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Human Intervention Study: Alkylresorcinol Metabolites as Potential Biomarkers for Grain Intake and the Occurrence of Alkylresorcinols in Commonly Consumed Foods in the German Retail Sector

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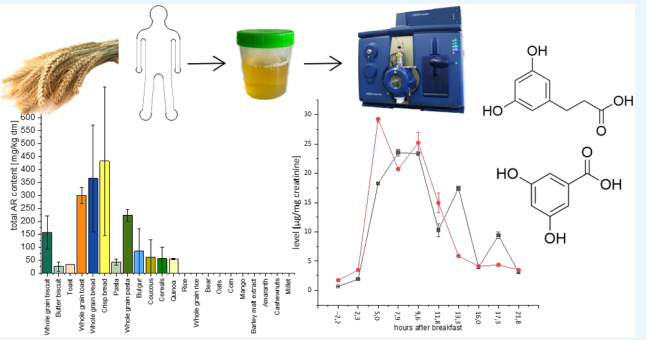
ABSTRACT: Grains are one of the primary nutrients and are associated with many health benefits. To reflect the intake of grain-based products, two promising potential biomarkers are alkylresorcinol (AR) metabolites 3,5-dihydroxybenzoic acid (3,5-DHBA) and 3-(3,5-dihydroxyphenyl)-propanoic acid (3,5-DHPPA). The aim of this study was to validate the occurrence of AR in food samples and investigate the suitability of their metabolites as potential biomarkers in human intervention studies. In the first step, the AR content in different grain products from the German retail sector was analyzed by GC−MS. ARs were found in higher concentrations in whole grain products and in moderate contents in refined grains and quinoa. Based on these results, human intervention studies were performed in the next step, and the AR metabolites 3,5-DHBA and 3,5-DHPPA were analyzed by LC-MS/MS in urine samples. The intake of only whole grain products leads to an increasing level of both potential biomarkers, while a refined grain diet shows decreasing levels of the AR metabolites. The excretion of 3,5-DHBA after a whole grain-rich diet differs significantly (p = 0.043) from no grain intake.



1. INTRODUCTION

The human diet is one of the crucial factors to sustain good health. Many chronic diseases such as obesity, cardiovascular diseases, metabolic syndrome, or some forms of cancer are directly linked to the human diet whereas other factors might also play a critical role.[1−3] However, to be able to correlate the diet and the occurrence of chronic diseases, more reliable evidence is required. Therefore, it is important, to provide a more reliable and accurate tool for dietary assessment.[4] Traditional methods such as food diaries or food-frequency questionnaires are often used. However, these methods are assumed to be prone to random and systematic errors, such as wrong estimations of portion size or misreporting of food choices. Metabolic biomarkers in biological matrices such as hair, blood, or urine which reflect the qualitatively as well as quantitatively intake with sufficient accuracy are required for reliable estimation of the nutritional intake.[4] Besides the objective assessment of food consumption, biomarkers provide insight into the intake of certain substances from food and can provide further information on biological effects and their potential impact on consumer health.[5]

Whole grain is known for its high quantity of fiber, vitamins, and minerals, especially in comparison to refined grain products, and is associated with health benefits. The intake of whole grains is linked to decreased risk of type-2 diabetes, cardiovascular diseases, and colon cancer.[6,7] Grain is one of the primary nutrients consumed in the world. In 2020/2021, the annual pro-capita consumption in Germany was 82,7 kg on average.[8] Grain provides around 56% of the energy and 50% of the protein intake. One aim to monitor a good health status is to reliably estimate whole grain intake in the human body.

To monitor the total intake of whole grain products, a selective and robust biomarker is necessary. One potential group of biomarkers is the 5-*n*-alkylresorcinols (AR). These are amphiphilic 1,3-dihydroxybenzene derivatives, with an odd-numbered alkyl chain in position five of the benzene ring, which is found in the bran fraction of most cereal grains (Figure 1). The highest amounts of ARs are found in wheat and rye with levels in a moderate to high mg/kg range.[9−11] Lower amounts can be found in barley, other grains, and pseudograin products. ARs occur exclusively in the outer part of a kennel which can be almost completely removed during the milling process leading to smaller amounts of ARs in refined products.[12,13] However, in refined rye products, the AR concentration is higher than in other refined flours due to the

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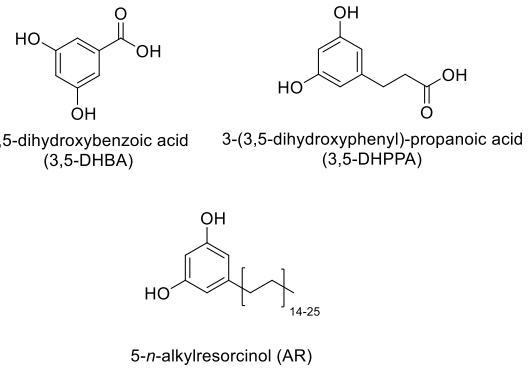

Figure 1. Structure of AR metabolites 3,5-DHBA and 3,5-DHPPA (above) and the general structure of ARs (below).

difficulty of separating the aleurone layer from the starchy endosperm.[7,10]

Wheat ARs have a saturated alkyl chain with a length between 17 and 25 carbon atoms, with C21:0 as the most abundant. In rye, the ARs have a chain length between 15 and 25 carbon atoms with C19:0 as the most abundant alkyl chain, which is saturated as well.[7,10] Figure 1 shows the basic structure of ARs and of the two most abundant metabolites 3,5-dihydroxybenzoic acid (3,5-DHBA) and 3-(3,5-dihydroxyphenyl)-propanoic acid (3,5-DHPPA).

Besides their occurrence in whole grains, ARs are also found in several families of higher plants, algae, mosses, bacteria, and fungi. Among the commonly consumed foods, they are almost exclusively found in whole grain products. Knödler et al. reported a total AR content in mango peels from 73.3 to 1851 mg/kg of dry mass (dm) and in mango pulp from 4.9 to 187.3 mg/kg dm.[14] They are also found in smaller amounts in cashew nuts and peas.[15] Following food supply data, the average per capita intake of total AR is 17.5 mg/d in Sweden and 11.9 mg/d in the UK.[16]

To estimate the intake of ARs based on the analysis of biological fluids, the metabolic pathway has to be elucidated. Ileostomy studies show that the absorption of the ARs is approximately 60%. The ARs are absorbed in the upper intestine via the lymphatic system. Feeding studies with radiolabeled C21:0 show that the maximum concentration of ARs in plasma in rats was reached after 10 h. The radiolabeled C21:0 was completely removed after 140 h, while in urine and feces, it peaked after 24 h. The excreted radioactivity was present as a polar compound. This suggests that the ARs are metabolized before the excretion in urine.[17]

The AR metabolism in the liver consists of two steps. The first step is ω-oxidation catalyzed by the CYP4F2 enzyme. Then, the hydroxy group is oxidized to a carboxylic acid, followed by ß-oxidation in the last step. In this manner, the alkyl chain is shortened into the two main metabolites 3,5-DHBA and 3,5-DHPPA (Figure 1). Both molecules can be detected in urine, where they also occur as glucuronides or sulfates.[18,19] Several studies proposed 3,5-DHBA and 3,5-DHPPA as potential biomarkers for whole grain intake.[18−21] Nevertheless, little is known about the influence of refined grain products and other foods that contain ARs in lower amounts as well as about the applicability of the potential biomarkers without bigger interventions. A number of studies quantifying the AR content in different grains and grain products have been conducted during the last decades, but currently, no data from the German retail sector are available.[10,17,22−24]

The aim of this study was first to quantify the total AR content in different commonly consumed grain products from the German market. Screening of different foods (grains, grain-based products, and pseudograins) was performed to get a better overview of the different sources of ARs as precursors for the potential biomarkers 3,5-DHPPA and 3,5-DHBA. Especially the nongrain products were of interest to provide further information about the specificity of the potential biomarkers for grain intake. The second goal was to investigate the kinetic excretion of the two most abundant metabolites 3,5-DHBA and 3,5-DHPPA in human intervention studies. The aim was to analyze the potential correlation between the main AR metabolites and the food intake with different ratios of whole grain products based on the normal eating habits of the volunteers. Furthermore, the suitability of the AR metabolites as biomarkers under normal eating habits was investigated.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Analytical Standard. The analytical standards of 3,5-dihydroxybenzoic acid and 3-(3,5-dihydroxyphenyl)-propanoic acid were purchased from Sigma-Aldrich (Steinheim, Deutschland). The analytical standard AR C15:0 (Adipostatin A) was purchased from Cayman Chemical (Ann Arbor, USA) and the internal standard methyl behenate was from Merck (Darmstadt, Deutschland). All solvents used for LC-MS/MS analyses were at least HPLC grade and were purchased from Fisher Scientific GmbH (Schwerte, Deutschland). The solvents used for the extraction of the ARs were at least analytical grade and have been purchased at Carl-Roth GmbH + Co. KG (Karlsruhe, Germany) and Sigma-Aldrich (Steinheim, Deutschland). ASTM type 1 quality water was produced using a Purelab Flex 2 system (Veolia Water Technologies, Celle, Germany).

2.2. Analysis of Alkylresorcinols in Foods. *2.2.1. Extraction with Ethyl Acetate.* The extraction of alkylresorcinols in different types of flours followed the protocol from Ross et al.[25] 250 mg of the sample were transferred into a 15 mL tube and 10 mL ethyl acetate and 50 μL internal standard (methyl behenate) were added. After homogenization, the samples were shaken at room temperature for 24 h. Subsequently, flour samples were centrifuged at 3200 *g* for 5 min at room temperature. An aliquot of 4 mL was taken from the supernatant; the organic solvent was removed using a vacuum concentrator at 40 °C and the residue was reconstituted using 200 μL of ethyl acetate. Subsequently, the sample was centrifuged at 15,000 *g* for 10 min at room temperature and the supernatant was transferred to the GC−MS.

2.2.2. Extraction with 1-Propanol/Water. This extraction method was used especially for processed products and followed the protocol from Menzel et al.[9] The samples were freeze-dried, ground, and dried at 105 °C for 16 h. Afterward, 500 mg of each sample were transferred into a round-bottom flask and mixed with 200 μL of the internal standard. The sample was extracted two times for 2 h and one time for 1 h using 10 mL of a mixture containing 1-propanol and water (3/1, v/v) each time under reflux. These conditions were chosen to extract the ARs from the starch-lipid complex.[11] The three extracts were combined and filled to 50 mL with the extraction solvent. The sample was centrifuged at 3200 *g* at room temperature for 5 min and subsequently, 5 mL from the

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ACS Omega 2024, 9, 23555−23566

supernatant was dried under vacuum at 40 °C. The residue was reconstituted using 200 μL ethyl acetate, centrifuged at 15,000 *g* for 10 min at room temperature, and the supernatant was used for GC−MS analysis.

2.2.3. GC−MS Measurement. The method was carried out on an HP 6890 Series GC system (HP, Böblingen. Germany) coupled to a 5973 mass selectivity detector (HP, Böblingen, Germany). The separation was performed on a Restec RTX-5 column (30 m × 0.25 mm, 0.1 μm). The flow rate was set to 1.4 mL/min of helium. The gradient started at 60 °C and was increased by 17.3 °C/min until 320 °C and held that temperature for 45 min. The total run time was 60.03 min. The injection volume was 1 μL with a split ratio of 20:1. The transfer line was set to 330 °C. Electron impact was used as the ionization mode. For quantification, single ion monitoring (SIM) was performed. The details for each analyte can be found in the Supporting Information (Table S1). Enhanced ChemStation G170BA Version B.01.00 and cycle Composer Version 1.5.2 were employed for data acquisition.

2.2.4. Calibration and Quantification. To quantify the analytes, an external calibration with the alkylresorcinol standard adipostatin A (C15:0) and a quadratic regression was used. The calibration ranged from 1 to 100 μg/mL. Concentrations of the other ARs were calculated considering their molecular masses using the calibration curve of adipostatin A.

2.2.5. Method Validation. A complete in-house validation was not carried out due to the absence of a suitable blank matrix that fits all of the analyzed foods. The limit of decision (LODC) for adipostatin A was calculated as S/N = 6 using 9 measurements of the lowest calibration point and integrating over a period of time that resembles the spread of the peak at the lowest concentration. The limit of decision was set to 2261 counts per second (cps). The used calibration ranged from 1 to 100 μg/mL with a coefficient of determination (R^2) of 0.998. The apparent recovery was 71%. To calculate the recovery, whole grain bread was used and spiked with 100 μL of C15:0 (0.1 μg/mL).

2.3. Human Study Design. All of the performed studies were conducted in Münster, Germany. The studies were approved by the responsible research ethical committee of the University Hospital Münster Germany, under the file reference 2014-632-f-S. The following Table 1 contains all relevant information about the volunteers. All volunteers are of legal age, did not have any inflammatory bowel diseases, are not allergic to any of the consumed food, and are nonsmokers.

Table 1. Average BMI, Gender, and Age of All Volunteers

study	average BMI	female	male	age
1	23.7 ± 1.7 kg m^{-2}	3	4	23−41
2	24.3 ± 1.6 kg m^{-2}	6	4	23−42
3	−[a]	2	1	−[a]

[a]Not collected due to the size of the cohort.

Figure 2 summarizes the study design of all three studies. The volunteers from study 1 took part in a washout period for 4 days, followed by giving a morning urine sample on day 5. Afterward, the volunteers consumed one whole-grain-rich meal and collected an aliquot of each urine sample until the next morning. On day 6 (24h after the whole grain consumption), the volunteers gave a sample of the morning urine. Over 6 days, the volunteers were asked to keep a food diary.

For study 2, all volunteers were divided into two individual groups characterized by either a whole grain (group 2.1) or a refined grain (group 2.2) intake, which was conducted over 1 week. The volunteers from group 2.1 were instructed to consume just whole grain products over a period of 6 days. On each day, the volunteers collected an aliquot of their morning urine and documented the time of the urine sample collection. The volunteers from group 2.2 were instructed to just consume refined grain products over 6 days. On each day, the volunteers collected an aliquot of their morning urine and documented the time. All volunteers from the two groups were further instructed to keep a food diary about the consumed grain products, the time of the meals, and the approximately consumed amount.

Study 3 aimed at long-term detection of the potential biomarkers 3,5-DHBA and 3,5-DHPPA. Therefore, volunteers were chosen who had abstained from gluten-containing grain consumption for an extended period of time, at least one year. The volunteers from Study 3 kept for 4 days a food diary and collected an aliquot of their morning urine on days 4 and day 5. In addition, they were asked to fill out a questionnaire about which grain products they do not consume and which they highly consume, in general.

All urine samples from studies 1−3 were stored at −20 °C for short-term storage and −80 °C for long-term storage. The volunteers from study 1 also participated in study 2.1 or 2.2, and these two studies were performed within a short interval of time.

2.4. Urine Sample Treatment and Creatinine Determination. After defrosting and homogenizing using a vortex shaker, the urine samples were centrifuged at 15,000 *g* at room temperature for 5 min. Subsequently, 20 μL of the supernatant was diluted with 180 μL of water containing 0.1% FA. Ten μL of the diluted samples were injected into the LC-MS/MS system. If samples exceeded the calibration range of at least one of the analytes, they were prepared a second time with a higher dilution factor (see Section 2.5.2 for details).

To normalize the determined concentrations of the analytes in urine, the creatinine level was used. The creatinine level was determined based on a modified Jaffe method.[26−29] The samples were defrosted and homogenized by using a vortex shaker. Afterward, the samples were centrifuged at 15,000 *g* for 5 min at room temperature and diluted with 0.01 M HCl-solution (1/25 *v*/*v*). To determine the creatinine concentration, 120 μL Na_2HPO_4 buffer (12.5 mM Na_2HPO_4; 313 mM NaOH) were added to a microtiter plate (96-well). Furthermore, 60 μL of diluted urine or blind sample (0.01 M HCl) was added followed by 120 μL 0.2% picric acid solution. The absorption was measured after 1 and 5 min at 492 nm and 30 °C using the FLUOstar Omega microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). Each analysis was performed in duplicate.

The quantification was performed using external calibration in the concentration range from 0.002 to 0.1 mg creatinine/mL and 60 μL from each concentration was used. To calculate the creatinine concentration, a linear regression was used with the difference between the extinctions after 1 and 5 min.

2.5. LC-MS/MS Measurement. LC-MS/MS was carried out using a 1260 Infinity LC system consisting of a HiP Degasser G5225A, binary pump G1312B, thermostated column compartment G1316A, hip automated liquid sampler G1367E, automated liquid sampler thermostat G1330B and hand-held controller G4208A (Agilent, Waldbronn, Germany)

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ACS Omega 2024, 9, 23555−23566

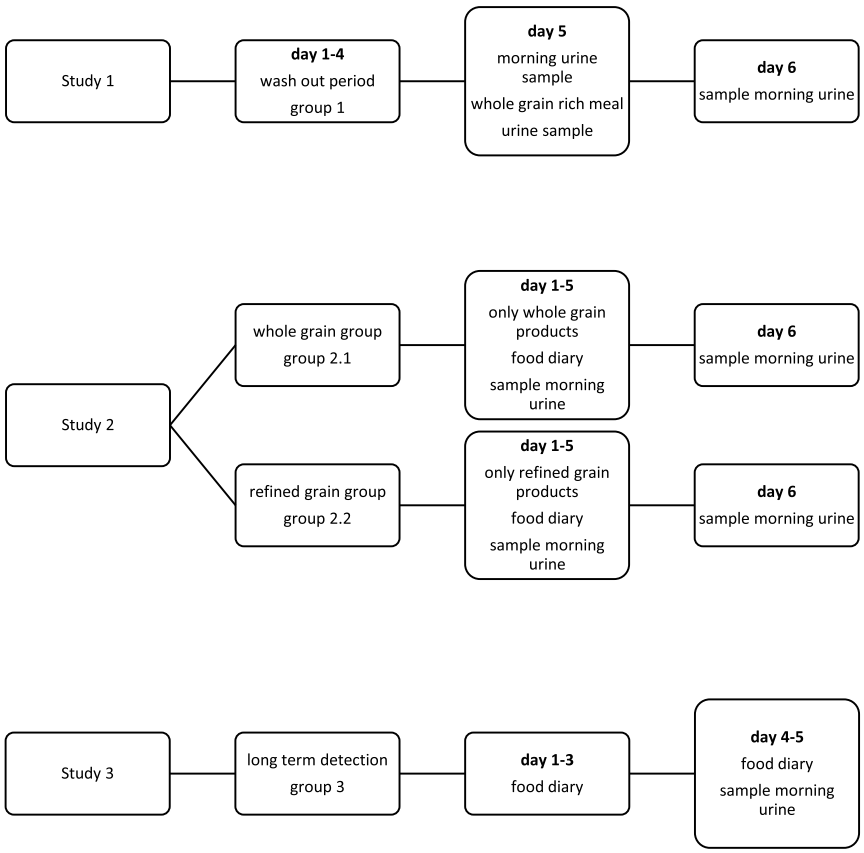

Figure 2. Overview of the design of the three human intervention studies.

Table 2. Performance of the LC-MS/MS Method

analyte	LOD[a] (ng/mL)	LOQ[b] (ng/mL)	calibration range (ng/mL)	R^{2c}	R_A[d] (%)	RSD[e] (%)
3,5-DHPPA	7.4	26.3	10−500	0.995	13	7.2
3,5-DHBA	8.0	28.7	10−500	0.995	14	7.8

[a]Limit of detection. [b]Limit of quantification. [c]Coefficient of determination. [d]Apparent recovery. [e]Relative standard deviation over whole calibration curve.

coupled to a QTRAP 6500 mass spectrometer (SCIEX, Darmstadt, Germany). The chromatographic separation was conducted on a Nucleodur C_{18} Pyramid column (100 × 2 mm, 3 μm) equipped with a precolumn of the same material (4.0 × 2.0 mm) (both Macherey-Nagel, Düren, Germany). The mobile phase for gradient elution consisted of acetonitrile containing 0.1% formic acid (A) and H_2O containing 0.1% formic acid (B). The flow rate was set to 400 μL/min and the analytical column was equilibrated at the initial conditions of 100% B for 2 min. After 2 min, the percentage of A was increased to 95% in 6 min and maintained for 2 min. Within 0.1 min, the percentage of B was increased to 100% and kept constant for another 5 min. The total run time was 16 min. During the analysis, the column oven temperature was set to 40 °C. A diverter valve at the mass spectrometer was incorporated in order to discard the first 2 min of each run. Electrospray ionization was carried out at an ion spray voltage of −4500 V in negative ionization mode. The temperature of the source was set to 500 °C. The curtain gas was set to 35 psi, the nebulizer gas to 35 psi, and the heating gas to 45 psi. The collision gas was set to "medium". The detection of the analytes was performed using scheduled multiple reaction monitoring (sMRM) with a targeted scan time of 0.2 s. The sMRM transitions as well as the respective declustering potential (DP) and the collision energy (CE) were optimized for each analyte by using direct injection of a standard solution. The detailed MRM transitions for each analyte can be found in the Supporting Information (Table S2). Analyst 1.6.2 Software was employed for data acquisition, and data processing was performed using SCIEX OS 3.0.0.3339 Software.

2.5.1. Calibration and Quantification. To determine the concentrations of the analytes, a matrix-matched calibration was used. Urine from different volunteers after a 4-day washout period was used. The urine was mixed in the same ratio and was added to each calibration point. The dilution (1:10, *v*/*v*) was identical to the dilution for each sample. For quantification, a standard solution with 3,5-DHBA and 3,5-DHPPA was used. The calibration ranges from 10 to 500 ng/mL. Glucuronides and sulfates of 3,5-DHBA and 3,5-DHPPA were not considered (see Section 3 for further information).

2.5.2. Method Validation. To evaluate the performance of the method, an in-house validation was performed. The LOD, LOQ, working range, linearity, reproducibility, and recovery were determined. The following Table 2 shows the performance data from the used LC-MS/MS-Method. The apparent recoveries are very low, but no specific cause of this was found.

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ACS Omega 2024, 9, 23555−23566

Matrix-matched calibration was used to compensate for the low recovery.

3. RESULTS AND DISCUSSION

3.1. Food Screening. The quantitative analysis of ARs in widely consumed grain and pseudo-grain products was performed to evaluate which grain or pseudo-grain product should led to high urinary concentrations of 3,5-DHBA and 3,5-DHPPA. Besides grain and pseudo-grain products, mangos, cashew nuts, and nutmegs were also analyzed for the total AR concentration (sum of C15:0, C17:0, C19:0, C21:0, C23:0, C25:0 alkylresorcinols).[15]

3.1.1. Flours. Different types of refined and whole grain flours made out of wheat, rye, spelt, buckwheat, and barley malt were analyzed. The total AR levels are summarized in Table 3 and Figure 3 (the detailed concentrations of each individual AR can be found in the Supporting Information (Table S3).

Table 3. Total AR Concentration (mg/kg Fresh Mass) (Sum of C15:0, C17:0, C19:0, C21:0, C23:0, and C25:0 Alkylresorcinols) in Analyzed Flour Samples (the Concentration of Each Individual AR Can Be Found in Table S3, Supporting Information)[a]

food	number analyzed samples	mean (mg/kg fm)	lowest concentration (mg/kg fm)	highest concentration (mg/kg fm)
wheat flour	3	25	13	39
whole wheat flour	3	596	464	771
spelt flour	3	35	20	52
whole spelt flour	3	508	472	567
rye flour	3	346	232	402
whole rye flour	3	736	728	745
buckwheat flour	2	nd		
barley malt flour	1	15		

[a]nd: not detected; fm: fresh mass.

The average concentration of total ARs in wheat flour is 25.3 ± 11.8 mg/kg of fresh mass (fm) in comparison to 596 ± 140 mg/kg of fm in whole wheat flour. The water content in flour is between 10 g/100 g and 14 g/100 g depending on the type of grain and the origin.[30,31] The determined total AR content in wheat flour is comparable to those found in the literature. Andersson et al. reported a total AR content in whole grain wheat flour of 625 ± 115 mg/kg dry mass (dm) and of 36.2 ± 6.4 mg/kg dm in refined wheat flour.[13] The levels of ARs in North American wheat products (300−700 mg/kg dm) are similar.[23] Other concentrations reported in the literature for refined wheat flour are ranging from not detectable to 25 mg/kg fm.[10,17,32] The difference might be due to different extraction rates during the milling process in various countries and due to natural fluctuations in plants.[33] Since labels for extraction rates of flours differ between countries and are in some places regulated by residual ash mass and in others by protein content, differences, e.g., for products labeled "whole grain flour" might be explainable by disparate types of samples. The extraction rate indicates the amount of flour percentage of the total milled grain.[34] Following data from the literature, the total AR content in wheat varies between 300 and 1000 mg/kg.[10,17,32,35,36]

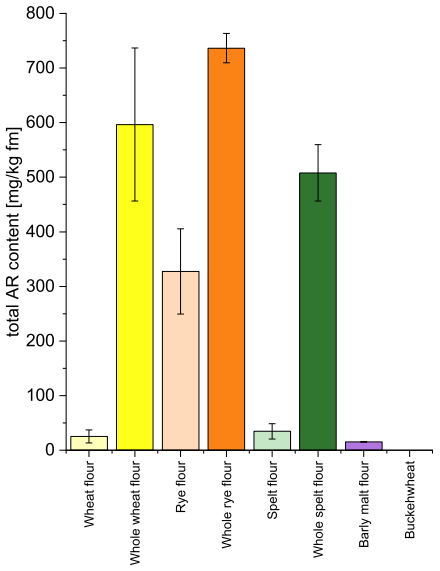

Figure 3. mean of the total AR concentration (mg/kg fresh mass) (sum of C15:0, C17:0, C19:0, C21:0, C23:0, and C25:0 alkylresorcinols) in various whole grain and refined flours.

The analyzed spelt flours had a total AR content of 34.6 ± 14.1 mg/kg fm. The total AR concentration in the analyzed whole spelt flour is 508 ± 51.5 mg/kg fm. These levels are similar to those reported in the literature. Andersson et al. reported a content of 650 mg/kg dm on average in whole grain spelt flours and 79.9 ± 28.1 mg/kg dm in refined flours.[13] Ross et al. analyzed AR levels of 819 mg/kg dm in whole grain spelt flour, which is higher than the results obtained here.[17] As mentioned above, the AR content is significantly affected by different cultivars and environmental factors.[36]

In rye flour, the average content of all ARs is 327 ± 78.1 mg/kg of fm. The analyzed whole rye flours had a total AR content of 736 ± 26.7 mg/kg fm. Compared with the other analyzed flours, the refined products have very high AR levels. This can be explained by different extraction rates between refined rye flour and wheat flour. The refined rye flour used (type 1150) has an extraction rate of 79−83%, while whole grain rye flour has an extraction rate of almost 98%. In comparison, refined wheat flour has an extraction rate of 40−56% and whole grain wheat flour has an extraction rate of 98%. The determined total AR contents in whole grain rye flours is lower compared to previously reported data (972 mg/kg dm and 1030 mg/kg dm).[13,37] In comparison, Ross et al. reported AR levels of 726 mg/kg dm and 865 mg/kg dm in Swedish rye flour products, which are comparable to the results of the present study.[17,25] The contents in refined flours are much higher (69 mg/kg dm−99 mg/kg dm) than described in the literature. This might be explainable due to different extraction rates. The reported content in the literature is based on refined rye flour with an extraction rate of 70−73%.[10,11,13,38] Ross et al. reported a steep incline of the total AR level with an increasing relative yield. While the total AR content is about 2000 μg/g dm at an extraction rate of 70%, the content is about 2500 μg/g dm at a rate of 80%.[11]

Aside from these flours, barley malt flour and buckwheat flour were analyzed, as well. Whereas Buckwheat flour did not contain any ARs, low levels of 15 mg/kg fm were detectable in

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ACS Omega 2024, 9, 23555−23566

barley malt flour. AR content in barely malt flour has not been reported yet.

3.1.2. Other Grain Products. The total AR levels of grain products and other food samples are summarized in Table 4 and Figure 4 (the detailed concentrations of each individual AR can be found in the Supporting Information (Table S4).

Table 4. Total AR Concentration (mg/kg Dry Mass) (Sum of C15:0, C17:0, C19:0, C21:0, C23:0, C25:0, Alkylresorcinols) in Grain Products and Other Food Samples (the Concentration of Each Individual AR Can Be Found in Table S4, Supporting Information)[a]

food	number analyzed samples	mean (mg/kg dm)	lowest concentration (mg/kg dm)	highest concentration (mg/kg dm)
whole grain bread	5	365	118	608
crispbread	3	431	64	653
whole grain toast	2	299	277	322
toast	3	32	31	33
whole grain pasta	4	222	201	256
pasta	4	41	22	50
whole grain biscuit	2	157	103	210
butter biscuit	2	25	10	40
couscous	2	61	23	138
bulgur	2	84	10	158
cereals	2	56	20	92
oats/oat bran	2	nd		
millet	3	nd		
quinoa	2	54	nd	54
corn	4	nd		
rice	2	nd		
whole grain rice	2	nd		
beer	3	nd		
mango	1	nd		
cashew	1	nd		
nutmeg	1	nd		
barley malt extract	1	nd		
amaranth	2	nd		

[a]nd: not detected; dm: dry mass.

In rice, whole grain rice, corn, beer, oats, millet, amaranth, barley malt extract, nutmeg, and mango, no AR was detected. However, in the literature, ARs are reported for cashew nuts and mango.[15,25] All analyzed groups of products have a high variation between the different samples in common, as can be seen from the large standard deviations (Figure 4). This is due to the different composition of the products. This trend is already visible for products from the same country and increases with products from different countries. As described above for the flour the milling grades differ between countries leading to different starting materials for the production of processed foods. Furthermore, the AR content is affected by environmental factors such as soil composition and fertilization as well.[35,36,39]

Five different whole-grain breads were analyzed. The total concentration of all ARs is 365 ± 205 mg/kg dm. Reported

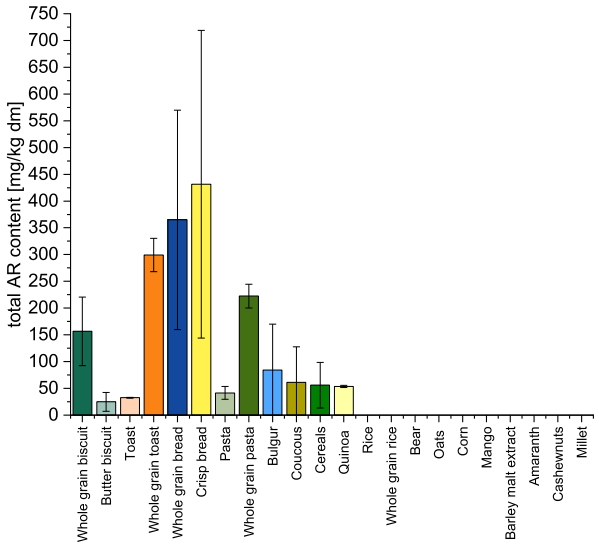

Figure 4. Mean of the total AR concentration (mg/kg dry mass) (sum of C15:0, C17:0, C19:0, C21:0, C23:0, C25:0 alkylresorcinols) in various grain products and other food samples.

total contents of AR in the literature vary a lot depending on the composition and the percentage of whole grain. In bread with a high rye content (>50%), the reported content is 491 mg/kg fm. The total AR content in bread with an intermediate rye content (11−50%) varies between 618 mg/kg fm (53% whole grain content) and 23 mg/kg dm (35% whole grain content).[9] In Scandinavia, soluble dietary fiber is often used. This might also have an influence on the total AR content and explain the differences between bread from the German and Scandinavian retail sectors.

The average total AR concentration among whole grain pasta products is 222 ± 22 mg/kg dm. The results are similar to those of previously published data. Landberg et al. determined a total AR content in whole grain durum wheat pasta of 232 ± 23 mg/kg dm.[40] Among Swedish whole grain durum wheat pasta products, the AR content varies between 240 and 21 mg/kg fm.[9] In comparison to the whole grain durum pasta products, the determined content in refined durum pasta is 41.4 ± 12.0 mg/kg of dm. This is higher than previously reported, as among Swedish refined pasta products, no ARs were detectable.[9,40]

The average total AR content found in crisp bread is 431 ± 288 mg/kg dm. Menzel et al. reported a total AR content between 1182 mg/kg fm (rye content >50%) and 46 mg/kg fm (wheat content <10%).[9] Anderson et al. reported a total content in whole grain crisp bread between 950 and 759 mg/kg dm.[13] In the present study, large variations between the samples were observed. Two of the three analyzed samples had similar AR contents of 577 and 653 mg/kg dm, respectively, while the third sample had a concentration of 63.6 mg/kg dm. All three samples were made with whole-grain rye flour. The only difference between the first two samples and the third one is that besides whole grain rye flour, sourdough containing rye was used as well. The observed variation might be connected to the different compositions of the samples.

In total, three different refined toasts (32.5 ± 0.7 mg/kg of dm) and two different whole grain toasts (299 ± 31 mg/kg of dm) were analyzed. Following data from the literature, different kinds of soft bread show total AR levels between 548 mg/kg dm (whole grain spelt wheat bread) and 20.5 mg/

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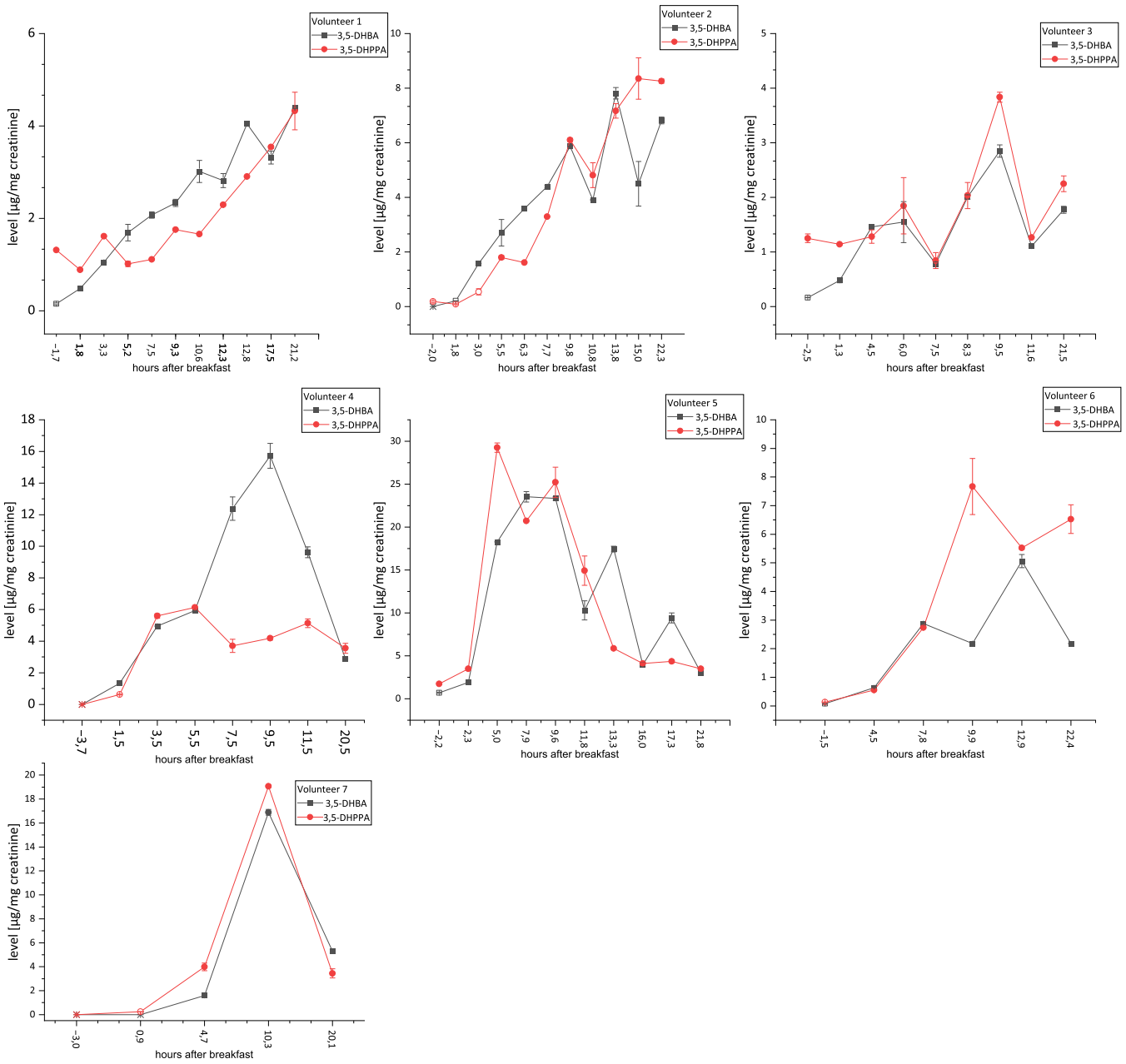

Figure 5. Kinetic profiles of 3,5-DHBA (black line) and 3,5-DHPPA (red line) in urine samples of all volunteers in study 1 after a whole grain rich breakfast. The urine samples were analyzed by HPLC-MS/MS and normalized to creatinine (*: under LOD, unfilled symbol: > LOD, but < LOQ; negative values for points in time on the *x*-axis indicate the blank sample before the whole grain breakfast (t = 0)).

kg dm (wheat bread).[13] The reported content for the wheat soft bread with no whole grain is lower than that determined in this study. This might be due to the different compositions of Norwegian and German soft breads and the flours used.

Four different biscuits were analyzed. Two of them were made out of 72 and 70% refined wheat grain. The other two were made out of 67 and 70% whole wheat grain. The determined total AR content is 157 ± 64 mg/kg dm in the whole grain products and 25.0 ± 17.6 mg/kg dm in the products of refined grain. Menzel et al. reported a total AR content in cookies between 696 mg/kg fm (47% whole grain) and 52 mg/kg fm (13% whole grain).[9]

The contents in the two analyzed cereal products (92.2 and 19.6 mg/kg dm) are very different. Reported total content of ARs in literature also varies between 1118 mg/kg fm and 33 mg/kg fm depending on the ratio of whole grain and bran fractions in the products.[9] The compositions of both cereals are similar.

Bulgur and couscous are made from durum wheat. The difference between these two products is in the manufacturing process. The total AR content in couscous is 22.9 mg/kg dm in the first sample and 138 mg/kg dm in the second sample. The determined total AR content in bulgur is 9.6 and 159 mg/kg dm. This broad spread for both products provides a hint that the choice of raw material is of much higher importance for the AR content compared to the differences in the respective manufacturing processes.

3.1.3. Conclusions. In the present food screening, ARs were found in the analyzed flours and grain-based products but also in higher amounts in the pseudo-grain quinoa. Furthermore,

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ACS Omega 2024, 9, 23555−23566

the contents in refined flours and products based on refined grains, especially in rye-based products, are higher than expected and might have an influence on the excretion of 3,5-DHBA and 3,5-DHPPA. The spread between similar products is very high, and based on the labeling alone, no reliable AR content can be derived. The determination of the urinary excretion of these analytes might more accurately reflect the intake of grain-based products.

3.2. Human Intervention Studies. The aim of this study was to elucidate how different shares of whole grain affect the urinary concentrations of 3,5-DHBA and 3,5-DHPPA. Four different eating habits were compared with each other. The first and the second studies are food intervention studies which differ in the degree of influence over the participants' choices for food (see Figure 1 for study design). In study 1, a washout period of 4 days was performed. On the morning of day five, the volunteers gave a morning urine sample to determine the baseline concentration for both biomarkers (blank samples). Afterward, the volunteers consumed a whole grain-rich breakfast followed by sample collection for the following 24 h. The volunteers participating in study 2 were asked to eat either exclusively whole grain (group 2.1) or exclusively refined grain products (group 2.2) on six consecutive days and to collect a morning urine sample on each day. The third study was performed to analyze the long-term detection of potential biomarkers. The volunteers had abstained from gluten-containing grains for at least one year. They kept a food diary for 5 days and collected a morning urine sample on days 4 and day 5. Additionally, they had to fill out a questionnaire about which grain products they do not eat and what kind of foods they consume frequently (see the Supporting Information for Questionnaire S1).

All samples were analyzed by LC-MS/MS and the results were normalized to the creatinine levels in each sample. Figure 5 shows the kinetic profiles between the urinary concentrations of both biomarkers over the time of the study from the volunteers (1−7) participating in study 1.

The blank urine samples were taken on day five before the whole grain-rich breakfast (see negative values for points in time, Figure 5). In three of the blank urine samples, 3,5-DHPPA (red line) was detected while no blank urine sample contained 3,5-DHBA (black line) above the limit of quantification (LOQ). While Ross et al. reported the maximum excretion after 24 h, Zhu et al. gave 6.0 h for 3,5-DHBA and 5.4 h for 3,5-DHPPA as time for the maximum excretion, no information on the total time for full excretion is given.[11,17,19] Thus, the detected levels in the blank might be due to some of the absorbed ARs being stored or delayed in the body or to a higher intake of AR-rich foods prior to the washout period.[17,41]

In this study, the maximum excretion for both compounds was reached mostly between 5 and 15 h, although two volunteers showed the highest excretion after 20 h (Figure 5). Volunteer 1 did not reach the maximum excretion within 24 h regarding both biomarkers. Volunteer 6 did not reach the maximum excretion of 3,5-DHPPA within 24 h. In literature, different t_{max} from 5 h to more than 24 h are reported.[19,41] The results from the present study are in line with the data reported in the literature. The t_{max} seems to be affected by various factors influencing intestinal uptake, human metabolism, and clearance.

Volunteer 5 showed, in comparison to the other volunteers, a higher excretion level. Also, the blank urine samples had a higher concentration in comparison to the other blank samples. This might also be due to the variations described above. Furthermore, this volunteer had by a factor of approx. 3 smaller creatinine concentrations compared to the other volunteers, which directly affects the calculated excretion levels. It is known, that creatinine concentration is, among other things, affected by drinking quantity, creatine supplementation, high muscle rate and meat consumption.[42]

The presented kinetic data clearly demonstrate the relationship between the intake of whole-grain-rich food and the urinary excretion of 3,5-DHBA and 3,5-DHPPA. Furthermore, the data showed that the excretion is also affected by individual differences in the uptake, metabolism, and clearance as can be seen from the differences in the maximum excretion time.

All obtained data are based on the direct analytes 3,5-DHBA and 3,5-DHPPA. Glucuronides and sulfates thereof can also occur in urine and were included during the method development. However, all tested forms of enzymatic treatment for cleaving glucuronides and/or sulfates during sample preparation led to an unexpected decrease in the concentrations of both analytes. As the determined concentrations of both aglycones were high and the main metabolic pathway was not proposed to be glucuronidation, the analysis was limited to these two biomarkers.

A potential biomarker needs to fulfill several criteria like plausibility, dose-response, and robustness to serve as a reliable biomarker.[43] To further investigate the dose-response relationship of these two biomarkers, two separate groups of volunteers were compared. The first group (group 2.1) ate exclusively whole grain products over 6 days, while the second group (group 2.2) consumed exclusively refined grain products (Figure 2). In order to be as close as possible to a realistic scenario, a washout period was not performed and the diet was not controlled or standardized. The volunteers were asked to eat according to their normal habits and to collect their morning urine for six consecutive days. Figure 6 shows the determined urinary levels of 3,5-DHBA and 3,5-DHPPA normalized to the creatinine concentration in each sample.

The concentration of both biomarkers from group 2.1 (Figure 6, above) is, apart from fluctuations, increasing within 6 days. The fluctuations between the single days are explainable due to the variation of the meals and the different intake times throughout the days. This is especially the case for volunteer 2. The peak in the urinary levels on day four can be explained by a very high consumption of whole grains on day three. The concentrations between the different volunteers vary a lot. This is explainable due to no washout period or further restriction in the diet. Every volunteer followed their normal eating habits with the exception of exclusively consuming whole grain products.

Volunteer 3 had a smaller average excretion with 0.87 ± 0.21 μg/mg creatinine 3,5-DHBA and 0.62 ± 0.14 μg/mg creatinine 3,5-DHPPA in comparison with the other volunteers in this group. Based on the food diary, the consumed amounts of grain-based products do not differ much from the other volunteers, and the creatinine levels are also in an expected range. Interestingly, volunteer 3 also had much lower concentrations of both biomarkers in Study 1 (Figure 5). Since no abnormalities were observed for the food intake, the reason for the low concentration might be due to individual differences in the uptake, metabolism, and clearance.

Group 2.2 consumed exclusively refined grain-based products. On day one, in all of the samples, despite volunteer

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9, at least one of both biomarkers was above the LOD but below the LOQ (Figure 6, below). These data are comparable to the excretion levels from group 2.1. Within 6 days, the excretion decreased and on day six, all levels were below the LOD. The differences between these two groups further show the influence of whole grain and refined grain product consumption. However, there are still volunteers with similar urinary concentrations in comparison to the whole grain group. Volunteer 8 had an average urinary concentration of 1.47 μg/mg creatinine 3,5-DHBA and 3.5 μg/mg creatinine 3,5-DHPPA. Nevertheless, volunteer 8 already showed different excretion levels during intervention study 1 (volunteer 5, Figure 5). As mentioned above, this volunteer also had very small creatinine concentrations which directly affect the excretion levels. Furthermore, this is the only volunteer who excreted 3,5-DHBA on another day than day one.

In order to approve for statistical differences, an ANOVA analysis was performed. The morning urine samples from study 1 (no grain diet) and the two different eating habits from studies 2.1 (whole grain diet) and 2.2 (refined grain diet) are compared with each other. The variance of the homogeneity of the three different eating habits was not calculated because the size of the cohort was too small. Therefore, the more robust Welch test was performed to test the equality of mean values.

In the case of 3,5-DHBA, the Welch test was not performed because the variance of one of the groups is 0. Therefore, the Games-Howell posthoc analysis was performed which shows no significant ($p = 0.054$) differences between a whole grain and a refined grain diet based on 3,5-DHBA. Nevertheless, a no-grain and a whole-grain diet differ significantly ($p = 0.043$) from each other. As expected, a diet based on refined grain and a diet with no grain does not differ significantly ($p = 0.615$).

The Welch test regarding the second potential biomarker 3,5-DHPPA, shows no significant differences between the three groups ($p = 0.162$). Also, the Games-Howell analysis for 3,5-DHPPA shows no significant differences between a whole-grain-rich diet and a refined-grain diet ($p = 0.179$). The no-grain diet and the refined-grain diet show no significant differences ($p = 0.992$). Surprisingly, based on the Games-Howell analysis, a whole-grain-rich diet and a diet without grain do not differ significantly ($p = 0.154$). Especially between these two groups, significant differences were expected.

Even without statistically significant differences between some of the groups, a comparison of the mean values still shows a high difference between a whole grain diet and a refined grain diet for both biomarkers. Volunteer 8 stands out with very high levels of the two potential biomarkers, which has a high impact on the results, especially on the mean and variance of this group, which strongly influences the statistic tests. In order to obtain a more reliable statistical significance, the study should be performed in the future with a bigger cohort.

In summary, the three different eating habits do lead to different urinary concentration of 3,5-DHBA and 3,5-DHPPA, even though a refined-grain-rich diet does not differ significantly from no grain at all.

To further confirm the results described above, urine samples from people who barely eat grain-based products were analyzed. The three volunteers did not consume any gluten-containing grain for at least one year. All volunteers were consuming oat products on a daily basis, but this grain does not contain any ARs. The volunteers were asked to keep a food diary for 4 days and collect a sample of the morning urine of days 4 and day 5. Table 5 shows the concentrations of both biomarkers in urine normalized to the creatinine level.

Table 5. Concentrations of Biomarkers 3,5-DHBA And 3,5-DHPPA in Urine Samples from Volunteers ($n = 3$) Not Eating Gluten Containing Grains for At Least One Year; Samples Were Analyzed with LC-MS/MS and the Results Normalized to Creatinine (First Line Showing the Results of Day 4, Second Line of Day 5)

volunteer	concentration 3,5-DHBA μg/mg creatinine	concentration 3,5-DHPPA μg/mg creatinine
1	<LOD	<LOD
	<LOD	<LOD
2	<LOD	0.20
	<LOD	<LOD
3	<LOD	3.73
	<LOD	<LOD

3,5-DHBA could not be determined in any of the samples. Volunteer 1 did not excrete any 3,5-DHPPA within the 2 days of sampling. Volunteers 2 and 3 excreted 3,5-DHPPA on one of the 2 days. Volunteer 2 ate a quinoa-containing dinner the evening prior to the first urine sample, which is known to contain ARs.

The first morning urine sample from volunteer 3 contained 3.73 μg/mg of creatinine 3,5-DHPPA. The volunteer was drinking at least 100 mL of a cashew drink each day. In this study, ARs were not found in cashew nut samples. However, according to literature, cashew nuts can contain ARs.[15,25] Nevertheless, it is questionable whether the intake of cashew nuts has a noticeable influence if regular grain products are consumed. It should be noted that the urine on day two did not contain any of the biomarkers. However, this volunteer had very low creatinine levels. In the first sample, the creatinine level was 0.19 mg/mL, and in the second one 0.02 mg/mL, which is very low for a morning urine sample. This very low concentration can also strongly affect the actual concentration.

Also, little is known about the AR content in barley. The food screening showed a total AR content of 16 mg/kg fm for barley malt flour, otherwise, no AR content for barley is reported in the literature. All volunteers from Study 3 abstained from barley, as it contains gluten. However, barley malt flour or barley malt extract is used in gluten-free products. Nevertheless, the intake of those products is very small and might not have an influence on the excretion of these biomarkers.

In the first intervention study, the relationship between a whole grain-rich meal and the excretion of AR metabolites 3,5-DHBA and 3,5-DHPPA was shown. In an intervention study, two different shares of whole grain and refined grain were analyzed without a prior washout period. The data demonstrate that while eating exclusively refined grain, the excretion levels of 3,5-DHBA and 3,5-DHPPA are very low compared to a whole grain-rich diet. Furthermore, data from volunteers abstaining from grain consumption suggest that cashew nuts, quinoa, and/or barley malt flour might have an influence on the excretion levels of 3,5-DHPPA when larger amounts are consumed. This needs to be further investigated with a larger cohort and by the analysis of additional samples. In summary, the results show the possibility of distinguishing between a whole grain-rich diet and a refined-rich diet based on the excretion levels of 3,5-DHBA and 3,5-DHPPA. A clear

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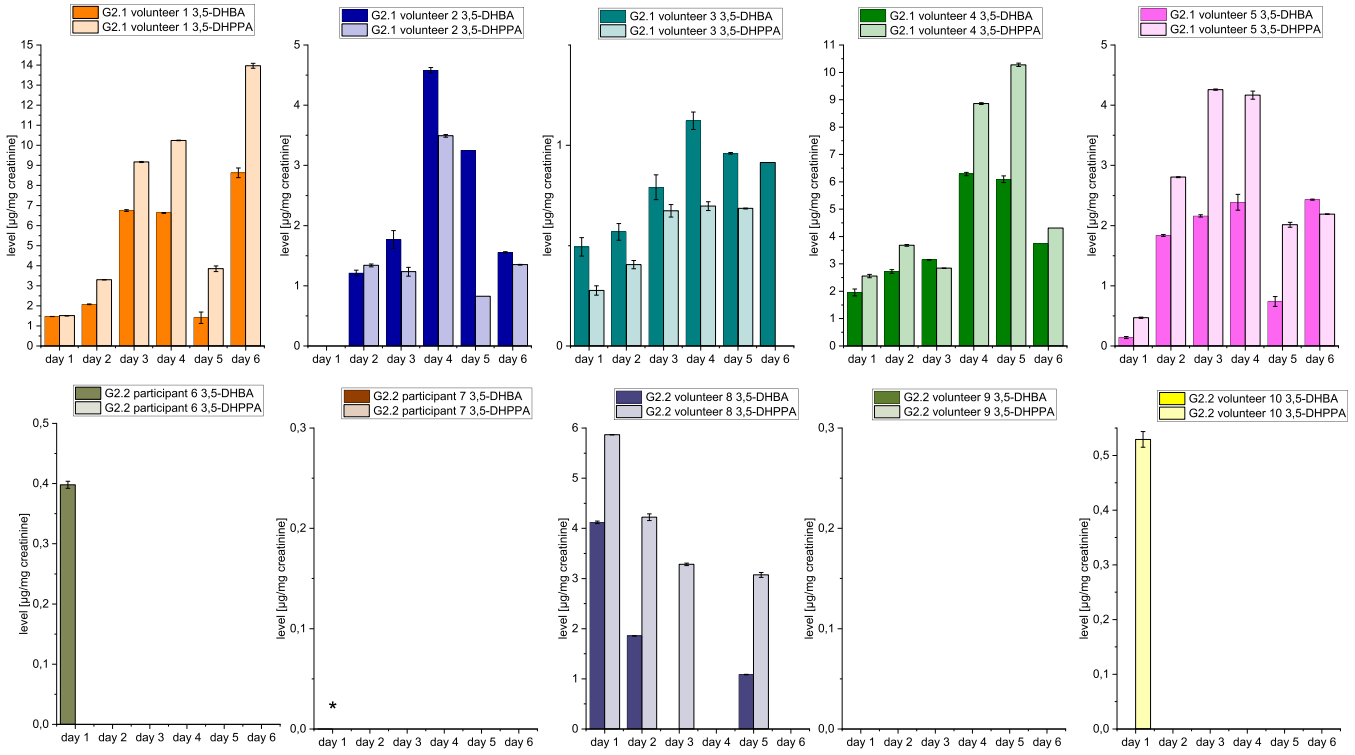

Figure 6. Concentration of 3,5-DHBA (dark color) and 3,5-DHPPA (light color) in urine samples of volunteers in group 2.1 (above) after whole grain product intake and group 2.2 (below) after refined grain product intake. The urine samples were analyzed by LC-MS/MS and normalized to creatinine (*: > LOD, but < LOQ).

limitation of this study is that only a small cohort was used due to the time-consuming urine sample analysis, and a larger cohort should be used to confirm these results. A bigger cohort would especially be beneficial in regard to volunteers who are not eating any grain-containing foods to obtain more information about other sources of ARs. For the other studies, a larger cohort would be mostly beneficial for statistical significance. First, no strong correlation between the intake of ARs and the excretion of the two biomarkers was shown. Second, the absolute concentration of the ARs is highly dependent on the consumed foods. The food screening clearly shows that the AR content in different food samples from the same category can differ strongly leading to strong variations in urine levels of the respective biomarkers. Furthermore, no data on the differences in the bioavailability of AR from different food samples is available. The effects of co-consumption of other foods together with the AR-containing foods, however, seem to have little influence on the bioavailability since the spread between individuals in study 1 is not smaller compared to the other studies, although all volunteers ate a very similar breakfast. Furthermore, the metabolism of consumers is influenced by many different factors such as genetics, diet, liver function, body mass index, medication, bodily activity, and age. All of these factors can have an influence on the metabolism and excretion levels of single individuals. Thus, each individual will still be within a large range of possible levels, and a direct correlation between the intake of ARs and the excretion of the two AR metabolites required for a quantitative biomarker will still not be fulfilled. Nevertheless, the suitability of both biomarkers in a mixed diet with even shares of whole grain and refined grain needs to be further investigated in future studies. Besides the use of the metabolites of AR as potential grain-specific biomarkers, other compounds such as benzoxazinoids and cinnamic acids which have been described in the literature (summarized in Landberg et al.) could also be considered in future studies as biomarkers for grain and grain products.[21]

■ ASSOCIATED CONTENT

Data Availability Statement

Data are included in the article and Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c00733.

Parameters of the GC−MS analysis of the alkylresorcinols; sMRM transitions and their corresponding RT, CEs, DPs, Eps, and CXPs; AR concentration [mg/kg fresh mass] in the analyzed flours; AR concentration [mg/kg fresh mass] in the analyzed grain and pseudograin products; and food questionnaire for Study 3 (PDF)

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Author Contributions

A.F., F.H., and H.-U.H. conceived and designed the experiments; A.F. performed the human intervention studies 1 and 3 analyzed the samples, interpreted the final data including food analysis data, and wrote the draft manuscript; H.G. performed the majority of food analyses including data analysis and performed the human intervention study 2; F.H. supported instrumental analysis and data interpretation; F.H. and H.-U.H. supervised the study and cowrote the manuscript; H.-U.H. provided all resources.

Notes

The authors declare no competing financial interest.

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