most softwood lignins). Incorporation of coniferyl aldehyde into lignin is particularly observed in plants with a deficit in cinnamyl alcohol dehydrogenase activity (*11*) but also in "normal" plants, for example, in pear insoluble fiber (*25*). 5-Hydroxyconiferyl alcohol is mainly present in plants with downregulated caffeic acid *O*-methyl transferase (*11*).

Nonlignified cell walls from maize cell suspensions contained 14.6 mg/g of total ferulates, about 10% of which were diferulates (**Table 1**). Treatment of isolated walls with H_2O_2 to stimulate dehydrodimerization by wall-bound peroxidases increased the proportion of diferulates to about 30%. The concentrations of ferulate monomers (10 mg/g) and diferulates (4 mg/g) in H_2O_2-treated cell walls were comparable to that reported for insoluble dietary fiber of rye (7 mg/g ferulates and 4 mg/g diferulates) and rice (8 mg/g ferulate and 4 mg/g diferulates) grains but were lower than the concentrations reported for maize grains (25 mg/g ferulates and 13 mg/g diferulates) (*18*, *27*). The degree of cell wall feruloylation was manipulated by growing cell suspensions with AIP, a specific inhibitor of phenylalanine ammonia lyase (*28*). Growing cell suspensions with 0.4 and 10.6 mg/L of AIP reduced total ferulate concentrations by 57% and 84%, respectively, without markedly altering the proportion of diferulates in cell walls treated with and without H_2O_2 (**Table 1**).

Adsorption of Heterocyclic Aromatic Amines by Cell Walls. Adsorption studies were performed with the four heterocyclic aromatic amines shown in **Figure 1**. These heterocyclic aromatic amines, especially amines **3** and **4** (**Figure 1**), are the most abundant types found in cooked food, detected in amounts of up to 500 ng/g. Amine **1** is the most frequently observed heterocyclic aromatic amine (*29*). The investigated amines represent different compositional groups (amine **1** as aminoimidazo-quinoxaline, amine **2** as aminoimidazo-quinoline, amine **3** as aminoimidazo-pyridine, and amine **4** as α-carboline), and they also cover the range of hydrophobicity, with amine **1** being one of the least hydrophobic and amine **4** being one of the most hydrophobic heterocyclic aromatic amines.

Although estimation of heterocyclic aromatic amine intake is difficult and depends on personal eating habits, previous adsorption studies have often used heterocyclic aromatic amine concentrations (e.g., in the range of 0.1−1 μg of heterocyclic aromatic amine/mg of cell wall) that are probably in great excess of concentrations found in human diets. Calculations of heterocyclic aromatic amine concentrations for our studies were based on data summarized by Jägerstad et al. (*29*). A model meal of 200 g of meat and 200 g of maize may contain up to 7 μg of heterocyclic aromatic amine and 4.0 g of dietary fiber (*29*, *30*), leading to a heterocyclic aromatic amine−dietary fiber ratio of 1.75 ng/mg. In our studies, 15 mg of cell walls were incubated with 180 ng of heterocyclic aromatic amine, resulting in a heterocyclic aromatic amine−cell wall ratio of 12 ng/mg. This ratio is still high, but lower ratios could not be used, limited by the quantitation limits of the investigated amines in our HPLC methodology. Swelling of cell walls during incubation prevented us from using smaller volumes for the assay.

Under conditions simulating the small intestine (pH 6.5, 37 °C, 1 h, continuous agitation), all four heterocyclic aromatic amines were adsorbed by all cell walls investigated, as shown in **Figures 3** and **4**. The extent of adsorbed heterocyclic aromatic amines varied between 8% and 72% and was influenced by the amines' hydrophobicity, lignin concentration, lignin composition, and ferulate concentration of cell walls as follows.

Hydrophobicity of Heterocyclic Aromatic Amines and Cell Wall Adsorption. Across all types of cell walls, adsorption was greatest with amine **4** (up to 72%), intermediate with amine **3** (up to 58%), and lowest with amines **1** and **2** (up to 29%) (**Figure 3**). Previous studies (*9*, *10*) suggest hydrophobicity of heterocyclic aromatic amines influences their adsorption to fiber. The hydrophobicity of a substance can be estimated from the calculated log of the partition coefficient (ClogP). ClogP is based on the partitioning of a substance between 1-octanol and water, and ClogP values increase with increasing hydrophobicity. ClogPs of the four investigated heterocyclic aromatic amines were calculated using a database of Advanced Chemistry Development (ACD/Labs). On the basis of these calculations, amine **4** is the most hydrophobic heterocyclic aromatic amine (ClogP = 3.05), followed by amine **3** (ClogP = 1.26) and amine **2** (ClogP = 0.85), while amine **1** is the least hydrophobic heterocyclic aromatic amine (ClogP = 0.52). The relation between heterocyclic aromatic amine hydrophobicity and adsorption indicates that adsorption is mainly based on hydrophobic interactions with cell wall components.

Lignin Concentration and Heterocyclic Aromatic Amine Adsorption. The Klason lignin concentrations of the investigated DHP−cell walls varied between 4.8% and 20.4%. To permit direct comparisons between different DHP−cell walls, adsorption data were interpolated to lignin levels of 4.0%, 7.5%, and 11.0% by linear regression. The measured adsorption values were plotted against lignin concentrations for each DHP−cell wall type as illustrated for amine **4** adsorption in **Figure 5**. Although a quadratic relationship between lignin concentration and adsorption could not be fully excluded for some treatments, linearity was demonstrated for most of the treatments. Therefore, linear regression was used uniformly for all treatments. Correlation coefficients were generally between 0.915 and 1.000. However, in six cases correlation coefficients were worse (0.724−0.899), especially for the poorly adsorbed amines **1** and **2**.

The interpolated data in **Figure 3** demonstrate that the adsorption of all four heterocyclic aromatic amines, especially amines **3** and **4**, increased as lignin concentrations of DHP−cell walls increased. For example, the adsorption of amine **4** to guaiacyl−DHP−cell walls (DHP−CW 1) (**Figure 3d**) increased from 39% to 58% as Klason lignin increased from 4% to 11%. The lignin concentration seems to be less important for the adsorption of the less hydrophobic amines **1** and **2**. With these compounds, increasing the Klason lignin concentration of guaiacyl−DHP−cell walls from 4% to 11% only increased heterocyclic aromatic amine adsorption from 19% to 24% for amine **2** and from 13% to 18% for amine **1**. However, it has to be considered that cereal dietary fibers are best represented by DHP−cell walls with 4% Klason lignin, whereas high lignin contents such as 11% are not common for cereals, although some fruit and vegetable insoluble dietary fibers contain higher lignin amounts than cereal fibers (*25*).

Cell walls that were not artificially lignified were used as control cell walls. These nonlignified cell walls, containing only trace amounts of Klason lignin (*20*), also adsorbed heterocyclic aromatic amines, but in smaller amounts. The adsorption to

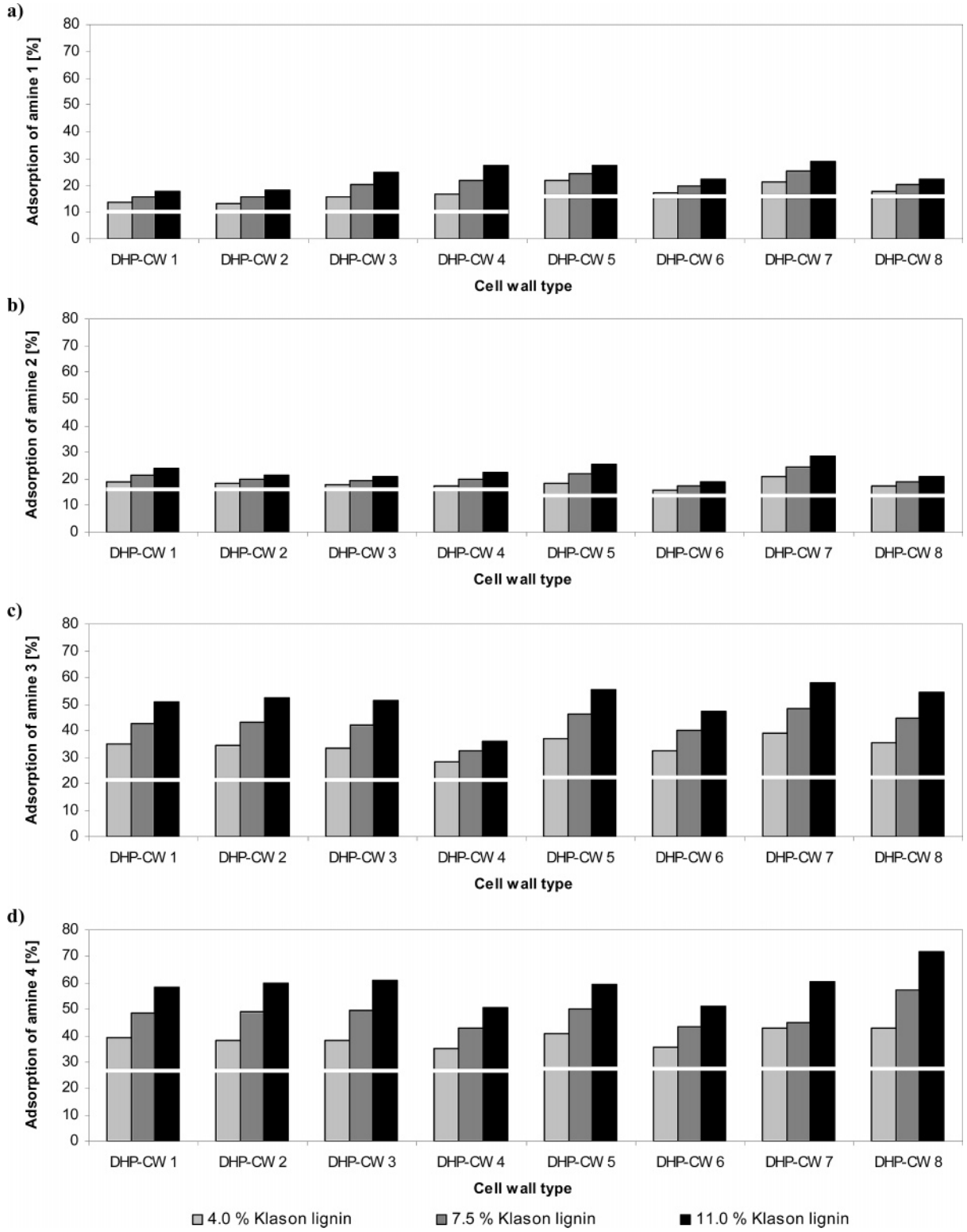

Figure 3. Adsorption of (**a**) 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx, amine **1**), (**b**) 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ, amine **2**), (**c**) 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP, amine **3**), and (**d**) 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC, amine **4**) (**Figure 1**) by dehydrogenation polymer (DHP)–cell walls (**Table 2**), interpolated to Klason lignin concentrations of 4.0, 7.5, and 11.0%. The white lines mark the adsorption by nonlignified control cell walls.

nonlignified cell walls is demonstrated by white lines in **Figure 3**. Adsorption to nonlignified cell walls was lowest for amine **1** (on average 13%) and amine **2** (15%), intermediate for amine **3** (22%), and highest for amine **4** (26%). When the adsorption of "nonlignified" cell walls with the adsorption of artificially lignified DHP−cell walls is compared, the significance of the lignin concentration of the cell walls for the adsorption of heterocyclic aromatic amines especially becomes clear for amines **3** and **4**. For example, guaiacyl−DHP−cell walls with 4% Klason lignin (DHP−CW 1) adsorbed 52% more amine **4** (increase from 26% to 39% adsorption) and 60% more amine **3** (increase from 22% to 35% adsorption) than the "nonlignified" control cell walls (**Figure 3c**,**d**). However, especially for the adsorption of amine **2**, the cell wall matrix seems to be more

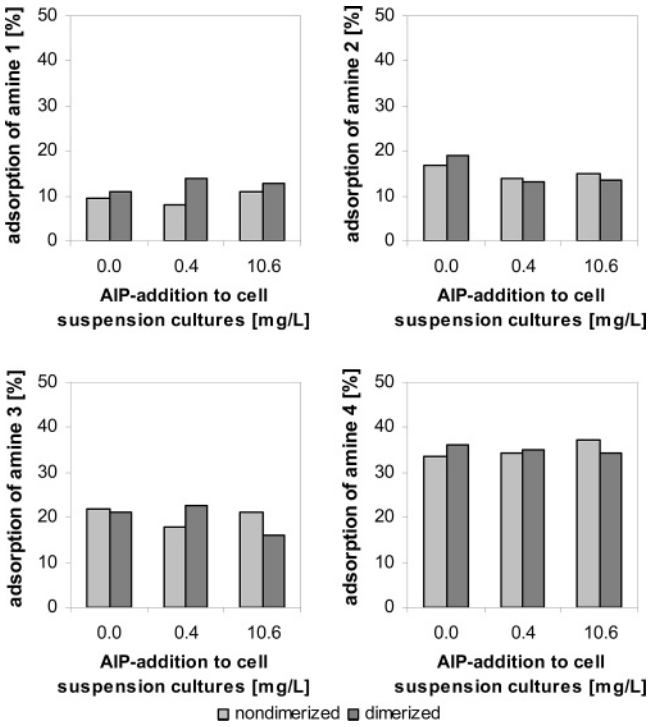

Figure 4. Adsorption of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx, amine **1**), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ, amine **2**), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP, amine **3**), and 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC, amine **4**) (**Figure 1**) by cell walls with normal and reduced (di)ferulate concentrations (**Table 1**). Reduction of ferulate levels was achieved by treatment of the growing cells with 0.4 and 10.6 mg of 2-aminoindan-2-phosphonic acid (AIP)/L of suspension culture. Ferulate monomers of AIP–cell walls were partly dimerized by H_2O_2 addition.

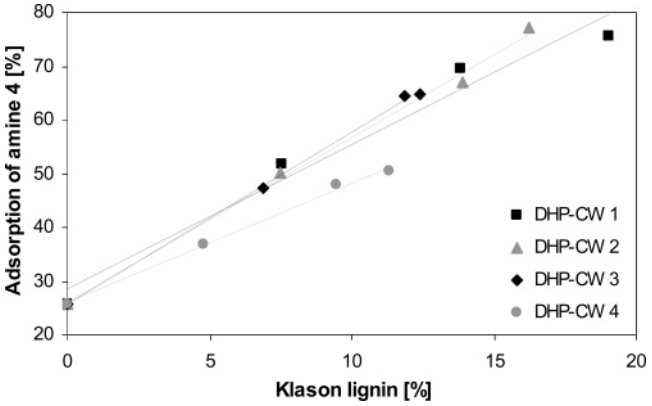

Figure 5. Adsorption of 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC, amine **4**) (**Figure 1**) by dehydrogenation polymer (DHP)–cell walls 1–4 (**Table 2**). Correlation coefficients of linear regression are 0.984 (DHP–CW 1), 0.998 (DHP–CW 2), 0.999 (DHP–CW 3), and 0.998 (DHP–CW 4).

effective. In comparison to nonlignified control cell walls, guaiacyl−DHP−cell walls with 4% Klason lignin (DHP−CW 1) (**Figure 3b**) increased the adsorption of amine **2** only by 16% (from 16% to 19% adsorption). Although the effect of lignin on the adsorption of amines **3** and **4** is very distinct, the matrix should also be considered as potent adsorber for these heterocyclic aromatic amines. Thus, in future studies the adsorption properties of different matrix components, especially matrix polysaccharides, have to be investigated more detailed.

Lignin Composition and Heterocyclic Aromatic Amine Adsorption. The impact of guaiacyl−syringyl ratios on the adsorption of heterocyclic aromatic amines was investigated, using DHP−cell walls formed with varying ratios of coniferyl alcohol (**1**) and sinapyl alcohol (**2**). Additional DHP−cell wall complexes were formed by adding 5-hydroxyconiferyl alcohol (**3**), γ-acetylated sinapyl alcohol (**4**), dihydroconiferyl alcohol (**5**), or coniferyl aldehyde (**6**) with coniferyl alcohol (**1**) and sinapyl alcohol (**2**) to investigate how lignins formed with more unusual monolignols may influence heterocyclic aromatic amine adsorption (**Figure 2**).

Lignin composition influenced the adsorption of all four heterocyclic aromatic amines (**Figure 3**). Among the DHP−cell walls with guaiacyl−syringyl lignins, the more hydrophobic amines **3** and **4** were better adsorbed to lignins containing guaiacyl units; variation from 100% guaiacyl units (DHP−CW 1) to 1:2 guaiacyl to syringyl (DHP−CW 3) did not affect adsorption, but shifting lignin composition to 100% syringyl units (DHP−CW 4) decreased adsorption to cell walls with 11% Klason lignin by 30% for amine **3** and 13% for amine **4** (**Figure 3c**,**d**). Adsorption of amine **2** showed no clear preference for guaiacyl or syringyl units (**Figure 3b**), whereas amine **1** had greater affinity to syringyl-rich DHP−cell wall complexes (**Figure 3a**). These shifts might be explained by the differing hydrophobicity of coniferyl alcohol (ClogP = 0.92) compared to that of sinapyl alcohol (ClogP = 0.57). For example, amine **1**, the least hydrophobic heterocyclic aromatic amine, prefers adsorption to DHP−cell walls mainly derived from the less hydrophobic sinapyl alcohol. However, it has to be considered that lignin is a complex macromolecule, and the adsorption properties probably do not only depend on the hydrophobicities of the monolignols themselves but also on the lignin structures resulting from the coupling positions of the lignin units.

This assumption is supported by the results of the experiments run with the DHP−cell walls deriving from "special" monolignols. Hydrophobicity of the monolignols increases from 5-hydroxyconiferyl alcohol (ClogP = 0.59), over dihydroconiferyl alcohol (ClogP = 0.85) and coniferyl aldehyde (ClogP = 1.35), to γ-acetylated sinapyl alcohol (ClogP = 1.50). Even though dihydroconiferyl alcohol has an intermediate hydrophobicity, DHP−cell walls that incorporated dihydroconiferyl alcohol (DHP−CW 6) (**Figure 3**) had the least adsorption of heterocyclic aromatic amines. The adsorption effect of DHP−cell walls containing γ-acetylated sinapyl alcohol (DHP−CW 8) (**Figure 3**) increased with growing hydrophobicity of the investigated heterocyclic aromatic amines and was best for the adsorption of amine **4**. Cell walls containing 11% Klason lignin, derived from coniferyl alcohol, sinapyl alcohol, and γ-acetylated sinapyl alcohol (1:1:1), adsorbed 72% of amine **4**, more than the corresponding DHP−cell walls with a 1:2 ratio of guaiacyl to syringyl units (61%, DHP−CW 3) (**Figure 3d**).

Ferulates and Heterocyclic Aromatic Amine Adsorption. Ferulates may interact with heterocyclic aromatic amines, for example by forming π−π interactions between the aromatic systems. Furthermore, diferulates are able to cross-link polysaccharides, thus possibly forming more hydrophobic regions by extensive cross-linking. As already shown for the much more expanded lignin complex, hydrophobicity enhances the adsorption of some heterocyclic aromatic amines. The influence of ferulates on the adsorption of heterocyclic aromatic amines was investigated with maize cell walls isolated from cell suspensions containing normal and reduced ferulate levels as well as varying degrees of ferulate dimerization (**Table 1**).

The adsorption properties of these cell walls are presented in **Figure 4**. As already shown for the adsorption to DHP−cell walls, the adsorption of the four investigated heterocyclic

aromatic amines increased with their growing hydrophobicity. However, there was no clear effect of cell wall feruloylation or ferulate dimerization on the adsorption of heterocyclic aromatic amines.

In conclusion, synthesis of DHP−cell walls with varying, defined lignin concentrations and compositions and synthesis of cell walls with normal and reduced ferulate concentrations enabled us to investigate the influence of lignification and feruloylation on the in vitro adsorption of the four most abundant heterocyclic aromatic amines in human nutrition (**Figure 1**). The results demonstrated that all heterocyclic aromatic amines were adsorbed to all cell walls and adsorption of heterocyclic aromatic amines was related to their hydrophobicity. While different degrees of feruloylation of cell walls had no effect, both lignin concentration and variations in the syringyl and guaiacyl makeup of lignin units influenced the adsorption of heterocyclic aromatic amines. Adsorption of heterocyclic aromatic amines increased with greater lignin deposition into cell walls. The more hydrophobic amines **3** and **4** were better adsorbed by guaiacyl-rich DHP−cell wall complexes, whereas amine **1**, the least hydrophobic heterocyclic aromatic amine, showed an increased affinity to syringyl-rich DHP−cell walls complexes. Incorporation of other 4-hydroxyphenylpropanoids into the DHP−cell wall complexes indicated that unusual monolignols, found in some types of plant lignins, also influence adsorption of heterocyclic aromatic amines; all amines had greater affinity to DHP−cell walls containing 5-hydroxyconiferyl alcohol and coniferyl aldehyde than to DHP−cell walls containing dihydroconiferyl alcohol. The affinity of the more hydrophobic heterocyclic aromatic amines was greatly enhanced when DHP−cell walls were formed with γ-acetylated sinapyl alcohol.

Lignified fiber is very effective for adsorbing the more hydrophobic heterocyclic aromatic amines **3** and **4**. This effect is even large for relatively low lignin concentrations that are typically found in cereal grain dietary fibers. However, lignified fiber is rather poor for adsorbing the less hydrophobic heterocyclic aromatic amines **1** and **2**. The adsorption of the less hydrophobic heterocyclic aromatic amines is mainly affected by the nonlignified cell wall matrix and not by the lignin amounts usually found in cereal grain fibers. Since considerable amounts of the more hydrophobic heterocyclic aromatic amines were also adsorbed to nonlignified fiber, adsorption to the cell wall matrix should not be underestimated and needs to be further investigated.

ABBREVIATIONS USED

AIP, 2-aminoindan-2-phosphonic acid; DHP, dehydrogenation polymer; PIPES, 1,4-piperazinediethanesulfonic acid.

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SAFETY

The International Agency of Research on Cancer classified the investigated heterocyclic aromatic amines as to their carcinogenic risk to humans as follows.

2-Amino-3-methylimidazo[4,5-*f*]quinoline is probably carcinogenic to humans. The exposure circumstance entails exposures that are probably carcinogenic to humans.

2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, and 2-amino-9*H*-pyrido[2,3-*b*]indole are possibly carcinogenic to humans. The exposure circumstance entails exposures that are possibly carcinogenic to humans.

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