GrainMark

Newcastle University

Biomarkers of whole-grain intake; contribution of alkylresorcinols and mammalian lignans to the metabolome (the GrainMark study).

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EXECUTIVE SUMMARY

The strong observational evidence showing that wholegrain foods are beneficial for health has been used to support the introduction of specific dietary recommendation for whole grain-intake in the US and Denmark. There is no such recommendation in the UK, although the Food Standards Agency advocates that whole grains should be part of a healthy diet. Accurate assessment of intake of wholegrain foods has been difficult because of poor definitions of wholegrain foods, limited information on portion/serving sizes and lack of detail on the whole-grain content of individual foods. In particular, the intake level at which health benefit can be achieved is not proven, presenting a major obstacle to the generation of sound public health targets. Thus there is a need to identify a specific biomarker for wholegrain foods which can be used to accurately assess intake for better correlation with health outcomes and to give strength to observational and intervention studies.

Three potential candidates for biomarkers of whole grain-intake are proposed.

- Firstly, alkylresorcinols (AR). These are 1,3-dihydroxy-5-alkylbenzenes with an odd numbered alkyl chain, usually in the range C17:0 to C25:0. AR are found exclusively in the bran fractions of some but not all cereals and are stable during cereal processing, including baking. Not only are these compounds unique to some cereal grains (wheat, rye, triticale and barley), but the AR content varies between grain types and varieties and different grains contain different patterns of alkyl chain homologues. This suggests that the AR content of body fluids could not only provide a measure of total whole grain-consumption, but could give additional information on the type of dietary grain consumed. .
- Secondly, wholegrain cereals are also a source of plant lignans which can be metabolised in the gut forming the mammalian lignans enterodiol (END) and enterolactone (ENL). It has been suggested that ENL, the dominant product of lignan metabolism, can be used as a biomarker of whole grain-consumption. However, lignans are also found in oilseeds such as flax, soy and rapeseed, legumes, various vegetables and fruit, especially berries, contributing to the ENL pool. Thus, while they may have some potential as biomarkers of whole grain-intake, it may be problematic to rely on them exclusively, since there may be interference from other dietary constituents.
- Thirdly, recent advances in analytical technologies and bioinformatics have opened the opportunity for exploring the metabolome – the dynamic set of (all the) small molecules present in biological samples. Changing steady state concentrations and fluctuations of metabolites that occur as a result of dietary modification can be identified and can be used to

complement profiling of single (or small numbers of) molecules such as AR or ENL. Using the metabolomics approach is distinct from metabolite targeted analysis, which is restricted to a target compound or biomarker. Metabolomics opens up the opportunity to identify any novel characteristics of the biological fluid that may be due to the intervention.

The aims of the GrainMark Study were to assess and compare these three different 'biomarkers' in response to changes in whole grain-intake in a UK population. The specific objectives of the study were:

1. To quantify the impact of increased intake of wholegrain wheat or rye on plasma concentrations of alkylresorcinols.
2. To quantify the impact of increased intake of wholegrain wheat or rye on plasma and urinary concentrations of mammalian lignans.
3. To describe the impact of increased intake of wholegrain wheat or rye on the pattern of metabolites (the metabolome) in plasma and urine.

Methods. 68 healthy, non-smoking, normal weight volunteers (48% male) average age 54.5 years were recruited. After a wash-out period of 4 weeks during which volunteers were asked to avoid all wholegrain foods (using a list provided), they were randomly allocated into two groups; a wheat group and a rye group. The volunteers were asked to consume 3 servings of whole grain per day (equivalent to 48g whole grain per day) for 4 weeks (Dose 1) from a selection of foods provided. After 4 weeks they were asked to double their intake of the same foods (Dose 2) for a further 4 weeks. Volunteers were asked to avoid all wholegrain foods except those provided for the duration of the study. At the beginning and end of intervention Doses 1 and 2 volunteers visited the clinical facility for blood and urine sampling and anthropometric measurements. Anthropometric measurements only were taken at the start of the wash-out period. AR were analysed by gas chromatography mass spectroscopy (GC-MS); mammalian lignans were analysed by high performance liquid chromatography with Coularray analysis. Metabolite fingerprints and profiles were determined by a combination of flow injection mass spectroscopy (FIE-MS) and gas chromatography time-of-flight mass spectrometry (GC-TOFMS), respectively.

Intervention foods were provided in pre-weighed individual packages. These included two wholegrain wheat breakfast cereals, wholegrain wheat pasta and wholewheat bread for the wheat group and two wholegrain rye breakfast cereals, wholegrain rye pasta and rye bread for the rye group. Participants were instructed on how to achieve their target level of intake and were provided

with calendars (to record intervention wholegrain foods consumption) to aid compliance. Food frequency questionnaires (FFQ) were completed in the middle of wash-out and each intervention periods to assess total food and wholegrain food intake.

Results. Self-reported intervention food records and FFQ indicated good compliance with dietary goals throughout the study. Volunteers lost weight during the wash-out period and gained some of this weight back during the intervention, although there were no significant differences in total energy consumption. Dietary fibre intake fell significantly during the wash-out period but rose during Dose 1 and again during Dose 2 periods. Intake of some minerals fell during the wash-out period (Mn and Mg for wheat group; Mn, Mg, Fe and Zn for the rye group) but these were restored during Dose 1 and Dose 2 periods. Plasma concentrations of individual and total AR were strongly (R^2>0.4) and significantly (P<0.0001) correlated with estimates of whole grain-intake from both intervention food records and FFQ. The ratio of concentrations of C17:C21 in plasma were significantly different for the wheat (0.06) and rye (0.36) groups (p<0.001). Plasma END, ENL and END+ENL concentrations showed far weaker associations with whole grain-intake than AR concentrations and there was no significant difference in the concentrations between each phase of the intervention. Plasma ENL concentration was weakly (R^2=0.02) but significantly (p=0.03) correlated with wholegrain intake for rye, but not for wheat. Concentrations of lignans were much higher (50-fold) in urine than in plasma, and there was a weak (R^2=0.05) but highly significant linear relationship between ENL concentration (p=0.0006) and ENL daily excretion (p=0.004) and wholegrain rye consumption. The relationships for wheat were similar but less strong (R^2<0.04) than for rye (p=0.0026 for ENL concentration and p=0.0077 for ENL daily excretion). Total mammalian lignan concentrations in urine and daily excretion showed similar responses to ENL. Non-targeted, high throughput metabolomics analysis using metabolite fingerprinting (FIE-MS) and profiling (GC-TOFMS) found that metabolite changes following increased whole grain-intake were only detectable using GC-TOFMS. The number of metabolites extracted, which strongly discriminate the three intervention phases suggest that urine is the more informative biofluid for high-throughput metabolomics analysis. Although number and discrimination strength was generally higher in 24 h urine samples, spot fasting urine samples revealed equally high relative concentrations of the main discriminatory metabolites indicative of increased whole grain-intake. Two of the metabolites, 3,5-dihydroxy-benzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA), have recently been suggested as potential biomarkers for whole grain-intake in the literature. The accessibility of these compounds in non-targeted GC-TOFMS high throughput screens of spot urine samples is of potential advantage to current targeted approaches as it allows the detection of other food-related metabolites other than just the target compounds at the

same time. The usefulness of these potential biomarkers has been demonstrated also after volunteer acute exposure to wholegrain wheat intake in the related MEDE project. Other discriminatory metabolites exhibiting a dose response to whole grain intake are currently of unknown structure, but mass spectra suggest that most of these compounds are bio-transformation products of bran components. At least one of these 'unknowns' is of particular interest as it is characteristic for whole-grain rye intake.

Conclusions. The results of the GrainMark study clearly demonstrate the usefulness of plasma alkylresorcinols concentrations as potential biomarker(s) of wholegrain wheat and rye consumption. The relative concentrations of different alkylresorcinol homologues (C:17 and C21) further gives a method for discriminating intake of the two whole grain sources. Since the whole grain intervention was very controlled with each group consuming only one source of whole grain, this discriminatory power needs to be further evaluated in samples from subjects consuming mixed grains (such as WHOLEheart N02036), or other dietary interventions where intake of whole grain can be quantified accurately. In contrast, mammalian lignans were much less strongly correlated with whole grain-intake. Plasma END and ENL concentrations were weakly correlated only with wholegrain rye intake suggesting that measurement of these compounds in plasma would not be useful as a biomarker of whole grain-intake in a UK population where rye consumption is not common. Urinary output of END and ENL was correlated with whole grain-intake but the relationship was much less strong that that seen for plasma alkyresorcinols. This suggests that urinary output of END and ENL may provide alternative, but less effective, biomarkers for whole grain-intake if plasma were not available. High throughput metabolomic analysis of plasma and urine samples showed that GC-TOFMS profiling was a useful tool for discriminating groups of participants according to whole grain-intake. In particular, specific metabolites of alkylresorcinols were identified as strong discriminators. Further analysis of some other discriminatory molecules may identify other metabolites of bran components which will strengthen the discriminatory power for identifying whole grain-intake.

1.0 Introduction

There is strong observational 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 (Jacobs *et al.*, 1998; Slavin, 2000; Slavin *et al.*, 2001; Marquart *et al.*, 2003; Murtaugh *et al.*, 2003; Seal, 2006). This observational evidence has been endorsed in the US, with a specific dietary recommendation for a minimum intake of three 16 g servings of whole grain per day being central in the current Dietary Guidelines for Americans 2005 [for example, 1 whole grain serving is equivalent to 1 slice wholemeal bread or 1.5 bowls of breakfast cereal] (US Department of Health and Social Services, 2005). Denmark also has a specific recommendation for 75 g whole grain/ 10 MJ energy per day (DTU, 2008). In the UK, it is recognised that whole grains should be part of a healthy diet (for example in the Food Standards Agency's Eat Well Guide), although not specifying any particular quantity.

Accurate assessment of intake of wholegrain foods has been difficult, with major limitations in the dietary assessment methods used in the larger epidemiological studies. These include poor definition of wholegrain foods, limited information on portion/serving sizes and lack of detail on the whole grain-content of individual foods. As a result it is difficult to make definitive conclusions about the protective role of wholegrain foods. In particular, the intake level at which health benefits can be achieved is not proven, with most estimates ranging from as little as one serving per day to more than three; although the Danish recommendation would suggest that an intake of 4.5 x 16 g servings/day are required. The lack of an appropriate ‘dose’ of wholegrain cereals is a major obstacle for the generation of sound public health targets. Thus, there is a need to identify specific biomarkers for the intake of wholegrain foods which can be used to help accurately assess intake to improve correlations with health outcomes and to give strength to observational and intervention studies.

Alkylresorcinols (AR) are a family of compounds which are found in the bran fractions of some cereals; wheat and rye, triticale and barley but not in oats, rice, maize, sorghum or millet (Ross *et al.*, 2003b). AR can be extracted from bread and cereals and have been shown to be stable during manufacture of cereal−based foods (Liukkonen *et al.*, 2003; Ross *et al.*, 2003b). The AR content varies between grain types and varieties and different grains contain different homologues (different chain lengths) of AR (Ross *et al.*, 2003b), suggesting that the AR content of body fluids could not only provide a measure of total whole grain-consumption, but could give additional information on

the type of whole grain consumed (Linko-Parvinen *et al.*, 2007). These different AR are generally chain length homologues, with odd-numbered saturated alkyl chains between C17:0 and C25:0. Rye also contains a relatively high proportion of unsaturated homologues, though these are generally not measured as biomarkers in plasma. Sensitive methodologies such as gas−chromatography linked to mass−spectrometry (GC−MS and GC-MS/MS) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) or electrochemical detection (HPLC-CAD) have been used to identify AR and AR metabolites in plasma, red cells and urine clearly demonstrating that these compounds are absorbed and metabolised in the human body (Linko *et al.*, 2002; Ross *et al.*, 2003a; Linko & Adlercreutz, 2005; Koskela *et al.*, 2007; Linko-Parvinen *et al.*, 2007; Landberg *et al.*, 2009c). Estimates of AR intake suggest potentially large differences between population groups which could be reflected in measurable differences in AR concentrations in blood and urine (Ross *et al.*, 2005).

Wholegrain cereals are also food sources of plant lignans, which can be metabolised by the intestinal microflora to produce the mammalian lignans enterodiol (END) and enterolactone (ENL). It has been suggested that ENL, the dominant product of lignan metabolism, can be used as a biomarker of whole grain-consumption. There have been a number of studies showing positive associations between whole grain-intake and plasma or urinary ENL concentration (Johnsen *et al.*, 2004; Adlercreutz, 2007; Linko-Parvinen *et al.*, 2007; Kuijsten *et al.*, 2009). However, cereal grains are not the sole dietary source of lignans; other sources include oilseeds such as flax, soy and rapeseed, legumes, various vegetables and fruit, especially berries (Milder *et al.*, 2005) as well as plant based beverages such as coffee and red wine. Thus, while they may have some potential as biomarkers of whole grain-intake, it is problematic to rely on them exclusively, since there may be interference from other food sources and this is yet to be tested in a UK situation.

Recent advances in analytical technologies and bioinformatics have opened the opportunity for exploring the 'metabolome' – the dynamic set of all small molecules (‘metabolites’, less than 1000 Daltons) present in biological samples. Using this approach, changes in steady state concentrations and fluctuations of metabolites that occur as a result of dietary modification can be identified and can be used to complement profiling of single (or small numbers of) molecules such as AR or ENL. A number of analytical methods can be used in combination to detect hundreds of individual chemical structures which can be used to derive an overall 'metabolic fingerprint' or ‘profile’ for the sample. This metabolomics approach is distinct from ‘metabolite targeted analysis’, which is restricted only to groups of target compounds (such as blood lipids, phenolics, lignans etc), and thus

opens up the opportunity to identify any novel characteristics of the biological sample that may be due to changes in the whole diet (such as from a diet based on refined cereals to a diet based on wholegrain cereals).

1.1 Objectives

The objectives of the study, as defined in the scope of work were as follows:

Objective 01 – To undertake a controlled dietary intervention based on a two group parallel design with increasing intake of wholegrain wheat or rye and to determine plasma and urinary concentrations of alkylresorcinols and mammalian lignans as biomarkers of whole grain-intake.

Objective 02 – To recruit a total of 62 normal, healthy subjects into the controlled dietary intervention at Newcastle University.

Objective 03 – To conduct a dietary intervention study aimed at increasing consumption of wholegrain foods based on wheat or rye in a dose-dependent manner over an eight week period. 62 subjects will be recruited and allocated randomly to one of two groups depending on cereal source of wholegrain foods.

Objective 04 – To evaluate alkylresorcinols as biomarkers of wholegrain cereal food consumption compared to mammalian lignans (enterolactone and enterodiol). Samples determined were as follows:

- Plasma alkylresorcinols
- Plasma and urinary mammalian lignans

Objective 05 – To evaluate the contribution of alkylresorcinols and mammalian lignans to the plasma and urinary metabolome during a dose-dependent increase in whole-grain consumption and to evaluate metabolomic analysis as a screening tool to identify alternative biomarkers to alkylresorcinols and mammalian lignans.

2.0 Methods

2.1 Project Staffing and Steering Group

The project commenced with the establishment of a steering group and the appointment of staff to manage the day to day running of the study. This was undertaken in May/June 2007 and Dr. Sumanto Haldar was appointed full-time to the project as a Research Associate and Mrs. Wendy Bal appointed (part-time) as the Research Technician. The steering group for the project consisted of Prof. Chris Seal (PI), Dr. Kirsten Brandt (Co-investigator), Dr. Sumanto Haldar, Mrs Wendy Bal – all from Newcastle University, Prof. John Draper (Co-investigator), Dr. Manfred Beckman, Dr. Shaobo (Peter) Zhou from Aberystwyth University and Dr. Alastair Ross from Nestlé Research Centre, Lausanne, Switzerland. The statisticians who assisted in the project were Dr. Lee Fawcett and Dr. Peter Avery (Newcastle University).

2.2 Protocol Establishment/Refinement

The standard operating procedures (SOPs) for the project were established between July 2007 and December 2007. These included the finalisation and refinement of the intervention study design, formulating a time-line for the dietary intervention and establishment of a calendar for volunteer visits and associated tasks. The logo was designed (as above) and the acronym "GrainMark" for the study was formulated. Sourcing, finalisation and packaging of intervention foods for volunteers were organised at this stage (See 2.2.1 below) along with the establishment of pre-sampling 'evening set-meals' (See 2.2.2). A list of common foods containing whole grains was also drawn up to give to each volunteer to help them avoid wholegrain foods except those given as 'study foods' during the dietary intervention period (Appendix 1).

2.2.1 Intervention Foods

The wholegrain (WG) foods used in this study were sourced from various suppliers and, unless stated otherwise, these were provided 'in kind'. For each intervention group, the foods were a choice of two breakfast cereals, bread and pasta. For the **WG wheat intervention group** the foods supplied to the volunteers comprised of WG wheat bread, Shredded Wheat Fruitful, Weetabix and WG wheat pasta. For the **WG rye intervention group** the choice of foods included rye bread, rye porridge, rye muesli and WG rye pasta. The rye pasta contained 20% WG rye and 80% refined grain wheat and was selected as the nearest commercial equivalent to WG wheat pasta. No 100% WG rye pasta could be sourced. The WG wheat bread was provided by Allied Bakeries, Gateshead, UK and the rye bread was provided by the Village Bakery, Penrith, UK. 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. The transportation cost for rye foods shipment from Finland was paid for from the project budget, although the foods themselves were provided in kind. Shredded Wheat Fruitful and WG wheat pasta were provided by CPUK. Shredded Wheat Fruitful and rye muesli were formulated from the same batch and with the same proportion of dried mixed fruit and were thus otherwise identical except for the whole grain source. Weetabix was provided in kind by the Weetabix Ltd., UK. Breakfast cereals and pasta were all produced from single batches of raw materials. All foods except WG wheat and rye breads and Weetabix were packed individually in sealed foil containers by CPUK. Each packet had pre-measured amounts of different WG foods based on the number of servings of whole grain present in them as shown in Table 2.1 below. Each serving of WG food approximated 16g of whole grains to mimic the serving size advocated by the USDA in its Dietary Guidelines for Americans (USDA, 2005).

Table 2.1

Food & Human Nutrition	**Serving Size**	**Portion weight (g)**	**Average wholegrain content per portion (g)**
Wholemeal wheat bread	1 slice	36	20
Shredded Wheat Fruitful	2 servings packet	55	32
Shredded Wheat Fruitful	3 servings packet	83	48
Weetabix	1 ‘biscuit’	19	15
Wholegrain wheat pasta	3 servings packet	54	48
Rye bread	1 slice	25	20
Rye muesli	2 servings packet	55	34
Rye muesli	3 servings packet	83	50
Rye porridge	2 servings packet	35	32
Rye pasta	1 serving packet	90	18

Examples of the packaged foods are shown in Figure 2.1.

Figure 2.1 GrainMark study foods pre-packaged in foil packets by Cereal Partners UK. Labels indicate the number of wholegrain servings contained within each packet.

2.2.2 Pre-sampling evening set-meals

To reduce random intra-individual and inter-individual variations in blood/urine samples caused by the acute influence of the evening meal before sampling days, all volunteers were provided with the same 'set-evening meal' that was consumed the evening before every measurement visit (for bio-fluid sampling, anthropometry etc.). The meals were chosen to provide approximately 30% of daily energy and nutrient needs for an 'average' adult volunteer. The components of the meal were chosen so that they exerted minimal effects on the biological markers measured in the biofluid samples collected the following day. The set-evening meal contained no whole grain ingredients.

The set-meal consisted of a pre-cooked chicken dinner, 'Chicken in a Pot' (Sainsbury's Supermarkets, UK), one chocolate éclair (Marks & Spencer Plc, UK) and one 500ml bottle of mineral water. The 'Chicken in a Pot' was made from one roasted chicken breast, roast potatoes and vegetables in a gravy sauce. The volunteers were instructed to consume the set-evening meal by 8 pm the evening before their measurement visit and fast until the measurements/sample collection

are completed the following day. Volunteers were provided with a second 500ml bottle of mineral water which they could use during their fasting period until their Clinical Research Facility (CRF) visit and the volume of additional water consumed from this bottle (if any) was recorded.

2.3 Ethical & Trust Approval

The project obtained ethical approval from the Northumberland Research Ethics Committee (NHS REC) on the 15th of November 2007. The REC reference number was 07/H0902/53. Since the dietary intervention part of the study was undertaken at the Newcastle NIHR CRF, it required NHS Trust Approval which was obtained on the 11th of December 2007 (Ref. No. 4349).

2.4 Volunteer Recruitment

The volunteers for the study were mainly recruited using a recruitment database of interested volunteers held at Newcastle University. In addition to this, potential volunteers were also identified using a variety of advertising means such as posters, adverts on the Newcastle University website, email circulation to staff within the university, leaflets through letterboxes and word of mouth. At the same time, a website for the study was created (Appendix 4; www.grainmark.org) which contained the participant information sheet and exclusion criteria (see below). All advertising materials contained a link to this website.

Each potential participant that met the principal inclusion criteria (i.e., males/females above 45 years of age) were interviewed over the telephone to assess their suitability for the study using the 'pre-screening questionnaire' (Appendix 3). Before the pre-screening questionnaire was administered, each volunteer was briefed on the study design and the commitments that were required of them in order to take part in the study. Once volunteers gave their verbal consent for participation, they were invited to complete the pre-screening questionnaire over the telephone.

The pre-screening questionnaire was based mainly on the exclusion criteria for the study which were as follows:

• Known allergies or intolerances to intervention foods (wheat, gluten etc).

• Individuals receiving any form of clinical treatment, and/or taking prescribed medications, since clinical treatment could affect metabolic profile and/or bioavailability of nutrients from the intervention diet, potentially masking true dietary effects.

• Individuals taking any form of dietary supplements since dietary supplements may affect metabolic profile and/or bioavailability of nutrients from the intervention diet, potentially masking true dietary effects.
• Having dietary restrictions, apart from being a vegetarian (for example being on a detox or slimming diet), since some dietary restrictions could interfere with metabolic profile in response to the whole grain diet
• Planning to change dietary habits, increase physical activity, change body weight, move away from the study centre/locality or to take a lengthy vacation during the duration of the study.
• Smokers, whose metabolic profile may be affected through increased oxidative stress.
• History of alcoholism or substance abuse, since these may affect compliance to dietary intervention and/or metabolic profile.
• Body Mass Index < 20 kg/m^2 or > 32 kg/m^2 as underweight or obese individuals are likely to have altered metabolic profiles and/or metabolic response to dietary manipulation.
• Being pregnant, planning pregnancy or having had a baby in the past 12 months.

On establishing suitability, the volunteers were invited in writing to attend the induction visit during which they could give their informed consent. Along with the induction visit invitation letter, volunteers were sent the Participant Information Sheet (Appendix 2) so that they had an opportunity to read this in detail and in their own time. Participants were also sent directions to attend the CRF.

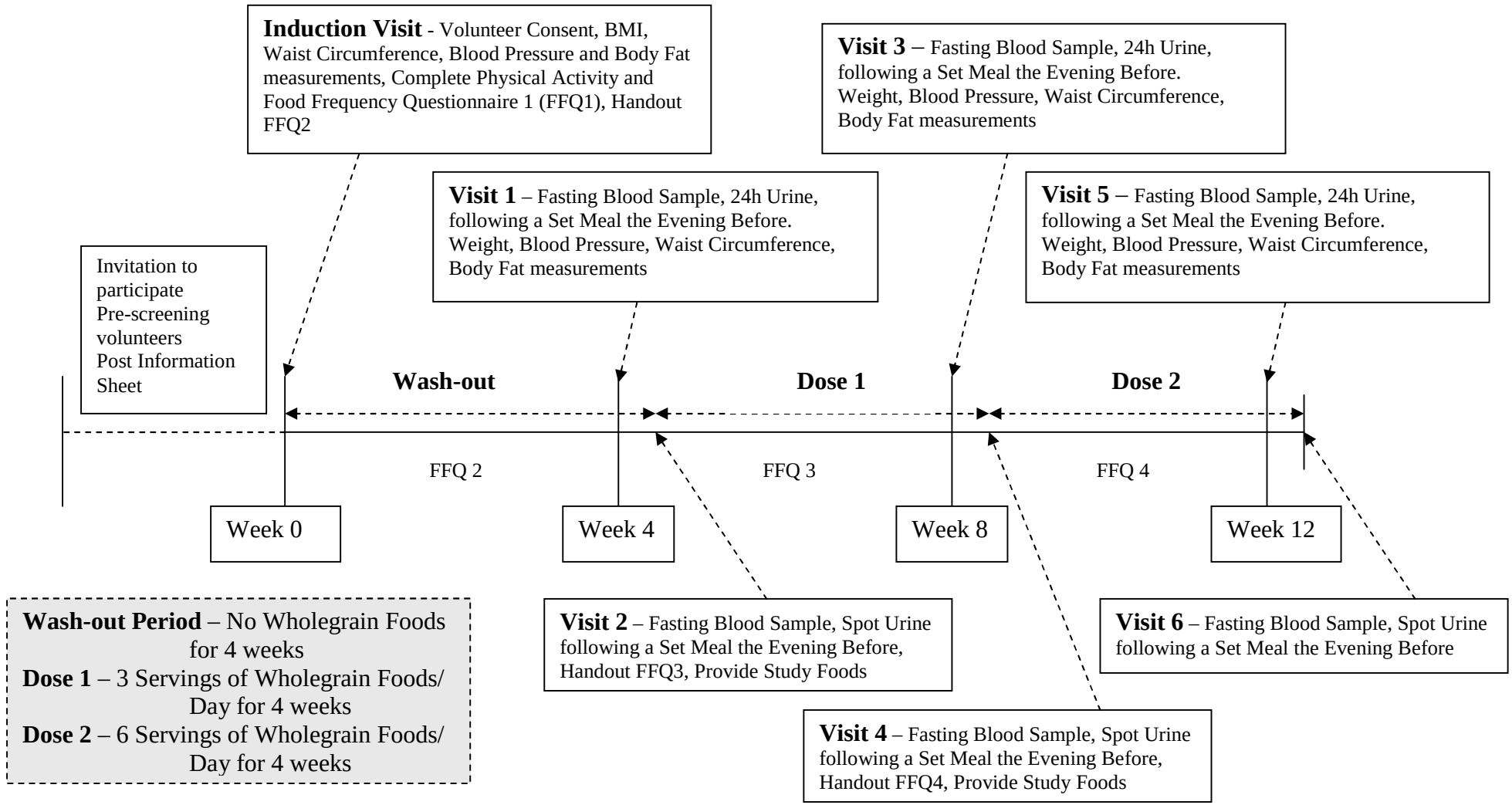
2.5 Dietary Intervention

The study was a randomised controlled dietary intervention based on a two-group parallel design with increasing intake of WG wheat or WG rye to determine biomarkers of whole grain-intake and changes in metabolite profile resulting from their consumption.

In brief, the volunteers were asked to avoid all wholegrain foods from their diet during a run-in/wash-out period for the first 4 weeks of the study, following which they were randomly allocated to either WG rye or WG wheat groups. After the completion of the wash-out period, volunteers were asked to consume 3 servings per day (equivalent to about 48 g of WG/d) of either WG rye or WG wheat foods for 4 weeks (Dose 1). In the final phase (Dose 2) of the study, the volunteers were asked to consume 6 servings per day of the same WG foods they had during the Dose 1 period, for another 4 weeks. During the dietary intervention, all wholegrain foods as listed in Section 2.2.1 above were provided to the volunteers. The volunteers were asked to avoid any other wholegrain foods in their diet apart from those provided for them during the dietary intervention. To aid

compliance, the volunteers were provided with a list of common foods consumed in the UK that contain whole grains (Appendix 1) and were given verbal guidance on identifying wholegrain foods. At the beginning and end of intervention Doses 1 and 2, volunteers visited the study facilities (CRF) for biofluid sampling and anthropometric measurements. At the beginning of the wash-out phase, anthropometric data (but no biofluid samples) were also collected. The various study visits and the measurements taken at each visit are schematically shown in Figure 2.2 and described below.

Figure 2.2 GrainMark Study Visits



Induction Visit

At the Induction Visit, the study protocol and the commitments required were explained to the volunteers. They were also asked if they had read and understood the participant information sheet that had been provided to them. Following this, if the volunteers agreed to participate, they were asked to complete the consent form.

After consent, at the Induction Visit, volunteers were asked to complete a food frequency questionnaire (FFQ 1) in the presence of a qualified researcher, who assisted with any volunteer queries if required. Height, weight and body composition (% Body Fat, Waist Circumference) measurements (Section 2.7) were taken, after which volunteers completed a physical activity questionnaire. Finally, resting blood pressure measurements were also taken.

At the end of the Induction Visit, volunteers were instructed to avoid all wholegrain foods for the following 4 weeks. In order to aid compliance, the volunteers were provided with the list of wholegrain foods commonly consumed in the UK diet that they must avoid (Appendix 1). Appointments for subsequent visits were then made.

Visits 1, 3 and 5

These identical visits took place after each period (Wash-out, Dose 1 and Dose 2 respectively) of dietary intervention. These were the first of two visits after each dietary intervention period during which volunteers provided one fasting blood sample, a 24 hour urine sample collected the day before the visit and a fasting ‘spot’ urine sample. During these visits, the volunteers returned the food frequency questionnaires covering one week prior to the visit, corresponding to the middle of each period of dietary intervention (FFQ 2, FFQ 3 and FFQ 4 respectively; see Section 2.6 for further details). Weight, body composition (% Body Fat, Waist Circumference) and blood pressure measurements were taken at each of these visits and the volunteers also completed a physical activity questionnaire. Until the duplicate visit for each intervention period (Visits 2, 4 and 6 respectively), the volunteers were asked to continue the same dietary regime for that phase.

Visits 2, 4 and 6

Visits 2, 4 and 6 took place two days after visits 1, 2 and 3 respectively. These were duplicate visits to obtain repeat biological samples representing each dietary intervention period. At each of these visits, the volunteers provided one fasting blood sample and one fasting spot urine

sample. At visits 2 and 4, the volunteers also received some/all of the intervention study foods for the dietary intervention period that followed. At visits 2 and 4 respectively, the volunteers received FFQ 3 and FFQ 4 and Food Record Form 1 and Food Record Form 2, for recording their overall diet and intervention study foods intake respectively (Section 2.6).

At the end of every visit, volunteers were provided with breakfast at the study site and were refunded for any travel expenses incurred.

2.6 Dietary Assessment

A validated 7-day **Food Frequency Questionnaire** (used in WHOLEheart Study (Brownlee *et al.*, 2010), FSA N02036, Appendix 6) was used to assess dietary intake at various stages during the study. These included one taken at induction visit (FFQ1 – representing habitual diet), one in the middle of the Wash-out period (FFQ2 – representing 0 servings/d WG intake), one in the middle of Dose 1 period (FFQ3 – representing 3 servings/d WG intake) and finally, one in the middle of Dose 2 period (FFQ4 – representing 6 servings/d WG intake). The FFQ were used to assess energy and nutrient intake at the start and during various periods of dietary intervention using an established nutrient database based on McCance & Widdowson 6th Edition food tables (Food Standards Agency, 2002) .

2.6.1 Wholegrain intake assessment

2.6.1.1 Food frequency questionnaire

The FFQ were used to assess volunteers' wholegrain intake. The wholegrain foods that were included in this analysis and the whole grain-contents of each portion for each gender are listed in Table 2.2. Wholegrain food portion sizes were based on data from National Diet and Nutrition Surveys (1991-2000) and data collected in Newcastle (Jones & Seal, unpublished) and have previously been used to estimate whole grain-intake in British adults (Lang & Jebb, 2003; Lang *et al.*, 2003; Thane *et al.*, 2007).

Table 2.2 Whole grain contents (g) of wholegrain food portions used to quantify whole grain-intake.

Wholegrain Food Item	**Portion size (Male)**	**Portion size (Female)**
Branflakes (1 bowl)	25	23
Brown rice (1/2 plateful, or in a dish e.g. rice salad, risotto etc)	75	62
Cheerios (1 bowl)	28	23
Crispbreads e.g. Ryvita, Ryvita currant crunch (one)	8	8
Flapjacks (each)	23	23
Muesli (1 bowl)	49	32
Oatcakes (one)	12	12
Porridge, Readybrek (1 bowl)	28	24
Rye bread (per slice/roll)	20	20
Shredded Wheat, Shreddies (1 bowl)	39	34
Weetabix (1 bowl)	34	29
Wholegrain cereals with fruit (1 bowl) e.g. Sultana Bran, Fruit n Fibre	36	25
Wholemeal bread/rolls (per slice/roll)	20	20
Wholemeal pasta/spaghetti (1/2 plate)	79	62
Wholemeal pita bread (each)	39	39

2.6.1.2 Food Record Form

To assess the intake of the whole grain intervention foods, during Dose 1 (3 servings whole grain/d) and Dose 2 (6 servings whole grain/d) periods of the study, each volunteer was provided with customised **Food Record Forms** for each period (for examples see Appendices 5A and 5B). These forms were used to record the type and frequency of the different intervention foods consumed during each phase of dietary intervention. They also served to help volunteers achieve the required amounts of either wholegrain rye or wholegrain wheat foods in their daily diet during each dietary intervention period and were subsequently used as compliance checks for the dietary intervention. In addition to investigating the average whole grain-intake in each phase from the Food Record Forms it was also possible to use the forms to quantify whole grain-intake the day before each measurement visit. These ‘pre-visit whole grain-intake’ measures were used to compare biomarker response to changes in pre-visit intake with ‘chronic whole grain-intake’ (average for phase).

2.6.1.3 Combined FFQ and Food Record Form

In addition to assessing whole grain-intake using FFQ and Food Record Forms separately, data from both assessment methods were combined to derive ‘Combined whole grain-intake’ data. Combined WG intake was calculated for the wash-out period (0 serving whole grain/d phase),

Dose 1 period (3 servings whole grain/d phase) and Dose 2 period (6 servings whole grain/d phase) of the study as follows: For the wash-out period, only whole grain-intake data from FFQ was used; for Dose 1 and Dose 2 periods, the sum of the Food Record Form whole grain-intake (study wholegrain foods) and non-study wholegrain food intake from FFQ were calculated.

To derive non-study whole grain-intake from FFQ, from the foods listed in Table 2.2 above, for volunteers in the wholegrain wheat group, Wholemeal Bread, Weetabix, Shredded Wheat, Wholegrain Cereals with Fruit and Wholemeal Pasta were classified as study wholegrain foods and the remaining foods as 'non-study wholegrain foods'. Similarly, for the wholegrain rye group, Muesli, Porridge, Flapjacks, Wholegrain Cereals with Fruit, Rye Bread and Wholemeal Pasta were classified as study wholegrain foods, with the remaining as non-study wholegrain foods. 'Wholegrain Cereals with Fruit' was included as study foods in both diet groups because this descriptor was commonly used by volunteers to record either Shredded Wheat Fruitful (a study food given to the wholegrain wheat group) and Rye Muesli (given to the wholegrain rye group). In addition, volunteers in the wholegrain rye group were provided with recipes for rye flapjacks in order to aid compliance, particularly for the 6 servings whole grain/d intake phase. Therefore, flapjacks were included as a study food for the wholegrain rye group.

2.6.2 Alkylresorcinols intake assessment

The average dietary alkylresorcinols (AR) intake from intervention study wholegrain foods were calculated for Dose 1 and Dose 2 periods using Food Record Form data and the AR content of the study foods provided to the volunteers in the two wholegrain groups (Ross & Kochhar, 2009). In addition to obtaining the average AR intake in each period, it was also possible to use the Food Record Forms to quantify AR intake the day before each measurement visit. These 'pre-visit AR intake' measures were used to compare biomarker dose-response relationships to changes in pre-visit AR intake with 'chronic AR intake' (average for each dietary intervention periods). The AR intake was calculated for separate AR homologues (C17:0, C19:0, C21:0, C23:0 and C25:0) based on their respective content in the study foods and the frequency of consumption of each study food during each period or day before biofluid collection.

2.7 Anthropometry Measurements

2.7.1. Height

The volunteers' height was measured using a wall mounted stadiometer. Volunteers were asked to remove shoes and stand upright with heels and shoulders against the measuring rod, knees and back straight and looking forward.

2.7.2 Body composition (weight, % body fat)

Body composition was assessed using a Tanita BC-418 segmental body composition analyzer. Measurements were taken first thing in the morning, after an overnight fast (except for induction visit) and with an empty bladder. Standard settings were used across all volunteers for weight of clothes (1.0 Kg) and body type (normal, not athletic).

2.7.3. Waist circumference

Waist circumference was taken at the widest point between the lower rib margin and the iliac crest. The waist circumference was measured on subjects with their waist uncovered to the nearest 0.1 cm. Volunteers were asked to stand with their shoulders-width apart with their weight equally distributed on each leg and with the arms to the sides. They were also asked to breathe normally and the reading was taken at the end of a normal exhalation. The tape measure was held closely against the body and placed horizontally at the desired height. The length of the tape measures was checked at the start of the study and at least once a month against a standard measure.

2.8 Fasting Blood Sample Collection and Processing

2.8.1 Venepuncture

Appropriately trained phlebotomists performed venepuncture ensuring local health and safety protocols were followed. Blood samples were collected in 6 ml lithium heparin blood tubes (BD Vacutainers, UK). All blood collection tubes were labeled appropriately and the volunteer was briefed about the procedure before venepuncture took place. Following blood collection, all vacutainers were inverted gently three times and placed on ice (i.e. placed horizontally) in an appropriate container and then transported to the laboratory.

2.8.2 Blood sample processing

Blood samples were processed in a Category 2 laboratory in the CRF, adhering to the local Bio-COSHH regulations. Blood samples collected in blood tubes were centrifuged at 1200 x g for 10 min, at 4 °C to separate plasma from whole blood.

After centrifugation, all samples were placed unopened into racks and the height of the meniscus of each blood tube was measured to take into account potential differences in the anticoagulant (heparin) concentration in them. Each tube lid was then opened carefully without disturbing the layers in the blood tube. Using fresh 1000 µl Gilson pipette tips for each sample, the plasma layer was removed and placed into labeled aliquot tubes (Sarstedt Micro tube 2ml, product no. 72.608) for metabolomic profile analyses. From two separate 6 ml Li-Hep tubes, 2 x 0. 5 ml duplicate (x2) aliquots of plasma were obtained. After removing plasma aliquots for metabolomic analyses, the remaining plasma from all Li-Hep tubes was pooled into 1 sterile universal, mixed carefully and then subsequently aliquoted to the remaining tubes (for other targeted analyses such as alkylresorcinols, mammalian lignan analyses etc) as required. In the pooling process, care was taken to avoid red cell-lysis. When red cell lysis did occur in a particular tube, the plasma from that tube was not pooled, but was stored separately if required. Care was also taken to avoid contamination with platelets and that the buffy coat was not disturbed when removing the plasma samples. All plasma samples were processed within 2 hours of blood collection and immediately stored at -80 ^{0}C following processing until analysed in batches at the end of the dietary intervention.

2.9 Urine Collection and Processing

2.9.1. 24 hour Urine Collection

The 24 hour urine samples were collected the days before visits 1, 3 and 5 respectively in two separate containers. The first container (Container A) was used to collect all urine passed during the day before the CRF visit, except for the first pass in that morning, up to and immediately before consuming the set evening meal. All urine passed following the evening meal and until the visit to the CRF the following morning (including the first pass) was collected in Container B. Container A was sourced from VWR, catalogue number 15-1102, Urisafe 3L 24 hr Urine Collection Container and Container B was a 2L container. No preservatives were used in any container, and the volunteers were provided with cool bags and ice packs to in order to keep the

samples cold throughout the collection period. To aid compliance, the volunteers were provided with detailed instructions for urine collection (Appendix 7).

2.9.2. Fasting 'Spot Urine' Collection

During each measurement visit (Visits 1-6), the volunteers were asked to provide a 'spot urine' sample whilst emptying their bladder prior to their weight/body composition measurements. These samples were collected in sterile 'Universal' tubes (25ml). Fasting urine samples for visits 1, 3 and 5 were labeled 'Sample C' and those from Visits 2,4 and 6 were labeled 'Sample D' as they represented duplicate samples from each period of the dietary intervention.

2.9.3. Urine sample processing

The volumes of 24 hr urine from Containers A and B were measured separately using measuring cylinders. A proportionally pooled sample was prepared using samples A and B (Sample P; 24 hr Urine) in a 50ml Falcon tube. Aliquots of all urine samples (Sample A, B, P, C and D) from each volunteer were then stored at -80 ^{0}C for further analyses.

2.10 Blood/Urine sample storage

All blood and urine samples, unless stated otherwise were stored in appropriate containers at -80 ^{0}C until analysed in batches at the end of the dietary intervention.

2.11 Plasma Alkylresorcinols (AR) Analysis

Plasma AR were measured using a gas-chromatography-mass spectrometry method based on the method by Landberg et al (2009c). In brief, plasma samples were thawed at room temperature in racks and vortexed for 30 seconds. 200 µl of plasma was then added to labelled Pyrex culture tubes containing 10 µl internal standard solution (AR C20:0, 1µg/ml in methanol). To this, 1 ml of 50% ethanol was added to precipitate protein and mixed using a multi-tube vortex mixer (VWR, UK). Following this, 3 ml diethyl ether was added to each tube and the tubes vortexed for 2 min. The tubes were then placed in an ethanol/dry ice bath to freeze the aqueous phase. The organic layer was poured off into clean correspondingly labelled culture tubes. This organic extraction step was repeated an additional two times (3 x 3 ml diethyl ether extraction in total) and the diethyl ether extracts pooled after every extraction. The pooled extract was then dried under nitrogen in a heating block at 45 ^{0}C after which the samples were resuspended in 1 ml methanol. The samples were then cleaned using Oasis MAX solid phase extraction columns. To

do this, each column was first primed with 1 ml 0.1M NaOH in 70% methanol at a flow rate of 1ml/min. The sample extract, previously dissolved in 1 ml methanol was then applied on to the column. The columns were further washed with 2 ml methanol and dried under vacuum for 5 min. AR were then eluted from the column by adding 1 ml 2 % acetic acid in methanol at a flow rate of approximately 1.0 ml/min. The eluted fraction was then evaporated to dryness under nitrogen in a heating block at 50 ^{0}C and silylated with 100 µl silylation mixture (MSTFA + 1 % TMCS; Thermo Fisher Scientific, UK) for 30 minutes. The silylated samples were then transferred to labelled GC vials for GC-MS analysis. The separation was performed on a TR-5 column (15 m x 0.25 mm x 0.25 µm, Thermo Fisher Scientific, UK). The alkylresorcinol (AR C17:0, AR C19:0, AR C20:0, AR C21:0, AR C23:0 and AR C25:0) standards were obtained from ReseaChem Lifesciences, Switzerland.

2.12 Lignans Analysis

2.12.1 Plasma lignan analysis

Plasma lignans were measured by HPLC using previously published methods (Nurmi *et al.*, 2003; Penalvo *et al.*, 2004). In brief, 0.4ml of each sample was added to 0.4ml hydrolysis reagent consisting of *Helix pomatia* enzyme mixture giving a concentration of 2500 U/ml of β-glucuronidase (Sigma) in sodium acetate buffer pH 5.0. The samples were mixed gently and incubated for 16h at 37 ^{0}C. After incubation the samples were cooled, and 20µl of 6 M HCl was added and gently mixed. 5 ml of diethyl ether was added and the tubes mixed using a multi-tube vortex mixer (VWR) for 2 minutes. The organic layers were transferred into clean Pyrex tubes. This diethyl ether extraction was repeated twice, giving a final volume of 15 ml. The samples were then evaporated to dryness under nitrogen at 40 ^{0}C and resuspended in 250 µl methanol. 200 µl of sample was then put through a QEA-Sephadex A-25-Acetate pre-prepared column and eluted using 4 ml methanol. The eluted samples were evaporated to dryness under nitrogen at 45 ^{0}C, then re-suspended in 100 µl HPLC buffer (40 parts 50mM sodium acetate pH 5.0, 40 parts methanol and 20 parts acetonitrile) and transferred to HPLC vials for analysis on an ESA HPLC-Coularray (ESA Corporation, USA). Lignans were separated using an ESA microdialysis MD-150 analytical 3 mm x 15 cm column. Enterodiol and Enterolactone standards were obtained from Sigma.

2.12.2 Urinary Lignan analysis

As for plasma, but re-suspended in 400µl HPLC buffer.

2.13 Statistical Analyses

The dietary differences between various stages of dietary intervention in wholegrain wheat and rye groups were compared using ANOVA and Tukey's HSD. The anthropometric data within each diet group were compared using paired t-test and between diet groups were compared using an independent t-test.

The effects of increasing wholegrain dose in wholegrain rye and wheat groups on various measurements were calculated using linear mixed effect models as described below.

2.13.1 Linear Mixed Effects Model (LMEM) to investigate dose-response effects of whole grain or alkylresorcinols intake and biomarker concentrations in plasma or urine

A *linear mixed effects model* was used to investigate effects of whole grain-intake at different doses on various anthropometric, blood lipid profile, alkylresorcinols and lignan (both plasma and urine) measurements. The possibility of various covariates having a *fixed effect* on these dependent measurements, whilst simultaneously allowing both a fixed and *random effect* for the wholegrain dose was investigated. The fixed effect for dose models the overall slope (if any) of the dependent variable through different doses of wholegrain intake; the random effect for dose models the variability about this overall slope, allowing different subjects in the study to have different dose responses. Such *random effects analyses* are commonly used with longitudinal data as obtained in this study. Although repeated measures ANOVA could have been used, the assumption of sphericity (constant variance of all within-subject differences) is often difficult to justify. Generally, such a model takes the following form:

$$Y_i = \beta \mathbf{X}_i + U_i \mathbf{Z}_i + \varepsilon_i,$$

where Y_i is the dependent variable (e.g., plasma total alkylresorcinols), $\mathbf{X}_i$ is a matrix of covariates which are assumed *not* to vary between subjects with associated parameter vector β (i.e. the fixed effects), $\mathbf{Z}_i$ is a matrix of covariates that *are* allowed to vary between subjects, U_i is a vector of unobservable random effects associated with the covariates in $\mathbf{Z}_i$, and ε_i is a vector of errors. It is common to assume that U_i is normally distributed with zero mean and variance matrix Σ, i.e. $U_i \sim N(0, \Sigma)$; similarly, as in standard regression models, ε_i is also assumed to be

normally distributed with zero mean, i.e., $\varepsilon_i \sim N(0, V_i)$, where V_i represents pure measurement error.

For example, if the dose-response relationship of wholegrain rye on plasma total alkylresorcinol concentration were to be investigated, then X_1 might be the dose of wholegrain rye; X_2 could be gender; X_3 the cohort group; X_4 might be age and X_5 could be body mass index (at Induction Visit). Y would represent the corresponding plasma total alkylresorcinol concentration measurement of participants who were in the wholegrain rye arm of the study (since there were repeat measurements for each individual corresponding to each dose of wholegrain rye, the mean of the two measurement would be taken). A standard regression analysis might fit the following model:

$$Y_i = \beta_0 + \beta_1 X_{1_i} + \beta_2 X_{2_i} + \beta_3 X_{3_i} + \beta_4 X_{4_i} + \beta_5 X_{5_i} + \varepsilon_i ;$$

the importance of predictor variables X_i in the model could then be checked via "backwards elimination"; i.e. when the above "full model" is fitted, if we retain any of the null hypotheses $H_0^{(j)} : \beta_j = 0$, j = 1, …, 5, then the predictor variable whose coefficient is least significant is removed from the model and the model re-fitted with the remaining four predictor variables. This process is repeated until the model is left with *all* the predictor variables having coefficients significantly different from zero.

However, to allow for subject-specific trends in dose, this model was extended to allow for dose random effects. Specifically, for the above example, which uses plasma total alkylresorcinol concentration as the dependent variable, the "full model" would comprise:

$$Y_i = \underbrace{\beta_0 + \beta_1 X_{1_i} + \beta_2 X_{2_i} + \beta_3 X_{3_i} + \beta_4 X_{4_i} + \beta_5 X_{5_i}}_{\text{Fixed effects}} + \underbrace{U_1 1_{n_i} + U_2 X_{1_i}}_{\text{Random effects for subject-specific dose effects}} + \underbrace{\varepsilon_i}_{\text{White noise}}.$$

There are two random effects in this model – U_1 and U_2. **1** is a vector of 1's and has the effect of adjusting the overall intercept β_0 for each subject i = 1, …, n, and X_1 represents the dose of

wholegrain rye. Thus, the random effects U_1 and U_2 allow for a random intercept and slope respectively, allowing each subject in the study to have their own mean level and rate of change in response to the dose of wholegrain rye. These random effects parameters are unobservable, but it is usual to assume a normal distribution, i.e.:

$$U_1 \sim N(0,\sigma_1^2) \text{ and } U_2 \sim N(0,\sigma_2^2),$$

where σ_1 and σ_2 are parameters to be estimated. An estimate of σ_2 will indicate the amount of variability about the fixed slope effect β_1 after allowing for subject-specific variations in the dose effect; an estimate of σ_1 will indicate the amount of variability in the *Y*–intercept of this slope. A process of backwards elimination, as described above, was used to remove covariates from the model where necessary.

The **linear mixed effects model** analyses were performed using the **R** statistical software (R_Development_Core_Team, 2006). The software was also used to check the goodness-of-fit of the models using standard regression diagnostic tools (normality of residuals etc.).

3.0 Results

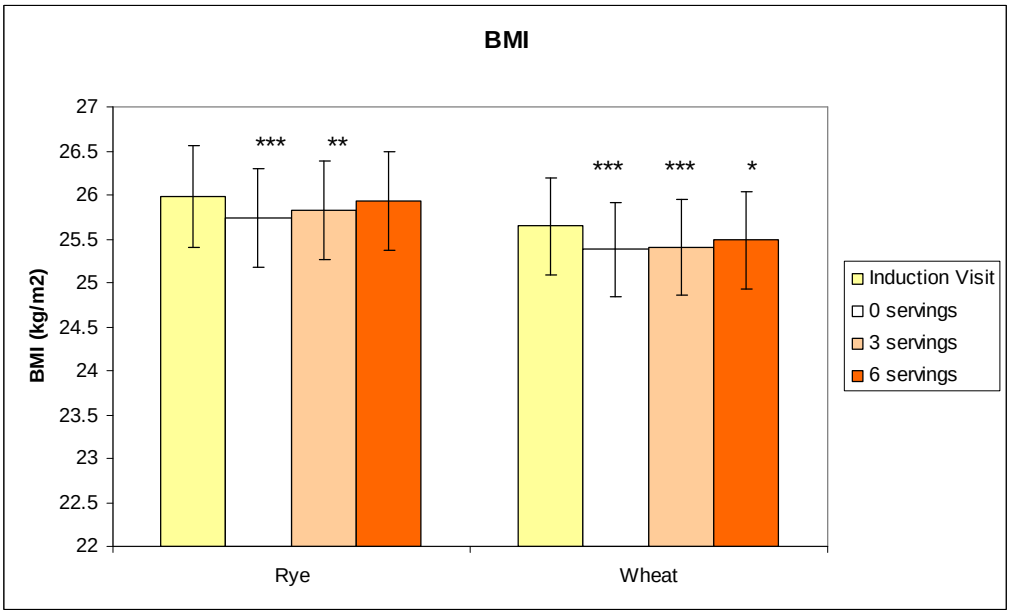
3.1 Volunteer Recruitment

Sixty-eight volunteers participated in the study in two batches. Therefore the original recruitment target of 62 as set out in Objective 02 was exceeded. Thirty eight volunteers participated in Batch 1 starting January 2008 and finishing May 2008, while in Batch 2 there were 30 volunteers who participated in the study between June 2008 and October 2008. In total, 70 volunteers were recruited into the study following pre-screening and 2 volunteers dropped out due to medical reasons not related to the study, equating to a drop-out rate of less than 3%. There were 35 (17 M, 18 F) volunteers in the wholegrain wheat group and 33 (16M, 17F) volunteers in the wholegrain rye group, ensuring an equal gender mix and number of volunteers in both intervention groups. The mean (±SD) age of the volunteers at the start of the intervention was 55.03 (±6.48) years in the wholegrain wheat group and 54.24 (±5.22) years in the wholegrain rye group.

3.2 Anthropometry

The mean (± SEM) Body Mass Index of volunteers in the wholegrain rye and wheat groups at various stages of intervention are shown in Figure 3.1

Figure 3.1 Mean BMI (± SEM) of volunteers at various stages of dietary intervention in wholegrain wheat and rye groups. BMI significantly different from Induction Visit; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.



There were no significant differences in BMI between the two intervention groups at each of the measurement time points of intervention (using separate independent samples t-tests at respective time points). However, for both diet groups, the volunteers had a significantly lower BMI after the wash-out (0 servings whole grain/d) period compared with that during the Induction Visit (baseline; $p<0.001$ for both wheat and rye groups). BMI increased in both groups but was still lower after Dose 1 (3 servings whole grain/d) period in both rye ($p<0.01$) and wheat ($p<0.001$) groups compared with BMI at baseline. For the wheat group only, BMI was lower after the Dose 2 period compared with baseline ($p<0.05$). Compared with the wash-out period, BMI was significantly higher in the wholegrain rye group after the Dose 2 period ($p<0.05$).

The body composition data for volunteers at various stages of dietary intervention is shown in Table 3.1. There were no significant differences between the groups or between various time points within each intervention group.

Table 3.1 Body Composition of subjects in the GrainMark study.

Measurement	**Visit**	**Wholegrain Wheat group**		**Wholegrain Rye group**	
		Mean	**SD**	**Mean**	**SD**
Body Fat (%)	Induction Visit	28.3	7.55	29.2	6.67
Body Fat (%)	Visit 1	29.1	8.02	29.4	6.37
Body Fat (%)	Visit 3	28.7	7.62	29.0	6.43
Body Fat (%)	Visit 5	28.8	7.54	29.7	6.55
Waist Circumference (cm)	Induction Visit	87.3	11.85	88.2	13.81
Waist Circumference (cm)	Visit 1	87.0	11.04	88.4	12.73
Waist Circumference (cm)	Visit 3	87.2	11.11	88.0	12.52
Waist Circumference (cm)	Visit 5	87.5	10.99	88.8	12.76

3.3 Dietary Intake
3.3.1 Energy/Nutrient Intake

The mean daily energy and nutrient intakes as assessed using FFQ are shown in Tables 3.2a and 3.2b for wholegrain wheat group and wholegrain rye group respectively. Statistical differences between various stages of dietary intervention were compared using ANOVA and Tukey's HSD. There were no significant differences in energy or macro-nutrient intake between various dietary intervention stages except for the higher intake of carbohydrates and starch in the wholegrain rye group during Dose 2 (6 servings whole grain/d). Dietary fibre intake was significantly lower after the wash-out period (0 serving whole grain/d phase) compared with the volunteers' habitual intake (measured at the Induction Visit). Dietary fibre intake was significantly higher in Dose 1 (3 servings whole grain/d) and Dose 2 (6 servings whole grain/d) periods of dietary intervention compared with that during the wash-out period, as a result of increasing whole grain-intake in both diet groups. In the wholegrain wheat group only, there was also an increase in lignin intake during Dose 2 compared with the Induction Visit and after the wash-out period.

With regards to micronutrients, there were significant increases in thiamin, riboflavin, iron, manganese, magnesium, phosphorus and zinc intake with increasing whole grain-intake in the wholegrain wheat group, particularly at Dose 2 compared with the wash-out period (Table 3.2a). Similarly, thiamin, vitamin E, iron, manganese, magnesium, phosphorus and zinc intake were greater in Dose 2 compared with the wash-out period in the wholegrain rye group (Table 3.2b).

Table 3.2a Mean (and standard deviation) of daily energy and nutrient intake during various stages of dietary intervention in wholegrain wheat group (n=35)

Mean Daily Intake	Induction Visit		Wash-out		Dose 1		Dose 2	
	Mean	(St. Dev.)	Mean	(St. Dev.)	Mean	(St. Dev.)	Mean	St. Dev.
Energy (kJ)	12190	(3951)	11869	(4780)	11723	(3554)	12932	(3556)
Carbohydrate (g)	368.1	(140.03)	353.6	(174.32)	350.3	(119.29)	408.9	(120.79)
Starch (g)	213.6	(100.35)	207.9	(117.52)	210.4	(79.82)	266.8	(91.65)
Total Fat (g)	90.9	(33.58)	85.2	(43.66)	80.8	(31.26)	88.7	(32.54)
- SFA (g)	26.6	(10.68)	27.1	(15.23)	25.4	(12.12)	27.0	(12.62)
- PUFA (g)	8.8	(2.88)	9.1	(4.08)	8.6	(3.59)	9.5	(4.00)
- MUFA (g)	21.1	(7.05)	22.3	(11.67)	20.6	(8.88)	22.6	(10.34)
Protein (g)	100.0	(32.06)	98.4	(35.98)	97.0	(28.39)	108.8	(31.61)
Fibre (g)[a]	28.3	(12.03)	**18.2**	**(6.73)^{III}**	**24.3**	**(7.08)^{O}**	**36.0**	**(10.66)$^{III/OOO}$**
Lignin (g)	2.1	(1.11)	1.9	(1.15)	2.2	(0.85)	**3.0**	**(1.16)$^{II/OOO}$**
Phytic_Acid (g)	0.2	(0.08)	0.1	(0.07)	0.1	(0.07)	0.1	(0.05)
Alcohol (g)	41.6	(38.22)	46.8	(43.31)	50.2	(55.86)	42.5	(47.89)
Folate (µg)	402.9	(132.01)	375.5	(125.40)	371.9	(146.71)	431.5	(125.60)
Vitamin B6 (mg)	2.8	(0.85)	2.8	(0.92)	2.8	(0.89)	3.2	(0.86)
Thiamin (mg)	2.3	(0.85)	2.0	(0.76)	2.2	(0.66)	**2.9**	**(0.84)$^{II/OOO}$**
Riboflavin (mg)	2.1	(0.74)	2.0	(0.75)	2.3	(0.74)	**2.9**	**(0.72)$^{III/OOO}$**
Vitamin_C (mg)	159.4	(64.46)	146.8	(57.97)	144.6	(72.18)	140.2	(64.26)
Vitamin E (mg)	7.9	(2.86)	6.6	(2.34)	6.6	(2.11)	7.4	(2.10)
Sodium (g)	4.1	(1.56)	4.0	(1.98)	3.6	(1.21)	4.3	(1.48)
Potassium (g)	4.8	(1.31)	4.4	(1.30)	4.4	(1.13)	4.8	(1.26)
Calcium (mg)	955.7	(298.97)	978.3	(504.80)	875.5	(335.55)	854.4	(261.75)
Iron (mg)	20.3	(8.34)	17.0	(6.26)	19.2	(6.84)	**24.2**	**(6.41)^{OOO}**
Manganese (mg)	7.6	(3.26)	**4.9**	**(1.63)^{III}**	**5.9**	**(2.01)^{I}**	**8.5**	**(2.97)^{OOO}**
Magnesium (mg)	489.8	(175.90)	**369.6**	**(124.87)^{II}**	427.3	(117.82)	**537.8**	**(142.52)^{OOO}**
Phosphorus (g)	1.8	(0.56)	1.5	(0.58)	1.6	(0.46)	**2.0**	**(0.55)^{OO}**
Zinc (mg)	12.7	(4.91)	10.2	(3.88)	11.0	(3.41)	**13.8**	**(4.19)^{OO}**

[1]as measured by Englyst method. Bold face indicate significant difference from either Induction Visit (I= p<0.05, II= p<0.01 or III= p<0.001) or from Wash-out (O=p<0.05, OO=p<0.01 or OOO=p<0.001)

Table 3.2b Mean (and standard deviation) of daily energy and nutrient intake during various stages of dietary intervention in wholegrain rye group (n=33)

Mean Daily Intake	Induction Visit		Wash-out		Dose 1		Dose 2	
	Mean	(St. Dev.)	Mean	(St. Dev.)	Mean	(St. Dev.)	Mean	St. Dev.
Energy (kJ)	12596	(4199)	12382	(3484)	12983	(3796)	14125	(4036)
Carbohydrate (g)	376.1	(127.25)	369.2	(118.35)	374.9	(105.27)	**450.7**	**(141.19)**^{O}
Starch (g)	224.2	(81.11)	220.3	(92.26)	232.0	(79.83)	**290.1**	**(101.00)**$^{I/O}$
Total Fat (g)	96.4	(45.33)	91.3	(36.03)	97.0	(32.11)	100.6	(39.05)
- SFA (g)	26.9	(13.33)	27.6	(13.36)	27.9	(10.07)	27.7	(12.74)
- PUFA (g)	10.6	(4.23)	10.1	(3.81)	11.1	(4.12)	11.0	(3.54)
- MUFA (g)	23.6	(10.48)	23.7	(9.43)	24.7	(8.21)	23.1	(9.09)
Protein (g)	105.5	(28.30)	97.6	(28.28)	101.2	(26.03)	111.2	(28.98)
Fibre (g)[a]	27.9	(8.46)	**20.1**	**(6.04)**^{III}	23.3	(6.20)	**30.5**	**(9.22)**^{OOO}
Lignin (g)	2.2	(1.04)	2.0	(1.04)	1.8	(0.72)	1. 7	(0.80)
Phytic_Acid (g)	0.2	(0.07)	0.2	(0.09)	0.2	(0.11)	0.1	(0.08)
Alcohol (g)	40.9	(33.14)	48.6	(41.39)	56.8	(64.62)	44.2	(33.65)
Folate (μg)	411.6	(125.99)	383.0	(109.85)	351.9	(105.95)	378.0	(121.44)
Vitamin B6 (mg)	2.9	(0.82)	2.9	(0.66)	2.7	(0.69)	2.8	(0.76)
Thiamin (mg)	2.4	(0.60)	2.0	(0.50)	2.1	(0.54)	**2.5**	**(0.70)**^{O}
Riboflavin (mg)	2.1	(0.65)	2.0	(0.56)	1.9	(0.53)	2.1	(0.64)
Vitamin_C (mg)	159.3	(76.61)	153.3	(63.69)	139.9	(49.93)	147.0	(56.90)
Vitamin E (mg)	8.7	(3.10)	7.5	(2.48)	8.8	(2.82)	**11.1**	**(2.92)**$^{II/OOO}$
Sodium (g)	4.4	(1.40)	4.1	(1.34)	4.3	(1.46)	**5.1**	**(1.72)**^{O}
Potassium (g)	4.9	(1.39)	4.5	(0.99)	4.7	(1.21)	5.0	(1.19)
Calcium (mg)	978.6	(299.96)	952.7	(306.02)	1008.0	(327.85)	1127.9	(342.26)
Iron (mg)	20.9	(5.80)	17.9	(4.66)	19.6	(6.06)	**22.2**	**(5.97)**^{O}
Manganese (mg)	7.7	(3.03)	**5.2**	**(1.58)**^{III}	6.3	(1.98)	**8.1**	**(2.65)**^{OOO}
Magnesium (mg)	508.7	(150.03)	**390.9**	**(93.24)**^{II}	457.8	(128.72)	**538.5**	**(137.25)**^{OOO}
Phosphorus (g)	1.8	(0.51)	**1.5**	**(0.40)**^{I}	1.7	(0.43)	**2.0**	**(0.50)**^{OOO}
Zinc (mg)	12.6	(3.81)	**10.0**	**(2.86)**^{I}	11.5	(3.08)	**13.5**	**(3.51)**^{OOO}

[1]as measured by Englyst method**.** Bold face indicate significant difference from either Induction Visit (I= $p<0.05$, II= $p<0.01$ or III= $p<0.001$) or from Wash-out (O=$p<0.05$, OO=$p<0.01$ or OOO=$p<0.001$)

3.3.2 Wholegrain food intake using FFQ and Food Record Form

The mean daily intake of wholegrain foods for both the diet groups is shown in Table 3.3. The whole grain-intake of both diet groups was comparable at each stage of dietary intervention. As expected, whole grain-intake was significantly reduced during the wash-out period compared with their intake at the Induction Visit, indicating good reported compliance to the dietary intervention regime. Reported study food whole grain-intake at Dose 2 period for both diet groups were approximately two times that in Dose 1 period, again suggesting good compliance to the advised study regime.

Table 3.3 Reported whole grain-intake using Food Frequency Questionnaires in wholegrain wheat and wholegrain rye groups (mean values with standard deviation, n=35 for wheat group and n=33 for rye group)

Mean Daily Intake	Induction Visit		Wash-out		Dose 1		Dose 2	
	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.
Total whole grain-intake wheat WG group (g)	52.8	39.65	0.9	3.12	58.1	29.91	127.0	59.87
Total whole grain-intake rye WG group (g)	55.3	34.29	1.5	4.78	57.8	39.14	114.3	54.53
Non-study food whole grain-intake wheat WG group (g)	5.0	7.29	0.3	1.49	0.7	2.50	0.7	2.31
Non-study food whole grain-intake rye WG group (g)	7.7	9.08	0.4	1.91	0.1	0.29	0.5	2.67
Study food whole grain-intake wheat WG group (g)	N/A	N/A	N/A	N/A	57.4	29.76	126.3	59.99
Study food whole grain-intake rye WG group (g)	N/A	N/A	N/A	N/A	57.7	39.13	113.8	54.01

Whole grain-intake as assessed using the food record form and the combined method is shown in Table 3.4. Once again the measured whole grain-intake in Dose 2 period was approximately twice that seen during Dose 1, indicating good (reported) compliance to the study regime. As shown in tables 3.3 and 3.4, non-study wholegrain foods contributed less than 2 % to the total whole grain-intake during Dose 1 and Dose 2 periods of the dietary intervention.

Table 3.4 Reported whole grain-intakes using Food Record Form and combined FFQ/Food Record Form during the dietary intervention periods (Dose 1 and Dose 2) in wholegrain wheat and wholegrain rye groups.

Mean Daily Intake	Dose 1		Dose 2	
	Mean	St. Dev.	Mean	St. Dev.
Study food whole grain-intake from Food Records – wholegrain wheat group (g)	49.9	2.16	102.2	4.08
Study food whole grain-intake from Food Records – wholegrain rye group (g)	51.3	1.98	104.6	5.65
Total whole grain-intake using combined method – wholegrain wheat group (g)	50.7	3.26	102.9	4.19
Total wholegrain-intake using combined method – wholegrain rye group (g)	51.4	2.01	105.0	4.26

3.3.3 Alkylresorcinol Intake

The calculated mean daily intake of different AR homologues in the wholegrain wheat and wholegrain rye groups is shown in Table 3.5. As with whole grain-intake, the calculated intake of all AR homologues during Dose 2 period was approximately twice that during Dose 1 period of dietary intervention. Moreover, intake of C17:0 AR in the wholegrain rye group was significantly higher during both intervention periods compared with their respective intake for the wholegrain wheat group, reflecting the difference in homologue distribution between wheat and rye whole grains. The composition of different homologues in wholegrain rye foods and wholegrain wheat foods as provided during the study is shown in Tables 3.6 and 3.7. Finally, the mean ratio of C17:0 to C21:0 intake remained constant within each diet group for both intervention periods, although as expected, the ratio was significantly different between the two diet groups (Table 3.5).

Table 3.5 Calculated intake of various AR homologues from intervention study foods in wholegrain wheat and wholegrain rye groups.

Mean Daily Intake (mg/d) or Ratio	Wholegrain Wheat group				Wholegrain Rye group			
	Dose 1		Dose 2		Dose 1		Dose 2	
	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.
C17 alkylresorcinol intake	1.4	0.23	2.9	0.41	11.0	0.99	23.6	2.58
C19 alkylresorcinol intake	9.6	1.92	20.9	3.37	13.1	1.46	28.6	3.78
C21 alkylresorcinol intake	16.0	2.81	34.7	4.59	12.1	1.29	26.3	3.32
C23 alkylresorcinol intake	4.5	0.82	9.8	1.19	7.4	0.77	16.1	1.99
C25 alkylresorcinol intake	1.8	0.35	3.8	0.51	6.3	0.69	13.7	1.78
Total alkylresorcinol intake	33.2	5.97	72.1	9.86	49.9	5.19	108.3	13.40
C17 to C21 AR intake ratio	0.1	0.01	0.1	0.01	0.9	0.02	0.9	0.03

Table 3.6 Alkylresorcinol content of wholegrain wheat foods provided in the study.

Wheat Foods	AR (ug/g) C17:0	AR (ug/g) C19:0	AR (ug/g) C21:0	AR (ug/g) C23:0	AR (ug/g) C25:0	C17/ C21 ratio
Weetabix	23.5	145.8	238.3	65	26.8	0.10
Shredded Wheat Fruitful	18.2	108.9	181.7	48.9	17.1	0.10
WG Wheat Bread	16.9	133.7	208.5	56.5	22.8	0.08
WG Wheat Pasta	3.6	54.5	228.2	108.2	41	0.02

Table 3.7 Alkylresorcinol content of wholegrain rye foods provided in the study.

Rye Foods	AR (ug/g) C17:0	AR (ug/g) C19:0	AR (ug/g) C21:0	AR (ug/g) C23:0	AR (ug/g) C25:0	C17/ C21 ratio
Rye Porridge flakes (100% rye)	170.9	192.9	178.7	109.45	92.55	0.96
WG Rye Bread	134.9	175.2	156.9	94.6	83	0.86
WG Rye Pasta	51.6	65.3	70.5	45.6	33.9	0.73

3.4 Biomarker Measurements

3.4.1 Plasma Alkylresorcinol Concentrations

Figures 3.2 to 3.7 show plasma AR concentrations in wholegrain rye and wheat groups at various stages of dietary intervention. Since most of the AR data had a skewed distribution, the interquartile range and median (indicated by horizontal line in each box plot) are shown for the concentration of each separate AR homologue as well as for the total AR concentration of plasma. Visits 1 and 2 represent samples taken two days apart at the end of the wash-out period, corresponding to 0 serving whole grain/d intake. Similarly, Visits 3 and 4 represent the end of Dose 1 period (3 servings whole grain/d) and visits 5 and 6 represent the end of Dose 2 period (6 servings whole grain/d). In general, there were no statistical difference between duplicate visits except for marginal difference between Visits 3 and 4 in the wholegrain rye group for C19:0, C21:0 and C23:0 AR (all $p<0.05$). With the exception of AR C21:0, there were higher plasma AR concentrations for all homologues in response to increasing whole grain-intake in the wholegrain rye group compared with the wholegrain wheat group. As a result, the total AR concentration in response to increasing dietary whole grain-intake was higher in the wholegrain rye group compared with the wholegrain wheat group (Figure 3.7). For AR C21:0, the wholegrain wheat group had a significantly higher plasma concentration of this homologue than that in the wholegrain rye group at both whole grain doses. Subsequently, the plasma C17:0 to C21:0 ratio was significantly different between the two diet groups at the end of Dose 1 and Dose 2 periods, although the ratio remained constant within each diet group, irrespective of the dose of whole grain as shown in Figure 3.8 (a-c). As expected, plasma concentrations of all AR homologues were low and similar in both the diet groups at the end of the wash-out period (representing 0 serving/d phase).

Figure 3.2 Plasma C17:0 AR concentrations in wholegrain rye and wholegrain wheat groups at the different phases of the dietary intervention.

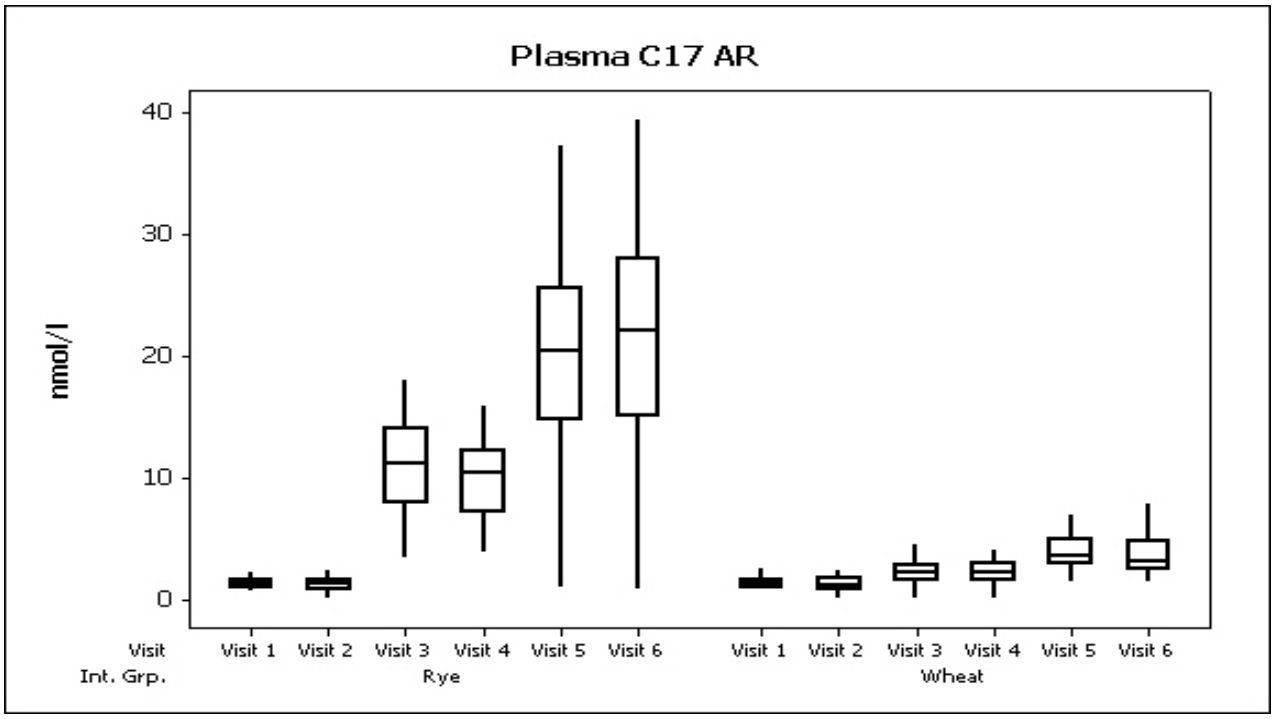


Figure 3.3 Plasma C19:0 AR concentrations in wholegrain rye and wholegrain wheat groups at the different phases of the dietary intervention.

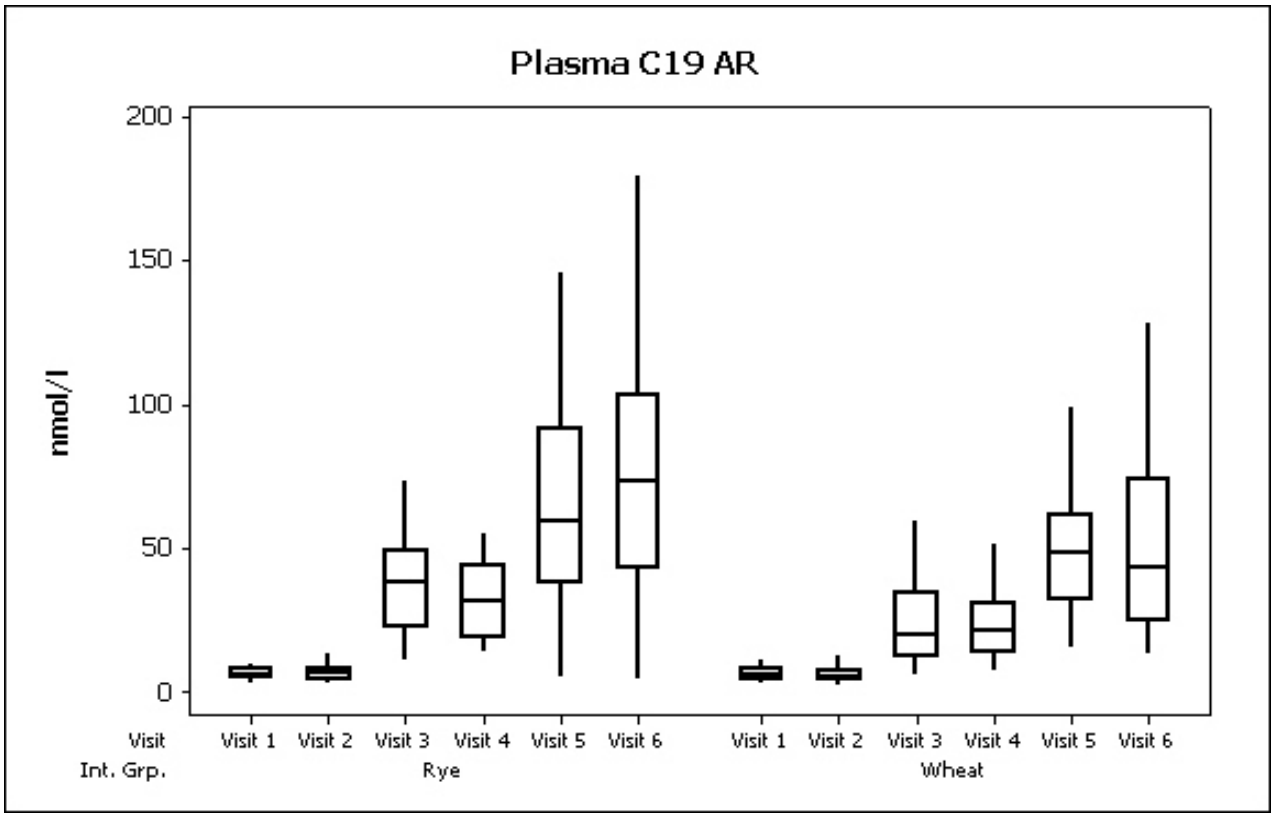


Figure 3.4 Plasma C21:0 AR concentrations in - wholegrain rye and wholegrain wheat groups at the different phases of the dietary intervention.

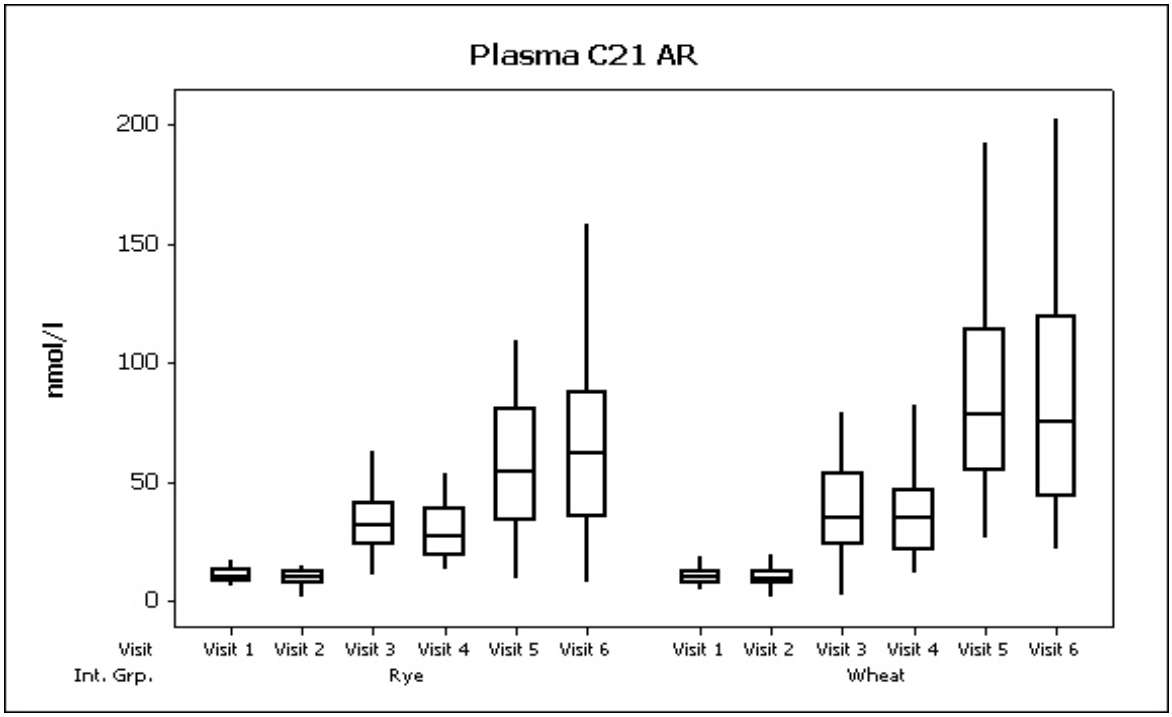


Figure 3.5 Plasma C23:0 AR concentrations in wholegrain rye and wholegrain wheat groups at the different phases of the dietary intervention.

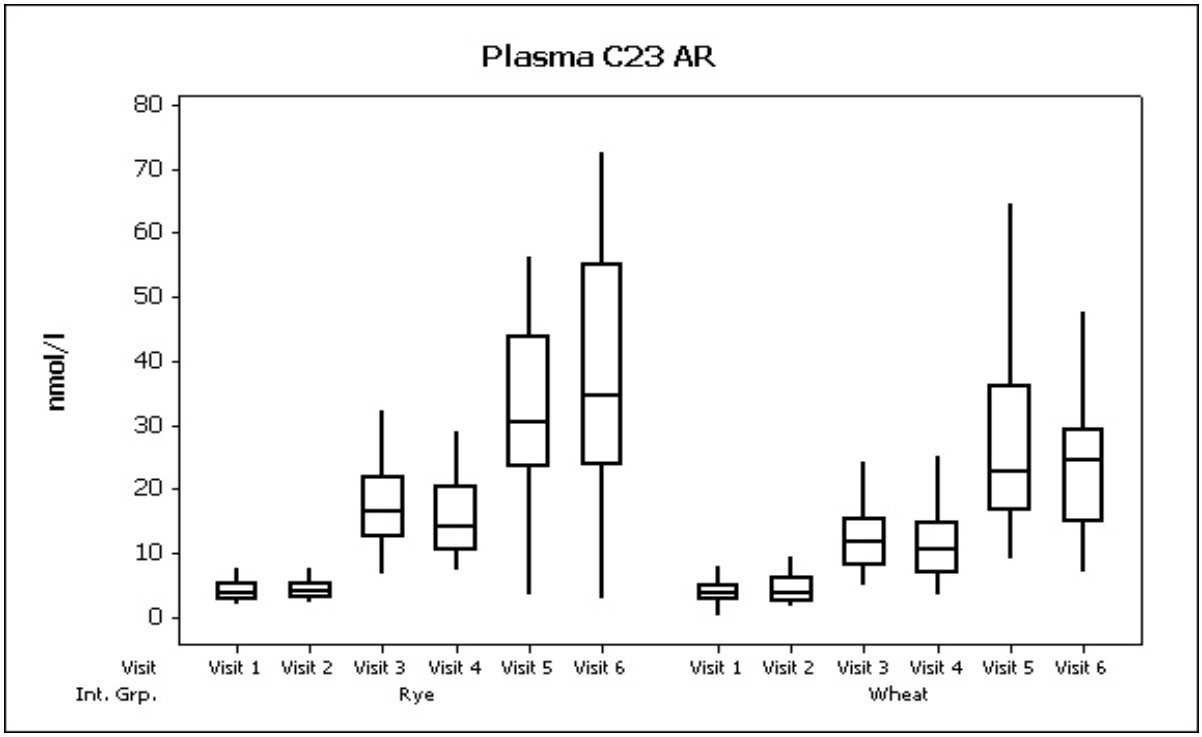


Figure 3.6 Plasma C25:0 AR concentrations in wholegrain rye and wholegrain wheat groups at the different phases of the dietary intervention.

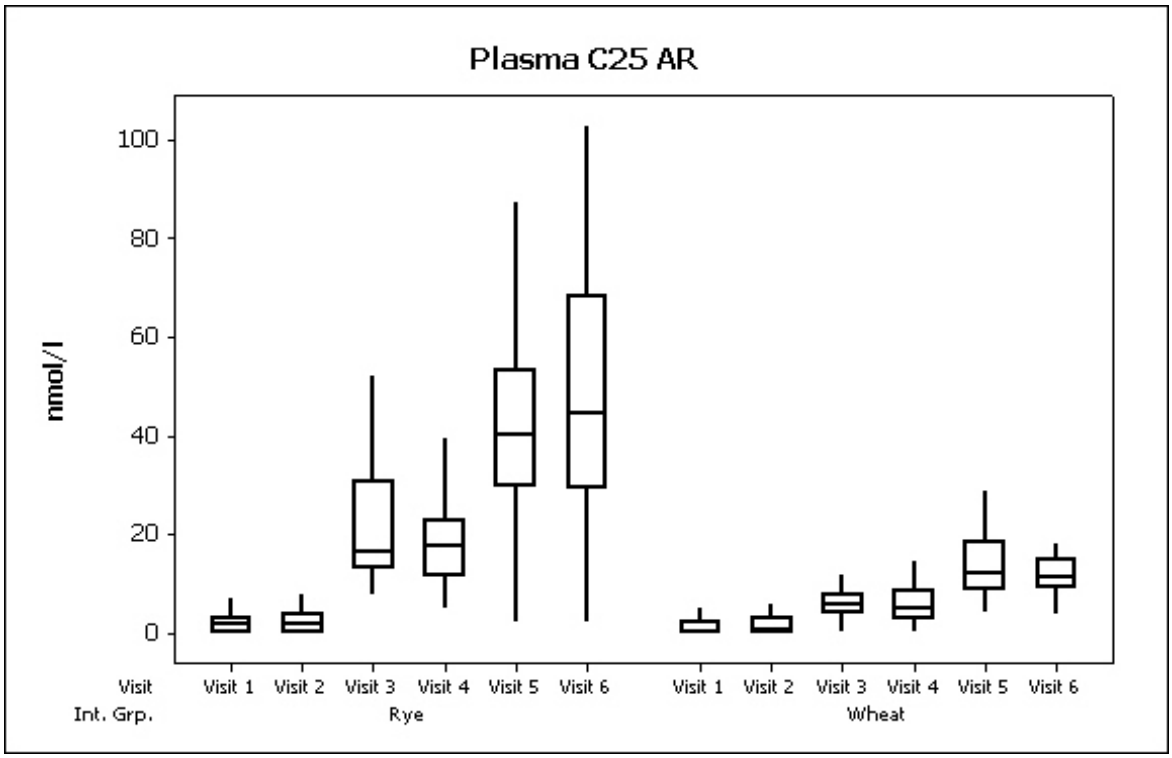


Figure 3.7 Plasma total AR concentrations in wholegrain rye and wholegrain wheat groups at the different phases of the dietary intervention.

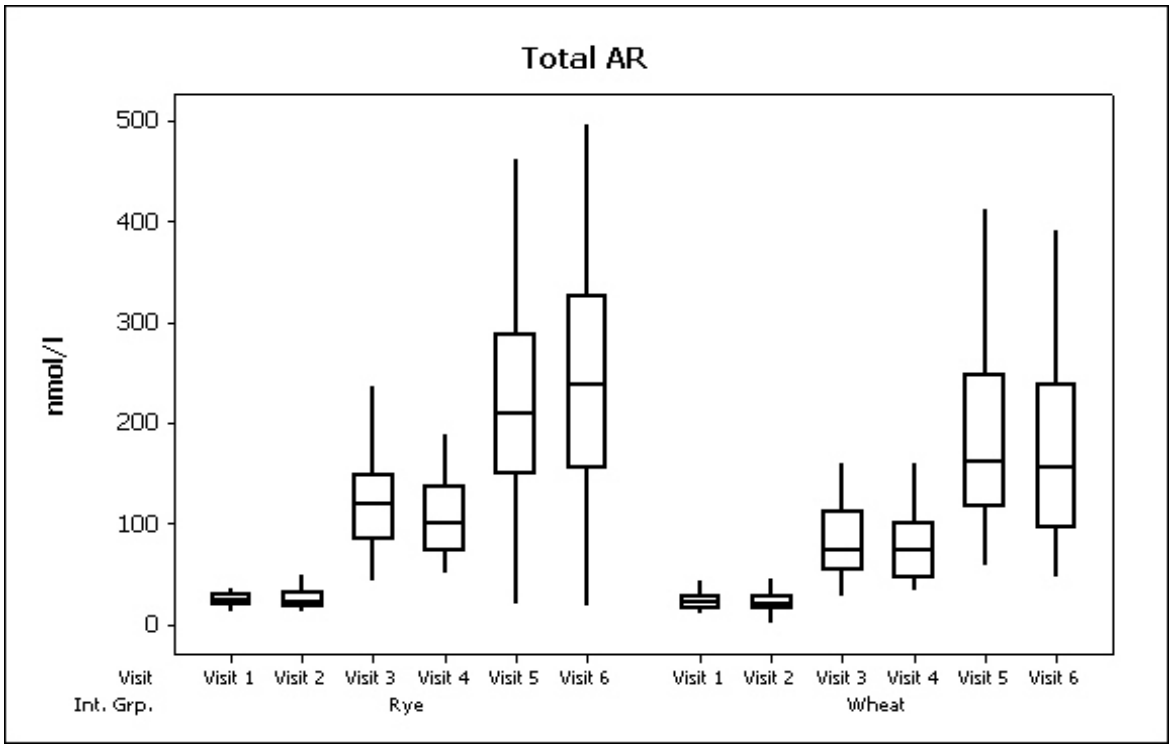
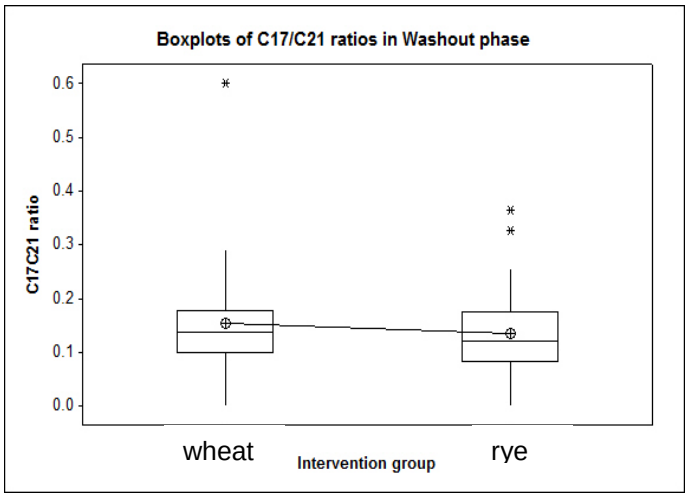
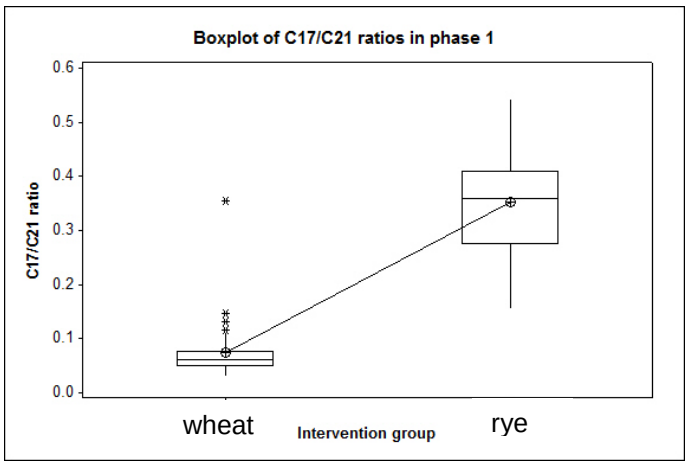


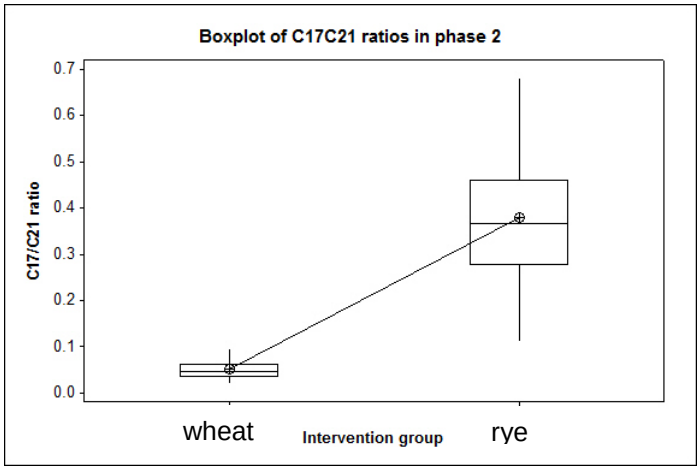
Figure 3.8 (a-c) Plasma C17:0 to C21:0 AR ratio for wholegrain rye and wholegrain wheat groups at the different phases of dietary intervention.



a). Mean plasma C17:0 to C21:0 AR ratio at the end of wash-out (0 servings WG/d) period



b). Mean plasma C17:0 to C21:0 AR ratio at the end of Dose 1 (3 servings WG/d) period



c). Mean plasma C17:0 to C21:0 AR ratio at the end of Dose 2 (6 servings WG/d) period

3.4.2 Plasma Mammalian Lignan Concentrations

As with plasma AR measurements above, the data for plasma mammalian lignan concentrations had a skewed distribution. Therefore the results are presented as inter-quartile range and median (horizontal line in each box plot) as shown in Figures 3.9 to 3.11. In general, for plasma lignan concentrations, the variations between wholegrain doses were nearly as great as the variations within the duplicate visits, particularly in the wheat group. This variability was greater for enterodiol (END, Figure 3.10) than for enterolactone (ENL, Figure 3.9). There were no significant differences in ENL, END or total plasma lignan concentrations between duplicate visits at each stage of the dietary intervention, as measured using paired samples t-tests.

Figure 3.9 Plasma ENL concentrations in wholegrain rye and wholegrain wheat groups at various phases of dietary intervention.

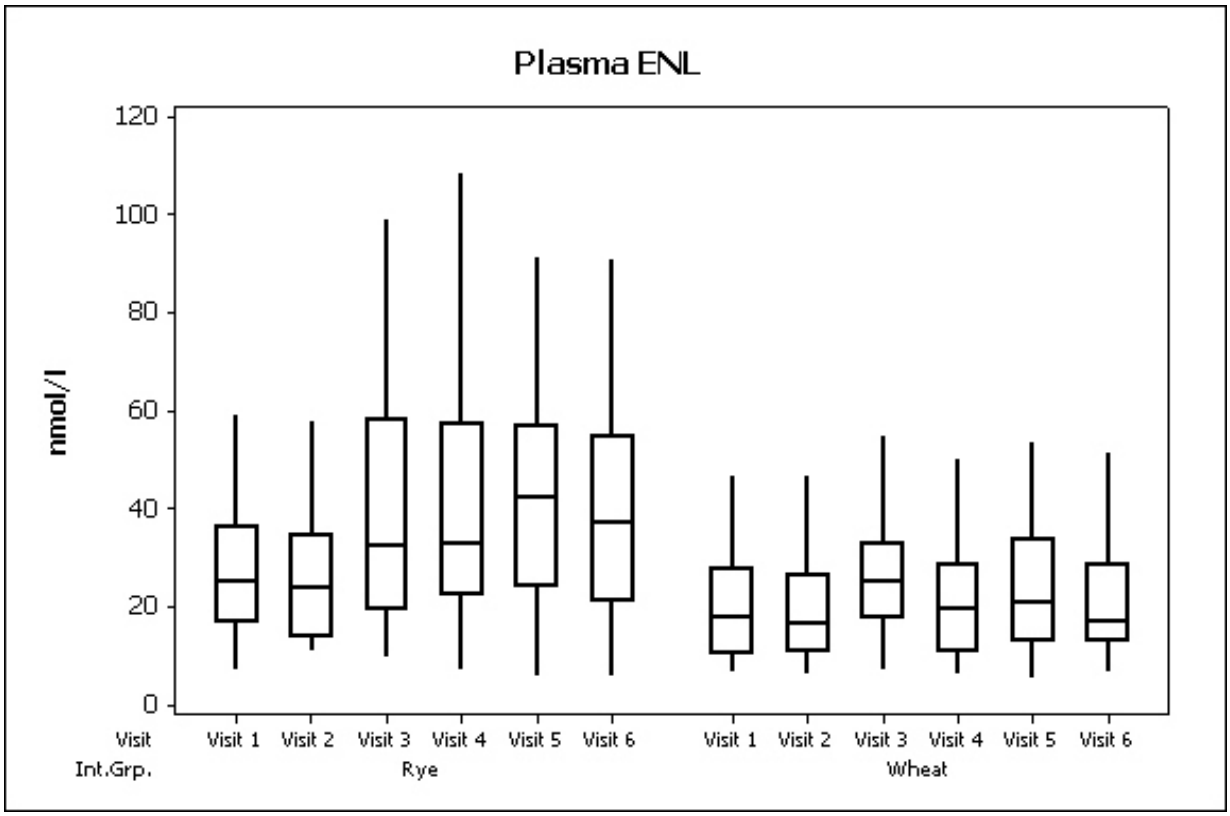


Figure 3.10 Plasma END concentrations in wholegrain rye and wholegrain wheat groups at various phases of dietary intervention.

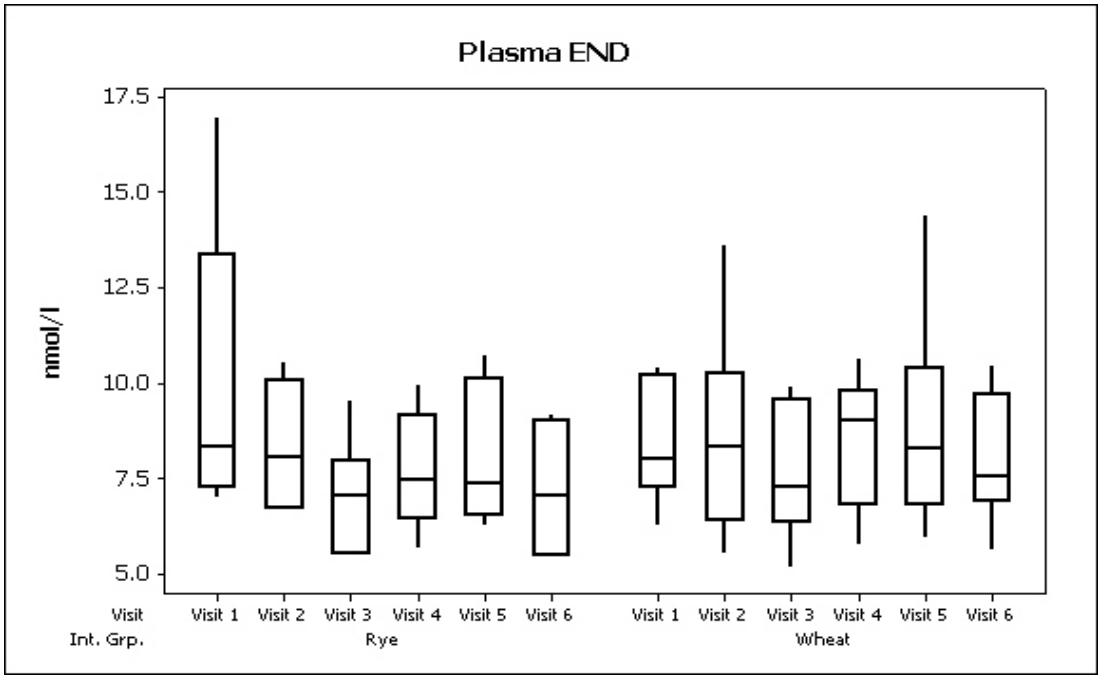
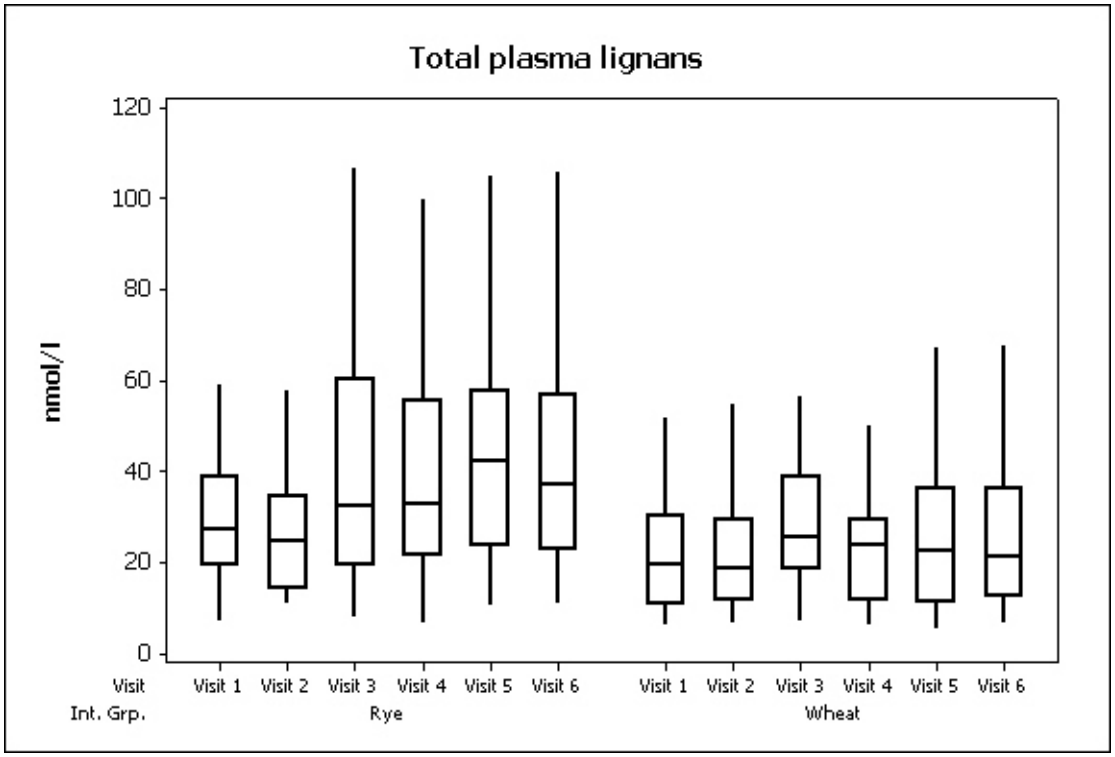


Figure 3.11 Plasma total lignans concentrations in wholegrain rye and wholegrain wheat groups at various phases of dietary intervention.



3.4.3 24 hour Urine Mammalian Lignan Concentrations

The data for 24 h urine ENL, END and total lignan concentrations are shown in Figures 3.12, 3.13 and 3.14 respectively. Since 24 h urine samples were collected only once after each phase of dietary intervention, only one set of data is shown. The concentrations of the two mammalian lignans in urine were much greater (more than 50 fold) than those in plasma. The concentration of lignans in urine were also much higher in response to increasing whole grain-intake in both diet groups compared with plasma lignans.

Figure 3.12 Urine (24 h) ENL concentrations in wholegrain rye and wholegrain wheat groups at various phases of dietary intervention.

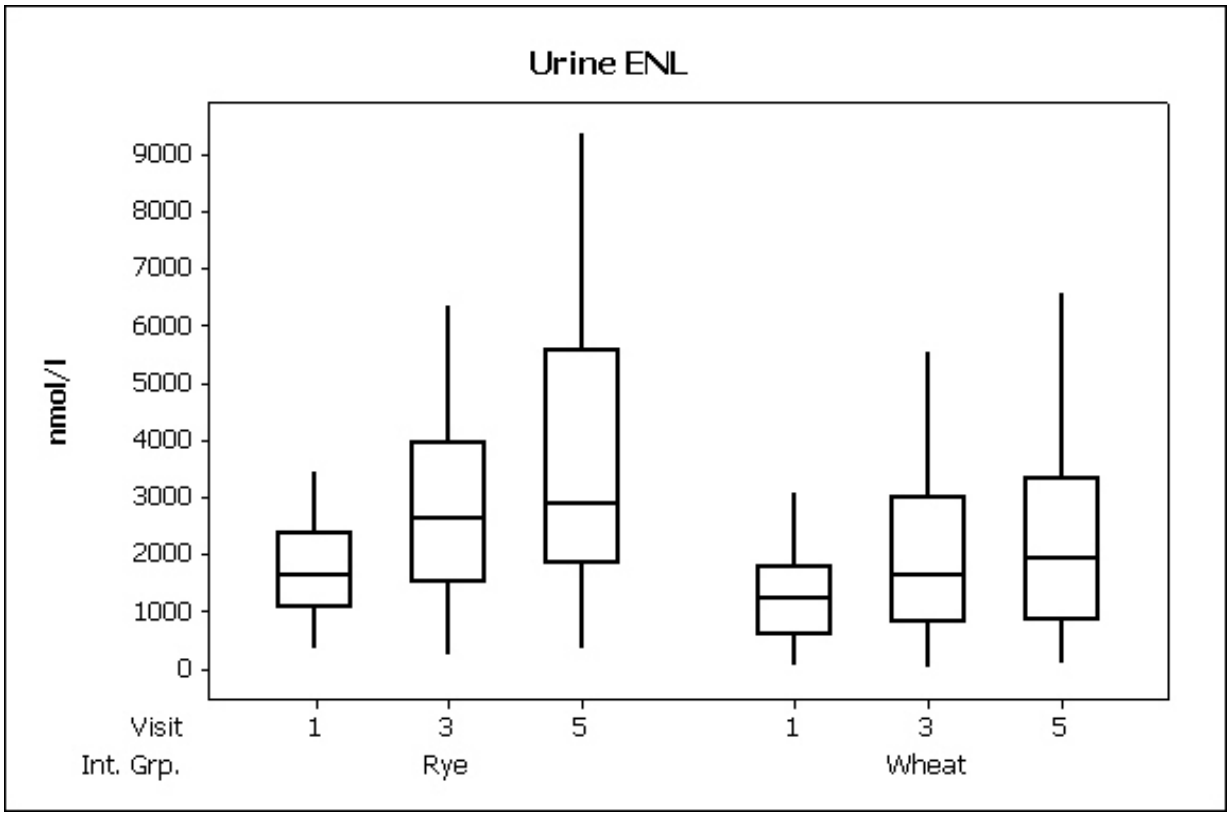


Figure 3.13 Urine (24 h) END concentrations in wholegrain rye and wholegrain wheat groups at various phases of dietary intervention.

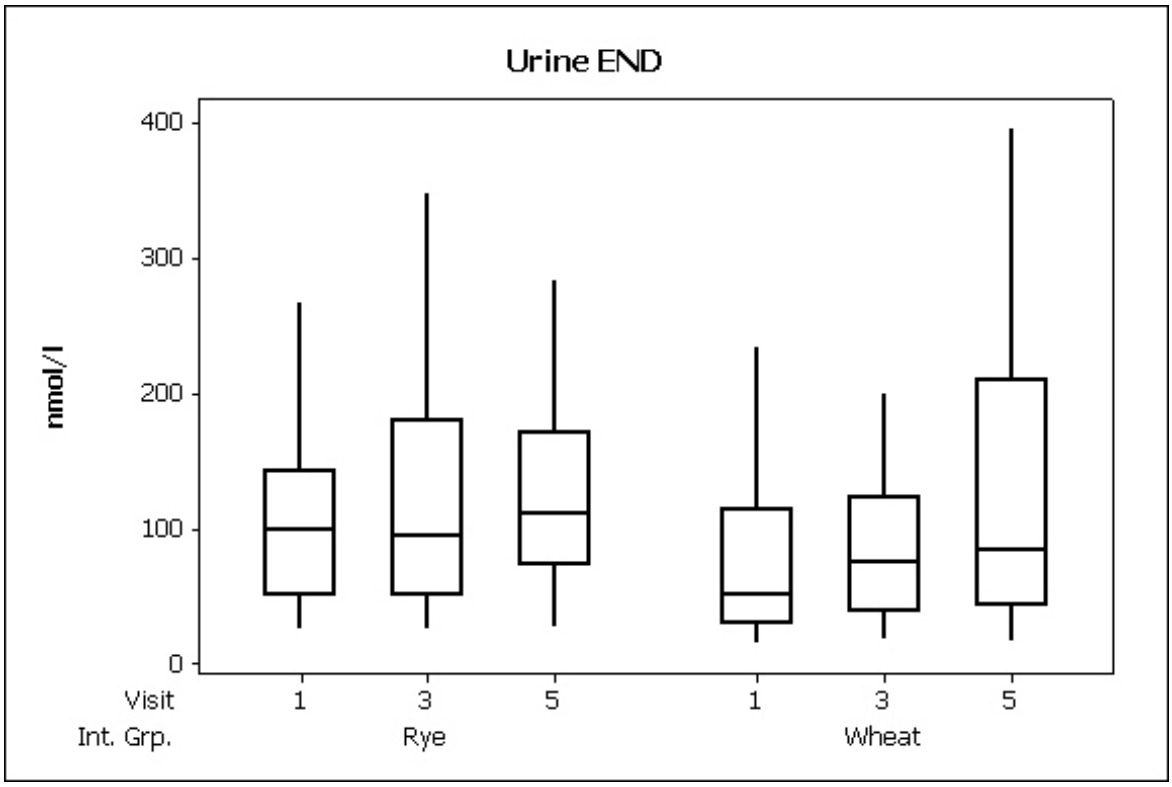
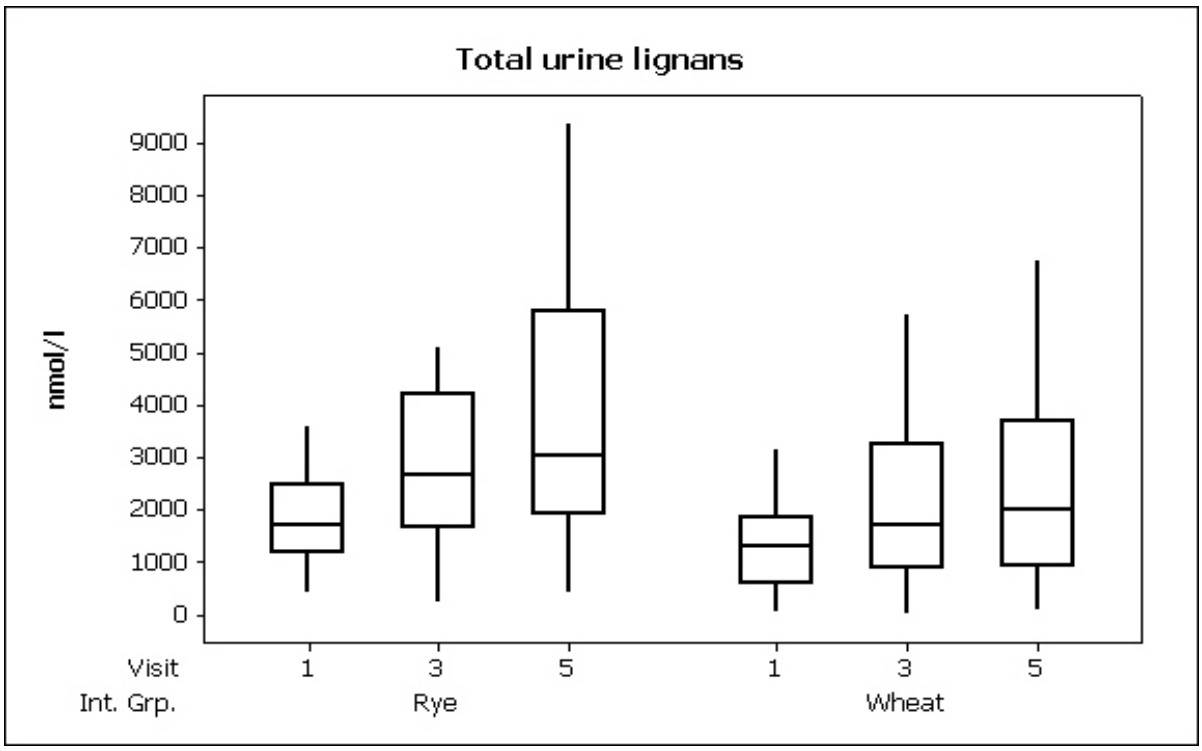


Figure 3.14 Urine (24 h) total lignan concentrations in wholegrain rye and wholegrain wheat groups at various phases of dietary intervention.



3.5 Dose-response trends (associations) between mean dietary whole grain/AR intake for each period of dietary intervention and the concentrations of biomarkers (in plasma/urine)

The mean daily intake of whole grain and AR for each phase (chronic intake) of dietary intervention were estimated with FFQ and food record forms respectively, and using linear mixed effect models (as described previously), and the dose-response trends were investigated, adjusting for various potential confounders including age, gender and BMI.

3.5.1 Whole grain-intake and plasma AR concentration

Figures 3.15 to 3.20 show separate regression plots indicating changes in plasma concentrations of separate (as well as total) AR concentrations with increasing wholegrain rye or wholegrain wheat intake, as assessed using the FFQ. There were significant linear dose-response trend between wholegrain rye or wholegrain wheat intake and all individual AR and total AR (all $p<0.0001$). The correlation coefficients (R^2) for the dose-respond trend for all AR were much greater in the wholegrain wheat group than the wholegrain rye group (Figures 3.15 to 3.20). Similarly, the dose-response relationship variability, as indicated by the number of points falling outside the 95% confidence interval of the regression line, indicates greater variability in the wholegrain rye group than the wholegrain wheat group for all plasma AR. However, it should be noted that the slopes of the regression line for the majority of AR (except for C21:0) were greater in the wholegrain rye group than the wholegrain wheat group, reflecting the higher AR content (except for C21:0) in rye foods than in wheat foods (see Tables 3.6 and 3.7).

Figure 3.15 Regression plots of plasma C17:0 AR concentrations versus mean whole grain-intake (AVDI; FFQ data) for wholegrain rye and wholegrain wheat groups.

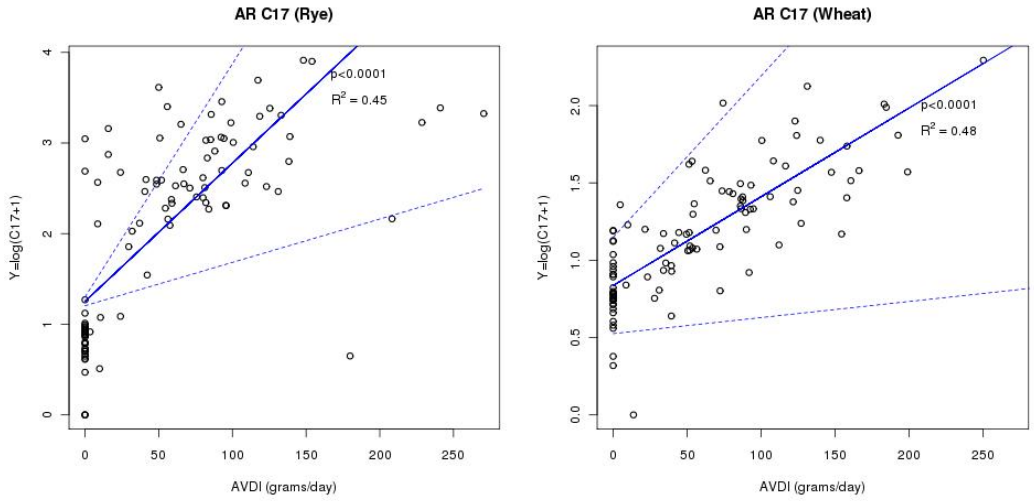


Figure 3.16 Regression plots of plasma C19:0 AR concentrations versus mean whole grain-intake (AVDI; FFQ data) for wholegrain rye and wholegrain wheat groups.

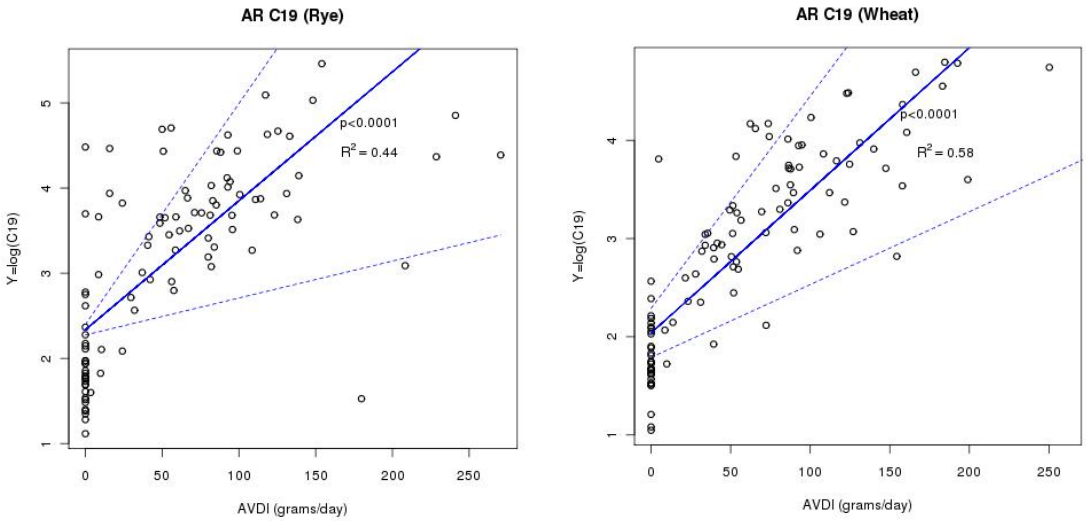


Figure 3.17 Regression plots of plasma C21:0 AR concentrations versus mean whole grain-intake (AVDI; FFQ data) for wholegrain rye and wholegrain wheat groups.

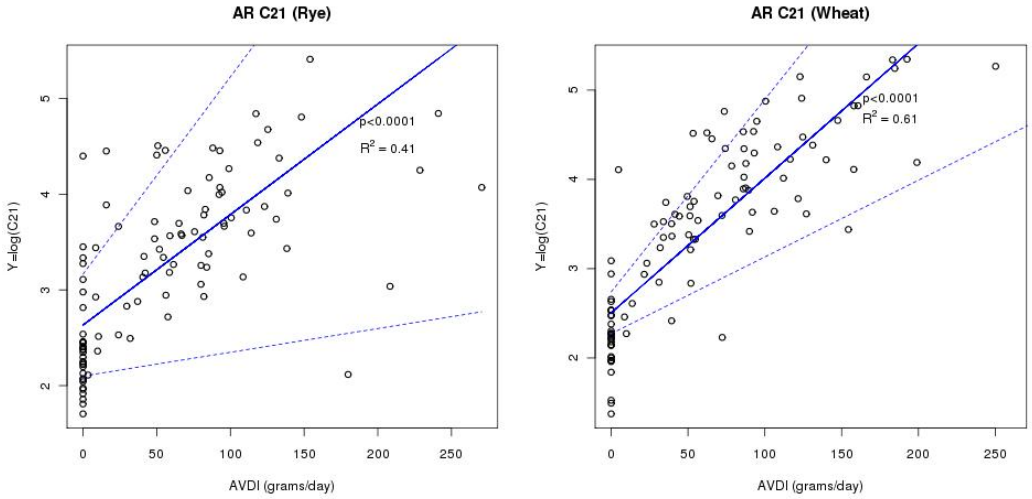


Figure 3.18 Regression plots of plasma C23:0 AR concentrations versus mean whole grain-intake (AVDI; FFQ data) for wholegrain rye and wholegrain wheat groups.

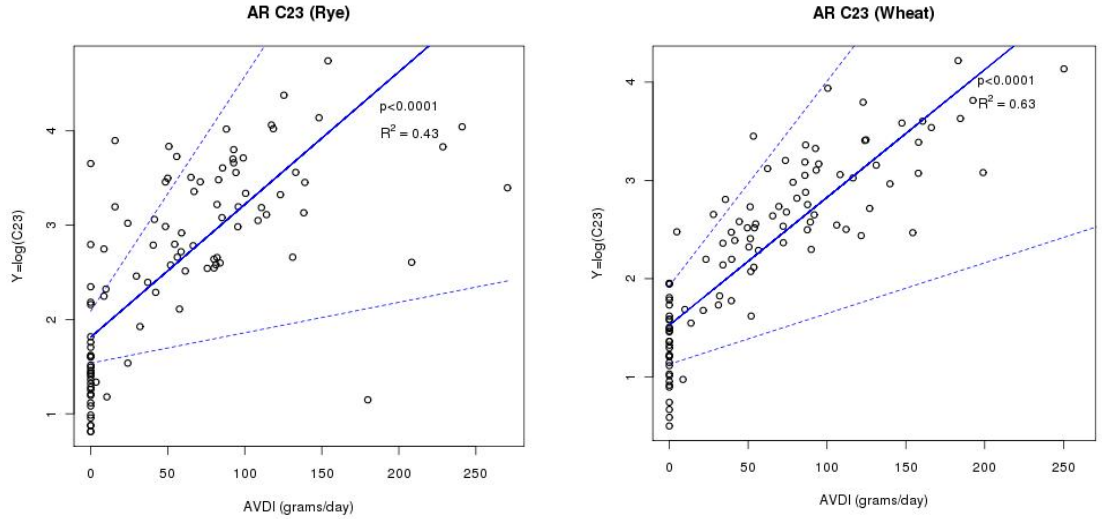


Figure 3.19 Regression plots of plasma C25:0 AR concentrations versus mean whole grain-intake (AVDI; FFQ data) for wholegrain rye and wholegrain wheat groups.

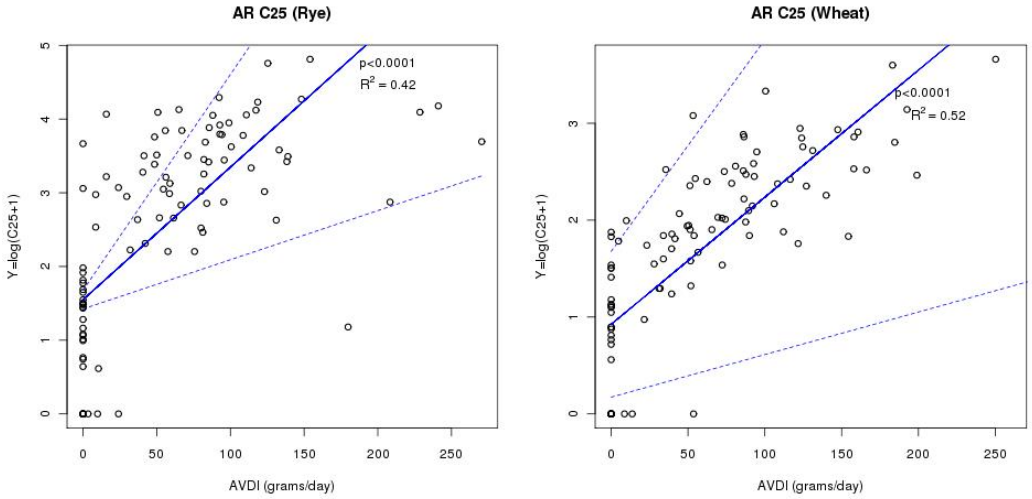
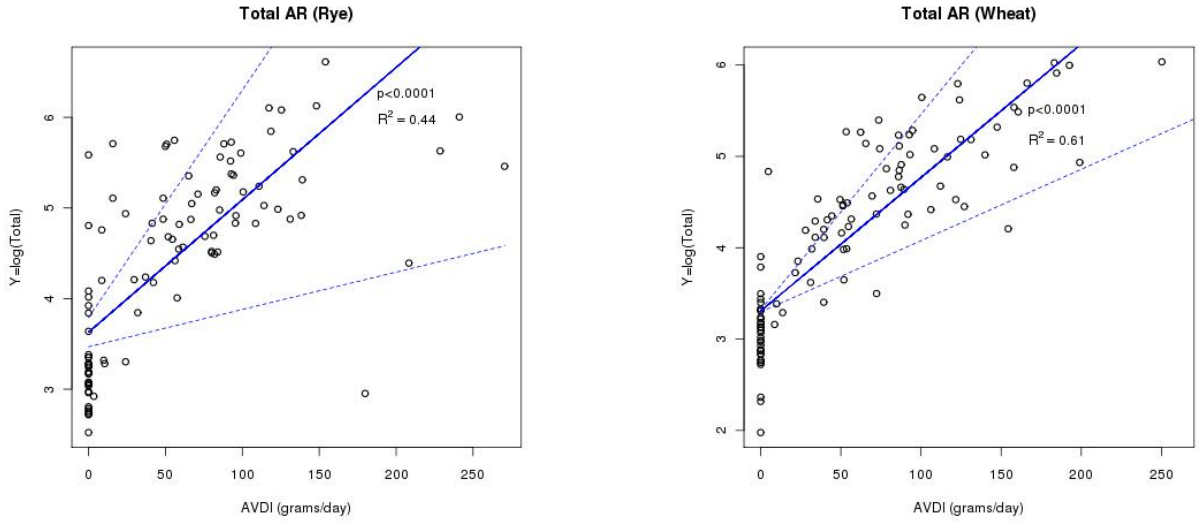


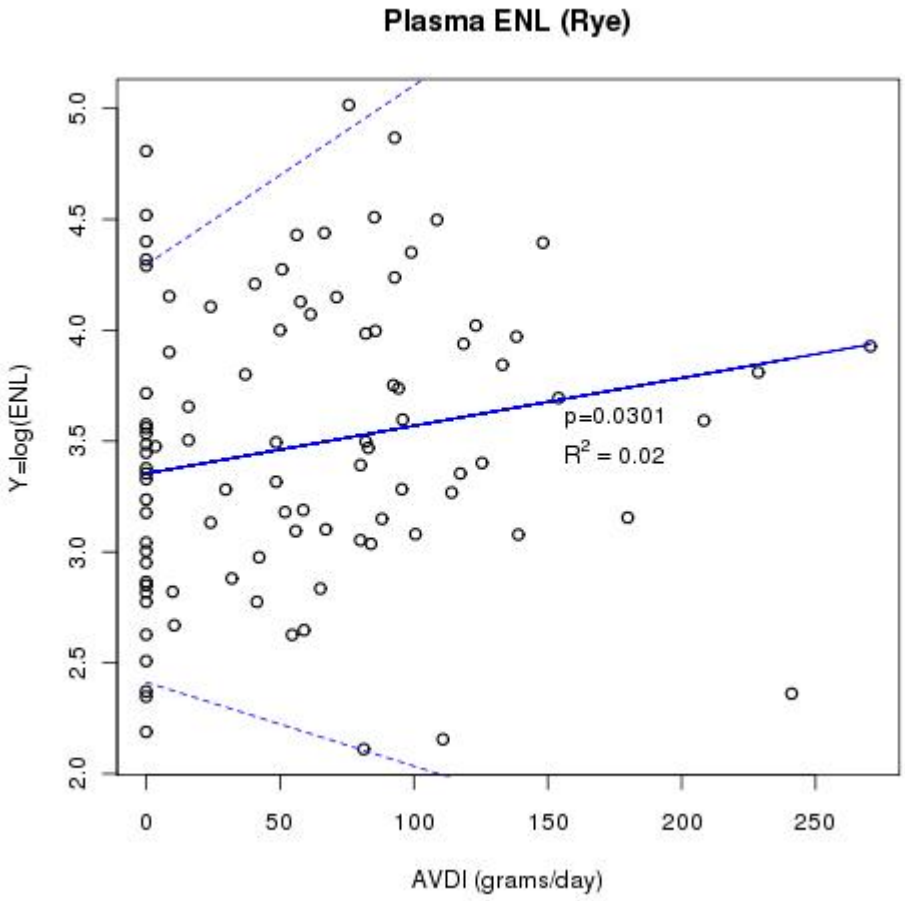
Figure 3.20 Regression plots of plasma total AR concentrations versus mean whole grain-intake (AVDI; FFQ data) for wholegrain rye and wholegrain wheat groups.



3.5.2 Whole grain-intake and plasma lignan concentration

There were no significant (linear or otherwise) dose-response trends between mean wholegrain wheat intake (using FFQ) and plasma lignans, including plasma enterolactone (ENL), plasma enterodiol (END) and plasma total lignans (ENL+END). However, intake of wholegrain rye foods were significantly associated with plasma ENL ($p<0.05$) but not END or total lignans. Figure 3.21 shows the regression plot indicating changes in plasma concentrations of ENL concentrations with increasing wholegrain rye intake.

Figure 3.21 Regression plot of plasma total enterolactone (ENL) concentrations versus mean whole grain-intake (AVDI; FFQ data) for the wholegrain rye group.



3.5.3 Wholegrain intake and 24h urine lignan concentration/excretion

No significant (linear or otherwise) dose-response trends could be found between mean whole grain-intake (in both groups) and 24 h-urinary END concentration as well as excretion. However, there were significant positive linear dose-response trends between wholegrain rye intake and urinary ENL concentration/excretion (both $p<0.001$) as shown in Figure 3.22. There were also significant positive linear dose-response trends between wholegrain rye intake and total urinary lignan concentration/excretion (both $p<0.0001$) as shown in Table 3.23. Similar to the rye group, there were significant positive linear dose-response trends between wholegrain wheat intake (using FFQ) and urinary ENL concentration/excretion (both $p<0.01$) and with total lignan concentration/excretion (both $p<0.01$) as shown in Figures 3.24 and 3.25 respectively.

Figure 3.22 Regression plots of ENL concentration and excretion versus mean whole grain-intake (AVDI; FFQ data) for the wholegrain rye group.

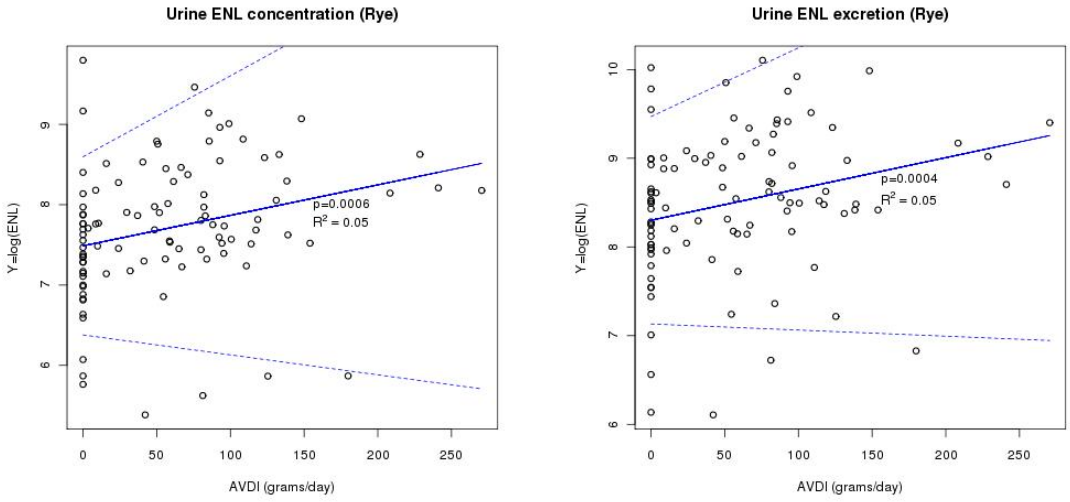


Figure 3.23 Regression plots of total lignan concentration and excretion versus mean whole grain-intake (AVDI; FFQ data) for the wholegrain rye group.

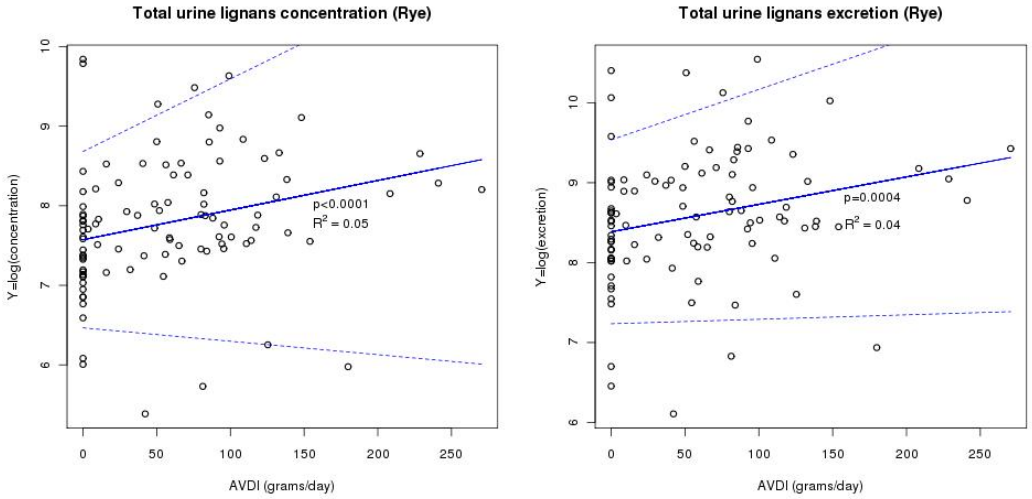


Figure 3.24 Regression plots of ENL concentration and excretion versus mean whole grain-intake (AVDI; FFQ data) for the wholegrain wheat group.

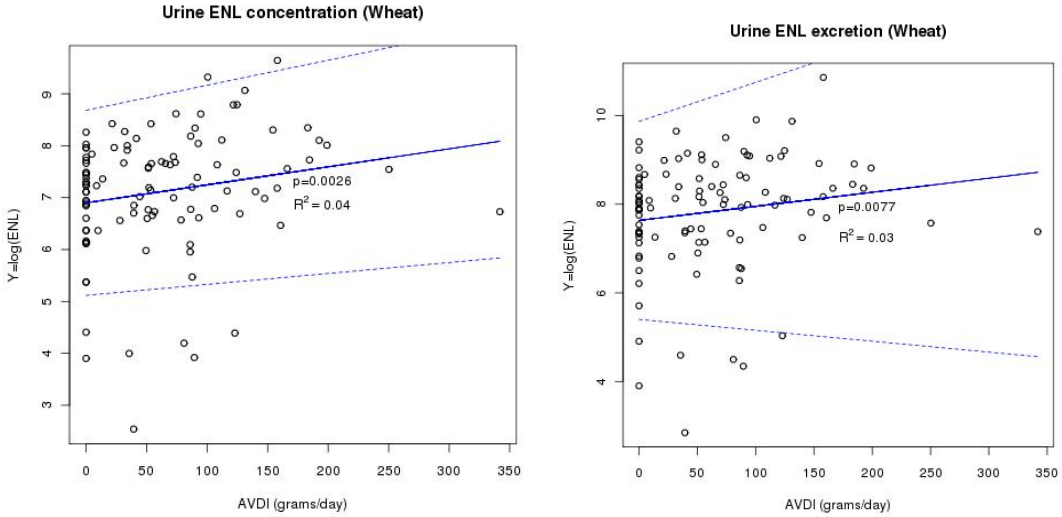
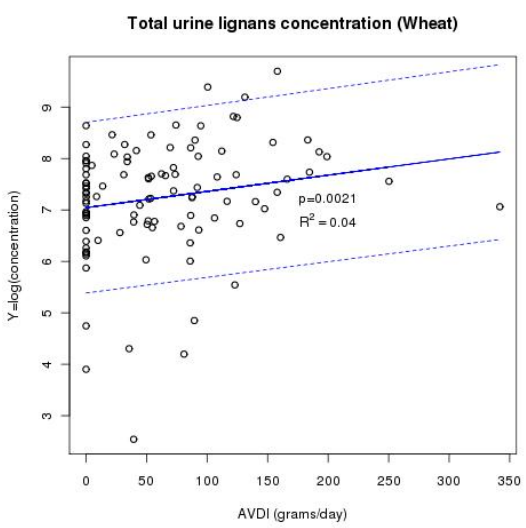
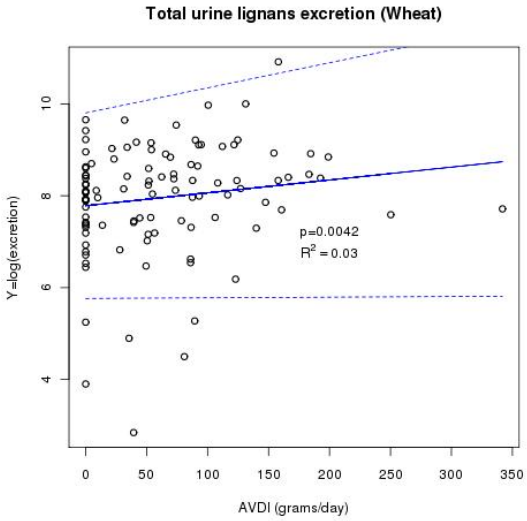


Figure 3.25 Regression plots of total lignan concentration and excretion versus mean whole grain-intake (AVDI; FFQ data) for the wholegrain wheat group.





3.5.4 Mean AR intake and plasma AR concentration

As with whole grain-intake, there were significant positive linear dose-response trends between the mean AR intake of separate homologues at the end of Dose 1 and Dose 2 periods of dietary intervention and their corresponding AR concentration in plasma (all $p<0.0001$) as shown in Figures 3.26 to 3.31.

Figure 3.26 Regression plots of plasma C17:0 AR concentrations versus mean C17:0 AR intake for wholegrain rye and wholegrain wheat groups.

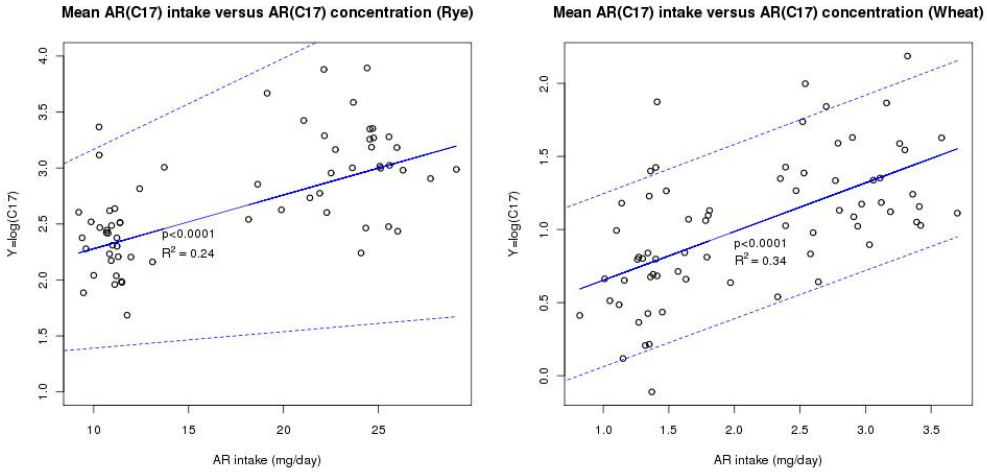


Figure 3.27 Regression plots of plasma C19:0 AR concentrations versus mean C19:0 AR intake for wholegrain rye and wholegrain wheat groups.

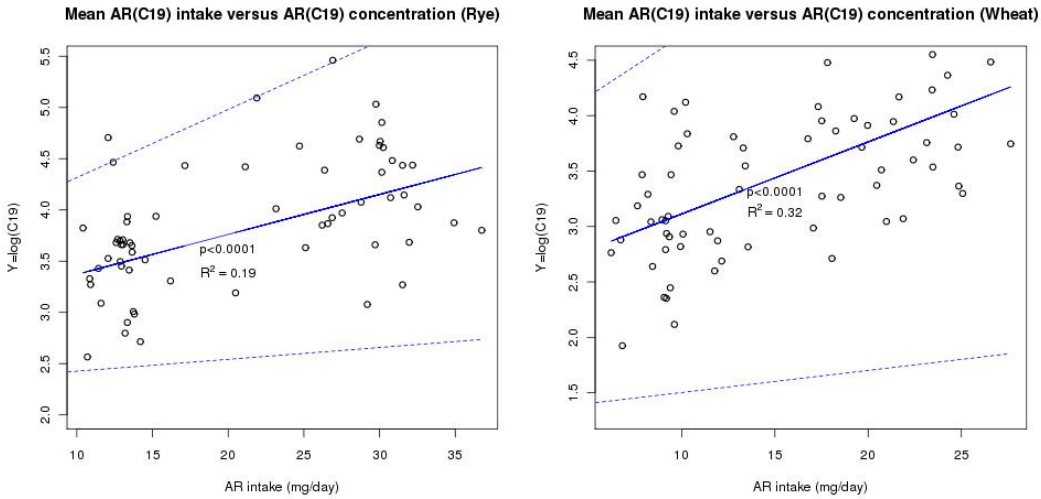


Figure 3.28 Regression plots of plasma C21:0 AR concentrations versus mean C21:0 AR intake for wholegrain rye and wholegrain wheat groups.

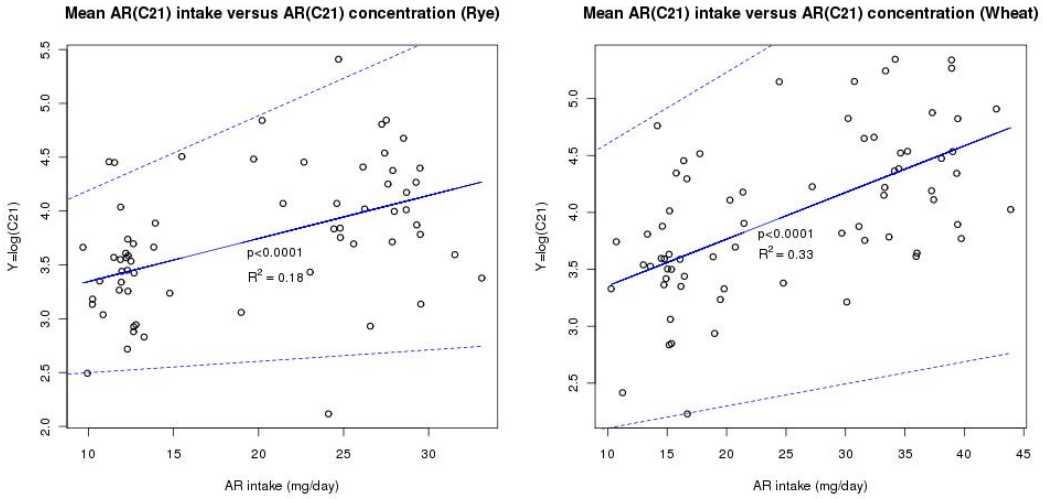


Figure 3.29 Regression plots of plasma C23:0 AR concentrations versus mean C23:0 AR intake for wholegrain rye and wholegrain wheat groups.

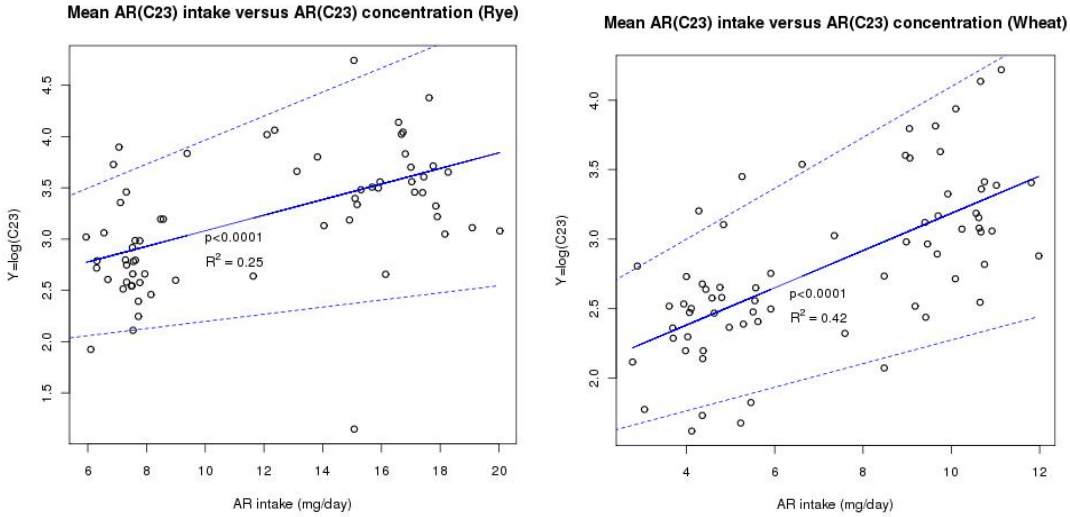


Figure 3.30 Regression plots of plasma C25:0 AR concentrations versus mean C25:0 AR intake for wholegrain rye and wholegrain wheat groups.

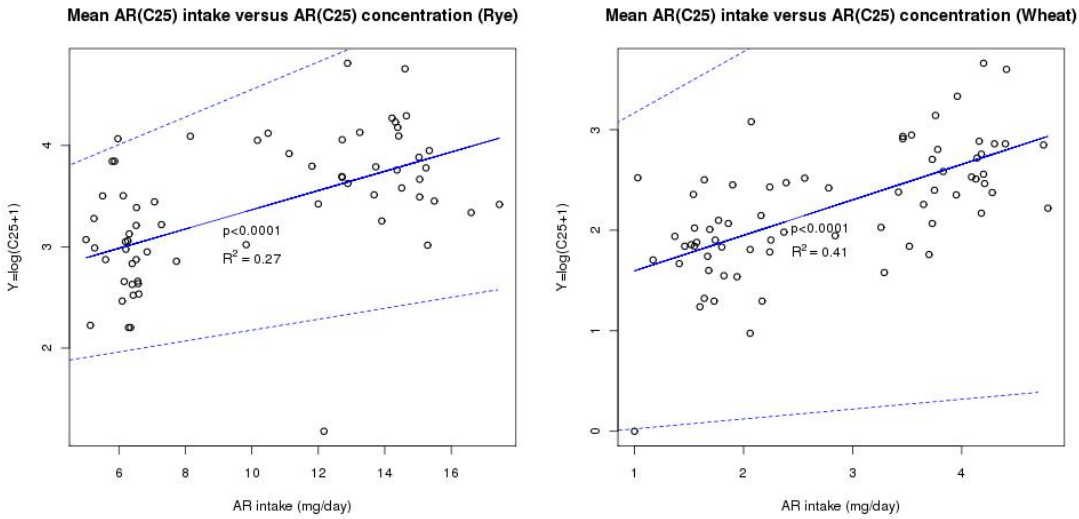
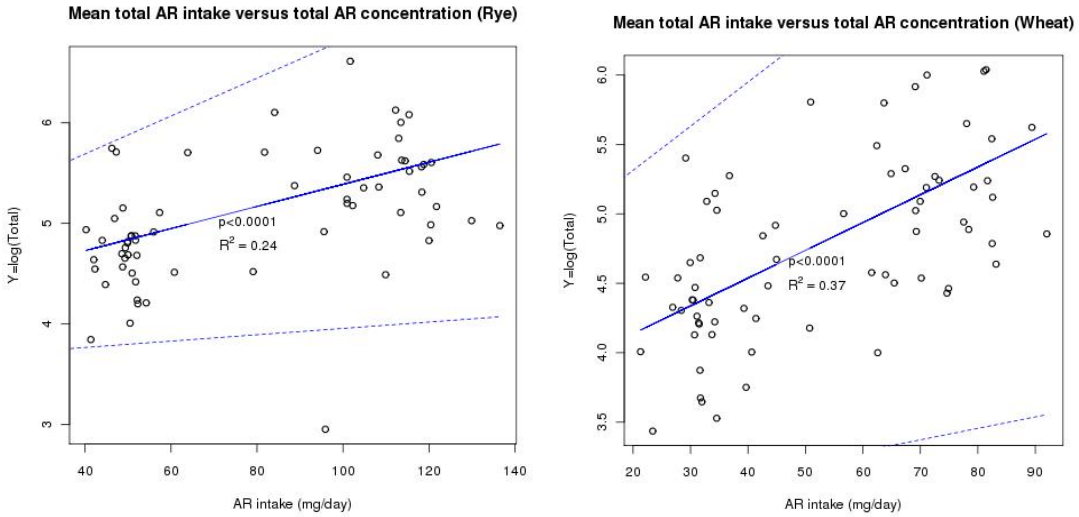


Figure 3.31 Regression plots of plasma total AR concentrations versus mean total AR intake for wholegrain rye and wholegrain wheat groups.



3.6 Dose-response trend comparisons between pre-visit dietary whole grain/AR intake with mean whole grain/AR intake for each phase and the concentrations of biomarkers in plasma and urine

The 'pre-visit' (the day before blood/urine sampling) intake of whole grain/AR during Dose1 and Dose 2 were calculated with food record forms and using linear mixed effect models (described previously), the dose-response trends were investigated, adjusting for potential confounders including age, gender and BMI. This enabled the comparisons of the dose-response trends between pre-visit dietary whole grain/AR intake and mean whole grain/AR intake against the concentrations/excretions of biomarkers in plasma and urine. The results for whole grain-intake are shown in Tables 3.8 and 3.9 for each diet group respectively and the results for AR intake are shown in Tables 3.10 and 3.11.

Table 3.8 Comparisons of dose-response trends between pre-visit whole grain-intake and mean whole grain-intake against measured biomarkers for the wholegrain wheat group.

Biomarker	Pre-visit WG intake			Mean WG intake		
	Slope (ln)	R^2	p value	Slope (ln)	R^2	p value
Plasma AR C17:0	+0.007	0.310	<0.0001	+0.006	0.480	<0.0001
Plasma AR C19:0	+0.013	0.267	<0.0001	+0.015	0.578	<0.0001
Plasma AR C21:0	+0.014	0.289	<0.0001	+0.015	0.607	<0.0001
Plasma AR C23:0	+0.013	0.374	<0.0001	+0.013	0.629	<0.0001
Plasma AR C25:0	+0.013	0.350	<0.0001	+0.013	0.517	<0.0001
Plasma AR total	+0.013	0.315	<0.0001	+0.015	0.612	<0.0001
Plasma ENL	-	-	NS	-	-	NS
Plasma END	-	-	NS	-	-	NS
Plasma total lignans	-	-	NS	-	-	NS
24h urine ENL conc.	-	-	NS	+0.004	0.039	0.0022
24h urine END conc.	-	-	NS	-	-	NS
24h urine total lignans conc.	+0.006	0.017	0.044	+0.003	0.044	0.0019
24h urine ENL excr.	-	-	NS	+0.003	0.028	0.0048
24h urine END excr.	-	-	NS	-	-	NS
24h urine total lignans excr.	-	-	NS	+0.003	0.031	0.0031

Table 3.9 Comparisons of dose-response trends between pre-visit whole grain-intake and mean whole grain-intake against measured biomarkers for the wholegrain rye group.

Biomarker	Pre-visit WG intake			Mean WG intake		
	Slope (ln)	R^2	p value	Slope (ln)	R^2	p value
Plasma AR C17:0	+0.010	0.276	<0.0001	+0.015	0.454	<0.0001
Plasma AR C19:0	+0.011	0.184	<0.0001	+0.015	0.435	<0.0001
Plasma AR C21:0	+0.010	0.179	<0.0001	+0.012	0.406	<0.0001
Plasma AR C23:0	+0.012	0.272	<0.0001	+0.014	0.431	<0.0001
Plasma AR C25:0	+0.013	0.305	<0.0001	+0.018	0.417	<0.0001
Plasma AR total	+0.011	0.245	<0.0001	+0.015	0.444	<0.0001
Plasma ENL	-	-	NS	+0.002	0.020	0.0301
Plasma END	-	-	NS	-	-	NS
Plasma total lignans	-	-	NS	-	-	NS
24h urine ENL conc.	+0.003	0.023	0.0259	+0.004	0.054	0.0004
24h urine END conc.	-	-	NS	-	-	NS
24h urine total lignans conc.	+0.004	0.036	0.010	+0.004	0.050	<0.0001
24h urine ENL excr.	+0.004	0.029	0.020	+0.003	0.046	0.0004
24h urine END excr.	-	-	NS	-	-	NS
24h urine total lignans excr.	+0.004	0.043	0.0071	+0.003	0.043	0.0005

Table 3.10 Comparison of dose-response trends between pre-visit AR intake and mean AR intake of specific AR homologues against plasma concentrations of the corresponding AR homologue for the wholegrain wheat group.

Biomarker	**Pre-visit AR intake (corresponding AR homologue)**			**Mean AR intake (corresponding AR homologue)**		
	Slope (ln)	**R^2**	**p value**	**Slope (ln)**	**R^2**	**p value**
Plasma AR C17:0	+0.208	0.331	<0.0001	+0.333	0.341	<0.0001
Plasma AR C19:0	+0.046	0.243	<0.0001	+0.065	0.324	<0.0001
Plasma AR C21:0	+0.031	0.240	<0.0001	+0.041	0.334	<0.0001
Plasma AR C23:0	+0.107	0.336	<0.0001	+0.134	0.421	<0.0001
Plasma AR C25:0	+0.279	0.324	<0.0001	+0.353	0.407	<0.0001
Plasma AR total	+0.014	0.270	<0.0001	+0.019	0.366	<0.0001

Table 3.11 Comparison of dose-response trends between pre-visit AR intake and mean AR intake of specific AR homologues against plasma concentrations of the corresponding AR homologue for the wholegrain rye group.

Biomarker	**Pre-visit AR intake (corresponding AR homologue)**			**Mean AR intake (corresponding AR homologue)**		
	Slope (ln)	**R^2**	**p value**	**Slope (ln)**	**R^2**	**p value**
Plasma AR C17:0	+0.042	0.231	<0.0001	+0.048	0.241	<0.0001
Plasma AR C19:0	+0.035	0.149	<0.0001	+0.039	0.191	<0.0001
Plasma AR C21:0	+0.037	0.147	<0.0001	+0.040	0.180	<0.0001
Plasma AR C23:0	+0.071	0.217	<0.0001	+0.076	0.251	<0.0001
Plasma AR C25:0	+0.088	0.265	<0.0001	+0.095	0.271	<0.0001
Plasma AR total	+0.010	0.204	<0.0001	+0.011	0.238	<0.0001

3.7 Correlations between biomarkers

Bivariate correlations (with pairwise exclusion) were used to determine correlations between the main biomarkers for every individual and all time points. The data were normalised using log transformation before performing statistical analyses. As shown in Tables 3.12 and 3.13, there were significant positive correlations between a) plasma AR and plasma/urine lignans, b) plasma lignan concentrations and urinary lignan concentrations/excretion, and c) urinary lignan concentrations and urine lignan excretion in both diet groups.

Table 3.12 Bivariate correlations between biomarkers in the wholegrain wheat group.

Biomarker	Plasma total AR conc.	Plasma total lignans conc.	Urine (24h) total lignan conc.	Urine (24h) total lignan exc.
Plasma total AR conc.	R=1	R=0.162 p=0.022	R=0.247 p=0.011	R=0.193 p=0.049
Plasma total lignans conc.	R=0.162 p=0.022	R=1	R=0.608 p<0.0001	R=0.608 p<0.0001
Urine (24h) total lignan conc.	R=0.247 p=0.011	R=0.608 p<0.0001	R=1	R=0.958 p<0.0001
Urine (24h) total lignan exc.	R=0.193 p=0.049	R=0.608 p<0.0001	R=0.958 p<0.0001	R=1

Table 3.13 Bivariate correlations between biomarkers in the wholegrain rye group.

Biomarker	Plasma total AR conc.	Plasma total lignans conc.	Urine (24h) total lignan conc.	Urine (24h) total lignan exc.
Plasma total AR conc.	R=1	R=0.252 p<0.0001	R=0.343 p=0.001	R=0.333 p=0.001
Plasma total lignans conc.	R=0.252 p<0.0001	R=1	R=0.765 p<0.0001	R=0.785 p<0.0001
Urine (24h) total lignan conc.	R=0.343 p=0.001	R=0.765 p<0.0001	R=1	R=0.931 p<0.0001
Urine (24h) total lignan exc.	R=0.333 p=0.001	R=0.785 p<0.0001	R=0.931 p<0.0001	R=1

4.0 Discussion

4.1 Dietary Intervention

The project was successful in meeting objectives 01 to 04 as set out in Section 1.1. The recruitment target for the project was exceeded with 68 volunteers completing the study with almost equal numbers of male and female in both wholegrain rye and wholegrain wheat groups (Section 3.1). The dietary intervention was carried out successfully with minimal difficulty for the subjects, as indicated by the very low drop-out rate (<3%). The reported compliance to the dietary intervention regime has also been excellent as indicated by the recorded changes in wholegrain intake during the 3 intervention periods using both FFQ and food record forms (Tables 3.3 and 3.4 respectively). The choice of two breakfast cereals, pasta and wholegrain bread for each wholegrain diet (rye and wheat) group helped volunteers with compliance, particularly at the higher dose of intake (6 servings whole grain/d). The list of common wholegrain foods supplied at the start of the study also helped volunteers to avoid all wholegrain foods other than those provided as part of the study which also enabled a better control of whole grain-intake in volunteers during various stages of dietary intervention. No adverse effects were reported by any volunteers as a result of the wholegrain dietary intervention throughout the study. Finally, having pre-evening set meals before sampling visits probably also reduced intra-subject and inter-subject variation of the acute effects of the pre-visit evening meal on the measured analytes in biological samples.

4.2 Anthropometric Changes

The reduction of BMI following the wash-out period compared with their initial baseline measure in both diet groups was most likely because the volunteers were habitual consumers of wholegrain foods, and they did not replace these foods in their diet with non-wholegrain equivalents during the wash-out period. At recruitment the mean daily intake of whole grain by the study volunteers was approximately 54g of whole grain per day (equivalent to more than 3 x 16 g servings whole grain/d). This was much higher than previous data showing a population mean intake of 22.2 g whole grain/d for a North East UK population (median 12.3 g WG/d, 38% non-consumers, *n*=741 Seal CJ unpublished data). Therefore, these self-selected volunteers were probably not representative of the general NE population and may have had an interest in consuming wholegrain foods and/or healthy nutrition. Once the volunteers started to consume wholegrain foods again they regained some of the weight lost during the wash-out period during

the subsequent intervention periods of increasing wholegrain dose (Dose 1 and Dose 2 periods respectively).

4.3 Dietary Changes

The changes in dietary fibre intake (Tables 3.2 and 3.3) during the wash-out period and during the periods of increasing whole grain-intake were as expected. The increase in starch and carbohydrate intake in the wholegrain rye group but not in the wholegrain wheat group during the Dose 2 period (6 servings whole grain/d) may indicate that some of the rye wholegrain foods were eaten by the volunteers in addition to their normal diet, rather than substituting these foods for refined grain foods or other foods consumed as part of their diet. In contrast, the lack of changes in macronutrients or energy intake with wholegrain wheat foods (particularly at the higher dose) might indicate that these foods, being more common in the UK diet, were more easily substituted for other foods consumed as part of volunteers' normal diet. The dietary assessment using FFQ also found increased intake of several micronutrients with increasing whole grain-intake including thiamine, riboflavin, vitamin E, magnesium, manganese, iron, phosphorous and zinc, supporting previous findings that increase whole grain-intake in the diet could lead to greater intake of several micronutrients (Anderson *et al.*, 2000; Melanson *et al.*, 2006; Brownlee *et al.*, 2010)

The reported whole grain-intake of volunteers using FFQ and food record forms, (Tables 3.3 and 3.4), showed excellent compliance to the dietary intervention regime in both diet groups with less than 2 g /d of total whole grain-intake during the wash-out period and approximately 60 g/d and 120 g/d intake of whole grain in Dose 1 and Dose 2 periods, respectively as recorded using FFQ. Food record forms (Table 3.4) showed a slightly lower whole grain-intake for both Dose 1 and Dose 2 periods than that using FFQ, which could either be due to an overestimation of dietary intake in general using the FFQ method and/or better record keeping and accurate portion size estimation of study wholegrain foods (since these were already pre-weighed) using the food record forms. Furthermore, the volunteers successfully avoided all wholegrain foods other than those supplied during the study, since recorded intake of non-study wholegrain foods using the FFQ contributed to less than 2% of total whole grain-intake during Dose 1 and Dose 2 periods (Table 3.3). The individualised food record forms also enabled monitoring the intake and amounts of the different wholegrain foods supplied to each volunteer during Dose 1 and Dose 2 periods. These data were then used to calculate the intake of the different AR homologues during Dose 1 and Dose 2 periods as shown in Table 3.5, based on the measured AR contents

(Tables 3.6 and 3.7 and Ross & Kochhar, 2009). The intake of AR in both diet groups for both doses was much greater than the median estimated per capita consumption of AR intake in the UK of 12 mg/d (Ross *et al.*, 2005) with intakes in this study in the range of the 90-99th percentile previously reported for AR intake in the UK. The higher content of AR in wholegrain rye compared with wholegrain wheat reported here has been observed in previous publications (Ross *et al.*, 2003b; Kulawinek *et al.*, 2008; Kamal-Eldin *et al.*, 2009). The mean AR C17:0 to AR C21:0 intake ratios for wholegrain rye and wholegrain wheat groups were 0.9 and 0.1 respectively (Table 3.5), findings similar to those reported previously (Chen *et al.*, 2004; Linko-Parvinen *et al.*, 2007; Kulawinek *et al.*, 2008).

4.4 Plasma AR as biomarkers of wholegrain rye or wholegrain wheat intake

AR have been suggested as specific biomarkers of the intake of wholegrain foods (Ross *et al.*, 2004c). To our knowledge, the GrainMark study is the first to investigate the effects of increased dietary doses of wholegrain wheat and wholegrain rye foods on plasma AR concentrations in a UK population and the largest study of its type world wide. The significant linear dose-response between wholegrain rye and wholegrain wheat intake and all AR homologues and total AR in plasma (all $p<0.0001$), strongly support the use of plasma AR as 'concentration biomarkers' for wholegrain rye and wholegrain wheat intake. A similar observation was made in another dose-response study in a Swedish population which reported a significant increase in plasma AR and their metabolites in urine with increasing rye bran intake at 3 intake doses, equivalent to 7.5 g, 15 g and 30 g per day of rye bran (Landberg *et al.*, 2009a). The present study showed a dose-dependent increase with the two types of whole grain cereals that are rich in AR (rye and wheat) consumed separately. In another study by the same Swedish group, significantly higher plasma AR concentration were observed after consuming a combined wholegrain wheat/rye diet compared with an equivalent period of consuming a refined-grain diet (Landberg *et al.*, 2008), again supporting the use of AR as a suitable biomarker for whole grain-intake. Another crossover study also found significant increases in plasma, erythrocyte and lipoprotein AR following dietary intervention with wholegrain rye or wholegrain wheat crispbread (each lasting 1 week), with the wholegrain rye period resulting in almost twice the plasma concentration of AR compared with the wholegrain wheat period (Linko-Parvinen *et al.*, 2007). Plasma AR concentrations were well correlated ($r=0.57$) with normal wholegrain intake among Swiss subjects (Ross *et al.*, 2009), a notable finding as the population measured had a habitually low intake of whole grain, similar to what could be expected in the UK. These

observations were confirmed in the current study which showed significantly higher plasma AR concentrations for most AR homologues, except for AR C21:0, in the wholegrain rye group compared with the wholegrain wheat group during both Dose 1 and Dose 2 periods of dietary intervention. Linko-Parniven et al. (2007) also found significantly different plasma C17:0 to C21:0 ratios between the wholegrain rye and wheat periods; the lower ratio was confirmed in the wholegrain wheat group of the present study for both intake doses of the wholegrain foods (Dose 1 and Dose 2), although the ratios for a particular type of wholegrain diet were fairly consistent irrespective of the dose of whole grains. Thus plasma C17:0 to C21:0 AR homologue ratios could be used to discriminate between various types and/or contents of wholegrain diets (rye vs. wheat) as suggested previously (Linko & Adlercreutz, 2005; Linko-Parvinen *et al.*, 2007). It was noted in the present study that plasma AR concentrations for duplicate fasting plasma samples after each period of dietary intervention were reproducible for both grain groups and for all AR homologues as shown in Figures 3.2-3.7. This is important with regards to the application of plasma AR as biomarkers of whole grain-intake in epidemiological settings, which have limitations on the number of samples that can be collected from each individual participating in such studies. The high inter-day reproducibility of plasma AR has also been shown in a recent study (Landberg *et al.*, 2009b) which found significant intra-class correlation coefficient (ICC) between plasma AR from individuals collected 2 weeks apart in treatment periods of 6 weeks duration with wholegrain foods. The authors found that a single fasting plasma sample gave a precision of ± 20 %, with an 80 % confidence interval. Based on these figures, the authors suggested that a single fasting plasma sample is suitable for use as a short/medium term biomarker of wholegrain rye or wholegrain wheat intake, a finding supported by the present study.

With respect to the absorption and metabolism of AR, it has been shown in a study with ileostomists that about 60 % of AR are taken up in the small intestine (Ross *et al.*, 2003a) and absorbed into the systemic circulation via the lymphatic system (Linko *et al.*, 2006). The half-life of AR in plasma is approximately 5 hours against a background of no AR intake (Landberg *et al.*, 2006), suggesting AR may be a reasonable short to medium term biomarkers of whole grain-intake, though studies in pigs suggest that the pharmacokinetics of AR appearance in plasma may be different for chronic AR consumption (Linko *et al.*, 2006) . In the present study, the plasma samples were collected after 12 hours of fasting and therefore the measured plasma AR concentrations most likely reflect levels following homeostatic saturation. This is further

supported by the stronger dose-response linear trend in plasma AR concentrations with the mean whole grain/AR intake after each period of intervention than the dose-response linear trends observed with pre-visit whole grain/AR intake (see below). Once in the body, AR are metabolised via β-oxidation to produce 1,3-dihydroxybenzoic acid (DHBA) or 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) (Ross *et al.*, 2004a). These AR metabolites have also been shown to be potentially good biomarkers in plasma as a measure of whole grain-intake (Soderholm *et al.*, 2009) and of cereal fibre intake (Aubertin-Leheudre *et al.*, 2010) from WG rye and wheat. As these metabolites have longer half-lives (Aubertin-Leheudre *et al.*, 2010; Soderholm *et al.*, 2009) they are good candidate biomarkers of whole grain-intake. Another observation study showed good correlation between whole grain-intake and 12 h urinary DHPPA excretion (Guyman *et al.*, 2008). Therefore, samples from the present study warrant the measurement of DHPPA and DHBA in plasma and urine samples.

4.5 Plasma mammalian lignan concentration and urine mammalian lignans concentration/excretion as biomarker of wholegrain rye or wholegrain wheat intake

Several studies have shown a positive association between dietary plant lignan intake and mammalian lignan concentrations in plasma (Kilkkinen *et al.*, 2001; Milder *et al.*, 2007; Morisset *et al.*, 2009) or their excretion in urine (Horn-Ross *et al.*, 2006; French *et al.*, 2007). The mammalian lignans, also referred to as enterolignans are enterolactone (ENL) and enterodiol (END) (Adlercreutz, 2007). These enterolignans constitute about 80% of total lignans in plasma and about 90% of total lignans in urine (Laerke *et al.*, 2009). Wholegrain foods are an important source of plant lignans in diet (Milder *et al.*, 2005; Adlercreutz, 2007) which makes mammalian lignans in plasma and/or urine potential biomarkers of whole grain-intake. Consequently, in this present study, concentrations of ENL and END were also measured in fasting plasma and in 24 h urine samples. The data for both lignans, and in particular END, were highly variable for all periods (wash-out, Dose 1 and Dose 2) of dietary intervention. There was a significant linear dose-response trend in the present study between wholegrain rye intake and plasma ENL concentrations ($p<0.05$, Figure 3.21) but no dose-response relationships were found for wholegrain wheat intake. This finding is similar to a study by McIntosh et al., (2003) which found significant increase in plasma ENL after a 4 week dietary intervention with foods containing wholegrain rye flour but not with wholegrain wheat flour compared with refined cereal foods Another study also found an increase in serum ENL concentrations after one week of a wholegrain rye crisp bread intervention, but not with wholegrain wheat crisp bread (Linko-

Parvinen *et al.*, 2007). Taken together, the results from these and the present study suggest that plasma/serum ENL concentration may serve as a biomarker for wholegrain rye intake but not for wholegrain wheat intake. This may either be due to the lower plant lignan content of wholegrain wheat compared with wholegrain rye (Kamal-Eldin *et al.*, 2009) or alternatively, the soluble fibre (mostly arabanoxylan) in rye may have a prebiotic effect which encourages colonic microbiota adaptation to better conversion of plant lignans to mammalian lignans (Bach Knudsen *et al.*, 2003). Colonic microbiota plays an important role in the metabolism of plant lignans to mammalian lignans (Pettersson *et al.*, 1996; Bowey *et al.*, 2003), and therefore potential prebiotic effects of WG rye may lead to a better microbial metabolic ability to convert plant ligans into mammalian lignans. In addition, the high variability in plasma lignans in both diet groups at the end of three wholegrain intervention periods in the present study could be due to the variety of other sources of plant lignans in the volunteers' diet (Milder *et al.*, 2005), which could potentially confound dose-response relationships. These foods include fruits, particularly berries (Mazur *et al.*, 2000) and vegetables (Stumpf *et al.*, 2000; Kilkkinen *et al.*, 2001; Johnsen *et al.*, 2004; Peeters *et al.*, 2007), tea and coffee (Mazur *et al.*, 1998; Kuhnle *et al.*, 2008), wine (Pellegrini *et al.*, 2010), nuts, seeds and oils (Kuhnle *et al.*, 2008). Nonetheless, one intervention study with a high whole grain-intake found a significant increase in serum ENL concentrations as a result of increased whole grain-intake (Jacobs *et al.*, 2002). However, a recent study in an Italian population estimated that cereals only contribute to <8% of total lignans in the diet (Pellegrini *et al.*, 2010). Taken together, the results from these studies and the present study suggest that plasma lignans are indicators of a diet rich in plant foods, including fruits, vegetables, plant-based beverages, nuts and seeds as well as wholegrain cereals and therefore, the mammalians lignans resulting from their intake cannot be used as specific biomarkers of whole grain-intake. However, plasma enterolignans may be of use for determining dietary patterns indicative of a healthy diet and/or diet rich in plant foods (Lampe, 2003). For example, higher plasma/urine concentrations are found in vegetarians compared with non-vegetarians (Adlercreutz *et al.*, 1993).

Several observational studies show good correlations between lignan intake and urinary lignan excretion (Horn-Ross *et al.*, 2006; French *et al.*, 2007). Compared with plasma, lignan concentration/excretion in 24 h urine showed stronger and significant linear dose-response trends with both wholegrain rye and wholegrain wheat intake in the present study. These results support previous suggestions that mammalian lignan excretion in urine can be used as a biomarker for

whole grain or dietary fibre intake (Lampe, 2003; Horn-Ross *et al.*, 2006). There were greater increases in urinary lignan excretion (assessed using the slope of the regression line) and stronger dose-response correlation (using R^2 values) in the wholegrain rye group than those in the wholegrain wheat group which may be due to the greater bioavailability and/or higher content of plant lignans in wholegrain rye foods than wholegrain wheat foods as discussed above. This is further supported by data from a study in pigs fed diets with equivalent amounts of either rye or wheat resulting in higher lignan excretion with the rye diet (Bach Knudsen *et al.*, 2003). However, an intervention study with 16 pre-menopausal females consuming 28 g of wheat bran for 2 months did not result in a significant increase in urinary ENL excretion (Frische *et al.*, 2003). As with plasma, END excretion was much more variable than ENL excretion and the dose-response relationships in the present study were only found for ENL excretion.

With respect to the metabolism and absorption of plant lignans, it has been shown that plant lignans are metabolised by bacteria and absorbed in the colon (Pettersson *et al.*, 1996). However, the absorptive ability is highly variable between individuals and a recent study estimated that up to one third of the population are low convertors of dietary plant lignans (Bolca *et al.*, 2009). The high inter-individual variability in dietary lignan metabolism could result from either variation in gut-microflora content and/or genetic variability (Lampe *et al.*, 2006; Adlercreutz, 2007). The half life for ENL is approximately 12 hours (Kuijsten *et al.*, 2009) and the high intra-/inter-individual variability renders the requirement of multiple sampling for the measurement of plasma/urine lignans (Stumpf & Adlercreutz, 2003; Hausner *et al.*, 2004) if they are to be used as biomarkers for dietary intake.

4.6 Comparisons of dose-response trends between whole grain/AR intake and biomarkers for pre-visit day intakes versus the mean daily intakes during the middle of each phase of dietary intervention period

The stronger dose-response trends for mean daily intake for each period of dietary intervention compared with the pre-visit day intake for virtually all biomarkers in both diet groups indicate that all of these targeted biomarkers measured in the study reflected chronic intake rather than acute intake of whole grains and/or AR. This is particularly useful in epidemiological settings, which are generally interested in assessing medium to long term dietary intake within populations. However, it should be noted that actual pre-visit intake data for wholegrain intervention foods were only obtained at the end of Dose 1 and Dose 2 periods, but not at the end

of the wash-out period. Therefore, the weaker dose-response trend using pre-visit intake data in comparison to the mean intake data (using FFQ) may just be a result from there being a bigger data-set for the mean intake data.

4.7 Correlation between biomarkers

Significant correlations between biomarkers in the present study suggest that concentrations of both lignans and AR increased as a result of whole grain-intake. It would be interesting to investigate further whether there are inter-individual differences in the dose-response trends for mammalian lignans and AR concentrations and whether the high responders for AR intake are also the high responders for conversion and uptake of mammalian lignans.

5. Metabolomics Analysis (Task 05)

The aim of Objectives 01 – 04 (see previous Sections) was to evaluate the use of alkylresorcinols as biomarkers for wholegrain food intake in comparison with mammalian lignans in a UK setting through quantifying alkylresorcinols and mammalian lignan concentrations in biological fluids from participants in a controlled dietary intervention aimed at delivering different types and amounts of whole grains. Objectives 01 – 04 (intervention study and targeted metabolite analysis) were performed by the Newcastle University group led by Prof Seal.

The aim of Objective 05 was to apply metabolomics by the High Resolution Metabolomics Laboratory (HRML) team at Aberystwyth University (AU) to scan for complementary compounds and determine if there is a metabolite profile indicative of a diet containing whole grain:

- "To evaluate the contribution of alkylresorcinols and mammalian lignans to the plasma and urinary metabolome during a dose-dependent increase in whole-grain consumption."
- "Metabolomic analysis of physiological samples provides a comprehensive analytical mechanism for low molecular weight molecules present in biological samples. Coupled with advanced bioinformatic analysis, this method can be used as a screening tool to identify alternative biomarkers to alkylresorcinols and mammalian lignans."

This section focuses on reporting the three tasks as outlined in the project proposal:

- Task 05-01 Transport of frozen samples to AU for analysis (section 5.2);
- Task 05-02 Analysis of metabolite profile in urine and plasma samples (section 5.3);
- Task 05-03 Evaluation of the metabolite profile (section 5.4).

5.1 Introduction to Metabolomics workflow

The following gives a brief overview to a general metabolomics workflow which has been applied successfully to identify metabolite differences in a variety of different investigations at Aberystwyth University. Metabolomics aims to analyse as many metabolites as possible (typically low molecular weight molecules < 1000 Daltons) in a specific tissue at a given time, with the aim of elucidating exogenous exposure and endogenous metabolism. The metabolome is defined as the total biochemical composition of a cell, tissue, biofluid or organisms at any given time. The metabolomics workflow consists of the five main sequential steps:

1. Experiment (what will be investigated and experimental design);
2. Sample (sampling, sample handling and preparation);
3. Instrumental analysis (acquiring metabolite signatures);
4. Data analysis (data pre-processing, classification and comprehensive data mining);
5. Interpretation (signal annotation, data compilation, pathway analysis, hypothesis generation).

Steps 1 to 3 are the main sources of variability which can be evaluated in step 4 using classification analysis. One of the main objectives in step 2 is to keep sample handling at a minimum. As indicated in standard operating procedures for non-targeted urine and plasma sample preparation for metabolomics analysis (SOPs, Appendix) a single supernatant is generated where this 'global extract' ideally contains all metabolites solubilised for metabolomics analyses in step 3. Preliminary classification analysis in step 4 is required to evaluate pre-processed data (binary instrument software code converted into *e.g.* accessible ASCII *m x n* data matrices). If classification measures are adequate, data will be subjected to further comprehensive data analysis, data mining and subsequent interpretation. If data quality does not meet classification thresholds, samples need to be re-analysed or freshly prepared, or if results are still unsatisfactory the whole experiment has to be re-designed and repeated if appropriate.

Since there is at present no single analytical procedure and instrument which can capture all metabolites at different concentrations and polarity ranges, a metabolomics laboratory requires a

variety of different instrumentations. Therefore, metabolomics analysis is a combination of three main methodologies and associated instrumental technologies:

- Metabolite fingerprinting
  - Flow injection or direct infusion mass spectrometry (FIE-MS, DI-MS);
  - Nuclear magnetic resonance spectrometry (NMR);
  - Fourier transform infrared spectroscopy (FT-IR);
- Metabolite profiling
  - Gas chromatography-mass spectrometry (GC-MS);
  - Liquid chromatography-mass spectrometry (LC-MS);
- Metabolite targeted analysis
  - Various sample preparation protocols and instrumentation allowing up-scaling, fractionation and analysis of target metabolites, including bespoke chromatographic conditions and/or accurate mass measurements using *e.g.* Fourier transform ion cyclotron mass spectrometry (FT-ICR-MS).

The list is not comprehensive because there is a diverse range and combination of hyphenated sample introduction and detection techniques applicable. From a metabolomics perspective Objective 04 in this project can be considered as 'metabolite targeted analysis' because the targeted quantification of compounds in specific sample fractions and its evaluation as potential biomarkers for whole grain consumption is a direct consequence of results obtained from previous investigations.

In the High Resolution Mass Spectrometry Laboratory at Aberystwyth University metabolomic analysis focuses on the combination of metabolite fingerprinting and profiling applying respectively high through-put FIE-MS on a LTQ linear ion trap (Thermo Electron Corporation, San Jose, CA, US) and GC-TOFMS on a Pegasus III GC-TOFMS system (Leco Inc., St Joseph, MI, USA). Generally, all samples are analysed first using FIE-MS and only a subset of the experimental sample set are then analysed on GC-TOFMS to measure as many metabolites as possible. FT-ICR-MS on selected samples is routinely used to further identify metabolites found to be explanatory in FIE-MS data sets.

5.1.1 Metabolite Fingerprinting: FIE-MS

Standard operating procedures for urine and plasma/serum sample preparation for instrumental metabolomics analysis were piloted initially on the MEDE project (Studies 1 and 2, FSA-Project N05073) and validated on the present project and have been attached (Appendices 8 and 9). The application of Flow Injection Electrospray Mass Spectrometry has been published recently in Nature Protocols (Beckmann *et al.*, 2008) . Further modifications for mammalian biofluid analysis have been introduced since during the MEDE project. Briefly, prepared urine and plasma samples (Section 2.8 and 2.9 above) were randomized and analysed in batches of 25 samples. 50 µl of sample was dispensed in autosampler vials with glass insert, and aliquots of 20 µl were injected into a flow of 60 µl/min methanol/water (70/30 v/v) using a Surveyor (Thermo Finnigan) liquid chromatography (LC) system. Ionisation conditions were set to +15 V (ESI+) and -13 V (ESI-) capillary voltage, 4.5 kV (ESI+) and 4.0 kV (ESI-) Spray voltage, 380°C transfer capillary. Sheath and auxiliary gas was nitrogen at pressures of 40 and 5 arbitrary units, respectively.

The linear ion-trap mass detector (LTQ, Thermo Finnigan) collected data in profiling mode concurrently in alternating positive and negative ionisation for 6 min per sample from m/z 15 to 1200 in 4 mass ranges per ionisation mode. For data analysis (Andersson *et al.*, 2008; Beckmann *et al.*, 2008) the resulting eight FIE-MS mass ranges (4 for each positive and negative ionisation mode, **Table 5.1.1-1**) were pre-processed separately resulting in eight mass spectra for each sample and, therefore, eight data matrices (runs x m/z value) for each instrumental analysis. This approach allows differentiating between mass ranges with distinct information content regarding percent explained variance and main variability, as well as goodness and direction of discrimination (see below).

Table 5.1.1-1: Sequence of subsequent scan events in FIE-MS experiments used to analyse plasma and urine samples. The mass range is defined by the mass to charge ratios (m/z) from 'Scan Start' to 'Scan End'.

Scan Event	Scan Start	Scan End	Polarity	Scan Range	Data Type	Scan Type
1	15	110	Pos1	1	profile	full
2	15	110	Neg1	1	profile	full
3	100	220	Pos2	2	profile	full
4	100	220	Neg2	2	profile	full
5	210	510	Pos3	3	profile	full
6	210	510	Neg3	3	profile	full
7	500	1200	Pos4	4	profile	full
8	500	1200	Neg4	4	profile	full

5.1.2 Metabolite Profiling: GC-TOFMS

For GC-MS metabolite profiling the protocols developed under the FSA-G02006 project were adopted. The adjustments for sample derivatization of urine and plasma samples were further developed on the MEDE study. Briefly, biofluid supernatants (50ml) were dried in vacuo and GC-TOF-MS analysis performed as previously described (Catchpole *et al.*, 2005). Methoximation of carbonyl moieties in 100ml of a 20 mg/ml solution of methoxyamine hydrochloride in pyridine (Fluka, St Gallen, Switzerland) was carried out at 30 °C for 90 min. Acidic protons were subsequently derivatised with 100ml N-methyl-N-(trimethylsilyl)-trifluoroacetamide (Machery-Nagel GmbH, Düren, Germany) at 37 °C for 30 min. One µL of the resulting solution was injected (injector temperature 250°C; 1:2 split ratio) into a Pegasus III GC-TOFMS system (Leco Inc., St Joseph, MI, USA) and separated on a 20m DB5-MS column (20m x 0·25 mm internal diameter x 0·25 mm film) using a temperature gradient (80–330°C over 17 min). Mass spectra were recorded after a solvent delay of 112 s over an m/z range of 54–500 at an acquisition rate of 20 spectra/s and ion source temperature of 230°C. Peak finding and deconvolution were performed using Leco ChromaTof software. Mass spectra of all detected compounds were compared with in-house standards and spectra in the National Institute of Standards and Technology library (http://www.nist.gov/srd/nist1.htm), and other publicly

available databases. All data pre-treatment procedures, such as baseline correction, chromatogram alignment, data compression and curve resolution, were performed using custom scripts in Matlab v.6.5.1 (The Math Works Inc., Natick, MA, USA). Targeted peak lists were generated, and the peak apex intensity of a characteristic mass in a retention time window for each GC-MS dataset was saved in the form of an intensity matrix (run x metabolite) using Matlab.

5.1.3 Multivariate Classification

One of the main objectives of the project is to understand differences in metabolite fingerprints and profiles between the 3 phases of the dietary intervention and to identify sources of variability. The first step in a hierarchical data analysis approach is to look for inherent variance in the data set using Principal Component Analysis (PCA) as an un-supervised multivariate classification tool. For example, clustering of individuals, samples and/or time points in the first few dimensions of PCA can be obscured by various sources of variability: sampling, sample treatment and preparation, analytical instrument drifts, diet or individual metabolic state related differences. PC-LDA is used as a supervised multivariate classification tool in this investigation. In principle, Linear Discriminant Analysis (LDA) uses the class information provided to minimize within group variance whilst the between group variance is maximised. Because of the high dimensionality of metabolomics datasets, we use uncorrelated Principal Components (PCs) as input for the LDA. The discriminant functions (DF) provide the direction in which the groups are best separated and are used to visualise the 'goodness of separation'.

In the following we present one example of PCA and/or PC-LDA score plots produced using statistical functions in Matlab (The MathWorks) of one of the eight matrices produced in FIE-MS analysis (**Table 5.1.1-1**). The class membership in score plots is colour and symbol coded. If appropriate, we additionally provide tables containing Eigenvalues (Tw-values: measure for goodness of discrimination in PC-LDA) and/or the percentage explained variance for the first 5 PCs. A major criterion for submitting data sets from Flow-Infusion Electrospray Mass Spectrometry (FIE-MS) experiments for further comprehensive in-depth data analysis (e.g. accuracy testing, data mining and model validation) using R-packageFIEmspro (Andersson *et al.*, 2008, http://users.aber.ac.uk/jhd) is a Tw-value greater than 2.0 for, ideally, more than one mass range. Tw-values between 1.0 and 1.5 indicate that there is a trend in the data suggesting the presence of poor models, which cannot be validated using the existing experiment.

5.1.4 Univariate and Multivariate Data-mining

Similarly, Tw-values were used to evaluate the goodness of multivariate classification in GC-MS data. Due to the 3-dimensional data structure, peak aligned GC-MS data were first filtered by univariate data analysis using analysis of variance (function ANOVA1 in Matlab, The Mathworks Inc.) to reduce their size. Metabolite signals with p-values $p>1x10^{-4}$ (ANOVA) were removed from the data set. The main criterion for good model selection (*i.e.* discriminatory metabolites) in multi-dimensional GC-MS metabolomics data sets is $p<1x10^{-10}$ (ANOVA). However, a p-value threshold of $1x10^{-7}$ is acceptable considering a False Discovery Rate (FDR) level of 0.005. Further in-depth analysis and data mining was performed in R-package FIEmspro (Andersson *et al.*, 2008) using Random forest (RF), Welsh-test and Area Under the receiver-operator Curve (AUC) bootstrapping in pair-wise comparisons.

5.2 Transport of frozen samples to AU for analysis (Task 05-01)

Urine and plasma samples were shipped on dry ice in two batches (Lot1: 30/04/2008 and Lot2: 04/11/2009) to Aberystwyth University (AU) for metabolome analysis (**Table 5.1.4-1**). Sample shipment of 1836 biofluid samples was completed on 05/11/2008 and comprised 1020 urine and 816 plasma samples.

Table 5.1.4-1: Stratification of volunteers in GrainMark study.

Volunteers	**Lot1**	**Lot2**	**Total**
Total	37	31	68
Female (pre-menopausal)	21(6)	14(5)	35(11)
Male	16	17	33
Rye-group	18	15	33
Female	11(3)	6(3)	17(6)
Male	7	9	16
Wheat-group	19	16	35
Female	10(3)	8(2)	18(5)
Male	9	8	17

The following were used to distinguish between 3 phases (or 'periods') of the intervention study:

- **Wash-out period**: 0 servings a day of wholegrain (WG) foods for 4 weeks;
- **Dose 1 period**: 3 servings a day of WG rye or WG wheat foods for 4 weeks;
- **Dose 2 period**: increase to 6 servings a day of same WG foods for further 4 weeks.

Pooled urine, fasting spot urine and fasting plasma samples were collected after each intervention period at 4, 8 and 12 weeks. 24 h urine samples were collected before and after a standardized pre-evening meal (discussed in Section 2.2.2) as follows.

- **Sample A** (Day 0): Combined urine sample (total voids) from 06:00h to set evening meal (eaten between 18.00-20.00h), excluding first void day 0;
- **Sample B** (Day 0/1): Combined urine sample (total voids) after set evening meal until and including first void the following morning (day 1);
- **Sample P**: Pooled 24 h urine sample: an aliquot of proportionally mixed pooled urine samples **A** and **B**;
- **Sample C** (Day1): Fasting 'spot' urine and plasma fasting samples before breakfast;
- **Sample D** (Day3): Fasting 'spot' urine and plasma fasting samples before breakfast.

The total number of samples equates to number of volunteers x 3 phases x number of samples per phase (**Table 5.1.4-2**). The number of urine samples per phase was 5 (**Samples A, B, P, C and D**); the number of plasma samples per phase was 4 (**Samples C and D** from 2 LiHep vacutainers **a** and **b**);

Table 5.1.4-2: Number of samples per bio-fluid in GrainMark study.

Samples	Lot1	Lot2	Total
Urine	555	465	1020
Plasma	444	372	816

5.3 Metabolomics analysis of urine and plasma samples (Task 05-02)

5.3.1 Preliminary discrimination of whole grain exposure in FIE-MS data using Lot1 urine samples

In order to obtain information regarding data structure and variance of sample collected in the GrainMark intervention study all urine samples of Lot 1 were preliminary analysed in 15 batches on an LTQ linear ion trap (Thermo Electron Corporation, San Jose, CA, US). A total of 555 samples were extracted using the standard operating procedure developed in MEDE Study 1 and Study 2 (FSA-Project N05073).

Preliminary clustering and discrimination results are shown in **Figure 5.3.1-1**. FIE-MS results show reasonable overall classification of the 5 urine sample classes, but there was no distinction possible between the three different phases of the study.

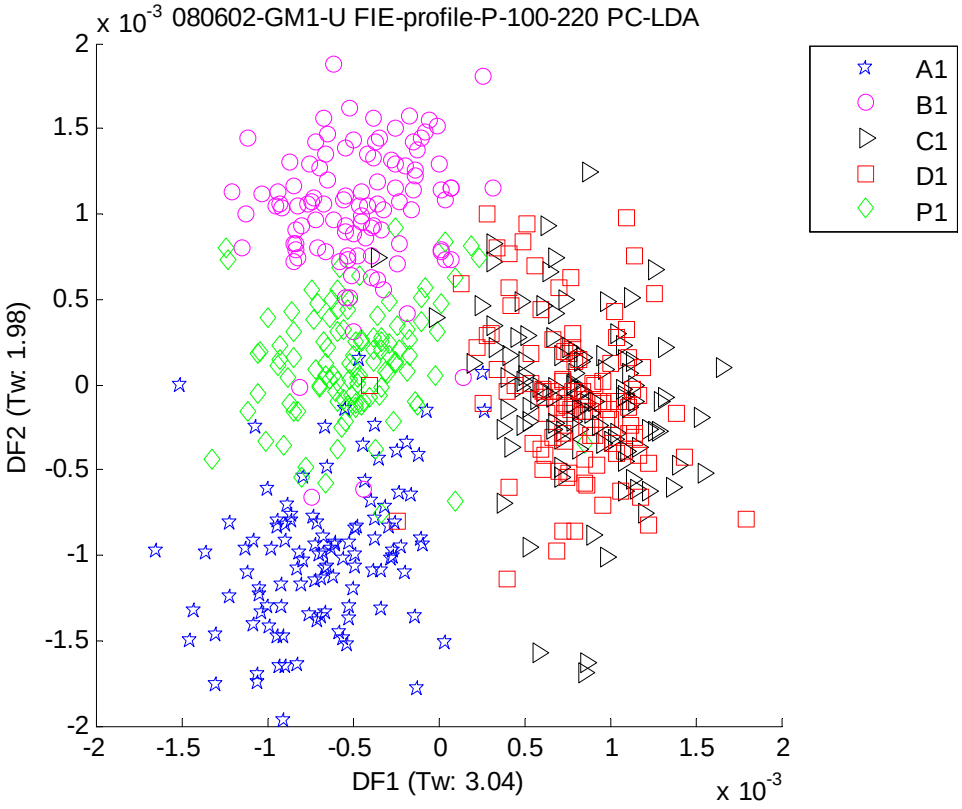


Figure 5.3.1-1: Comparison of all 5 urine Lot1 sample classes by ESI-MS fingerprinting. Shown is a representative PC-LDA scores plot for FIE-MS mass range Pos2 (*m/z* 100-220). No distinction has been made between the three phases of the study.

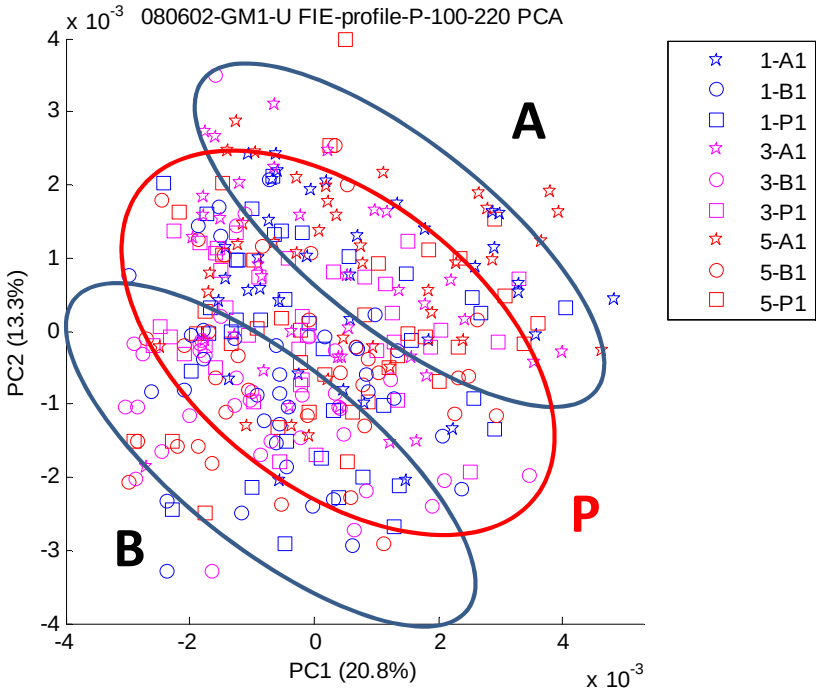


Figure 5.3.1-2: Comparison of Lot1 urine samples A, B and P by ESI-MS fingerprinting. Shown is a representative PCA scores plot for FIE-MS mass range Pos2 (*m/z* 100-220). The three phases of the study are indicated by colour coded symbols: after wash-out period, blue; after 4 weeks of 3 servings, magenta; after 4 weeks 6 servings, red.

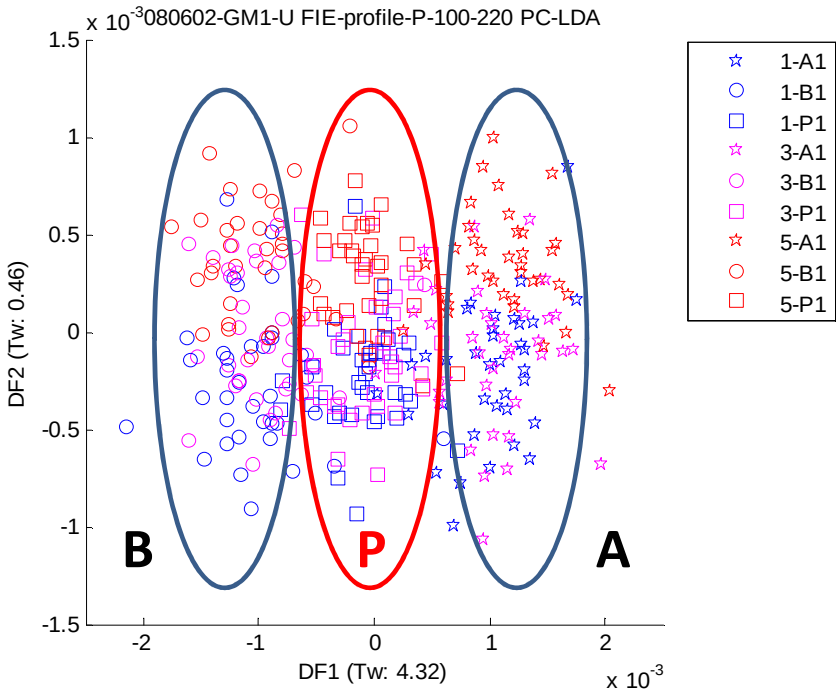


Figure 5.3.1-3: Comparison of Lot1 urine samples A, B and P by ESI-MS. Shown is a representative PC-LDA score plot for FIE-MS mass range Pos2 (*m/z* 100-220). The three phases of the study are indicated by colour coded symbols: after wash-out period, blue; after 4 weeks of 3 servings, magenta; after 4 weeks 6 servings, red.

The pooled urine samples (*i.e.* samples **A**, **B** and **P**) could be discriminated from the spot fasting samples **C** and **D**. Further FIE-MS results show that pre-and post-evening meal samples **A** and **B** are different, clustered in PCA (**Figure 5.3.1-2**) and were discriminated in the PC-LDA score plot (**Figure 5.3.1-3**). The pooled 24 h sample **P** is located in the centre of the score plot indicating that sample **P** consisted of representative fractions of samples **A** and **B**. Similar behaviour was found for true Master-Mix samples, which were always centred (coordinates [0,0] in score plots).

The spot samples **C** and **D** however could not be discriminated (**Figure 5.3.1-4**), which suggests that fasting urine samples collected within a single phase separated by two days were comparable. Similarly, samples from different phases of intervention did not cluster and could not be discriminated. A discriminant analysis of all single sample time points using the three phases of intervention as class information found Tw-(Eigen) values of below 1.4. This clearly indicates that there were no significantly inherent differences between samples from the wash-

out phase (0 servings, 1-C1 and 2-D1) and phase three (6 servings of whole grain, 5-C1 and 6-D1) determined using this technique.

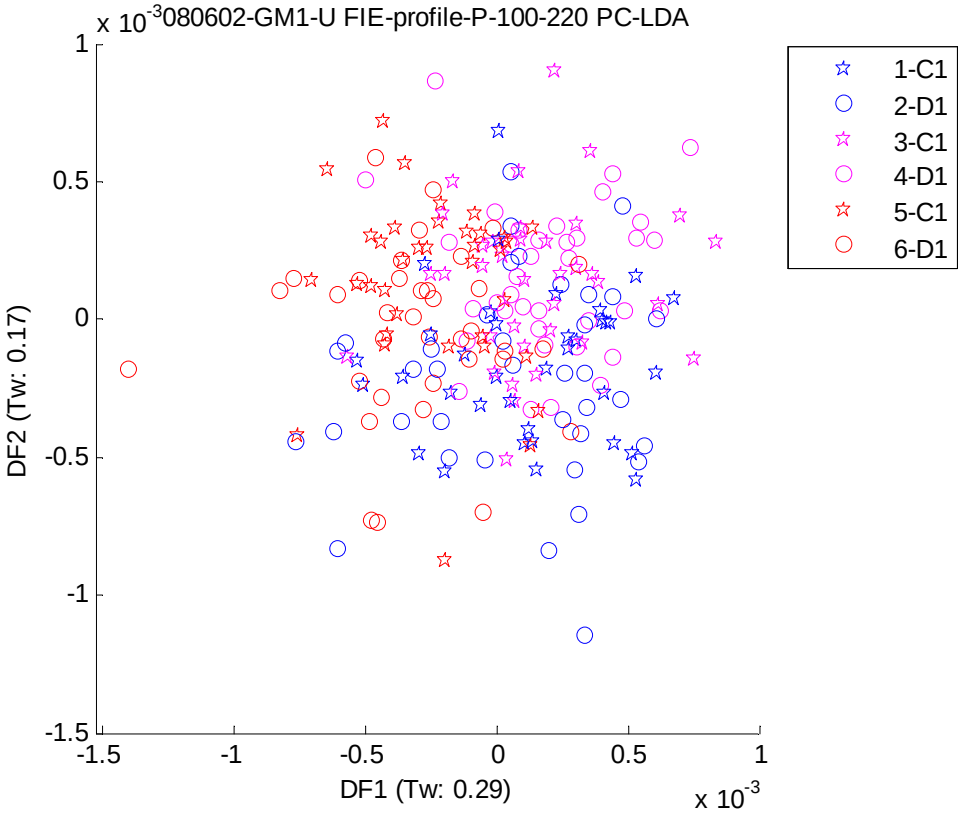


Figure 5.3.1-4: Comparison of Lot1 urine samples C and D by ESI-MS fingerprinting. Shown is a representative PCA score plot for FIE-MS mass range Pos2 (m/z 100-220). The three phases of the study are indicated by colour coded symbols: after wash-out period, blue; after 4 weeks of 3 servings, magenta; after 4 weeks 6 servings, red.

Table 5.3.1-1 shows the results of PC-LDA analyses aiming to answer the question if one of the 5 urine samples classes showed discrimination between the three phases (wash-out, 3 and 6 servings of whole grain foods). Tw-values were found to be below 1.4 which indicated that there was no adequate discrimination in either of the 5 urine sample classes and, therefore, no metabolite signal apparent in flow infusion fingerprint data which could be used as a biomarker for whole grain-intake.

Table 5.3.1-1: PC-LDA Eigenvalues for all FIE-MS mass ranges showing discrimination of three phases (time-points) for pooled and spot urine sample.

ScanEvent	Pos1	Pos2	Pos3	Pos4	Neg1	Neg2	Neg3	Neg4
MassRange	15-110	100-220	210-510	500-1200	15-110	100-220	210-510	500-1200
Sample A								
DF1	0.45	0.50	0.65	**1.01**	0.25	**1.22**	**1.20**	**1.00**
Sample B								
DF1	0.65	0.60	0.42	0.88	0.24	**1.03**	0.64	**1.01**
Sample P								
DF1	0.65	0.50	0.57	0.99	0.21	**1.39**	0.95	**1.00**
Sample C								
DF1	0.46	0.36	0.40	0.84	0.19	0.76	0.51	0.95
Sample D								
DF1	0.38	0.38	0.39	0.86	0.17	0.58	0.43	**1.00**
Sample ABP								
DF1	0.87	0.85	0.81	0.96	0.41	**1.95**	**1.50**	**1.00**
Sample C&D								
DF1	**1.24**	0.57	0.54	**1.07**	0.26	0.99	0.66	**1.09**

Highlights indicate presence of **green** = adequate, **amber** = nearly adequate, **red** = poor and white = no models.

The inter-individual differences (**Figure 5.3.1-5**) were from a discrimination point of view strong, but from an overall variance perspective moderate and were expected for urine samples. There were no extreme or outlying subjects indicating a homogenous set of volunteers recruited for the first cohort of the GrainMark study. The split in the PC-LDA score plot between males and females is only apparent in the negative mass range between *m/z* 210 and *m/z* 510 and is anticipated.

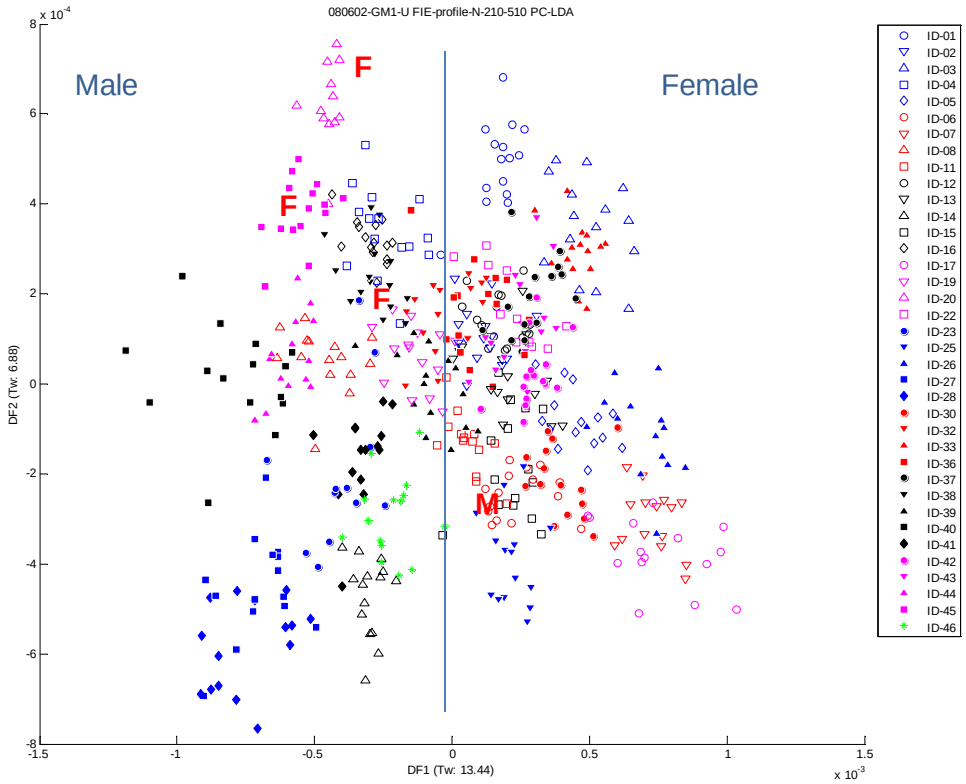


Figure 5.3.1-5: Discrimination of volunteers by ESI-MS fingerprinting of Lot1 urine samples. Shown is a representative PC-LDA score plot for FIE-MS mass range Neg3 (*m/z* 210-510). Symbols of same colour represent all samples taken for one volunteer during the intervention study. Red letters (F: female, M: male) indicate volunteer samples which are the exception to general discrimination of males and females.

5.3.2 Preliminary conclusions

Multivariate analyses (PCA and PC-LDA) of FIE-MS fingerprinting data using Lot1 urine samples showed good discrimination between pooled samples A and B and between pooled samples and spot fasting samples (C and D) whereas the duplicate fasting 'spot' urine samples C and D, collected two days apart were indistinguishable. The 24 h urine sample (Sample P) was a representative mix of pooled samples A and B and is located as expected between samples A and B in multivariate score plots of the first two dimensions. Using all 15 samples (3 phases x 5 different urine samples) for each individual allowed discrimination between individuals. Although discrimination was strong there were no outlying individuals apparent, which indicates that the first cohort of volunteers participating in the GrainMark project was homogenous. The urine collection methodology can be assessed as excellent giving stable compositions and that the A and B and C/D samples gave the opportunity to distinguish signals when the volunteers were in different physiological states at different times of the day. Some urine classes (*e.g.* Sample B) may be more dominated by gut microbial flora fermentation (*i.e.* after overnight fast) whilst other would have more impact from recent meals (*i.e.* sample A).

The first flow injection fingerprinting analysis of urine samples was designed to provide an insight into the experimental variance and general data structure. Besides the positive results there was, however, a lack of adequate discrimination between the three different doses of whole grain-intake. There are four possible reasons for this result:

1. The number of biological replicates (*i.e.* number of volunteers and therefore urine samples) was not sufficient.
2. The inherent variance, for example, the sum of instrument and individual variability, was greater than the anticipated changes to be observed in urine samples following increased wholegrain food intake.
3. Plasma rather than urine might be a more appropriate source for finding metabolite changes with increased wholegrain food intake.
4. There is no discriminating metabolite detectable using FIE-MS technology.

5.3.3 Revised strategy for GrainMark metabolomics analysis

The aim of metabolomics is to provide comprehensive data sets, which not only allow addressing as many questions as possible for hypothesis generation (including investigation of potential correlation with meta-data), but also to allow discovery of unanticipated results. Limiting the number of sample classes therefore reduces the list of questions to address. Since data analysis of FIE-MS fingerprinting data using Lot1 urine samples indicated that changes in the urine metabolome following increased whole grain-intake were extremely subtle we tailored instrumental urine analysis to particularly focus on metabolites indicative for wholegrain foods by reducing potential instrument variance.

We therefore decided that the best strategy for GrainMark metabolomics was to analyse urine samples in 5 separate randomized sets each belonging to only one sample class (*i.e.* either sample A, B, P, C/D). This facilitates comparing the three phases of the study at an increased number of replicates (*i.e.* all individuals) and potentially reduced overall variance. Within a sample class wheat and rye based urine samples were randomized together to allow a comparison between the two different whole grain interventions. To further address the general problems discussed at recent NUGO conferences that LC-MS fails to discriminate between rye and wheat based biofluids, and the fact that proposed biomarkers are accessible using targeted GC-MS technology, the 5 urine sample sets (total of 1020 samples) were analyzed using both metabolomics technologies, FIE-MS metabolite fingerprinting and GC-TOFMS metabolite profiling.

5.3.4 Urine and plasma FIE-MS metabolite fingerprinting

The results of supervised multivariate PC-LDA analyses following metabolite fingerprinting of separately randomized urine sample classes clearly show no discrimination between the three phases (**Table 5.3.4-1**). Tw-values were found to be below 1.2. Thus, more comprehensive metabolite analysis suggests that there are no urine biomarkers for general whole grain-intake detectable in flow infusion fingerprint data.

The analysis of plasma samples using flow infusion metabolite fingerprinting has focussed on using all spot samples C and D from the rye group only to further reduce the batch size and therefore minimize potential machine variance. Multivariate discrimination analysis results in Tw-values below 1.0 indicating that there are no biomarkers for rye whole grain-intake detectable in plasma FIE-MS data (**Table 5.3.4-2**).

Table 5.3.4-1: PC-LDA Eigenvalues for all FIE-MS mass ranges showing discrimination of the three wholegrain intervention phases following separate FIE-MS analysis of pooled and spot urine sample using all volunteers (n=68). 'Pos' and 'Neg' refer to ionisation modes used.

ScanEvent	Pos1	Pos2	Pos3	Pos4	Neg1	Neg2	Neg3	Neg4
Mass Range	15-110	100-220	210-510	500-1200	15-110	100-220	210-510	500-1200
Sample A								
DF1	0.39	0.60	0.85	**1.08**	0.13	0.89	**1.19**	**1.03**
Sample B								
DF1	0.38	0.45	0.71	**1.09**	0.13	0.59	0.84	**1.05**
Sample P								
DF1	0.47	0.45	0.69	**1.06**	0.15	0.63	**1.07**	**1.12**
Sample C								
DF1	0.35	0.31	0.69	**1.06**	0.14	0.51	0.81	**1.03**
Sample D								
DF1	0.40	0.49	0.71	**1.06**	0.19	0.66	**1.03**	**1.04**

Highlights indicate presence of **green** = adequate, **amber** = nearly adequate, **red** = poor and white = no models

Table 5.3.4-2: PC-LDA Eigenvalues for all FIE-MS mass ranges showing discrimination of the three wholegrain intervention phases following FIE-MS analysis of spot plasma sample using all volunteers in the rye group only (n=33). 'Pos' and 'Neg' refer to ionisation modes used.

ScanEvent	Pos1	Pos2	Pos3	Pos4	Neg1	Neg2	Neg3	Neg4
Mass Range	15-110	100-220	210-510	500-1200	15-110	100-220	210-510	500-1200
Sample C								
DF1	0.20	0.25	0.55	0.93	0.13	0.31	0.65	0.96
Sample D								
DF1	0.24	0.17	0.62	0.84	0.15	0.22	0.54	0.98

Highlights indicate presence of **green** = adequate, **amber** = nearly adequate, **red** = poor and white = no models.

5.3.5 Urine GC-TOFMS metabolite profiling

For GC-TOFMS metabolite profiling urine samples A, B, P, C and D of all 3 phases have been analysed in separately randomized sets. Data analysis of a single sample class will, therefore, allow the investigation of metabolite changes in response to increased whole grain intake (3 intervention phases) and a direct comparison between wheat and rye treatments.

The main results are summarized in the form of a heat map. **Figure 5.3.5-1** shows the relative concentration changes of single metabolites in binary comparisons of two phases in the study (*e.g.* Wash-out *vs*. 3 whole grain servings a day = 1~2) for each sample class and whole grain group (wheat and rye) based on ANOVA F-statistic (values = ratio of between group variance and within group variance). Therefore, high F-values indicate high between group variance and the darker the orange/red colour the higher the significance ($p<1x10^{-7}$). Although metabolite between-group variances highlighted yellow to bright orange ($1x10^{-5}>p>1x10^{-7}$) are statistically not significant with respect to a fixed threshold (*i.e.* one pair-wise comparison single data) the observation of the same variable/metabolite becoming significant (or still being 'borderline') in another binary comparison, different treatment and/or a similar data set gives confidence that the variable is not selected by chance. The metabolites selected for **Figure 5.3.5-1** on the basis of

ANOVA p-values, and bootstrapping using RF, Welch test and AUC in FIEmspro are listed in **Table 5.3.5-1**.

Figure 5.3.5-2 to Figure 5.3.5-6 visualize the discrimination of whole grain groups and dietary intervention phases in the GrainMark study for each respective sample based on all preselected variables ($p<1x10^{-4}$, ANOVA). The Eigenvalues are summarized in **Table 5.3.5-2**. The strongest discrimination in all sample classes is shown in the first dimension (DF1) and is attributed to the differences between the rye group urine samples and those of the wash-out. Discrimination of urine samples of the wash-out phase and the wheat whole grain group is achieved in the second dimension (DF2). For comparison, **Figure 5.3.5-7** shows the discrimination of whole grain groups and dietary intervention phases based on AR concentrations as obtained in Objective 04 (log_{10}-transformed concentration values, Section 3.4.1). Whereas discrimination between intervention phases is strong (Tw = 4.02) discrimination of whole grain groups is weak (Tw = 0.89) indicating that discrimination between wheat and rye group achieved using GC-TOFMS profiling data cannot be achieved using plasma AR concentration differences between rye and wheat group. The strong discrimination of whole grain groups using spot fasting urine Sample C suggests inherently different chemistry in whole grain wheat and rye foods. However, the finding that the rye group showed better discrimination using metabolomics data in response to increasing WG dose compared with the wheat group was similar for both, AR and lignan concentrations (i.e. dose-response slopes were steeper for the rye group than the wheat group, Sections 3.5).

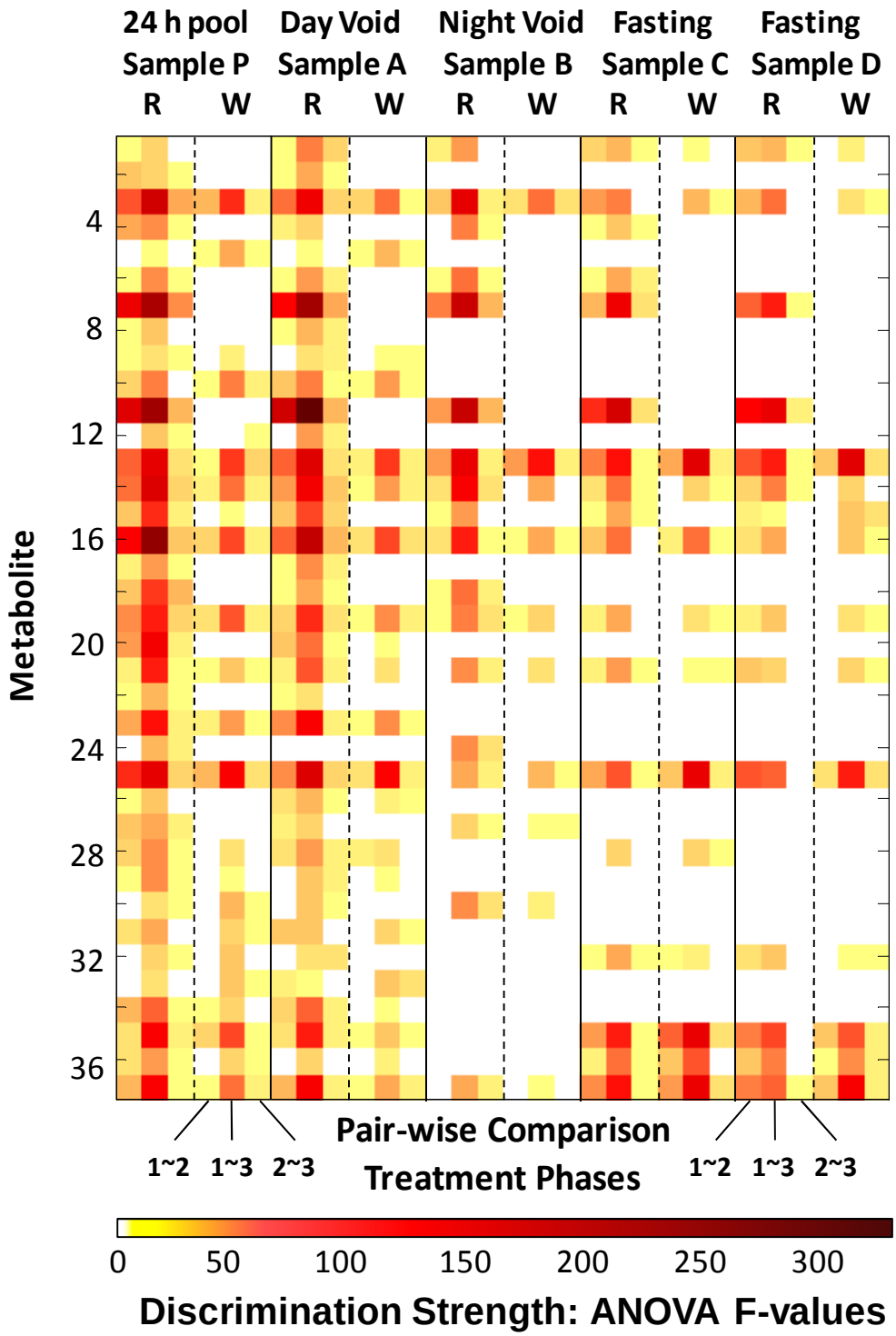


Figure 5.3.5-1: Heatmap of ANOVA F-values showing the strength of metabolite signals in urine GC-MS data to discriminate between the three treatment phases following pair-wise comparison. For example 'Metabolite 11 in Sample A, R, 1~3' is statistically the strongest discriminator (highest F-value, p-value=0) of whole grain rye intake following a pair-wise comparison between urine metabolites after 3 servings and after 6 servings in the pooled sample taken on Day 0 before the standardized evening meal. The list of metabolites is shown in **Table 5.3.5-1**. Treatment Phases: 1 = Wash-out; 2 = 3 servings WG; 3 = 6 servings WG. R=rye; W=wheat.

Table 5.3.5-1: GC-TOF-MS metabolites in urine explanatory of whole grain food intake discriminating treatment phases in retention time order (see also **Figure 5.3.5-1**).

Peak	R.T. [s]	Discriminatory in group	Metabolite
1	273.8	R[a]	U-274
2	274.6	R	U-275
3	309.2	R & W[b]	U-309 (most likely aromatic)
4	312.1	R	U
5	338.2	W > R	U
6	348.8	R	U
7	352.2	R[d]	U-352 (organic acid of unknown structure)
8	356.3	R	U
9	369.0	R > W	U
10	370.9	R & W	U-370 (most likely aromatic)
11	389.3	R[e]	U-389 (organic acid of unknown structure)
12	395.7	R	U
13	402.0	R & W[b,c]	3,5-dihydroxy-benzoic acid (DHBA)[f]
14	433.9	R & W[b]	3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA)[f]
15	446.0	R > W[d]	U-446 (aromatic)
16	451.4	R & W[b]	U-451 (aromatic, metabolized alkylresorcinol?)
17	454.2	R	U
18	455.7	R	U-455 (very low abundant)
19	462.9	R & W[b]	U-463 (most likely glucosilated compound)
20	463.8	R	U-463 (very low abundant)
21	468.6	R & W[b]	U-468 (aromatic, metabolized alkylresorcinol?)
22	479.9	R	U
23	489.2	R & W	U-498 (aromatic, metabolized alkylresorcinol?)
24	496.6	R > W	U
25	502.4	R & W[b,c]	U-502 (most likely aromatic; conjugated?))
26	516.7	R & W	U
27	521.6	R > W	U
28	528.4	R & W	U
29	529.5	R & W	U
30	538.1	R & W	U
31	554.3	R & W	U
32	555.4	R > W	U
33	557.5	W > R	U
34	558.7	R & W	U
35	569.8	R & W[b,c]	U-569 (most likely aromatic; conjugated?)
36	575.3	R & W[c]	U-575 (most likely aromatic; conjugated?)
37	591.1	R & W[c]	U-591 (most likely aromatic; conjugated?)

R=rye, W=wheat, U=unknown metabolite; highlighted: strongest discriminators.
a: also explanatory in plasma and spot fasting urine samples; b: explanatory in all samples; c: highly discriminatory in spot fasting urine samples; d: metabolite identified in rye bread; e: most likely derivative (biotransformation product) of metabolite peak 7; f: identity of metabolite confirmed by standard

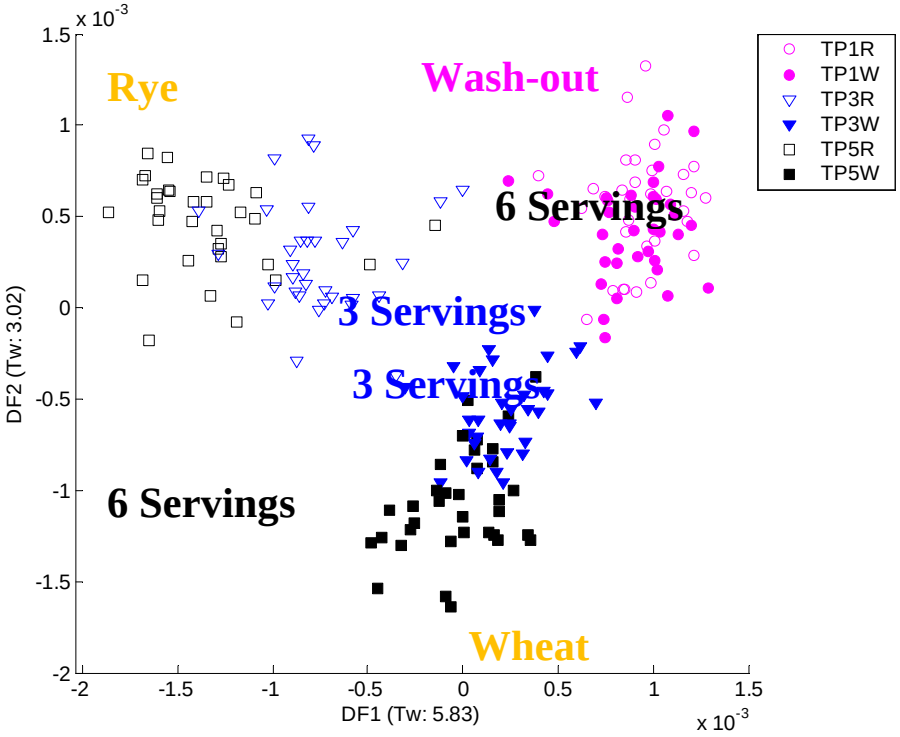


Figure 5.3.5-2: Discrimination between rye and wheat urine **Samples P** (24 h pooled urine samples) in GC-MS data. Shown is the PC-LDA scores plot for all signals with a $p < 1x10^{-4}$. The three phases of the study are colour coded.

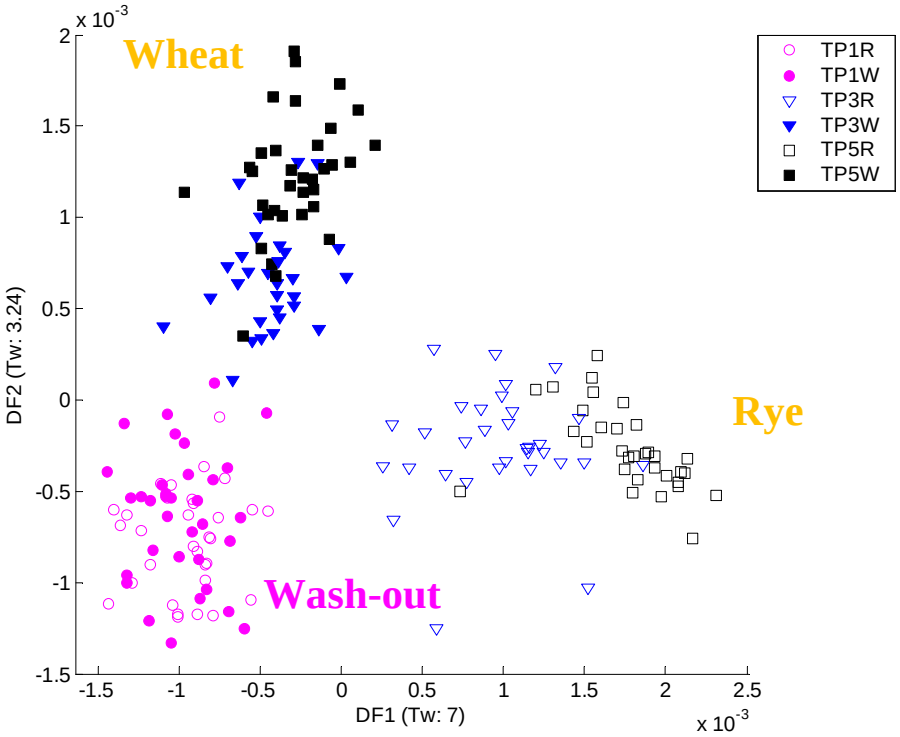


Figure 5.3.5-3: Discrimination between rye and wheat urine **Samples A** (day void) in GC-MS data. Shown is the PC-LDA scores plot for all signals with a $p < 1x10^{-4}$. The three phases of the study are colour coded.

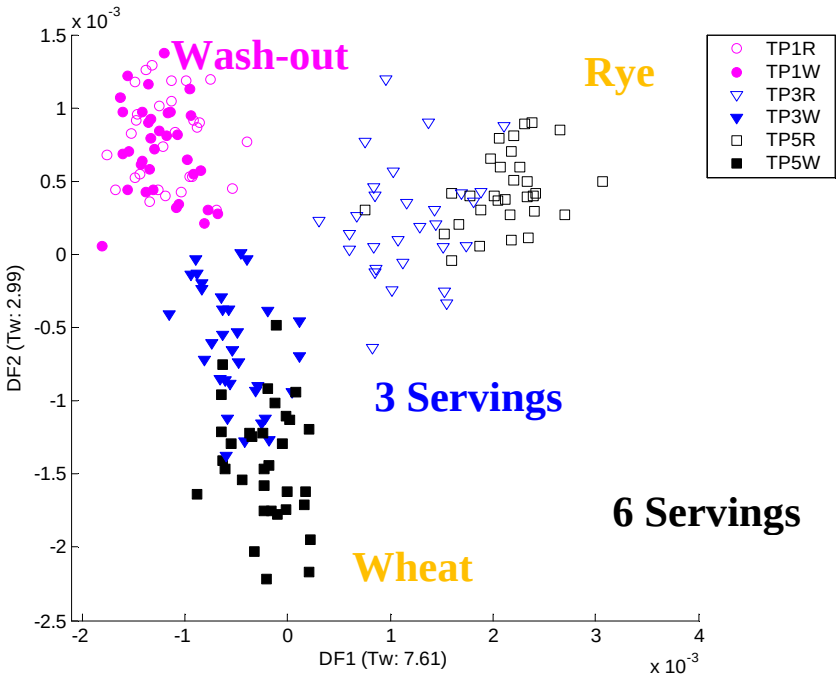


Figure 5.3.5-4: Discrimination between rye and wheat urine **Samples B** (night void) in GC-MS data. Shown is the PC-LDA score plot for all signals with a $p < 1x10^{-4}$. The three phases of the study are colour coded.

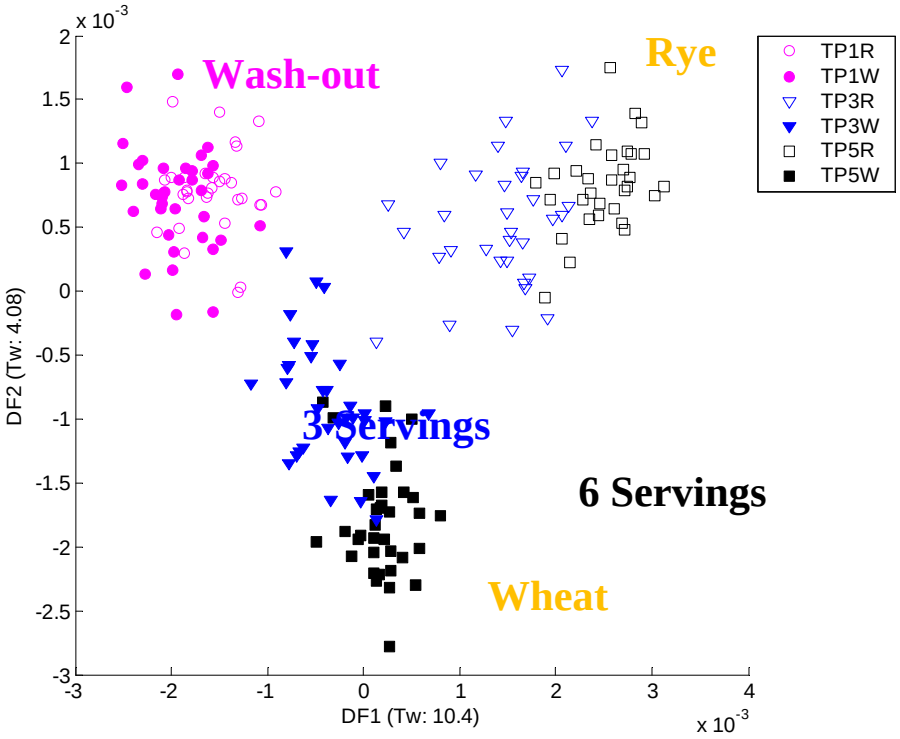


Figure 5.3.5-5: Discrimination between rye and wheat urine **Samples C** (fasting) in GC-MS data. Shown is the PC-LDA score plot for all signals with a $p < 1x10^{-4}$. The three phases of the study are colour coded.

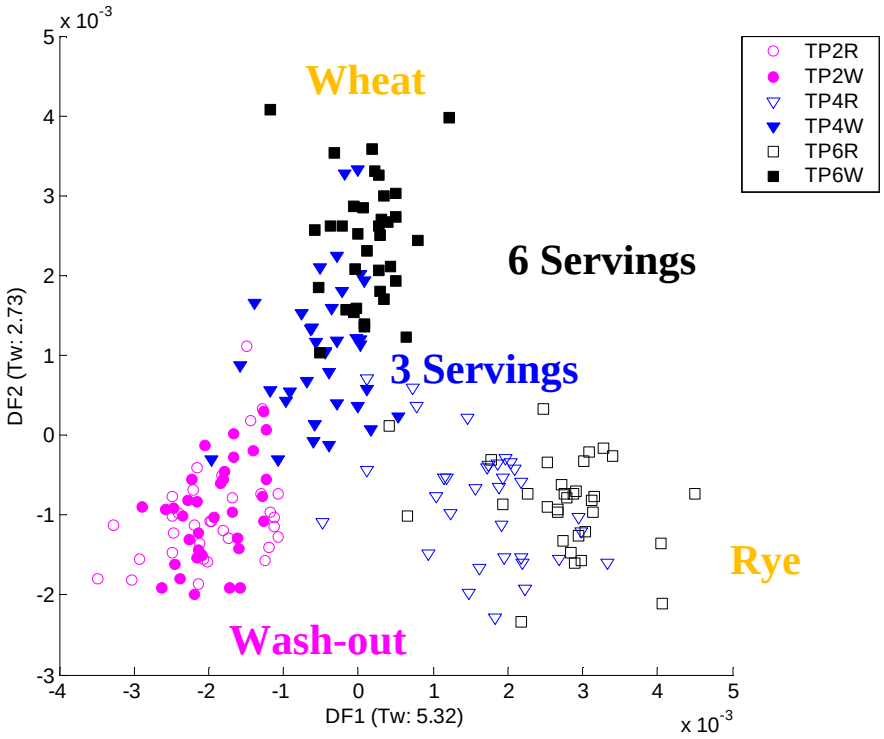


Figure 5.3.5-6: Discrimination between rye and wheat urine **Samples D** (fasting) in GC-MS data. Shown is the PC-LDA score plot for all signals with a $p < 1x10^{-4}$. The three phases of the study are colour coded.

Table 5.3.5-2: PC-LDA Eigenvalues for the 5 sample classes showing discrimination of whole grain groups and three intervention phases.

Sample	A	B	C	D	P
DF1	7.00	7.61	10.40	5.32	5.83
DF2	3.24	2.99	4.08	2.73	3.02
DF3	0.50	0.68	0.83	0.55	0.49
DF4	0.39	0.51	0.68	0.41	0.34
DF5	0.29	0.45	0.47	0.20	0.25

Highlights indicate presence of **green** = adequate, **amber** = nearly adequate, **red** = poor and **white** = no models.

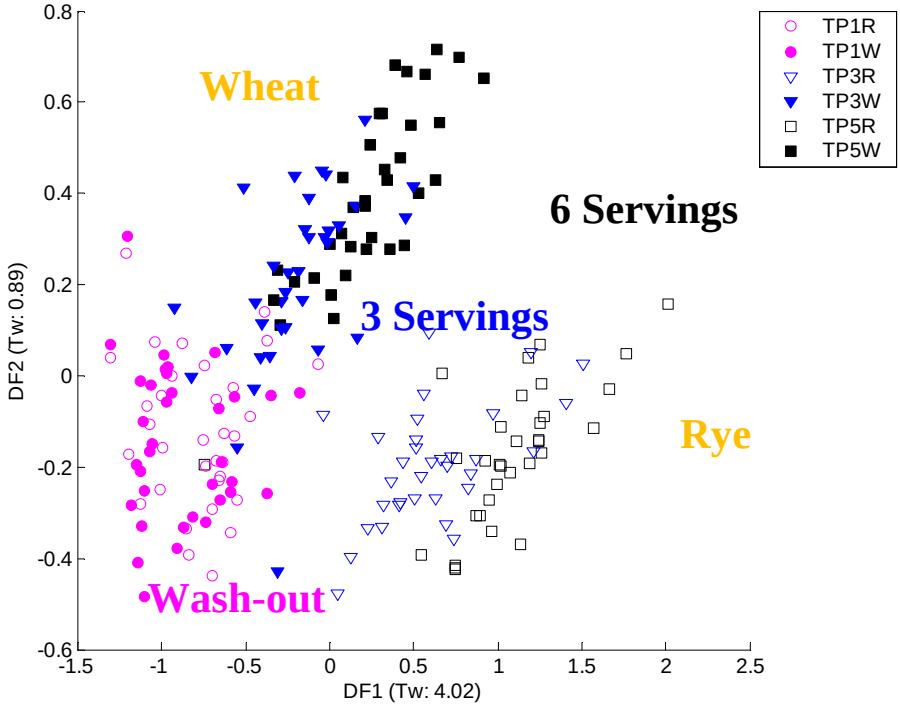


Figure 5.3.5-7: Discrimination between rye and wheat plasma **Samples C** (fasting) based on plasma alkylresorcinols concentration (see Section 3.4.1). Shown is the PC-LDA score plot for log10-transformed concentration values. The three phases of the study are colour coded.

Discrimination between meta-classes of explanatory metabolite signals, and associated inter- and intra-group variances in metabolomics data sets, can be best visualized in boxplots where a box indicates the interquartile range, a red horizontal bar the median and vertical bars the extent of the maximum and minimum values up to 1.5 x interquartile range. If present, an outlier is indicated by a red star (> 1.5 x interquartile range).

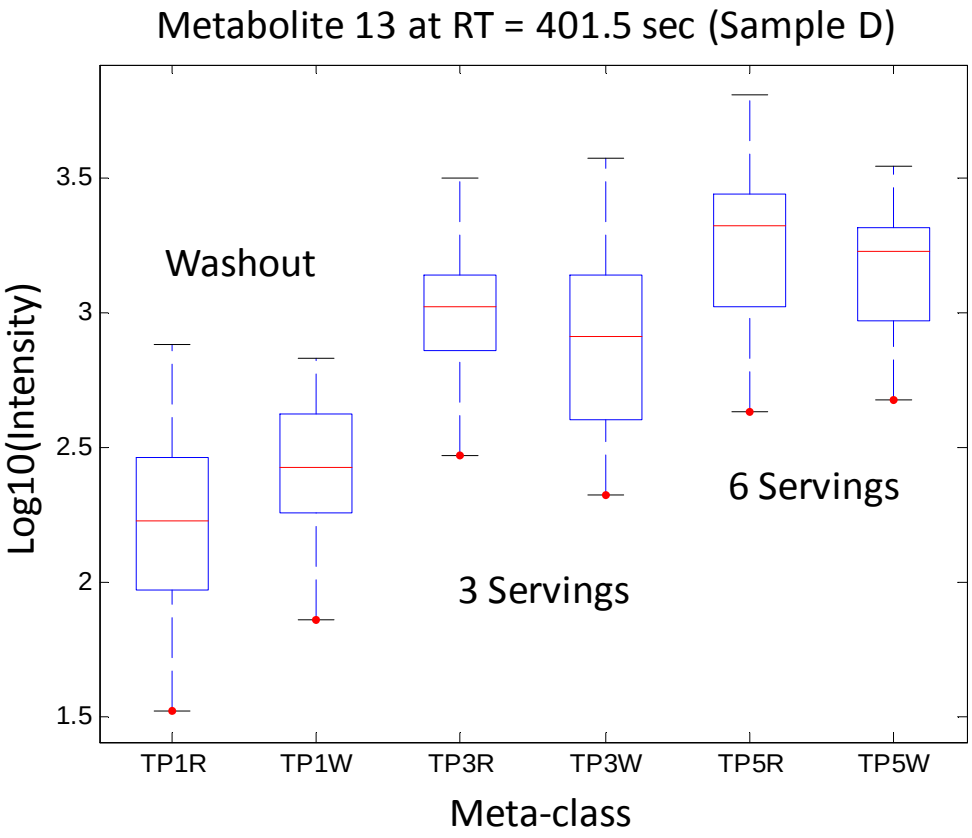


Figure 5.3.5-8: Log10-transformed intensity values of metabolite 13 (DHBA) discriminating three treatment phases of whole grain intake: repartitioning of data by whole grain type and treatment phase.

Figure 5.3.5-8 shows the boxplot of 3,5-dihydroxy-benzoic acid (DHBA, metabolite 13, see **Table 5.3.5-1**) as a representative example for all metabolite signals which increase in response to increased whole grain intake. DHBA, as well as DHPPA potentially good candidates for biomarkers of whole grain-intake (Ross *et al.*, 2004a; Soderholm *et al.*, 2009; Aubertin-Leheudre *et al.*, 2010).

The unknown organic acids (metabolites 7 and 11, **Figure 5.3.5-9**) and those metabolites assigned with an 'R' in **Table 5.3.5-1** may lead to the identification of metabolites with potential as biomarkers specific for rye intake. More specifically, metabolite 7 has been positively identified in the rye bread used in the study and so appears to have been excreted into the urine unaltered. This compound again may be a good candidate for further investigation if it can be purified from rye bread and structurally identified.

Interestingly, although an increase in metabolite 15 concentrations indicates increased whole grain-intake (albeit higher intensities in rye compared with wheat intake) this most likely aromatic compound has been identified only in rye bread, but not found in other wholegrain food items provided to volunteers. It is possible that this result reflects a concentration effect (i.e. present at too low levels to be detected in other rye or wheat products) or the fact that the signal in urine samples has derived mainly from the biotransformation of a more abundant precursor metabolite and not simply from excretion in an unaltered state in urine from dietary sources.

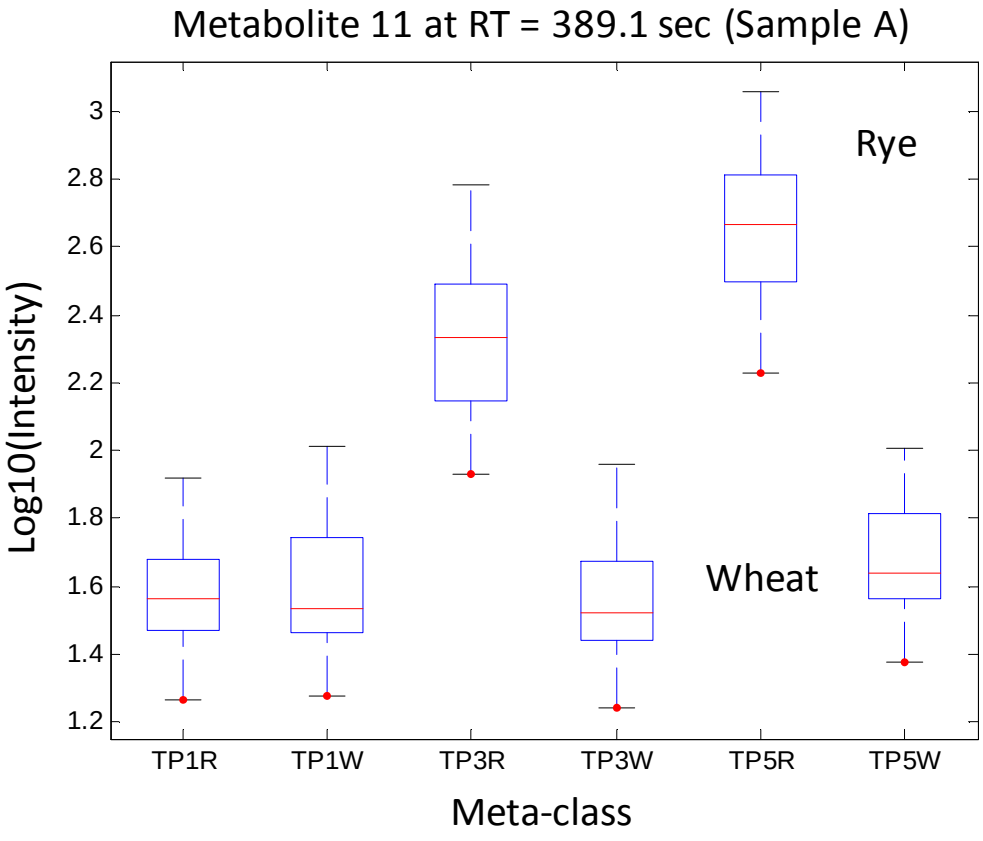


Figure 5.3.5-9: Log10-transformed intensity values of metabolite 11 discriminating rye and wheat whole grain group: repartitioning of data by meta class whole grain type and treatment phase.

5.3.6 Plasma GC-TOFMS metabolite profiling

For GC-TOFMS metabolite profiling of plasma all samples C and D of the rye and those of the wheat intervention have been analysed in separately randomized sets. From a biomarker discovery point of view there are only very few metabolites present in plasma GC-MS metabolite profiling data sets which correlate with increased wholegrain food intake. The main reasons for this is due to greater homeostatic control in blood and the overall within meta-class variance due to strong individual differences of metabolite concentrations in homoeostatic biofluids, which can be as strong as the between class variance, as subject metabolism seem to respond variably to changes in food intake. As a result, no discrimination between intervention phases can be achieved for the wheat whole grain group (Tw < 0.7). Spot fasting plasma samples obtained from the rye group however showed nearly adequate discrimination of wash-out and 6 servings in the first dimension (DF1) for both sample classes C and D (**Figure 5.3.6-1**).

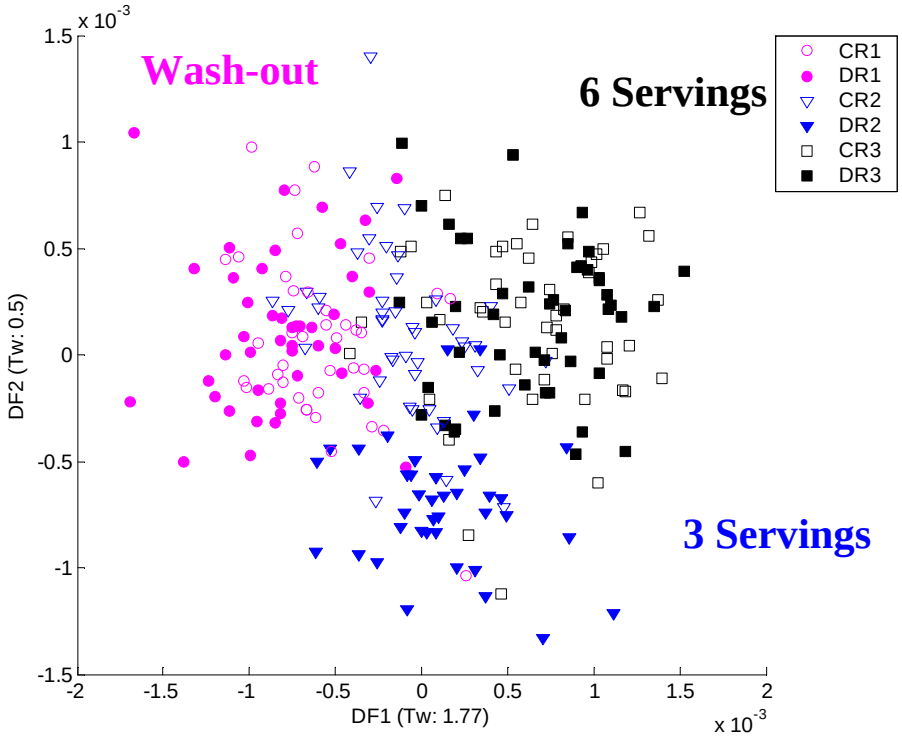


Figure 5.3.6-1: Discrimination between rye WG servings in GC-MS data of fasting plasma **Samples C and D**. Shown is the PC-LDA score plot for all signals with $p < 1x10^{-4}$. The three phases of the study are colour coded.

Three metabolites clearly responded to an increase of whole grain-intake. The overall strongest explanatory metabolite in plasma samples has been identified to be the same as unknown metabolite 1 in urine samples (see **Table 5.3.5-1**). The within and between meta-class variance of log_{10}-transformed signal intensities is shown as a boxplot (**Figure 5.3.6-2**). The second metabolite also showing raised signal intensities in GC-MS data with increased whole grain-intake has been positively identified as nonadecylresorcinol (AR C19:0).

Decreasing abundance in plasma has been found for a compound peak tentatively assigned as monostearin (stearoylglycerol). With regard to fatty acid metabolism there were weak indications in GC-MS data sets of increasing abundance of poly-unsaturated fatty acids (*e.g.* 8,11,14-eicosantrienoic acid) and decreasing saturated fatty acids of currently unknown structure.

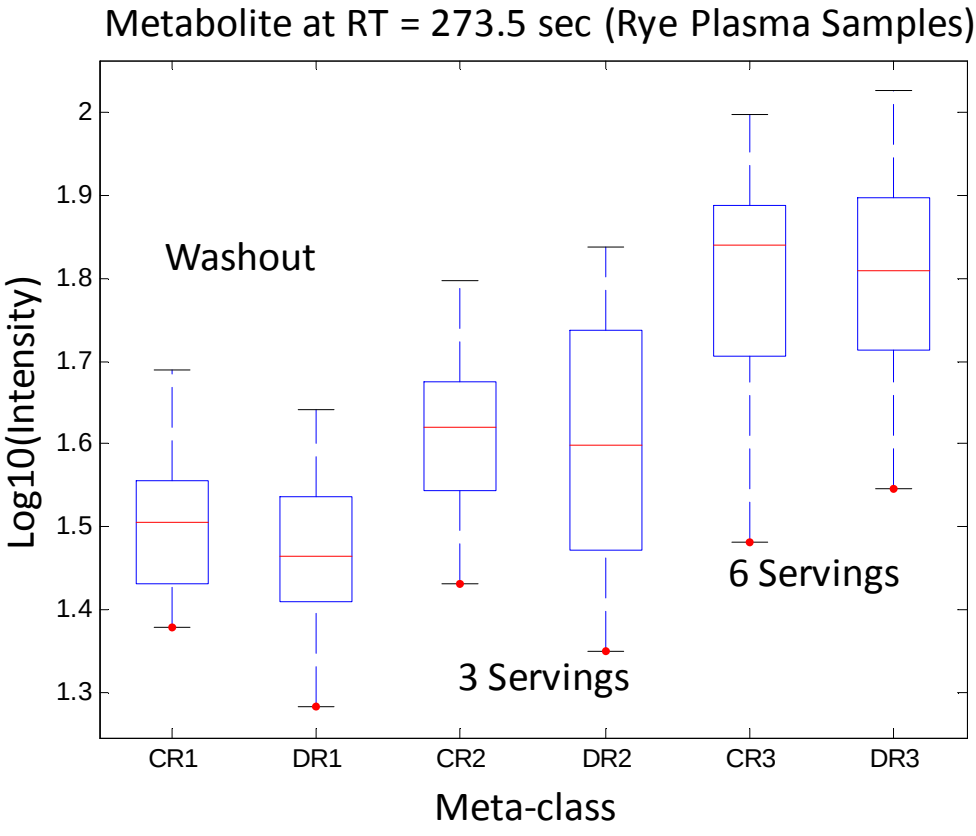


Figure 5.3.6-2: Log_{10}-transformed intensity values of an unknown metabolite discriminating three intervention phases in wholegrain rye group: repartitioning of data by meta-class spot fasting samples and treatment phase.

5.4. Evaluation of metabolite profiles (Task 05-03)

High throughput metabolite fingerprinting using flow infusion electrospray mass spectrometry (FIE-MS) coupled with advanced bioinformatics data analysis has been proven to be a quick and reliable way for knowledge and hypothesis generation in complex experiments. Therefore, FIE-MS has been applied to urine and plasma samples as a screening tool to identify alternative biomarkers in comparison with the targeted extraction, concentration and analysis of alkylresorcinols and mammalian lignans as described in sections 4.4 and 4.5. Although the analysis of metabolite fingerprinting data shows good discrimination of pooled and spot fasting urine samples none of the metabolites detected with electrospray ionisation in urine and plasma samples responded to a dose-dependent increase of whole grain consumption. Without looking into possible reasons (*e.g.* variance, ionisation capability and efficiency, detection thresholds *etc*) we have to accept that there were no biomarkers of whole grain-intake detectable using FIE-MS technology. Although there are indications of novel rye biomarkers emerging the results of this investigation and the general problem identifying new biomarkers for whole grain is shared by many other investigators using LC-ESI-MS technology as discussed in recent NUGO conferences (EU-funded Network of Excellence shaping The European Nutrigenomics Organisation).

The second technology used in the metabolomics workflow at AU is GC-TOFMS metabolite profiling. The results found that pooled (12 h and 24 h) and spot fasting urine samples showed stronger discrimination than spot plasma samples based on several metabolite signals following the dose-dependent increase of whole grain-intake. Although a low intensity nonadecylresorcinol peak has been identified to be discriminatory in plasma samples, none of the other targeted alkylresorcinols or lignans could be identified in metabolite profiling data sets. This is because they are present at concentrations too low to be detected with a sufficient signal to noise ratio above background noise in crude extracts. However, most of the explanatory metabolite peaks are categorized as being of aromatic character (*i.e.* phenolic compounds: hydroxylated and/or methoxylated benzene ring).

Two of the discriminatory metabolites (DHBA and DHPPA, **Table 5.3.5-1**) have been previously suggested as biomarkers for whole grain-intake (Ross *et al.*, 2004a; Soderholm *et al.*, 2009; Aubertin-Leheudre *et al.*, 2010). Although quantification of single compounds in

metabolomics data sets is usually not attempted (mainly due to its complexity and generally missing calibration standards in sample batches) concentrations can be estimated reasonably well in some cases because GC-TOFMS is a semi-quantitative technique.

DHBA and DHPPA have been quantified using commercially available standards which have been used to unequivocally identify the presence of these metabolites in urine samples. The concentrations of DHBA and DHPPA in all urine samples are shown in **Figure 5.3.6-1** and **Figure 5.3.6-2**, respectively, following 1-point external calibration. The concentration ranges found in GC-TOFMS metabolite profiling data are in the order of magnitude of those previously reported for both metabolites using HPLC coupled to electrochemical detection (Koskela *et al.*, 2007). Moreover, it will be possible to normalise these metabolomics data for urine creatinine concentrations once creatinine analyses of all urine sample classes (A, B, P, C and D) are completed [task additional to original contract].

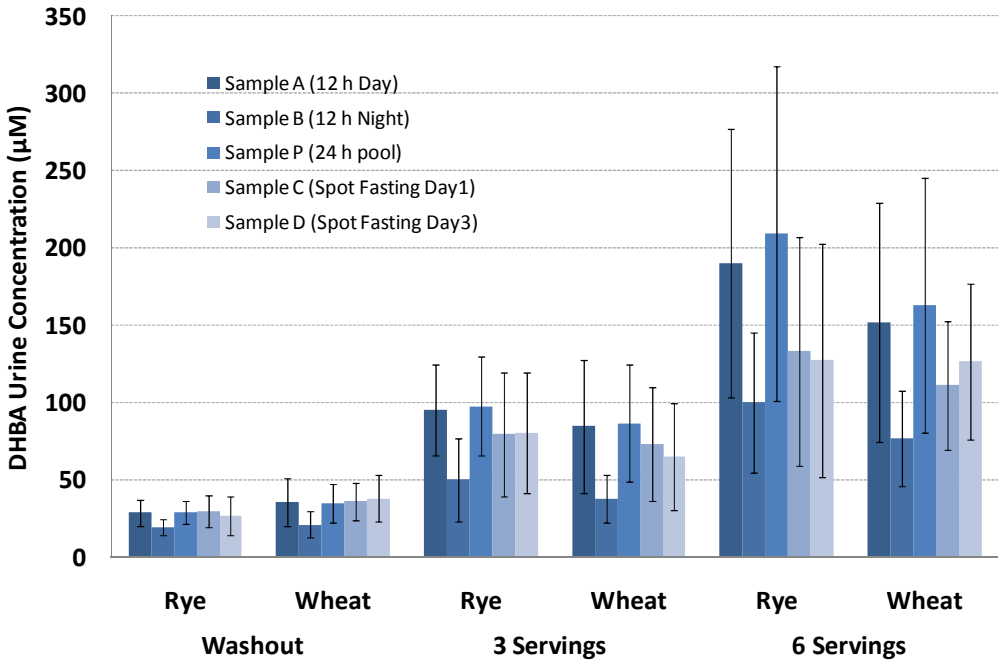


Figure 5.3.6-1: Estimated 3,5-dihydroxy-benzoic acid (DHBA) concentration in urine samples following 1-point external calibration.

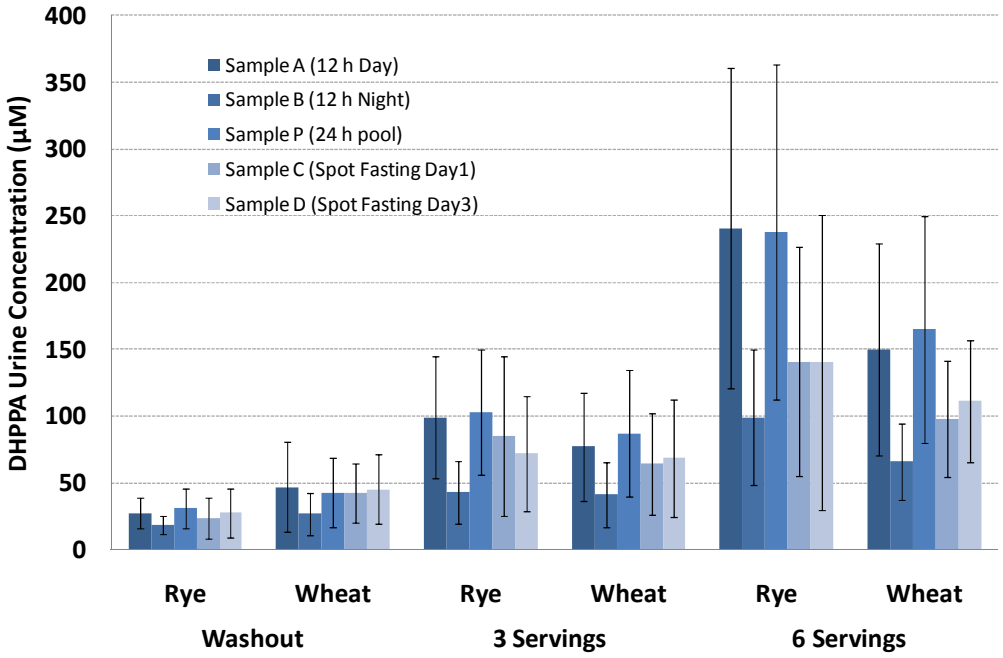


Figure 5.3.6-2: Estimated 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) concentration in urine samples following 1-point external calibration.

DHBA and DHPPA concentrations were highly correlated (correlation coefficient r = 0.90 in *e.g.* Sample P). Both metabolites are additionally correlated with metabolite peaks 16, 21 and 23 (**Table 5.3.5-1**) with correlation coefficients between 0.70 and 0.91 (Sample P). A common characteristic mass fragment of these three metabolites is m/z 268, which is a strong indication for a methyl-bis[(trimethylsilyl)oxy]benzene-fragment, which is also the most stable fragment in derivatized C15 to C25-alkylresorcinols (Ross *et al.*, 2004c). Unfortunately, these metabolites coelute with other urine metabolites and are too low in abundance to allow the extraction of the molecular ion. Further analytical work is required to allow tentative assignments.

Of particularly interest are urine metabolites 7 and 15 (**Table 5.3.5-1**) as these have been positively identified only in rye bread and not in other rye products (*e.g.* cereals, porridge, pasta) or wheat products, including bread. Both metabolites may therefore be potential biomarkers for rye bread consumption. Metabolite peak 7 is most likely a hydroxylated organic acid with an additional olefinic double bond. Interestingly, metabolite peak 7 appears to be correlated with peak 11, which is not present in rye bread. It is most likely that metabolite 11 is the saturated

form of metabolite 7. The mass spectrum of metabolite 15 shows aromatic (or phenolic) characteristics and has also been identified in some urine samples of the wheat group. Hypothetically, metabolite 15 is a bran fermentation product formed from bran constituents during metabolism by gut microbes similar to those reactions present in sour dough. Although metabolite concentrations appear generally higher in urine samples from the rye group, metabolite peaks 7, 11 and 15 are the main discriminators between the rye and wheat group (**Figure 5.3.5-2 to Figure 5.3.5-6**).

Metabolite peaks 25, 35, 36 and 37 are not only explanatory for increased whole grain-intake in pooled day urine sample **A** they are also strong discriminators in spot fasting urine samples. Mass spectra of chromatographic peaks indicate that these metabolites contain aromatic and pyranose functionalities suggesting these metabolites are potentially products of physiological biotransformation reactions. These metabolites exhibit weak or no discrimination of treatment phases in overnight urine sample **B** (**Figure 5.3.5-1**) and are present in at least half of the relative concentration when compared with spot fasting urine samples **C** and **D**. Theoretically, there are two reasons for this effect: 1) a dilution of wholegrain food-related metabolites by those originating from the standardised evening meal; 2) reduced digestion, general organ activity and, consequently, phase 2 biotransformation activities during the resting phase.

5.5. Metabolomics Conclusions

5.5.1. Expected chemistry versus discriminatory metabolites

High throughput metabolite profiling of pooled and fasting spot urine samples using GC-TOFMS found the presence of compounds indicative of increased whole grain food intake. From the list of compounds suggested in current literature 3,5-dihydroxy-benzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) have been positively identified as potential biomarkers for whole grain-intake. Higher concentrations may have been found had the urine samples been enzymatically deconjugated, as they are known to be conjuaged with glucuronide and sulphate groups in order to increase their water solubility (Ross *et al.*, 2004a; Koskela *et al.*, 2007) Free C17:0 to C25:0-alkylresorcinols are not expected to be present in urine at important concentrations as they are too lipophillic (Ross *et al.*, 2004a). Similarly, free lignans (*e.g.* enterodiol or enterolactone) were not detected (compared to authentic standards)

because urine samples for metabolomics analysis were not enzymatically treated to deconjugate metabolites; lignans are thought to be present as sulphates, glucuronides and/or sulfoglucuronides in urines. At present, the detection of conjugated lignans in GC-MS data is not confirmed. However, most of the explanatory compound peaks are compounds with aromatic or phenolic groups which suggests that these metabolites are breakdown or biotransformation products of lignans, alkylresorcinols or some of the other many phenolic bran constituents.

The peak list (**Table 5.3.5-1**) indicates potential biomarkers for wholegrain food intake in urine voids collected during the day (Sample A, metabolites 3, 13 [DHBA], 14 [DHPPA], 16, 25) and in spot fasting urines (Samples C and D, metabolites 13 [DHBA], 25, 35, 36, 37). Rye samples can be discriminated from wheat urine samples using metabolites 7 and 11 (potentially organic acids) for all samples classes, and metabolite 15 (aromatic or phenolic compound) for sample A. Metabolites 7 and 15 have been positively identified in rye bread as the sole food source of products provided to volunteers and could be therefore indicative for rye bread consumption.

Thus, from a list of 37 metabolite GC-TOFMS peaks discriminating the three phase of rye and/or wheat whole-grain intervention 12 metabolites are strongly discriminatory with minimum p-values of $0 \leq p < 1 \times 10^{-11}$ in one of sample classes A, B, C, Dor P. Only two metabolite peaks (i.e. DHBA and DHPPA) have been previously considered as potential biomarkers for whole grain intake. Another two metabolite peaks (7 and 11) are indicative for rye whole grain intake and could be potentially indicative for just rye-bread consumption. The remaining 8 strongly discriminatory metabolite peaks are most likely metabolized compounds from the bran or germ fraction of whole grain and characteristic mass fragments suggest aromatic or phenolic compounds. All other metabolite peaks exhibit less strong discrimination power partly due to their low abundance. No identification is possible for most of these metabolites because the number and value of their stable ion-fragments either do not allow any conclusions regarding molecule properties or indicate chemistry not yet observed in GC-MS data (i.e. not in NIST library). However, as indicated in column '*Discrimination*' in **Table 5.3.5-1** there are 13 potential metabolites which are indicative for rye whole grain intake. Particularly in urine samples **A** (and sample **P**, but not sample **B**) two metabolites (i.e. 5, 33) have been found potentially indicative for wheat whole grain-intake at 6 servings whole grain-intake.

5.5.2. Use of 24 hour urines versus spot fasting or '12 hour' urines

Especially useful is the demonstration that a range of sample types other than 24 h urine had metabolite signals that could be linked to whole grain-intake. In general the 24 h urines had the largest number of discriminatory signals and high statistical relevance followed by sample **A**, which is a 12 h day urine sample. The number of discriminatory signals in the overnight void (Sample **B**) for example was lower than those found in the 24 h urine or day void: some discriminatory peaks were missing or weaker in sample **B**. Conversely, some signals were equally (peaks 3, 7, 11, 13, 14, 19) or more discriminatory in sample **B** (peaks 18, 24, 30) than in 24 h urine. Despite this, concentrations of metabolite peaks are found to be below those in samples **A** and **P**. However, as sample **B** was collected when volunteers were at home, this sample was much easier to collect, a factor that may be important for compliance in epidemiological studies, especially if peaks 13 and 14 (DHBA and DHPPA, respectively) will be used as biomarkers for whole grain-intake.

It is also of great interest to see that some peaks in the spot fasting sample (**C or D**) are in fact more abundant than (peak 35 and 37, **Figure 5.5.2-1**) or as strong (peak 7, 11, 13, 25**, Figure 5.5.2-2**) as those peaks found to be discriminatory in 24 hour pooled urines (Sample **P**). This and the fact that AR have been quantified in spot plasma samples (Objective 04) is an indicator that much of the chemistry is conserved in spot samples taken after an over-night fast most likely because of food metabolites were biotransformed or have a relatively slow clearance rate. As it is generally a major challenge getting representative samples for studies of habitual diet a spot urine sample is clearly a very simple sample to obtain for epidemiological studies. Furthermore, urines are the most non–invasive samples obtainable and in conjunction with MEDE we showed that use of a standard evening meal probably helps to normalize in particular spot fasting samples in terms of the impact of recent food.

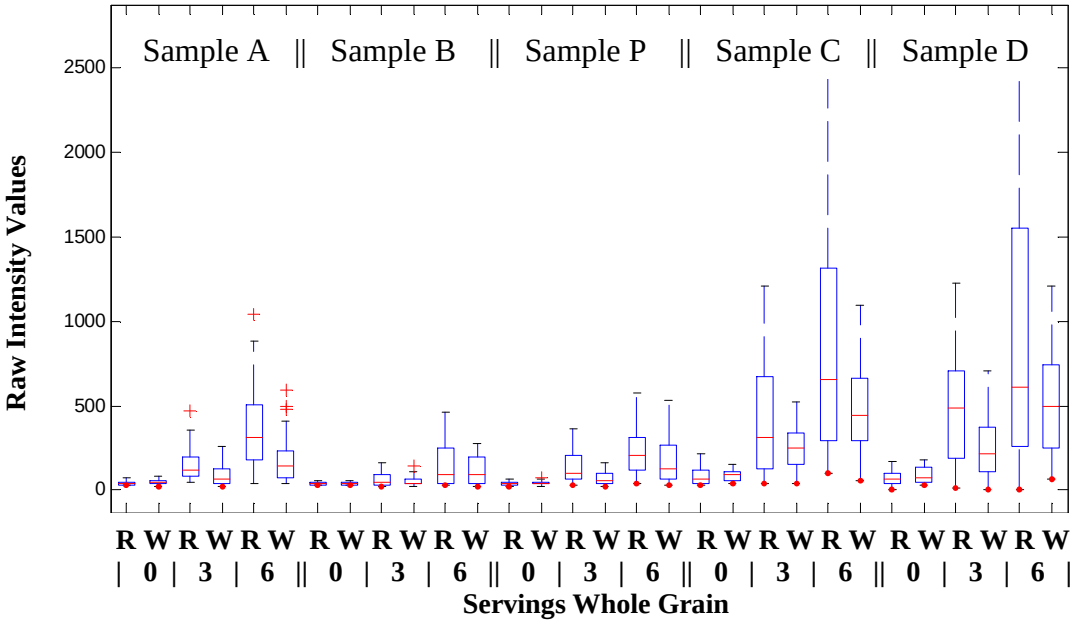


Figure 5.5.2-1: Raw intensity values of unknown metabolite peak 37 discriminating three treatment phases of whole grain-intake particularly in spot fasting urine samples **C** and **D**: repartitioning of data by whole grain type and treatment phase over all sample classes.

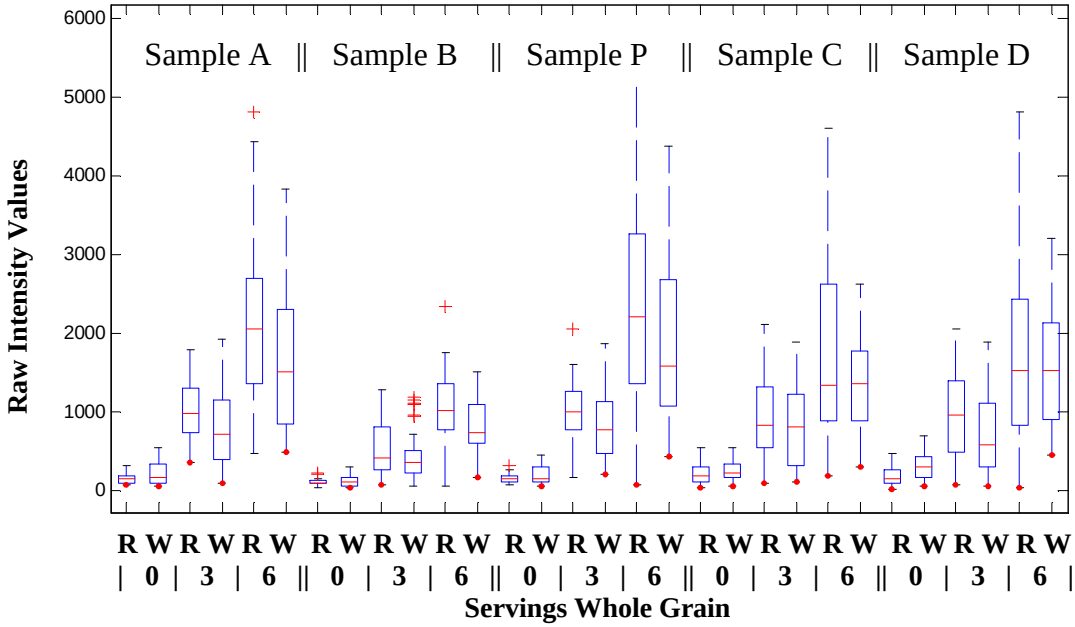


Figure 5.5.2-2: Raw intensity values of metabolite 13 (DHBA) discriminating three treatment phases of whole grain-intake: repartitioning of data by whole grain type and treatment phase over all sample classes.

5.5.3. Towards a metabolite barcode

A possible conclusion of the metabolomics project could be one or more of the following taken from past and recent investigations concerned with identifying biomarkers for whole grain-intake:

1. "Further studies are needed to confirm that these metabolites come from alkylresorcinols, and to develop quantitative methods to test for their suitability as biomarkers of wholegrain wheat and rye intake." (Ross *et al.*, 2004a)
2. "However, before AR 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." (Landberg *et al.*, 2008)
3. "In conclusion, DHBA and DHPPA have potential for use as biomarkers, but further research is needed to evaluate the reproducibility of these findings and to assess the feasibility of their use in large epidemiologic studies." (Soderholm *et al.*, 2009).
4. "Further research in larger free-living populations in other countries is needed to generalise and confirm the present findings." (Aubertin-Leheudre *et al.*, 2010)

From a scientific point of view there will be always a rational for performing new intervention studies to prove suitability of a potential biomarker. Especially from a metabolomics perspective there are further investigations required because metabolomics is data driven to discover new leads and to generate hypotheses which need validation. A very recent review by Jones and Engleson (Jones & Engelson, 2010) summarises general benefits and challenges of whole grains and lists current issues and additional further work needed.

One of this project's objectives was the targeted analysis and quantification of AR in plasma; urine and plasma samples were additionally analysed using metabolomics techniques. With the exception of DHBA and DHPPA most of the explanatory metabolites in urine GC-TOFMS metabolite profiles are currently unknown, but all are characterized by one or more diagnostic mass ions and are therefore identifiable in chromatograms. The relative long list of explanatory urine metabolites and the overall successful outcome of the project can be attributed to volunteers' adherence to the study protocol. The use of the proposed biomarker compounds still needs to be tested in epidemiological cohorts, preferably from studies where links with wholegrain cereal intake and health have already been established.

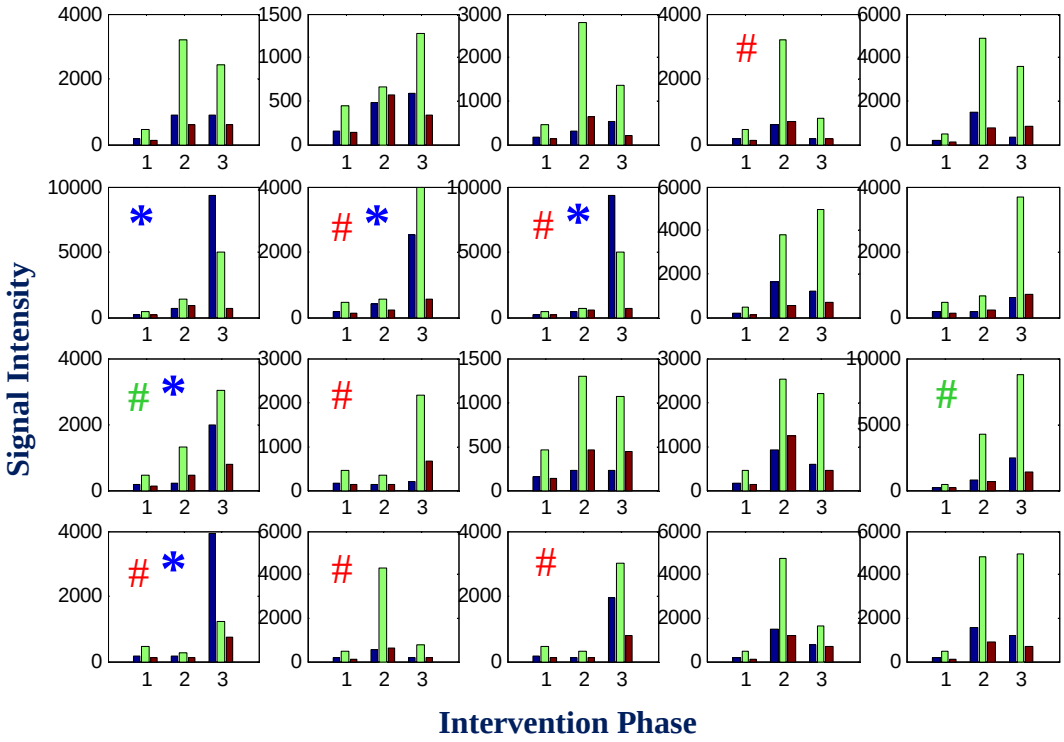


Figure 5.5.3-1: Simplified metabolite barcode to classify individual spot fasting urine samples **C** according to intervention phase. Twenty bar graphs show urine metabolite raw-intensities where one volunteer after Wash-out phase (1) has been compared in all graphs with two randomly selected volunteers after respectively 3 Servings (2) and 6 Servings (3) intervention phase. Therefore, data for phase 1 is the same in all plots and data for phases 2 and 3 are different subjects. **Blue**: sum of metabolite peaks **7**, **11** (indicative for rye/rye bread); **Green**: sum of **DHBA**, **DHPPA**; **Red**: sum of metabolite peaks **25**, **35**, **36**, **37**. **#:** ideal and expected increase of WG intake; #: potential outlier; *: strong rye signal; all others: anticipated behaviour.

To answer these questions the relative abundance of explanatory urine metabolites in GC-TOFMS profiles has been used to demonstrate the main source of variability: individuals. In contrast to most investigations, which use 24 hour urine samples, the focus here is on fasting spot urine samples **C** which are more easily accessible. Each of the 20 sub-plots in **Figure 5.5.3-1** shows 3 metabolite bars (i.e. blue, green, red) with different intensities for each of the 3 intervention phases (i.e. Wash-out, 3 Servings, 6 Servings, respectively). The blue bars represent the intensities of metabolite peaks 7, 11 (indicative for rye/rye bread); green bars are sum of DHBA, DHPPA intensities and red bars are the combined intensities of metabolite peaks 25, 35, 36, 37. The metabolite intensities for wash-out (phase 1) are taken from a randomly chosen

urine sample (i.e. volunteer X) and has been kept the same in all sub-plots. The metabolite intensities for 3 Servings (phase 2) and 6 Servings (phase 3) are taken from two randomly selected urine samples (i.e. volunteers Y and Z, respectively).

In this random selection of samples only two sub-plots (green #) show ideal behaviour which is expected for increased whole grain-intake. Sub-plots assigned with a red # show extreme outlier behaviour because either phase 2 or phase 3 metabolite intensities are similar to those in phase 1 (*i.e.* Wash-out). A blue star indicates the presence of samples following high rye or rye bread intake because of high intensities of metabolite peaks **7** and **11**. All the other sub-plots show however trends which are anticipated in this context because lower intensities of metabolites in phase 3 could just indicate that this sample has been taken from an individual in the wheat group compared with a rye sample for phase 2, and *vice versa*. Additionally, most explanatory metabolite signals are probably linked to components within the bran and the germ of wholegrain food sources. As can be seen in **Figure 5.5.3-2** the actual AR abundance varied with food items volunteers were provided with.

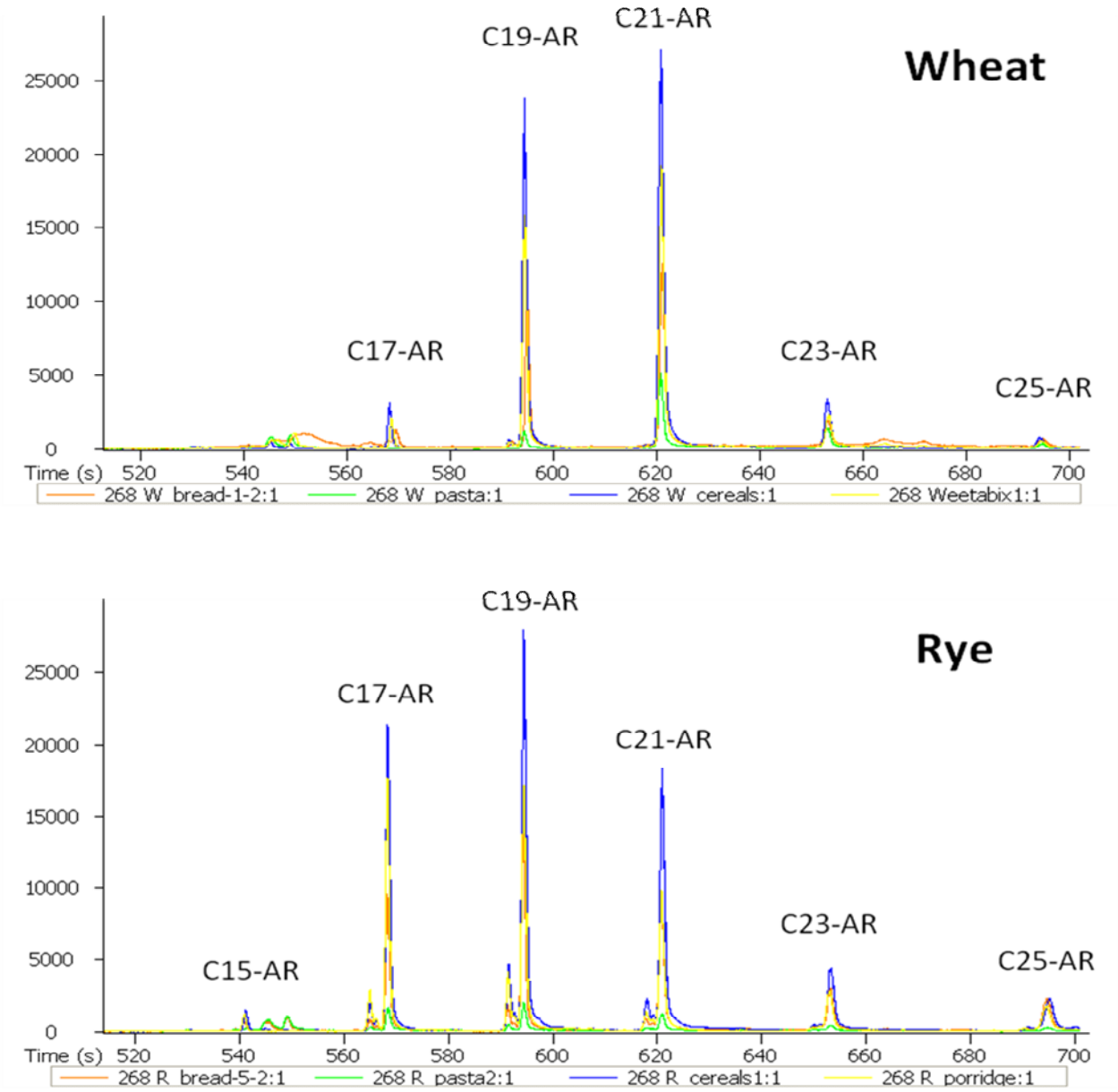


Figure 5.5.3-2: Raw intensities of alkylresorcinols (AR) in different food sources (~20 mg) indicated by diagnostic mass ion m/z 268. Ratios of AR with different chain lengths are largely conserved within Wheat and Rye wholegrain food products.

It becomes obvious that volunteers who prefer pasta have to eat approximately 10 times the amount to equal their AR intake with those individuals who prefer breakfast cereals. Thus,

individual eating habits or preferences for different food products contribute to the overall variance in urine metabolite concentrations (see also Section 3.4.1 Plasma AR concentrations).

The approach outlined in **Figure 5.5.3-1** is feasible for epidemiological studies where we have to expect 1) a mix of wholegrain food products based on rye and wheat, and 2) getting only a single sample per individual. The systematic comparison of metabolites indicative for whole wheat and rye intake ('barcode') allows categorizing volunteers into different groups of habitual wholegrain consumers. From a perspective of investigating wholegrain wheat and rye intake it is not of importance if 24 h or fasting spot urine samples have been or will be collected because GC-TOFMS metabolite profiling found that both samples are of great value. However, spot fasting urine samples are much easier accessible than 24 h urine samples and could guarantee improved compliance. Fasting spot urine samples (e.g. sample **C**) are potentially of greater value as the effects of slow and fast metabolisers and recent or acute whole grain-intake on variance might be reduced which favours investigation of more habitual whole grain-consumption (see below).

From a metabolomics perspective urine samples are much more informative than plasma samples. The main reason is the homeostatic regulation of blood to avoid fluctuations in concentrations. The excess of metabolites, electrolytes and water is removed through the kidneys and then into the urine. Metabolites, which cannot be removed directly, because they are for example more lipophilic, undergo biotransformation reactions (e.g. glucuronidation in liver P450 system) to become more hydrophilic and excreted as urine. What are then the benefits of plasma? With respect to expected chemistry in plasma GC-TOFMS metabolite profiles only nonadecylresorcinol (AR C19:0) has been positively identified to follow the dose-dependent increase of whole grain intake. The abundance of AR was very low using this general method, and only just reached a signal-to noise ratio of 10:1, the threshold for quantification. However, targeted AR analysis (Objective 04) successfully quantified all AR in plasma samples, which suggests that more method development is required to achieve detection of these and other nutritionally characteristic metabolites (i.e. biomarkers) in plasma using non-targeted high-throughput metabolite profiling. Therefore, besides the successful identification of biotransformed whole grain bran or germ components in urine, plasma is an important source for unadulterated food metabolites (e.g. AR17:0 to AR25:0).

Metabolite fingerprinting and profiling as it now stands have their limitations because there are potentially many compounds that might have been missed with the technological and methodological limitations that we have at present. One of the main conclusions of this project is the importance of a comprehensive metabolomics approach, which also includes LC-MS metabolite profiling (e.g. lipidomics approaches) and, most importantly, metabolite targeted analysis (see Section 5.1) for reportedly important and discriminatory metabolites. A summary of the results obtained in Objective 04 show that the AR intensity pattern of whole rye and wheat food products (**Figure 5.5.3-2**) is similar to the plasma AR concentration profile (**Figure 5.5.3-3**). Individuals in the rye and wheat groups can be discriminated using for example the AR21:0-to-AR19:0-ratio. Similar to the urine metabolite barcode (**Figure 5.5.3-1**) the barcode of one volunteer (here phase 2) has been compared 20 times to those of randomly chosen individuals in the Wash-out and 6 Servings group (**Figure 5.5.3-4**). The AR21:0-to-AR19:0-ratio identifies the volunteer as belonging to the rye group. Although individual concentration differences are large, a comprehensive regression analysis would categorize this individual as a phase 2 (3 servings/d) member. Of interest is that all individuals in phase 1 (wash-out) show the characteristic pattern for wheat, which is not surprising for volunteers within the UK.

Although it might prove to be challenging to deconvolve different wholegrain foods like rye, barley, different species of wheat etc in an epidemiological context using a saturated long-chain AR plasma 'barcode', the GrainMark project has delivered a proof of principle of its feasibility. These findings corroborate the results of others that found distinct alkylresorcinol homologue profile between rye and wheat diet (Linko-Parvinen *et al.*, 2007) The combination of barcodes applied to results of comprehensive plasma and urine spot fasting sample metabolite analyses might give the confidence needed when evaluating and categorizing wholegrain food intake of individuals at the epidemiological scale.

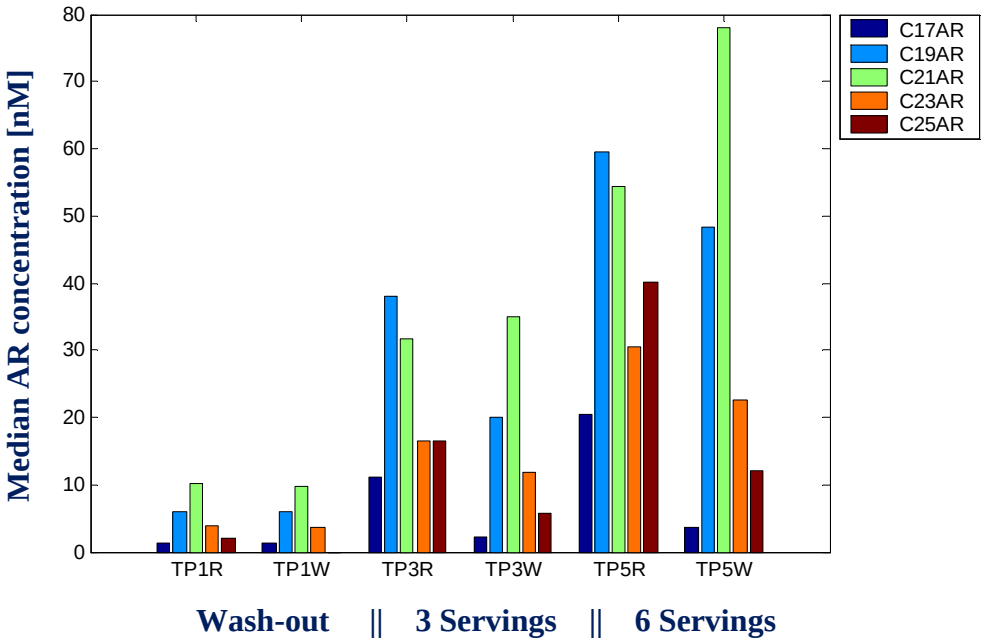


Figure 5.5.3-3: Median concentration (nM) of plasma alkylresorcinols (AR) in fasting sample **C**. The ratio of AR with different chain lengths as found for different food products (**Figure 5.5.3-2**) are largely conserved in plasma samples. TP1: Wash-out, TP3: 3 Servings, TP5: 6 Servings; R: Rye and W: Wheat group.

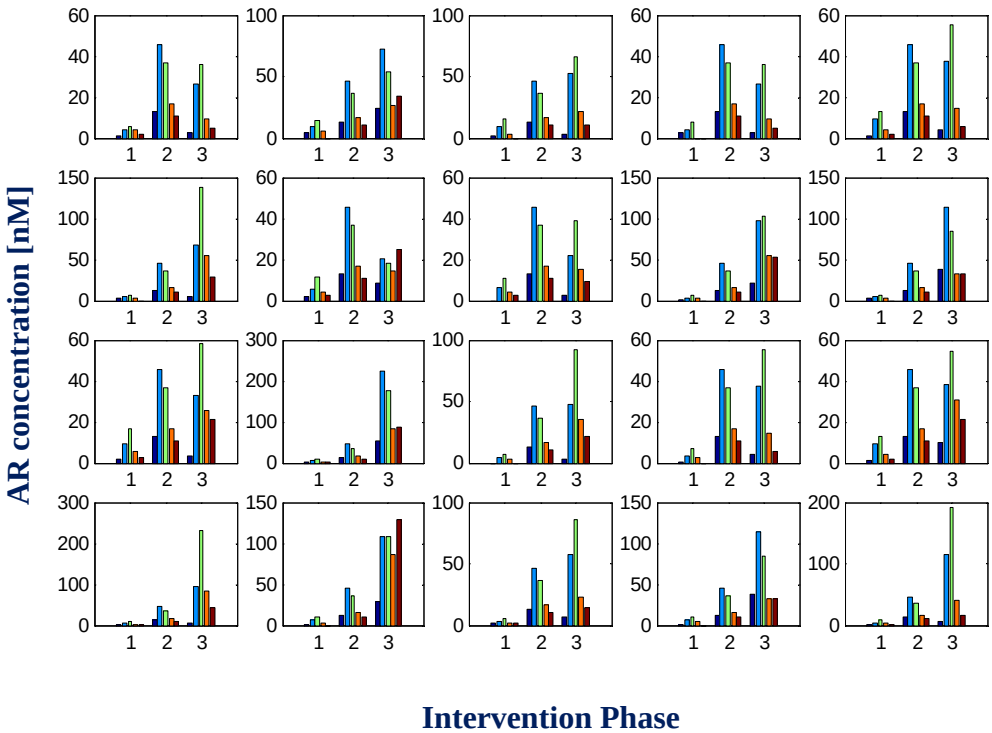


Figure 5.5.3-4: Alkylresorcinol (AR) barcode to classify individual plasma samples C according to intervention phase. Twenty comparisons of plasma AR concentrations (nM) of one volunteer in phase 2 (3 Servings) with two randomly selected volunteers, one each after Wash-out (2) and 6 Servings (3) intervention phase. Therefore, data for phase 2 is the same in all plots and data for phases 1 and 3 are different subjects. Colour code of AR as in **Figure** 5.5.3-3.

5.5.4 Weetabix, a MEDE3 Study example

On average the strongest signal in sub-plots of **Figure 5.5.3-1** is attributed to the abundance to DHBA and DHPPA (combined intensities shown as green bars), which indicates excellent diagnostic value in spot fasting urine samples using high-throughput GC-TOFMS metabolite profiling (**Figure 5.5.2-2**). Their usefulness as biomarkers is demonstrated in the following example using spot urine samples collected during Study 3 (Test Foods) of the MEDE project (see Report of FSA Project N05073: "Development of metabolomics as novel approach to biological indicators which characterise and quantify dietary exposure", Sections 3.4.1 and 3.5).

Due to stringent thresholding of $\log_{10}$-transformed GC-TOFMS urine data (MEDE3 Study) DHBA and DHPPA have not been labelled as discriminatory metabolite peaks in pair-wise comparisons of Weetabix (Weet) and the Standard Breakfast (SB). However, given 4 – 5 h half-life of AR and clearance through hepatic cytochrome P450-dependent metabolism (Ross *et al.*, 2004b; Landberg *et al.*, 2009a) these metabolites are expected to be more abundant in 4.5 h post-prandial spot urine samples (e.g DHPPA, **Figure 5.5.3-5, A**). The same data plotted using raw-intensity values as a semi-quantitative measure show up to 6 volunteers with high urine concentrations of DHPPA (red +) in other test food groups suggesting habitual or recent (e.g. the day before test day) whole grain-intake of individuals (**Figure 5.5.3-5, B**). The presence of these in this context strong 'outliers' weakens the models of DHPPA and DHBA as explanatory metabolites for acute wholegrain wheat (Weet) intake in metabolite profiling data. The number of volunteers per breakfast (n = 24) allows the removal of these outliers, which drastically improves significance for DHBA and DHPPA as discriminators between Weetabix and other test foods (**Figure 5.5.3-6**).

This example demonstrates the advantages of using a barcode approach where more than just one metabolite is used to answer specific questions like acute whole grain-intake. Filtering the data set (*i.e.* removing individual outliers with potentially high habitual whole grain intake) improves confidence in extracted models which would have been rejected otherwise as not significant. Thus, the outcome of the present, related (e.g. MEDE project) and future projects is to further develop barcodes with metabolites of diagnostic value for many food items to be used

instead of a single biomarker. The list of specific explanatory metabolites can be used in parallel to the more generalized but robust metabolomics approach.

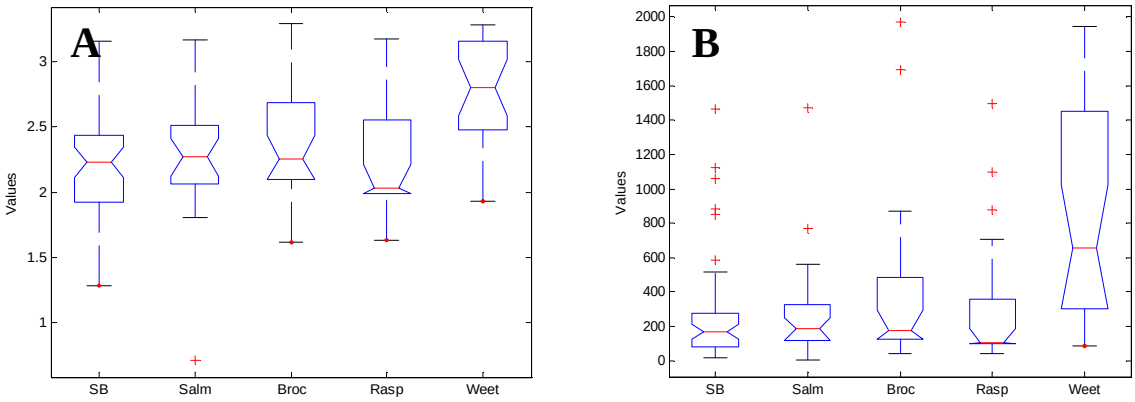


Figure 5.5.3-5: Boxplot of log_{10}-transformed (**A**) and raw intensity values (**B**) of DHPPA in 4.5 h post-prandial urine samples (MEDE3 Study). Repartitioning of data by changed breakfast item cornflakes in SB with smoked salmon (Salm), broccoli (Broc), raspberry (Rasp) and Weetabix (Weet) (**A**: $p = 1.21 \times 10^{-5}$; **B**: $p = 5.43 \times 10^{-6}$, ANOVA, n=24). Red +: outlier (>1.5 times inter-quartile range).

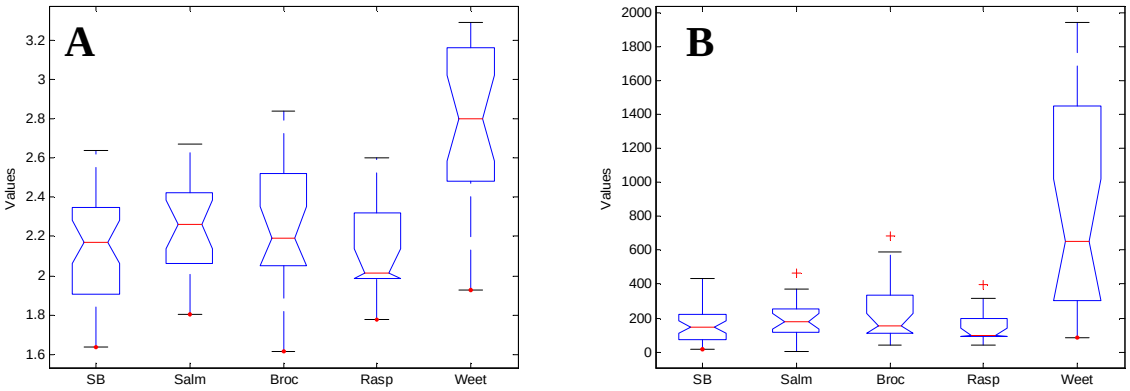


Figure 5.5.3-6: Boxplot of log_{10}-transformed (**A**) and raw intensity values (**B**) of DHPPA in 4.5 h post-prandial urine samples after removal of outliers (MEDE3 Study). Repartitioning of data by changed breakfast item cornflakes' in SB with smoked salmon (Salm), broccoli (Broc), raspberry (Rasp) and Weetabix (Weet) (**A**: $p = 2.0 \times 10^{-13}$; **B**: $p = 2.0 \times 10^{-15}$, ANOVA). Red +: new outlier (>1.5 times inter-quartile range).

6. Conclusions and scope of future work

The GrainMark study was a well controlled dietary intervention which provided samples for targeted biomarker analysis (alkylresorcinols and mammalian lignans) and for metabolomics screening of biofluid samples. The design of the intervention allowed for comparison between two different wholegrain cereals and two levels of intake (US recommended 48g whole grain/d and twice this amount). Careful instruction and monitoring of volunteers ensured satisfactory completion rates and very good compliance with consumption of the intervention foods. The volunteers consumed a limited range of wholegrain wheat and wholegrain rye foods to reduce variability in whole grain-consumption patterns.

Plasma concentrations of alkylresorcinols were identified as the best targeted biomarker for whole grain-intake with highly significant correlations between plasma alkylresorcinol homologues and whole grain-intake. Plasma concentrations of the mammalian lignans, enterodiol and enterolactone, were less strongly correlated with whole grain-intake, being weakly significant in the case of wholegrain rye foods only. Urinary concentrations and 24h excretion of mammalian lignans were significantly correlated with whole grain-intake, but the relationships were less strong than for plasma alkyresorcinols. Changing ratios of different homologues of alkyresorcinols in plasma samples from wholegrain wheat and rye groups provides a mechanism for screening samples based on consumption of mixed-grain diets and would be a logical next step in confirming their value as biomarkers of whole grain-intake.

Metabolites of alkyresorcinols (DHBA and DHPPA) were identified in GC-TOFMS as strong discriminators in urine samples, together with other unidentified biomolecules likely to be breakdown products of bran components. DHBA and DHPPA may represent more accessible biomarkers of whole grain-intake as their analysis is easier than that for alkylresorcinols. The strong discriminatory power of these biomolecules also raises the potential of using these biomolecules in development of a ‘bar code’ approach to categorising biofluid samples. There is scope for further evaluation of both these methodologies using samples from the GrainMark study.

A major conclusion of from task 5 is that metabolomics is capable of making new discoveries of potential biomarkers derived from whole grain-intake that may be more abundant and thus much easier to measure in urine samples. Purification and structural analysis of the potentially explanatory unknown metabolite peaks detected in urine and blood samples was beyond the scope of the present project. In future it would be valuable to carry out more work to structurally identify unknown metabolites and to prove the hypotheses stated above.

In the present project there was additionally a suggestion that the intake of whole grain was affecting endogenous metabolism related to saturated lipid composition of plasma. However, it was beyond the scope of the project to investigate this by targeted analysis following up additional leads gained from GC-MS analysis of plasma samples regarding increasing and decreasing concentrations of polyunsaturated and saturated fatty acids respectively following an increase of whole grain-intake. As the overall analysis methods were restricted to 'global' extraction procedures it may be possible in the future to gain further insight into the potential benefits of whole grain-intake by application of lipidomics techniques to analyse the plasma samples obtained in the GrainMark project to further corroborate biochemical analysis results.

7.0 Acknowledgements

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9.0 Appendices

Appendices 1 to 9 as attached below.

GrainMark

Newcastle University

Appendix 1: List of Foods to Avoid

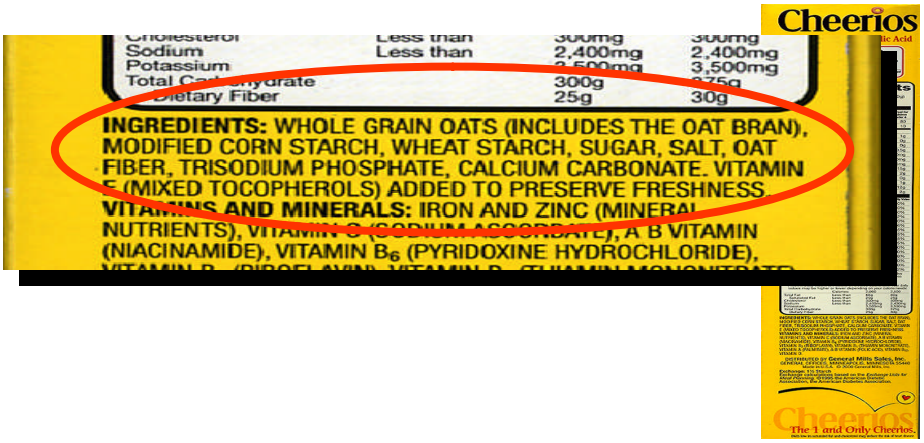
During the study we need you to avoid ALL wholegrain foods *except* those we provide for you.

Please try not to eat any food which contains:

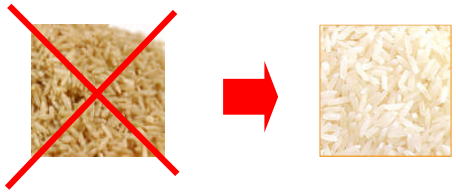
OATS, RYE, BARLEY, WHOLE-WHEAT, FLAX SEED

How to identify foods containing whole grains:

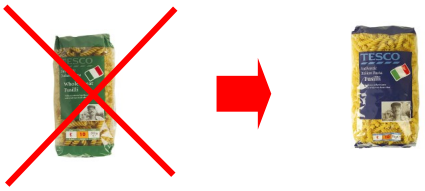
Look for the "Whole Grain" logo or banner on some breakfast cereals and products



Look for "Whole Grain" or "Wholemeal" as a leading ingredient



Use WHITE rice NOT BROWN rice



Use WHITE pasta NOT WHOLMEAL pasta

The following table will help you choose the right options

Foods to avoid	Food you can eat
Breakfast cereals	*Breakfast cereals*
Shredded Wheat	Cornflakes
Weetabix/Weetaflakes	Frosties
Oatabix	Rice Krispies
Muesli	
Puffed Wheat	
Shreddies	
Cheerios	
Bran flakes	
All bran	
Ready Break	
Porridge	
Breads	*Breads*
Any wholemeal bread/roll/pita	Any standard 'white' bread/roll/pita
Granary bread	
Warburton's 'Best of Both'	
Staffordshire oat cakes	
Kingsmill 50-50	
Rice and Pasta	*Rice and Pasta*
Any 'brown' rice	Any 'white' or polished rice
Any wholemeal (or whole-wheat) pastas	Any standard pasta
Wholemeal cous cous	
Millet	
Bulgar wheat	
Cakes and Pastries	*Cakes and Pastries*
Flapjacks	Sponge cake
Parkin	Scones (not wholemeal scones)
Fruit crumbles made with wholemeal flour	Apple pie
Crisps and Snacks	*Crisps and Snacks*
Rice cakes	Standard Crisps
Ryvita crispbreads	Kettle crisps
Oatcakes	Prawn crackers
Twiglets	
Cereal bars	
Tortilla chips	

If you are not sure about a product please give us a call:

Study Contacts: Dr. Sumanto Haldar, Mrs Wendy Bal
Telephone: 0191 222 6619
GrainMark@ncl.ac.uk

You are being invited to take part in a research study. Before you decide it is important you understand why the research is being done, and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of this study?

This study will investigate possible indicators (biomarkers) of whole-grain intake. The aim of the study is to try and find out whether certain substances present in whole grains are absorbed in the gut and appear in blood and urine, and how the mixture of metabolites in blood changes after eating different wholegrain foods.

Why have I been chosen?

We are looking for men and women who are non-smokers and over the age of 18 years to take part in this study. We will be recruiting 64 volunteers in total from the Newcastle upon Tyne area.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form on your 'Induction Visit'; you will be given a copy of this to keep. However, you will be free to withdraw from the study without giving a reason anytime up to the end of your final visit. Shortly after this, all data will be fully annonymised, and therefore, from this point forward it will not be possible to withdraw any data from the study.

What will happen to me if I take part?

If you decide to take part and you are a suitable volunteer for the study, we will ask you to exclude any wholegrain foods from your diet for a period of 4 weeks (we will give you a list of foods to avoid). After this 'washout period', you will be randomly allocated to one of two different groups. For each of the two groups we will provide you with a number of different foods including bread, breakfast cereals, and pasta and ask you to substitute these for similar foods you normally eat in your diet. In one group these foods will be made from wholegrain wheat, in the other group the foods will be made from wholegrain rye. For the first 4 weeks following the washout period, we will ask you to consume 3 sevings/day of these foods (for example, 1 serving = 1 slice wholemeal bread or ½ a bowl of cereal) and for the subsequent 4 weeks we will ask you consume 6 servings/day. We will provide you with the study foods regularly (every 2 weeks) and will also advise you on the amounts of these foods we would like you to eat. In total, the study will last for about 3 months.

What else do I have to do?

If you agree to take part we will ask you to visit **the Clinical Research Facility, RVI** on six occasions, twice after the four-week washout period, then twice each after a further 4 and 8 weeks (12 weeks in total) respectively. Each pair of visits after every 4 week periods will usually take place within 2 days of each other. On the evening before each visit we will ask you to eat a standard meal (and water) which we will provide and then you will need to fast from 9 pm; this means that you should not eat or drink anything except water until you complete your visit the following morning.

At the first visit of each occasion, we will take a blood sample (30ml/6 teaspoons of blood) from your arm and will also measure your height, weight, waist circumference, body fat and blood pressure using non-invasive procedures and will ask you to complete a physical activity questionnaire. We will also ask you to collect your urine for 24 hours the day before and bring it to this visit (appropriate containers and full instructions on how to do this will be provided). This visit will last approximately 45 minutes.

At the second visit, on each occasion, we will take a small blood sample (20ml/4 teaspoons of blood) and a small urine sample (20ml). This visit will last approximately 30 minutes.

We will ask you to collect foods from **the Clinical Research Facility, RVI** after the wash-out period and then after every 2 weeks.

Finally, we will ask you to complete a questionnaire on 4 occassions to record how often you have eaten certain foods at specific periods during the study (known as a 'Food Frequency Questionnaire'). We would also like you to tell us about your general wellbeing after meal times during these 4 occassions by completing an additional 'After Meal Wellbeing Questionnaire'. Whether you decide fill this questionnaire is entirely up to you and your decision will not affect the rest of your participation in this study.

What will happen to the samples I provide?

Blood and urine samples provided will be tested for substances present in wholegrain foods. Lipid profile (e.g., cholesterol) and glucose will also be measured in blood. A small amount of the blood taken at study visits will be stored (for up to 10

years) for future tests to confirm results. DNA samples from blood will also be stored to carry out genetic tests if necessary as part of this study. These genetic tests will be used to help understand how different people respond differently to eating wholegrain foods. All stored plasma and DNA samples will be coded so that no one can be identified from these samples.

What are the possible disadvantages and risks of taking part?
Taking blood samples may cause minor discomfort and there is a small chance of minor bruising afterwards. If a new diagnosis of high blood pressure is made, this could affect your future insurance status (e.g. for life insurance or private medical insurance).

What are the possible benefits of taking part?
If we discover any abnormalities of significance in your lipid profile, blood glucse or blood pressure, we will inform you and your GP. Although you will derive no further individual benefit, the knowledge gained from this study will help our research into identifying biological indicators of whole-grain intake in the diet that will prove valuable for future research.

What will happen if anything goes wrong?
Any complaints you have about this study should be made to Dr. Chris Seal, Newcastle University (chris.seal@ncl.ac.uk or 0191-2227650) and will be fully investigated.

Will my taking part in this study be kept confidential?
Any information which is collected about you during the course of the research will be kept strictly confidential. Your GP will be notified that you are participating in this study. He/she will be notified if any abnormal results of significance to your health are found.

What will happen to the study results?
We will publish the results of the study in a scientific journal and on the project website. You will not be personally identified in any publications. We will be happy to discuss the overall results with you when the study is completed, and will let you know where you can obtain a copy of the published results if you wish.

Who is organising and funding the study?
This study is being organised by Newcastle University. The Food Standards Agency is funding the research. In recognition of your time commitment, you will be paid an honorarium of £90 at the completion of the study. Any travel expenses will also be re-imbursed.

Who has reviewed the study?
This study has been reviewed by the Food Standards Agency, Newcastle NHS Trust and a Research Ethics Committee.

Contact for further information
If you would like any further information about this study, please do not hesitate to contact **Dr. Sumanto Haldar on 0191 222 6619**

And finally…
Thank you for having taken the time to read this information sheet and for your interest in the study.

Newcastle University

Appendix 2

GrainMark Study

GrainMark

Information Sheet for Participants

Newcastle University
School of Agriculture, Food & Rural Development
Agriculture Building
Newcastle upon Tyne
NE1 7RU

Study Contact: Dr. Sumanto Haldar

Telephone: 0191 222 6619
e-mail: GrainMark@ncl.ac.uk
www.grainmark.org

Appendix 3: Pre-Screening Questionnaire

Please explain more about the study – assure confidentiality of personal information at the start of the telephone interview

	YES	NO
Are you over 18 years of age?	☐	☐
Do you smoke?	☐	☐
Are you allergic to wheat/rye/gluten?	☐	☐
Do you have any other food allergies or intolerances?	☐	☐

If yes, please give details.

.....

	YES	NO
Do you have any dietary restrictions? (such as being on 'detox' or other slimming diets)	☐	☐
Are you currently suffering from any illness *(prompt volunteers with – heart disease, diabetes, cancer, celiac disease, high blood pressure, anaemia)*	☐	☐
Are you currently taking any prescribed medications?	☐	☐
Are you currently taking any other medications that can be purchased over the counter?	☐	☐
Are you currently taking any dietary supplements (vitamins/minerals etc.)?	☐	☐
If taking dietary supplements, would you be prepared to stop taking them for 3 months during the study period?	☐	☐
Do you drink alcohol?	☐	☐
If yes, do you drink more than the recommended amount of alcohol per week? *[prompt – 21 units recommended for men and 14 units recommended for women. One unit = ½ pint beer or 1 spirit (25 ml) or 1 small glass of wine]*	☐	☐

Exclude if yes

Diet and BMI			
Estimate of current weight	St/lb kg	**Estimate of current height**	Ft/in m

Estimated BMI:

Exclude if estimated BMI < 20 kg/m ^{2} or > 30 kg/m^2

	YES	NO
Have you had a weight change of more than 3 kg (7lbs) in the past 2 months?	☐	☐
Are you planning to loose/gain weight in the next 3-4 months?	☐	☐

If yes, Exclude

	YES	NO
Do you eat breakfast cereal?	☐	☐
Do you eat bread?	☐	☐
Do you eat pasta?	☐	☐
Do you eat wholegrain foods?	☐	☐
Would you be willing to stop eating whole grain foods for one month during the study?	☐	☐

Supplementary Information *(to be used to allocate volunteers to different study groups)*

1. For Women only:

Are you; pre-menopausal ☐ post-menopausal ☐

If pre-menopausal :	YES	NO
Are you or have you been pregnant within the last 12 months?	☐	☐
Are you planning a pregnancy within the next 12 months?	☐	☐

If yes, Exclude

	YES	NO
2. Are you a vegetarian/vegan?	☐	☐

Availability Information

	YES	NO
Would you have any anticipated difficulties with your availability or transport arrangements to attend each of the study visits?	☐	☐

	YES	NO
Are you planning to go away on a holiday in the next 3-4 months?	☐	☐

If yes, if you will be interested in taking part after your holiday and please state when.

…….

Suitability

	YES	NO
Suitable?	☐	☐

If yes, book Induction Visit

Date ……. Time …….

ID code ☐☐☐☐☐☐☐

	YES	NO
If no, ask volunteers if they wish to take part in any future human nutrition studies and have their details retained within our confidential database	☐	☐

Participant details

Name ……. **M** ☐ **F** ☐

Address

……

…..

…… **Post Code** ……

Age …… **Date of Birth** ……..

Phone Number: Day …….. Evening ……..……

Mobile ……

Best time to phone……..

Preferred Telephone Number (circle) Day/Evening/Mobile

General Practitioner Name……

Of (medical practice)……..……..

……

GP telephone number……

☐

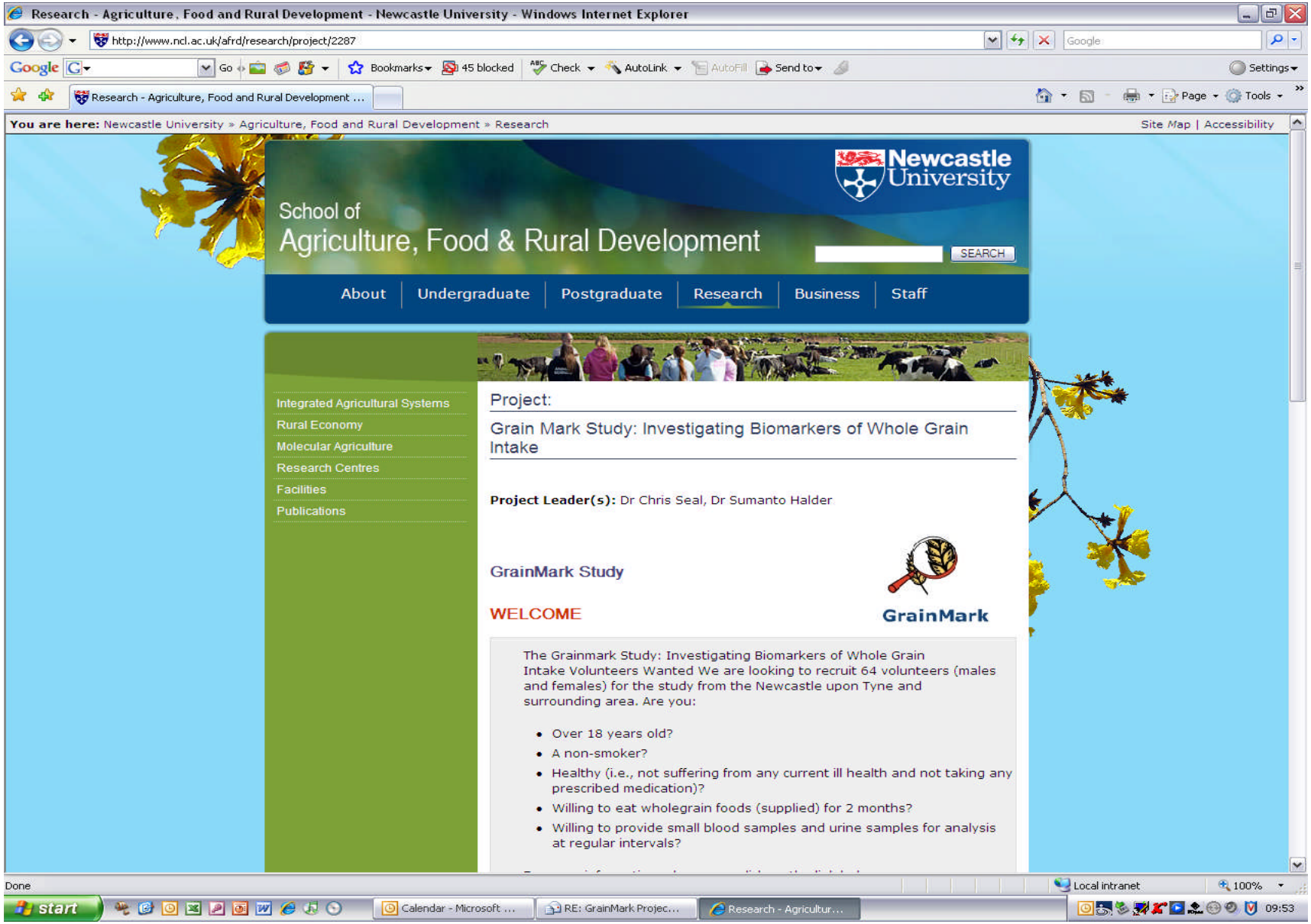
Please mention to interested volunteers that they will be sent:

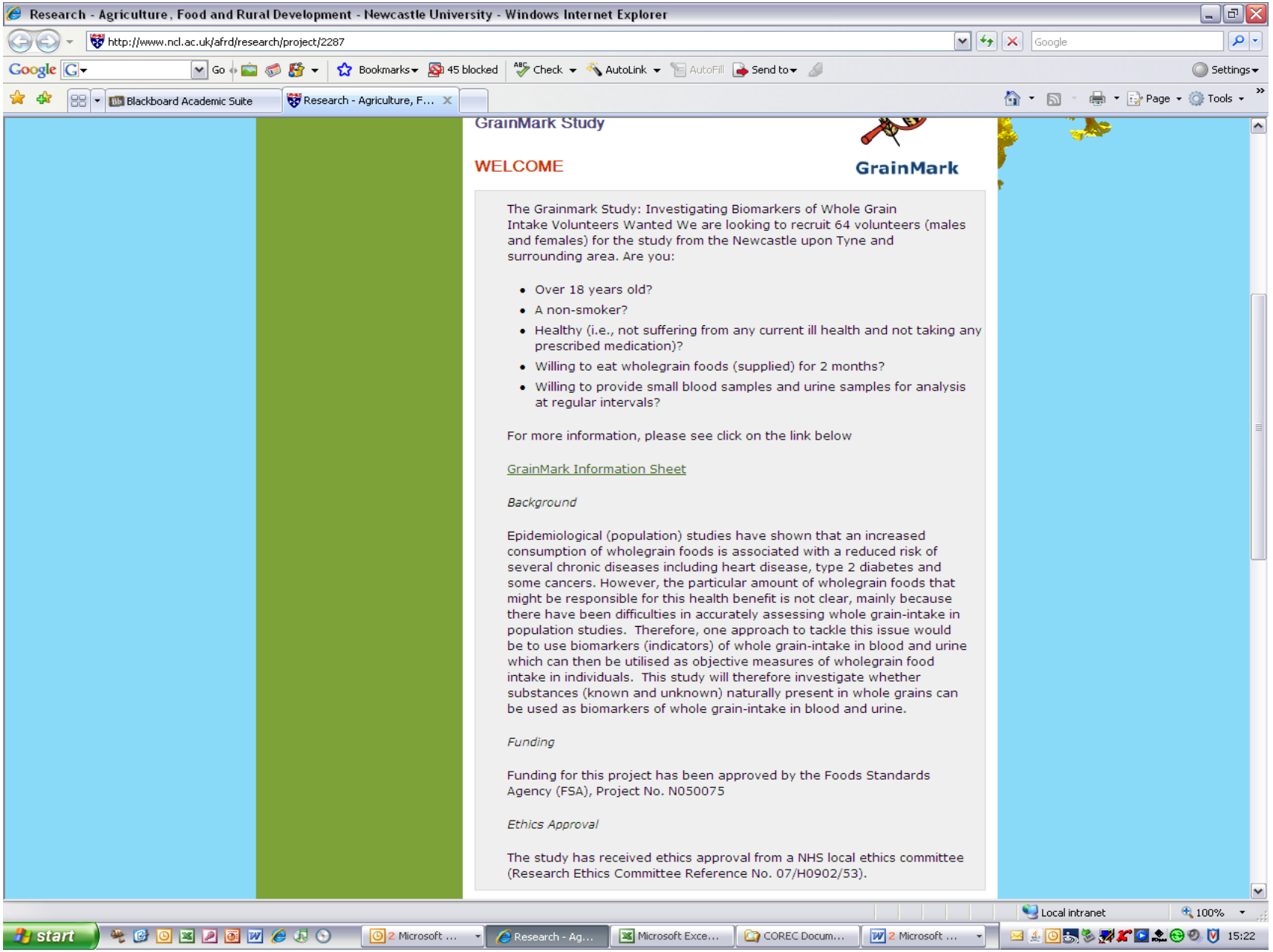
(i) an official Invitation Letter, to attend the Induction Visit
(ii) the Study Information Sheet, containing more information about the study

1. **File this form in `Pending Induction Visit Folder** ☐
2. **On obtaining consent, file form in individual folder** ☐
3. **If no consent, shred this form using a shreder**

Signature …… **Date** ……

Appendix 4: GrainMark Project Website (www.grainmark.org) – screendump

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Research - Agriculture, F...
GrainMark Study
WELCOME
GrainMark
The Grainmark Study: Investigating Biomarkers of Whole Grain Intake Volunteers Wanted We are looking to recruit 64 volunteers (males and females) for the study from the Newcastle upon Tyne and surrounding area. Are you:
Over 18 years old?
A non-smoker?
Healthy (i.e., not suffering from any current ill health and not taking any prescribed medication)?
Willing to eat wholegrain foods (supplied) for 2 months?
Willing to provide small blood samples and urine samples for analysis at regular intervals?
For more information, please see click on the link below
GrainMark Information Sheet
Background
Epidemiological (population) studies have shown that an increased consumption of wholegrain foods is associated with a reduced risk of several chronic diseases including heart disease, type 2 diabetes and some cancers. However, the particular amount of wholegrain foods that might be responsible for this health benefit is not clear, mainly because there have been difficulties in accurately assessing whole grain-intake in population studies. Therefore, one approach to tackle this issue would be to use biomarkers (indicators) of whole grain-intake in blood and urine which can then be utilised as objective measures of wholegrain food intake in individuals. This study will therefore investigate whether substances (known and unknown) naturally present in whole grains can be used as biomarkers of whole grain-intake in blood and urine.
Funding
Funding for this project has been approved by the Foods Standards Agency (FSA), Project No. N050075
Ethics Approval
The study has received ethics approval from a NHS local ethics committee (Research Ethics Committee Reference No. 07/H0902/53).
Local intranet
100%
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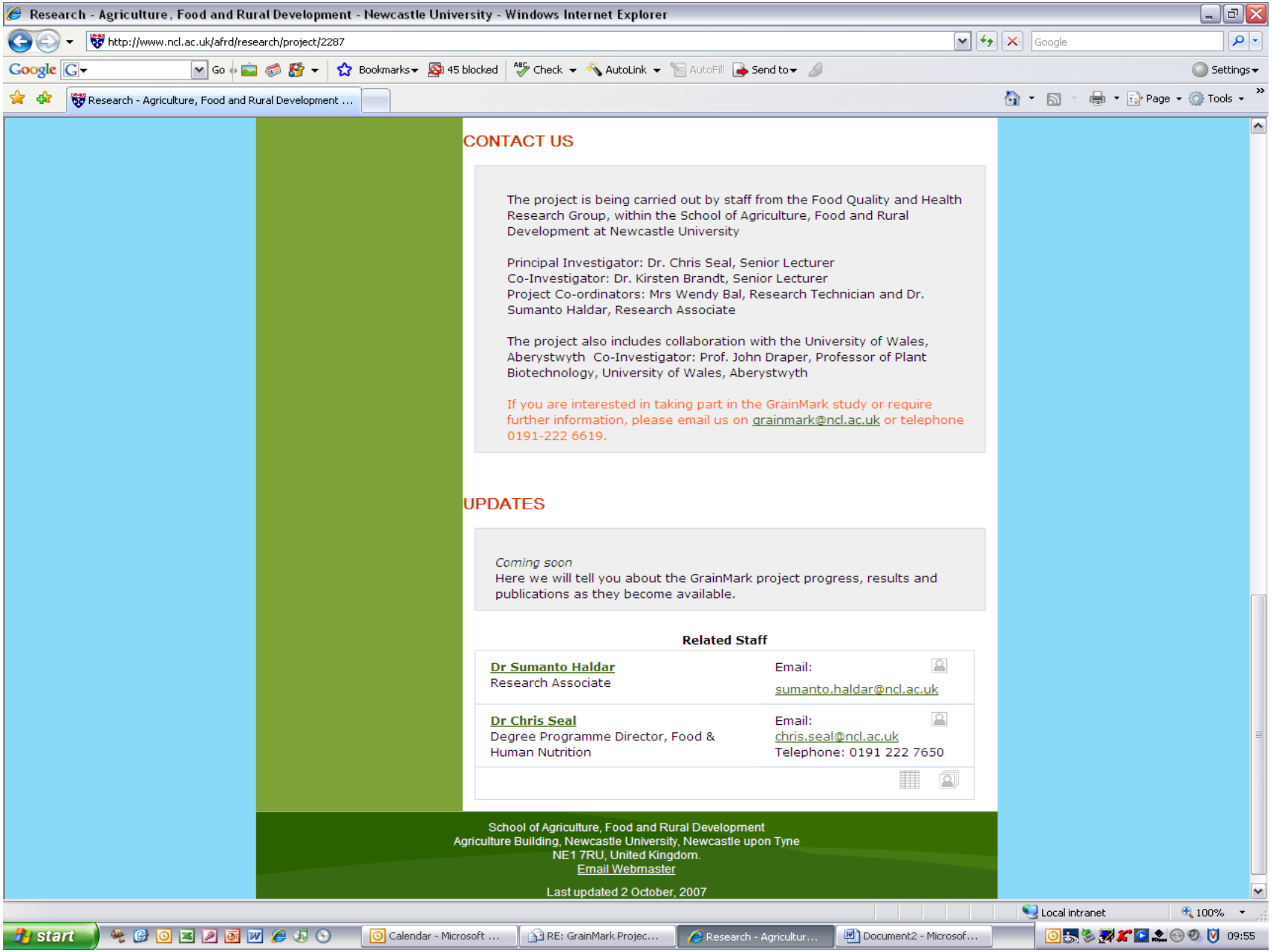
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CONTACT US
The project is being carried out by staff from the Food Quality and Health Research Group, within the School of Agriculture, Food and Rural Development at Newcastle University
Principal Investigator: Dr. Chris Seal, Senior Lecturer
Co-Investigator: Dr. Kirsten Brandt, Senior Lecturer
Project Co-ordinators: Mrs Wendy Bal, Research Technician and Dr. Sumanto Haldar, Research Associate
The project also includes collaboration with the University of Wales, Aberystwyth Co-Investigator: Prof. John Draper, Professor of Plant Biotechnology, University of Wales, Aberystwyth
If you are interested in taking part in the GrainMark study or require further information, please email us on grainmark@ncl.ac.uk or telephone 0191-222 6619.
UPDATES
Coming soon
Here we will tell you about the GrainMark project progress, results and publications as they become available.
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Appendix 5A: Example of Rye Group Food Record for Dose 1 Period

GrainMark

Newcastle University

Volunteer ID: _____

<u>PHASE 1 Intervention (rye group)</u> – Please keep a record of the actual number(s) servings of each types whole-grain foods eaten each day from the study foods supplied.

You may choose to make up your 3 servings/day using any combination of these foods provided, BUT PLEASE DO NOT eat more than 3 servings of these rye foods/day

Day of Study	*Date*	*Number of Servings*				
		Rye Porridge	**Rye Muesli**	**Rye Bread**	**Rye Pasta**	**<u>Total</u>**
30	11 July 2008					
31	12 July 2008					
32	13 July 2008					
33	14 July 2008					
34	15 July 2008					
35	16 July 2008					
36	17 July 2008					
37	18 July 2008					
38	19 July 2008					
39	20 July 2008					
40	21 July 2008					
41	22 July 2008					
42	23 July 2008					
43	24 July 2008					
44	25 July 2008					
45	26 July 2008					
46	27 July 2008					
47	28 July 2008					
48	29 July 2008					
49	30 July 2008					
50	31 July 2008					
51	01 August 2008					
52	02 August 2008					
53	03 August 2008					
54	04 August 2008					
55	05 August 2008					
56	06 August 2008					
57	07 August 2008					

***<u>N.B.:</u>* Please continue to avoid all other whole-grain foods (from the list supplied previously) other than the foods we have provided as above. *Please return this completed record sheet at Visit 4.* Thank you.**

Appendix 5B: Example of Wheat Group Food Record for Dose 2 Period

GrainMark

Newcastle University

Volunteer ID: _____

<u>PHASE 2 Intervention (wheat group)</u> – Please keep a record of the actual number(s) servings of each types whole-grain foods eaten each day from the study foods supplied.

You may choose to make up your <u>6 servings/day</u> using any combination of these foods provided, BUT PLEASE ENSURE you complete (but not exceed) 6 servings/day

Day of Study	*Date*	*Number of Servings*				
		Weetabix	**Shredded Wheat Fruitful**	**Wholemeal Bread**	**Wholemeal Pasta**	**<u>Total</u>**
58	08 August 2008					
59	09 August 2008					
60	10 August 2008					
61	11 August 2008					
62	12 August 2008					
63	13 August 2008					
64	14 August 2008					
65	15 August 2008					
66	16 August 2008					
67	17 August 2008					
68	18 August 2008					
69	19 August 2008					
70	20 August 2008					
71	21 August 2008					
72	22 August 2008					
73	23 August 2008					
74	24 August 2008					
75	25 August 2008					
76	26 August 2008					
77	27 August 2008					
78	28 August 2008					
79	29 August 2008					
80	30 August 2008					
81	31 August 2008					
82	01 September 2008					
83	02 September 2008					
84	03 September 2008					
85	04 September 2008					

***<u>N.B.:</u>* Please continue to <u>avoid all other whole-grain foods</u> (from the list supplied previously) other than the foods we have provided as above. *Please return this completed record sheet at Visit 6.* Thank you.**

Volunteer ID						
Date						

GrainMark

Newcastle University

GrainMark Study

Appendix 6

Food Frequency Questionnaire

- Please read the instructions on pages 2 and 3 before completing the questionnaire.
- All information collected will be kept completely confidential.
- Thank you for taking time to complete this questionnaire.

If you have any queries please contact:

Dr. Sumanto Haldar Tel: 0191 222 6619
or email GrainMark@ncl.ac.uk

How to answer the questions

There are several types of question in this booklet. Most of them can be answered by ticking one box (ONLY) beside each food types.

For example:

FOODS & AMOUNTS	AVERAGE USE IN THE LAST WEEK							
FISH *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Fried fish in batter, as in fish and chips		✓						

Please put ONE tick in the appropriate box (✓) on each line to indicate how often, **on average**, you have eaten each food **during the past week.**

- Answer every question by putting ONE tick (✓) on every line
- Do not leave **ANY** lines blank.

Another example of questions requiring boxes to be ticked:

Q. Do you usually add salt to food while cooking? Yes……☑
No……❒

Some of these questions have several boxes and you may be asked to tick ONE only.

For example:

What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter……❒
Lard/dripping……❒
Solid vegetable fat……❒
Margarine……❒
Vegetable Oil……❒
Olive oil……☑
Walnut Oil……❒
Soya Oil……❒
None……❒
Other……❒

Some of these questions have several boxes and you may be asked to tick all the boxes you think apply to you.

For example:

14. What kind of fat did you use for cooking?

Please tick all that apply

Butter……☑
Lard/dripping……❒
Solid vegetable fat……❒
Margarine……❒
Vegetable oil……☑
Olive oil……❒
Walnut Oil……❒
Soya Oil……❒
None……❒
Other……❒

If "other" selected in question 14, please state……

What do I do if I make a mistake?

Cross out the incorrect answer, and put a tick where you think the right answer should be. We'll verify your answers at your next appointment visit.

If you have any problems filling in this dietary questionnaire, we will discuss them at your next visit. If you have any questions do not hesitate to contact Dr Sumanto Haldar or Mrs Wendy Bal, contact details are provided on the front cover of this questionnaire.

For **Questions 1-12**, please put **ONE tick** in the appropriate box (✓) on each line to indicate how often, **on average**, you have eaten each food **during the past week.** Please **DO NOT leave any lines blank.**

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
1. MEAT ***(medium serving)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: e.g. roast, steak, mince, stew, casserole, curry, Bolognese								
Beefburgers *(single burger)*								
Corned beef, Spam, luncheon meats *(2 slices – a sandwich's-worth)*								
Lamb: e.g. roast, chops, stew, curry								
Chicken, turkey or other poultry: e.g. casserole, sliced, curry								
Breaded or fried poultry products: e.g. chicken nuggets, deep fried chicken pieces *(1 breaded chicken portion or c.6 nuggets)*								
Pork: e.g. roast, chops, stew, curry								
Bacon and ham *(2 rashers/slices – a sandwich's-worth)*								
Sausages *(one)*								
Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls, scotch egg *(single pie/savoury)*								
Game and Wild-fowl: e.g. duck, rabbit, grouse								
Kidneys or liver; including liver pate, liver sausage								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
2. FISH and SEAFOOD ***(medium serving)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White fish- not coated e.g. cod, halibut, haddock, whiting, plaice, sole, etc *(per portion)*								
White fish- in batter or crumbs e.g. cod, haddock, plaice, etc *(per portion)*								
Oily fish e.g. herring, mackerel, *(tinned)* salmon- not tinned, trout, kippers etc *(per portion)*								
Tinned fish e.g. Sardines, Pichards, Tuna, Salmon etc *(per can, or portion)*								
Prawns, shellfish and other fish *(within dish or one sandwich's-worth)*								
Fish cakes, Fish fingers *(one)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
3. BREAD & SAVOURY BISCUITS ***(one slice or biscuit)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls, white pitta bread (per slice/roll)								
Scones, teacakes, crumpets, muffins or croissants (each)								
Brown bread and rolls (per slice/roll)								
Wholemeal pitta bread (each)								
Wholemeal bread/rolls (per slice/roll)								
Granary bread (per slice/roll)								
Rye bread (per slice/roll)								
Naan bread, chapatti (each)								
Garlic bread (per serving)								
Cream crackers, cheese biscuits (each)								
Wholemeal crackers (per cracker)								
Crispbreads e.g. Ryvita, Ryvita currant crunch (one)								
Oatcakes (one)								
Other speciality breads (each) (please state and tick for frequency) 1. 2.								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
4. CEREALS ***(one bowl)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Porridge, Readybrek								
Sugar coated cereals e.g. Sugar Puffs, Cocoa Pops, Frosties								
Non-sugar coated cereals e.g. Cornflakes, Rice Crispies								
Muesli								
Bran containing cereals e.g. All Bran								
Cheerios								
Branflakes								
Weetabix								
Shredded Wheat, Shreddies								
Wholegrain cereals with fruit e.g. Sultana Bran, Fruit n Fibre								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
5. POTATOES, RICE & PASTA *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Boiled, mashed, instant or jacket potatoes *(about 1/3 of a plate)*								
Chips, potato waffles *(side order with meal – chip-shop portions count as 2)*								
Roast potatoes *(3 – 5 potatoes)*								
Yorkshire pudding, pancakes, dumpling *(each medium)*								
Potato salad *(per small tub, c. 2 tablespoons)*								
White rice *(1/2 plateful, or in a dish e.g. rice salad, risotto etc)*								
Brown rice *(1/2 plateful, or in a dish e.g. rice salad, risotto etc)*								
White or green pasta, e.g. spaghetti, macaroni, noodles, *(1/2 plate)*								
Tinned pasta, e.g. spaghetti, ravioli, macaroni *(1/2 standard tin)*								
Super noodles, pot noodles, pot savouries *(per pot)*								
Wholemeal pasta/spaghetti *(1/2 plate)*								
Pasta dishes e.g. Lasagne, moussaka, cannelloni *(as individual ready-meal)*								
Pizza *(10" = 1, 12" = 2, 12"+ = 3-4)*								
Wholegrain dishes not mentioned (Please state and tick for frequency) 1. 2. 3.								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
6. (a) DAIRY & EGG PRODUCTS	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream *(tablespoon)*								
Double or clotted cream *(tablespoon)*								
Low fat yoghurt, fromage frais *(125g carton)*								
Full fat or Greek yoghurt *(125g carton)*								
Dairy desserts *(125g carton),* e.g. mousse								
Cheese, e.g. Cheddar, Brie, Edam *(medium serving)*								
Cottage cheese, low fat soft cheese *(medium serving)*								
Eggs as boiled, fried, scrambled, omelette etc. *(one)*								
Quiche *(medium serving = 1/6 of pie)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
6.(b) DAIRY PRODUCTS & FATS used on bread *(teaspoon/curl)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter								
Margarines/spreads (state type and tick for frequency) 1. 2. 3.								
Reduced/Low fat spreads (state type and tick for frequency) 1. 2. 3.								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
6.(c) DAIRY PRODUCTS & FATS used on vegetables *(teaspoon/curl)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter								
Margarines/spreads (state type and tick for frequency) 1. 2. 3.								
Reduced/Low fat spreads (state type and tick for frequency) 1. 2. 3.								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
7. SWEETS & SNACKS *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chocolate coated sweet biscuits, e.g. Penguin, kit-kat, chocolate digestive *(one)*								
Sweet biscuits, plain, e.g. Nice, ginger *(one)*								
Cakes e.g. fruit, sponge, sponge pudding *(medium slice)*								
Sweet buns & pastries e.g. doughnuts, Danish pastries, cream cakes *(each)*								
Flapjacks (*each)*								
Fruit pies, tarts, crumbles (per individual pie/medium serving)								
Milk puddings, e.g. rice, custard, trifle *(medium serving)*								
Ice cream, choc ices *(one)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
7. SWEETS & SNACKS (continued)	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chocolates,, toffee, sweets and other confectionary *(medium bar of chocolate, one snack bar, one packet)*								
Sugar added to tea, coffee, cereal *(teaspoon)*								
Crisps or other packet snacks e.g. Wotsits *(one packet)*								
Peanuts *(one packet)*								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
8. SOUPS, SAUCES AND SPREADS	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Vegetable soups *(medium bowl)*								
Meat soups *(medium bowl)*								
Sauces, e.g. white sauce, cheese sauce, gravy *(1/3 of plate or in dish)*								
Tomato based sauces e.g. pasta sauces *(1/3 of plate or in dish)*								
Tomato ketchup, brown sauce *(per tablespoon)*								
Relishes e.g. pickles, chutney, mustard *(per tablespoon)*								
Salad cream, mayonnaise, other salad dressings *(per tablespoon)* (state type and tick for frequency) 1. 2. 3.								
Marmite, Bovril *(per teaspoon/slices of bread)*								
Jam, marmalade, honey, syrup *(per teaspoon/slices of bread)*								
Peanut butter *(per teaspoon/slices of bread)*								
Chocolate spread, chocolate nut spread *(per teaspoon/slices of bread)*								
Dips e.g. houmous, cheese and chive *(per tablespoon/slices of bread)*								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
9. DRINKS	None	Once A Week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea *(cup)*								
Coffee, instant or ground *(cup)*								
Coffee whitener, e.g. Coffee-mate *(teaspoon)*								
Cocoa, hot chocolate *(cup)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
9. DRINKS (continued)	None	Once A Week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Horlicks, Ovaltine *(cup)*								
Wine *(glass)*								
Beer, lager or cider *(half pint)*								
Port, sherry, vermouth, liqueurs *(glass)*								
Spirits, e.g. gin, brandy, whisky, vodka *(single)*								
Low calorie or diet fizzy soft drinks *(glass)*								
Fizzy soft drinks, e.g. Coca cola, lemonade *(glass)*								
Pure fruit juice (100%) e.g. orange, apple juice *(glass)*								
Fruit squash or cordial *(glass)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
10. FRUIT *(1 fruit or medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Apples *(each)*								
Pears *(each)*								
Oranges *(1x)*, satsumas, mandarins, tangerines, clementines *(all 2x)*								
Grapefruit *(1/2 a fruit)*								
Bananas *(each)*								
Grapes *(per small handful)*								
Melon *(1 medium slice)*								
Peaches *(1x)*, plums, apricots, nectarines *(2 – 3x)*								
Strawberries, raspberries *(per small handful)*, kiwi fruit *(each)*								
Tinned fruit *(1/2 tin)*								
Dried fruit, e.g. raisins, prunes, figs *(per small handful)*								
Other fruit or fruit dishes / products not mentioned (please state and tick for frequency) 1. 2. 3.								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
11. VEGETABLES Fresh, frozen or tinned *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots *(2-3 table spoonfuls)*								
Spinach *(major ingredient in dish (e.g. curry) or per 2 – 3 tablespoonfuls)*								
Broccoli *(per 4 – 5 florets)*								
Brussels sprouts *(2-3 tablespoonfuls)*								
Cabbage *(2-3 tablespoonfuls)*								
Peas *(2-3 tablespoonfuls)*								
Green beans, broad beans, runner beans *(2-3 tablespoonfuls)*								
Marrow, courgettes *(major ingredient in dish or 2-3 tablespoonfuls)*								
Cauliflower *(major ingredient in dish (e.g. curry) or 2-3 tablespoonfuls)*								
Parsnips, turnips, Swedes *(2-3 tablespoonfuls)*								
Leeks *(2-3 tablespoonfuls)*								
Onions *(per onion)*								
Garlic *(2 cloves)*								
Mushrooms *(handful of uncooked mushrooms, or 2-3 tablespoonfuls)*								
Sweet peppers *(per ½ pepper)*								
Beansprouts *(major ingredient in dish or (2-3 tablespoonfuls)*								
Green salad, lettuce, cucumber, celery *(side-salad or per 1/3 plate)*								
Mixed vegetables (frozen or tinned) *(2-3 tablespoonfuls)*								
Watercress *(per bunch, or as a major ingredient in salad)*								
Tomatoes *(2 medium tomatoes, ½ can of tomatoes)*								
Sweetcorn *(2-3 tablespoonfuls)*								
Beetroot, radishes *(2-3 tablespoonfuls)*								
Coleslaw *(2-3 tablespoonfuls)*								
Avocado *(per ½ fruit)*								
Baked Beans *(per ½ tin)*								
Dried lentils, beans, peas *(2-3 tablespoonfuls, or major ingredient)*								
Tofu, soya meat, TVP, (*in dish e.g. curry),* Vegeburger *(each)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
11. VEGETABLES Fresh, frozen or tinned (continued)	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Other vegetables or vegetable dishes / products not mentioned (please state and tick for frequency) 1. 2. 3.								

YOUR DIET IN THE LAST WEEK, *continued*

12. What type of milk did you most often use?

Select one only

Full cream……❒
Channel Islands……❒
Dried milk……❒
Semi-skimmed……❒
Skimmed……❒
Soya……❒
Other……❒
None……❒

13. Approximately, how much milk did you drink each day, including milk with tea, coffee, cereals etc?

None……❒
Quarter of a pint (roughly 125mls)……❒
Half a pint (roughly 250mls)……❒
Three quarters of a pint (roughly 375mls)……❒
One pint (roughly 500mls)……❒
More than one pint (more than 500mls)……❒

14. What kind of fat did you use for cooking?

Please tick <u>all</u> that apply

Butter……❒
Lard/dripping……❒
Solid vegetable fat……❒
Margarine……❒
Vegetable oil……❒
Olive oil……❒
Walnut Oil……❒
Soya Oil……❒
None……❒
Other……❒

If "other" selected in question 14, please state……

15. Do you usually add salt to food while cooking?

Yes……❒
No……❒

16. Do you usually add salt to any food at the table?

Yes……❒
No……❒

17. Do you usually eat the fat on cooked meats?

Yes……❒
No……❒

18. Do you usually eat the skin on cooked meats?

Yes……❒
No……❒

19. Do you usually add sugar to drinks i.e. tea/coffee?

Yes……❒
No……❒

20. On average, in the past week, how many portions of fruit and vegetables did you eat per DAY?

Please estimate:…….……

21. On average, in the past week, how many servings of wholegrain foods did you eat per DAY?

Please estimate:…….……

22. Have you taken any of the following during the past week?

	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day
Vitamins (e.g. multivitamins, vitamin B, vitamin C, folic acid)						
Minerals (e.g. iron, calcium, zinc, magnesium)						
Fish oils (e.g. cod liver oil, omega-3)						
Other food supplements (e.g. oil of evening primrose, starflower oil, royal jelly, ginseng)						

- Did you use any other food supplements? Please state below:

1_____
2_____
3_____
4_____

Thank you for taking the time to complete this questionnaire!!

Appendix 7: Instructions for making a 24-hr urine collection (*NEW METHOD: using gel packs*)

Prior to your Xth Visit, we will ask you to do a 24-hr urine collection as before. You will be provided with a jug, 2 plastic containers, 4 gel packs. *Please place the gel packs in your freezer as soon as you get them*. **On starting your urine collection please keep urine containers in the cool bag provided along with a pair of frozen gel packs (placed upright on either sides) AT ALL TIMES.** Please start your urine collection on the morning of the day before your Xth visit, after you have emptied your bladder into the toilet on waking up. Please keep your 24 hour urine collection cool in the cool bag provided along-side two frozen gel packs at all times!

Please follow these instructions:

1. On the morning of the **day before** your Xth Visit, when you wake up, please empty your bladder (i.e., the 'first pass') into the toilet. Please make a note on the collection container of the **date** and **time** you did this.
2. At this point please take out the first pair of frozen gel packs from the freezer and place them upright on two sides of the cool bag.
3. **After this, collect and store in the first container (Container A) all urine you pass during the day** up until you eat your set-meal that evening, keeping the container cool in the cool bag provided at all times. **Please remember to empty your bladder into Container A before you have your set-meal**.
4. At this point, please insert the second pair of frozen gel packs (previously kept frozen in your freezer) to the cool bag, and keep them upright on the sides.
5. **After you finish your set-meal, any time you need to empty your bladder (that evening or throughout the night), please collect urine into Container B**.
6. The following morning when you awake, (that is 24 hours after you started) **complete the urine collection by emptying your bladder into Container B**. Make a note of the **time** you complete the collection (make sure you tick off the appropriate box on Container B label). If this is not exactly 24 hours later don't worry.
7. When you feel the need to have a **bowel movement**, first try to pass urine into the jug for transfer to the appropriate container (Containers A or B, depending on the time of the day). That is, please avoid passing urine directly into the toilet with a bowel motion.
8. ***Please keep the top of the collection containers closed, and keep the containers in the cool bag provided, with at least one pair of gel packs at all times.***

If you pass urine but forget to collect it, please tell us when you hand your collection in (and if possible try to estimate how much was lost). Please ask us if you have any queries and let us know if you have any difficulties in making the collection.

PRIFYSGOL ABERYSTWYTH UNIVERSITY

Appendix 8

IBERS Institute of Biological, Environmental & Rural Sciences

Standard Operating Procedure EX-05-v1.0

Non-targeted plasma/serum sample preparation for metabolomics analysis

Valid from:	19/10/2009		
Validity area:	High Resolution Mass Spectrometry Laboratory IBERS, Aberystwyth University		
Responsible:	Kathleen Tailliart (sample processing and preparation for instrumental analysis)		
SOP supersedes:	Plasma/serum sample preparation protocols		
Prepared by:	Dr. Manfred Beckmann		Date: 19/10/2009
Approved by:	Dr. Manfred Beckmann		Date: 19/10/2009

Contents

1. HISTORY / BACKGROUND

1.1 Background

Protocols for blood sample preparation were developed starting in 2005 in preparation for the FSA-N05073-MEDE and FSA-N05075-GrainMark project. This SOP is a result of procedures which provided robust models following data analysis of high through-put metabolite fingerprinting and profiling data. The procedure is concerned specifically with the preparation of human plasma and serum samples used in above named projects.

1.2 Changes in current version

This SOP is based on previous protocols and replaces those protocols which have been used to prepare human plasma and serum samples for metablomics analysis.

2. PURPOSE

This SOP describes the procedure of protein precipitation and/or extraction of mammalian plasma and serum samples for non-targeted high through-put metabolomics analyses. Adhering to the procedure increases reproducibility and consistency of sample handling, preparation, and storage and instrument performance.

3. SCOPE

This SOP includes sample preparation of raw extracts for further GC-tof-MS, FIE-MS and nano-flow FT-icr-MS analysis. A minimum of twelve biological replicates per class or treatment are required for successful data analysis and data mining of multi-dimensional data sets. The scope of the method is to provide data analysts with data matrices of different instrumentation to identify differences in treatment classes (*e.g.* food metabolites and biotransformation products in post-prandial samples), individual differences (*e.g.* high and low responders) and outliers.

This SOP is appropriate for equine, canine and rodent plasma samples, but volumes subjected to instrumental analysis might need adjustment.

4. DEFINITIONS AND ABBREVIATIONS

Metabolomics	short for comprehensive, non-targeted analysis of all metabolites; generally refers to application of metabolite profiling and fingerprinting techniques;
GC-tof-MS	Gas Chromatography-time-of-flight-Mass Spectrometry;
FIE-MS	Flow Infusion Electrospray Mass Spectrometry;
nano-flow	refers here to the use of a NanoMate (Advion) for direct infusion experiments;
FT-icr-MS	Fourier Transform ion cyclotron resonance Mass Spectrometry;
PPE	Personal Protective Equipment;
IS	Internal GC-tof-MS standard.

5. PRINCIPLE OF THE METHOD

This method describes sampling handling, extraction and sample preparation for instrumental analysis of mammalian plasma and serum samples. The solvent mix together with water content of blood samples results in a Bligh and Dyer extraction mix which has been proven suitable for metabolomics analyses in previous years. Although protein precipitation will never be quantitative the application of pre-chilled solvent mix and keeping the extraction mix in a freezer for additional 20 min at -20 °C supports protein precipitation. The resulting supernatant is a single phase liquid.

The procedure includes breakpoints for sample storage. Generally samples are stored with the supernatant and its precipitation pellet to reduce waste from transferring supernatants into new disposable tubes. Unlike plant extracts blood extracts appear to be stable in metabolomics experiments with respect to metabolite composition for at least two years. It is anticipated that light and oxygen sensitive compounds (*e.g.* fats, micronutrients) reacted already as precautionary measures are not in place to stabilise targets. For metabolite analysis on GC-tof-MS supernatant has to be dried. For direct or flow infusion experiments using electro-spray mass spectrometry instrumentation supernatants are reconstituted in methanol/water mixes to improve metabolite ionisation.

6. MATERIALS AND EQUIPMENT

6.1 Chemicals

- Chloroform: GLC—pesticide residue grade (C/4963/15; Fisher)
- Methanol for extraction: trace analysis grade (M/4020/17; Fisher)
- Methanol for mobile phase: HPLC grade (M/4056/PB17; Fisher)
- L-threo-tert.-butylserine monohydrate (99%, 25500 5000, Acros)
- Gas Helium 5.0 (BOC)
- O-Methylhydroxylamine hydrochloride; store in desiccators (67546, Fluka)
- Dry Pyridine, puriss./absolute, over molecular sieve, H_2O < 0.005% (82704, Fluka)
- N-methyl-N-trimethylsilyltrifluoroacetamide, MSTFA, store at 4-8 ºC (701 270.510, Macherey-Nagel)
- Quality control (QC) solution (Fridge 1)
- Gas Nitrogen (BOC)

6.2 Water

- Water (≤18 MΩ x cm; Elga Purlab Ultra, room B1.11)

6.3 Solutions, standards and reference materials

Master Mix (MM) samples: A MM sample is ideally a mix of aliquots of extracts from every blood sample in the experiment that can be used to monitor instrument performance throughout a long-run series or multi-batch work. For practical purposes, it has been proven advantageous to first prepare MM samples for each biological class separately. For each sample class, pipette a minimum of 50 µl solvent extract (*i.e.* supernatant, see 7.2) of each biological replicate into a fresh 2-ml Eppendorf tube and mix thoroughly. In a second step, pipette a minimum of 100 µl of the single class MMs into a fresh 2-ml Eppendorf tube to obtain the MM for the experiment. Mix thoroughly and store all MM samples at -80 °C.

GC-tof-MS internal standard (IS): An IS stock solution is kept in Freezer 1. If required weigh 1.87mg L-threo-*tert*-butylserine monohydrate (99%) and dissolve in 100 mL methanol/water [70/30] using a volumetric flask.

Methoxymation solution: Prepare in a screw-cap glass bottle 10 ml of 20mg/ml methoxyamine hydrochloride (Fluka) in dry pyridine (Fluka) and sonicate for at least 15 min. Check all crystals are dissolved. Store solution in a dark place.

6.4 Plasticware

- 1,000-µl pipette and pipette tips (Gilson)
- 100-ml pipette and pipette tips (e.g. Gilson)
- 1 l container to collect used pipette tips
- Eppendorf 'safe-lock' tubes 2 ml (Eppendorf AG, 0030 120.094)

6.5 Glassware

Rinse clean glassware at least once with a few ml of solvent to be used.

- Glass measuring cylinders 500, 250 and 100 ml
- 100ml volumetric flask
- Clean screw-cap glass bottles (50 ml to 2 l, solvent resistant, *e.g*, Schott Duran)
- Micro inserts, 200 µl FB micro-serts (C4012-465, National Scientific)
- Crimp caps 11mm aluminium silicone/PTFE liner (11-AC7 820, Chromacol)
- Micro vials 200 µl clear glass, crimp cap (02-CTVG, Chromacol)
- Crimp caps 8mm aluminium silicone/PTFE liner (8-AC-ST101, Chromacol)
- Support sleeves, long necked version for HP7673A (SV-S14, Chromacol)

6.6 Equipment

- Laminar flow hood
- Dewar (thermally insulated flask) for liquid nitrogen, 2 x 1 L (Dilvac)
- Personal protective equipment (PPE) for lab work
- Brady IP Series Label Printer
- Balance (e.g., BP 211 D; Sartorius)
- Glass beads (0.5-0.75 mm, Retsch)
- Refrigerated centrifuge (EBA 12R; Hettich)
- Orbital shaker (IKAVibrax VXR) in cold room at 4 °C
- Thermo shaker, microtubes (FATSM002, Favorgen Biotech Corp.)
- Mixer-mill, MM200 or MM301 (Retsch)

- Teflon adaptor for 1.5–2.0 ml micro-vials (Retsch)
- Incubator shaker for up to 48 samples (PROGR, HLC)
- Incubator shaker for more than 48 samples (Innova4400, Brunswick Scientific)
- SpeedVac; Centrifugal vacuum concentrator Univapo 150H with Unijet II
- Refrigerated Aspirator (UniEquip)
- Microspoon
- HPLC-glass vials, 2-ml crimp top (2-CV; Chromacol)
- Micro-vial insert, 200 ml (02-NV; Chromacol)
- Aluminum crimp cap, rubber/PTFE seal (11-AC7; Chromacol)
- Cap crimper and de-crimper (11 mm)
- Cap crimper (8 mm)
- Cap de-crimper (20 mm)
- Fridge 4-8 °C
- Freezer -20 °C
- Ultra deep freezer -80 °C

7. PROCEDURES

7.1 Preparation of extraction mix and material before experiment:

- Wear PPE and work in designated area when handling biofluids!
- Pre-label Eppendorf tubes.
- Check Gilson pipettes for accuracy and adjust for correct volume.
- Switch on Thermo Shaker and pre-cool at 4 °C .
- Switch on refrigerated centrifuge and close lid to start refrigeration at 4 °C.
- Switch on SpeedVac to refrigerate water bath at 3 °C.
- Prepare sufficient solvent mix for the experiment: methanol/chloroform [4/1]:
    - prior mixing degas methanol with $He_{(g)}$ for 10 min before adding chloroform by using HPLC-solvent bottle frits.
- Pre-chill solvent in freezer at -20 °C.

7.2 Sample preparation

- Defrost samples on ice
- Make a note if sample appears red to dark red (cell lysis)
- Add 1 micro-spoon of glass beads into new pre-labelled 2 ml Eppendorf tube
- Vortex raw plasma/serum sample (approx. 5 sec)
- pipette 200 µl raw plasma or serum sample
- pipette 1520 µl pre-chilled solvent mix (methanol/chloroform [4/1] v/v)
- Vortex (approx. 5 sec)
- Shake for 15 min at +4°C
- Keep sample at -20 °C for 20 min.
- Centrifuge at 14 000 rpm and 4 °C for 5 min
- Breakpoint:
    - Store plasma samples at -80 °C until further analysis.
    - For further analysis:
        - Defrost plasma sample on ice.
        - Vortex
        - Centrifuge at 14 000 rpm and 0 °C for 5 min.

- For GC-MS analysis:
  - Transfer 420 µl of the plasma supernatant into new 2 mL Eppendorf tube.
  - Add 50 µl IS.
  - Dry in speed vac.
  - Breakpoint:
    - Store dried sample at -80 °C until further analysis.
    - For further analysis defrost dried sample at RT for at least 30 min.
  - 1st Derivatization: Methoximation
    - Add 20 µl of methoxymation solution (10 ml of 20mg/ml methoxyamine hydrochloride (Fluka) in dry pyridine (Fluka)) and incubate and shake solution for 90 min at 30°C and 200 rpm.
  - 2nd Derivatization: Silylation
    - Add 40 µl N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, Macherey-Nagel) to the solution and incubate and shake mixture for 30 min at 37°C and 200 rpm.
    - Allow mixture to cool to room temperature for 10 min.
  - Transfer 60 µl of the supernatant into micro vials.
  - Immediately crimp-cap micro vial and place into PTFE support sleeve.
  - After the 2nd derivatization wait 120 min prior to the first injection.
  - Sample is ready for GCMS analysis.

- For FIE-MS analysis:
  - Transfer 100 µl of the plasma supernatant into a new 2 mL Eppendorf tube.
  - Dry in speed vac.
  - Reconstitute in 100 µl methanol/water [70/30].
  - Vortex.
  - Shake for 15 min at +4°C.
  - Centrifuge at 14 000 rpm and +4 °C for 5 min.
  - Transfer 60 µl of the supernatant into HPLC vial with new micro insert.
  - Store remaining reconstituted sample at -80 °C until further analysis.
  - Sample is ready for FIE-MS analysis.
    - Mobile phase: Methanol/Water [70/30].

- For nano-flow FT-icr-MS analysis:
  - Transfer 100 µl of the plasma supernatant into a new 2 mL Eppendorf tube.
  - Dry in speed vac.
  - Reconstitute in 100 µl methanol/water [80/20].
  - Sonicate for 15 min.
  - Vortex.
  - Shake for 15 min at +4°C.
  - Centrifuge at 14 000 rpm and +4 °C for 5 min.
  - Prepare a dilution series (D1 to D5) in 5 new 2 mL Eppendorf tubes.
  - Sample and dilution series is ready for nano-flow FT-icr-MS analysis.
  - Store reconstituted sample at -80 °C until further analysis.

7.3 Problems

Plasma and serum samples take at least 3 hours to defrost on ice when taken from a -80 °C freezer. Samples can be highly viscous. In order to pipette the correct amount vortex well and use the plunger of the pipettor to take up and release sample several times.

7.4 Disposal of waste

After sample preparation discard used disposable plasticware in autoclave bag.
Collect all chemicals in appropriate bottles and follow the disposal rules for blood samples.

7.5 Quality Assurance

Every time you need to use a new batch of Eppendorf tubes, pipette tips or glass vials prepare a negative blank control following the procedure (*i.e.* all materials and plastic ware) without biological sample. Insure that consumables of the same batch are used for all samples of an experiment. Randomize all samples to be processed.

8. CALCULATIONS AND DATA ANALYSIS

Please see:

- Beckmann M, Parker D, Enot DP, Chareyron E, Draper J (2008). High throughput non-targeted metabolite fingerprinting using Flow Injection Electroctrospray Mass Spectrometry. *Nature Protocols*, 3(3): 486-504 (DOI: 10.1038/nprot.2007.500).
- Enot DP, Lin W, Beckmann M, Parker D, Overy DP, Draper J (2008). Pre-processing, classification modelling and feature selection using Flow Injection Electrospray Mass Spectrometry (FIE-MS) metabolite fingerprint data. *Nature Protocols*, 3(3): 446-470 (DOI: 10.1038/nprot.2007.511).
- Enot DP, Beckmann M, Overy D, Draper J (2006). Predicting interpretability of metabolome models based on behavior, putative identity, and biological relevance of explanatory signals. *Proc Natl Acad Sci USA*; 103(40):14865-70.
- Enot DP, Beckmann M, Draper J (2007). Detecting a difference – Assessing generalisability when modelling metabolome fingerprint data in longer term studies of genetically modified plants. *Metabolomics*, 3(3): 335-347.

9. RELATED PROCEDURES

Urine sample preparation SOP-EX-04-v1.0

10. APPENDICES

NA

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PRIFYSGOL ABERYSTWYTH UNIVERSITY

Appendix 9

IBERS Institute of Biological, Environmental &Rural Sciences

Standard Operating Procedure EX-04-v1.0

Non-targeted urine sample preparation for metabolomics analysis

Valid from:	20/10/2009		
Validity area:	High Resolution Mass Spectrometry Laboratory IBERS, Aberystwyth University		
Responsible:	Kathleen Tailliart (sample processing and preparation for instrumental analysis)		
SOP supersedes:	Urine sample preparation protocols		
Prepared by:	Dr. Manfred Beckmann		Date: 20/10/2009
Approved by:	Dr. Manfred Beckmann		Date: 20/10/2009

Contents

1. HISTORY / BACKGROUND

1.1 Background

Protocols for urine sample preparation were developed starting in 2005 in preparation for the FSA-N05073-MEDE and FSA-N05075-GrainMark project. This SOP is a result of procedures which provided robust models following data analysis of high through-put metabolite fingerprinting and profiling data. The procedure is concerned specifically with the preparation of human urine samples used in above named projects.

1.2 Changes in current version

This SOP replaces previous protocols which have been used to prepare human urine samples for metablomics analysis.

2. PURPOSE

Although protein precipitation has been observed occasionally this SOP describes in principle the dilution of mammalian urine samples for non-targeted high through-put metabolomics analyses. Adhering to the procedure increases reproducibility and consistency of sample handling, preparation, storage and instrument performance.

3. SCOPE

This SOP includes sample preparation of raw extracts for further GC-tof-MS, FIE-MS and nano-flow FT-icr-MS analysis. A minimum of twelve biological replicates per class or treatment are required for successful data analysis and data mining of multi-dimensional data sets. The scope of the method is to provide data analysts with data matrices from different instrumentation to identify differences in treatment classes (*e.g.* food metabolites and biotransformation products in post-prandial samples), individual differences (*e.g.* high and low responders) and outliers. This SOP is appropriate for equine, canine and rodent urines, but volumes subjected to instrumental analysis might need adjustment.

4. DEFINITIONS AND ABBREVIATIONS

Metabolomics	short for comprehensive, non-targeted analysis of all metabolites; generally refers to application of metabolite profiling and fingerprinting techniques;
GC-tof-MS	Gas Chromatography-time-of-flight-Mass Spectrometry;
FIE-MS	Flow Infusion Electrospray Mass Spectrometry;
nano-flow	refers here to the use of a NanoMate (Advion) for direct infusion experiments;
FT-icr-MS	Fourier Transform ion cyclotron resonance Mass Spectrometry;
PPE	Personal Protective Equipment;
IS	Internal GC-tof-MS standard.

5. PRINCIPLE OF THE METHOD

This method describes sampling handling and sample preparation of mammalian urine samples for further instrumental analysis. The solvent mix together with water content of urine samples results in a methanol/water [70/30] extraction mix. Although protein precipitation is of minor importance the application of pre-chilled solvent mix and keeping the extraction mix in a freezer for an additional 20 min at -20 °C is a precautionary step. The resulting supernatant is a single phase liquid.

The procedure includes breakpoints for sample storage. Generally samples are stored with the supernatant and its precipitation pellet to reduce waste from transferring supernatants into new disposable tubes. Unlike plant extracts urine extracts appear to be stable in metabolomics experiments with respect to metabolite composition for at least two years. For metabolite analysis on GC-tof-MS the supernatant has to be dried. For direct or flow infusion experiments using electro-spray mass spectrometry instrumentation supernatants need not necessarily to be reconstituted in solvents with a higher methanol content (*e.g.* [80/20]), but it can improve metabolite ionisation.

6. MATERIALS AND EQUIPMENT

6.1 Chemicals

- Chloroform: GLC—pesticide residue grade (C/4963/15; Fisher)
- Methanol for extraction: trace analysis grade (M/4020/17; Fisher)
- Methanol for mobile phase: HPLC grade (M/4056/PB17; Fisher)
- L-threo-tert.-butylserine monohydrate (99%, 25500 5000, Acros)
- Gas Helium 5.0 (BOC)
- O-Methylhydroxylamine hydrochloride, store in desiccators (67546, Fluka)
- Dry Pyridine, puriss./absolute, over molecular sieve, H_2O < 0.005% (82704, Fluka)
- N-methyl-N-trimethylsilyltrifluoroacetamide, MSTFA, store at 4-8 ºC (701 270.510, Macherey-Nagel)
- Quality control (QC) solution (Fridge 1)
- Gas Nitrogen (BOC)

6.2 Water

- Water (≤18 MΩ x cm; Elga Purlab Ultra, room B1.11)

6.3 Solutions, standards and reference materials

Master Mix (MM) samples: A MM sample is ideally a mix of aliquots of extracts from every urine sample in the experiment that can be used to monitor instrument performance throughout a long-run series or multi-batch work. For practical purposes, it has been proven advantageous to first prepare MM samples for each biological class separately. For each sample class, pipette a minimum of 50 µl solvent extract (*i.e.* supernatant, see 7.2) of each biological replicate into a fresh 2-ml Eppendorf tube and mix thoroughly. In a second step, pipette a minimum of 100 µl of the single class MMs into a fresh 2-ml Eppendorf tube to obtain the MM for the experiment. Mix thoroughly and store all MM samples at -80 °C.

GC-tof-MS internal standard (IS): An IS stock solution is kept in Freezer 1. If required weigh 1.87mg L-threo-*tert*-butylserine monohydrate (99%) and dissolve in 100 mL methanol/water [70/30] using a volumetric flask.

Methoxymation solution: Prepare in a screw-cap glass bottle 10 ml of 20mg/ml methoxyamine hydrochloride (Fluka) in dry pyridine (Fluka) and sonicate for at least 15 min. Check all crystals are dissolved. Store solution in a dark place.

6.4 Plasticware

- 1,000-µl pipette and pipette tips (Gilson)
- 100-ml pipette and pipette tips (e.g. Gilson)
- 1 l container to collect used pipette tips
- Eppendorf 'safe-lock' tubes 2 ml (Eppendorf AG, 0030 120.094)

6.5 Glassware

Rinse clean glassware at least once with a few ml of solvent to be used.

- Glass measuring cylinders 500, 250 and 100 ml
- 100ml volumetric flask
- Clean screw-cap glass bottles (50 ml to 2 l, solvent resistant, *e.g*, Schott Duran)
- Micro inserts, 200 µl FB micro-serts (C4012-465, National Scientific)
- Crimp caps 11mm aluminium silicone/PTFE liner (11-AC7 820, Chromacol)
- Micro vials 200 µl clear glass, crimp cap (02-CTVG, Chromacol)
- Crimp caps 8mm aluminium silicone/PTFE liner (8-AC-ST101, Chromacol)
- Support sleeves, long necked version for HP7673A (SV-S14, Chromacol)

6.6 Equipment

- Laminar flow hood
- Dewar (thermally insulated flask) for liquid nitrogen, 2 x 1 L (Dilvac)
- Personal protective equipment (PPE) for lab work
- Brady IP Series Label Printer
- Balance (e.g., BP 211 D; Sartorius)
- Refrigerated centrifuge (EBA 12R; Hettich)
- Orbital shaker (IKAVibrax VXR) in cold room at 4 °C
- Thermo shaker, microtubes (FATSM002, Favorgen Biotech Corp.)

- Incubator shaker for up to 48 samples (PROGR, HLC)
- Incubator shaker for more than 48 samples (Innova4400, Brunswick Scientific)
- SpeedVac; Centrifugal vacuum concentrator Univapo 150H with Unijet II
- Refrigerated Aspirator (UniEquip)
- HPLC-glass vials, 2-ml crimp top (2-CV; Chromacol)
- Micro-vial insert, 200 ml (02-NV; Chromacol)
- Aluminum crimp cap, rubber/PTFE seal (11-AC7; Chromacol)
- Cap crimper and de-crimper (11 mm)
- Cap crimper (8 mm)
- Cap de-crimper (20 mm)
- Fridge 4-8 °C
- Freezer -20 °C
- Ultra deep freezer -80 °C

7. PROCEDURES

7.1 Preparation of extraction mix and material before experiment:

- Wear PPE and work in designated area when handling biofluids!
- Pre-label Eppendorf tubes.
- Check Gilson pipettes for accuracy and adjust for correct volume.
- Switch on Thermo Shaker and pre-cool at 4 °C .
- Switch on refrigerated centrifuge and close lid to start refrigeration at 4 °C.
- Switch on SpeedVac to refrigerate water bath at 3 °C.
- Prepare sufficient solvent mix for the experiment: methanol/water [3.5/1]:
    - degas with $He_{(g)}$ for 10 min.
- Pre-chill solvent in freezer at -20 °C.

7.2 Sample preparation

- Defrost urine samples on ice
- Make a note if sample shows a pellet (including colour)
- Transfer 450 µl pre-chilled methanol/water [3.5/1] into a new 2 mL Eppendorf tube
- Vortex raw urine sample (approx. 5 sec)
- Add 50 µl raw urine
- Vortex (approx. 5 sec)
- Shake for 15 min at +4°C
- Keep sample at -20 °C for 20 min.
- Centrifuge at 14 000 rpm and 4 °C for 5 min
- Breakpoint:
    - Store urine samples at -80 °C until further analysis.
    - For further analysis:
        - Defrost urine samples on ice.
        - Vortex
        - Centrifuge at 14 000 rpm and 0 °C for 5 min.

- For GC-MS analysis:
  - Transfer 300 µl of the urine supernatant into new 2 mL Eppendorf tube.
  - Add 50 µl IS.
  - Dry in speed vac.
  - Breakpoint:
    - Store dried sample at -80 °C until further analysis.
    - For further analysis defrost dried sample at RT for at least 30 min.
  - 1st Derivatization: Methoximation
    - Add 60 µl of methoxymation solution (10 ml of 20mg/ml methoxyamine hydrochloride (Fluka) in dry pyridine (Fluka)) and incubate and shake solution for 90 min at 30°C and 200 rpm.
  - 2nd Derivatization: Silylation
    - Add 60 µl N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, Macherey-Nagel) to the solution and incubate and shake mixture for 30 min at 37°C and 200 rpm.
    - Allow mixture to cool to room temperature for 10 min.
  - Transfer 60 µl of the supernatant into micro vials.
  - Immediately crimp-cap micro vial and place into PTFE support sleeve.
  - After the 2nd derivatization wait 120 min prior to the first injection.
  - Sample is ready for GCMS analysis.

- For FIE-MS analysis:
  - Transfer 60 µl of the supernatant into HPLC vial with new micro insert.
  - Store remaining urine sample at -80 °C until further analysis.
  - Sample is ready for FIE-MS analysis.
    - Mobile phase: Methanol/Water [80/20].

- For nano-flow FT-icr-MS analysis:
  - Urine sample supernatant is generally ready for analysis. However, preparation of a dilution series (D1 to D3) for analysis is recommended (last three steps).
  - Alternatively, the supernatant can be adjusted to methanol/water [80/20] either by adding 50 µl methanol (yields a diluted sample):
    - Transfer 100 µl of urine supernatant into a new 2 mL Eppendorf tube.
    - Add 50 µl methanol.

    Or:
    - Transfer 100 µl of the urine supernatant into a new 2 mL Eppendorf tube.
    - Dry in speed vac.
    - Reconstitute in 100 µl methanol/water [80/20].
    - Sonicate for 15 min.
  - Vortex.
  - Shake for 15 min at +4°C.
  - Centrifuge at 14 000 rpm and +4 °C for 5 min.
  - Prepare a dilution series (D1 to D3) in 5 new 2 mL Eppendorf tubes.
  - Sample and dilution series is ready for nano-flow FT-icr-MS analysis.
  - Store reconstituted sample at -80 °C until further analysis.

7.3 Problems

Urine samples take at least 2 hours to defrost on ice when taken from a -80 °C freezer.

7.4 Disposal of waste

After sample preparation discard used disposable plasticware in autoclave bag.
Collect all chemicals in appropriate bottles and follow the disposal rules for urine samples.

7.5 Quality Assurance

Every time you need to use a new batch of Eppendorf tubes, pipette tips or glass vials prepare a negative blank control following the procedure (*i.e.* all materials and plastic ware) without biological sample. Insure that consumables of the same batch are used for all samples of an experiment. Randomize all samples to be processed.

8. CALCULATIONS AND DATA ANALYSIS

Please see references:

- Beckmann M, Parker D, Enot DP, Chareyron E, Draper J (2008). High throughput non-targeted metabolite fingerprinting using Flow Injection Electroctrospray Mass Spectrometry. *Nature Protocols,* 3(3): 486-504 (DOI: 10.1038/nprot.2007.500).
- Enot DP, Lin W, Beckmann M, Parker D, Overy DP, Draper J (2008). Pre-processing, classification modelling and feature selection using Flow Injection Electrospray Mass Spectrometry (FIE-MS) metabolite fingerprint data. *Nature Protocols,* 3(3): 446-470 (DOI: 10.1038/nprot.2007.511).
- Enot DP, Beckmann M, Overy D, Draper J (2006). Predicting interpretability of metabolome models based on behavior, putative identity, and biological relevance of explanatory signals. *Proc Natl Acad Sci USA*; 103(40):14865-70.
- Enot DP, Beckmann M, Draper J (2007). Detecting a difference – Assessing generalisability when modelling metabolome fingerprint data in longer term studies of genetically modified plants. *Metabolomics,* 3(3): 335-347.

9. RELATED PROCEDURES

Plasma/Serum sample preparation SOP-EX-05-v1.0

10. APPENDICES

NA

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