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Title: Hydroxylated phenylacetamides derived from bioactive benzoxazinoids are bioavailable in humans **after** habitual consumption of wholegrain sourdough rye bread.

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Abbreviations

AAPO, 2-acetylamino-3H-phenoxazin-3-one

amu, atomic mass unit

AP, 2-aminophenol

APO, 2-amino-3H-phenoxazin-3-one

AUC, area under the ROC (receiver operating characteristic) curve

BOA, Benzoxazolinone

CRF, Clinical Research Facility

DF, Discriminant Functions

DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one

DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one

EPIC, European Prospective Investigation into Cancer and Nutrition

FFQ, Food Frequency Questionnaire

FDR, false discovery rate

FIE-MS, Flow Infusion Electrospray-Ionisation Mass Spectrometry

FIE-MSⁿ, Flow Infusion Electrospray-Ionisation tandem Mass Spectrometry

FT-ICR-MS, Fourier Transform-Ion Cyclotron Resonance Ultra-Mass-Spectrometry

GC-MS, Gas Chromatography-Mass Spectrometry

HBOA, 2-hydroxy-1,4-benzoxazin-3-one

HPAA, 2-hydroxy-N-(2-hydroxyphenyl) acetamide

HMBOA, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one

HPAA, N-(2-hydroxyphenyl) acetamide

HPLC, High-Performance Liquid Chromatography

HPLC-DAD, High-Performance Liquid Chromatography with Diode-Array Detection

LC-MS, liquid chromatography mass spectrometry

LDA, Linear Discriminant Analysis

MSⁿ, Tandem Mass Spectroscopy

MBOA, 7-methoxy-2-benzoxazolinone

m/z, mass-to-charge ratio

NMR, Nuclear Magnetic Resonance

PCA, Principal Component Analysis

Q, activation coefficient

RF, Random Forest

RF IS, Random Forest Importance Score

ROC, receiver operating characteristic

Tw, Eigenvalues

Key words

Benzoxazinoids/ bioactive/ metabolomics/ multivariate data analysis/ wholegrain sourdough
rye bread

Abstract

Scope: Understanding relationships between dietary whole grain and health is hindered by incomplete knowledge of potentially bioactive metabolites derived from these foods. We aimed to discover compounds in urine correlated with changes in amounts of wholegrain rye consumption.

Methods and Results: After a wash-out period, volunteers consumed 48 g wholegrain rye foods per day for 4 weeks and then doubled their intake for a further 4 weeks. 24 hour urines were analyzed by flow infusion electrospray MS followed by supervised multivariate data analysis. Urine samples from participants who reported high intakes of rye flakes, rye pasta or total wholegrain rye products could not be discriminated adequately from their wash-out samples. However, discrimination was seen in urine samples from participants who reported high wholegrain sourdough rye bread consumption. Accurate mass analysis of explanatory signals followed by fragmentation identified conjugates of the benzoxazinoid lactam 2-hydroxy-1,4-benzoxazin-3-one (HBOA) and hydroxylated phenyl acetamide derivatives. **Statistical** validation showed sensitivities of 84–96% and specificities of 70–81% (P values <0.05) for elevated concentrations of these signals after preferential wholegrain sourdough rye bread consumption.

Conclusion: Several **potentially** bioactive alkaloids **have been identified** in humans consuming **fermented** wholegrain sourdough rye bread.

1 Introduction

There is strong observational evidence, and some intervention evidence supporting the link between increased consumption of wholegrain foods and reduced risk of several diseases including cardiovascular diseases, type 2 diabetes and some cancers [1-6]. The intake level at which health benefits may be achieved is not proven and consequently recommendations for intake range from imprecise statements such as ‘choose whole grains whenever possible’ to precise recommendations such as 75g /10 MJ in Denmark [7, 8]. One confounding problem is accurate quantification of wholegrain food intake in epidemiological studies [e.g. 6] due to poor definition of wholegrain foods, limited information on portion/serving sizes and lack of detail on the wholegrain-content of individual foods, including type of grain (plant species). Thus it has proved difficult to make definitive conclusions about the protective roles of wholegrain foods in general or of specific wholegrain-containing foods.

Compared with cereal products produced from refined flour, the components unique to wholegrain foods are the fiber content and a spectrum of plant secondary metabolites associated with the bran fractions of specific grains. It would be particularly desirable to understand the bioavailability of metabolites with known or potential bioactivities and their biotransformation products originating from consumption of wholegrain foods, since they would represent prime candidates for identifying causal relationships between desirable health outcomes and individual compounds in these foods [9]. Previous studies of metabolites associated with wholegrain foods have focused on the concentration of alkylresorcinols in plasma and their breakdown products in urine, and shown their value as biomarkers of wholegrain exposure [10, 11, 12-14]. Benzoxazinoids are secondary metabolites with allelochemical properties found in many species of cereals including rye, maize and wheat [e.g. 15, 16]. They occur primarily in the vegetative parts of the plant [16, 17, 18] and have antimicrobial, antifeedant, and insecticidal effects [19, 20, 21]. Recently benzoxazinoids and

a range of novel conjugates have been shown to be present in grains of wheat, and particularly, rye [22, 23]. Benzoxazinoid chemicals include benzoxazolinones such as 2-benzoxazolinone (BOA), the lactam 2-hydroxy-1,4-benzoxazin-3-one (HBOA) and its corresponding hydroxamic acid 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). DIBOA is often the major compound and occurs as a glucoside in plant tissues [24], which on cellular injury is converted to an aglycone that undergoes non-enzymic degradation to BOA [15, 24]. The main microbial degradation pathway of benzoxazinoids is centred on the oxidation of 2-amino-3H-phenoxazin-3-one (APO) derived from the 2-aminophenol (AP) moiety of BOA and HBOA [21, 25, 26-28]. Benzoxazinoids represent a unique chemical structure with potential pharmaceutical activity, and a subgroup of benzoxazinoids, benzoxazolinones, have provided a “chemical scaffold” for the design of new drugs [29].

In pigs fed wholegrain rye buns containing high concentrations of DIBOA-hexoses along with lower amounts of HBOA-hexoses [30], plasma contained very high concentrations of HBOA- gluc and HBOA relative to DIBOA glucosides. Only low amounts of APO and AAPO were found in bile and the concentrations in either urine or feces were close to or below detection limits. However this targeted analysis was limited to only the phenoxazinone class of compounds derived from AP [30], so other types of relevant compounds may have been overlooked.

In the present study we examined 24 hr urine samples from free-living individuals after a wash-out period and intervention periods with 48 g wholegrain rye products per day for 4 weeks and then 96 g wholegrain rye products per day for 4 weeks. Non-targeted metabolite fingerprinting and targeted FT-ICR-MS accurate mass analysis were employed to determine types and amounts of compounds taken up and excreted in the course of human whole-grain rye consumption.

2 Materials and methods

2.1 Study design and volunteer recruitment

This research project was supported by the UK Food Standards Agency (Project N05075). This project constitutes part of The GrainMark Study (<http://www.ncl.ac.uk/afrd/research/project/2287>), which had ethical approval from the Northumberland Research Ethics Committee on 15th November 2007 (reference number 07/H0902/53) and was registered with the Newcastle upon Tyne Hospitals NHS Foundation Trust, UK (registration number 4349) and with isrctn.org (ISRCT number CCT-NAPN-A13175). All volunteers gave written informed consent. All procedures were carried out in the Clinical Research Facility (CRF), Royal Victoria Infirmary, Newcastle upon Tyne, UK. Healthy adult volunteers aged >45 years, taking no medication and with no history of recent illness were recruited from the local community.

The dietary intervention consisted of a 4-week wash-out period during which volunteers were asked to avoid all wholegrain foods (aided by a list provided). The volunteers were asked to consume 3 servings of wholegrain rye foods per day ‘dose 1’ which was equivalent to 48g wholegrain rye per day for 4 weeks from a selection of foods provided (where each serving of wholegrain rye food approximated 16 g of whole grains to mimic the serving size advocated by the USDA in its Dietary Guidelines for Americans [USDA, 2005], *see Supplemental data 1*, under “Supplemental data” in the online issue). Whole grain foods were substituted incrementally for refined flour products. Foods included were wholegrain sourdough rye bread (The Village Bakery, Penrith, UK, prepared using a sourdough recipe of Russian origin using 50/50 dark rye flour/light rye flour, **where the dark rye flour is milled from wholegrain and the light flour has 40% of the bran fraction sieved out**), rye flakes as either a porridge or muesli and rye pasta (80% wholegrain rye, 20% refined wheat). **The rye pasta and rye flakes (for porridge and muesli) were provided by Raisio Group, Raisio, Finland and shipped to Cereal Partners UK (CPUK, Welwyn Garden City, UK) for packaging.** After 4

weeks they were asked to double their intake of the same foods for a further 4 weeks ‘dose 2’. Volunteers were asked to avoid wholegrain foods throughout the study period except for the study wholegrain foods. **Compliance with dietary intervention was monitored by measuring total alkylresorcinol level in plasma [12] (see Supplemental data 2, under “Supplemental data” in the online issue).** At the end of each period (wash-out, 3 portions ‘dose 1’ and 6 rye wholegrain products per day ‘dose 2’) volunteers completed a validated Food Frequency Questionnaire (FFQ) [31, 32] based on the European Prospective Investigation into Cancer and Nutrition (EPIC) FFQ and asking for consumption of foods during the preceding 7 days only [32]. The system used to score the volunteers in terms of the frequency of consumption for each wholegrain rye product based on analysis of FFQ information is shown in Supplemental data 3 (see Supplemental data 3, under “Supplemental data” in the online issue).

2.2 Urine collection and processing

In line with our previous research [31, 33-36] we asked volunteers to minimize physical activity and avoid alcohol consumption on the day prior to the test day. To reduce random intra-individual and inter-individual variations in urine samples caused by the acute influence of the evening meal before sampling days [36-39] all volunteers were provided with a standardized evening meal that was consumed the evening before every measurement visit. The meal consisted of a pre-cooked chicken dinner, (Sainsbury’s Supermarkets, UK), one chocolate éclair (Marks & Spencer Plc., UK) and one 500ml bottle of mineral water. The chicken meal consisted of one roasted chicken breast, roast potatoes and vegetables in a gravy sauce was purposely chosen so that no component of the meal had any whole grains in it. The volunteers were instructed to consume the standardized evening meal by 8 pm the evening before their measurement visit and fast until the measurements/sample collection the

following morning. Study participants were provided with a second bottle of still mineral water to be consumed *ad lib* until arrival at the CRF in a fasted state on the morning of the study day. Twenty-four hour urine samples were collected the days before visits to the CRF at the end of each phase of the study at week 4, 8 and 12 respectively as described in Lloyd et al. 2012 [36] and stored in a 50ml Falcon tube at -80°C for further analyses.

2.3 Metabolite extraction from sourdough rye bread and component flours

Three replicates (20 mg) were taken from slices of The Village Bakery wholegrain sourdough rye bread and the two types of rye flour used in the baking of this bread, dark rye flour and light rye flour (kindly supplied by Mike Darlow from Nicholas and Harris Ltd. Salisbury). These samples were placed into Eppendorf tubes each containing a stainless steel ball and on ice, 200µl of extraction solvent A was added (Chloroform/ Methanol/ H₂O, 1:1:1) and milled using a Mixer mill (Retsch MM200) for 30s at 30Hz. Following milling, 800µl of extraction solvent B (Chloroform/ Methanol/ H₂O, 1: 2.5: 1) was added and samples were vortexed, shaken for 20min at 4°C using an Orbital shaker (Ika-Vibrax-VXR, Jankel & Kunkel) and then centrifuged at 13,000 g for 6 min at 4°C. 100 µl of the supernatant was placed into a new Eppendorf tube, dried down using a vacuum evaporator (Univapo 150H + Unijet II refrigerated aspirator) and reconstituted in 100µl 70/30 methanol/H₂O. Samples were stored at -80°C prior to analysis.

2.4 Flow Infusion Electrospray-Ionisation Mass Spectrometry (FIE-MS)

FIE-MS was carried out as described previously [33-36, 40, 41]. Aliquots of thawed urine (50µl) were diluted in 450µl of pre-chilled methanol/water [3.5/1], vortexed, shaken for 15 min at 4°C and then centrifuged for 5 min at 14,000 g. Data were acquired in alternating positive and negative ionization modes and over four scan ranges (15–110 mass-to-charge ratio (*m/z*); 100–220 *m/z*; 210–510 *m/z*; 500–1200 *m/z*), with an acquisition time of 5 mins, on

a LTQ linear ion trap (Thermo Electron Corporation, San Jose, CA, US). The resulting mass spectrum was the mean of 20 scans about the apex of the infusion profile. Raw data dimensionality was reduced by electronically extracting signals with ± 0.1 Da mass accuracy and (unless shown otherwise) are presented at 1 amu accuracy in Figures and Tables. Data were Log10 transformed and normalized to total ion current prior to analysis [41, 42].

2.5 Sample classification and selection of potentially explanatory signals

Data mining was carried out by following the FIEmSpro workflow validated previously in Aberystwyth [42] (URL <http://users.aber.ac.uk/jhd/>). Principal Component Analysis (PCA) was used to reduce data dimensionality and was followed by PC-Linear Discriminant Analysis (PC-LDA). Plots of the first two Discriminant Functions (DFs) allowed visualization the goodness of class separation. Random Forest (RF) was employed in the analysis of the multivariate data and the RF classification ‘margin’, along with the area under the ROC (receiver operating characteristic) curve (AUC) was used to assess classification performance [42].

Several feature selection techniques were used to highlight the potentially explanatory signals (i.e. nominal mass bins) responsible for discriminating between different sample classes. RF, AUC and student’s *t*-test, were used to produce a full feature rank list [42]. RF feature selection was performed by calculating Importance Scores, being the mean decrease in accuracy over all classes when a feature is omitted from the data. AUC used the area under curve of the sensitivity (true-positive rate) against the specificity (false-positive rate) and student’s *t*-test ranked the features by the absolute value of the P-values.

Randomized re-sampling strategies using bootstrapping were applied in the process of classification and feature selection to counteract the effect of any unknown, structured variance in the data. We used 100 bootstraps in pair-wise comparisons for each of the applied

statistical operations with 2/3 of data as training and 1/3 as test set. RF was set to $n_{tree} = 1000$ for each bootstrap which is adequate considering the dimensionality of data. Two independent RF cycles resulted in very slightly different Importance Score values for each variable (m/z value), but changes in rank of highly important variables is rare.

Pearson correlation coefficients between selected variables were calculated using the function *cor* in R version 2.5.1 [42]. Variables with correlation coefficients > 0.7 were considered to belong to a cluster indicative of different ionization or potential biotransformation/breakdown products of a single food-derived metabolite.

2.6 Targeted accurate mass analysis and annotation of FIE-MS signals

Selected nominal mass bins were investigated further using targeted Nano-Flow (TriVersaNanoMate, AdvionBioSciences Ltd, UK) LTQ- Fourier Transform-Ion Cyclotron Resonance Ultra-Mass-Spectrometry (FT-ICR-MS; where Ultra refers to the high-sensitivity ICR-cell) as reported previously [34, 35, 36]. Samples were prepared as for FIE-MS, but three urine pools from randomized groups of participants were prepared and reconstituted in methanol/water (80/20, v/v).

For metabolite signal identification, the accurate mass values were then queried using MZedDB, an interactive accurate mass annotation tool which can be used directly to annotate signals by means of neutral loss and/or adduct formation rules [43]. FIE-MSⁿ was employed for further metabolite signal identification with the scan window set for 20 scans, an isolation width of 1 m/z and using normalized collision energy of 30V. An activation coefficient 'Q' of 0.250 was chosen and an activation time of 30ms, with wideband activation turned on and a source fragmentation of 20 V. Mass range settings were dependent upon the molecular weight of the target ion. Standards were prepared by dissolving 1 mg of each metabolite in 1 ml of extraction solvent. The metabolite standards N-(2-hydroxyphenyl) acetamide (HPAA) and 2-

hydroxy-1, 4-benzoxazin-3-one (HBOA) were kindly donated by Prof. Damià Barceló and Dr Ethel Eljarrat (Dept. Environmental Chemistry, IDAEA-CSIC, Barcelona). Professor Alan Crozier (College of Medical, Veterinary & Life Sciences, University of Glasgow, UK) kindly supplied the N-feruloylglycine. HPLC grade phenylacetylglutamine and creatinine were obtained from Santa Cruz Biotechnology, Inc (Heidelberg, Germany) and Sigma-Aldrich UK respectively.

3 Results

3.1 Habitual exposure to a combination of rye food products is difficult to detect in the urine metabolome using FIE-MS fingerprinting

The 33 participants (16 M, 17 F) in the GrainMark study had a mean ($\pm$ SD) age of 54.2 years ($\pm$ 5.2) and a mean ($\pm$ SD) BMI of 26.1 kg/m² ($\pm$ 3.3). Biodata for participants are presented in [Supplemental data 2](#) (*see Supplemental data 2*, under “Supplemental data” in the online issue). Examination of FFQ data ([Supplemental data 2](#)) revealed that five participants did not adhere to the protocol and therefore only 28 individuals were included in the metabolomics analysis (13M, 15F). *Analysis of total alkylresorcinol levels in plasma confirmed that all of these volunteers complied with the dietary intervention (see Supplemental data 2, under “Supplemental data” in the online issue).*

24hr urine samples were subjected to non-targeted metabolite fingerprinting by FIE-MS and preliminary data modeling experiments revealed that the m/z range 100–510 in both ionization modes contained signals providing most of the power for discrimination between urine samples provided by participants at the end of each phase of the GrainMark study (i.e. wash-out, Dose 1 and Dose 2 of rye wholegrain products). This mass range was used for all further data modeling and both Random Forest (RF) margin values and AUC values were

calculated to assess classification performance [42]. Modeling on the basis of ‘total rye foods’ consumption produced a maximal RF margin value of 0.11 (in negative ion data) in compositional comparison of urine samples collected after the wash-out period and those collected after consumption of 6 portions of wholegrain ([Table 1](#)). This value was below our previously suggested RF margin threshold of 0.15 for an adequate model in urine analysis [36].

3.2 Supervised multivariate analysis of 24 hr urine metabolite fingerprints shows discrimination only of exposure to wholegrain sourdough rye bread

Although all foods were designed to contain a similar level of wholegrain rye each was manufactured using different processes, which could impact on the final chemical composition of individual dietary components. It was therefore anticipated that specific dietary choice could have a confounding influence on data modelling.

The GrainMark FFQ data were thus re-examined to highlight individuals who incrementally increased the consumption of a specific rye wholegrain food (i.e. either sourdough rye bread, rye pasta or rye flakes) to achieve eventually a frequency of >1 portion per day during the Dose 2 period ([Supplemental data 2](#)). A wholegrain rye sourdough bread group consisting of 15 participants, (5 M, 10F) was identified who consumed from 2-4 portions per week to 1 portion per day during the Dose 1 phase and consumed ≥ 2 -3 portions per day at the Dose 2 phase of the study. The wholegrain rye flakes (muesli and porridge) group consisted of 14 participants, (8 M, 6F). During the Dose 1 phase of the study this group consumed from 2-4 portions per week to just over 1 portion per day, and during the Dose 2 phase they consumed wholegrain rye flakes at ≥ 1 portion per day. There was an overlap of 4 participants between the wholegrain rye sourdough bread group and the participants chosen for the rye flakes

analysis. As wholegrain rye pasta was not favored by any of the participants there were not sufficient individuals to develop a rye pasta consumption group.

Further metabolome modeling was carried out on the basis of individual rye food consumption. The data indicated that only Dose 2 urines from the 15 participants who preferentially selected wholegrain sourdough rye bread could be discriminated adequately from their washout urine phase samples in negative ionization mode (**Table 1**), with a RF margin of 0.19 and an AUC value of 0.90 [42]. PC-LDA scores plots of positive mode and negative mode FIE-MS data representing the composition of 24 hr urine samples from wash-out, Dose 1 and Dose 2 of participants who preferentially selected wholegrain sourdough rye bread are shown in **Figure 1**. We observed separation of metabolite patterns from wash-out samples and wholegrain sourdough rye bread exposure samples along the axis of maximum discrimination (Discriminant Function 1; DF1) with slightly better clustering in negative ionization mode (**Figure 1B**). Relatively poor separation between the two wholegrain sourdough rye bread exposure classes was evident in DF2, as predicted by the data in **Table 1**.

3.3 Identification of urine metabolite fingerprint signals potentially explanatory of wholegrain sourdough rye bread consumption

Previously we have demonstrated, using a range of other FIE-MS data sets, that the threshold for significance in a comparison of two classes of urine samples (pair-wise analysis) lies within a RF Importance Score (IS) range of 0.0015 – 0.003 [34, 42]. The curve of Importance Scores in rank order for the top 50 explanatory features in RF models comparing wash-out urine samples and samples from participants who preferentially selected wholegrain sourdough rye bread (at the 6 rye wholegrain products per day level) (**Figure 2A**) shows that the top 10-22 of the m/z signals conferred the majority of discriminatory power in 24 hr urine. A correlation analysis (**Figure 2B**) indicated that the 2nd ranked signal (m/z 230, RF IS 0.012

and AUC 0.94) was correlated with m/z 246, which was ranked 20th (RF IS >0.002 and AUC 0.80), suggesting that these two signals may be related. In addition, the 4th ranked signal (m/z 401, RF IS 0.009 and AUC 0.89) clustered with m/z 330 (Ranked 8th, RF IS 0.005 and AUC 0.88) as well as several lower ranked signals (m/z 357, 445, 360). The remaining top explanatory features in RF models did not display strong correlations ($r < 0.7$).

Further investigation of the top ranked mass bins was performed by targeted ultra-high accurate mass analysis using FT-ICR-MS (Table 2) followed by FIE-MSⁿ fragmentation (Figures 3 and 4, and Supplemental data 4). In the first correlation cluster (see Figure 2B), m/z 230 was recognized to be a sulphonated molecule due to the presence of a ³⁴S isotope signal plus the fragment ion $[M-H SO_3]^{1-}$ [44] (Supplemental data 4). The remaining aglycone (m/z 150) was identified as the hydroxylated phenylacetamide, N-(2-hydroxyphenyl)acetamide (HPAA) $[M-H]^{1-}$ (Figure 3A) by accurate mass analysis (Table 2) and comparison of the FIE-MS³ spectra with that of the FIE-MS² spectra of an authentic standard (Figure 3B). The FIE-MSⁿ spectra of both the standard and m/z 230/150 showed a major ion at m/z 108, indicating the basic core structure 2-aminophenol, common to all benzoxazolinone and benzoxazinone secondary metabolites [e.g. 21, 25-28]. The correlated signal, m/z 246, was also a sulphonated molecule due to the signature MSⁿ ions and a ³⁴S isotope [44]. The remaining aglycone (m/z 166) had an accurate mass matching that of 2-hydroxy-N-(2-hydroxyphenyl) acetamide (HHPAA) $[M-H]^{1-}$ (Table 2). A standard was not available to confirm this identity, but the FIE-MS³ spectra (Figure 3C) again showed a major ion at m/z 108, indicating the common core structure 2-aminophenol. The nominal mass bin corresponding to the top ranked signal, m/z 342, was shown to contain a major signal with the signature MSⁿ glucuronide fingerprint ions including an aglycone $[M-H-176]^{1-}$ and an ion at m/z 175 ($[M-2H-aglycone]^{1-}$), MS³ fragment ions at m/z 113 (loss of CO₂ and H₂O from m/z 175), and an anion at m/z 85 (loss of CO from m/z 113) [43] (Supplemental data 4). FIE-MS³ ion fragmentation of the aglycone

m/z 166 showed the same spectra as m/z 246/166, suggesting it was highly probable that this signal was also HHPAA $[M-H]^{-1}$ (Figure 3D, Supplemental data 4). A minor sulphonated signal was also present in the nominal mass bin m/z 342 but the aglycone signal was too weak to be analyzed further. The feature ranked 5th, m/z 340, was demonstrated to be a HBOA glucuronide using FT-ICR-MS accurate mass analysis followed by FIE-MSⁿ fragmentation and comparison with an authentic HBOA standard (Figures 3E and F and Supplemental data 4).

The highly ranked signal m/z 330, belonging to the second cluster (m/z 401, 330, 357, 445, 360) was structurally identified as N-feruloylglycine sulphate $[M-H]^{-1}$ using accurate mass analysis, FIE-MSⁿ fragmentation and comparison with an authentic N-feruloylglycine standard (Figure 4A and B, and Supplemental data 4). The major ion in the other highly ranked correlated nominal mass bin potentially containing signals derived from metabolism of ferulate, m/z 401, could not be structurally identified. However after FIE-MSⁿ fragmentation it was shown to be a glucuronide by the identification of signature ions. A minor sulphonated molecule was also present in the same bin. Using accurate mass analysis together with the MZedDB Molecular Formula Generator [43] the formula $C_{13}H_{21}O_3 [M-H]^{-1}$ was deduced for the glucuronide. Using the same method, the 4th ranked signal was also identified as a glucuronide of structure $C_{14}H_{25}O_4 [M-H]^{-1}$. The exact structures of these glucuronidated signals were not determined.

A derivative of phenylacetylglutamine $[M-H]^{-1}$ was identified as the signal ranked 6th (Table 2, Figures 4C and D, Supplemental data 4). FT-ICR-MS accurate mass analysis followed by FIE-MSⁿ fragmentation and comparison with an authentic phenylacetylglutamine standard indicated a phenylacetylglutamine conjugate. The sample showed MS³ product ions of the MS² m/z 263 ion at 245, 145 and 127, whilst the standard showed the exactly the same MS² product ions m/z 245, 145 and 127 as reported previously [45]. Using the MZedDB

Molecular Formula Generator it was deduced that phenylacetylglutamine was conjugated to a moiety with the formula $C_7H_8N_2O$, however the exact structure of this conjugate could not be determined. The remaining signal in Table 2 was identified as creatinine $[M+Cl]^{1-}$ (Supplemental data 4).

3.4 The signal intensity of hydroxylated phenylacetamides, phenylacetylglutamine and ferulate derivatives FIE-MS fingerprints of urine demonstrates a semi-quantitative relationship with differential exposure to wholegrain sourdough rye bread

The analysis of the top explanatory features in RF models comparing 24 hr urines from the 15 participants in the wholegrain rye sourdough bread group representing wash-out, 3 wholegrain rye portions and 6 wholegrain rye portions demonstrated a semi-quantitative relationship between exposure level and signal intensity. In particular the benzoxazinoid lactam HBOA, the two hydroxylated phenylacetamide derivatives, HPAA and HHPAA, and the unknown $C_{14}H_{25}O_4$ glucuronide showed the clearest trend between wholegrain sourdough rye bread consumption and nominal mass signal intensity (Figure 5). Validation of these potential concentration trends between wash-out and high habitual consumption of wholegrain sourdough rye bread was performed by calculating the area under the ROC (receiver operating characteristic) curve (AUC) using the area under the curve of the sensitivity (true-positive rate) against the specificity (false-positive rate). Validation showed sensitivities of 61-96% and specificities of 60-81% for elevated amounts of all potential biomarkers (Table 3).

3.5 Fermentation-induced changes impact on the benzoxazinoid content of wholegrain sourdough rye bread

In an attempt to understand the origin of the excreted phenylacetamides the relative concentrations of several benzoxazinones and their degradation products in rye flour and wholegrain sourdough rye bread were examined (Figure 6A). These data suggested that DIBOA-diglucosides were abundant prior to fermentation, with higher concentrations evident in the dark rye flour, which contained more bran. The dark rye flour additionally contained smaller amounts of BOA together with trace amounts of HPAA (HBOA derivatives were below detection threshold). After fermentation and baking the main benzoxazinoid present in wholegrain sourdough rye bread was BOA, together with a small amount of DIBOA-gluc and elevated concentrations of HPAA (Figure 6A).

4 Discussion

It is becoming increasingly clear that to understand causal relationships between wholegrain consumption and desirable health outcomes will demand improved knowledge, not only of the origin of the bran component, but also the production processes used to manufacture wholegrain foods. Our present data suggest that the diversity of wholegrain rye products consumed was responsible for the less than adequate discrimination of wash-out samples from treatment samples when all participants were considered in metabolome modeling. Differences in rye varieties and processing of grains have been shown to have an impact on the benzoxazinoid content of individual wholegrain rye food products [22, 23]. For both rye and durum wheat bread, both the baking process and the use of malted grains for part of the flour additionally altered the relative proportion of benzoxazinoids in the final product [23]. Our current data demonstrate that DIBOA glycoside levels fall, whilst BOA and HPAA accumulate during the lengthy fermentation process used to generate sourdough, which involves bacteria as well as yeast and takes place at a low pH of 3.5-4.5 [46]. An *in vitro* simulated colonic fermentation of rye bran extracts was shown recently also to cause an

extensive alteration of phytochemical content [47, 48]. One particular nitrogen-containing metabolite with a m/z of 150 had greatly increased in abundance by the end of the fermentation period and it is possible that this represents the HPAA aglycone signal we describe in the present report. Zikmundova et al. [27] provided evidence using pure fungal cultures that HPAA can be formed from BOA via 2-aminophenol.

Adhikari et al. [2012] recently examined the absorption and metabolism of benzoxazinoids in pigs fed with buns made from a rye bran-rye flour mixture using short-term yeast fermentation [30]. In contrast to the present study, whereas our participants consumed a fully fermented wholegrain sourdough rye bread rich in BOA and HPAA, the buns used in the previous pig study contained high levels of DIBOA-gluc. The high concentration of HBOA-gluc observed in the portal vein of pigs 3 hours after consuming wholemeal rye buns suggested it was derived directly from dietary DIBOA-gluc-hex and DIBOA-gluc prior to absorption in the blood, but the site and mechanism of reduction was unknown [30]. In contrast, the present study using non-targeted metabolite fingerprinting discovered that a HBOA glucuronide was strongly discriminatory of habitual wholegrain sourdough rye bread exposure in humans. This observation is in agreement with a recent report in which a HBOA glucuronide was observed as a major metabolite in the urine of rats fed a rye bread-based diet [49]. A commonly reported pathway for the degradation of HBOA and BOA involves transformation to phenoxazinones via a highly unstable 2-aminophenol intermediate [21, 25, 27, 28]. In agreement with [30] we also could find no evidence that the phenoxazinone class of compounds were linked to a high wholegrain sourdough rye bread diet. **As neither urinary BOA nor any of its possible conjugates were highlighted as metabolites explanatory of wholegrain sourdough rye bread exposure we suggest that dietary BOA is rapidly metabolized after absorption.** This is in agreement with Adhikari et al. [30] who observed a substantial reduction in the concentration of BOA in the hepatic vein compared to the portal vein,

consistent with its metabolism in the entero-hepatic system. A novel discovery in the present study was the observation that 2-hydroxy-N-(2-hydroxyphenyl) acetamide (HHPAA) glucuronide was the signal found to be most discriminatory of wholegrain sourdough rye bread exposure. As HHPAA can be formed through a reduction of an intermediate of HBOA in which the heterocyclic ring is cleaved [27, 28] it is perhaps surprising that the HHPAA signal (m/z 342) intensity shows no correlation with that of the HBOA glucuronide (m/z 340). In contrast, the strong correlation in signal intensity between HHPAA sulphate (m/z 246) and HPAA sulphate (m/z 230) may be suggestive of a direct metabolic link between these two metabolites. Figure 6B illustrates the potential metabolic relationships between the benzoxazinoids and their metabolites described in the present research, although it should be stressed that further pharmacokinetic data are required to confirm these metabolite bioconversions.

Regarding a potential direct role of benzoxazinoids in the health benefits of wholegrain food consumption [22, 23, 30, 48, 49] our preliminary studies (see Supplementary data 5) have demonstrated that DIMBOA significantly enhanced cell proliferation in cultured primary mammary epithelial cells [50]. As wholegrain sourdough rye bread contained only modest amounts of DIBOA conjugates, but was specifically rich in BOA and HPAA, our results support, but do not prove, that its consumption may affect health differently than other rye foods as suggested previously [51]. It would therefore be beneficial in the future to undertake a deeper quantitative analysis of the fate of dietary benzoxazinoids. Such data may provide crucial background to studies attempting to link positive health outcomes to exposure to specific wholegrain foods, not all of which would be predicted to be particularly rich in benzoxazinoids.

Other researchers have previously linked phenylacetylglutamine (PAG) concentrations in urine to both vegetable [e.g. 38, 39] and wholegrain bread [39] intake. In the present study,

we identified a derivative of PAG and a strongly correlated cluster of 5 metabolite signals (of which one was identified as a feruloylglycine conjugate) associated with wholegrain sourdough rye bread consumption. Bondia-Pons et al. [52] observed a significant ($P = 0.00$) post-prandial rise in the concentration of plasma benzoic acid (phenylacetate) following intake of a sourdough-fermented endosperm rye bread rich in phenolics, including ferulate. We therefore suggest that a significant proportion of the PAG-derivative and the 5 compounds correlated with feruloylglycine in the present study may predominantly arise from metabolism of ferulates which are found generally at high amounts in the types of wholegrain foods used in the present study. Most previous studies examining the fate of dietary ferulate in mammals used synthetic foods containing excessive amounts of bran high in ferulate [e.g. 53, 54] or even isolated ferulic acid sugar esters (either synthesized or extracted from cereal bran e.g. 55-58] in short term exposure experiments. Generally these studies concentrated on targeted analysis of ferulate using HPLC-DAD on glucuronidase/sulphatase-treated samples to examine absorption and bioavailability of ferulate and diferulates. However, a complex range of ferulate-conjugates was evident when a non-targeted metabolomics LC-MS profiling method was employed to analyse urines from rats fed for two days on a high lignin diet [54]. We suggest that further detailed examination of the metabolic fate of free ferulic acid and wall-bound ferulate molecules (e.g. ferulic acid sugar esters) originating from specific wholegrain foods **derived by different manufacturing process (e.g. fermentation)** will be timely.

In summary, a wider than previously thought range of potentially bioactive phenolics are bio-available in humans consuming wholegrain rye sourdough bread specifically. Apart from benzoxazinoid derivatives, a range of currently structurally unknown compounds potentially derived from ferulate may also additionally be present in the circulatory system. As both the grain variety, bran content and the food processing methodology appear to greatly

influence specific benzoxazinoid content, great care will need to be taken to distinguish between different sources of wholegrain products in the diet in any future studies.

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References

1. Van Dam, R.M., De Munter, J.S.L., Spiegelman, D., Franz, M., Hu, F.B., Whole grain, bran, and germ intake and risk of type 2 diabetes. *Diabetologia*. 2007, *50*, S157-S157.
2. Seal, C.J., Brownlee, I.A., Whole grains and health: evidence from observational and intervention studies. *Cereal Chem*. 2010, *87*, 167-174.
3. Mellen, P.B., Walsh, T.F., Herrington, D.M., Whole grain intake and cardiovascular disease: A meta-analysis. *Nutr, Metab and Cardiovas*. 2008, *18*, 283-290.
4. Aune, D., Chan, D.S.M., Lau, R., Vieira, R., *et al.*, Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. *Brit Med J*. 2011, *343*, d6617.
5. Department of Health (2012) National Health Service Choices
<http://www.nhs.uk/Livewell/Goodfood/Pages/Healthyeating.aspx> Accessed 8th July 2012.
6. Jones, J.M., Engleson, J., Whole grains: benefits and challenges. *Annu Rev Food Sci Technol*. 2010, *1*, 19-40.
7. Kyrø, C., Skeie, G., Dragsted, L.O., Christensen, J., *et al.*, Intake of whole grain in Scandinavia: Intake, sources and compliance with new national recommendations. *Scand J Public Health*, 2012, *40*, 76-84.
8. United States Department of Agriculture, Centre for Nutrition Policy and Promotion
(2011) <http://www.cnpp.usda.gov/dgas2010-policydocument.htm> Accessed 8th July 2012.
9. Brandt, K., Christensen, L.P., Hansen-Møller, J., Hansen, S.L., *et al.*, Health promoting compounds in vegetables and fruits: A systematic approach for identifying

- plant components with impact on human health. *Trends Food Sci Tech.* 2004, *15*, 384-393.
10. Ross, A.B., Kamal-Eldin, A., Åman, P., Dietary alkylresorcinols: Absorption, bioactivities, and possible use as biomarkers of whole-grain wheat- and rye-rich foods. *Nutr Rev.* 2004, *62*, 81-95.
 11. Aubertin-Leheudre, M., Koskela, A., Samaletdin, A., Adlercreutz, H., Plasma alkylresorcinol metabolites as potential biomarkers of whole-grain wheat and rye cereal fibre intakes in women. *Brit J Nutr.* 2010, *103*, 339-343.
 12. Ross, A.B., Bourgeois, A., Macharia, H.N., Kochhar, S., *et al.*, Plasma alkylresorcinols as a biomarker of whole-grain food consumption in a large population: results from the WHOLEheart Intervention Study. *Am J Clin Nutr.* 2012, *95*, 204-11.
 13. Landberg, R., Townsend, M.K., Neelakantan, N., Sun, Q., *et al.*, Alkylresorcinol metabolite concentrations in spot urine samples correlated with whole grain and cereal fiber intake but showed low to modest reproducibility over one to three years in U.S. women. *J Nutr.* 2012, *142*, 872-877.
 14. Guyman, L.A., Adlercreutz, H., Koskela, A., Li, L., Beresford, S.A.A., Lampe, J.W., Urinary 3-(3,5-dihydroxyphenyl)-1-propanoic acid, an alkylresorcinol metabolite, is a potential biomarker of whole-grain intake in a US population. *J Nutr.* 2008, *138*, 1957-1962.
 15. Sicker, D., Frey, M., Schulz, M., Gierl, A., Role of natural benzoxazinoes in the survival strategy of plants. *Int Rev Cytol.* 2000, *198*, 319-346.
 16. Villagrasa, M., Guillamon, M., Labandeira, A., Taberner, A., Benzoxazinoid allelochemicals in wheat: Distribution among foliage, roots, and seeds. *J Agric Food Chem.* 2006, *54*, 1009-1015.

17. Carlsen, S.C.K., Kudsk, P., Laursen, B., Mathiassen, S.K., *et al.*, Allelochemicals in rye (*Secale cereale* L.): Cultivar and tissue differences in the production of benzoxazinoids and phenolic acids. *Nat Prod Commun.* 2009, 4, 199-208.
18. Prinz, S., Schauburger, D., Bauer, I.M., Knasmueller, S., Kopp, B., Aneugenic 2,4-dihydroxyl-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxyl-1,4-benzoxazin-3-one (DIBOA) in sprouts of *Triticum aestivum* cultivars – A ‘Safety health food’? *Food Chem.* 2010, 121, 973-979.
19. Macias, F.A., Marin, D., Oliveros-Bastidas, A., Molinillo, J.M.G., Rediscovering the bioactivity and ecological role of 1,4-benzoxazinones. *Nat Prod Rep*, 2009, 26, 478-489.
20. Niemeyer, H.M., Hydroxamic acids derived from 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one: Key defense chemicals of cereals. *J Agri Food Chem.* 2009, 57, 1677-1696.
21. Fomsgaard, I.S., Mortensen, A.G., Carlsen, S.C.K., Microbial transformation products of benzoxazolinone and benzoxazinone allelochemicals - a review. *Chemosphere.* 2004, 54, 1025-1038.
22. Hanhineva, K., Rogachev, I., Aura, A-M., Aharoni, A., *et al.*, Qualitative characterization of benzoxazinoid derivatives in whole grain rye and wheat by LC-MS metabolite profiling. *J Agri Food Chem.* 2011, 59, 921-927.
23. Pedersen, H.A., Laursen, B., Mortensen, A., Fomsgaard, I.S., Bread from common cereal cultivars contains an important array of neglected bioactive benzoxazinoids. *Food Chem.* 2011, 127, 1814-1820.
24. Cambier, V., Hance, T., de Hoffmann, E. Non-injured maize contains several 1,4-benzoxazin-3-one related compounds but only as glucosylconjugates. *Phytochem Analysis.* 1999, 10, 119-126.

25. Krogh, S.S., Mensz, S.J.M., Nielsen, S.T., Mortensen, A.G., *et al.*, Fate of benzoxazinone allelochemicals in soil after incorporation of wheat and rye sprouts. *J Agri Food Chem.* 2006, *54*, 1064-1074.
26. Villagrasa, M., Guillamon, M., Navarro, A., Eljarrat, E., Barcelo, D., Development of a pressurized liquid extraction-solid-phase extraction followed by liquid chromatography-electrospray ionization tandem mass spectrometry method for the quantitative determination of benzoxazolinones and their degradation products in agricultural soil. *J Chromatogr A.* 2008, *1179*, 190-197.
27. Zikmundova, M., Drandarov, K., Bigler, L., Hesse, A., Werner, C., Biotransformation of 2-benzoxazolinone and 2-hydroxy-1,4-benzoxazin-3-one by endophytic fungi isolated from *Aphelandra tetragona*. *Appl Environ Microb.* 2002, *68*, 4863-4870.
28. Zikmundova, M., Drandarov, K., Hesse, M., Werner, C., Hydroxylated 2-amino-3H-phenoxazin-3-one derivatives as products of 2-hydroxy-1,4-benzoxazin-3-one (HBOA) biotransformation by *Chaetosphaeria* sp., an endophytic fungus from *Aphelandra tetragona*. *Z Naturforsch C.* 2002, *57*, 660-665.
29. Poupaert, J., Carato, P., Colacillo, E., 2(3H)-benzoxazolone and bioisosters as "Privileged Scaffold" in the design of pharmacological probes. *Curr Med Chem.* 2005, *12*, 877-885.
30. Adhikari, K.B., Laursen, B.B., Lærke, H.N., Fomsgaard, I.S., Bioactive benzoxazinoids in rye bread are absorbed and metabolized in pigs. *J Agri Food Chem.* 2012; *60*, 2497-2506.
31. Brownlee, I.A., Moore, C., Chatfield, M., Richardson DP., *et al.*, Markers of cardiovascular risk are not changed by increased whole grain-intake. The WHOLEheart study: - a randomised, controlled dietary intervention. *Brit J Nutr.* 2011, *104*, 125-134.

32. Bingham, S.A., Gill, C., Welch, A., Cassidy, A., *et al.* Validation of dietary assessment methods in the UK arm of EPIC using weighed records, and 24-hour urinary nitrogen and potassium and serum vitamin C and carotenoids as biomarkers. *Int J Epidemiol.* 1997, 26, S137-S151.
33. Favé, G., Beckmann, M., Lloyd, A.J., Zhou, S., Development and validation of a standardized protocol to monitor human dietary exposure by metabolite fingerprinting of urine samples. *Metabolomics.* 2011, 7, 469-484.
34. Lloyd, A.J., Beckmann, M., Favé, G., Mathers, J.C., Draper, J., Proline betaine and its biotransformation products in fasting urine samples are potential biomarkers of habitual citrus fruit consumption. *Brit J Nutr.* 2011, 106, 812-824.
35. Lloyd, A.J., Favé, G., Beckmann, M., Lin, W., Draper, J., Use of mass spectrometry fingerprinting to identify urinary metabolites after consumption of specific foods. *Am J Clin Nutr.* 2011, 94, 981-991.
36. Lloyd, A. J., Beckmann, M., Halder, S., Seal, C., *et al.*, Data driven strategy for the discovery of potential urinary biomarkers of habitual dietary exposure. *Am J Clin Nutr.* 2012. doi: 10.3945/ajcn.112.048033.
37. Assfalg, M., Bertini, I., Colangiuli, D., Luchinat, C., *et al.*, Evidence of different metabolic phenotypes in humans. *P Natl Acad Sci USA.* 2008, 105, 1420-1424.
38. Holmes, E., Loo, R.L., Stampler, J., Bictash, M., *et al.*, Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature.* 2008, 453, 396-401.
39. O'Sullivan, A., Gibney, M.J., Brennan, L., Dietary intake patterns are reflected in metabolomic profiles: potential role in dietary assessment studies. *Am J Clin Nutr.* 2011, 93, 314-321.

40. Beckmann, M., Enot, D.P., Overy, D.P., Scott, I.M., *et al.*, Metabolite fingerprinting of urine suggests breed-specific dietary metabolism differences in domestic dogs *Brit J Nutr.* 2010, *103*,1127-1138.
41. Beckmann, M., Parker, D., Enot, D.P., Duval, E., Draper, J., High-throughput, nontargeted metabolite fingerprinting using nominal mass flow injection electrospray mass spectrometry. *Nat Protoc.* 2008, *3*, 486-504.
42. Enot, D.P., Lin, W., Beckmann, M., Parker, D., *et al.*, Preprocessing, classification modeling and feature selection using flow injection electrospray mass spectrometry metabolite fingerprint data. *Nat Protoc.*2008, *3*, 446-470.
43. Draper, J., Enot, D.P., Parker, D., Beckmann, M., *et al.*, Metabolite signal identification in accurate mass metabolomics data with MZedDB, an interactive m/z annotation tool utilising predicted ionisation behaviour 'rules'. *BMC Bioinformatics.* 2009, *10*, 24.
44. Levsen, K., Schiebel, H.M., Behnke, B., Dotzer ,R., *et al.*, Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview. *J Chromatogr A.* 2005, *1067*,55-72.
45. Laryea, M.D., Herebian, D., Meissner, T., Mayatepek, E., Simultaneous LC-MS/MS determination of phenylbutyrate, phenylacetate benzoate and their corresponding metabolites phenylacetylglutamine and hippurate in blood and urine. *J Inherit Metab Dis.* 2010, DOI 10.1007/s10545-010-9172-9.
46. Vogelmann, S.A., Hertel, C., Impact of ecological factors on the stability of microbial associations in sourdough fermentation. *Food Microbiol.* 2011, *28*, 583-589.
47. Katina, K, Liukkonen K-H, Kaukovirta-Norja H, Adlercreutz H., *et al.*, Fermentation-induced changes in the nutritional value of native or germinated rye. *J Cereal Sci.* 2007, *46*, 348-355.

48. Hanhineva, K., Aura, A.-M., Rogachev, I., Matero S., *et al.*, In vitro microbiotic fermentation causes an extensive metabolite turnover of rye bran phytochemicals. *PLoS One*. 2012, 7, e39322.
49. Adhikari, K.B., Lærke, H.N., Mortensen, A.G., Fomsgaard, I.S., Plasma and urine concentrations of bioactive dietary benzoxazinoids and their glucuronidated conjugates in rats fed a rye bread-based diet. *J Agri Food Chem*. 2012, 60, 11518-11524.
50. Brandt, K., Christensen, L.P., Hansen, S.L., Young, J.F., *et al.*, Functions of bioactive plant compounds and effect on humans. *Innov Food Tech*. 2001, 10, 60-62.
51. Katina, K., Arendt, E., Liukkonen, K.-H., Autio, K., Flander, L., Poutanen, K., Potential of sourdough for healthier cereal products. *Trends in Food Sci & Tech*. 2005, 16, 104–112.
52. Bondia-Pons, I., Nordlund, E., Mattila, I., Katina, K., *et al.*, Postprandial differences in the plasma metabolome of healthy Finnish subjects after intake of a sourdough fermented endosperm rye bread versus white wheat bread. *Nutr J*. 2011, 10, 116.
53. Rondini, L., Peyrat-Maillard, M.N., Marsset-Baglieri, A., Fromentin, G., *et al.*, Bound ferulic acid from bran is more bioavailable than the free compound in rat. *J Agri Food Chem*. 2004, 52, 4338-4343.
54. Fardet, A., Rock, E., Remesy, C., Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? *J Cereal Sci*. 2008, 48, 258-276.
55. Kern, S.M., Bennett, R.N., Mellon, F.A., Kroon, P.A., Garcia-Conesa, M.T., Absorption of hydroxycinnamates in humans after high-bran cereal consumption. *J Agri Food Chem*. 2003, 51, 6050-6055.
56. Zhao, Z., Egashira, Y., Sanada, H., Digestion and absorption of ferulic acid sugar esters in rat gastrointestinal tract. *J Agri Food Chem*. 2003, 51, 5534-5539.

57. Zhao, Z., Egashira, Y., Sanada, H., Ferulic acid sugar esters are recovered in rat plasma and urine mainly as the sulfoglucuronide of ferulic acid. *J Nutr.* 2003, *133*, 1355-1361.
58. Zhao, Z., Egashira, Y., Sanada, H., Ferulic acid is quickly absorbed from rat stomach as the free form and then conjugated mainly in liver. *J Nutr.* 2004; *134*:3083-3088.

Tables

Table 1 Discrimination of wholegrain rye foods exposure by FIE-MS fingerprint analysis of 24 hr urine

Dietary exposure intervention phase comparisons in multivariate model	Positive ionization mode		Negative ionization mode	
	AUC	RF margin	AUC	RF margin
Wash-out ^a vs Dose 1 ^b Total rye foods ^c	0.31	-0.06	0.46	-0.02
Wash-out vs Dose 2 ^d Total rye foods	0.58	0.02	0.76	0.11
Dose 1 vs Dose 2 Total rye foods	0.32	-0.06	0.41	-0.04
Wash-out vs Dose 1 Rye bread	0.41	-0.05	0.51	-0.02
Wash-out vs Dose 2 Rye bread	0.61	0.03	0.90	0.19
Dose 1 vs Dose 2 Rye bread	0.36	-0.06	0.43	-0.03
Wash-out vs Dose 1 Rye flakes ^e	0.24	-0.09	0.36	-0.06
Wash-out vs Dose 2 Rye flakes	0.51	-0.01	0.64	0.04
Dose 1 vs Dose 2 Rye flakes	0.37	-0.05	0.43	-0.05

Area under the (ROC; receiver operating characteristic) curve (AUC) and Random Forest (RF) margins following classification of Flow Infusion Electrospray-Ionization Mass Spectrometry (FIE-MS) in positive and negative ionization mode data representing 24 hr urine samples collected after differential exposure to rye food products.

a, Wash-out, 4-week wash-out period where volunteers avoided all wholegrain foods

b, Dose 1, volunteers consumed 3 servings of wholegrain rye foods per day for 4 weeks (~ 48g wholegrain rye per day).

c, Total rye foods, sum of rye bread, rye pasta and rye flakes consumption.

d, Dose 2, volunteers consumed 6 servings of wholegrain rye foods per day for 4 weeks (~ 96 g wholegrain rye per day).

e, Rye flakes, porridge or muesli.

Table 2 Structural identification of metabolite signals explanatory of wholegrain sourdough rye bread consumption

RF rank ¹	Nom. mass	Measured mass	Putative ID	Theoretical mass	Δ PPM	Corr. m/z ⁷	Mass of aglycone
1	342	342.08288	2-hydroxy-N-(2-hydroxyphenyl) acetamide [HHPAA] glucuronide ² [M-H] ¹⁻	342.08305	0.5	-	166.05097 ⁴
2	230	230.01274	N-(2-hydroxyphenyl) acetamide [HPAA] sulfate ^{3, 6} [M-H] ¹⁻	230.01287	0.6	230, 246	150.05606 ⁵
3	433	433.20776	C ₁₄ H ₂₅ O ₄ glucuronide ² [M-H] ¹⁻	433.20792	0.4	-	257.17567 ⁴
4	401	401.18154	C ₁₃ H ₂₁ O ₃ glucuronide ² [M-H] ¹⁻	401.18170	0.4	401, 330, 357, 445, 360	225.14962 ⁴
5	340	340.06724	2-hydroxy-1,4-benzoxazin-3-one (HBOA) glucuronide ^{2,6} [M-H] ¹⁻	340.06740	0.5	-	164.03532 ⁴
6	399	399.03681	Phenylacetylglutamine derivative ⁶ [M-H] ¹⁻	399.16739	0.6	-	263.10374 ⁴
7	148	148.02842	Creatinine ⁶ [M+Cl] ¹⁻	148.02831	0.7	-	-
8	330	330.02923	N-feruloylglycine sulfate ^{3,6} [M-H] ¹⁻	330.02891	1.0	401, 330, 357, 445, 360	250.07203 ⁵
20	246	246.00783	2-hydroxy-N-(2-hydroxyphenyl) acetamide (HHPAA) sulfate ³ [M-H] ¹⁻	246.00778	0.2	230, 246	166.05100 ⁵

¹ Rank in a Random Forest classification of wash-out versus participants who consumed up to 6 rye wholegrain products per day and preferentially selected sourdough rye bread.

² Identified as a glucuronide with the aglycone [M-H-176]¹⁻ and an ion at m/z 175 ([M-2H-aglycone]¹⁻). MS³ fragment ions at m/z 113 (loss of CO₂ and H₂O from m/z 175), and anion at m/z 85 (loss of CO from m/z 113).

³ Identified as a sulphonated molecule due to the presence of a ³⁴S isotope plus the fragment ion $[M-H SO_3]^{1-}$ (loss of SO_3 , m/z 80).

⁴Calculated mass.

⁵ Measured using Fourier Transform-Ion Cyclotron Resonance Ultra -Mass- Spectroscopy.

⁶ Identified by comparison to a standard by FIE-MSⁿ.

⁷ Pearson correlation method, correlation coefficient > 0.7

Table 3 Validation of signals potentially explanatory of exposure to wholegrain sourdough rye bread.

RFrank	Nominal mass (m/z)	Sensitivity	Specificity	AUC	P-value
1	342	0.96	0.70	0.94	0.0000
2	230	0.84	0.81	0.94	0.0026
3	433	0.61	0.72	0.92	0.0007
4	401	0.73	0.60	0.89	0.0095
5	340	0.75	0.67	0.81	0.2391
6	399	0.71	0.73	0.86	0.0057
8	330	0.82	0.64	0.88	0.0066

AUC (area under the ROC (receiver operating characteristic) curve), the sensitivity (true-positive rate) and the specificity (false-positive rate) results for the top explanatory features in RF models potentially explanatory of wholegrain sourdough rye bread exposure. Where $n=15$; randomized re-sampling with 100 bootstraps was used.

Legends for Figures

Figure 1 Discrimination of wholegrain sourdough rye bread consumption by metabolite fingerprinting of 24 hr urine. Principal Component-Linear Discriminant Analysis of Flow Infusion Electrospray-ionization Mass Spectrometry (FIE-MS) fingerprints (100-510 m/z) of (A) positive ionization mode; (B) Negative ionization mode. Eigenvalues (Tw values) are given in brackets. Where $\circ$ wash-out; Δ 3 rye wholegrain products per day (Dose 1); + 6 rye wholegrain products per day (Dose 2).

Figure 2 Analysis of signals discriminating wash-out from urines collected from participants whom preferentially chose high wholegrain sourdough rye bread consumption. (A) Random Forest Importance Scores plotted against rank for the top 50 features selected in a pair-wise comparison between wash-out 24 hr urine samples and 24 hr urines from participants preferentially selecting wholegrain sourdough rye bread in the dietary intervention phase with consumption of 6 portions of wholegrain rye food product per day (Dose 2); (B) Hierarchical cluster analysis based on the correlation coefficient (Pearson correlation method) of the same samples.

Figure 3 Confirmation of benzoxazinoid-related signals explanatory of the consumption of wholegrain sourdough rye bread following analysis by Flow Infusion Electrospray-ionisation tandem Mass Spectrometry (FIE-MSⁿ) of pooled 24 hr urine samples from participants preferentially selecting wholegrain sourdough rye bread in the dietary intervention phase with consumption of 6 portions of wholegrain rye food product per day (Dose 2). (A) FIE-MS³ m/z 230/150; (B) FIE-MS² of a N-(2-hydroxyphenyl) acetamide [M-H]¹-standard; (C) FIE-MS³ of

m/z 246/166; (D) FIE-MS³ of m/z 342/166; (E) FIE-MS³ m/z 340/164; (F) FIE-MS² of a 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one [M-H]¹⁻ standard.

Figure 4 Confirmation of feruloylglycine and phenylacetylglutamine-related signals explanatory of the consumption of wholegrain sourdough rye bread following analysis of 24 hr pooled urine samples by Flow Infusion Electrospray-ionization tandem Mass Spectrometry (FIE-MSⁿ) from participants preferentially selecting wholegrain sourdough rye bread in the dietary intervention phase with consumption of 6 portions of wholegrain rye food product per day (Dose 2). (A) FIE-MS³ of m/z 330/250; (B) FIE-MS² feruloylglycine [M-H]¹⁻ standard; (C) FIE-MS³ of m/z 399/263; (D) FIE-MS² phenylacetylglutamine [M-H]¹⁻ standard.

Figure 5 Box plots of the top negative ionisation mode explanatory metabolite signals in **nominal mass fingerprints** between wash-out urine samples and urine from participants preferentially selecting wholegrain sourdough rye bread in the dietary intervention phase with consumption of 6 portions of wholegrain rye food product per day (Dose 2). (A) m/z 340, HBOA glucuronide; (B) m/z 230, HPAA sulphate; (C) m/z 342, HHPAA glucuronide; (D) m/z 246, HHPAA sulphate; (E) m/z 433, C₁₄H₂₅O₄ glucuronide. Where: the box indicates the interquartile range; the red horizontal bar, the median; vertical bars, the maximum and minimum values up to 1.5 x interquartile range; error bars represent the standard error of 15 participants. Log10/TIC, normalization using sample total ion count (TIC) and logarithmic transformation of data.

Figure 6 The relative amounts of several benzoxazinones and their degradation products in rye flour and wholegrain sourdough rye bread and the potential metabolic relationships between selected benzoxazinoids in 24 hr urine after consumption of wholegrain sourdough

rye bread. (A) Fourier Transform-Ion Cyclotron Resonance Ultra Mass-Spectrometry (FT-ICR-MS) signal intensity plots of DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) diglucoside, (m/z 504.13506); DIBOA glucoside, (m/z 342.08251); BOA, 2-benzoxazolinone (m/z 134.02475) and HPAA, N-(2-hydroxyphenyl) acetamide (m/z 150.05606) in wholegrain sourdough rye bread (Bread), Dark rye (DR) flour and light rye (LR) flour. A mean signal intensity of three extraction replicates is displayed together with the standard error. (B) Suggested fate of selected benoxazinoids during wholegrain sourdough rye bread production and following consumption by humans and eventual excretion of specific metabolites in urine. DIBOA; HPAA; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; 2-AP, 2-aminophenol; APO, 2-Amino-3H phenoxazin-3-one; HHPAA, 2-hydroxy-N-(2-hydroxyphenyl)acetamide; HMPA, N-(2-hydroxyphenyl) malonamic acid. Pathway (solid arrows) adjusted from known metabolic inter-conversions described previously by Zikmundová et al. (2002) [27], Adhikari et al. (2012) [30, 49] and Pedersen et al. (2011) [23]. Block arrow indicates potential absorption of metabolite directly from diet. Dotted arrows represent a suggested chemical transition whilst ‘?’ denotes uncertainty if the metabolic step occurs during the bread making process or in humans.