Alkylresorcinols as biomarkers of whole-grain wheat and rye intake: plasma concentration and intake estimated from dietary records

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ABSTRACT
Background: Alkylresorcinols (ARs), phenolic lipids exclusively present in the outer parts of wheat and rye grains, have been proposed as specific dietary biomarkers of whole-grain wheat and rye intake.
Objective: The objective was to validate plasma ARs as a biomarker of whole-grain wheat and rye intakes by studying the correlation between their plasma concentration and intake calculated from food records.
Design: In a randomized crossover study, 22 women and 8 men were given a defined amount of either whole-grain or refined-cereal-grain products to be included in their habitual diets for two 6-wk periods. Blood samples were collected and food intakes were recorded before and after each intervention period.
Results: Plasma AR concentrations were significantly higher after the whole-grain diet period than after the refined-grain period (P < 0.0001) and were well correlated with average daily AR intake estimated by self-reported weighed food records (Spearman’s r = 0.58, P < 0.001).
Conclusion: Plasma AR concentrations are correlated with intake assessed by food records, which suggests that ARs are selective nutritional biomarkers for the intake of whole-grain wheat and rye.

INTRODUCTION
Epidemiologic studies have shown a relatively strong inverse correlation between whole grain intake and risk of heart disease (1, 2). More recently, reduced risks of type 2 diabetes and certain cancers have also been found (3). A major problem in nutritional epidemiology is the relatively low accuracy in measuring the intake of foods and nutrients (3), which might weaken the diet-disease association. The assessment of whole grain intake is further complicated by the fact that consumers may have difficulties identifying products containing whole grain among other products. Using a biomarker of whole grain intake would overcome some of the problems associated with dietary assessment methods and could be used to validate other methods (4, 5). A group of phenolic lipids, alkylresorcinols (ARs), seem to fit many of the general criteria for biomarkers of whole-grain wheat and rye intake (6–8).

ARs, 1,3-dihydroxy-5-alkylbenzene homologues with alkyl side chains in the range of 17–25 carbon atoms (Figure 1), are mainly present in wheat (400 μg/g), rye (700 μg/g), and barley (40 μg/g) among foods commonly consumed by humans (9, 10). AR homologues are exclusively found in the outer parts of these cereals, with high concentrations in bran fractions (up to 4000 μg/g), whereas refined flours contain no ARs or only trace amounts, but the variation in content is large within and between species. The homologue profile is rather consistent within species but different between species, which results in a C17:0/C21:0 ratio of ≈1.0 in rye, 0.1 in wheat, and 0.01 in durum wheat (11, 12). The source of grain used in a food product can be identified by the C17:0/C21:0 ratio. The measured AR content and C17:0/C21:0 ratio are strongly correlated with the calculated content, and the C17:0/C21:0 ratio is based on lists of ingredients for products generally available on the Swedish market (11).

The physiologic functions of AR in plants are to great extent unknown, but various bioactivities in different models including animals have been demonstrated (13). AR are absorbed (40–60%) from the small intestine (14, 15), transported via the lymphatic system (16), and carried in lipoproteins and erythrocytes in blood (16, 17). In humans, the terminal AR half-life is rather short, about 5 h for all homologues, suggesting that plasma AR might reflect short- to medium-term intake (6). Plasma AR concentration has been found to respond to differences in intake (7, 16) and to correlate to the amount of rye bread intake (7). The plasma C17:0/C21:0 ratio was found to be ≈0.1 in subjects consuming a wheat-bread diet and =0.6–0.9 in subjects consuming a rye-bread diet (7, 16). Until now, no study has attempted to correlate calculated AR intakes with plasma AR concentrations, which is a crucial step in the validation of plasma AR as a marker of whole grain intake.

The aim of this study was to investigate the relation between plasma AR concentration and AR intake calculated from 3-d self-reported weighed food records in a controlled whole grain crossover intervention study.

SUBJECTS AND METHODS
Subjects and study design
A total of 30 subjects (22 postmenopausal women and 8 men) with a mean (±SD) age of 59 ± 5 y and a body mass index (in

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Received March 1, 2007.
Accepted for publication October 8, 2007.
kg/m²) of 28.3 ± 2.0 were included. The present study forms part of an intervention study primarily designed for the investigation of whole-grain foods in the context of insulin sensitivity and inflammation (18). All volunteers were provided with written and oral information about the study before it began. The study was performed as a randomized, nonblind, crossover dietary intervention trial with treatments during 2 consecutive 6-wk periods separated by a 6–8 wk washout period. It was approved by the local ethics committee in the Uppsala region of Sweden.

Diets

Subjects were instructed to adhere to their habitual diet during both treatment periods, except for cereal products, which were provided. During treatment periods, subjects were instructed to replace their habitual cereal foods with a defined amount of several whole-grain or refined-grain products to eat every day. These products included soft bread, crisp bread, pasta, rice, and muesli and were described in detail elsewhere (18). A whole-grain product in this study is defined as a product containing all of whole-grain foods in the context of insulin sensitivity and inflammation (18). All volunteers were provided with written and oral information about the study before it began. The study was performed as a randomized, nonblind, crossover dietary intervention trial with treatments during 2 consecutive 6-wk periods separated by a 6–8 wk washout period. It was approved by the local ethics committee in the Uppsala region of Sweden.

Dietary fiber intake

The dietary fiber contributions from intervention diets were 18 g (whole grain) and 6 g (refined grain), respectively. Total dietary fiber intake and cereal dietary fiber intake from wheat and rye for the 2 intervention periods and for the baseline periods were estimated from the 3-d weighed food records and from dietary fiber contents listed in the database of the Swedish National Food Administration (PC-kost 1/2000). Dietary fiber fractions in test products were analyzed and found to be similar to database values (18). Total dietary fiber intake and cereal fiber intake from wheat and rye were correlated with both the calculated total AR intake and the total plasma AR concentration.

Samples and analytic methods

Blood samples were collected in the morning after the subjects had fasted overnight and before and after the two 6-wk intervention periods. Blood drawn from an antecubital vein into heparin-coated vacuum tubes was immediately centrifuged in a Hettich Rotina 48R centrifuge (Bach, Germany) (2000 × g for 10 min at 4 °C) to separate plasma and erythrocytes. Plasma samples were portioned into 2-mL cryotubes and stored at −80 °C until analyzed.

Total AR content and relative homologue composition of food products used during the whole-grain and refined-grain diet periods were analyzed by gas chromatography (GC). Briefly, samples were milled and extracted with a hot 1-propanol:water mixture (3:1, by vol) and analyzed with GC without further purification. Samples were analyzed in triplicate on a fresh batch, and the intraassay precision expressed as CV was <5% and the interassay precision was <8%.

Plasma samples were analyzed for total AR concentration and relative AR homologue composition according to a method slightly modified from Linko et al (20). The AR C20:0 (Researcher Life Sciences, Burgdorf, Switzerland), which does not exist naturally, was used as an internal standard. Briefly, plasma (0.5 mL) was mixed with the internal standard (45 ng) and incubated with water (0.5 mL) at 37 °C overnight. Samples were extracted with diethyl ether (3 mL × 3), evaporated to dryness, and dissolved in 0.5 mL methanol. The ARs present were separated from nonpolar lipids on a diethyl-amino-ethyl (DEAE)-Sephadex A-25 ion exchange gel (Amersham Biotech, Uppsala, Sweden) in free base form dissolved in methanol and packed in Pasteur pipettes to a final height of 2 cm. Columns were washed with 6 mL MeOH to elute neutral compounds, and then ARs were eluted via the addition of 0.1 mol acetic acid/L in 6 mL MeOH. Eluted ARs were evaporated to dryness, silylated with 0.2 mL pyridine:hexamethyldisilazane:trimethylchlorosilane 9:3:1 (vol:vol:vol), and analyzed with a Finnigan Trace GC Ultra Gas chromatograph coupled to a Finnigan Trace DSQ mass detector (Thermo Fisher Scientific, Waltham, MA). The column and temperature program were the same as for the quantitative analysis performed on a GC-FID system described elsewhere (9).
Inlet and ion source temperatures were set at 325 and 280 °C, respectively, and the ionization energy was 70 eV. ARs were identified by their molecular ions, the typical base ion at a mass-to-charge ratio of 268, and by comparing their retention times with those of a mixture of synthetic AR standards (C15:0–C21:0) (Researchem Life Sciences). AR homologues C17:0-C25:0 were quantified by using their molecular ions, the typical base ion at a mass-to-charge ratio of 268. A multipoint (n = 8; 5–500 pg/µL) standard curve was prepared for homologues C17:0-C25:0 within each batch of samples. Known concentrations of ARs were set versus the ratio of ARs to internal standard area ratio, and linear regression was applied. Homologues C23:0 and C25:0 were quantified by using the standard curve of C21:0 because of the lack of reference compounds. Plasma samples were analyzed as single samples because of the scarcity of sample. A blank and a control sample were included; the intraassay CV was <12%, and the interassay CV was <15%.

**Statistical analysis**

Differences in AR intakes, total plasma AR concentrations, and C17:0/C21:0 ratios between periods were evaluated by an analysis of variance model appropriate for crossover design experiments. Treatment sequence, subjects nested within sequence, period, and treatment were included as factors in the model. The residuals from the model were examined for normality, and if the residuals were extremely skewed (Shapiro-Wilk’s test), the variables were transformed to their natural logarithms before analyses. This was the case for total plasma AR concentration, plasma C17:0/C21:0 ratios, and average daily AR intakes. The statistical analysis was conducted in 26–28 subjects, because of the lack of sample in some cases. One individual was excluded from all statistical analysis because of an extreme fasting plasma AR concentration (>800 nmol/L). Carryover effects were evaluated by comparing the treatment sequences with patient nested within treatment as an error term. These tests were 2-sided and were performed at 10% significant level. No carryover effects were found. The tests for treatment effect were 2-sided, and P values < 0.05 were considered significant. All P values were Bonferroni corrected for multiplicity. In addition to an evaluation of treatment effects, correlation analysis was performed by using data reported during the baseline and treatment periods, separately and together. The plasma AR concentration was correlated with total AR, cereal, and total dietary fiber (excluding oats and rice) intakes reported during the intervention periods and baseline periods, individually and together. In correlation analysis, only P values for correlations where data from all periods were included were Bonferroni corrected. Plasma AR concentrations, adjusted for total lipids [plasma AR/(total cholesterol + triacylglycerols)], were also tested as suggested by Linko-Parvinen et al (16). In addition, dietary fiber intake was correlated to AR intake. All correlation coefficients reported are Spearman’s rank correlation coefficients, unless otherwise stated. Pearson’s correlation coefficients were also calculated, which were similar (data not shown). A general linear model (GLM), with subject as a factor, was applied to take repeated measurements of each subject into account when the data from all periods was used together.

The validity of plasma AR concentration as a reflector of intake was tested in terms of its ability to accurately rank individuals into quartiles according to their AR intake. Therefore, quartile agreement between total plasma AR concentration and the intake estimated from 3-d records of intervention and baseline periods was calculated, as described by Bhakta et al (5). Agreement between plasma AR concentration and weighed food records was evaluated by dividing the intake and plasma AR concentrations of treatment and baseline periods into quartiles.
and then calculating the percentage of observations classified into the correct or the correct ± nearest adjacent quartiles for each period. Additionally, an average of quartile agreement estimated within each treatment and baseline period, separately, was calculated. All statistical analyses were performed with SAS (version 9.1; SAS, Chicago, IL) and Minitab 14.0 (Minitab Inc, State College, PA) software.

RESULTS
Test foods and intake of AR and dietary fiber
The total AR contents in the products provided were in the range of not detected to 776 μg/g fresh weight (Table 1), and the relative homologue composition was comparable with data published previously for similar types of products. The C17:0 to C21:0 ratio was in the range 0.02–1.0, which reflected the source of whole grain used in the products (11).

Compliance during the study was good; 30 individuals completed the intervention and 28 completed diaries and 3-d food records. The 3-d weighed food records and the diary checklist showed good adherence to the dietary advice (18). The calculated intake from the advised diets corresponded to 63 ± 11 and 6 ± 1 mg AR/d during the whole-grain and refined-grain periods, respectively. The relative homologue composition calculated from the advised whole-grain diet was 13% (C17:0), 28% (C19:0), 36% (C21:0), 15% (C23:0), and 8% (C25:0). For the advised refined-grain period, it was 10% (C17:0), 30% (C19:0), 30% (C21:0), 26% (C23:0), and 4% (C25:0). Hence, the average C17:0/C21:0 ratios were 0.4 and 0.3 for the whole-grain and refined-grain intervention periods, respectively.

There was no difference in total energy intake between the 2 intervention periods or compared with baseline. As expected, total dietary fiber intake differed significantly (P < 0.001) between the whole-grain period (30 ± 4.7 g) and the refined-grain period (18.3 ± 7.4 g). Additionally, total fiber intake was lower (P < 0.05) during the refined-grain period than at baseline (21.4 ± 9.1 g) and higher (P < 0.001) during the whole-grain period than at baseline (22.1 ± 8.0). Cereal fiber corresponded to 64.7 ± 10.5% and 18.3 ± 7.4% of total fiber during the whole-grain and refined-grain periods and to 45.7 ± 14.0% and 47.6 ± 14.2% during the respective baseline periods. Total dietary fiber and cereal fiber of wheat and rye were both well correlated with calculated AR intake [Pearson r = 0.64 (P < 0.01) and Pearson r = 0.81 (P < 0.01), respectively]. Detailed information about the test foods was published elsewhere (18).

Plasma AR concentration compared with reported intake
All subjects showed the same response pattern, with a significantly higher plasma AR concentration after the whole-grain diet than after the refined-grain diet (P < 0.0001) (Table 2). The total AR concentration in plasma was also significantly higher after the whole-grain period and lower after the refined-grain period than at baseline (P < 0.0001; Table 2). There was no carryover effect in any of the studied variables between the study periods.

A comparison of total AR intake calculated from weighed food records with the plasma total AR concentration from the 2 interventions periods and from the baseline periods together showed a good correlation (r = 0.58, P < 0.001). The GLM with subject as a factor showed a slope of 0.53 (P < 0.001). As expected, no correlation was found when data from the whole-grain and refined-grain periods were analyzed separately, but significant correlations were found for the baseline periods (Table 3). When data from all periods were used, the correlations were better for longer than for shorter AR homologues and were marginally improved by relating the total AR concentration in plasma to the total plasma lipid concentration (total cholesterol concentration + triacylglycerols) (r = 0.32–0.62, P < 0.05) (Table 4). Combined total plasma AR concentrations from the 2 intervention periods and baseline periods also correlated with the daily total dietary fiber intake (r = 0.35, P < 0.011) and with cereal fiber intake from wheat and rye (r = 0.47, P < 0.011). Here too, the correlations were marginally improved when ARs were related to total cholesterol and triacylglycerols before the analysis: r =

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total AR intake from diaries mg/d</th>
<th>Total AR intake from food records mg/d</th>
<th>Total plasma AR concentration nmol/L</th>
<th>AR homologue ratio (C17:0/C21:0)</th>
<th>Baseline</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole grain</td>
<td>63.9 ± 11.0^7 (53.6–111.7)</td>
<td>36.1 ± 19.0 (12.8–89.9)</td>
<td>65.0 ± 12.2^7,8 (43.1–98.8)</td>
<td>102 ± 70 (21–251)</td>
<td>0.37 ± 0.23 (0.04–0.95)</td>
<td>0.30 ± 0.23 (0.05–0.87)</td>
</tr>
<tr>
<td>Refined grain</td>
<td>6.5 ± 0.5^7 (5.4–7.8)</td>
<td>44.0 ± 31.6 (4.0–136.9)</td>
<td>6.8 ± 1.9^7,8 (1.7–11.0)</td>
<td>104 ± 77 (9–220)</td>
<td>0.32 ± 0.20 (0.02–0.80)</td>
<td>0.34 ± 0.23 (0.09–0.96)</td>
</tr>
</tbody>
</table>

1 All values are x ± SD; range in parentheses. Plasma AR concentrations are single measurements from 28 individuals.
2 No significant carryover effects were found for any of the studied variables at the level (P < 0.10) tested by ANOVA appropriate for crossover designs.
3 Diary not filled in during the baseline periods.
4 Intake during baseline periods was estimated from pooled 3-d dietary records and by matching reported products to similar products analyzed previously (10, 18).
5 No time-by-treatment interaction was found at the level (P < 0.05) tested.
6 Intake during treatment periods was estimated from pooled 3-d dietary records and by analyzing all reported products.
7 Significant difference between treatment periods, P < 0.0001 (Bonferroni corrected). Data were analyzed by ANOVA appropriate for crossover designs.
8 Significantly different from corresponding baseline period, P < 0.0001 (Bonferroni corrected). Data were analyzed by ANOVA appropriate for crossover designs.

TABLE 2
Daily average total alkylresorcinol (AR) intake estimated from diaries and pooled 3-d weighed food records, total AR concentrations, and AR C17:0 to C21:0 homologue ratios in plasma

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TABLE 3
Spearman’s correlation coefficients (r) between alkylresorcinol (AR) intake and plasma total AR concentration during baseline and the whole-grain and refined-grain diet periods (n = 28 per period) and for all periods together (n = 4 × 28)¹.

<table>
<thead>
<tr>
<th>Diet period</th>
<th>r</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline before whole grain</td>
<td>0.33</td>
<td>0.018</td>
</tr>
<tr>
<td>Whole grain</td>
<td>0.25</td>
<td>0.210</td>
</tr>
<tr>
<td>Baseline before refined grain</td>
<td>0.40</td>
<td>0.042</td>
</tr>
<tr>
<td>Refined grain</td>
<td>0.21</td>
<td>0.280</td>
</tr>
<tr>
<td>All periods</td>
<td>0.58</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

¹ Intake was estimated from 3-d food records.
² Bonferroni corrected.

0.36 (P < 0.010) and r = 0.52 (P < 0.010) for total and cereal fiber, respectively.

In contrast with what was found previously (16), there was no statistically significant change from baseline in the AR C17:0/C21:0 ratio after the whole-grain diet or after the refined-grain period after Bonferroni correction of the P value.

When the estimated AR intake and the plasma AR concentrations for all individuals and all periods were divided into quartiles, ≈51% of the plasma AR concentrations were classed into the correct average daily intake quartile and 87% were classified into the correct ± the nearest adjacent quartiles. The corresponding averages of agreement determined for each treatment and baseline period separately were ≈30% and 70%. The distribution of plasma AR concentrations in each intake quartile from all periods together is shown in Figure 2.

DISCUSSION

This is the first study to show a correlation between plasma AR concentration and calculated AR intake from self-reported weighed food records. It was found that the C17:0/C21:0 ratio in plasma followed that of intake, in agreement with previous reports (7, 16). The results strongly suggest that plasma AR concentrations reflect intake and can therefore serve as a dietary biomarker of whole-grain wheat and rye intake, as previously suggested (6–8).

Diets and intake

Products consumed during the refined-grain period contained only low levels of AR, except for a mistaken crisp bread, which was found to contain 121 μg/g. This product was included on the basis of labeled ingredients (sifted rye flour) and was not expected to contain the amounts of AR found by analysis. The relative homologue composition of some refined-grain products was not in agreement with what could be expected because of the fact that the measured amounts were very close to the detection limit of the method, which made integration of the peaks difficult, especially those of C23:0 and C25:0 (Table 1).

The average habitual AR intake of subjects during baseline periods was ≈40 mg/d, which is higher than the estimated per capita intake of the Swedish population (≈20 mg/d) (21). The intake of ARs during the whole-grain period was 1.5 times that of the habitual intake. There was good agreement between the calculated AR intake and that reported in both the 3-d weighed food records and the diaries (Table 2). Compared with the whole-grain and refined-grain periods, the precision in food recording was less during the baseline periods, possibly caused by consumption of a wider range of products for which AR contents were estimated and not analyzed.

As expected, the correlation between cereal fiber intake from wheat and rye and AR intake was high, because ARs are located in the bran, which is also the main source of cereal fiber. Because
cereals were the dominant source of dietary fiber in this study, AR intake was also fairly well correlated with total dietary fiber intake.

**Plasma AR in relation to estimated intake**

The baseline AR concentration observed was slightly higher than that found in postmenopausal Finnish women (7) and was slightly lower than that reported for 15 healthy young Finnish subjects (16). The individual variation was rather high (21–340 nmol/L), in agreement with 2 Finnish studies (7, 16). This large variation might have been due to differences in intake, absorption, elimination, and demographics or to a combination thereof (22). The plasma AR concentration was significantly higher after the whole-grain period than after the refined-grain period, but the magnitude of the difference was not as large as that observed in the 2 previous studies (7, 16), because of the inclusion of refined ingredients other than sifted wheat flour during the refined-grain diet period in the present study. Another explanation might be the slight differences in analytic determinations at low concentrations or a systematic underreporting of AR intake during the refined-grain period. However, the average plasma concentration after 6 wk of the whole-grain diet (with a rather high intake) was similar to values found after a 1-wk rye bread intervention with a corresponding daily AR intake (16), which suggests that, at the level tested, 1 wk would be sufficient to obtain plasma AR concentrations that reflect intake. This finding is in line with the relatively short half-life of plasma AR, which implies that a steady state level would be established within a few days when ARs are consumed regularly (6).

The lack of a correlation between total AR intake and plasma concentration within each intervention period was probably due to the small variation in intake caused by the study design (Table 3). A good correlation was found when data from the intervention periods were used, and baseline periods together and GLM showed a highly significant slope of 0.53 (P < 0.001), which indicated that there was a subject-independent increase in plasma AR concentration caused by intake.

It was recently shown that plasma AR is carried in lipoproteins and that plasma AR should therefore be adjusted for total plasma lipids (16). In the present study, the best correlation between estimated total AR intake and plasma concentration was obtained by adjusting AR for total plasma lipid (r = 0.59, P < 0.001). However, the effect of adjustment was small and no significant correlation between plasma AR concentration and total lipoprotein concentration was found, which contradicts results reported by Linko-Parvinen et al (16). When individual plasma AR homologues were correlated with the total AR intake, correlations were better for the longer homologues (C23:0 and C25:0); the best correlation was found for C25:0 (r = 0.60 and lipid-adjusted r = 0.62, P < 0.001), despite the fact that C25:0 is the most difficult AR homologue to analyze in plasma because of a low concentration and long retention in the GC column (Table 4). We previously showed that the relative bioavailability of AR homologues increases with homologue length, probably because of differences in absorption and elimination (6). Our current results indicate that longer AR homologues with high relative bioavailability are better correlated with intake. This may be of importance for deciding whether any specific AR homologue or total AR concentration in plasma should be used as the biomarker of whole-grain wheat and rye intake.

The C17:0/C21:0 ratio in plasma has been suggested to reflect the source of whole grain used in the diet, with a value near 1.0 when rye is consumed and 0.1 when wheat is consumed (7, 16, 23). In the present study, the C17:0/C21:0 ratio was marginally higher in the calculated whole-grain diet than in the refined-grain diet and was thus not expected to differ in plasma samples (Table 2). The C17:0/C21:0 ratios in plasma were similar after the baseline periods, while the subjects were consuming their habitual diet, but were lower than those reported for Finnish subjects (7, 16), who seem to have more rye in their habitual diet than did the Swedish subjects (7, 16, 21). The finding of a lower C17:0/C21:0 ratio in plasma than in the diet might have been related to differences in the absorption and elimination of AR homologues, as suggested previously (6). The ratio might be affected by the duration of intake (16). However, both the C17:0/C21:0 ratios in the calculated diets and in plasma were almost the same for both diet intervention periods. Long-term compared with short-term intake studies as well as interventions with different intakes are needed to better understand how the C17:0/C21:0 ratio is affected and how it should be interpreted.

The ability to classify AR intake into quartiles depending on plasma AR concentration was fairly good. About 70% were classified in the correct or the correct ± the nearest adjacent quartiles when repeated measurements in each subject were taken into account, which was about the same as for the other dietary biomarkers studied (5, 24).

Results of the present study and previous studies (6, 7, 16, 17, 23) showed that ARs fulfill many of the criteria set for a specific dietary biomarker. However, before ARs are used for the evaluation of other dietary assessment methods, large-scale, well-controlled calibration studies are needed to evaluate dose-response relations and to explore possible factors other than intake that affect plasma concentrations.

We thank Sylvia Olofsson (Biostatistics Unit of Uppsala Clinical Research Centre) for statistical support, Åsa Ramberg (Department of Environmental Assessment, Swedish University of Agriculture Science) for kind help with the GC-MS instrument, and Lantmännen AB and Wasabröd AB for donating the cereal products for the intervention periods.

The authors’ responsibilities were as follows—RL: performed the laboratory and data analysis; RL and AK-E: wrote the paper; AA: coordinated the intervention trial and provided information on nutrient calculations and dietary record data; BV, AK-E, and PA: initiated and designed the study and obtained the funding. All authors critically reviewed the manuscript. None of the authors had a potential or actual conflict of interest. This work has not been published before.

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