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ORIGINAL CONTRIBUTION

Comparison of plasma alkylresorcinols (AR) and urinary AR metabolites as biomarkers of compliance in a short-term, whole-grain intervention study

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Abstract
Purpose Alkylresorcinols (AR) are phenolic lipids present in the bran of wheat and rye. Plasma AR and their urinary metabolites may be suitable biomarkers of whole-grain (WG) wheat and rye consumption. The objective of this study was to examine plasma AR and urinary AR metabolites in response to WG wheat consumption.
Methods In a randomized crossover study, 19 subjects (10 males, 9 females; BMI 22.0 kg/m^2; age 26 years) incorporated either 3 servings (48 g) or 6 servings (96 g) of WG wheat daily into their regular diet for 1 week. Subjects completed a 2-week washout period, abstaining from all WG consumption, before each intervention. Fasting blood and 24-h urine were collected before and after each intervention. Plasma AR homologues (C19:0, C21:0, C23:0) were quantified by GC–MS after diethyl ether and solid phase extraction and derivatization. Urinary AR metabolites [3,5-dihydroxybenzoic acid and 3-(3,5-dihydroxyphenyl)-propanoic acid] were determined using HPLC with electrochemical detection after enzymatic deconjugation and ethyl acetate extraction.
Results Urinary total AR metabolites were significantly higher after 6 compared with 3 servings of WG wheat (56 vs. 32 μmol/day, $P < 0.001$). This dose–response relationship was independent of age, sex, energy intake, and baseline urinary AR metabolite concentration. Plasma total AR tended to be higher after 6 compared with 3 servings of WG wheat (103.0 vs. 86.9 nmol/L), but this difference was not significant ($P = 0.42$).
Conclusion The results suggest that urinary AR metabolites from 24-h urine collections may be useful as biomarkers of compliance in intervention studies of WG wheat.

Keywords Alkylresorcinols · Biomarkers · Whole grain · Whole wheat

Introduction

Several epidemiological studies have linked higher whole-grain (WG) intake to improved cardiometabolic risk factors and reduced risk of chronic disease using a variety of dietary assessment methods to capture intake [1, 2]. However, there are a multitude of factors that may bias self-reports of WG intake in observational and intervention studies and confound research examining the health effects of WGs. Thus, there is a need for reliable, independent biochemical markers of WG intake in research studies.

Alkylresorcinols (ARs) are a group of phenolic lipids with a polar head and a long, hydrophobic alkyl chain that are abundantly found in the bran layer (e.g., pericarp, testa, and aleurone) of wheat, rye, and triticale. ARs are

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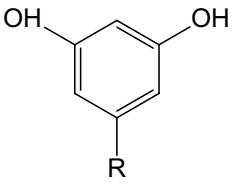
Table 1 Structure of alkylresorcinols commonly found in barley, rye, triticale, and wheat



R (alkyl chain)	Full name	Abbreviation	Average percentage by weight in common wheat
$C17H_{35}$	5-*n*-heptadecylresorcinol	C17:0	3
$C19H_{39}$	5-*n*-nonadecylresorcinol	C19:0	32
$C21H_{43}$	5-*n*-heneicosylresorcinol	C21:0	50
$C23H_{47}$	5-*n*-tricosylresorcinol	C23:0	14
$C25H_{51}$	5-*n*-pentacosylresorcinol	C25:0	5

Unsaturated and oxygenated derivatives are also found in minor amounts in these cereals but account for <5 % of total alkylresorcinols in wheat, the most commonly consumed alkylresorcinol-containing cereal in the USA

also found in low amounts in barley [3, 4]. Dietary ARs are predominantly present in WG and bran products of these cereals, although minor amounts of ARs have been reported in refined grain foods, as well [4]. There are five major homologues of cereal ARs which vary by alkyl chain length (Table 1), and the relative abundance differs between cereals. Upon consumption, ARs are readily absorbed from the small intestine and can be measured in plasma, erythrocytes, and adipose tissue [5–8]. Plasma ARs are rapidly eliminated, presumably through extensive hepatic metabolism. Although minor metabolites exist [9], the phase I metabolism of AR mainly results in the formation of two phenolic acids: 3,5-dihydroxybenxoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-propanoic acid (DHPPA). DHPPA and DHBA can be measured in plasma and urine either as free molecules or as conjugates (glucuronides and sulfonates) resulting from phase II metabolism [10, 11]. Conjugated AR and DHPPA identified in pig bile, as well as AR metabolites in human ileostomal effluent, not only suggest biliary elimination of AR and their metabolites, but also indicate that they are available for enterohepatic circulation [12].

In free-living populations, plasma ARs have been shown to distinguish daily consumers of whole wheat and/or rye from non-WG consumers [13], and evidence for the utility of plasma ARs as biomarkers of WG wheat and rye intake has increased in recent years [14]. However, ARs in plasma mainly reflect short-term WG intake in free-living individuals because they are generally eliminated from plasma 24 h after consumption [15, 16]. In addition, AR and AR metabolites in plasma and spot urine are largely determined by the temporal relationship between intake, blood sampling time, and capacity of individual phase I and II metabolisms [8, 17, 18]. Since the number of studies using ARs to assess compliance in WG intervention studies is scarce [19–22], especially in populations fed <100 g of WG/day, we conducted a randomized, crossover pilot study to compare the short-term, dose–response of WG wheat intake on plasma AR concentrations and urine metabolites.

Methods

Subjects

Healthy adults aged 18 and 40 years were recruited for this study, and potential study subjects underwent an in-person screening visit to determine eligibility. A total of 24 subjects (13 women and 11 men) were enrolled. Subjects were required to be healthy, non-smokers, and with a BMI between 19 and 26 kg/m^2 for inclusion in the study. Additional exclusion criteria are detailed in supplemental Table 1. Written informed consent was obtained from all subjects prior to study enrollment. The study was reviewed and approved by the Tufts Medical Center and Tufts University Health Sciences Campus Institutional Review Board.

Study design

The study was a randomized open-label, crossover intervention study in which subjects were assigned to consume for a 1-week period (a) 3 daily servings of WG wheat and 3 daily servings of refined grains and (b) 6 daily servings of WG wheat, separated by a 2-week washout period. Subjects were asked to otherwise eat their normal diet, replacing some foods with the assigned grains and refraining from any other WG foods for the entire course

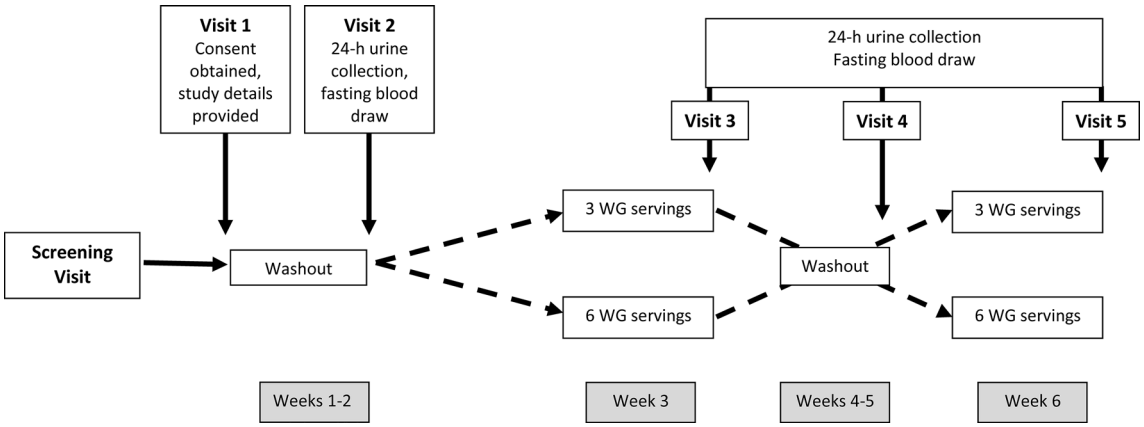


Fig. 1 Summary of study design. *WG* whole grain

Table 2 Whole-grain (WG) and alkylresorcinol (AR) content of grain foods provided to study participants

Study grains	Portion			Total AR	
	Size	Weight (g)	WG (g/serving)[a]	(μg/g)	(mg/serving)
Whole grain					
100 % whole-wheat bread[b]	1 slice	28.3	24.0	277.5	7.853
Whole-wheat pasta, dry weight[c]	1/2 cup, cooked	25.0	25.0	237.5	5.938
Whole-wheat ready-to-eat cereal[d]	1 cup	30.0	29.3	279.8	8.394
Whole-wheat crackers[e]	6 crackers	28.0	24.0	305	8.540
Whole-wheat muffins, homemade[f]	1 small muffin	17.1 g 100 % whole-wheat flour	17.1	199.7	10.225
Refined grain					
White bread[g]	1 slice	28.0	0	19.8	0.554
Regular pasta, dry weight[h]	1/2 cup, cooked	25.0	0	44.0	3.080
Refined grain ready-to-eat cereal[i]	1 cup	28.0	0	0.2	0.006
Saltine crackers[j]	7 crackers	28.4	0	16.8	0.476

[a] The WG content of the foods was calculated based on nutritional information available through the Whole Grains Council (www.wholegrains.org) and/or the product's manufacturer

[b] Pepperidge Farms natural 100 % whole-wheat bread

[c] Hodgson Mill whole-wheat penne pasta

[d] Wheaties cereal (General Mills)

[e] Triscuit crackers (Kraft)

[f] Prepared by Tufts HNRCA metabolic research unit kitchen using King Arthur 100 % whole-wheat flour

[g] Sysco vendor

[h] Pasta LaBella Spaghetti

[i] Cornflakes cereal (Kellogg's)

[j] Saltine crackers (Sysco vendor)

of the study. Prior to randomization, subjects completed a 2-week run-in period where they were instructed not to consume WG foods, to establish a comparable baseline status. Subjects completed a 3-day diet record and 24-h urine collection on the final day of the run-in, intervention, and washout periods. To prevent microbial growth, 5 mL 35 % HCl was added to the urine collections. Urine volume was measured, and aliquots of 50 mL urine samples were then stored at −80 °C until analysis. At each study visit, subjects completed a blood draw (30 mL) following an overnight fast (4 total time points). Figure 1 illustrates the study design and visit schedule. After centrifugation, plasma was collected, aliquoted, and stored at −80 °C until analysis.

Diets

The WG wheat products (Table 2) used in this study were selected because they contain all parts of the grain and >50 % WG wheat on a dry matter basis. All WG wheat and refined grain products were analyzed for AR content as described below. Average daily WG intakes and AR intakes were estimated based on the amount of study-assigned grain servings. Estimates of overall dietary intake were derived from the 3-day food records analyzed using the Nutrition Data System for Research software version 2010, developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN.

Analytical methods

Extraction and analysis of ARs in food

The content of ARs in the study intervention foods was determined as previously described [23]. In brief, the grain samples were extracted using hot 1-propanol/water (3:1 v/v) [3]. An internal standard, alkylresorcinol C20:0 (20 μg/sample, not present naturally), was added to each WG sample prior to extraction. Samples were then analyzed using reverse phase ultra-high-performance liquid chromatography (UHPLC) on a C_{18} column and detected using fluorescence detection as described by Ross et al. [23].

Determination of ARs in plasma

Plasma concentrations of the individual AR homologues C19:0, C21:0, and C23:0 were quantified to determine the total plasma AR concentration, using the method of Marklund et al. [24] with slight modifications. Briefly, 500 μL plasma was spiked with 50 μL of 500 ng/mL internal standard (C20:0), followed by protein precipitation using 500 μL 100 % ethanol. After vortexing for 1 min and centrifugation for 10 min at 11,000×*g*, ARs were extracted twice using diethyl ether. The diethyl ether supernatant was combined, dried under N_2 gas and reconstituted with methanol for further solid phase extraction using a Waters Oasis Max cartridge (6 cc, 150 mg), which was activated with 3 mL 0.1 M NaOH in 70 % methanol. After the reconstituted samples were loaded on the activated cartridge, the cartridge was washed with 5 mL methanol, followed by the elution of alkylresorcinols with 8.5 mL methanol containing 4 % acetic acid. ARs eluted from the cartridge were dried and then derivatized with trifluoroacetic acid at room temperature for 1 h. After solvent removal under N2 gas and reconstitution in undecane, ARs were analyzed by GC–MS using negative chemical ionization mode and selected ionization monitoring. AR C17:0 was below the level of detection, consistent with the consumption of a wheat-based diet (this homologue is largely found in rye), and AR C25:0 was detected, but its plasma concentrations in the study subjects were below the limit of quantification. Four ARs (C19:0, C21:0, C23:0, and C25:0) and internal standard (C20:0) were monitored at mass-to-charge ratio (*m*/*z*) of 471, 499, 527, 555, and 485, respectively. Plasma concentrations of ARs were calculated with standard curves constructed with authentic standards spiked into quality control plasma with adjustment for the internal standard. Linearity of standard curves was evident with R^2 values >0.9928. Lower limit of quantification for all four AR homologues in plasma was 2.5 ng/mL (<7.0 nmol/L). All samples were analyzed in duplicate. Intraday CV for C19:0, C21:0, and C23:0 were 0.8, 2.0 and 3.3 %, respectively; inter-day CV were 5.3, 8.0, and 12.4 %, respectively.

Determination of ARs in urine

The two major AR metabolites, DHBA and DHPPA, were measured in urine by high-performance liquid chromatography (HPLC) equipped with coulometric electrode array detection after enzymatic deconjugation and ethyl acetate extraction, as described elsewhere according to the methods of Marklund et al. and Koskela et al. with slight modifications [11, 24]. The lower limit of quantification (LLOQ) was 1.0 and 0.4 μmol/L for DHBA and DHPPA, respectively. Standard curves for quantification were constructed separately for each batch by spiking a low-concentration (DHBA < LLOQ and DHPPA ≈ 1 μmol/L) urine sample with known amounts ($n = 7$; 0.2–20 nmol) of DHBA and DHPPA. A pooled urine sample was analyzed in quintuplicate in each assay to assess intra- and inter-assay CV for DHBA (3 and 7 %, respectively) and DHPPA (5 and 6 %, respectively). Recovery was calculated by dividing the sum of DHPPA and DHBA excreted over 24 h by the treatment period's mean daily intake.

Statistics

Change between baseline and intervention periods was examined using a paired *t* test. Mixed models were used to analyze the treatment effects, with subject being treated as a random effect factor, and treatment and period representing the dietary regimens and sequence of the regimens. The interaction between treatment and period was used to examine whether a carryover effect existed. We adjusted for age, sex, energy intake, and/or baseline values of outcomes. Spearman's rank correlation coefficients were calculated between DHBA and DHPPA excretion, as well as between total plasma AR and total urinary AR metabolite excretion. Log-transformation was performed to normalize the skewed distribution for plasma and urinary AR

Table 3 Mean nutrient intake estimated from 3-day diet records during run-in, washout, and whole-grain (WG) intervention periods ($n = 19$)[a]

	Run in		3 WG servings[b]		Washout		6 WG servings	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Energy (Kcal)	2118	658	2122	585	2041	559	2229	645
Energy (KJ)	8860	2754	8879	2446	8540	2337	9326	2698
Protein (g)	87.1	26.1	84.2	28.5	85.9	32.5	91.7	25.9
Protein (% kcal)	17.1	3.9	16.0	3.7	17.5	5.2	16.7	3.6
Carbohydrate (g)	279	83	280	74	249	70	286	89
Carbohydrate (%)	52.9[c]	7.3	52.3	8.3	48.2	9.3	50.0	8.3
Fat (g)	74.3	36.1	77.9	31.8	79.7	32.3	84.4	32.7
Fat, % calories	29.8[c]	7.0	31.4	6.1	34.0	7.2	32.7	6.8
Sugar (g)	115	60	101	42	88	46	105	62
Dietary fiber (g)	18.9	7.8	21.3[d]	5.3	15.9	6.1	26.1[e]	6.9
Magnesium (mg)	257	58	300[f]	62	252	64	367[e]	73
Folate (µg)	481.2	162.8	555.5	195.5	407.1	143.4	517.1	295.0
Niacin (mg)	25.0	8.8	29.1[c]	9.7	21.7	7.1	33.0 [g]	14.3
Thiamin (mg)	1.9	0.6	2.2	0.7	1.7	0.7	2.2	1.1
Riboflavin (mg)	2.1	0.8	2.5	0.9	2.0	0.6	2.5[c]	1.3
Calcium (mg)	842	430	869	317	856	355	850	364
Vitamin E (IU)	12.4	5.7	13.0	5.4	13.4	6.5	15.3	6.5

[a] 3-Day diet records were kept at the end of run-in, washout, and each diet intervention period; diets were recorded on the final 2 weekdays/1 weekend day

[b] One subject did not return a food record for the 3-serving diet intervention period, so sample is $n = 18$; p values are from repeated measures ANOVA and based on $n = 18$ subjects with complete diet records

[c] Significantly differs ($P < 0.05$) from washout

[d] Significantly differs ($p < 0.05$) from washout and 6-serving arm

[e] Significantly differs ($P < 0.05$) from run in, washout, and 3-serving arm

[f] Significantly differs ($P < 0.05$) from run in, washout, and 6-serving arm

[g] Significantly differs ($P < 0.05$) from run in and washout

concentrations. A two-tailed $P < 0.05$ was considered statistically significant. Values were presented as means and 95 % confidence intervals (95 % CI) for whole-grain and AR intake variables and as geometric means and 95 % CI for plasma and urinary metabolite variables. All analyses were performed using SAS, version 9.3 (SAS institute), and STATA, version 11 (StataCorp LP).

Results

Subjects

Of the 24 subjects enrolled, three dropped out due to personal reasons (conflicts of time), while two did not comply with the dietary interventions (failing to attend study visits). A total of 19 subjects (10 males and 9 females) completed the study.

The average age of subjects was 25.6 ± 5.8 years, 53 % of subjects were male, and the majority of subjects were Caucasian (37 %) and Asian-American (37 %). Mean BMI was 22.0 ± 1.7 kg/m^2, and mean fasting blood levels were within normal ranges.

Food intake

Although the AR content is variable among WG products (Table 2), depending on the actual amount of WG wheat present, these foods contain at least 200 µg/g of total ARs and, as such, are clearly distinguishable from refined grain products which contain <50 µg/g of total ARs. Dietary intake was not significantly different during the intervention periods (3 vs. 6 servings) with the exception of the WG intake and nutrients attributed to WG such as dietary fiber, niacin, and magnesium (Table 3). There was no significant difference in total energy intake, macronutrient composition, total sugar intake, vitamin E, and calcium intakes between the intervention periods and the run-in or washout periods. Dietary fiber, niacin, and magnesium intakes were higher by 22.5, 13.4, and 22.3 % ($P \leq 0.05$), respectively, during the 6 servings/day WG intervention period as compared to the 3 servings/day

Table 4 Intake of whole grains (WG) and alkylresorcinols (AR), plasma ARs, and urinary excretion of AR metabolites during run-in, treatment, and washout periods ($n = 19$)[a]

		Run in	3 servings/day	Washout	6 servings/day
Assigned intake	Whole grain, g/day	N/A	66.4 (61.4, 71.8)	N/A	142 (136, 148)[c]
	Total AR, mg/day	N/A	22.6 (21.1, 24.3)	N/A	43.6 (41.4, 45.8)[c]
	C17:0, %	N/A	4.1 (3.6, 4.7)	N/A	4.1 (3.6, 4.7)
	C19:0, %	N/A	31 (29, 33)	N/A	31 (29, 33)
	C21:0, %	N/A	51 (50, 52)	N/A	51 (50, 52)
	C23:0, %	N/A	9.9 (9.0, 11)	N/A	9.7 (8.9, 11)
	C25:0, %	N/A	3.4 (3.1, 3.9)	N/A	3.2 (2.9, 3.5)
	C17:0/C21:0 ratio	N/A	0.081 (0.070, 0.094)	N/A	0.081 (0.070, 0.094)
Plasma	Total ARs, nmol/L	20.6 (13.2, 32.1)	86.9 (59.7, 126)[b]	19.1 (13.1, 27.9)	103 (69.1, 154)[b]
	C19:0, nmol/L	5.04 (3.04, 8.04)	23.2 (15.5, 34.7)[b]	5.50 (3.77, 7.87)	28.2 (19.3, 40.8)[b]
	C21:0, nmol/L	14.0 (9.08, 21.5)	53.0 (36.6, 76.7)[b]	12.1 (8.5, 17.7)	61.6 (40.6, 93.4)[b]
	C23:0, nmol/L	1.54 (0.704, 2.80)	9.07 (5.35, 15.0)[b]	1.19 (0.421, 2.37)	12.2 (7.47, 19.5)[b]
Urine	Total AR metabolites, µmol/day	9.9 (6.4, 15)	32 (25, 41)[b]	8.6 (6.5, 11)	56 (43, 73)[b,c]
	DHPPA, µmol/d	8.1 (5.1, 13)	24 (19, 30)[b]	6.9 (5.1, 9.2)	41 (31, 53)[b,c]
	DHBA, µmol/day	2.2 (1.4, 3.3)[d]	7.3 (5.4, 10)[b]	1.6 (1.1, 2.3)[e]	15 (11, 25)[b,c]
	DHPPA/DHBA ratio	4.0 (2.9, 5.6)[d]	3.3 (2.5, 4.2)[b]	4.5 (2.8, 7.0)[e]	2.8 (2.3, 3.3)[b,c]
Recovery[f]	Total AR metabolites, %		56 (43, 73)		51 (40, 64)

[a] DHBA, 3,5-dihydroxybenzoic acid; DHPPA, 3,5-dihydroxyphenylpropanoic acid. Values are geometric mean (95 % CI). $n = 19$ for assigned intakes and urinary AR metabolites if not stated elsewhere. $n = 18$ for plasma AR if not stated elsewhere

[b] Significantly different ($P < 0.05$) from washout and run in

[c] Significantly different ($P < 0.001$) from 3-serving period

[d] Quantifiable amounts in $n = 16$ participants

[e] Quantifiable amounts in $n = 15$ participants

[f] Calculated as the percentage of assigned daily AR intake excreted as metabolites in urine

period and higher by 12.7, 16.4, and 16.7 %, respectively, during the 3 servings/day WG period as compared to the run in. Based on the selected foods assigned, mean WG intake was 66.4 (95 % CI 61.4, 71.8) and 142 (95 % CI 136, 148) g/day on the 3- and 6-serving arm of the intervention, respectively. As expected, the assigned daily AR intake was significantly (93 %) higher ($P < 0.0001$) when 6 daily WG servings were consumed compared with 3 daily WG servings (Table 4). The relative homologue composition of dietary AR did not differ between the two treatment periods and was typical for wheat-based products. Among the three ARs, C21:0 was most abundant, followed by C19:0 and C23:0, and their sum represented 92 ± 0.4 % of total dietary ARs.

Plasma AR and urinary AR

Geometric mean plasma AR concentrations after 3 and 6 daily servings of WG wheat were ≥3.1-fold higher ($P < 0.001$) than concentrations at run in and washout when adjusted for sex, age, and energy intake (Table 4). Plasma AR concentration was not significantly different between the 3 and 6 serving periods, and no significant differences in plasma AR between baseline and washout were observed ($P = 0.79$).

Urinary AR metabolites (DHBA, DHPPA, and their sum) excreted after WG wheat consumption were significantly higher ($P < 0.001$) compared with baseline and washout (Table 4), and excretions were significantly higher ($P = 0.004$) when the WG intake increased from 3 to 6 servings daily in a sex-, age-, and energy-intake-adjusted model. Compared with the run-in and washout periods, the total amount of AR metabolites excreted in urine after 6 WG servings was 5.7- and 6.5-fold greater, respectively. Similarly, the excretion of metabolites after 3 WG servings was 3.2- and 3.7-fold greater compared with the run-in and washout periods, respectively. The mean percent increase for 3 WG servings compared with 6 WG servings was 75 %. Urinary DHBA and DHPPA were strongly correlated (Spearman correlation coefficient = 0.77, $P < 0.001$), and the excretion of DHPPA was more than double that of DHBA (i.e., DHPPA to DHBA ratio ≥2). The proportion of assigned AR intake recovered in urine as DHPPA or DHBA was 53 and did not differ between treatments. During the washout period, plasma ARs were significantly correlated with urinary AR metabolites ($r = 0.51$; $P = 0.028$); however, no significant

correlation was observed between biomarker concentrations during the intervention with 3 ($r = 0.01$; $P = 0.95$) or 6 ($r = 0.21$; $P = 0.39$) servings of WG (data not shown).

Discussion

In the present study, plasma ARs and 24-h urinary AR metabolite excretion were assessed in healthy US volunteers after two doses of WG wheat consumption to compare the usability of ARs as biomarkers for compliance in WG wheat intervention studies. Although plasma AR concentrations were significantly higher after both arms of WG consumption compared with baseline and washout, no statistically significant difference was observed between the 3 and 6 serving periods, perhaps due to the small sample size. Of note, the plasma ARs in this study were comparable to those observed in the UK WHOLEheart study where participants consumed 3 and 6, mainly wheat-based, WG servings [19]. In line with that study, the significantly higher plasma AR concentrations during WG consumption compared with baseline and washout suggest that plasma AR can be used to distinguish between frequent whole-wheat consumers and non-consumers. Similar to the WHOLEheart study [19], plasma ARs could not differentiate 3 servings from 6 servings in the present study, likely due to large intra-individual variation in amount of WG foods consumed, timing at which WG foods were consumed, and AR absorption, metabolism, disposition, and elimination after consumption.

Excretion of AR metabolites DHPPA and DHBA was higher after WG consumption compared to baseline and, unlike plasma AR, increased when the WG intake was doubled from 3 to 6 servings. This suggests that 24-h urinary excretion of AR metabolites cannot only distinguish between WG wheat consumers and non-consumers but can also be used to differentiate between levels of WG wheat intake. Shorter collection intervals (e.g., spot urine samples) may reflect WG intake less effectively and be less reproducible than 24-h collections [17, 25], although a WG serving (16 g of WG) increment increased 12-h DHPPA urinary excretion by 67 % based on a 12-h urine sample in a US population [26], against a background of very low overall WG intake.

As far as we are aware, only one other study has compared plasma AR concentrations to excretion of urinary AR metabolites under controlled settings with fixed doses; however, this was after consumption of three 1-week doses of whole-grain rye rather than whole-grain wheat [16]. In the study conducted by Landberg et al. [16], the homologues C17:0 and C25:0 contributed sufficiently (31 %) to the total AR content compared with our study (8 %). Although the two studies differ in the number of AR homologues quantified in plasma, study comparisons are unlikely to be substantially affected by this as the abundance of dietary C17:0 and C25:0 was low in the present study. Landberg et al. observed that a daily AR intake of 33 mg/day resulted in apparently greater plasma AR concentration (148 ± 60 nmol/L) and 24-h urinary AR metabolite (sum of DHBA and DHPPA) excretion (76 ± 15 μmol/24 h) than after 3 daily WG servings in the present study (mean assigned AR intake = 22.6 mg/day, total plasma AR = 86.9 nmol/L; total urinary AR metabolites = 32 μmol/day).

Landberg et al. [16] also observed dose differences in plasma AR after increasing AR intake from 33 to 66 mg/day and a positive correlation between plasma AR and urinary AR metabolites. However, the AR intake in that study was strictly controlled in terms of dose amount and dose frequency, and AR was consumed exclusively as rye bran, while a diversity of wheat-based AR sources was consumed in the present study. Differences between WG wheat foods and rye bran (e.g., fiber composition and content, food matrix, AR homologue distribution) may affect AR bioavailability [3, 5, 15, 27] in addition to variation in absorption due to other foods co-consumed with the cereal foods. Furthermore, AR homologue composition has also been suggested to affect AR metabolism [28–30] and may influence plasma AR concentrations. In addition, non-dietary determinants of plasma ARs, including sex and blood lipids, have been reported previously [19, 22, 31] and could have affected the dose–response relationship in the present study.

DHBA and DHPPA could potentially be the end products of minor AR derivatives (e.g., AR with unsaturated alkylchains or keto groups), of which rye is richer (15–30 % of all AR derivatives) than wheat (5 %) [32], and hence, greater AR metabolite excretion after rye bran consumption could partly be due to non-quantified intake of minor AR derivatives. In addition, two novel AR metabolites [5-(3,5-dihydroxyphenyl)-pentanoic acid and 2-(3,5-dihydroxybenzamido)-acetic acid] were recently detected in human urine after a single dose of WG wheat bread and accounted for ~12 % of all AR metabolites in urine [9]. So far, no study has reported the presence of these novel metabolites in urine after rye consumption, and it is possible that the urinary AR metabolite composition differ after wheat and rye intake. This is supported by the DHPPA/DHBA ratio in urine which is usually >2 after wheat-based AR intake as reported here and elsewhere [9, 25], while the ratio in studies where rye is the primary AR source commonly <2 after rye-based AR consumption [16, 17, 33, 34]. Unlike previous studies [16, 17], no inverse relationship between AR intake and urinary AR metabolite recovery was observed. Again, this condition may be attributed to differences between cereal AR intake in the previous studies investigating AR metabolite recovery originated

mainly from WG rye and not wheat. Additionally, 18–26 % of consumed ARs were present as DHPPA and DHBA in 24-h effluent from ileostomy-operated individuals consuming 147 mg/day ARs, and in the same study, DHPPA was quantified in pig bile after WG rye bread consumption [12]. Although the AR metabolites identified in human ileostomal effluent could partly be due to AR degradation during storage (>5 years), these results indicate the possibility of non-urinary elimination of AR and their metabolites [12]. It should be noted that recovery difference between studies may, to some extent, be explained by the analytical methods employed, although a previous method comparison study displayed negligible practical differences between the compared methods [35].

Previously published methods for plasma AR quantification [7, 36] could not be employed without modifications due to sensitivity issues of the analytical equipment, and only three of the five major AR homologues could be quantified in the present study. However, the three homologues measured in plasma (C19:0, C21:0, and C23:0) represented the majority (92 %) of dietary AR in the present study due to the wheat-based WG intake and the absence of rye in the diet. The mean daily AR intake during the 3-serving period of the present study (mean = 22.6, 95 % CI 21.1–24.3 mg/day) was comparable to mean intake of Swedes consuming their habitual diet (36 ± 30 mg/day) [37]. However, the habitual AR intake in the USA is likely considerably lower due to a lower intake of WG wheat and rye compared with Nordic countries, as suggested by the intake differences between high-WG-consuming Swedes and low-WG-consuming British [38]. The presence of low amounts of ARs in refined wheat foods is known and did contribute to AR intake during all the dietary periods, though the overall contribution of refined products to the total intake of ARs during the 3-serving period was small (6.9 ± 4.1 %). The presence of low amounts of ARs in refined wheat likely explains the low amount of ARs measured in plasma during these periods.

In conclusion, the present study shows that both plasma ARs and urinary AR metabolites (DHBA and DHPPA) can be used to differentiate between non-consumers and consumers of WG wheat and that the 24-h urinary AR metabolite excretion can additionally distinguish levels of WG wheat intake. Although plasma ARs were increased with WG wheat intake, no significant dose–response relationship was observed, perhaps due to the small sample size, and thus, it was not possible to distinguish between 3 and 6 daily servings of WG wheat in fasting plasma samples.

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Ethical standard Written informed consent was obtained from all subjects prior to study enrollment. The study was reviewed and approved by the Tufts University Health Sciences Campus Institutional Review Board and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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