LC-MS metabolomics of pig plasma and urine

Changes in biochemical profiles of biofluids after consumption of diets with contrasting dietary fiber composition

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Preface

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Abstract

Epidemiological studies have indicated that diets high in dietary fiber (DF) are associated with positive nutritional and physiological effects with importance to health and wellbeing for both animal and humans. Results obtained by classical biochemical and clinical methods on the health promoting effects of DF and phytochemicals have already been reported. These results, however, cannot explain all the mechanisms behind the protective effects of DF and phytochemicals, and therefore there is a demand for novel approaches to study their mechanism of action and to elucidate their synergetic effects. Metabolomics is an emerging field of ‘omics’, which aims to study global metabolic changes in biological systems. LC-MS platform allows the measurements of hundreds to thousands of compounds in one sample, and therefore is ideally suited for metabolomics studies. In this PhD project we used LC-MS metabolomics to study the metabolome of plasma and urine of catheterized and hypercholesterolemic pigs after consumption of diets with contrasting DF composition.

Cereals such as wheat and rye are good sources of DF and bioactive compounds. However, bioactive compounds are unevenly distributed in the grain with highest concentrations within bran and germ. Therefore, we tested breads based on whole grain wheat, whole grain rye with added rye bran and wheat aleurone versus refined wheat flour to elucidate the biochemical effects and identify the biomarkers of this dietary intervention in plasma and urine using pig as a model for human subjects. In study I, we used a catheterized pig model with catheters in portal vein and mesenteric artery to study the absorption of nutrients/phenolic acids in the gastrointestinal tract. Two LC-MS metabolomics approaches were used in this study: targeted (paper I) and non-targeted (paper II). In study II, we used a non-targeted multi-compartmental approach to explore the metabolic changes in plasma and urine of hypercholesterolemic pigs (paper III).

The results of targeted metabolomics on the absorption and bioavailability of phenolic acids from wheat showed good agreement with previous findings. Low bioavailability of ferulic acid was measured after consumption of both whole grain wheat and wheat aleurone diets. The absorption profile of phenolic acids, however, differed between cinnamic and benzoic acid derivatives. Cinnamic acid derivatives such as ferulic acid and caffeic acid had a hyperbolic curve shape with increasing concentration as a result of several days exposure to the diet, whereas benzoic acids were mainly characterized by a constant concentration in plasma. In general, the difference between the artery and the vein for all phenolic acids was diminutive, indicating that the release of
phenolic acids in the large intestine was not sufficient to create a porto-arterial concentration difference. It was therefore concluded that plant phenolic acids undergo extensive inter-conversion in the colon and that their absorption profiles reflected their low bioavailability in the plant matrix (paper I).

The results of non-targeted LC-MS metabolomics showed that diverse pool of metabolites discriminated between the diets in plasma and urine of pigs, and that metabolites could be summarized into two classes: fatty acids/oxylipins and phenolics. In study I, oxylipins discriminated between whole grain wheat versus aleurone wheat diets, whereas bile acids discriminated between portal vein and mesenteric artery. The elevated level of oxylipins such as 13-hydroxy-9,11-octadecadienoic acid (13-HODE) and 9-hydroxy-10,12-octadecadienoic acid (9-HODE) in plasma of pigs fed whole grain wheat diet was explained by the presence of germ rich in lipids. 13-HODE and 9-HODE may, therefore, be potential lipid markers of whole grain wheat consumption (paper II). The porto-arterial difference was mainly represented by conjugated bile acids, which indicated the reabsorption of the bile acids during entero-hepatic circulation (paper II). In study II, 13-HODE and 9-HODE also discriminated between the diets, however with higher concentration after refined wheat (WB) consumption compared to whole grain rye with added rye bran (RB), which may indicate that the presence of germ was probably overruled by the presence of antioxidants/penolics in the RB, which could inhibit oxidation, and that the germ has only a minor effect. In urine, the effect of the diets (RB versus WB) was characterized by phenolic compounds such as enterolactone, hippuric acid, 2-methylhippuric acid and ferulic acid and with higher concentrations after RB consumption. This is in accordance with the higher concentration of phytochemicals in RB compared to WB diets (paper III). Enterolactone is already considered as a biomarker for a plant lignan rich diet and the other phenolic compounds can also be potential biomarkers of polyphenol rich diets. Medium chained di- and monocarboxylic fatty acids also discriminated between the diets and with higher concentration in urine of pigs fed RB. Two novel dicarboxylic acids were identified and their metabolic pathway and the link to the consumption of RB diet have to be examined in future experiments. Moreover, dicarboxylic acids may be potential targets for future research on hypocholesterolemic effect of rye components (paper III).

In conclusion, LC-MS metabolomics provided valuable information on the metabolic responses in plasma and urine of pigs fed diets with contrasting DF composition. Moreover, LC-MS proved to be
useful in detection and identification of novel biomarkers of whole grain wheat and rye consumption.
Resume

Epidemiologiske studier har vist, at diæter med højt indhold af kostfibre har positive næringsmæssige og fysiologiske effekter og er vigtige for sundhed og velfærd hos både dyr og mennesker. Resultater, som er opnået med traditionelle biokemiske og kliniske metoder vedrørende sundhedsfremmende effekter af kostfibre og fytokemikalier, er allerede blevet beskrevet. Resultater fra disse studier kan imidlertid ikke forklare alle de mekanismer som ligger til grund for de positive sundhedsmæssige effekter af kostfibre og fytokemikalier, og derfor efterspørges nye fremgangsmåder til at studere disse mekanismer og belyse deres synergier.

Metabolomiks er et nyt felt indenfor ‘omics’, hvis formål er at studere de globale ændringer i biologiske systemer. Ved at bruge en LC-MS platform er det muligt at måle hundred- til tusindevis af molekyler bare i en prøve, og derfor er denne metode velegnet til metabolomiske studier. I dette PhD projekt, har vi brugt LC-MS metabolomiks til at studere plasma- og urinmetabolomet i grise med kateter og hypercholesterolæmiske grise, som har indtaget diæter med varierende indhold og sammensætning af kostfibre.

Cerealer, som hvede og rug, er gode kilder til kostfibre og bioaktive forbindelser. Disse bioaktive forbindelser er ujævnt fordelt i kornet med den højeste koncentration i klid og kim. I den forbindelse har vi testet brød bagt af fuldkornshvede, fuldkornsrug tilsat rugklid og hvedealeurone overfor raffineret hvedebrød for at belyse de biokemiske effekter og identificere biomarkører af denne diætintervention i plasma og urin. I studierne blev grise benyttet som model for mennesker. I studie I, brugte vi grise med kateter i portåren og den mesenteriske arterie til at studere absorptionen af næringsstoffer/fenoliske syrer i mave-tarmkanalen. To LC-MS fremgangsmåder blev brugt i dette studie: en målrettet metode (artikel I) og en ikke målrettet metode (artikel II og III). I studie II, brugte vi endvidere en ikke målrettet multikompartment metode til at udforske de metaboliske ændringer i plasma og urin i hypercholesterolæmiske grise, sammenholdt med de metabolitter som kunne detecteres i brødet (artikel III).

Resultaterne af målrettet metabolomiks, som gik ud på at måle absorption og biotilgængelighed af fenoliske syrer fra hvede, understøttede tidligere fund. Lav biotilgængelighed af ferulinsyre blev målt efter indtagelsen af både en fuldkornshvedediæt og en hvedealeuronediæt. Absorptionsprofilen af de fenoliske syrer var dog forskellige afhængigt af, om de var afledt af cinnaminsyre eller benzoinsyre. De fenoliske syrer afledt af cinnaminsyre, såsom ferulinsyre og kaffeinsyre, havde hyperbolisk kurveforløb med en stigende concentration som repons på flere
dages esponering til diæten, hvorimod de fenoliske syrer, afledt af benzoinsyre, havde en konstant koncentration i plasma. Generelt var forskellen mellem arterien og venen for alle fenoliske syrer minimal, hvilket indikerer at frigivelse af fenoliske syrer i tyktarmen ikke var tilstrækkelig til at skabe porto-arterial koncentrationsforskel. Det blev derfor konkluderet, at fenoliske syrer fra hvede gennemgår omfattende omdannelse i tyktarmen, og at deres absorptionsprofil reflekterede lav biotilgængelighed i plantematricen (artikel I).

Konklusionen er derfor, at LC-MS metabolomiks gav brugbar information omkring de biokemiske effekter i plasma og urin efter intage af diæter med varierende indhold af kostfibre. Derudover, har LC-MS vist sig at være en brugbar metode til detektion og identifikation af nye biomarkører for indtag af fuldkornshvede og –rug.
List of included papers


**Abbreviations**

DF  
Dietary fiber

ROS  
Reactive oxygen species

OSC  
Orthogonal signal correction

PCA  
Principal Component analysis

PLS-DA  
Partial least square – discriminant analyses

LC-MS  
Liquid chromatography – mass spectrometry

LC-MS/MS  
Liquid chromatography – tandem mass spectrometry

LDL-C  
Low density lipoproteins-cholesterol

PUFA  
Polyunsaturated fatty acids

LOX  
Lipoxygenase

PGs  
Prostaglandins

LKs  
Leukotrienes

13-HODE  
13-hydroxy-9,11-octadecadienoic acid

9-HODE  
9-hydroxy-10,12-octadecadienoic acid

5-HETE  
5-hydroxy-6,8,11,14-eicosatetraenoic acid

LTB₄  
Leukotriene B₄

9,12,13-TriHOME  
9,12,13-trihydroxy-10-octadecenoic acid

9,10,13-TriHOME  
9,10,13-trihydroxy-11-octadecenoic acid
Introduction

Epidemiological studies have indicated that diets high in dietary fiber (DF) are associated with decreased incidence of chronic diseases, such as cardiovascular diseases, type 2 diabetes and some forms of cancer. Cereals such as wheat and rye are good sources of DF, and they have a special role in the daily life of people in most parts of the world since they are suitable for bread-making. In industrial countries, the nutrients provided by the bread consumption account for 50 % of the intake of carbohydrates, 50-60 % of vitamin B and one third of the proteins (1). However, while total carbohydrate only varies slightly between the breads made of refined flour and whole grain flour, there is a big difference in the provision of the nutritive and non-nutritive compounds. Whole grain flour is not only a good source of DF, but also a rich source of minerals, vitamins and phytochemicals, which have been suggested to contribute to the protective effects of whole grain cereals. On the contrary, bread derived from refined flour is primarily an energy source depleted of most of the DF and bioactive components. The bioactive components are mainly situated in the outer layers such as aleurone, pericarp and testa, and they are therefore an important part of whole grain. Nevertheless, the inverse relationship between consumption of DF and development of chronic diseases identified in epidemiological studies does not mean a direct connection and provides no information about the health promoting mechanisms involved. Today, some health promoting mechanisms are nevertheless well recognised. High content of some DF increases the viscosity of the food matrix thereby influencing satiety and the slow postprandial absorption of glucose and is therefore recommended for type 2 diabetic subjects. The increased viscosity also leads to impaired absorption of fat and cholesterol and increased excretion of bile acids, which was previously suggested as a mechanism for the hypcholesterolemic effects of DF (2) (3) (4). The anti-carcinogenic effects are attributed to the presence of antioxidants such as phenolic acid. Ferulic acid is the most abundant phenolic acid in both wheat and rye, and it may possess antioxidative activity along the digestive tract by trapping reactive oxygen spices (ROS). However, not all mechanisms of action of bioactive compounds are yet elucidated nor are their synergetic effects explained.
Chapter 1: Background

1.1 Chemical composition of the whole grain wheat and rye

Wheat is the second largest food cereal after rice and with a production that takes up the greatest part of land cultivated with cereals (1). Rye, in comparison to wheat, is a minor crop and it is mainly grown in Northern, Central and Eastern Europe, where it is traditionally used in production of bread and feeds (5) (6). In most Western countries whole grain cereals such as wheat and rye are therefore important contributors of DF, vitamins, minerals and phytochemicals.

Wheat and rye have a similar cell tissue composition and are approximately composed of 10-14 % bran, 2.5-3.0 % germ and 80-85 % endosperm, where the bran and the germ contain highest concentration of bioactive compounds, Figure 1 (7). The bran of the grain covers pericarp, seed coat and aleurone layer of the endosperm. The pericarp and testa layers accounts for the highest content of DF followed by the aleurone layer. In the aleurone layer the major components of DF in both wheat and rye are arabinoxylans, cellulose and small amounts of lignin (5). The aleurone layer also account for the major content of phytochemicals such as phenolic acids, lignans, carotenoids, anthocyanins and isoflavonoids (7). The germ of whole grain is an important source of lipids and

![Figure 1](image.png)

**Pericarp**
- Epidermis
- Hypodermis
- Mesocarp
- Endocarp

**Seed coat**
- Testa/spermoderm (1%)
- Alklyresorcinols
- Hyaline layer

**Endosperm**
- Aleurone layer (6-9 %)
- Soluble and insoluble dietary fibre (xylans, β-glucans, raffinose)
- Proteins and enzymes
- Antioxidants (phenolic acids, lignans, carotenoids, anthocyanins, isoflavonoids)
- Vitamin E and B vitamins
- Minerals
- Phytic acid
- Betaine and choline
- Starch endosperm/flour (80-85 %)
- Starch and proteins
- B-glucans and arabinoxylans
- Carotenoids and flavonoids
- Thiamin (B1) and vitamin E

**Germ** (3 %)
- Lipids and enzymes
- Sucrose and monosaccharides
- Sulfur amino acids
- Glutathione
- Insoluble and soluble fibre, raffinose
- Flavonoids and phytosterols
- Betaine and choline
- Vitamins and minerals

**Figure 1.** Whole grain wheat with the main bioactive compounds and different layers of the pericarp, seed coat and endosperm.
enzymes (1). The main lipids in both wheat and rye grains are polyunsaturated fatty acids (PUFAs) accounting for 63.7% and 68.6% of the total lipid content respectively (8). Since there is a great diversity of bioactive compounds in the whole grain the focus of this study will be on the lipid and phenolic acid content of whole grain wheat and rye.

1.2 Polyunsaturated fatty acids and oxylipins

1.2.1 Polyunsaturated fatty acids and oxylipins in plants

Linoleic acid (18:2) and α-linolenic acid (18:3) are the main PUFAs present in wheat and rye with linoleic acid accounting for 60% and α-linolenic acid accounting for 4-8% of the total lipid content (8). They are preferentially stored in the germ and to a smaller extend in the aleurone layer (1). During plant growth, the composition and turnover of these lipids can be altered by for example environmental changes. The oxidation of these PUFAs to different oxylipins is one of the main reactions in the lipid alteration (9). In plants, oxylipins serves as signal molecules in developmental processes such as pollen formation. They are also signal molecules in defense mechanisms and their increased production is a response to pathogenesis, wounding and herbivores (10) (11) (12). The enzymes, which are responsible for the conversion of PUFAs into the plethora of oxylipins, are lipoxygenase (LOX), peroxygenase (POX), α-dioxygenase (α-DOX), epoxide hydrolases (EH) and enzymes belonging to the family of cytochrome P450 such as allene oxide synthase (AOS), divinyl ether synthase (DES), hydroperoxide lyase (HPL), epoxy alcohol synthase (EAS) and reductase. Modified after Feussner et al., (9).

**Figure 2.** The lipoxygenase (LOX) pathway in plants. Metabolism of polyunsaturated fatty acids (PUFAs) leading to formation of (9S)-hydroperoxy and (13S)-hydroperoxy derivatives and further metabolism yielding plethora of oxylipins. Enzymes that are involved: peroxygenase (POX), α-dioxygenase (α-DOX), epoxide hydrolases (EH), allene oxide synthase (AOS), divinyl ether synthase (DES), hydroperoxide lyase (HPL), epoxy alcohol synthase (EAS) and reductase. Modified after Feussner et al., (9).
synthase (AOS), divinyl ether synthase (DES), hydroperoxide lyase (HPL) and epoxy alcohol synthase (EAS), Figure 2. The start reaction is production of two LOX products (9S)-hydroperoxy and (13S)-hydroperoxy derivatives of PUFA, and with the preferential substrate for LOX activity being linoleic acid (9) (12) (13). One of the best known enzymes, allene oxide synthase, the key enzyme in production of jasmonic acid, has an important cell signaling function in plant development (13) (14). Another enzymatically catalyzed pathway, which produces many antifungal compounds, and which is comparable to the mammalian immune system cascade of arachidonic acid, is the linoleic acid cascade. The linoleic acid cascade involving LOX, POX and EH explains the biosynthesis of the epoxy-, hydroxy-, dihydroxy-, and trihydroxy-oxylipins, Figure 3 (12). These highly bioactive, antifungal compounds are found in cereals such as rice, oat, wheat and rye (12) (11;15).

Figure 3. The linoleic acid cascade which leads to formation of epoxy, epoxy-hydroxy, tri-, di- and monohydroxy fatty acids. Enzymes involved: lipoxygenase (LOX), peroxygenase (POX), reductase and epoxide hydrolases (EH). Compound abbreviations: 9-hydroperoxy-10,12-octadecadienoic acid (9-HpODE), 13-hydroperoxy-9,11-octadecadienoic acid (13-HpODE) 13-hydroxy-9,11-octadecadienoic acid (13-HODE), 9-hydroxy-10,12-octadecadienoic acid (9-HODE), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME) and 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-TriHOME), 9,10-dihydroxy-12-octadecenoic acid (9,10-DiHOME), 12,13-dihydroxy-9-octadecenoic acid (12,13-DiHOME).
However, oxylipins can be formed not only by enzymes but also in the presence of ROS. In contrast to the enzymatic reaction which leads to formation of pure enantiomers, lipid peroxidation leads to the production of racemic mixtures. LOX inserts molecular oxygen only at C9 or C13, whereas ROS can lead to formation of hydroperoxides at C8, 10, 12 and 14 (16). Though, besides the known LOX- and ROS products, few studies have shown the existence of other C-positions of oxygen addition, such as 15(R)-hydroxylinoleic acid, an oxylipin identified in oat seeds (17). Similarly, 15-hydroxy-octadecadienoic acid was also identified in the pollen of *Brassica campestris* L. var. *oleifera* DC. (18).

Many studies with fungi have shown the existence of non-LOX derived oxylipins such as 8(R)-hydroxy-octadecadienoic acid, which is synthetized by an enzyme named 7,8-linoleate diol synthase, and oxylipins with still unknown biosynthetic pathways (13) (10).

### 1.2.2. Polyunsaturated fatty acids and oxylipins in mammals

The enzymatic addition of molecular oxygen to PUFAs is a reaction that occurs not only in plants but also in the animal kingdom. While the preferential substrate for LOX activity in plants is linoleic acid, it is arachidonic acid in mammals (10). The arachidonic acid cascade, Figure 4, leads to production of eicosanoids such as prostaglandins (PGs) and leukotrienes (LKs), which constitute the best characterized group of mammalian oxylipins (10). These two classes of eicosanoids play important roles as signal molecules that exert complex control over processes such as inflammation and immunity, and therefore their overproduction is associated with diseases such as atherosclerosis, type 2 diabetes and cancer. PGs are synthetized by most cells in the body and may act as both anti- and pro-inflammatory lipid mediators. On the contrary, LKs are well known pro-inflammatory lipid mediators and are formed predominantly by inflammatory cells like leukocytes, macrophage and mast cells (19) (20). LKs are the main products of the 5-LOX pathway, which leads to formation of 5-hydroperoxy derivatives of arachidonic acid. The 5S-hydroperoxyeicosatetraenoic acid (5S-HPETE) undergo further transformation by 5-LOX and by leukotriene A₄ hydrolase (LTA₄H) to form leukotriene B₄ (LTB₄), Figure 4, a potent stimulator of leukocyte adhesion to endothelial cells (21). Since LOX is an enzyme that is found in both plants and mammals, consumption of linoleic acid may lead to the formation of oxylipins in mammals similar to the ones that are found in the plant kingdom. However, in mammals and humans, the role of oxylipins derived from linoleic acid is not clear (22) (23). These oxylipins can add to the in vivo oxygenated lipid pool as a part of the lipoproteins, known as oxidized low-density the lipoproteins (OX-LDL). Their release from
Figure 4. The arachidonic acid cascade which leads to formation of eicosanoids, prostaglandins (PGs) and leukotrienes (LTs). Enzymes involved: lipoxygenase (LOX), cyclooxygenase (COX) and leukotriene A₄ hydrolase (LTA₄H). Compound abbreviations: 5-hydroperoxy-6,8,11,14-eicasatetraenoic acid (5-HPETE), 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 5,11-dihydroxy-6,8,12,14-eicosatetraenoic acid (5,11-DiHETE), 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid (5,15-DiHETE) and 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (5,15-DiHETE).

the lipoprotein matrix by lipoprotein lipase and delivery to the endothelia of heart, skeletal muscles, and adipose tissue can occur along with arachidonic acid derived oxylipins. Recent studies suggest that oxylipins derived from linoleic acid may play an important role in the inflammatory processes of the endothelium together with arachidonic acid derived oxylipins and can also be used as markers of pro-inflammation in atherosclerosis (24) (25) (26).

1.2.3 Polyunsaturated fatty acids and health effects

Linoleic acid (ω-6 fatty acid) and α-linolenic acid (ω-3 fatty acid) are termed essential fatty acids because mammals cannot synthesize them. They are required as building blocks for biologically active membranes. In the body, linoleic acid can be metabolized to arachidonic acid and α-linolenic acid can be metabolized to eicosapentaenoic acid (27). The arachidonic acid derived oxylipins are generally referred to as pro-inflammatory lipid mediators whereas oxylipins derived from eicosapentaenoic acid are mainly anti-inflammatory compounds. Today it is well recognized that the ratio between the consumption of ω-6 and ω-3 fatty acids is essential in maintaining a healthy lifestyle. A higher consumption of ω-6 fatty acids and therefore increased ratio between ω-6 and ω-
3 fatty acid may lead to higher production of PGs, LKs and hydroxy fatty acids, and thereby disturb the sensitive balance between anti- and pro-inflammatory mediators, Figure 5 (27) (28). Inflammation is the organism’s response to local injury or bacterial invasion, and is a self-limiting and controlled process of the innate immune system (29) (28). The initiation phase is started by the production of pro-inflammatory eicosanoids such as LTB₄ and PGE₂ and/or PGD₂. Although PGE₂ and/or PGD₂ are initially pro-inflammatory, they determine the switch to the next phase, the resolution of the inflammation. When the level of PGE₂ and/or PGD₂ is equal to the level of LTB₄, the production of LTB₄ is inhibited and production of anti-inflammatory mediators like lipoxins and resolvins is activated. Sufficient level of anti-inflammatory mediators turns on the Stop Signal to stop the pro-inflammatory process (28).

![Figure 5](image)

**Figure 5.** Inflammation as a self-limiting process with initiation, resolution and termination phases. Initiation phase: starts by the production of pro-inflammatory eicosanoids such as LTB₄ and PGE₂ and/or PGD₂. These mediators increase inflammation until the Eicosanoid Switch, the end of initiation phase. Resolution phase: the level of PGE₂ and/or PGD₂ is equal to the level of LTB₄, the production of LTB₄ is inhibited and production of anti-inflammatory mediators is activated. Termination phase: the total level of anti-inflammatory mediators exceeds the level of LTB₄ the Stop Signal takes place. The inflammation is terminated by clearing the inflamed area. Modified after Bosma-den Boer et al., (28).

Prolonged and high production of pro-inflammatory mediators may lead to the development of chronic inflammation diseases like cardiovascular diseases, diabetes, respiratory diseases, autoimmune diseases and cancer (28). For example arachidonic acid derived oxylipins contribute to the formation of atherosclerotic plaques by promoting pro-inflammatory cells adhesion (30). LTB₄ is also associated with tumor cell adhesion leading to the spread of cancer cells in the body (31). It is also recognized that high dietary intake of linoleic acid favors the oxidative modification of LDL-cholesterol and increases platelet response to aggregation promoting formation of atherosclerotic plaques (32) (33). In the propagation of arteriosclerosis OX-LDL play an important role since scavenger receptors internalize OX-LDL in the presence of cholesterol. The consequence is the
formation of intracellular cholesterol pools and the development of macrophage to lipid loaded foam cells, which are the markers of arteriosclerosis (34) (32). It is also reported that oxylipins derived from linoleic acid like 13- and 9-HODE are the major components of OX-LDL and have different physiologic effects, including accumulation of macrophages in atherosclerotic plaques (25) (24). Therefore, there is a growing evidence that 13- and 9-HODE play an active role in atherosclerotic plaques formation and can be used as pro-inflammatory markers of this disease (24). On the contrary, consumption of α-linolenic acid is associated with inhibitory effects on the platelet aggregation and their response to thrombin and on the metabolism of arachidonic acid (27). Therefore, as a result of changes in the Western diet, such as higher dietary consumption of cholesterol and saturated fatty acids together with refined carbohydrates and increased ratio of ω-6 and ω-3 fatty acid and, on the contrary, lower intake of vitamins D and antioxidants may lead to increased number of people suffering from chronic inflammation diseases (28).

1.3 Phenolic acids

1.3.1 Phenolic acids in plants

Phenolic acids are secondary metabolites produced through the shikimic acid pathway in plants (35). The shikimic acid pathway converts simple carbohydrate precursors derived from glycolysis and pentose phosphate pathway to aromatic amino acids, which are further metabolized to alkaloids having common features of C₆C₃ units (36) (35). Phenolic acids are hydroxylated derivatives of cinnamic acid and benzoic acid (37). The key reaction in formation of phenolic acids is the removal of amino group from phenylalanine by phenylalanine ammonia lyase (PAL) yielding trans-cinnamic acid. The aromatic ring of cinnamic acid can be hydroxylated, methylated and O-methylated to yield p-coumaric acid, ferulic acid, caffeic acid and sinapic acid. Moreover, benzoic acid and its derivative can be formed from cinnamic acid by β-oxidation or from chorismic acid, Figure 6 (35). Hydroxycinnamic acids are intermediates in the formation of lignin, the major non-carbohydrate component of the plant cell wall (35). Hydroxycinnamic acids are highly abundant in wheat and rye and other cereals, while the hydroxybenzoic acid content is generally very low. The most abundant phenolic acid in both wheat and rye is ferulic acid, which accounts for 72-90 % of total phenolic acid content, followed by sinapic acid (9-10 %) (38) (39). Most phenolic acids in wheat and rye occur cross-linked with plant cell wall macromolecules such as cellulose, arabinoxylan and other non-cellulosic polysaccharides (40). Ferulic acid is bound via ester and ether bonds to lignin or linked to the O-5 position of the arabinofuranose substituents (38). Caffeic acid is
ester linked to quinic acid, which together constitute chlorogenic acid (37). Small proportions of phenolic acids exist as free compounds conjugated with sugars and other low molecular weight molecules (40) (37).

**Figure 6.** The shikimic acid pathway in plants converts carbohydrate precursors derived from glycolysis and pentose phosphate pathway to aromatic amino acids such as phenylalanine which is further metabolized to trans-cinnamic acid by phenylalanine ammonia lyase (PAL). Cinnamic acid can be hydroxylated, methylated and O-methylated to yield p-coumaric acid, ferulic acid, caffeic acid and sinapic acid, which are intermediates in the formation of lignin.

### 1.3.2 Phenolic acids in mammals

The shikimic acid pathway does not exist in mammals and humans and therefore dietary intake is an important provider of the products derived from the shikimic acid pathway, for example essential aromatic amino acids and phenolic acids known as important antioxidants (41) (37). There is growing evidence that when consumed, plant phenolic acids undergo substantial interconversion. Since the majority of phenolic acids are bound to plant cell walls, they escape the absorption in the small intestine, but can be released from the plant matrix by intestinal esterases or, the main part, by bacterial esterases in the colon. Their metabolism in the colon is still not fully explained, but it is agreed that the main microbial degradations are reduction, demethylation and dehydroxylation (42). For example ferulic acid can undergo reduction, demethylation and dehydroxylation at C4 to yield 3-hydroxyphenylpropionic acid (43). Further metabolism of phenolic acids may occur in the intestinal mucosa, liver and kidney, where they can undergo hydrogenation, methylation and O-methylation, β-oxidation and conjugation with glycine, sulphate and glucuronic.
Conjugation of phenolic acids increases their hydrophilicity and facilitates their biliary and urinary excretion (37). The major products of colonic metabolism and conjugation in the liver are typically excreted in urine. Many studies refer to hippuric acid as being the main end product excreted in urine after a polyphenol rich diet (44) (45) (46). The proposed formation of hippuric acid and hippuric acid derivatives from hydroxycinnamic acids is shown in Figure 7.

Figure 7. The proposed formation of hippuric acid, 3-hydroxyhippuric acid and 4-hydroxyhippuric acid from hydroxycinnamic acids through colonic fermentation and β-oxidation and conjugation with glycine in the liver. Modified after Rechner et al., (44).

1.3.3 Phenolic acids and health effects

Since plant phenolic acids are now recognized for their antioxidative properties, their consumption is related to prevention of chronic diseases associated with oxidative stress such as cancer and cardiovascular disease. The link between the antioxidative properties and probable role in the prevention of various diseases is, however, not well established (37). Several in vivo and in vitro studies have aimed to establish evidence for the health effects of phenolic acids in the context of preventive nutrition. However, there are controversial results regarding the bioaccessibility and bioavailability of plant phenolic acids versus the concentration of these compounds required to induce the antioxidative effect. Several animal and human in vivo studies report low bioaccessibility and bioavailability of plant phenolic acids in the plant matrix and their high metabolic conversion in the gastrointestinal tract together with rapid urinary excretion and hence low circulating levels of
plant phenolic acids in the blood has been observed (47) (48) (49). However, in vitro studies on the antioxidative activity of plant phenolic acids report high concentrations required to induce the antioxidative effect (50) (47). It has been shown in vitro that hydroxycinnamic acids, like ferulic acid and caffeic acid possess antioxidative activity at micro molar concentration (1-100 µM) (47). The antioxidative activity decreases in the order of: caffeic acid > sinapic acid > ferulic acid > p-coumaric acid (50). The bioavailability of ferulic acid after consumption of cereals, particular bran, varies from 0.4-0.5 % in rats to 3 % in humans. The relative bioavailability of the phenolic acids decreases in the order: p-coumaric acid > ferulic acid > caffeic acid > chlorogenic acid (42) (49) (48). The correlation between dietary intake and physiological effects is therefore not fully understood and more research on the nutritional efficacy of phenolic acid-rich food is warranted (51).

1.4 Pig as a model for human subjects

Experimentally, the pig has been described as the “best non-primate model for studying human nutrition” as it is a large single-stomached omnivore and has a comparable gut physiology to humans (52) (53). It has also been used extensively as a model for nutritional and physiological research (53). Since the digestive physiology of pig is comparable to human, the catheterised pig model is a well-established model for studying the uptake of water soluble compounds to the portal vein system after digestion in the gastrointestinal tract. The uptake of nutrients can be measured using permanent catheters placed in the portal vein and the mesenteric artery and with a flow probe around the portal vein, Figure 8 (54) (55) (56).

Figure 8. Schematic representation of catheterised pig model with permanent catheters placed in the portal vein and the mesenteric artery and with the flow probe around the portal vein (55).

The main site of absorption of digested nutrients is the small intestine, whereas the large intestine is the main site for microbial fermentation. The small intestine is covered with villi, which are lined
by epithelial cell. These cells are responsible for the absorption of nutrients from the gut lumen. Each villus contains an arteriole and venule together with lacteal, a drainage tube of the lymphatic system. The water soluble compounds are absorbed to the epithelium and further transported to the venule that drains into the hepatic portal vein (57). Absorption of lipids is facilitated by an emulsification processes, in which bile salts form lipid-bile salt complexes called micelles (58). When a micelle contacts the intestinal epithelium, lipids can diffuse across the cell membrane and form protein coated complexes, chylomicrons. Due to their size chylomicrons cannot diffuse into capillaries, but are able to diffuse into lacteals, which have large gaps between adjacent endothelial cells. Chylomicrons are drained along the lymphatic vessels and through the thoracic duct, entering the bloodstream at the left subclavian vein (59). Most of the bile salts within micelles are reabsorbed and recirculate via entero-hepatic circulation (58).

1.5 Liquid Chromatography Mass Spectrometry metabolomics

1.5.1 Approaches and applications

Metabolomics has initially been defined as the comprehensive quantification and identification of all metabolites present in a biological system/sample (60) (61). The goal of metabolomics is to measure all the metabolites in a given sample with one analytical procedure. The combination of Liquid Chromatography and Mass Spectrometry (LC-MS) is an analytical platform, which allows the measurements of hundreds to thousands of compounds or spectral features in one sample. However, it is now acknowledged that due to the complexity and diversity of physical and chemical properties of metabolites, no single analytical platform can be applied to detect all metabolites in a biological sample. The application of multiple analytical platforms, larger sample sets and collection of samples of multiple compartments can be a better strategy to increase the sensitivity and coverage of metabolite detection. Since metabolomics is a core area of system biology the aim of metabolomics is also to study global metabolic changes in biological systems and, more importantly, their complex interactions (62) (61). For example consumption of metabolites constituting the food matrix may result in their excretion from the body unmodified or, bio-transformed by the body and/or gut microorganisms into further metabolites that can be detected in the blood, urine and feces. Metabolomics analyses of these samples therefore provide a snapshot of the state of an organism and its metabolic processes. Reduced or elevated levels of particular metabolites may offer an indication, a biomarker of the metabolic state and link to the health status of the organism (63). Typically, metabolomics studies are designed for hypothesis
generation or knowledge discovery. There are, however, different approaches that can be applied in metabolomics studies. The starting point can be a limited biological knowledge with the overall objective of measuring and identifying the metabolites in a sample, a non-targeted metabolomics approach. Another approach is based on some knowledge about a sample’s biology, where particular set of metabolites are measured, a targeted metabolomics approach (62) (60).

1.5.2 Metabolomics workflow

Depending on the approach a metabolomics workflow can be designed. There are several steps in a typical metabolomics workflow: study design, sample preparation, sample measurements, data pre-processing, statistical data analyses and identification of metabolites/biomarkers, Figure 9.

![Figure 9. Schematic representation of metabolomics workflow, showing stepwise procedure. Abbreviations: liquid-liquid extraction (LLE), solid-phase extraction (SPE), Liquid Chromatography-Mass Spectrometry (LC-MS), Principle Component Analyses (PCA), Partial List Squares (PLS) and tandem mass spectrometry (MS/MS).](image)

To ensure statistically validated data and to reduce the influence of biological variability a sufficient number of samples are required. Therefore, in metabolomics studies a large number of samples are commonly analysed to detect biologically relevant sample clustering. Sample preparation procedure varies depending on the sample’s nature, plasma, urine, feces or tissue. Solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are the most used sample preparation procedures.
SPE is mainly used in targeted metabolomics approach when a sufficient separation of analytes from interfering matrix is required. The goal of LLE is to extract a large number of metabolites and therefore it is a method of choice for non-targeted metabolomics approach. Further steps are sample measurements on the LC-MS instrument and data handling, including pre-processing prior to statistical analyses. Pre-processing includes deconvolution, alignment of the chromatograms, normalization and filtering of the data. The biggest difference between non-targeted and targeted approaches lies within the statistical data analyses and identification. To reduce the dimensions of large data sets of non-targeted metabolomics multivariate data analyses such as Principle Component Analyses (PCA) and Partial Least Squares (PLS) are typically used, whereas univariate data analyses can be applied to both targeted and non-targeted metabolomics. Moreover, no identification is needed in a targeted approach since the measured metabolites are known. In contrast to targeted metabolomics, the identification step is a complex process in non-targeted metabolomics and not all metabolites are possible to identify. This is because the libraries and databases applied for identification are far from being complete to reflect all known metabolites. Identification can therefore be a time consuming process requiring commercially available standards and tandem mass spectrometry. However, identification is an important step in the biological interpretation and biomarker elucidation.
Chapter 2: Hypothesis and aim

The overall aim of this PhD project was to use LC-MS metabolomics to investigate the metabolic profiles of plasma and urine after consumption of diets with contrasting DF composition using pig as a model for human subjects. The hypothesis was that pig’s metabolic processes would respond differently depending on the diet consumed which would be reflected in urine and plasma (explorative study). This difference in metabolic responses would be detected using LC-MS platform combined with statistical analyses, which would allow the biological interpretation and biomarker elucidation. Another hypothesis was that absorption profiles of phenolic acid present in the diets would differ between the diets with contrasting DF structure, which could be measured using a targeted metabolomics approach.

Two pig studies were used for this purpose. In Study I, pigs were surgically fitted with catheters in the portal vein and mesenteric artery to study the absorption of nutrients. The pigs were fed contrasting diets in a repeated cross-over design, and blood samples were collected during the fasted and the fed state of the pigs. Two LC-MS metabolomics approaches were used, a targeted approach (paper I, chapter 4) and non-targeted approach (paper II, chapter 5). In the Study II, pigs were fed two diets high in dietary cholesterol to provoke hypercholesterolemia. The effect of the DF was measured in plasma, spot urine and 24 hours urine using a non-targeted metabolomics approach (paper III, chapter 6).
Chapter 3: Methods overview

3.1 Study I

The pigs used in the study were from the swine herd at Aarhus University, Department of Animal Science. The pigs were fed three times daily at 09.00 (breakfast), 14.00 (lunch) and 19.00 hours (dinner) with an amount of 40, 40 and 20% of the daily supply, thereby mimicking the diurnal variation in cereal intake experienced by humans. Six female pigs (Landrace x Yorkshire) with a bodyweight of 56.5 kg (±1.8 kg) were included in the experiment, which was designed as a repeated 3 x 3 cross-over design. The pigs were adapted to the pen for 5 days, and then the animals were surgically fitted with a flow probe around the portal vein, a catheter in the portal vein and a catheter in the mesenteric artery as described previously by Jørgensen et al., (56).

![Diagram of experimental design](image)

**Figure 10.** Experimental design. Crossover design where each pig was fed three experimental diets during one week. The breads were made of whole-wheat grain flour (WWG), wheat aleurone flour (WAF) and rye aleurone flour (RAF). The blood samples were collected at mesenteric artery and portal vein.

After, the surgery, the animals were allowed a 5-7 days recovery period before entering a 21-day experimental period (three consecutive experimental weeks). In each experimental week, the pigs were fed washout bread on day 1-3 (Friday-Sunday), and then the pigs were fed one of three experimental breads on days 4-7 (Monday-Thursday). The breads were made of standard white wheat flour (WFL), whole-wheat grain flour (WWG), wheat aleurone flour (WAF) and rye aleurone-rich flour (RAF). The pigs had access to water ad libitum, whereas no straw was supplied. Fasting blood samples (30 min before the first daily meal) were collected from the portal vein and the
mesenteric artery on day 4-7 (Monday-Thursday). On day 7 (Thursday), consecutive blood samples were drawn in the fed state from the portal vein and the mesenteric artery during 0-10 h after the first daily meal (breakfast), but with a re-feeding 5 hours after the breakfast (lunch).

**Figure 11.** Schematic representation of the sampling time. Fasting blood samples (30 min before the first daily meal) were collected on day 4-7 (Monday-Thursday). On day 7 (Thursday), consecutive blood samples were drawn in the fed state during 0-10 h after the first daily meal (breakfast), but with a re-feeding 5 hours after the breakfast (lunch). Three plasma samples were pooled to represent the mean content of metabolites in plasma for the periods: Pool 1, 0-150 min after breakfast (samples collected at 15, 60 and 120 min); Pool 2, 150-300 min after breakfast (samples collected at 180, 240 and 300 min); Pool 3, 300-450 min after breakfast (samples collected at 315, 360 and 420 min (pigs were re-fed at 300 min)); Pool 4, 450-600 min after breakfast (samples collected at 480, 540 and 600 min). The pigs were fed washout diet between collections (Friday-Sunday).

Blood samples were collected in 9 mL Na-heparinised vacutainers, centrifuged and plasma was harvested. Three arterial plasma samples and three venous plasma samples, respectively, were pooled to represent the mean content of metabolites in plasma for the periods: Pool 1, 0-150 min after breakfast (samples collected at 15, 60 and 120 min); Pool 2, 150-300 min after breakfast (samples collected at 180, 240 and 300 min); Pool 3, 300-450 min after breakfast (samples collected at 315, 360 and 420 min (pigs were re-fed at 300 min)); Pool 4, 450-600 min after breakfast (samples collected at 480, 540 and 600 min). Thus, Pool 1-Pool 4 represents a 10 h postpradial period relative to breakfast. The samples were stored at -20 °C.

Only two experimental diets were used for metabolomics measurements, WWG and WAF. In paper I, washout diet WFL was called refined wheat flour (RWF) and WWG was changed to whole grain wheat (WGW). In paper II, washout diets WFL was called “refined flour”, WWG was called “whole grain wheat” and WAF was called “wheat aleurone flour”.

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3.2 Study II

A total of seventeen, hyper-responders, Duroc x Danish Landrace x Yorkshire gilts (weighing approximately 70 kg), age 4 months and obtained from the swineherd at Aarhus University (Foulum, Denmark), were used for this study. Before the start of experiment the pigs were fed a wash-out diet (low-fat and low-DF) for 3 weeks. The low-fat and low-DF diet (LF) consisted of 867 g/kg refined wheat flour, 73 g/kg whey protein concentrate, 30 g/kg rapeseed oil, 30 g/kg vitamin-mineral mixture and 60 g/kg cellulose in the form of Vitacel WF 600. The breads fed during the experimental period were prepared from whole grain rye and with added rye bran (RB) or wheat flour with added refined wheat cellulose rich ingredient (Vitacel WF 600) (WB) and with added egg power, lard, rapeseed oil, sugar and whey protein concentrate (paper III). The diets were iso-DF and high in fat and were fed to the pigs for 9,5 weeks; 8 pigs were fed WB and 9 pigs were fed RB. The pigs were housed individually in 4 m² smooth-walled pens with a concrete floor. In the second week the pigs were transferred to metabolic cages for 7 days, where 24 hour urine was collected. Urine was collected over 4 g ascorbic acid per bottle, and pH was measured on a daily basis. The urine samples were pooled 7 x 24 h and stored frozen. The feed intake during the period, where the pigs ate the experimental bread-based diets, was restricted to 2 kg/d increasing to 3 kg/d for the last 2.5 weeks of the study. Postprandial blood samples from the jugular vein and lateral auricular artery were taken 3 h after the morning meal on the day of slaughter. The pigs were euthanized with an overdose of sodium pentobarbital by exsanguination and blood samples were collected in EDTA vacutainers, centrifuged and plasma was harvested and stored at – 20 °C. The pigs were weighed once weekly and before the slaughtering. When the pigs were slaughtered a spot urine was taken directly from the urine bladder and stored frozen.
3.3 Liquid Chromatography Mass Spectrometry platform

The main advantage of combining Liquid Chromatography with Mass Spectrometry is the chromatographic separation of metabolites prior to detection. In this study, High Pressure Liquid Chromatography (HPLC) was compelled to quadrupole Time of Flight Mass Spectrometer (qTOF-MS). The HPLC was equipped with a C18 column, which allowed the separation of metabolites according to their hydrophobic properties. The mobile phase was introduced into the qTOF-MS by electro spray ionization (ESI). ESI is the ionization method, which is used to produce gaseous ionized molecules from a liquid solution. Dry gas and heat cause droplets to decrease in size, while N2 evaporates the solvent. Ions pass through the capillary funnels, hexapol and quadrupole to the collision cell, where the fragmentation of the ions can be performed. Before detection, ions pass through the TOF analyser, which improves the separation of the ions and calculates the accurate masses. Due to its good resolution, accurate masses and moderate sensitivity LC-MS platform gained its status as powerful screening technique for metabolomics analyses.

3.4 Statistical methods

3.4.1 Univariate data analyses

Univariate data analyses was applied to the metabolomics data using Welch’s two-sample t test, which can be applied to the data to compare whether two independent variables differ (paper I and III). The P values of < 0.05 were considered to be statistically significant. For correction of false-positives (FDR) q values were calculated, with significance threshold set at q < 0.25. Moreover, a linear mixed-model was applied to obtain least-squares means values (LSMeans) of the diet and time effects and the diet x time interaction (paper II).

3.4.2 Multivariate data analyses

The first objective of the multivariate data analysis is to reduce the dimensionality of the complex data sets to enable easy visualization of any metabolic clustering. This was achieved by an unsupervised method of PCA and a supervised method for pattern recognitions Partial Least Squares Discriminant Analyses (PLS-DA) (Paper I and III). The Orthogonal Signal Correction (OSC) filter was applied to the data set prior PCA and PLS-DA. It was applied to the data to remove inter-subject variation among the pigs and to enhance the relevant information. OSC selectively removes the variation of data X that have no correlation with Y (paper I).
Chapter 4: Paper I
Phenolic acids from wheat show different absorption profiles in plasma – a model experiment with catheterized pigs

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The concentration and absorption of the nine phenolic acids of wheat were measured in a model experiment with catheterized pigs fed whole grain wheat and wheat aleurone diets. Six pigs in a repeated crossover design were fitted with catheters in the portal vein and mesenteric artery to study the absorption of phenolic acids. However, the difference between the artery and the vein for all phenolic acids was diminutive, indicating that the release of phenolic acids in the large intestine was not sufficient to create a porto-arterial concentration difference. Although, the porto-arterial difference was diminutive, their concentrations in the plasma and the absorption profiles differed between cinnamic and benzoic acid derivatives. Cinnamic acids derivatives such as ferulic acid and caffeic acid were metabolically linked and their absorption profiles differed depending on the diet consumed. Benzoic acid derivatives showed low concentration in the plasma and in the diets. The exception was p-hydroxybenzoic acid, with a plasma concentration much higher than the other plant phenolic acids likely because it is an intermediate in the phenolic acid metabolism. It was concluded that plant phenolic acids undergo extensive inter-conversion in the colon and that their absorption profiles reflected their low bioavailability in the plant matrix.

Keywords: phenolic acids, dietary fiber, whole grain wheat, wheat aleurone, plasma, pigs, ferulic acid
Phenolic acids are considered ubiquitous among vascular plants, where they occur in most tissues. In cereals, phenolic acids are present in all cell tissues, but with much higher concentrations in the aleurone and pericarp/testa layers compared to the endosperm (1). Epidemiological studies have linked whole-grain cereal consumption with a reduced risk of developing colonic and breast cancer, arteriosclerosis and type-2 diabetes (1) (2) (3). The underlying physiological mechanism behind the protective effects of whole-grain, however, are unclear but is most likely assigned to a concerted action of a wide variety of bioactive compounds, many of which are associated with the dietary fiber (DF) matrix (1). Among the possible mechanisms, the antioxidative capacity of phenolic acids most likely play a role even though the concentration level induced in vitro and ex vivo to obtain an effect appears much higher than detected in vivo in both animals and humans (4) (5) (6) (7).

Phenolic acids are hydroxylated derivatives of cinnamic and benzoic acids, Figure 1. Cinnamic acid derivatives found in wheat are ferulic acid, caffeic acid, p-coumaric acid and sinapic acid, whereas protocatechuic acid, p-hydroxybenzoic acid, salicylic acid, vanillic acid and syringic acid are derivatives of benzoic acid. Ferulic acid is the predominant phenolic acid in wheat accounting for 70-90 % of total phenolic acid content (8). A small proportion of free phenolic acid is located in the outer layer of the pericarp. Free phenolic acids are absorbed in the small intestine and conjugated in the intestinal epithelium or in the liver (9). Most phenolic acids in cereals, however, occur bound to plant cell walls, which consist of cellulose, arabinoxylan and β-glucan. Ferulic acid, for instance, is linked to the O-5 position of the arabinofuranose substitutes in the arabinoxylan but may also be linked by ester and ether bonds to lignin (10). In contrast to the free phenolic acids, bound phenolic acids have to be released from the plant matrix by intestinal esterases in the mucosa or, the main part, by bacterial esterases in the colon (11). The bioavailability of phenolic acids depends largely on their bioaccessibility in the plant matrix (8). In the process of microbial fermentation, however, plant phenolic acids are further metabolized to, i.e. derivatives of phenylpropionic, phenylacetic, hippuric and benzoic acids with different hydroxylation patterns. For instance, ferulic and caffeic acids can be de-esterified to 3-(3-
hydroxyphenyl)-propionic acid and subsequently $\beta$-oxidized to benzoic acid. The efficiency of release by intestinal and bacterial esterases and further metabolism of phenolic acids affect their absorption pattern and their route of excretion, i.e. through feces or urine (11) (9). More knowledge concerning absorption and bioavailability of phenolic acids in plasma is therefore required to fully understand their physiological properties and potential health effects in vivo.

To our knowledge previous studies on bioavailability and absorption of phenolic acids from wheat have primarily been performed with rats. However, since the digestive physiology of pigs is more similar to human than it is the case for rats, we used porto-arterial catheterized pigs to measure the net absorption of the nine phenolic acids present in wheat. We hypothesized that bound phenolic acids were released during active fermentation in the large intestine and that the absorption profiles may differ between the phenolic acids after consumption of whole grain wheat and wheat aleurone.

Materials and Methods

Chemicals. Acetonitrile and methanol of HPLC grade were from Rathburn (Mikrolab Aarhus A/S, Denmark). Acetic acid was from J.T.Baker (Deventer, Holland) and formic acid was from Merck (Darmstadt, Germany). Sulfatase (EC 3.1.6.1) type H-1 from Helix pomatia was purchased from Sigma-Aldrich (S. Louis, MO, USA) and $\beta$-glucuronidase (EC 3.2.1.31) from E.coli K12 was purchased from Roche (Mannheim, Germany). Sodium acetate was obtained from Prolabo (Leuven, Belgium). Following phenolic acid standards were used: syringic acid, salicylic acid, and caffeic acid (Sigma-Aldrich, S. Louis, MO, USA), vanillic acid, sinapic acid, protocatechuic acid, $p$-coumaric acid, ferulic acid, $p$-hydroxybenzoic acid (Fluka, Sigma-Aldrich, S. Louis, MO, USA). Water was purified with an A10 system from Millipore (Merck Millipore, MA, USA).

Diets. Breads were made of standard refined wheat flour (RWF), whole grain wheat (WGW) and wheat aleurone flour (WAF). The RWF and WGW breads were produced at Holstebro Technical College (Holstebro, Denmark), and the WAF breads were baked in a local bakery (Konditor-Bager Ørum, Denmark). The ingredients for the RWF breads were refined wheat flour (Triticum aestivum L.
Cv. Tiger), purified wheat fiber (Vitacel R200; J. Rettenmaier and Söhne GmbH, Rosenberg, Germany), rapeseed oil, sugar, salt, wheat gluten (LCH A/S, Frederiksberg, Denmark) and yeast, while in WGW and WAF breads, whole wheat grain (BFEL Karlsruhe, Germany) and wheat aleurone flour (Bühler AG, Uzwil, Switzerland) replaced the refined wheat flour and purified wheat fiber on an iso-DF basis. After production, the breads were cut into pieces. After weighing, meal portions were frozen at -20 °C and thawed immediately before consumption. Information on the ingredients content of the breads can be found in Table 1. The breads were balanced with regard to starch, proteins, fat and DF, but they varied in DF characteristics and the ratio between DF and phenolic acids as shown in Table 2.

**Study design and animals.** Experiments were in compliance with the guidelines of the Danish Ministry of Justice and regulations for humane care and use of animals in research (The Danish Ministry of Justice, Animal Testing ACT (Consolidation Act no. 726 of 9 September 1993 as amended by Act no. 1081 of 20 December 1995)). The pigs were fed three times daily at 09.00 (breakfast), 14.00 (lunch) and 19.00 hours (dinner) with an amount of 40, 40 and 20 % of the daily supply, thereby mimicking the diurnal variation in cereal intake experienced by humans. Daily feed allowance supplied 193-211 g DF/d and 76-779 mg/d of phenolic acids, Table 3. The pigs used in the study were from the swineherd at Aarhus University, Department of Animal Science. Six female pigs (Landrace x Yorkshire) with a bodyweight of 56.5 kg (±1.8 kg) were included in the experiment. The pigs were adapted to the pen for 5 days, and then the animals were surgically fitted with a flow probe (Transonic, 20A probe, 20 mm; Transonic System, Inc., Ithaca, NY, USA) around the portal vein, a catheter in the portal vein and catheter in the MA. After, the surgery, the animals were allowed a 5-7 days recovery period before entering the experimental crossover design. In each experimental week, the pigs were fed the wash-out diet, RWF on day 1-3 (Friday-Sunday), and then the pigs were fed either WGW or WAF on days 4-7 (Monday-Thursday). The pigs had access to water ad libitum, whereas no straw was supplied. Fasting blood samples (30 min before the first daily meal) were collected from the portal vein and the mesenteric artery on day 4-7 (Monday-Thursday). Blood samples were collected in 9 mL Na-heparinised vacutainers, centrifuged and plasma were harvested and stored at -20 °C.
**Proximate analyses of the breads.** The dry matter content was measured by drying to a constant weight for approximately 20 h at 103 °C, and ash was analyzed by AOAC method (12). Nitrogen was measured according to DUMAS (13), and calculation of protein was performed using N x 6.25. Fat was extracted by Bligh & Dyer (14) after hydrochloric acid hydrolysis. Sugars such as glucose, fructose, sucrose and fructans were analyzed as described by Larsson & Bengtsson (15), and starch and NSP were measured as described by Bach Knudsen (16). Klason lignin was analyzed as the sulphuric acid-insoluble residue according to Theander and Åman (17).

**Extraction of bound phenolic acids in breads.** To 0.1 g finely milled bread was added 5 mL of 0.08 M Na phosphate buffer pH 6 and shaken for 30 sec. Afterwards 5 mL of 4 M NaOH was added and the solution was shaken again for 30 sec and placed in the tailored mixer for approximately 20 hours at room temperature. After incubation the solution was adjusted to pH 2 with HCl and placed in the freezer overnight and freeze-dried afterwards. The freeze-dried samples were extracted using Accelerated Solvent Extraction.

**Accelerated Solvent Extraction (ASE).** Free and bound phenolic acids were extracted using ASE. The samples were extracted on an ASE 350 system from Thermo Scientific Dionex (Hvidovre, Denmark) as described previously by Petersen et al., (18). Briefly, after inserting a cellulose filter, 5 grams of glowed chemically inert Ottawa sand (particle size 20–30 mesh, Fisher Chemicals) was added to the 33-mL extraction cells. Approximately 0.1 g of pulverized flour or fine milled bread were weighed, sample was transferred to the extraction cell followed by 5 grams of glowed chemically inert Ottawa sand. A filter was placed on top of the sample and the extraction cell was filled with glowed glass balls. The cycles for the ASE extraction was as following: temperature 80 ºC, preheat for 5 min, heat for 5 min, static-extraction for 3 min, flush volume was 80% of cell volume, purge with N2 for 60 s, pressure 1500 psi, totally 4 cycles. Phenolic acids were eluted with 80% methanol in water containing 1% acetic acid (v/v). The extracts were collected in vials and stored at -20 ºC until analysis. All extractions were performed in duplicate. The extracted samples were diluted 1:1 with water and subjected to LC-MS/MS analyses.
Recovery of phenolic acids after ASE. The recovery of nine phenolic acids was measured in the breads spiked with standards with the concentration of 2 µg/g. The recovery experiments were repeated six times and gave mean values for syringic acid 67.7 % ± 0.49, salicylic acid 109.67 % ± 0.13, vanillic acid 94.5 % ± 0.30, sinapic acid 58.9 % ± 0.31, protocatechuic acid 54.3 % ± 0.43, p-coumaric acid 76.9 % ± 0.31, ferulic acid 61.8 % ± 0.59, caffeic acid 72.8 % ± 0.10, p-hydroxybenzoic acid 103.9 % ± 0.28.

Deconjugation of phenolic acid in plasma. Hydrolysis of plasma samples were performed with an enzyme mix containing β-glucuronidase with β-glucuronidase activity of ≥ 140 U/mg and sulfatase type H-1 with sulfatase activity of ≥ 10 U/mg. Enzymes were dissolved in 0.1 M NaOAc buffer with pH 5. Plasma (0.5 mL) was incubated at 37 °C for 2 h with 2 mL of enzyme mix containing 1.3 U/mL sulfatase and 0.13 U/mL β-glucuronidase.

Solid Phase Extraction (SPE). SPE of phenolic acids was performed on OASIS HBL column from Waters (Milford, MA, USA) using an extraction chamber equipped with a pump. For determination of free phenolic acids 0.5 mL of plasma was added 4 mL of 0.2 % of formic acids in water. To determine the concentration of conjugated phenolic acids hydrolyzed plasma samples were extracted on OASIS HBL columns without addition of 0.2 % of formic acids in water. Before the extraction the OASIS HBL columns were equilibrated with 2 mL methanol and 2 mL of water. After addition of the sample, the column was washed with 5 % methanol and the sucked to dryness. The elution of phenolic acids was performed twice with 1 mL of 80 % methanol containing 1 % acetic acid. The elution solution was added to the column and the valve was closed. After 2.5 min the valve was opened and the eluent was collected. The procedure was repeated one more time. The extracted samples were diluted 1:1 with water and subjected to LC-MS/MS analyses.

Recovery of phenolic acids in plasma. Recovery of phenolic acids were determined by spiking 0.5 mL blank plasma (plasma of pig after consumption of refined wheat) with 400 ng/mL standard stock solution to obtain the concentration of 20 ng/mL and then subjecting the samples to the described SPE procedure. The recovery experiments were repeated six times and gave mean values for syringic acid 69.9 % ± 6.2, salicylic acid 82.5 % ± 8.1, vanillic acid 91.7 % ± 5.1, sinapic acid 58.9 % ± 12.6,
protocatechuic acid 56.4 % ± 2.3, \( p \)-coumaric acid 94.4 % ± 10.6, ferulic acid 80.5 % ± 2.0, caffeic acid 81.2 % ± 10.5, \( p \)-hydroxybenzoic acid 84.9 % ± 13.1. The influence of hydrolyses on the recovery of phenolic acids was also investigated by spiking the blank plasma samples prior to enzymatic hydrolysis and subjecting the samples to SPE. The values for recovery of phenolic acids after the hydrolysis were similar to the values without the enzymatic hydrolysis.

**Calibration curves.** Calibration curves were prepared from standards with concentration ranging from 0.195-100 ng/mL. The calibration curves were linear with the mean \( r^2 \) of 0.998 for nine phenolic acid standards.

**LC MS/MS analysis.** Measurements were performed on LC-MS system from Agilent Technologies (UK, Workinham). Chromatographic separation was performed on HPLC Agilent 1200 equipped with Polar-RP column (Polar-RP 80 Å 250 x 2.0 mm) from Phenomenex (Torrance, CA, USA) and with column oven set to 30 °C. The flow of the system was 0.200 µL/min. Solvent A consisted of 7 % acetonitril containing 20 mM acetic acid and solvent B consisted of 78 % acetonitril containing 20 mM acetic acid. The gradient started at 16 % of solvent B and continued to 30 % of solvent B during 17 minutes, and then it continued from 30 % to 50 % of solvent B during 8 minutes and from 50 % to 70 % of solvent B during 2 minutes. It was kept isocratically for 3 minutes at 70 % of solvent B. The column was equilibrated for 5 minutes in the beginning of each injection and 20 µL of sample was injected each time. HPLC system was connected to 3200 Linear Ion Trap Quadrupole mass spectrometer (Agilent Technologies, UK, Workinham). The eluent was introduced into the mass spectrometer by TurbolonSpray probe operating at 475 °C, with ion spray voltage set in negative mode to -4500 V. Both the nebulizer gas pressure (GS1) and turbo heater gas (GS2) were set to 60 psi. The curtain gas flow was set to 12 L/min. The MS data were collected in MRM scan mode with compound-dependent parameters listed in **Table 4.**

**Calculations and statistics**

The net absorption of phenolic acids into the portal vein was calculated from the porto-arterial (AV) differences and the portal flow measurements according to Rérat et al (19) using the following equation:
\[ q = (C_p - C_a) \cdot F(dt) \]

where \( q \) is the amount of phenolic acids absorbed within the time period \( dt \), \( C_p \) is the concentration (nM) of phenolic acids in the portal vein, \( C_a \) is the concentration (nM) of phenolic acids in the mesenteric artery, \( F \) is blood flow (L/min) in the portal vein, and \( Q \) is the amount absorbed (nmol/h) from \( t_0 \) to \( t_n \).

Statistical analyses were performed in SAS (version 9.2; SAS Institute Inc., Cary, NC, USA). The effects of diet and time on the concentration of phenolic acids in plasma were examined by PROC MIXED repeated measurements (20).

\[
Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (U)_{ik} + \epsilon_{ijkl}
\]

Where \( Y_{ijkl} \) is the dependent variable, \( \alpha_i \) denotes the effect of diet \((i = WGW, WAF)\), \( \beta_j \) is the effect of time \((j = Mon, Tue, Wed, Thu)\), \( \gamma_k \) is the effect of week \((k = 1, 2)\), \( (\alpha\beta)_{ij} \) is the interaction between diet and time, \( (U)_{ik} \) term accounted for repeated measurements being performed on the same pig within week, whereas \( \epsilon_{ijkl} \) describes the random error. The level of significance was set at \( P < 0.05 \). The concentration results were reported as the least squares means (LSMeans) with standard errors of the means (SEM).

**Results and Discussion**

The concentration of total phenolic acids varied substantially between the three diets, from 57 mg/kg DM in RWF bread to 550 mg/kg DM in the WAF bread and the WGW bread being in-between with 327 mg/kg DM, Table 2. It was expected that WGW and WAF breads would have a higher content of phenolic acids than RWF bread since the bran and aleurone layer account for the highest concentration of phenolic acids in the grain (1). The higher concentration of phenolic acids in WAF than WGW is a
reflection of a higher concentration of phenolic acids as a proportion of the DF in the aleurone cells relative to the whole grain. It was also clear that the main phenolic acids detected in the breads were ferulic acid and sinapic acid and that these phenolics were mainly present as bound phenolic acids. Similar results were achieved in other studies with wheat, where bound ferulic acid was the main phenolic acid found in wheat bran and bread enriched with aleurone (21) (8).

Absorption

The bioavailability of phenolic acid in the body depends to a large extent on the bioaccessibility of these compounds in the food matrix with free phenolic acids more easily available for absorption than bound phenolic acids (10). In the current study, the proportion of free phenolic acids account for only approximately 6% in the WAF and WGW diets, whereas it represented 19% of total phenolics in the RWF diet, Table 2. The majority of phenolic acids, therefore, have to be released by microbial esterases in the caecum and colon along with the fermentation of carbohydrates. Theil et al., (22) reported that the net absorption of short-chain fatty acids, the products deriving from the anaerobic fermentation of carbohydrates in the large intestine, was 33 mmol/h and 39 mmol/h for WGW and WAF diets respectively. For comparison, the absorption of ferulic acid varied from 240 nmol/h on Monday after consumption of the RWF diet to 297 nmol/h and 458 nmol/h after consumption of WGW and WAF diets respectively. These data correspond to a bioavailability of only 0.4-2.9% of ingested phenolic acids. Similar low bioavailability have been reported in studies with humans and rats (5) (23) (24).

In spite of an active fermentation in the large intestine at the time of sampling we were not able to identify a significant AV difference with any of the diets (P > 0.05) most likely because the load of phenolic acids was not sufficient. For instance, the daily supply of DF was approximately 200 g/d whereas phenolic acids were in the range of 37-500 mg/d, Table 3, which may not be sufficient to create a significant concentration difference between the artery and the vein.

Cinnamic acid derivatives

The absorption profiles of the cinnamic acid derivatives, ferulic acid and caffeic acid, differed depending on the diet consumed. When consuming the WAF diet the absorption profile over time
reflected the continuous increase until the concentration became nearly constant after 72 h (Thursday) Figure 2a and b, whereas the concentration was already constant after 24 h (Tuesday) when consuming the WGW diet. This difference in the shape of the curves can be explained by the higher digestibility of the DF in WAF compared to WGW diet, which makes phenolic acids more accessible (25) (26). However, neither a time nor a diet effect was identified to be significant for ferulic acid, Table 5. Opposite to ferulic acid, diet and diet x time interaction affected plasma concentration of caffeic acid in both mesenteric artery and portal vein, Table 5. Moreover, in contrast to ferulic acid, the concentration of caffeic acid was negligible in the diet, Table 2, but in plasma the concentration of caffeic acid reached a maximum of 200 nM; more than the double of ferulic acid of 82 nM. Increase in the concentration from Monday to Thursday was observed for both caffeic acid and ferulic acid, but since no caffeic acid was detected in the breads, we speculated of a metabolic link between these two compounds. Moreover if these phenolic acids are metabolically related, the higher amount of ferulic acid in the WAF bread than WGW bread can explain the dietary concentration difference of caffeic acid found in plasma. It is known that the colon is the main site for the metabolic conversion of phenolic acids; e.g. ferulic acid can undergo reduction, demethylation and dehydroxylation to form a majority of related phenolic acid metabolites (27) (11). Therefore, a proportion of ferulic acid may be metabolized to caffeic acid.

Ferulic acid was only detected in the plasma as conjugates, whereas the majority of caffeic acid was present as non-conjugated. That is in agreement with in vivo and in situ studies, which reported that the most abundant form of ferulic acid in plasma and bile is glucurono- and sulfoconjugated (23) (5). It has also been reported that conjugation of phenolic compounds can occur in both enterocytes and hepatocytes (9) (28). Conjugation increases the hydrophilicity of these compounds, which can facilitate their biliary and urinary excretion; a feature of metabolic detoxification known for many phenolic compounds (9). The low concentration of conjugated ferulic acid (27 to 82 nM) indicates that the enterohepatic circulation did not allow the plasma concentration of this compound to remain high. It has also been reported that the half-life of caffeic acid in the plasma is longer than that of ferulic acid (29),
which is in agreement with our result indicating that free caffeic acid is more stable than free ferulic acid in the plasma of pigs.

Another cinnamic acid derivative present at high level in the diets was sinapic acid, Table 2. However, this phenolic acid was not detected in the plasma indicating total metabolic conversion to other phenolic acids and/or rapid elimination to the urine. We cannot completely exclude that this compound could be present in the plasma at concentrations below the detection limit of our method (0.195 ng/mL). The concentration of p-coumaric acid in the diets was low compared to ferulic acid and sinapic acid, but the concentration in plasma of this metabolite varied largely among the pigs; from 0 nM to approximately 200 nM. The recovery of p-coumaric acid in the extraction procedure was high, 94.4 %, indicating that the main reason for large variation in the concentration of this phenolic acid is more likely related to the diversity of the micro flora and/or hepatic conversion rather than the analytical procedure.

**Benzoic acid derivatives**

The concentration of salicylic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid and protocatechuic acid in the WGW, WAF and RWF breads was all low or negligible compared to ferulic acid and sinapic acid, Table 2. In the plasma, the concentration of salicylic acid, vanillic acid and protocatechuic acid after consumption of WAF and WGW diet was similarly low, ranging between 6 – 28 nM, Figure 3a, c and d. However, time and diet affected plasma concentration of salicylic acid, Table 6. In general, vanillic acid and protocatechuic acid were not affected either by diet or time. The low concentration of the free and conjugated salicylic acid, vanillic acid and protocatechuic acid and the absence of syringic acid in the plasma can be related to their low abundance in the diets and/or to the rapid metabolism of these phenolics to the other phenolic acids and elimination to the bile and urine.

The capacity to catalyze demethylation and dehydroxylation reactions of phenolic acid such as vanillic acid and protocatechuic acid is restricted to the colonic microbiota.

In contrast to the other benzoic acid derivatives the plasma concentration of p-hydroxybenzoic acid was high, and much higher than the concentration of any other phenolic acid measured in this study;
fold greater than the concentration of caffeic acid and ferulic acid and 100 fold greater than the concentration of salicylic acid, vanillic acid and protocatechuic acid. Since the diets were not the source of \(p\)-hydroxybenzoic acid, Table 2, the presence of this compound in plasma may be only due to the metabolism of other phenolic acids. For instance hydroxycinnamic acids derivatives such as ferulic acid, \(p\)-coumaric acid and caffeic acid can undergo \(\beta\)-oxidation in the colon or in the liver and be converted to hydroxybenzoic acid derivatives (9). Although \(\beta\)-oxidation may take place in the colon, the main site for benzoic acid, 3-hydroxybenzoic acid and \(p\)-hydroxybenzoic acid formation, is the liver (30). Thus, \(p\)-hydroxybenzoic acid is a phenolic acid intermediate formed from other phenolic acid, and with no specific link to the experimental diets, Table 6.

**Health effects**

The metabolic fate of plant phenolic acids in the body is crucial for understanding their biological function *in vivo* such as antioxidative activity against LDL oxidation. Their bioactivity depends on their bioavailability and it is therefore also important to consider the concentration of the phenolic acids in the plasma. *In vitro* it has been shown that hydroxycinnamic acids, such as ferulic acid and caffeic acid possess antioxidative activity against LDL oxidation at micro molar concentration (1-100 \(\mu\)M) (5). The antioxidative activity decreased in the order: caffeic acid > sinapic acid > ferulic acid > \(p\)-coumaric acid (7). In the present study, however, the maximum concentrations of caffeic acid and ferulic acid reached 200 nM and 87 nM respectively, and no sinapic acid was detected. Thus, the concentration of caffeic acid and ferulic acid was considerably lower than the concentration required to induce the response *in vitro*. Too low concentration of phenolic acids in plasma was also one of the explanations for the lack of effect of rye bran on LDL oxidation, in the study of Harder et al., (6). Conjugation of phenolic acids is another factor to consider. Ohta et al., (31) reported that conjugated ferulic acid possessed higher antioxidative activity against LDL oxidation than non-conjugated ferulic acid *in vitro*. Of the measured phenolic acids, \(p\)-hydroxybenzoic acid was the only phenolic acid with plasma concentration high enough to potentially affect the LDL oxidation. However, it was reported that hydroxybenzoic acid derivatives, such as \(p\)-hydroxybenzoic acid have lower antioxidative activity than hydroxycinnamic
Thus, the levels of plant phenolic acids in plasma of pigs measured in this study was much lower than the concentration needed to induce the inhibition, when measured *in vitro*. One may therefore suggest that the protective effect of whole grain cereals against cardiovascular diseases may not only come from the action of plant phenolic acids, but as a result of a synergetic action of different phytochemicals.

In summary, the concentration of the nine phenolic acids of wheat in the plasma of fasted pigs after overnight fermentation was measured to be low. In spite of an active fermentation in the large intestine at the time of sampling we were not able to identify any significant AV difference with any of the diets probably because the load of phenolic acids was not sufficient to create a concentration difference between the artery and the vein. The plasma concentration of phenolic acids therefore represented the net balance between their transformation in the colon and elimination to the urine and feces. However, the concentration of ferulic acid in the plasma increased when feeding the diets with higher content of ferulic acid. More interestingly the concentration of caffeic acid was higher than that of ferulic acid even though the concentration of caffeic acid in the diets was negligible. That indicated that caffeic acid was metabolized endogenously and that the precursor to caffeic acid was ferulic acid. It was also clear from the results that free caffeic acid was more stable than free ferulic acid, since only conjugated ferulic acid was detected in the plasma. The concentration of salicylic acid, vanillic acid and protocatechuic acid in the plasma was low with limited time and diet effects, the exception being *p*-hydroxybenzoic acid the only phenolic acid with plasma concentration high enough to potentially affect LDL oxidation. In conclusion, we found big differences in the plasma concentrations and the absorption profiles between the cinnamic and benzoic acid derivatives, which most likely were related to extensive inter-conversion in the colon.
Acknowledgment

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**Figure captions**

**Figure 1.** Chemical structures of cinnamic acid derivatives, a: ferulic acid, b: caffeic acid, c: sinapic acid, d: *p*-coumaric acid and benzoic acid derivatives, e: salicylic acid, f: vanillic acid, g: syringic acid, h: protocatechuic acid, i: *p*-hydroxybenzoic acid.

**Figure 2.** Concentration of cinnamic acid derivatives, ferulic acid a. caffeic acid b. in the plasma of pigs after overnight fermentation, taken as a fasting value Monday, Tuesday, Wednesday and Thursday. Monday is represented by the wash-out diet (RWF). The total acid concentration (solid line) and the free acid concentration (dash line). Wheat aleurone-rich flour (WAF) diet is represented with (blue) and whole-wheat grain (WGW) diet is represented with (red). Mesenteric artery (MA) is represented with (light red) and portal vein (PV) with (dark blue). Values are least square means with standard error (SEM), n=6. Standard error is illustrated Monday using error bars. In the case of ferulic acid, a standard error for PV (WGW) is given on Tuesday due to missing values.

**Figure 3.** Concentration of benzoic acid derivatives, salicylic acid a. *p*-hydroxybenzoic acid b. vanillic acid c. and protocatechuic acid d. in the plasma of pigs after overnight fermentation, taken as a fasting value Monday, Tuesday, Wednesday and Thursday. Monday is represented by the wash-out diet (RWF). The total acid concentration (solid line) and the free acid concentration (dash line). Wheat aleurone-rich flour (WAF) diet is represented with (blue) and whole-wheat grain (WGW) diet is represented with (red). Mesenteric artery (MA) is represented with (light red) and portal vein (PV) with (dark blue). Values are least square means with standard error (SEM), n=6. Standard error is illustrated Monday using error bars.
### Table 1. Ingredients list of experimental diets

<table>
<thead>
<tr>
<th>Ingredients (g/kg, as-fed basis)</th>
<th>RWF</th>
<th>WGW</th>
<th>WAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat whole grain</td>
<td></td>
<td>813</td>
<td></td>
</tr>
<tr>
<td>Standard wheat flour</td>
<td>711</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat aleurone 2&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td>214</td>
<td></td>
</tr>
<tr>
<td>Wheat starch&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>516</td>
<td></td>
</tr>
<tr>
<td>Wheat gluten&lt;sup&gt;2&lt;/sup&gt;</td>
<td>59</td>
<td>36</td>
<td>116</td>
</tr>
<tr>
<td>Vitacel R200 (99.5% cellulose)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapeseed oil</td>
<td>86</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td>Sugar</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Baker's yeast</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Vitamin-mineral mixture&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>1</sup> ASP02, Bühler AG, Uzweil, Switzerland.

<sup>2</sup> LCH A/S, Peter Bangs Vej 33, DK-2000 Frederiksberg

<sup>3</sup> J. Rettenmaier and Söhne GmbH, Rosenberg, Germany.

<sup>4</sup> Supplying per kg diet: retinol 660 µg, cholecalciferol 12.5 µg, α-tocopherol 30 mg, menadione 11 mg, thiamin 1 mg, riboflavin 2 mg, d-pantothenicacid 5.5 mg, niacin 11 mg (available), biotin 27.5 µg, cyanocobalamin 11 µg, pyridoxine 1.65 mg, Fe 25 mg, Cu 10 mg, Zn 40 mg, Mn 13.9 mg, Co 0.15 mg, I 0.01 mg, Se 0.15 mg and maize Ca<sub>2</sub>(PO<sub>4</sub>)<sub>3</sub>, K<sub>2</sub>PO<sub>3</sub>, NaCl, CaCO<sub>3</sub> as a carrier (SolivitMikro 106, Løvens Kemiske Fabrik, Vejen, Denmark).
Table 2. Chemical composition and phenolic acid content of the experimental diets

<table>
<thead>
<tr>
<th></th>
<th>RWF</th>
<th>WGW</th>
<th>WAF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical composition (g/kg DM)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM (g/kg as is)</td>
<td>639</td>
<td>654</td>
<td>704</td>
</tr>
<tr>
<td>Ash</td>
<td>37</td>
<td>43</td>
<td>41</td>
</tr>
<tr>
<td>Protein (Nx6.25)</td>
<td>168</td>
<td>173</td>
<td>177</td>
</tr>
<tr>
<td>Fat</td>
<td>37</td>
<td>43</td>
<td>58</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>641</td>
<td>630</td>
<td>589</td>
</tr>
<tr>
<td>Sugars</td>
<td>11</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>Starch</td>
<td>513</td>
<td>506</td>
<td>470</td>
</tr>
<tr>
<td>Total NSP(^1)</td>
<td>117</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td>Cellulose</td>
<td>66</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Arabinoxylan</td>
<td>26</td>
<td>61</td>
<td>62</td>
</tr>
<tr>
<td>B-glucan</td>
<td>1</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Klason lignin</td>
<td>10</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Total dietary fiber</td>
<td>125</td>
<td>124</td>
<td>125</td>
</tr>
<tr>
<td><strong>Phenolic acid (mg/kg DM)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total phenolic acids</td>
<td>57 (11)(^2)</td>
<td>327 (21)</td>
<td>550 (33)</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>28 (2)</td>
<td>204 (8)</td>
<td>353 (20)</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>21 (1)</td>
<td>97 (1)</td>
<td>164 (3)</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>1 (1)</td>
<td>6 (b.d.)</td>
<td>12 (1)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>b.d.(^3)</td>
<td>b.d.</td>
<td>b.d.</td>
</tr>
<tr>
<td>p-hydroxybenzoic acid</td>
<td>4 (4)</td>
<td>5 (5)</td>
<td>5 (2)</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>2 (2)</td>
<td>11 (5)</td>
<td>11 (4)</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>b.d.</td>
<td>2 (1)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>b.d.</td>
<td>1 (b.d.)</td>
<td>1 (b.d.)</td>
</tr>
</tbody>
</table>

\(^1\)Non-starch polysaccharides (NSP)  
\(^2\)Values in brackets are free phenolic acids  
\(^3\)Below detection limit (b.d.)
Table 3. Intake of feed and phenolic acids when fed the refined wheat flour (RWF), whole grain wheat (WGW) or wheat aleurone (WAF) diets.

<table>
<thead>
<tr>
<th></th>
<th>RWF</th>
<th>WGW</th>
<th>WAF</th>
</tr>
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<tbody>
<tr>
<td><strong>Intake of feed (g/d)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Feed</td>
<td>2333</td>
<td>2550</td>
<td>2185</td>
</tr>
<tr>
<td>DM</td>
<td>1543</td>
<td>1669</td>
<td>1539</td>
</tr>
<tr>
<td>Dietary fiber</td>
<td>193</td>
<td>207</td>
<td>211</td>
</tr>
<tr>
<td><strong>Intake of phenolic acids (mg/d)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total phenolic acids</td>
<td>76</td>
<td>501</td>
<td>779</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>37</td>
<td>312</td>
<td>500</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>28</td>
<td>149</td>
<td>233</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>1</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p-hydroxybenzoic acid</td>
<td>6</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>0</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4. Compound-dependent LC MS/MS parameters, Declustering potential (DP), Entrance potential (EP), Cell entrance potential (CEP), Collision energy (CE) and Cell exit potential (CEP).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Q1 mass</th>
<th>Q3 mass</th>
<th>DP (V)</th>
<th>EP (V)</th>
<th>CEP (V)</th>
<th>CE (V)</th>
<th>CEP (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulic acid</td>
<td>193.0</td>
<td>149.0</td>
<td>-22</td>
<td>-2</td>
<td>-18.80</td>
<td>-16</td>
<td>-1.0</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>223.1</td>
<td>164.0</td>
<td>-24</td>
<td>-3</td>
<td>-19.94</td>
<td>-22</td>
<td>-1.0</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>163.2</td>
<td>119.2</td>
<td>-22</td>
<td>-5</td>
<td>-17.69</td>
<td>-20</td>
<td>-2.0</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>179.0</td>
<td>135.0</td>
<td>-22</td>
<td>-4</td>
<td>-18.28</td>
<td>-22</td>
<td>-3.0</td>
</tr>
<tr>
<td>p-hydroxybenzoic acid</td>
<td>137.2</td>
<td>92.8</td>
<td>-24</td>
<td>-4</td>
<td>-16.73</td>
<td>-23</td>
<td>-1.5</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>153.1</td>
<td>109.0</td>
<td>-28</td>
<td>-10</td>
<td>-17.32</td>
<td>-22</td>
<td>-2.0</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>167.2</td>
<td>152.0</td>
<td>-24</td>
<td>-2</td>
<td>-17.84</td>
<td>-20</td>
<td>-3.0</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>197.2</td>
<td>121.2</td>
<td>-22</td>
<td>-2</td>
<td>-18.95</td>
<td>-22</td>
<td>-1.0</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>137.2</td>
<td>92.8</td>
<td>-24</td>
<td>-4</td>
<td>-16.73</td>
<td>-23</td>
<td>-1.5</td>
</tr>
</tbody>
</table>
Table 5. *P* values of diet, time and diet and time interactions for cinnamic acids derivatives for portal vein (PV) and mesenteric artery (MA). *P* values were calculated using PROC MIXED repeated measurements.

<table>
<thead>
<tr>
<th></th>
<th>Ferulic acid</th>
<th></th>
<th></th>
<th>Caffeic acid</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P</em> diet</td>
<td><em>P</em> time</td>
<td><em>P</em> diet x time</td>
<td><em>P</em> diet</td>
<td><em>P</em> time</td>
<td><em>P</em> diet x time</td>
<td></td>
</tr>
<tr>
<td>PV</td>
<td>Total</td>
<td>0.35</td>
<td>0.069</td>
<td>0.32</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>Free</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>MA</td>
<td>Total</td>
<td>0.38</td>
<td>0.086</td>
<td>0.81</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.008</td>
</tr>
<tr>
<td>Free</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Table 6. *P* values of diet, time and diet and time interactions for benzoic acids derivatives for portal vein (PV) and mesenteric artery (MA). *P* values were calculated using PROC MIXED repeated measurements.

<table>
<thead>
<tr>
<th></th>
<th>Salicylic acid</th>
<th>p-hydroxybenzoic acid</th>
<th>Vanillic acid</th>
<th>Protocatechuic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P</em> diet</td>
<td><em>P</em> time</td>
<td><em>P</em> diet x time</td>
<td><em>P</em> diet</td>
</tr>
<tr>
<td><strong>PV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.001</td>
<td>0.011</td>
<td>0.076</td>
<td>0.44</td>
</tr>
<tr>
<td>Free</td>
<td>0.021</td>
<td>0.017</td>
<td>0.15</td>
<td>0.80</td>
</tr>
<tr>
<td><strong>MA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.0001</td>
<td>0.001</td>
<td>0.47</td>
<td>0.15</td>
</tr>
<tr>
<td>Free</td>
<td>0.010</td>
<td>0.017</td>
<td>0.39</td>
<td>0.38</td>
</tr>
</tbody>
</table>


Figure 1. Chemical structures of cinnamic acid derivatives, a: ferulic acid, b: caffeic acid, c: sinapic acid, d: p-coumaric acid and benzoic acid derivatives, e: salicylic acid, f: vanillic acid, g: syringic acid, h: protocatechuic acid, i: p-hydroxybenzoic acid.
Figure 2. Concentration of cinnamic acid derivatives, ferulic acid a. caffeic acid b. in the plasma of pigs after overnight fermentation, taken as a fasting value Monday, Tuesday, Wednesday and Thursday. Monday is represented by the wash-out diet (RWF). The total acid concentration (solid line) and the free acid concentration (dash line). Wheat aleurone-rich flour (WAF) diet is represented with (blue) and whole-wheat grain (WGW) diet is represented with (red). Mesenteric artery (MA) is represented with (light red) and portal vein (PV) with (dark blue). Values are least square means with standard error (SEM), n=6. Standard error is illustrated Monday using error bars. In the case of ferulic acid, a standard error for PV (WGW) is given on Tuesday due to missing values.
Figure 3. Concentration of benzoic acid derivatives, salicylic acid a. p-hydroxybenzoic acid b. vanillic acid c. and protocatechuic acid d. in the plasma of pigs after overnight fermentation, taken as a fasting value Monday, Tuesday, Wednesday and Thursday. Monday is represented by the wash-out diet (RWF). The total acid concentration (solid line) and the free acid concentration (dash line). Wheat aleurone-rich flour (WAF) diet is represented with (blue) and whole-wheat grain (WGW) diet is represented with (red). Mesenteric artery (MA) is represented with (light red) and portal vein (PV) with (dark blue). Values are least square means with standard error (SEM), n=6. Standard error is illustrated Monday using error bars.
Oxylipins discriminate between whole grain wheat and wheat aleurone intake: a metabolomics study on pig plasma

Natalja P. Nørskov · Mette Skou Hedemann · Peter K. Theil · Knud Erik Bach Knudsen

Abstract A pig model was used to investigate the difference in metabolic response of plasma between whole grain wheat and wheat aleurone. Six pigs were fed in a cross-over design iso dietary fiber (DF) breads prepared from whole grain wheat and wheat aleurone and with a wash-out diet based on bread produced from refined wheat flour made iso-DF by adding Vitacel. The pigs were fitted with catheters in the mesenteric artery and the portal vein, which allow studying the enrichment of nutrient in plasma after passing the gastrointestinal tract. LC–MS measurements showed the presence of oxygenated fatty acids (oxylipins) in the plasma of pigs and with discrimination between whole grain wheat versus wheat aleurone and refined flour. The oxylipin-marker of this difference was identified as a mixture of 13-hydroxy-9,11-octadecadienoic and 9-hydroxy-10,12-octadecadienoic acid (13-HODE and 9-HODE). Similar oxylipins were also found in the flour and the bread consumed by pigs. Since the germ is part of the whole grain flour, the germ is most likely responsible for the elevated level of oxylipins in plasma after whole grain wheat consumption. This finding may also point towards bioactive compounds, which can be used as novel lipid candidate biomarkers of whole grain intake versus aleurone. Principal component analysis discriminated between venous and arterial plasma samples. We identified glycine conjugated and unconjugated bile acids to be responsible for this discrimination. Moreover we discovered the existence of unsaturated bile acid in the plasma of pigs.

Keywords Dietary fiber · Metabolomics · Oxylipins · Polyunsaturated fatty acids · 13-HODE and 9-HODE · Unsaturated bile acids

Abbreviations DF Dietary fiber · PUFA Polyunsaturated fatty acids · OSC Orthogonal signal correction · PCA Principal component analysis · PC Principal component · PLS–DA Partial least square–discriminant analyses · LC–MS Liquid chromatography–mass spectrometry · LC–MS/MS Liquid chromatography–tandem mass spectrometry

1 Introduction

Several epidemiological studies have indicated that consumption of whole grain cereals is protective against many chronic diseases, such as cardiovascular diseases, type 2 diabetes, and some forms of cancer (Aune et al. 2011; de Munter et al. 2007; Fardet 2010; Jensen et al. 2004; Slavin 2004). Whole grain cereals are a rich source of dietary fiber (DF) and bioactive compounds but bioactive compounds are unevenly distributed within the grain with bran (aleurone and pericarp/testa) and germ containing the highest levels of bioactive compounds (Brouns et al. 2012; Fardet 2010; Hemery et al. 2007). Products deriving from refined flour are therefore primarily an energy source depleted for most DF and bioactive components. The renewed interest in the consumption of whole grain cereals has inspired the cereal processing industry to investigate the possibilities of producing new cereals.
fractions rich in DF and bioactive components, e.g. fractions rich in aleurone from wheat (Fardet 2010; Hemery et al. 2007). The aleurone layer is a part of the bran and accounts for the highest content of DF and antioxidants (Hemery et al. 2007), whereas the germ has the highest content of lipids and enzymes (Belitz et al. 2004). The aleurone is a single cell layer at the inner side of the bran and it contains most of the minerals, vitamins, phenolic antioxidants, and lignans of the wheat grain (Brouns et al. 2012). The aleurone may therefore exert different nutritional and physiological effects relative to whole grain wheat.

The bioactive compounds in the aleurone have numerous physiological functions, and may therefore exert different nutritional and physiological effects (Price et al. 2010, 2012). To study these aspects, an approach which provides an overview of the global metabolite profile of complex biological samples can potentially be used (Manach et al. 2009; McGhie and Rowan 2012). Different metabolomics studies on plasma and urine have shown changes in metabolic responses after consumption of whole grain versus refined products (Bertram et al. 2006; Fardet et al. 2007, 2008). However, which compounds responsible for the beneficial health effects and their mechanisms of action is still largely unexplored (Fardet et al. 2008).

In present study we used nontargeted LC–MS metabolomics to study the biological changes in plasma following dietary interventions with breads prepared from whole grain wheat, wheat aleurone and refined flour. Multivariate data analysis was used to discover the metabolic differences and to elucidate markers of wheat consumption.

2 Materials and methods

2.1 Chemicals

All solvents were of HPLC or analytical grades. Acetonitrile and formic acid were purchased from Fluka (Fluka, Sigma-Aldrich, St. Louis, MO, USA), ethyl acetate was from Merck (Darmstadt, Germany) and methanol was from Pro-labo (Leuven, Belgium). The following fatty acids standards were used: 13-hydroxy-9,11-octadecadienoic acid (13-HODE) (Sigma-Aldrich, St. Louis, MO, USA), 9-hydroxy-10,12-octadecadienoic acid (9-HODE) (Cayman Chemical Co., Ann Arbor, MI, USA), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME) (Larodan, Malmo, Sweden), oleic acid, linoleic acid, arachidonic acid, linolenic acid (all from Sigma-Aldrich, St. Louis, MO, USA). The following bile acids standards were used: cholic acid (CA), deoxycholic acid (DCA), hyodeoxycholic acid (HDCA), hyocholic acid (HCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), glycocholic acid (GCA), glycodeloxycholic acid (GDCA) (all from Sigma-Aldrich, St. Louis, MO, USA).

2.2 Breads

The breads were made of refined flour, whole grain wheat flour and wheat aleurone flour. The refined flour and whole grain wheat breads were produced at Holstebro Technical College (Holstebro, Denmark), and the wheat aleurone bread was baked in a local bakery (Konditor-Bager Ørum, Denmark). The ingredients for the refined flour bread was refined wheat flour (Triticum aestivum L. cv. Tiger), purified wheat fibre (Vitacel R200; J. Rettenmaier and Söhne GmbH, Rosenberg, Germany), rapeseed oil, sugar, salt, wheat gluten (LCH A/S, Frederiksbek, Denmark) and yeast, while in whole grain and aleurone breads, whole-grain wheat (BFEL Karlsruhe, Germany), wheat aleurone flour (Bühler AG, Uzwil, Switzerland) replaced the refined flour and purified wheat fibre on an iso-DF basis. After production, the breads were cut into pieces. After weighing, meal portions were frozen at –20 °C and thawed immediately before consumption. The breads were balanced with regard to starch, proteins, fat and DF, but they varied in DF characteristics, Table 1.

2.3 Study design and animals

The experiment was in compliance with the guidelines of the Danish Ministry of Justice concerning regulations for human care and use of animals in research (The Danish Ministry of Justice, Animal Testing ACT (Consolidation Act no. 726 of 9 September 1993 as amended by Act no. 1081 of 20 December 1995)). The pigs used in the study were from the swineherd at Aarhus University, AU-Foulum, Department of Animal Science. The pigs were fed three times daily at 09.00 (breakfast), 14.00 (lunch) and 19.00 h (dinner) with an amount of 40, 40 and 20 % of the daily supply. Six female pigs (Landrace × Yorkshire) with a bodyweight of 56.5 kg (±1.8 kg) were included in the experiment, which was designed as a repeated cross-over design. The pigs were adapted to the pen for 5 days, and then the animals were surgically fitted with a flow probe (Transonic, 20A probe, 20 mm; Transonic System, Inc., Ithaca, NY, USA) around the portal vein, a catheter in the portal vein and a catheter in the mesenteric artery as described previously by (Jorgensen et al. 2010). After the surgery, the animals were allowed a 5–7 days recovery period before entering an experimental period. In each experimental week, the pigs were fed wash-out bread of refined flour on day 1–3 (Friday–Sunday), and then the pigs were fed one of the experimental breads (whole grain or...
wheat aleurone) on days 4–7 (Monday–Thursday) using cross-over design. The pigs had access to water ad libitum, whereas no straw was supplied. Fasting blood samples (30 min before the first daily meal) were collected from the portal vein and the mesenteric artery on day 4–7 (Monday–Thursday). On day 7 (Thursday), consecutive blood samples were drawn in the fed state from the portal vein and the mesenteric artery during 0–10 h after the first daily meal (breakfast), but with a re-feeding 5 h after the breakfast (lunch). Three arterial plasma samples and three venous plasma samples were pooled to represent the mean content of metabolites in plasma for the periods: 0–150 min after breakfast (samples collected at 15, 60 and 120 min); 150–300 min after breakfast (samples collected at 180, 240 and 300 min); 300–450 min after breakfast (samples collected at 315, 360 and 420 min (pigs were re-fed at 300 min)); 450–600 min after breakfast (samples collected at 480, 540 and 600 min). Blood samples were collected in 9 mL Na-heparinised vacutainers, centrifuged and plasma was harvested and stored at −20 °C.

2.4 Chemical analyses of breads

All chemical analyses of breads were performed in duplicate on freeze-dried materials. The dry matter content was measured by drying to a constant weight for approximately 20 h at 103 °C, and ash was analyzed by AOAC method (AOAC 1990). Nitrogen was measured according to DUMAS (Hansen 1989), and calculation of protein was performed using N × 6.25. Fat was extracted by Bligh & Dyer (Bligh and Dyer 1959) after hydrochloric acid hydrolysis. Sugars such as glucose, fructose, sucrose and fructans were analysed as described by (Larsson and Bengtsson 1983), and starch and NSP were measured as described by (Knudsen 1997). Klason lignin was analyzed as the sulphuric acid-insoluble residue according to Theander and Åman (1979). Available carbohydrates were the sum of sugars and starch. For the conversion of available carbohydrates and DF into energy, the energy convention factors as reported in FAO Food and Nutrition paper 77 was used (FAO 2003).

2.5 Preparation of samples and standards for LC–MS

Plasma samples, 0.5 mL, or 0.8 g of finely milled flours and breads were mixed with 2 mL MILLI-Q water and 2.5 mL or 5 mL ethyl acetate respectively, shaken for 1 min and centrifuged (Rotixa 50 RS, Andreas Hettich GmbH & Co.KG, Tuttingen, Germany) at 45 g for 15 to 30 min at 30 °C. After centrifugation the upper layer of

| Table 1 | Ingredients list and chemical composition of experimental breads |
|---------------------------------|-----------------------------|-----------------------------|
| Ingredients (g/kg, as-fed basis) | Whole grain wheat | Wheat aleurone | Refined flour |
| Wheat whole grain | 813 | – | – |
| Standard wheat flour | – | – | 711 |
| Wheat aleurone 2 | – | 214 | – |
| Wheat starch b | – | 516 | – |
| Wheat gluten b | 36 | 116 | 59 |
| Vitacel R200 (99.5 % cellulose) c | – | – | 69 |
| Rapeseed oil | 76 | 79 | 86 |
| Sugar | 15 | 15 | 15 |
| Baker’s yeast | 15 | 15 | 15 |
| Vitamin-mineral mixture d | 4 | 4 | 4 |
| Chemical composition (g/kg DM) | | | |
| Protein (N × 6.25) | 173 (16) c | 177 (17) | 168 (16) |
| Fat | 137 (28) | 134 (29) | 128 (27) |
| Available carbohydrates | 528 (50) | 487 (48) | 526 (51) |
| Total dietary fibre (NSP + lignin) | 124 (6) | 125 (6) | 125 (6) |

a ASP02, Bühler AG, Uzwil, Switzerland
b LCH A/S, Peter Bangs Vej 33, DK-2000 Frederiksberg
c J. Rettenmaier and Sohne GmbH, Rosenberg, Germany
d Supplying per kg bread: retinol 660 μg, cholecalciferol 12.5 μg, α-tocopherol 30 mg, menadione 11 mg, thiamin 1 mg, riboflavin 2 mg, p-pantothenic acid 5.5 mg, niacin 11 mg (available), biotin 27.5 μg, cyanocobalamin 11 μg, pyridoxine 1.65 mg, Fe 25 mg, Cu 10 mg, Zn 40 mg, Mn 13.9 mg, Co 0.15 mg, I 0.01 mg, Se 0.15 mg and maize Ca₃(PO₄)₂, K₂PO₃, NaCl, CaCO₃ as a carrier (SolivitMikro 106, Løvens Kemiske Fabrik, Vejen, Denmark)

Values in bracket is relative energy contribution according to (FAO 2003)
ethyl acetate was removed by use of an ethanol-dry ice bath. The extraction procedure was repeated twice for plasma samples and once for flours and breads samples. The organic layers were taken to dryness under a flow of nitrogen gas and redissolved in 200 l of 60 % methanol. Further the samples were centrifuged (Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany) at 20,800 x g through 0.22 µm Spin-X centrifuge tube filters and pipetted to 200 µL HPLC vials to be analyzed on the LC–MS system.

All standard were dissolved in 60 % of methanol in concentration of 15 µM and pipetted to 200 µL HPLC vials and measured on the LC–MS system. Standards of linoleic acid, linolenic acid and oleic acid were also tested for auto-oxidation during the sample preparation procedure. Linoleic acid, linolenic acid and oleic acid standards were dissolved in methanol in concentration of 15 µM, and dried under a flow of nitrogen gas and redissolved in 200 µL of 60 % methanol. Their chromatograms were compared with chromatograms of the standards that did not undergo the sample preparation procedure.

2.6 LC–MS and LC–MS/MS analysis

Chromatographic separation was performed on a HPLC system UltiMate 3000 (Thermo Scientific Dionex, Sunnyvale, CA, USA)—equipped with a C18 silica coated column (3 µm 150 × 2.1 mm) from (Supelco, Sigma-Aldrich, St. Louis, MO, USA) and with column oven set to 30 °C. The temperature of the auto sampler was 10 °C. The flow of the system was 0.200 mL/min. Solvent A consisted of 0.1 % formic acid in water and solvent B consisted of 0.1 % formic acid in acetonitrile. The gradient started at 10 % and continued to 100 % of solvent B during 20 min. It was kept isocratically for 5 min at 100 % of solvent B. The column was equilibrated for 5 min in the beginning of each injection. Five microlitre of sample was injected each time. Each sample was injected twice. Subsequent blank samples (solvent mixture of 60 % methanol) were injected between the runs to ensure that there was no carry-over effect between the samples. The HPLC system was connected to a microOTOF-Q II mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). The eluent was introduced into the mass spectrometer by electrospray ionization, with capillary and end plate offset voltage set in the positive and negative mode to 4,500 and −500 V, respectively. The dry gas flow was 8 l/min at temperature of 200 °C and nebulizer pressure was set to 1.8 bar. Mass to charge ratio of quadrupole’s scan ranged from 50 to 1,000. Collision energy during MS scan was set to 10 eV. MS/MS fragmentation was used for the identification of compounds. The collision energy of MRM scan varied from 15 to 35 eV optimized according to the chemical characteristics of compound analysed. Optimization of the collision energy was performed with direct injection of the standard into the mass spectrometer. Lithium formate was used as an external standard which was introduced into the mass spectrometer in the beginning of each chromatogram with an independent syringe pump. This approach allows mass correction of each chromatogram.

2.7 Data processing

The raw data was processed prior to statistical analysis. We used mzMine (Katajamaa and Oresic 2005; Pluskal et al. 2010) for this purpose. Centroid data was first cropped removing the external standard then peaks were detected, retention time (RT) normalized, deconvoluted and deisotoped. Join aligner was used to align the chromatograms with m/z tolerance of 0.025 and RT tolerance of 10 s. Duplicated peaks were removed and missing peaks were filled in using peak finder. Normalization was performed according to the average signal. Afterwards, the average of replicated samples was taken and averaged blank samples were subtracted from the data using Matlab (software version 7.11.0.584 (R2010b), Natick, MA, USA).

2.8 Multivariate data analysis

Due to the multiparametric data, having different variation within collected samples, the data was analyzed by dividing the samples in the fasted samples, representing fasted state of the pig and the fed samples, representing fed state of the pig. Furthermore these samples were divided in samples collected at the mesenteric artery and portal vein. The samples collected on Monday before the start of experimental week, represented wash-out bread/refined flour and therefore were used as reference. The data sets were subjected to multivariate data analyses using unsupervised PCA and supervised OSC filtered PCA and PLS–DA. OSC filter was applied to the data set using Matlab in accordance with (Beckwith-Hall et al. 2002; Wold et al. 1998). It was applied to the data to remove physiological differences among the pigs and to enhance the relevant information. OSC selectively removes the variation of data X that have no correlation with Y (Gavaghan et al. 2002; Wagner et al. 2006). Multivariate modeling was performed in LatentX (Iler et al. 2008). Prior to PCA and PLS–DA the data was Pareto scaled. The OSC–PLS–DA models were validated using an independent test set that was used for prediction of the samples which were not included in the OSC–PLS–DA model. The data set was randomly divided into a training set containing 67 % of the samples (n = 4 pigs) and a test set containing 33 % of the samples (n = 2 pigs). To ensure the robustness, the models were full cross validated and the model statistics and actual versus predicted plots were analyzed. The variables were selected.
using loadings plots and principle variable selection in LatentiX. The variables which described 99.9% of the total variation in the model were selected for further identification. Metabolites causing the discrimination were tested by Welch’s two-sample t test. For correction of false-positives (FDR) q values were calculated, with significance threshold set at q < 0.25 (Storey and Tibshirani 2003).

2.9 Biomarker identification

Both positive and negative modes of LC–MS data were evaluated for water loss, dimer formation and adduct formation with formic acid, sodium and potassium. After recognition of the parent ion, measured m/z value was subjected for identification to the METLIN database (http://metlin.scripps.edu/) and Human Metabolome Database (http://www.hmdb.ca/). RT and fragmentation pattern of commercially available standards and respective spectra from the literature were used to confirm the biomarker identity (Dufour and Loonis 2005; Lee et al. 2003; Oliw et al. 2006). The common names of oxylipins were written according to http://www.lipidmaps.org/.

3 Result

3.1 Quality control and evaluation of data

The quality of the data was evaluated by several methods. The possible occurrence of carry-over was studied by frequent injections of blank samples during each batch. PCA score plot of the blank samples and the six batches is represented in Fig. 1a. Blank samples were separated from the rest of the data set in a tightly clustered group, suggesting that carry-over did not occur. Re-injection of plasma samples was also used as quality control. PCA score plot showed that replicates were closely related, Fig. 1b. The blank samples were subtracted from the data set and the average of two injections was used for modeling. Shifts in masses were corrected by use of external standard and the chromatograms were calibrated with High Precision Calibration mode with mass accuracy deviating from 0.01 to 6 ppm. Shift in RT was not higher than 10 s and was corrected by use of RT normalization and alignment in mzMine.

Plasma samples were analyzed in positive and negative modes. In positive mass spectra, the parent ions mainly appeared as adducts ions of sodium, dimers and dehydrogenated forms. Therefore the data in positive ionization mode was mainly used for confirmation of parent ions. On the contrary negative mass spectra contained distinguished [M–H] ions and provided sufficient information for multivariate data analyses.

3.2 Statistical models

To analyze the difference between arterial and venous plasma samples of pigs in fasted and fed states, an unsupervised method for pattern recognition was performed. PCA analysis of the samples were suggestive of group separation, Fig. 2a and b, where PC1 was mainly responsible for the separation between artery and vein and PC2 was responsible for the separation between physiological differences among the pigs. To enhance the relevant information OSC filter was applied and new PCA model was constructed, Fig. 2c and d. OSC–PCA model revealed the clear disparity between artery and vein for both fasted and fed states. The ellipse marked the 95 % Hotelling T² control chart, showing possible outliers. PC1 accounted for 26.3 % and PC2 for 13.5 % of explained variance for the fasted samples and for the fed samples PC1 accounted for 29.9 % and PC2 for 12 % and of the variance. The score plot illustrated that PC1 was responsible for the discrimination between the artery and the vein, whereas PC2 was characterized by deviation among the samples. The metabolites, which had the highest influence in PC1 together with t test results, are listed in Table 2. The chromatographic comparison of the plasma sample collected at mesenteric artery and portal vein revealed the difference in metabolic profile of metabolites selected by the PCA model, which is shown in Fig. 2e.
To analyze the effect of dietary intervention with whole grain wheat and wheat aleurone, a supervised method for pattern recognition was performed on the plasma samples collected during the fed state of the pigs. OSC–PLS–DA score plots of whole grain versus aleurone for samples collected at mesenteric artery and portal vein are shown in Fig. 3a and c. Both score plots illustrated separation with PC1 responsible for the clustering between whole grain and aleurone with explained variance of 79.1 % for arterial samples and 76.3 % for venous samples, and PC2 responsible for the deviation among the samples. The ellipse marked the 95 % Hotelling T² control chart, showing possible outliers. Representative LC–MS base peak chromatograms in negative electrospray ionization mode showing visible differences in the chromatographic profiles of arterial (low intensity) and venous (high intensity) plasma samples (e). The peaks with the biggest difference in intensity between artery and vein are labeled with mz and retention time. Multivariate analysis was performed in LatentX. Visualization of chromatograms was performed in DataAnalysis from Bruker.

Fig. 2 PCA score plots of plasma samples taken at fasted state (a) and fed state of the pigs (b). OSC–PCA score plots of plasma samples taken at fasted state (c) and fed state of the pigs (d). (+) Samples collected at mesenteric artery, (empty square) samples collected at portal vein. The ellipse marked the 95 % Hotelling T² control chart, showing possible outliers. Representative LC–MS base peak chromatograms in negative electrospray ionization mode.
The model statistics showed that the optimal number of PC’s was 1 for both models with correlation coefficient $R^2 \approx 0.98$ and RMSE $\approx 0.54$ indicating robustness of the models. Moreover, robustness of the model was tested using an independent test set for prediction of the samples that were not a part of OSC–PLS–DA model. The predicted PC1 assigned samples with only one misclassification for arterial samples and with three misclassifications for venous samples.

The variables were selected for further metabolite identification using principle variables selection describing 99.9 % of total variance. However, the strongest marker for whole grain consumption describing 98 % of total variance for arterial samples and 85.7 % for venous samples was metabolite with $m/z$ and RT of 295.2264/18.34 respectively. Moreover the chromatographic comparison of the plasma sample after consumption of whole grain and aleurone revealed the difference in metabolic profile of metabolites selected by the OSC–PLS–DA model, which is shown in Fig. 3e. To analyze the effect of dietary intervention with whole grain wheat and wheat aleurone versus refined flour, samples collected on Monday were used as reference. OSC–PCA model for whole grain, aleurone and refined flour is shown in Fig. 4. OSC–PCA score plot showed that there was discrimination between whole grain and refined flour, and for the slight discrimination between aleurone and refined flour, whereas refined flour and aleurone were closely related. To investigate which metabolites were responsible for the discrimination between whole grain and refined flour and for the slight discrimination between aleurone and refined flour, OSC–PLS–DA models were constructed. OSC–PLS–DA model on aleurone versus refined flour was poor indicating that no significant difference was achieved between these two sets of samples (data not shown). OSC–PLS–DA models on whole grain versus refined flour showed significant discrimination and metabolites responsible for the differences were the same metabolites responsible for the differences between whole grain and aleurone. The selected metabolites and $t$ test results of the comparisons between the diets are listed in Table 3.

### 3.3 Identification of metabolites

#### 3.3.1 Mesenteric artery and portal vein

The metabolites, which discriminated arterial from venous samples, in both fasted and fed state of the pigs, were identified as glycine conjugated dihydroxy and trihydroxy bile acids, which were enriched in portal vein comparing to mesenteric artery. Unconjugated bile acids were also

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>$m/z$</th>
<th>Mass accuracy (ppm)</th>
<th>Metabolites</th>
<th>Portal vein vs. mesenteric artery (fasted state)</th>
<th>Portal vein vs. mesenteric artery (fed state)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fold change$^b$</td>
<td>$q$ value$^c$</td>
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<tr>
<td>13.62</td>
<td>448.3056$^a$</td>
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<td>Glycochenodeoxycholic acid</td>
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<tr>
<td>12.78</td>
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<td>$&lt;0.001$</td>
</tr>
</tbody>
</table>

Metabolites are listed according to the loadings of PCA models, from metabolites with the highest influence to metabolites with the less influence. The measurements were performed in negative electrospray ionization mode.

- $^a$ Indentified using commercial standard
- $^b$ Fold change was calculated by dividing the mean of normalized intensity of each plasma metabolite measured in the portal vein by the mean intensity of the same plasma metabolite measured in the mesenteric artery
- $^c$ FDR $q$ value was calculated for correction of false-positives
- $^d$ $P$ value was calculated by a two-sample $t$ test; $* >0.05$ ** $>0.01$ *** $>0.001$
enriched in the portal vein, but to a much lesser extent if comparing to glycine conjugated bile acids.

The ions with $m/z$ and RT of 464.3007/11.02, 448.3059/11.73, 407.2790/12.78, 448.3056/13.62, 391.2837/13.78 and 391.2839/16.16 were identified as GHCA, GUDCA, HCA, GCDCA, HDCA or UDCA and CDCA respectively, Table 2, with RT and fragmentation pattern similar to the standards (data not shown). No identification for the ion

Fig. 3 OSC–PLS–DA score plots of plasma samples collected at mesenteric artery (a) and plasma samples collected at portal vein, (c) during the fed state of the pigs. (empty square) plasma samples after consumption of whole grain wheat, and (empty circle) wheat aleurone. The ellipse marked the 95 % Hotelling T$^2$ control chart, showing possible outliers. OSC–PLS–DA model was used to predict the plasma samples of the test set. Predicted PC1 of the test set for arterial samples (b) and venous samples (d). Representative LC–MS base peak chromatograms in negative electrospray ionization mode showing visible differences in the chromatographic profiles of wheat aleurone (low intensity) and whole grain wheat (high intensity) plasma samples (e). The peaks with the biggest difference in intensity between whole grain wheat and wheat aleurone are labeled with $m/z$ and retention time. Multivariate analysis was performed in LatentX. Visualization of chromatograms was performed in DataAnalysis from Bruker.
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Fig. 4 OSC–PCA score plot of plasma samples after consumption of whole grain wheat (x), wheat aleurone (+) and refined flour (empty square) collected at mesenteric artery and portal vein. Multivariate analysis was performed in LatentXI

[M–H]− 446.2899/12.17 was suggested when submitting the mass to the METLIN database (http://metlin.scripps.edu/) and the Human Metabolome Database (http://www.hmdb.ca/). However, both the ion 448 and 446 formed dimers in negative ionization mode, Fig. 5a and b, and had a particular pattern for water loss which was representative for the loss of hydroxyl group in positive ionization mode, Fig. 5c and d. Moreover, the fragmentation pattern of 446 had similarities to the fragmentation pattern of 448, suggesting the two metabolites have similar structure. The fragmentation pattern of the ions 448 and 446 was represented by the intense MS/MS fragments of m/z 386 and 384, respectively, showing the loss of 62 amu, Fig. 5e and f. The loss of 62 amu can arise from the loss of carboxylic acid and a hydroxyl group. However, the ion 446 was also characterized by the fragment m/z of 344, showing the loss of 102 amu, which was not observed for the ion 448. The loss of 102 amu can represent the loss of a C3H4NO3 moiety. Nevertheless, the ion 446 has a difference of two mass units from the ion 448. This can indicate the presence of a carbon–carbon or a carbon–oxygen double bond in the structure of the molecule. Because of the similarity in the MS and MS/MS patterns the ion 446 was assigned to be an unsaturated glycine conjugated dehydroxy bile acid. A similar feature of two mass units difference was also noticed for the unconjugated dehydroxy bile acids with m/z value of 391 and 389. The ion 389 was therefore also assigned as an unsaturated unconjugated dehydroxy bile acid. This shows the presence of unsaturated bile acids in the plasma of pigs.

An important feature of unconjugated bile acids was their stability to fragmentation in negative ionization mode. It was not possible to produce stable fragments by increasing the collision energy. Therefore these compounds were fragmented in positive mode (data not shown). However, the fragmentation pattern was similar for all the ions with m/z of 391, and therefore it was not possible to
distinguish between HDCA and UDCA, which also had the same RT.

3.3.2 Whole grain versus aleurone and refined flour

The identified metabolites responsible for the major discrimination between plasma samples after consumption of whole grain versus aleurone and refined flour were oxylipins, oxidation products of polyunsaturated fatty acids (PUFA), Table 3. Most of the oxylipins were characterized by the MS/MS daughter ion with m/z of 171 containing [M−HCO(CH2)7CO2H–H]−. The neutral loss of 100 amu was also observed for the most of the oxylipins. RT was influenced by the number of hydroxyl groups and their position, degree of saturation and the position of the double bond. It was also noticed that oxylipins and PUFA contained stereoisomers, which eluted close to each other, making chromatographically irresolvable mixtures.

OSC–PLS–DA model showed that after consumption of the whole grain wheat bread plasma was enriched with oxylipins compared to the aleurone wheat and refined flour. The strongest marker for whole grain consumption was identified as being the chromatographically irresolvable mixture of 13-HODE and 9-HODE isomers. The MS/MS pattern of 13-HODE standard was represented by the fragments with m/z of 277 and 195 and 9-HODE standard was represented by the fragments with m/z of 277 and 171. The similar fragments were identified in the samples (data not shown).

To be sure that oxylipins were a result of the dietary intervention and not a result of auto-oxidation during the sample preparation, standards of linoleic acid, linolenic acid and oleic acid underwent similar sample preparation as the plasma samples. No oxylipins were observed for these samples (data not shown). Moreover, whole grain, aleurone and refined flour, the flours and the breads were extracted and analysed. Oxylipins similar to those found in plasma as well as linoleic acid, linolenic acid and oleic acid were detected in the flour and the bread extracts. Chromatographic comparison of plasma after consumption of the whole grain bread, and the extracts of whole grain flour and whole grain bread is illustrated in Fig. 6a, b and c. From the chromatograms in Fig. 6 it was striking that many of detected oxylipins had similar masses, though eluting at different times. That indicated that there are many positional and stereo-isomers of oxylipins. The main difference in Fig. 6 between plasma after consumption of the whole grain bread, and the extracts of whole grain flour and whole grain bread was the ion with m/z value of 313.2364 and the ion with m/z value of 319.2269. The ion 313.2364 was found in the flour and bread samples. The ion 319.2269 was detected in the plasma samples, but not in the flour and the bread extracts. By comparison of the
In the current study we discovered PUFA and oxylipins in the plasma of pigs after consumption of experimental breads, as well as in the flour and bread extracts. The composition of oxylipins in plasma compared to the flour and bread extracts was quite similar. However, multivariate data analysis on plasma showed that oxylipins discriminated between whole grain wheat, wheat aleurone and refined flour breads consumed by pigs, with the highest oxylipin concentration after consumption of whole grain wheat. This suggests that oxylipins may be potential lipid biomarkers of whole grain consumption. The strongest oxylipin-marker for this discrimination was identified as the mixture of 13-HODE and 9-HODE, which are oxygenated products of linoleic acid.

Oxylipins constitute a large family of bioactive metabolites abundant in both plants and animals. Cereal grains contain PUFA such as linolenic acid, linoleic acid and oleic acid, belonging to omega-3, -6 and -9 families as well as enzymes, such as lipoxygenase (LOX) and hydroperoxidase that are involved in the oxidation of PUFA to oxylipins (Belitz et al. 2004). In plants, oxylipins serves as signal molecules in developmental processes such as pollen formation and in defense mechanisms related to pathogenesis, wounding and herbivores (Brodhun and Feussner 2011; Levandi et al. 2009). In mammals and humans, the role of oxylipins derived from linoleic acid is not clear (Shearer et al. 2010; Fischer 1997). Cell culture and animal studies have shown that the intestine efficiently absorbs oxidized linoleic acid, and oxylipins can have different metabolic roles, such as promoting epithelial cell growth (Moreno et al. 2011; Khan-Merchant et al. 2002). Moreover, LOX is also found in mammals and humans and therefore oxylipins can also be synthesized endogenously.
Fig. 5  Mass spectra of ions 448 (a, c, e) and 446 (b, d, f). MS scan in negative mode (a, b), MS scan in positive mode (c, d) and MS/MS scan in negative mode (e, f). MS$^-$ scan shows a clear signal for the ions 448 and 446 and their dimers. MS$^+$ scan shows water loss for both ions. The loss of 62 amu is similar for both ions which is depicted in MS/MS$^-$ scan. The loss of 102 amu is only representative for the ion 446. Visualization of MS and MS/MS spectra were performed in DataAnalysis from Bruker.
However, multivariate data analysis showed clear clustering between whole grain wheat versus wheat aleurone and refined wheat which suggest that the oxylipins of linoleic acid originated from the experimental breads consumed by the pigs. Analysis of the flour and breads showed the same oxylipins as detected in plasma. This is in agreement with previous studies performed by (Levandi et al. 2009). They showed the presence of oxylipins in different varieties of wheat with the highest peak for 13-HODE and 9-HODE (Levandi et al. 2009). The cause of the elevated level of oxylipins in plasma of pigs after consumption of the whole grain bread is probably because the whole grain flour contains germ rich in lipids compared to the aleurone flour and refined flour where the germ is depleted. It is known that wheat germ contains 27.6 % lipids by dry weight whereas the aleurone cells contain 9.1 % lipids by dry weight (Belitz et al. 2004). The lipid content of the breads in this study was equalized using rapeseed oil, containing linoleic acid, which could lead to formation of additional oxylipins. However the analysis of the flour extracts showed similar oxylipins found in the bread even though no rapeseed oil was added to the flour. Moreover, multivariate analysis showed clear discrimination of plasma after consumption of whole grain wheat and wheat aleurone, which indicates that oxylipins were from the breads or they were synthesized endogenously as a result of germ intake.

Fig. 6 LC–MS base peak chromatograms in negative electrospray ionization mode of plasma sample after consumption of whole grain wheat (a), whole grain wheat bread (b) and whole grain wheat flour (c). Peaks are numbered according to the detected oxylipins, peak (1) \([\text{M-H}]^- = 327.2144\), peak (2) \([\text{M-H}]^- = 329.2323\), peak (3) \([\text{M-H}]^- = 325.2184\), peak (4) \([\text{M-H}]^- = 313.2364\), peak (6) \([\text{M-H}]^- = 295.2272\), peak (7) \([\text{M-H}]^- = 293.2095\), peak (8) \([\text{M-H}]^- = 319.2269\). Visualization of chromatograms was performed in DataAnalysis from Bruker.
Oxidation of flour containing the germ can be of problem, decreasing the shelf-life of the whole grain flour. The breads in this study were baked from the fresh flour. Moreover, the breads were frozen directly after baking. This handling of the flour and bread is comparable to the handling of the flour and bread by consumers. Our results may therefore concern the aspect related to the aging of flour, the role of auto-oxidation and the level of these oxidation products found in flour and bread. It is known that LOX in plants always forms pure S-enantiomers, whereas racemic mixtures are usually formed as a result of auto-oxidation (Gosset et al. 2008). However, Lee et al. (2003) reported that MS/MS spectra for R- and S-isomer of 9-HODE and 13-HODE were identical (Lee et al. 2003). Therefore it was not possible to distinguish whether 9-HODE and 13-HODE identified in this study had R- or S-configuration. Quantification of oxylipins and identification of racemic mixtures may be the objective of future experiments, which can contribute to the understanding of shelf-life of the bread and lead to the development of methods for prevention of lipid oxidation in cereals.

4.2 Bile acids in pig metabolome

Bile acids play an important role in the absorption of lipids as they are involved in the emulsification of lipids in the gut and with a major part undergoing entero-hepatic circulation. The majority of the bile acids, not absorbed in the terminal ileum, are deconjugated and converted to secondary bile acids by the microflora in the large intestine (Danielsson and Sjovall 1975; Griffiths and Sjovall 2010; Hofmann and Hagey 2008). In this study we identified glycine conjugated and unconjugated bile acids and in agreement with the absorption route for bile acids they were enriched in portal vein. That was especially the case for conjugated bile acids, which were the most influential metabolites in the PCA model. It reflects the fact that the uptake by the liver of bile acids returning from the intestine is not 100 % efficient (Hofmann and Hagey 2008). The preference of the hepatic uptake depends on the structure of the bile acids with conjugated bile acids being more easily taken up by the liver than unconjugated bile acids that spill over into systemic circulation and return to the liver via mesenteric artery (Hofmann and Hagey 2008).

The pigs in the current study were in a negative energy balance in the fasted state, whereas in the fed state nutrients provided from the food stimulated the excretion of bile from the gallbladder (Hofmann & Hagey 2008; Theil et al. 2011). However, with regard to bile acids no difference in the metabolites clustering between mesenteric artery and portal vein was observed in the PCA models of fasted and fed states.

In contrast to other mammals, pigs produce HCA instead of CA (Alvaro et al. 1986). Alvaro et al. (1986) mainly identified glycine conjugates of HCA, CDCA and HDCA and low concentration of taurine conjugates of CDCA and HDCA. In agreement with that we identified HCA and no CA was detected in the plasma of pigs. Moreover, we identified glycine conjugated and unconjugated HCA, CDCA and HDCA or UDCA. Glycine conjugated dihydroxylated and trihydroxylated bile acids predominated the plasma samples, which is in agreement with previous studies (Alvaro et al. 1986; Oomen et al. 2004; Qiao et al. 2011). The fact that we did not measure the presence of taurine conjugated bile acids, may be due to the hydrophobicity of the extraction solvent. Taurine conjugated bile acids are more hydrophilic compared to glycine conjugates and therefore the hydrophobicity of the extraction solvent can be an issue (Alvaro et al. 1986). However, we discovered unsaturated bile acids, which to our knowledge was not detected before in plasma of pigs. The unsaturated dihydroxy bile acids had the mass of 389.2682 m/z and RT of 14.41 min for the unconjugated bile acid and the mass of 446.2899 m/z and RT of 12.17 min for the glycine conjugated bile acid.

4.3 Biological interpretation and perspectives

Recent studies have shown that oxylipins, like lipids, are absorbed to the lymph and transported in the body as part of the lipoprotein matrix (Shearer and Newman 2008, 2009). Once absorbed they are endogenously released from the lipoprotein matrix by lipase action and can potentially influence the endothelial function. 13-HODE and 9-HODE are major components of oxidized low-density lipoprotein, which can accumulate in macrophages of atherosclerotic plaques (Shearer and Newman 2008, 2009). In vivo experiments performed by Khan-Merchant et al. (2002) showed that 13-HODE promoted atherosclerosis in the presence of dietary cholesterol. They suggested that the combination of a cholesterol rich diet and the consumption of oxylipins in fried food has deteriorating health effect such as promotion of atherosclerosis (Khan-Merchant et al. 2002). To our knowledge the health effect of oxylipins is still largely unexplored with many unknown factors in the bioactivity of the single oxylipins and their synergetic effects (Shearer and Newman 2009). Elevated level of oxidized fat in food may therefore be a cause of cardiovascular diseases and may even have toxic effects, causing diarrhoea and other symptoms, as a result of food poisoning (Gotoh et al. 2006). However, it is unlikely that the level of oxylipins in cereals will reach a concentration at which they will have detrimental effect. The epidemiological investigation by Jensen et al. (2004) did not identify any negative effects of germ consumption. Rather, the presence of oxylipins and the different isomers of oxylipins in bread can potentially be a marker of off-flavor formation.
and the shelf-life of the bread. It is clear from our study that LC–MS can be a sensitive method for detection of oxylipins in biological samples and in different context.

To our knowledge the bile acid composition of pigs was not analyzed since 1986, and when coupling LC together with MS, more sensitive and detailed analyses of bile acid composition can be performed. In this study, using LC–MS, unsaturated dihydroxy bile acids were detected in the plasma samples of pigs. The biological interpretation of the discovery of unsaturated bile acids at present is uncertain, but it is known that unsaturated bile acids are involved as intermediates in the bioconversion of primary to secondary bile acids by the bacteria in the large intestine (Aries and Hill 1970; Kuksis and Child 1980). Unsaturated unconjugated trihydroxy bile acids were reported by (Kuriyama et al. 1979) when analyzing the bile of rats. Also unsaturated unconjugated monohydroxy bile acid was detected in bile and feces of newborn and fetal guinea pigs (Li et al. 1977). The unsaturated bile acids were also found in the urine of children with extrahepatic-bile duct atresia and in adults with cholestatic liver disease (Kuksis and Child 1980). However, more research needs to be done in this area to investigate whether more metabolites of unsaturated bile acids can be detected in the bile, plasma, urine and feces of pigs and to elucidate their role in the metabolic processes.

5 Concluding remarks

In this study we used a nontargeted metabolomic approach to explore changes in the metabolome of pigs with catheters in mesenteric artery and portal vein after consumption of whole grain wheat and wheat aleurone breads, and pigs being in two metabolic states, fasted or fed. Based on our results we conclude that plasma of pigs contained less oxylipins after wheat aleurone consumption, compared to whole grain wheat. If oxylipins can be considered as being not beneficial for health, then flour enriched with aleurone can be considered more beneficial for health than whole grain flour with regard to its lipid content. The oxylipin-marker of this difference between wheat aleurone and whole grain wheat was identified to be a mixture of 13-HODE and 9-HODE isomers. These compounds can be used as novel lipid candidate biomarkers of whole grain intake versus aleurone, highlighting the importance of lipid components of the germ. Future experiments on the quantification of oxylipins and especially 13-HODE and 9-HODE in the flour, bread and plasma can give a better understanding whether these oxylipins may have health detrimental effects and/or they may just be considered as markers for off-flavor formation and shelf-life of bread.

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Conflict of interest There is no conflict of interest.

References


by ESI-mass spectrometry and NMR of the oxidation products. 


Erratum to: Oxylipins discriminate between whole grain wheat and wheat aleurone intake: a metabolomics study on pig plasma

Natalja P. Nørskov · Mette Skou Hedemann · Peter K. Theil · Knud Erik Bach Knudsen

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Erratum to: Metabolomics
DOI 10.1007/s11306-012-0465-x

The original version of the article unfortunately contained a mistake. In the Tables 2 and 3 all the signs “>” have to be “<”. In the Result section there is mistake in the sentence. The sentence should be written as: OSC-PCA score plot showed that there was discrimination between whole grain and refined flour, whereas refined flour and aleurone were closely related.

The online version of the original article can be found under doi:10.1007/s11306-012-0465-x.

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Published online: 16 November 2012
Table 2  List of metabolites discriminating between portal vein and mesenteric artery

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<td>12.78</td>
<td>407.2790&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2</td>
<td>Hyocholic acid</td>
<td>5.0***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>13.78</td>
<td>391.2837&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3</td>
<td>Hyodeoxycholic acid or Ursodeoxycholic acid</td>
<td>2.1***</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Metabolites are listed according to the loadings of PCA models, from metabolites with the highest influence to metabolites with the less influence. The measurements were performed in negative electrospray ionization mode

<sup>a</sup> Identified using commercial standard

<sup>b</sup> Fold change was calculated by dividing the mean of normalized intensity of each plasma metabolite measured in the portal vein by the mean intensity of the same plasma metabolite measured in the mesenteric artery

<sup>c</sup> FDR q value was calculated for correction of false-positives

<sup>d</sup> P value was calculated by a two-sample t test; * <0.05 ** <0.01 *** <0.001
Table 3 List of metabolites discriminating between whole grain wheat, wheat aleurone and refined flour

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Retention time (min)</th>
<th>m/z</th>
<th>Mass accuracy (ppm)</th>
<th>Metabolites</th>
<th>Whole grain wheat vs. refined flour</th>
<th>Wheat aleurone vs. refined flour</th>
<th>Whole grain wheat vs. wheat aleurone (artery)</th>
<th>Whole grain wheat vs. wheat aleurone (vein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fold change&lt;sup&gt;d&lt;/sup&gt;</td>
<td>q value&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Fold change&lt;sup&gt;d&lt;/sup&gt;</td>
<td>q value&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>18.34</td>
<td>295.2264&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0</td>
<td>13-HODE and 9-HODE</td>
<td>1.3**&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.07</td>
<td>0.9</td>
<td>0.41</td>
<td>1.5**</td>
</tr>
<tr>
<td>18.80</td>
<td>297.2419&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3</td>
<td>9,10-epoxy-stearic acid</td>
<td>33.3***</td>
<td>&lt;0.001</td>
<td>40.3***</td>
<td>&lt;0.001</td>
<td>0.8</td>
</tr>
<tr>
<td>11.68</td>
<td>329.2323&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0</td>
<td>9,12,13-TriHOME</td>
<td>1.04**</td>
<td>0.05</td>
<td>0.7</td>
<td>0.79</td>
<td>1.6**</td>
</tr>
<tr>
<td>18.64</td>
<td>293.2095&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.2</td>
<td>13-oxo-ODE</td>
<td>0.4</td>
<td>0.66</td>
<td>0.6</td>
<td>0.25</td>
<td>1.6*</td>
</tr>
<tr>
<td>21.45</td>
<td>309.2404</td>
<td>–</td>
<td>Unidentified</td>
<td>4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14</td>
<td>0.4*</td>
<td>0.10</td>
<td>4.3**</td>
</tr>
<tr>
<td>16.30</td>
<td>311.2207&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.7</td>
<td>13H-EpODE</td>
<td>1.04**</td>
<td>0.04</td>
<td>0.7</td>
<td>0.78</td>
<td>1.5**</td>
</tr>
<tr>
<td>22.02</td>
<td>277.2142&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2</td>
<td>Linolenic acid</td>
<td>1.4***</td>
<td>&lt;0.001</td>
<td>1.2*</td>
<td>0.05</td>
<td>1.2**</td>
</tr>
<tr>
<td>23.33</td>
<td>279.2306&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3</td>
<td>Linoleic acid</td>
<td>1.04</td>
<td>0.54</td>
<td>0.9</td>
<td>0.54</td>
<td>1.1</td>
</tr>
<tr>
<td>19.11</td>
<td>319.2274</td>
<td>1.5</td>
<td>11-HETE</td>
<td>1.1</td>
<td>0.83</td>
<td>0.8</td>
<td>0.25</td>
<td>1.5*</td>
</tr>
</tbody>
</table>

Metabolites are listed according to the % of described variation in the PLS-DA models, from high to low. The measurements were performed in negative electrospray ionization mode

<sup>a</sup> Identified using commercial standard

<sup>b</sup> Identified using spectra from the literature

<sup>c</sup> 13-Hydroxy-9,11-octadecadienoic acid (13-HODE), 9-hydroxy-10,12-octadecadienoic acid (9-HODE), 9,10-epoxyoctadecanoic acid (9,10-epoxy-stearic acid), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME), 13-keto-9,11-octadecadienoic acid (13-oxo-ODE), 13-hydroxy-9,10-epoxy-11-octadecenoic acid (13H-EpODE), 11-hydroxy-5,8,12,14-eicosatetraenoic acid (11-HETE)

<sup>d</sup> Fold change was calculated by dividing the mean of normalized intensity of each plasma metabolite after consumption of one experimental bread by the mean intensity of the same plasma metabolite after consumption of another experimental bread

<sup>e</sup> FDR q value was calculated for correction of false-positives

<sup>f</sup> P value was calculated by a two-sample t test; * <0.05 ** <0.01 *** <0.001
Multi-compartmental non-targeted LC-MS metabolomics: explorative study on the metabolic responses of rye intake in plasma and urine of hypercholesterolemic pigs

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF</td>
<td>Dietary fiber</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PLS-DA</td>
<td>Partial least square-discriminant analyses</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low density lipoproteins-cholesterol</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High density lipoproteins-cholesterol</td>
</tr>
<tr>
<td>RB</td>
<td>High-fat/high-DF rye bread</td>
</tr>
<tr>
<td>WB</td>
<td>High-fat/high-refined DF wheat bread</td>
</tr>
</tbody>
</table>
Multi-compartmental non-targeted LC-MS metabolomics approach was used to study the metabolic responses on plasma and urine of hypercholesterolemic pigs after consumption of diets with contrasting dietary fiber composition (whole grain rye with added rye bran versus refined wheat). To study the metabolic responses, we performed a supervised multivariate data analyses used for pattern recognition, which revealed marked effects of the diets on both plasma and urine metabolic profiles. Diverse pools of metabolites were responsible for the discrimination between the diets. Elevated levels of phenolic compounds and dicarboxylic acids were detected in urine of pigs after rye consumption compared to wheat. Whereas, the discrimination between the diets in plasma was characterized by oxylipins and cholesterol. Moreover, we performed an alignment of the metabolic profiles between the breads consumed by pigs, plasma and urine with the purpose to follow the metabolic fate of the compounds and to identify their pathways. One metabolite was identified in all three compartments, 16 metabolites were similar between bread and plasma, 3 between plasma and urine and 2 between bread and urine. The use of multi-compartmental metabolomics offered higher order information, including inter-compartment relationships and provided novel targets for future research.

**Keywords:** dietary fiber, wheat, rye, metabolomics, biomarker, phenolics, oxylipins, cholesterol, alignment
1. Introduction

Whole grain rye bread has gained much interest due to its high content of dietary fiber (DF) and bioactives, which are suggested to contribute to the beneficial health effects of these food items. Whole grain products differ from refined products by their bran content, which is rich in DF, vitamins, minerals and phenolics. These compounds are known to improve bowel physiology and lipid and antioxidant status and therefore are protective against chronic diseases such as atherosclerosis, diabetes and cancer. The hypocholesterolemic effect of some cereals is ascribed to DF, particularly soluble DF. Rye, which is the only grain traditionally used as whole grain, has a high content of soluble and insoluble DF in form of arabinoxylans. Lærke et al. reported that a diet consisting of whole grain rye enriched with rye bran reduced plasma cholesterol levels in hypercholesterolemic pigs compared to a diet with similar DF content consisting of refined wheat with added purified, insoluble wheat fiber. The mechanisms behind the protective effects of rye against metabolic diseases is still largely unknown, but it is most likely due to a synergetic actions of a large number of bioactive components. Therefore, further evidence of the health effects of rye consumption and knowledge of the mechanisms are needed. During the past few years, metabolomics analyses have provided new insights on the influence of whole grain versus refined products on metabolic profiles of plasma and urine. Recently, using a non-targeted metabolomics approach we reported that oxylipins were elevated in the plasma of pigs after consumption of whole grain wheat compared to wheat aleurone and refined flour, probably due to the presence of germ in the whole grain wheat diet. The result of the study suggested that oxylipins may be potential lipid biomarkers of germ/whole grain wheat consumption.

In the present study, we hypothesized that a non-targeted LC-MS metabolomics approach would provide new and more in-depth information on the hypocholesterolemic effect of rye components. In this respect the pig is considered an excellent model for humans as it can develop hypercholesterolemia and atherogenic lipoproteins within a few weeks. We therefore examined the response of high-fat/high-DF rye bread (RB) versus high-fat/high-refined DF wheat bread (WB) intake on the metabolic profiles
of plasma and urine and used the data to detect and identify novel markers of rye DF consumption. Moreover we used multi-compartmental metabolomics to investigate the metabolic fate of metabolites from their consumption to their excretion by profiling analyses of bread, plasma and urine.

2. Material and Methods

2.1 Chemicals

The following standards were used: enterolactone, ferulic acid, hippuric acid, 2-methylhippuric acid, DL-3-phenyllactic acid, DL-indole-3-lactic acid, 5-methoxy-3-indoleacetic acid, azelaic acid and 13-hydroxy-9,11-octadecadienoic acid (13-HODE) were all from Sigma-Aldrich (St. Louis, MO, USA), 9-hydroxy-10,12-octadecadienoic acid (9-HODE) was from Cayman Chemicals Co. (Ann Arbor, MI, USA), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME) and 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-TriHOME) were from Larodan (Malmö, Sweden), as internal standard was glycocholic acid (Glycine-1 $^{13}$C) Sigma-Aldrich (St. Louis, MO, USA) was used. Acetonitrile and formic acid were obtained from Fluka/Sigma-Aldrich (St. Louis, MO, USA) and ethyl acetate was from Merck (Darmstadt, Germany). All solvents were of HPLC or analytical grades.

2.2 Diets

A low-fat and low-fiber diet (LF) consisting of 867 g/kg refined wheat flour, 73 g/kg whey protein concentrate, 30 g/kg rapeseed oil, 30 g/kg vitamin-mineral mixture and 60 g/kg cellulose in the form of Vitacel WF 600 (LCH A/S, Frederiksberg, Denmark) was used as wash-out diet before the experimental period. The breads fed during the experimental period were prepared of rye or wheat ingredients and with added egg powder, lard, rapeseed oil, sugar and whey protein concentrate, Table 1. The rye ingredients in the RB diet (produced at Holstebro Technical College, Denmark) contained finely ground whole-grain rye flour (Valsemøllen, Esbjerg, Denmark) and ground rye bran (Nordmills, Uppsala, Sweden). The wheat ingredients in the WB diet (produced at Lantmännen Unibake, Karup, Denmark) contained refined wheat flour supplemented with refined cellulose-rich wheat fiber Vitacel WF 600 in order to achieve the same DF level as in the RB. Lard, egg powder (Danæg Production A/S, Roskilde,
Denmark) and cholesterol (Sigma-Aldrich Co., St Louis, MO, USA) were added to increase the fat and cholesterol content. Both diets were fortified with a vitamin and mineral mixture. The breads were stored at –20 °C until consumption.

2.3 Study design and animals

A total of seventeen, hyper-responders, Duroc x Danish Landrace x Yorkshire gilts (weighing approximately 70 kg), age 4 months and obtained from the swineherd at Aarhus University (AU-Foulum, Denmark), were used for this study.

Before the start of experiment the pigs were fed the LF wash-out diet for 3 weeks. Following this, the pigs were fed the experimental diets for 9.5 weeks, 8 pigs were fed WB and 9 pigs were fed RB. The pigs were housed individually in 4 m² smooth-walled pens with concrete floor. In the second week the pigs were transferred to metabolic cages for 7 days, where 24 hour urine was collected. Urine was collected on daily basis over 4 g ascorbic acid/bottle/day and pooled over 7 day of collection, and pH was measured on a daily basis. The urine samples were pooled 7 x 24 h and stored frozen. The feed intake during the period, where the pigs ate the experimental bread-based diets, was restricted to 2 kg/d increasing to 3 kg/d for the last 2.5 weeks of the study. Postprandial blood samples from the jugular vein and lateral auricular artery were taken 3 h after the morning meal on the day of slaughter. The pigs were euthanized with an overdose of sodium pentobarbital by exsanguination and blood samples were collected in EDTA vacutainers, centrifuged and plasma was harvested and stored at –20 °C. Spot urine was taken directly from the urine bladder and stored frozen. The pigs were weighed once weekly and before slaughtering.

The animal experiment was conducted according to protocols approved by the Danish Animal Experiments Inspectorate and complied with the guidelines of the Danish Ministry and Justice concerning animal experimentation and care of animals under study.

2.4 Chemical analyses of the breads

All analyses were performed in duplicate on freeze-dried materials. The dry matter content was measured by drying to constant weight at 105 °C for 20 h. Nitrogen was measured according to DUMAS
and calculation of protein was performed using N x 6.25. Fat was extracted according to the Stoldt procedure. Starch, non-starch polysaccharides (NSP), constituent sugars and Klason lignin were determined as described by Bach Knudsen K. E. et al. The content of plant lignans in the diets was analyzed by isotope dilution GC-MS as previously described Penalvo et al. The content of ferulic acid was measured as described by Kamal-Eldin et al.

2.5 Chemical analyses of plasma and urine

Plasma glucose, triglycerides, total cholesterol, LDL and HDL cholesterol and urinary creatinine were all measured using an auto-analyzer, ADVIA 1650 Chemistry System (Bayer Corporation, Tarrytown, NY, USA) using human standards and calibration materials. Glucose, triglycerides, total cholesterol and low density lipoproteins-cholesterol (LDL-C) and high density lipoproteins-cholesterol (HDL-C) were analyzed according to standard procedures (Bayer HealthCare LLC). The urinary creatinine concentration was measured according to Jaffe reaction.

2.7 Sample preparation for LC-MS

2.7.1 Plasma

Metabolites were extracted from 0.5 mL plasma using 2 mL ethyl acetate, shaken for 1 min and centrifuged at 45 x g for 15 min at 30 °C. After centrifugation the upper layer of ethyl acetate was removed using an ethanol-dry ice bath. The extraction was repeated twice. Combined organic layers were taken to dryness under a flow of nitrogen gas and redissolved in 200 µL of 40 % acetonitrile. Internal standard was added in the concentration of 8 µM and samples were centrifuged for 5 min at 20,800 x g and pipetted to HPLC vials to be analyzed on the LC-MS instrument.

2.7.2 Urine

Urine samples were diluted 1:1 with 5 % of acetonitrile. The internal standard was added in the concentration of 8 µM and samples were centrifuged for at 45 x g for 30 min at 4 °C and after that pipetted to HPLC vials to be analyzed on the LC-MS instrument.

2.7.3 Breads
Extractions of breads were performed on freezed-dried material, 0.5 g of finely milled breads were mixed with 2.5 mL of 40 % acetonitrile, mixed for 1 min and then sonicated for 30 min. After sonication samples were centrifuged at 45 x g for 15 min at 4 °C and the supernatant was pipetted to 200 µL HPLC vials for LC-MS analyses.

2.7.4 Standards

All standards, used for metabolite identification purpose, were dissolved in 40 % acetonitrile in concentration of 15 µM and analyzed on LC-MS instrument.

2.8 LC-MS and LC-MS/MS

Chromatographic separation was performed on a UltiMate 3000 HPLC system (Thermo Scientific Dionex, Sunnyvale, CA, USA) and equipped with a C\textsubscript{18} silica coated column 3 µm 150 x 2.1 mm (Supelco, Sigma-Aldrich, St. Lois, MO, USA), and a C\textsubscript{18} 4 x 2.0 mm pre-column (Phenomenex, USA). The column oven was set to 30 °C and the temperature of the auto sampler was 10 °C. The flow rate was 200 µL/min. Solvent A consisted of 0.1 % formic acid in water and solvent B consisted of 0.1 % formic acid in acetonitrile. Two different gradient systems were used for plasma/bread and urine samples. For plasma and bread samples the gradient started at 20 % and continued to 100 % of solvent B during 40 min and kept isocratically for 2 min. For urine samples the gradient started at 15 % and continued to 55 % of solvent B during 50 min and kept isocratic for 3 min. In both methods the column was re-equilibrated for 5 min in the beginning of each run. Fifteen microliter of plasma or bread sample or 25 µL of urine sample was injected. The mass spectrometric analyses were performed as described previously by Nørskov et al.,\textsuperscript{8}. The method is briefly as follows. Negative electrospray ionization mode was used for the MS and MS/MS analyses of plasma, bread extracts and urine. Capillary and end plate offset voltage were set to 4,500 and -500 V respectively. The dry gas flow was 8 L/min at 200 °C and nebulizer pressure was set to 1.8 bar. Mass to charge ratio of the quadrupole’s scan ranged from 50 to 1,000. MRM scan was used for fragmentation of compounds with collision energy varying according to the chemical characteristics of the compound analyzed.
2.9 Quality control of the data

The quality of the data was evaluated by several methods. Frequent blank samples (solvent mixture of 40% acetonitrile) and quality control samples (QC) were injected between the runs to ensure the quality of the data. In the case of urine QC sample were a mixture of RB and WB urine samples and in the case of plasma QC represented stock plasma at Aarhus University. The blank samples were used to study the occurrence of carry-over effects during LC-MS runs. The chromatograms of blank samples were studied, and since they contained only low intensity peaks, it was concluded that carry-over effect did not occur. Unsupervised multivariate data analysis was used to investigate the relationship between QC samples and data set samples of plasma and urine, Figure 1Sa, b and c (supporting information). On the PCA scores plots, QC samples clustered together showing that no variation occurred during day-to-day performance. Reinjection of samples was also used as quality control. PCA score plots showed the replicates were closely related, Figure 1Sa, b and c (supporting information). Moreover, shifts in masses were corrected by use of an external standard and the chromatograms were calibrated in High Precision Calibration mode with mass accuracy deviating between 0.01 and 6 ppm.

2.10 Data processing and statistics

Data processing was performed in MZmine. Centroid data were cropped removing the external standard and the peaks were detected using mass detection and chromatogram builder, thereafter deconvoluted, deisotoped and retention time (RT) normalized. Join aligner was used to align the chromatograms with m/z tolerance 0.025 and RT tolerance of 10 s. The data was filtered for duplicated peaks and individual bias, where common features found in at least 75% of the samples were selected for further data processing. The missing peaks were filled in using peak finder. Normalization of the data was performed with the standard compound, which was added to the samples during sample preparation. Join aligner was also used to align the chromatograms of the breads and plasma, spot urine and 24 h urine for heat map construction. The average of the bread, plasma and urine samples were used
for the construction of heat maps. Moreover, the data were scaled and log transformed when performing heat map analyses.

Furthermore, the data sets of urine and plasma were subjected to multivariate data analysis using PLS-DA, a supervised method for pattern recognition. Prior to multivariate modeling the plasma samples collected at jugular vein and lateral auricular artery were averaged to represent the average of metabolites in the peripheral blood circulation. The urine samples were normalized using the concentration of creatinine, measured for each sample. Multivariate modeling was performed in LatentiX. The data was scaled using Pareto scaling and cross validated. Robustness of the models was evaluated by models statistics and actual versus predicted plots. Outliers were removed after evaluation of X-Y relation Outliers plots (T vs. U scores). Root Mean Square Error of Cross Validation (RMSECV) was used to select the optimal number of PC’s for each model. The optimal number of PC’s was selected when deviation between cross validated values and real values was at its lowest. Regression coefficient ($r^2$) was selected based on the optimal number of PC’s. The variables were selected using scaled regression coefficients, which show the contribution of each variable to the score formation. The variables were also tested by Welch’s two-sample $t$ test, where $P$ values of < 0.05 were considered to be statistically significant. For correction of false-positives (FDR) $q$ values were calculated, with significance threshold set at $q < 0.25$.17

2.11 Biomarker identification

Tandem mass spectrometry was used to identify the metabolic of interest by comparison of fragmentation pattern of the metabolite with fragmentation pattern of the standard or searching for fragments and parent ions in METLIN database (http://metlin.scripps.edu/). LIPID MAPS database was used to assign oxylipins detected in the samples (http://www.lipidmaps.org/). The systematic and common names of oxylipins were written according to LIPID MAPS. Spectra from the literature were also used to confirm the biomarker identity.18 19 20 21 Bile acids and polyunsaturated fatty acids (PUFA) were identified according to Nørskov et al.8 Identification of phosphatidylcholines (PC) and
phosphoserines (PS) was performed in mzMine. The MS/MS spectra and proposed structure of novel identified metabolites can be seen in supporting information, Figure 2S.

3. Results and Discussion

3.1 PLS-DA models

The effect of RB versus WB on plasma and urine metabolite profiles was analyzed by multivariate data analyses. PLS-DA models of plasma, spot urine and 24 h urine are presented in Figure 1a, c and e. In all three models PC 1 discriminated between RB and WB and explained most of the variance. To ensure robustness of the models, the models were cross validated and the optimal numbers of PC’s, $r^2$ and RMSECV are summarized in Table 2. For creation of the plasma PLS-DA model the variables total cholesterol, LDL-C, HDL-C, LDL:HDL ratio, triglycerides, glucose and weight of the pigs were included. Inter-subject variation was higher for plasma samples, which resulted in a lower regression coefficient compared to urine samples. A difference between 24 h urine and spot urine was also observed in the model statistics. The variation among the samples for spot urine was higher than for 24 h urine, especially for RB, and therefore two PC’s was necessary to explain the variation in this model. One PC explained most of the variation in PLS-DA model for 24 h urine, which also had the highest regression coefficient.

3.1.1 Metabolites of plasma

The scaled regression coefficients of the PLS-DA model were used to investigate which metabolites discriminated between RB and WB intake in plasma, Figure 1b. The PLS-DA model showed that the main difference in plasma was associated with oxylipins and total cholesterol. In general, arachidonic acid derived oxylipins, eicosanoids such as 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and 5,12-dihydroxy-6,8-10,14-eicosatetraenoic acid (LTB₄) discriminated the plasma after RB consumption, whereas linoleic acid derived oxylipins such as an isomeric mixture of 13-hydroxy-9,11-octadecadienoic acid (13-HODE) and 9-hydroxy-10,12-octadecadienoic acid (9-HODE) discriminated plasma after WB consumption. Other markers of WB intake were total cholesterol, LDL-C, LDL:HDL ratio and 7α-
hydroxy-3-oxo-4-cholestenoic acid. An extended list of metabolites, their metabolic pathways and t test results are summarized in Table 3. Even though cholesterol was added to the diets in equal amount, the diets influenced the cholesterol metabolism differently, which was reflected in the PLS-DA model by the total cholesterol, LDL-C, LDL:HDL ratio and 7α-hydroxy-3-oxo-4-cholestenoic acid, which had higher regression coefficient after WB intake compared to RB, Figure 1b. The effect of RB on postprandial concentrations of total cholesterol, LDL-C and LDL:HDL ratio was reported before by Lærke et al.4 Lærke et al.4 also reported that the expression of the gene, CYP7A1, encoding cholesterol 7α-hydroxylase, was significantly lower in pigs fed RB compared to pigs fed WB. It is known that 7α-hydroxy-3-oxo-4-cholestenoic acid is a precursor for bile acid synthesis in the liver. The enzyme, which converts cholesterol to 7α-hydroxy-3-oxo-4-cholestenoic acid is 7α-hydroxylase.22 This supports the higher concentration of 7α-hydroxy-3-oxo-4-cholestenoic acid (P = 0.02) in the plasma of pigs after WB consumption. Bile acids such as chenodeoxycholic acid (CDCA) and hyodeoxycholic acid (HDCA) or ursodeoxycholic acid (UDCA) were also detected in the plasma, however, their concentrations were not significantly affected by the dietary intervention, which is in accordance with the results reported by Lærke et al.4

Other metabolic pathways that were affected by the diets, were oxylipin synthesis from arachidonic acid and linoleic acid. The pigs fed RB had significantly higher concentration of 5-HETE (P < 0.001) and LTB₄ (P = 0.04), oxylipins derived from arachidonic acid. LTB₄ and 5-HETE are known to be inflammatory lipid mediators, and their overproduction is associated with diseases such as atherosclerosis and cancer.23 24 The pigs fed WB had significantly higher concentration of 9-HODE and 13-HODE (P = 0.006), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME) and 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-TriHOME) (P = 0.02), 9-oxo-10,12-octadecadienoic acid (9-oxo-ODE) (P = 0.01), 13-oxo-9,11-octadecadienoic acid (13-oxo-ODE) (P = 0.01), 12,13-epoxy-9-hydroxy-10-octadecenoic acid (P = 0.008) and 9,10-epoxy-13-hydroxy-11-octadecenoic acid (P = 0.02) oxylipins derived from linoleic acid. In our previous study we measured higher concentration of 13-
HODE and 9-HODE in the plasma of pigs fed whole grain wheat compared to wheat aleurone and refined wheat, but no LTB₄ or 5-HETE were detected. We explained the higher concentration of 13-HODE and 9-HODE by the fact that whole grain wheat contained germ rich in lipids in contrast to aleurone and refined flour where the germ is depleted.⁸ However, mammals contain lipoxygenase (LOX), which can convert linoleic acid to oxylipins. We are therefore most tempted to believe that the higher concentration of linoleic acid derived oxylipins after WB intake is due to endogenous metabolism as the presence of germ in RB did not influence the concentration of linoleic acid derived oxylipins in this study the same way as it was seen in the previous study. However, the oxylipins that discriminated between the diets in plasma are known as pro-inflammatory markers ²⁵, and their presence in plasma was probably activated by the consumption of the diets high in cholesterol and saturated and unsaturated fatty acids in the form of lard, rapeseed oil and egg powder, Table 1.

### 3.1.2 Metabolites of urine

The scaled regression coefficients of the PLS-DA model were used to investigate which metabolites discriminated between RB and WB intake in urine, Figure 1d and f. Metabolites, which had the highest influence in the PLS-DA models and significant P values for both spot and 24 h urine, were enterolactone ($P_{\text{spot urine}} = 0.002$, $P_{24 \text{ h urine}} < 0.001$), hippuric acid ($P_{\text{spot urine}} = 0.002$, $P_{24 \text{ h urine}} < 0.001$), 2-methylhippuric acid ($P_{\text{spot urine}} = 0.003$, $P_{24 \text{ h urine}} < 0.001$) and ferulic acid ($P_{\text{spot urine}} = 0.011$, $P_{24 \text{ h urine}} < 0.001$), all with the highest concentration after RB intake. An extended list of metabolites, their metabolic pathways and t test results are summarized in Table 4. The majority of metabolites detected in urine were the end products of bacterial fermentation in the colon. Enterolactone is known as the mammalian fermentation product of plant lignans ²⁶ ²⁷, and was only detected in the urine of pigs fed RB. This is in agreement with the higher concentration of plant lignans in the RB, Table 1, and in line with previous findings.²⁸ Hippuric acid and 2-methylhippuric acid are associated with the metabolic conversion of plant phenolic acids including ferulic acid ⁷ ²⁹, which were substantially higher in RB than WB, Table 4. Hippuric acid was recently proposed as a potential biomarker of fruits and vegetables.
The results of this study suggest that hippuric acid but also 2-methylhippuric acid are major mammalian fermentation products of phenolic compounds from cereals, and therefore may be used as biomarkers of a polyphenol rich diet. Other phenolic compounds, which were detected in urine and with higher concentrations after RB versus WB, were \( p \)-cresol, 3-phenyllactic acid, 5-methoxy-3-indoleacetic acid, N-feruloylglycine, quinol and homovanillic acid, Figure 1d and f and Table 4. These metabolites are known to be a result of bacterial bioconversions and in the case of \( p \)-cresol and quinol which are also known as simple phenols, they are the end products of fermentation of phenolic compounds and aromatic amino acids.\(^{30-31}\)

Oxylipins/fatty acids were also responsible for the discrimination between the experimental diets in the urine samples, Figure 1d and f and Table 4. Both even and odd, di- and monocarboxylic fatty acids containing mono-, di- and trihydroxy and methyl groups were detected in urine with carbon chain length varying from 9 to 18 carbon atoms and with and without the presence of a double bound. These fatty acids were mainly conjugated with glucuronic acid, but some were also detected in unconjugated form. Production of dicarboxylic acids has mainly been studied in yeasts, but microbial bioconversion of rapeseed oil to dicarboxylic acids has been reported.\(^{32-33}\) The metabolic pathway for the production of dicarboxylic acids in microorganisms is known as \( \omega \)-oxidation.\(^{33}\) Omega-oxidation is also known as a minor metabolic pathway in the liver of mammals and humans, and can be an alternative to \( \beta \)-oxidation \(^{34-35}\). In this study we identified several dicarboxylic acids, which may originate from both microbial bioconversion and/or liver metabolism. There is an indication of bacterial conversion, reported by Hanhineva et al.\(^{36}\) where metabolite masses of \( m/z \) 329.23, 357.26, 229.24 and 373.26 were detected in an \textit{in vitro} simulated colonic fermentation model of rye bran fractions.\(^{36}\) One of the masses (329.23) match the metabolite mass of unconjugated ion (TriHOME) detected in the urine of our study, Table 4. Furthermore, the characteristic increase in 2 amu in the molecular ion, which was reported by Hanhineva et al.\(^{36}\), was also observed for example for the ions with \( m/z \) 241.1806 and 243.1951, which reflects the reduction of a carbon-carbon double bound; a saturation that is generally performed by the
microorganisms. Moreover, we found that the urine of pigs fed RB contained higher concentration of these different medium chained hydroxy fatty acid compared to urine of pigs fed WB. That may indicate that more extensive microbial conversion in the gastrointestinal tract characterized the pigs fed RB, which is a likely consequence of the lower ileal digestibility of fats reported by Lærke at al.4 We also identified novel medium chained dicarboxylic acids containing hydroxyl/methyl groups with \textit{m/z} values of 289.1634 and 255.1608, which discriminated between RB and WB consumption and which may possibly be novel lipid markers of rye consumption, Table 4. Tandem mass spectrometry and accurate masses were used to tentatively identify these novel fatty acids by comparison of MS/MS spectra with already identified fatty acids (supporting information, Figure 2Sa and b). It was furthermore noticed that higher concentration of 9,12,13-TriHOME was measured after WB compared to RB, which can be due to the higher concentration of this metabolite in plasma.

Other metabolites such as bile acids were also detected in urine in both conjugated and unconjugated form. The bile acids detected in urine were identical to the bile acids detected in plasma, although no significant dietary influence was observed for the bile acids in urine. However, there was a tendency of a higher bile acid excretion after RB consumption observed in the PLS-DA model for spot urine but not for 24 h urine. This conflicting result was tested with the \textit{t} test, where no significant dietary effect was achieved.

3.2 Multi-compartmental metabolomics: plasma, urine and bread

The advantage of measuring metabolic profiles in more than one compartment is a better understanding of the response to a given intervention.37 A heat map was constructed to compare the metabolic profiles of the breads (RB and WB), and plasma (after consumption of RB and WB), Figure 2. In general, from color patterns comparison, the metabolic profile of plasma after consumption of RB and WB was more similar than the metabolite profile of the corresponding breads. When comparing the color patterns of the breads and plasma, the major similarity can be assigned to four classes of metabolites; oxylipins, monounsaturated fatty acid (MUFA), PUFA and lysophosphatidylcholines
PUFA such as arachidonic acid, linolenic acid, linoleic acid and MUFA such as oleic acid and LPC (18:2), (18:1), (16:0) were conserved in both compartments (breads and plasma). On the contrary, not all the oxylipins were equally distributed across the compartments. The color dissimilarity shows that arachidonic acid derived oxylipins like the different isomers of HETEs, DiHETEs and LTB₄, were restricted to the plasma. On the contrary, linoleic acid derived oxylipins such as for example isomeric mixtures of 13-HODE and 9-HODE and 9,12,13-TriHOME and 9,10,13-TriHOME were detected across both compartments. The oxylipins, which were conserved across both compartments, belong to the linoleic acid cascade, which is a known enzymatic pathway in plant kingdom for production of antifungal compounds. Other HODEs, HOMEs and DiHOMEs were restricted to the breads. That indicates that the breads contained many more different isomers of oxylipins, compared to the plasma. Provided the presence of oxidized PUFA in plasma mainly comes from endogenous turnover of lipids within the animal, and not as dietary source, the existence of similar oxylipins in both plasma and bread is due to related enzymatic pathways. That is in agreement with the fact that LOX is an abundant enzyme in both the plant and animal kingdoms and the linoleic acid cascade can therefore also occur in mammals. On the contrary, when oxylipins are only detected in the breads, it may indicate the presence of plant specific enzymes.

Since the majority of metabolites in urine were conjugated with glucuronic acid it was not possible to align urine metabolite profiles with plasma and bread profiles. The heat map of spot urine and 24 h urine after consumption of RB and WB is shown in Figure 3. The hierarchical clustering and color pattern clearly showed the dietary influence on the metabolic profile of urine. Moreover, the alignment showed that many more rye-derived metabolites were detected, which may reflect the high content of phenolic compounds. It can be noticed that 9,12,13-TriHOME was conserved across all three compartments; breads, plasma and urine. However, detection of 9,12,13-TriHOME in urine may result both from the diet and endogenous oxidation of linoleic acid. Azelaic acid and hydroxysebacic acid were identified in the breads and urine. This indicates that a proportion of fatty acids from the diet can pass the
gastrointestinal tract and be excreted in urine without metabolic conversion. It has been reported that after oral administration more than 50% of azelaic acid was detected in urine, which indicated its high plasma and renal clearance.

Out of 103 identified metabolites, one metabolite was detected in all three compartments, 16 metabolites were similar between bread and plasma, 3 were similar between plasma and urine and 2 were similar between breads and urine, Figure 4.

3.3 Biological interpretation

The intake of RB and WB resulted in different metabolic profiles of plasma and urine. The presence of high content of DF (in the form of arabinosylns) and antioxidants in the RB clearly affected not only metabolism of lignans and phenolic compounds, but also triggered the lipid profiles of plasma and urine. It is known that the PUFAs are protected against free radical attack and oxidation by the presence of antioxidants. The presence of high content of antioxidants such as ferulic acid and plant lignans in the RB, Table 1, may have inhibited the endogenous oxidation of lipids. It is well established that dietary content of antioxidants is important with respect to prevention of lipid oxidation and oxidative LDL. Another aspect, which may have influenced the result, is the digestibility of lipids, which can be influenced by DF particularly soluble DF. In the study of Lærke et al., it was found that the digestibility of fat at the distal ileum and at all sites in the large intestine as well as total tract was consistently lower after consumption of RB compared to WB. The interaction of fatty acids with soluble DF may result in reduced absorption in the small intestine and consistently lower concentration in plasma, which may explain the lower concentration of linoleic acid derived oxylipins in the plasma of pigs fed RB.

The presence of cholesterol in the diets may have triggered inflammation processes, which can explain the presence of arachidonic acid derived oxylipins such as 5-HETE and LTB4, which are known to be pro-inflammatory markers. Detection of these oxylipins in plasma of pigs fed high cholesterol diet may be indicative of onset of inflammation and development of atherosclerosis. The higher concentration of these oxylipins after RB intake is, however, difficult to explain in view of the higher
content of antioxidants (ferulic acid and lignans) for this diet, and in contrast to what would be expected. However, it has to be emphasized that plasma samples analyzed in this study were taken at the end (after 9.5 weeks) of the experiment and no samples were analyzed, which could represent the beginning of the experiment. To be able to draw a conclusion it is important to know whether these metabolites were present from the start of the experiment or whether their concentration rose gradually during the experimental period and therefore could be linked to the development of inflammation.

Impaired absorption of fat and cholesterol and increased fecal excretion of bile acids is probably one of the important mechanism proposed for hypocholesterolemic effect of DF. We have measured a higher concentration of 7α-hydroxy-3-oxo-4-cholestenoic acid, precursor to bile acids, in plasma of pigs fed WB compared to RB. Since higher concentration of a precursor to bile acids was measured after WB intake, it would also be expected to measure a higher concentration of bile acids. However, the bile acids detected in plasma and urine did not show any significant dietary concentration difference. The result of this study therefore indicates that if the bile acids contribute to the hypocholesterolemic effect of rye DF, it was not reflected in plasma or urine concentrations. But since we did not perform metabolomics analyses of feces, it cannot be excluded that higher fecal excretion of bile acids could be measured after RB consumption.

The results of multi-compartmental alignment showed that fatty acids, i.e. 9,12,13-TriHOME, azelaic acid and hydroxysebacic acid were detected in the breads and further in the plasma (in the case of 9,12,13-TriHOME) and urine. That demonstrates that a proportion of fatty acids consumed was excreted into urine and depending on their hydrophobicity they can undergo glucuronization in the liver. This detoxification process is also known for phenolic acids, which is in accordance with the finding of conjugated ferulic acid in urine of pigs fed RB. In contrast to 9,12,13-TriHODE, 13-HODE and 9-HODE were only detected in the breads and plasma but not in the urine. That indicates that the presence of lower number of hydroxyl groups, which influences the hydrophobicity of oxylipins may diminish their excretion into urine. One may therefore suggest that the presence of oxylipins in the plasma and the
interference with the lipoprotein matrix can depend on their hydrophobic properties. For example, several studies have shown the involvement of 13-HODE and 9-HODE in formation of atherosclerotic plaques and stimulation of cell proliferation. Moreover, there is growing evidence that 13-HODE and 9-HODE promote arteriosclerosis in the presence of cholesterol and can be used as pro-inflammatory markers.

Different medium chained, di- and monocarboxylic fatty acids containing hydroxyl and methyl groups were detected at higher concentration in the urine of pigs fed RB compared to WB. Their presence in urine can reflect both the metabolism in the gastrointestinal tract and/or liver. It has been reported that medium chained fatty acids (MCFAs) are absorbed in the gastrointestinal tract to the portal vein system and directly to the liver, where they further can undergo hydroxylation, methylation and/or ω-oxidation and conjugation before their excretion in urine as it was reported in the case of phenolic acids. In this case MCFAs have to be present in the diet. We have detected azelaic acid and hydroxysebacic acid in the diets, but no other medium chained di- and monocarboxylic fatty acids were detected. Another possibility is β- and ω-oxidation of long chain oxylipin such as HODEs and TriHOMEs in the liver. It can be suggested that since they are present in the diets and plasma they can be absorbed in the gastrointestinal tract and that some proportion of these oxylipins may undergo β- and ω-oxidation to medium chained dicarboxylic acids in the liver and further modifications and conjugation with glucuronic acid before excretion in urine. Since 9,12,13-TriHOME was detected in urine conjugated with glucuronic acid, it indicates that a proportion of this oxylipin is transported to the liver and further excreted via the kidney. Yet another possibility can be the microbial bioconversion in the colon. However, the listed possibilities have to be examined by future hypothesis driven experiments using a more targeted approach. Moreover, whether higher level of medium chained di- and monocarboxylic aids after RB can be linked to the hypocholesterolemic effect of rye has to be examined by further experiments.
3.4 Applicability of non-targeted LC-MS metabolomics in nutritional research

In this study, the number of metabolites detected in the plasma, urine and breads was 379, out of which only 103 metabolites were identified and the largest portion of metabolites remained unidentified, which is a bottleneck in LC-MS profiling studies. On the other hand profiling of the large number of metabolites enhances the possibility to detect novel and potentially important metabolites that are not in the scope of targeted LC-MS metabolomics. Moreover the combination of LC-MS and multivariate data analyses helps to highlight the potential endogenous and exogenous markers of food intake. The limitation in biomarkers discovery may however lie within the concentration difference among the metabolites and/or instrumental detection limits. For example, in this study we did not detect ferulic acid and enterolactone in the plasma, but it was possible to detect them in urine. This is due to the concentration of these compounds being very low (nM) in the plasma \(^{28}\) (Nørskov N. P. unpublished), which requires better sample preparation/concentration or a combination of targeted and non-targeted metabolomics for the detection of these metabolites. In general, however, our results show applicability and usefulness of non-targeted LC-MS metabolomics in nutritional research.

4. Conclusions

Diverse pools of metabolites were detected in all three compartments, plasma, urine and the experimental breads. The comparison between the metabolic profiles of plasma, urine and breads showed that it is possible to follow the metabolic fate of compounds present in the diets, e.i. 9,12,13-TriHOME, which was detected in all three compartments. The effect of the RB diet on the metabolic profiles was more pronounced in the urine than in plasma, where many more rye-derived metabolites were measured. These metabolites were mainly fermentation products of the colon, which were excreted into urine via glucuronization in the liver. Novel medium chained, di- and monocarboxylic fatty acids containing hydroxyl or methyl groups were identified in urine. These metabolites may potentially be novel lipid markers of rye DF consumption. Their possible contribution to hypocholesterolemic effect of rye DF, however, has to be examined by future experiments. Markers of high polyphenol consumption
were enterolactone, ferulic acid, hippuric acid and 2-methylhippuric acid. The influence of the dietary intervention on the plasma metabolic profile was less obvious, most likely, because metabolites in the plasma are better regulated. However, the RB diet influenced the metabolism of cholesterol, which was reflected in lower concentration of 7α-hydroxy-3-oxo-4-cholestenoic acid, a precursor to bile acids, after RB compared to WB intake. That further pointed towards the hypocholesterolemic effect of rye DF.

In general, the present study showed that a multi-compartmental non-targeted LC-MS metabolomics approach provided useful information on the influence of rye components on plasma and urine metabolic profiles, which can be used to explain the hypocholesterolemic effects of rye. These findings offer novel targets for future research.
5. Acknowledgment

The Nordic Research Council (NKJ-121) project “Rye Bran for Health”, NordForsk (Nordic Center of Excellence Programme on Food, Nutrition and Health - System biology in dietary intervention and cohort studies SYSDIET; 070014), and the Research School in Animal Nutrition and Physiology RAN is gratefully acknowledged for co-funding the PhD project for N.P.N.. There is no conflict of interest.


Table 1. Ingredients list and chemical composition of RB and WB experimental breads

<table>
<thead>
<tr>
<th>Ingredients (g/kg, as-fed basis)</th>
<th>RB</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rye whole meal</td>
<td>310</td>
<td>-</td>
</tr>
<tr>
<td>Rye bran</td>
<td>400</td>
<td>-</td>
</tr>
<tr>
<td>Refined wheat flour</td>
<td>-</td>
<td>528</td>
</tr>
<tr>
<td>Vitacel WF 600&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>157</td>
</tr>
<tr>
<td>Whey protein concentration</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>Yeast</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Sugar</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Egg powder</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Rape seed oil</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Lard</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin-mineral mix&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

Chemical composition (g/kg DM)

<table>
<thead>
<tr>
<th></th>
<th>RB</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>172</td>
<td>182</td>
</tr>
<tr>
<td>Fat</td>
<td>159</td>
<td>153</td>
</tr>
<tr>
<td>Starch and sugar</td>
<td>384</td>
<td>437</td>
</tr>
<tr>
<td>Dietary fiber&lt;sup&gt;d&lt;/sup&gt;</td>
<td>203</td>
<td>194</td>
</tr>
<tr>
<td>Plant lignans (µg)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>41527</td>
<td>124</td>
</tr>
<tr>
<td>Ferulic acid (µg/g DM)</td>
<td>871.4</td>
<td>39.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Before water addition and baking

<sup>b</sup> J. Rettenmaier & Söhne GmbH (Rosenberg, Germany)

<sup>c</sup> Provided in mg/kg diet: 6642 Ca(H2PO4)2, 4122 NaCl, 16 580 CaCO3, 286 FeSO4.7H2O, 114 ZnO, 41 Mn3O4, 92 CuSO4.5H2O, 0.3 Kl, 0.8 Na2SeO3.5H2O, 2.1 retinoacetate, 0.03 cholecalciferol, 69 α-tocopherol, 2.52 menadione, 4.58 riboflavin, 12.59 D-pantothenic acid, 0.025 cyanocobalamine (B12), 2.52 thiamin (B1), 25.2 niacin, 3.78 pyridoxine (B6), 0.0063 biotin

<sup>d</sup> Dietary fiber was calculated as the sum of NSP and Klason lignin

<sup>e</sup> Plant lignans were calculated as the sum of secoisolariciresinol, matairesinol, lariciresinol, pinoresinol, syringaresinol and medioresinol
Table 2. Summary of model statistics (Root Mean Square Error of Cross Validation (RMSECV), Principal component (PC) for PLS-DA performed on plasma, 24 h urine and spot urine

<table>
<thead>
<tr>
<th></th>
<th>Optimal number of PC’s</th>
<th>$r^2$</th>
<th>RMSECV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>2</td>
<td>0.81</td>
<td>0.60</td>
</tr>
<tr>
<td>Spot urine</td>
<td>2</td>
<td>0.87</td>
<td>0.54</td>
</tr>
<tr>
<td>24 hour urine</td>
<td>1</td>
<td>0.90</td>
<td>0.52</td>
</tr>
</tbody>
</table>
Table 3. List of plasma metabolites discriminating between RB and WB intake

<table>
<thead>
<tr>
<th>m/z / RT</th>
<th>Ion</th>
<th>Mass accuracy (ppm)</th>
<th>Metabolitesf</th>
<th>Pathways</th>
<th>RB versus WB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fold change</td>
</tr>
<tr>
<td>257.226/29.76</td>
<td>Fragment</td>
<td>-</td>
<td>Fragment of 5-HETE</td>
<td>5-LOX arachidonic acid metabolism</td>
<td>1.14</td>
</tr>
<tr>
<td>277.179/24.58</td>
<td>[M-H]</td>
<td>4.6</td>
<td>12-oxo-5,8,10-heptadecatrienoic acidb</td>
<td>unidentified</td>
<td>1.16</td>
</tr>
<tr>
<td>277.216/27.43</td>
<td>Fragment</td>
<td>-</td>
<td>Fragment of 13-HODE and 9-HODE</td>
<td>13- and 9-LOX/reductase linoleic acid metabolism</td>
<td>0.76</td>
</tr>
<tr>
<td>293.211/28.45</td>
<td>[M-H]</td>
<td>4.1</td>
<td>13-OxoODEb</td>
<td>13-LOX linoleic acid metabolism</td>
<td>0.64</td>
</tr>
<tr>
<td>293.211/29.14</td>
<td>[M-H]</td>
<td>4.1</td>
<td>9-OxoODEb</td>
<td>9-LOX linoleic acid metabolism</td>
<td>0.70</td>
</tr>
<tr>
<td>295.226/27.40</td>
<td>[M-H]</td>
<td>6.4</td>
<td>13-HODE and 9-HODEa</td>
<td>13-and 9-LOX/reductase linoleic acid metabolism</td>
<td>0.77</td>
</tr>
<tr>
<td>295.248/25.28</td>
<td>[M-H]</td>
<td>-</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>0.57</td>
</tr>
<tr>
<td>301.215/29.76</td>
<td>Fragment</td>
<td>-</td>
<td>Fragment of 5-HETE</td>
<td>5-LOX arachidonic acid metabolism</td>
<td>1.14</td>
</tr>
<tr>
<td>311.221/22.49</td>
<td>[M-H]</td>
<td>5.7</td>
<td>12,13-epoxy-9-hydroxy-10-octadecenoic acidb</td>
<td>9-LOX/POX linoleic acid metabolism</td>
<td>0.62</td>
</tr>
<tr>
<td>311.221/23.18</td>
<td>[M-H]</td>
<td>5.7</td>
<td>9,10-epoxy-13-hydroxy-11-octadecenoic acidb</td>
<td>13-LOX/POX linoleic acid metabolism</td>
<td>0.71</td>
</tr>
<tr>
<td>319.226/29.76</td>
<td>[M-H]</td>
<td>5.9</td>
<td>5-HETEb</td>
<td>5-LOX arachidonic acid metabolism</td>
<td>1.24</td>
</tr>
<tr>
<td>321.263/29.55</td>
<td>[M-H]</td>
<td>-</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>0.83</td>
</tr>
<tr>
<td>329.232/13.87</td>
<td>[M-H]</td>
<td>3.0</td>
<td>9,12,13-TriHOME and 9,10,13-TriHOMEa</td>
<td>13- and 9-LOX/EH linoleic acid metabolism</td>
<td>0.60</td>
</tr>
<tr>
<td>335.221/22.02</td>
<td>[M-H]</td>
<td>3.5</td>
<td>LTBb</td>
<td>5-LOX arachidonic acid metabolism</td>
<td>1.17</td>
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<tr>
<td>343.251/28.53</td>
<td>[M-H]</td>
<td>-</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>0.71</td>
</tr>
<tr>
<td>429.299/26.50</td>
<td>[M-H]</td>
<td>3.2</td>
<td>7α-hydroxy-3-oxo-4-cholestoneic acidb</td>
<td>7α-hydroxylase cholesterol metabolism</td>
<td>0.60</td>
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<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Total cholesterol</td>
<td>-</td>
<td>0.57</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LDL-C</td>
<td>-</td>
<td>0.60</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LDL:HDL ratio</td>
<td>-</td>
<td>0.70</td>
</tr>
</tbody>
</table>

aIdentified using commercial standard
bTentatively identified using MS/MS and METLIN/LIPID MAPS databases

Fold change was calculated by dividing the mean of normalized intensity of each plasma metabolite after consumption of one experimental diet by the mean intensity of the same plasma metabolite after consumption of another experimental diet

P < 0.05 was assigned to be significant

FDR q value was calculated for correction of falls-positives

13-oxo-9,11-octadecadienoic acid (13-OxoODE), 9-oxo-10,12-octadecadienoic acid (9-OxoODE), 13-hydroxy-9,11-octadecadienoic acid (13-HODE), 9-hydroxy-10,12-octadecadienoic acid (9-HODE), hydroxyoctadecadienoic acid (HODE), 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME) and 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-TriHOME), 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (LTB5), low density lipoproteins-cholesterol (LDL-C), high density lipoproteins-cholesterol (HDLC), lipoxygenase (LOX), peroxygenase (POX), epoxide hydrolase (EH)
Table 4. List of urinary metabolites discriminating between RB and WB intake

<table>
<thead>
<tr>
<th>m/z / RT</th>
<th>Ion</th>
<th>Mass of unconjugated ion</th>
<th>Mass accuracy (ppm)</th>
<th>Metabolites</th>
<th>Pathways</th>
<th>RB versus WB (spot urine)</th>
<th>RB versus WB (24 h urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fold change&lt;sup&gt;c&lt;/sup&gt;</td>
<td><em>P</em> value&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>134.059/6.74</td>
<td>Fragment</td>
<td>-</td>
<td>-</td>
<td>Fragment of hippuric acid</td>
<td>-</td>
<td>34.4</td>
<td>0.002</td>
</tr>
<tr>
<td>158.081/5.14</td>
<td>[M-H]</td>
<td>-</td>
<td>-</td>
<td>Unidentified</td>
<td>-</td>
<td>0.41</td>
<td>0.090</td>
</tr>
<tr>
<td>165.055/10.58</td>
<td>[M-H]</td>
<td>-</td>
<td>0.6</td>
<td>3-phenyllacteic acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tyrosine metabolism/fermentation</td>
<td>n.d&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.50</td>
</tr>
<tr>
<td>178.049/6.76</td>
<td>[M-H]</td>
<td>-</td>
<td>3.9</td>
<td>Hippuric acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Phenolic acids metabolism/fermentation</td>
<td>2.92</td>
<td>0.002</td>
</tr>
<tr>
<td>187.006/10.26</td>
<td>[M-H+Sulfate]&lt;sup&gt;-&lt;/sup&gt;</td>
<td>107.0489</td>
<td>6.5</td>
<td>p-cresol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Tyrosine and 4-hydroxyphenylacetic acid metabolism/fermentation</td>
<td>3.06</td>
<td>0.166</td>
</tr>
<tr>
<td>187.096/14.94</td>
<td>[M-H]</td>
<td>-</td>
<td>6.5</td>
<td>Azelaic acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>7.99</td>
<td>0.006</td>
</tr>
<tr>
<td>192.065/8.35</td>
<td>[M-H]</td>
<td>-</td>
<td>2.6</td>
<td>2-methylhippuric acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Phenolic compounds metabolism/fermentation</td>
<td>2.47</td>
<td>0.003</td>
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<td>204.065/14.16</td>
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<td>-</td>
<td>5.8</td>
<td>5-methoxy-3-indoleacetic acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tryptophane metabolism/fermentation</td>
<td>5.69</td>
<td>0.026</td>
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<tr>
<td>217.108/11.39</td>
<td>[M-H]</td>
<td>-</td>
<td>2.3</td>
<td>Hydroxysebacic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>16.9</td>
<td>0.013</td>
</tr>
<tr>
<td>250.071/6.86</td>
<td>[M-H]</td>
<td>-</td>
<td>1.9</td>
<td>N-feruloylglycine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Phenolic acids metabolism/fermentation</td>
<td>42.2</td>
<td>0.003</td>
</tr>
<tr>
<td>261.128/6.15</td>
<td>[M-H]</td>
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<td>-</td>
<td>Unidentified</td>
<td>-</td>
<td>9.61</td>
<td>0.021</td>
</tr>
<tr>
<td>283.081/9.56</td>
<td>[M-H+Glucuronide]&lt;sup&gt;-&lt;/sup&gt;</td>
<td>107.0489</td>
<td>6.5</td>
<td>p-cresol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Tyrosine and 4-hydroxyphenylacetic acid metabolism/fermentation</td>
<td>2.82</td>
<td>0.115</td>
</tr>
<tr>
<td>285.061/4.93</td>
<td>[M-H+Glucuronide]&lt;sup&gt;-&lt;/sup&gt;</td>
<td>109.0295</td>
<td>5.5</td>
<td>Quinol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Tyrosine and isoflavone metabolism/fermentation</td>
<td>n.d.</td>
<td>19.6</td>
</tr>
<tr>
<td>289.038/7.81</td>
<td>[M-H+Sulfate]&lt;sup&gt;-&lt;/sup&gt;</td>
<td>209.0782</td>
<td>-</td>
<td>Unidentified</td>
<td>-</td>
<td>n.d.</td>
<td>28.2</td>
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<tr>
<td>289.166/13.63</td>
<td>[M-H]</td>
<td>-</td>
<td>3.8</td>
<td>9,12-dihydroxy-tetradecanedioic acid&lt;sup&gt;d&lt;/sup&gt; (novel)</td>
<td>unidentified</td>
<td>98.8</td>
<td>0.009</td>
</tr>
<tr>
<td>297.113/28.01</td>
<td>[M-H]</td>
<td>-</td>
<td>0.6</td>
<td>Enterolactone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Plant lignans metabolism/fermentation</td>
<td>n.d.</td>
<td>18.1</td>
</tr>
<tr>
<td>326.086/4.64</td>
<td>[M-H+Glucuronide]&lt;sup&gt;-&lt;/sup&gt;</td>
<td>150.0647</td>
<td>-</td>
<td>Unidentified</td>
<td>-</td>
<td>34.4</td>
<td>0.003</td>
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<tr>
<td>330.190/12.59</td>
<td>[M-H]</td>
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<td>-</td>
<td>Unidentified</td>
<td>-</td>
<td>0.34</td>
<td>0.062</td>
</tr>
<tr>
<td>345.227/27.84</td>
<td>[M-H]</td>
<td>-</td>
<td>1.7</td>
<td>Dihydroxy-octadecanedioic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>unidentified</td>
<td>22.9</td>
<td>0.027</td>
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<tr>
<td>349.199/25.38</td>
<td>[M-H]</td>
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<td>-</td>
<td>Unidentified</td>
<td>-</td>
<td>n.d.</td>
<td>3.18</td>
</tr>
<tr>
<td>357.081/3.35</td>
<td>[M-H+Glucuronide]&lt;sup&gt;-&lt;/sup&gt;</td>
<td>181.0505</td>
<td>2.2</td>
<td>Homovanillic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Tyrosine metabolism/fermentation</td>
<td>26.9</td>
<td>0.011</td>
</tr>
<tr>
<td>m/z</td>
<td>[M-H]</td>
<td>Glucuronide</td>
<td>Retention Time</td>
<td>Peak Area</td>
<td>Assignment</td>
<td>p Value</td>
<td>FDR q Value</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>-------------</td>
<td>----------------</td>
<td>-----------</td>
<td>------------</td>
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<td>-------------</td>
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<tr>
<td>363.251/32.30</td>
<td>[M-H]</td>
<td>-</td>
<td>Unidentified</td>
<td>-</td>
<td>n.d.</td>
<td>0.32</td>
<td>&lt;0.001</td>
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<td>361.150/15.86</td>
<td>[M-H+Glucuronide]</td>
<td>185.1177</td>
<td>0.3</td>
<td>Hydroxy-decenoic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>unidentified</td>
<td>3.59</td>
<td>0.009</td>
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<tr>
<td>369.080/3.97</td>
<td>[M-H+Glucuronide]</td>
<td>193.0500</td>
<td>0.5</td>
<td>Ferulic acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>12.3</td>
<td>0.011</td>
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<td>[M-H+Glucuronide]</td>
<td>209.1013</td>
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<td>Unidentified</td>
<td>-</td>
<td>66.8</td>
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<tr>
<td>387.165/21.59</td>
<td>[M-H+Glucuronide]</td>
<td>211.1544</td>
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<td>Unidentified</td>
<td>-</td>
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<td>0.032</td>
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<tr>
<td>405.170/15.00</td>
<td>[M-H+Glucuronide]</td>
<td>229.1455</td>
<td>6.9</td>
<td>Dodecanedioic&lt;sup&gt;b&lt;/sup&gt;</td>
<td>unidentified</td>
<td>0.52</td>
<td>0.049</td>
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<tr>
<td>413.147/13.76</td>
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<td>237.1351</td>
<td>-</td>
<td>Unidentified</td>
<td>-</td>
<td>18.8</td>
<td>0.023</td>
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<tr>
<td>417.211/29.88</td>
<td>[M-H+Glucuronide]</td>
<td>241.1806</td>
<td>1.2</td>
<td>Hydroxy-tetradecenoic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>unidentified</td>
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<td>0.093</td>
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<td>419.227/35.75</td>
<td>[M-H+Glucuronide]</td>
<td>243.1951</td>
<td>3.7</td>
<td>Hydroxy-tetradecanoic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>unidentified</td>
<td>n.d.</td>
<td>21.3</td>
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<td>431.194/18.53</td>
<td>[M-H+Glucuronide]</td>
<td>255.1608</td>
<td>4.7</td>
<td>2-methyl-9-tridecenedioic acid or 4-tetradecenedioic acid&lt;sup&gt;d&lt;/sup&gt; (novel)</td>
<td>unidentified</td>
<td>4.41</td>
<td>0.005</td>
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<tr>
<td>473.146/17.65</td>
<td>[M-H+Glucuronide]</td>
<td>297.1109</td>
<td>5.7</td>
<td>Enterolactone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Plant lignans metabolism/fermentation</td>
<td>35.9</td>
<td>0.002</td>
</tr>
<tr>
<td>489.139/12.67</td>
<td>[M-H+Glucuronide]</td>
<td>313.1066</td>
<td>2.8</td>
<td>Hydroxy-enterolactone&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Plant lignans metabolism/fermentation</td>
<td>7.32</td>
<td>0.029</td>
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<tr>
<td>505.262/23.83</td>
<td>[M-H+Glucuronide]</td>
<td>329.2323</td>
<td>3.0</td>
<td>9,12,13-TriHOME and 9,10,13-TriHOME&lt;sup&gt;g&lt;/sup&gt;</td>
<td>13-and 9-LOX/EH linoleic acid metabolism</td>
<td>0.42</td>
<td>0.018</td>
</tr>
</tbody>
</table>

<sup>a</sup>Identified using commercial standard

<sup>b</sup>Tentatively identified using MS/MS and METLIN/LIPID MAPS databases

<sup>c</sup>Fold change was calculated by dividing the mean of normalized intensity of each plasma metabolite after consumption of one experimental diet by the mean intensity of the same plasma metabolite after consumption of another experimental diet

<sup>d</sup><sup>P</sup> < 0.05 was assigned to be significant

<sup>e</sup>FDR q value was calculated for correction of false-positives

<sup>f</sup>not detected

<sup>g</sup>9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME) and 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-TriHOME), lipoxygenase (LOX), epoxide hydrolase (EH)
Figure 1. PLS-DA score plots and their corresponding regression coefficients plots for plasma (a and b), spot urine (c and d) and 24 h urine (e and f). The RB diet is represented with (□) and the WB diet is represented with (○). Ellipse marks 95% of Hotelling T^2 showing possible outliers. The highest regression coefficients are marked with identified metabolites. Abbreviations/systematic names: 13-oxo-9,11-octadecadienoic acid (13-OxoODE), 9-oxo-10,12-octadecadienoic acid (9-OxoODE), 13-hydroxy-9,11-octadecadienoic acid (13-HODE), 9-hydroxy-10,12-octadecadienoic acid (9-HODE), 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME) and 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-TriHOME), 5,12-dihydroxy-6,8-10,14-eicosatetraenoic acid (LTB₄), low density lipoproteins-cholesterol (LDL-C), high density lipoproteins-cholesterol (HDL-C)
Figure 2. Heat map alignment of the RB and WB breads and plasma (after consumption of RB and WB). Metabolite’s m/z / RT and the name of identified metabolites are listed in the heat map. The scale map for heat intensity is shown below. Abbreviations/systematic names: 13-oxo-9,11-octadecadienoic acid (13-OxoODE), 9-
oxo-10,12-octadecadienoic acid (9-OxoODE), 13-hydroxy-9,11-octadecadienoic acid (13-HODE), 9-hydroxy-10,12-octadecadienoic acid (9-HODE), 7-hydroxy-4,8-octadecadienoic acid (7-HODE), 8-hydroxy-5,9-octadecadienoic acid (8-HODE), 11-hydroxy-7,9-octadecadienoic acid (11-HODE), 8-hydroxy-6-octadecenoic acid (8-HOME), 9-hydroxy-7-octadecenoic acid (9-HOME), 9-hydroxy-10-octadecenoic acid (9-HOME), 8-hydroxy-9-octadecenoic acid (8-HOME), 9-hydroperoxy-10,12-octadecadienoic acid (9-HpODE), 13-hydroperoxy-9,11-octadecadienoic acid (13-HpODE), 9,10-dihydroxy-12-octadecenoic acid (9,10-DiHOME), 12,13-dihydroxy-9-octadecenoic acid (12,13-DiHOME), 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE), 11-hydroxy-5,8,11,14-eicosatetraenoic acid (11-HETE), 8-hydroxy-5,9,11,14-eicosatetraenoic acid (8-HETE), 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 9,12,13-trihydroxy-10,15-octadecadienoic acid (9,12,13-TriHODE), 9,12,13-trihydroxy-5,10-octadecadienoic acid (9,12,13-TriHOME), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME) and 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-TriHOME), 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid (5,15-DiHETE), 5-12-dihydroxy-6,8-10,14-eicosatetraenoic acid (LTB₄), 5,11-dihydroxy-6,8,12,14-eicosatetraenoic acid (5,11-DiHETE), phosphatidylcholines (PC), phosphoserines (PS), lysophosphatidylcholines (LPC), hyodeoxycholic acid (HDCA), ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA)
Figure 3. Heat map alignment of spot urine and 24 hours urine after consumption of the RB and WB diets. Metabolite’s m/z / RT and the name of identified metabolites are listed in the heat map. The scale map for heat intensity is shown below. Abbreviations/systematic names: hyocholic acid (HCA), hyodeoxycholic acid (HDCA), ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME)
Figure 4. Heat map alignment of the breads, plasma, spot urine and 24 hours urine after consumption of the RB and WB diets. One metabolite was detected in the breads, plasma and urine, 16 metabolites were similar between bread and plasma, 3 were similar between plasma and urine and 2 were similar between bread and urine. Identified metabolites are listed in the heat map. The scale map for heat intensity is shown below. Abbreviations/systematic names: 13-oxo-9,11-octadecadienoic acid (13-OxoODE), 9-oxo-10,12-octadecadienoic acid (9-OxoODE), 13-hydroxy-9,11-octadecadienoic acid (13-HODE), 9-hydroxy-10,12-octadecadienoic acid (9-HODE), hyocholic acid (HCA), hyodeoxycholic acid (HDCA), ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME), 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-TriHOME), phosphoserines (PS), lysophosphatidylcholines (LPC)
Figure 1S. PCA scores plots of quality control (QC) samples and first and second injections, a) plasma, b) spot urine, c) 24 hours urine. First injection (○), second injection (□), QC samples (+).
Figure 2S. MS/MS’ spectra of novel dicarboxylic acids tentatively identified in urine of pigs. Proposed structures of the acids are presented. a) 9,12-dihydroxy-tetradecanedioic acid, b) 4-tetradecenedioic acid or 2-methyl-9-tridecenedioic acid glucuronide.
**General discussion**

The main objective of the PhD study was to identify biomarkers in plasma and urine after consumption of diets with contrasting DF composition. Based on multivariate data analyses two classes of metabolites discriminated between the diets: fatty acids and phenolics. Fatty acids such as oxylipins discriminated between the diets in both plasma and urine, whereas phenolics were the major group of metabolites in urine that differentiated between the diets. Moreover, novel metabolites were detected and identified. This general discussion will connect the findings in paper I, II and III as well as supplementary data not included in the papers. Moreover, broad discussion on the metabolic pathways and bioconversion of detected metabolites as well as their possible health effects will be given.

**Linoleic acid and its oxylipins in the diets**

The discovery of oxylipins deriving from the linoleic acid cascade in cereals date back to 1970, where Craveland (64) analysed the oxidation products of extracts of wheat flour-water suspensions and dough. He came to the conclusion that the action of LOX led to the formation of two isomeric hydroperoxy-octadecadienoic acids (HPODE) and to two isomeric hydroxy-epoxy-octadecenoic acids. Reduction led to isomeric hydroxy-ocadacadienoic acids (HODEs), whereas hydrolyses of hydroxy-epoxy-octadecenoic acids led further to the formation of trihydroxy-octadecenoic acids (TriHOMEs), as an end product by an unknown enzyme. Few years later in 1975 Mann and Morrison (65) confirmed the conversion of linoleic acid to TriHOMEs. In 1988 Gardner published a book chapter on “Lipoxygenase pathway in cereals”, where the enzyme which converts hydroperoxides to TriHOMEs was called flour-isomerase. He wrote that two hydroperoxide-reactive enzymes were identified in cereals; one that is mainly resided in cereal germ and another one that is mainly localized in the endosperm and therefore caused the oxidation of flour (66).

Recently, Levandi et al., (11) used a LC-MS method to detect and identify oxylipins in different varieties of wheat. Oxylipins were mainly derivatives of the linoleic acid cascade. In our study, we detected and identified oxylipins in flour and breads made from wheat and rye (paper II and III). In addition to the oxylipins belonging to the linoleic acid cascade, we detected other isomers of HODEs and HOMEs, Figure 13. At least five isomers of HODEs and four isomers of HOMEs were identified (Appendix A). Other oxylipins such as dihydroxy-octadecadienoic acids (DiHODEs) and oxo-octadecadienoic acids (oxo-ODEs) were also identified and many more were not fully identified. This plethora of oxylipins point towards the existence of numerous enzymatic reactions,
but auto-oxidation is also important to consider. From the literature it is known that auto-oxidation can lead to the formation of hydroperoxides at C8, 9, 10, 12, 13 and 14, which can further auto-oxidize until stable products are formed, i.e. the end products of the linoleic acid cascade like isomeric HODEs and TriHOMEs (16). The main difference between enzymatic and auto-oxidative reaction is formation of \( R \)-isomers of auto-oxidation, which results in racemic mixtures. Lee et al., (67) reported that MS/MS spectra for \( R \)- and \( S \)-isomers of oxylipins such as 9-HODE and 13-HODE were identical, which is probably also factual for other oxylipins. Therefore, in our study it was not possible to distinguish between \( R \)- and \( S \)-configurations of oxylipins and identify whether these oxylipins were formed due to auto-oxidation, for example during samples storage or due to enzymatic reactions. If the oxylipins were formed due to storage conditions, then our results may concern the aspect related to the aging of flour and bread. Since both flour and bread contain PUFAs, they cannot be considered as stable food products. Storage conditions such as temperature, exposure to light and oxygen and moisture content influence stability of these lipids to a high degree. Moreover, the rate of auto-oxidation also depends on the degree of unsaturation. Model studies on auto-oxidation revealed that the induction period for linoleic acid is 19 h, whereas it is only 1,5 h for \( \alpha \)-linolenic acid at 25°C (16). Oxidation of PUFAs causes deterioration in taste, flavour, odour, colour and texture and therefore decreases the shelf-life of flour and bread (16). For example it is known that TriHOMEs elicit a bitter taste and therefore cause the off-flavour formation in flour (16) (66). From a food quality and food safety perspective the oxidation of PUFAs and the formation of oxylipins in cereals is therefore a major concern.

**Linoleic acid and its oxylipins in plasma**

LC-MS analyses of plasma after consumption of the breads also showed the presence of oxylipins (paper II and III). However, the chromatographic alignment of plasma and breads displayed that many more isomers of oxylipins were detected in the bread compared to plasma (paper III). In general, oxylipins that were conserved in both plasma and breads belonged to the linoleic acid cascade. In plasma, if these oxylipins have enzymatic origin they may have exogenous as well as endogenous sources. Since these oxylipins were present in the breads, they can be absorbed in the small intestine of the pig and further transported to the blood circulation. It is known that the absorption route of saturated long chain fatty acids is through the lymphatic pathway (68). On the other hand depending on their hydrophobic/hydrophilic properties linoleic acid and oxylipins may have two absorption routes; portal vein system and/or lymphatic system. McDonald et al., (69) reported that 58 % of linoleic acid bypassed the lymphatic pathway when infused in the rat.
Our result on porto-arterial difference of linoleic acid and its oxylipins like 9,12,13- and 9,10,13-TriHOME and 13- and 9-HODE showed negative absorption indicating that the major proportion of these acids were absorbed through the lymphatic pathway (unpublished results). Once absorbed, linoleic acid is transported to the tissue and liver for further metabolism whereas the role of oxylipins in the body is still largely unexplored (24).

Another source of oxylipins in plasma may come from the endogenous oxidation of linoleic acid by LOX. Linoleic acid is the major PUFA in wheat and rye as well as in the rapeseed oil, which was added to the diets, and found to be highly digestible in pigs (70). Rapeseed oil was added to the diets in both studies, I and II, and accounted for approximately 80% (Study I) and 20% (study II) of the total lipid content. Rapeseed oil may therefore, in addition to the wheat and rye, have been a source of linoleic acid and its oxidation products in plasma.

**Linoleic acid and 13- and 9-HODE, health effects**

The human consumption of linoleic acid (ω-6 fatty acid) increased rapidly since the introduction of vegetables oils in 1913, while the consumption of ω-3 fatty acids decreased (28). The increased consumption of linoleic acid is now associated with increased risk of developing chronic inflammation diseases (27) (28). It is believed that the composition of cell membranes especially those of platelets, erythrocytes, neutrophils, monocytes and liver cells depends greatly on the diet (28) (27). Moreover the fatty acid composition of HDL, LDL, VLDL and free fatty acid in plasma is also affected. Furthermore, linoleic acid can be oxidized by both LOX and ROS leading to the formation of oxylipins, which can interfere/contribute to the oxylipins produced from arachidonic and eicosapentaenoic acids that regulate the process of inflammation. The physiological role of linoleic acid derived oxylipins, however, is at present not fully elucidated and controversial results exist. Especially the role of 13- and 9-HODE in the development of cancer and arteriosclerosis is very much discussed (24). An *in vitro* model with Caco-2 cells showed, that exogenous addition of 13-HODE in the concentration range of 10-1000 nM induced a concentration dependent cell proliferation, where the enantiomeric form 13(R)-HODE presented higher mitogenic effects than 13(S)-HODE (71). Another *in vitro* model suggested that 13-HODE have anti-tumorigenic effect (72). The results of Buchanan et al., (73) showed that 13-HODE had anti-atherogenic properties mainly by inhibiting binding of platelets to the endothelium. Since many of the studies reported on 13-HODE were performed using *in vitro* models, the controversial results point towards that study design may play an important role on the outcome and result interpretation. Moreover, the *in vitro*
studies showed that the properties of 13-HODE were concentration dependent. It is reasonable to think that while oxylipins can be derived by coordinated enzymatic reaction, auto-oxidation or a combination of both, the effect of these compounds may depend on their local concentration rather than on their absence or presence. Since we did not perform quantification of oxylipins in our study it is not possible to conclude whether their concentrations could be considered as health detrimental or beneficial. Therefore, detection of linoleic derived oxylipins in plasma does not necessarily mean that the pigs developed a chronic inflammation disease, but it can be an indication of high linoleic acid consumption. Moreover, 13- and 9-HODE may be potential lipid biomarkers of oxidative stress and the risk of developing for example arteriosclerosis. Therefore there is a demand for controlled in vivo models to study the physiological effects of these highly bioactive molecules, and to establish the physiological level at which they may have health beneficial or deteriorating effects.

**Phenolic acids in the diets**

In study I, phenolic acid such as ferulic acid, sinapic acid, p-coumaric acid, caffeic acid, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, syringic acid and salicylic acid were quantified in the three diets, WGW, WAF and RWF, consumed by pigs (paper I). We found ferulic acid and sinapic acid to be the major bound phenolic acids in all three diets, which compares well with previous results on phenolic acids in wheat (39). Furthermore, we have measured that ferulic acid accounted for more than 60 % of total phenolic acids in the diets WGW and WAF and 49 % in the RWF diet, whereas sinapic acid was measured to be responsible for approximately 30 % of total phenolic acids content in all three diets. The most literature on phenolic acids in wheat reports higher values for ferulic acid and lower values for sinapic acid contents compared to measured values in our study (39) (40). The reason for this difference may be related to the large variations in the content of phenolic acids, which depends on the cultivation conditions, wheat genotype and wheat varieties, but also baking and storage conditions can influence the content of phenolic acid in the bread (39) (74) (40). Ferulic acid and sinapic acid belong to the cinnamic acid derivatives, which are also known to be the major phenolic acids in wheat compared to benzoic acid derivatives that are generally present at low levels (39). Cinnamic acid derivatives accounted for more than 90 % of total phenolic acid content in the diets WGW and WAF and for 88 % in the diet RWF. In general, our quantitative analyses on phenolic acids in wheat are in good agreements with previous results reported on cereal (38) (39).
**Phenolic acids in plasma and urine, health effects**

Plant phenolic acids are known as natural antioxidants and therefore their consumption is associated with beneficial effects on health, since they can capture ROS and thereby prevent lipid oxidation (16). However, numerous studies on the consumption of food rich in phenolic acids report low bioavailability and high conversion in the colon to other phenolic acids together with a rapid urinary excretion (47) (49) (48). In our study, using targeted LC-MS analyses we found an agreement with previous studies that the bioavailability of ferulic acid was low, 0.4-2.9 % of ingested phenolic acids (paper I). The bioavailability, however, depends to a large extent on the bioaccesability of phenolic acids in the plant matrix. For instance, Harris et al. (75) found that the aleurone cell walls of wheat bran were degraded to a higher degree than pericarp walls that were not degraded, when isolated from the feces of rats. In support of these findings our results showed that the plasma concentration of ferulic acid and caffeic acid was higher after WAF consumption compared to WGW, indicating a higher bioaccesability of phenolic acids in WAF compared to WGW (paper I).

Even though, ferulic acid is an effective natural antioxidant with high abundance in both wheat and rye, its low concentration in plasma is not sufficient to induce the antioxidative effect (47). Since there is a high inter-conversion to other phenolic acids it seems important to consider whether the metabolic products of plant phenolic acids such as hydroxyphenylpropionic acid, hydroxybenzoic acid and hippuric acid can be potential antioxidants and contribute to the antioxidative effect *in vivo*. Gontier et al., (76) reported that 3-hydroxyphenylpropionic acid and hippuric acid were the main phenolic acids detected in plasma after chlorogenic acid supplementation in rats. Moreover, hippuric acid was the only phenolic acid, which was detected in plasma after supplementation with chlorogenic acid, caffeic acid, quinic acid and control diet in concentration ranging from 41.1 to 98.2 µM and highest after chlorogenic acid supplemented diet. We have detected \( p \)-hydroxybenzoic acid, which was the only phenolic acid with plasma concentration of 4 µM that is high enough to potentially induce the antioxidative effect (paper I). It has to be emphasized that we did not performed the quantification of hippuric acid in plasma, however, low intensity peak of hippuric acid was detected during non-targeted metabolomics analyses of plasma samples (unpublished results). The analyses of urine in Study I (unpublished) and Study II showed that hippuric acid and 2-methylhippuric acid were the main mammalian phenolic acids discriminating between whole grain wheat versus wheat aleurone (Study I) and RB versus WB (Study II), with higher concentration of these two phenolic acids after wheat aleurone (unpublished) and RB intake.
This finding of hippuric acid in urine is in agreement with other studies on consumption of diets rich in polyphenols, which indicates that hippuric acids is a potential biomarker of high polyphenol intake (44) (46). Moreover, it indicates that microbial fermentation and metabolism in the liver result in conversion of plant phenolic acids and probably other plant phenolics such as flavonoids to mammalian phenolic acids as it was seen in the case of lignans (fermentation of plant lignans to enterolactone) (77) (78). Since phenolic acids such as ferulic acid are plant’s natural antioxidants, it can be suggested that hippuric acid possess antioxidative activity in mammals, and may contribute to the antioxidative pool in plasma. However, to the best of our knowledge its antioxidative activity has not been proposed or reported. Therefore there is a demand for more research in this area.

The interaction between metabolism of oxylipins and phenolic acids

The major markers (based on multivariate data analyses) of particular diet consumption in both study I and II, were oxylipins derived from the linoleic acid cascade, such as isomeric 13- and 9-HODE and isomeric 9,12,13- and 9,10,13-TriHOME, Table 1.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Whole grain wheat vs refined flour</th>
<th>Wheat aleurone vs refined flour</th>
<th>Whole grain wheat vs wheat aleurone (Artery)</th>
<th>Whole grain wheat vs wheat aleurone (Vein)</th>
<th>RB vs WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>13- and 9-HODE</td>
<td>1.3*** &lt;i&gt;c&lt;/i&gt;</td>
<td>0.9</td>
<td>1.5**</td>
<td>1.4**</td>
<td>0.77**</td>
</tr>
<tr>
<td>9,12,13- and 9,10,13-TriHOME</td>
<td>1.04**</td>
<td>0.7</td>
<td>1.6**</td>
<td>1.3*</td>
<td>0.60**</td>
</tr>
<tr>
<td>5-HETE</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.24***</td>
</tr>
<tr>
<td>LTB&lt;sub&gt;4&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.17*</td>
</tr>
</tbody>
</table>

<i>a</i>Fold change was calculated by dividing the mean of normalized intensity of each plasma metabolite after consumption of one experimental bread by the mean intensity of the same plasma metabolite after consumption of another experimental bread

<i>b</i>FDR q value was calculated for correction of false-positives

<i>c</i>P value was calculated by a two-sample <i>t</i> test; *<0.05  **<0.01  ***<0.001

In study I, 13-HODE and 9-HODE were important markers of whole grain wheat versus wheat aleurone and refined wheat consumption. Since linoleic acid is mainly resident in the germ of the grain, but is removed during purification of aleurone and flour (79), it is reasonable that higher level of linoleic acid derived oxylipins were measured in the plasma after consumption of whole grain wheat versus aleurone and refined wheat (paper II). In study II, oxylipins derived from the linoleic
acid cascade were also responsible for discrimination between WB and RB. However, these oxylipins were elevated after WB (refined wheat) compared to RB (whole grain rye with added rye bran) (paper III). These conflicting results point towards that there must be other reasons for the discrimination. If the presence of oxidized PUFA in plasma was mainly due to endogenous turnover of lipids within the animal, then the inhibition of LOX activity by the different content of antioxidants in the diets could be one of the reasons for this discrimination. The beneficial effect of phenolic compounds on oxidative stress is well known (80). Higher concentration of ferulic acid, which is known as natural antioxidant, was measured in the aleurone diet versus whole grain wheat (paper I) and RB versus WB (paper III). Moreover, higher concentration of ferulic acid and caffeic acid was measured in plasma after intake of aleurone wheat diet compared to whole grain wheat (paper I). This could result in more effective inhibition of linoleic acid oxidation, and thereby overruling the influence of the germ. Moreover, since rye and wheat contain variety of phenolic compounds their synergetic effects cannot be excluded. Another reason could be the interaction of fatty acids with DF, which may result in reduced absorption of fatty acids and consistently lower concentration of linoleic acid and its oxylipins in plasma, the mechanism also observed for bile acid and sterols (4) (3) (81). Lærke et al., (2) reported that the digestibility of fat in the small intestine and throughout the colon was significantly reduced in the pigs fed RB compared to WB. Le Gall et al., (82) also reported that total tract digestibility of lipids in the wheat aleurone fraction was significantly lower than of the diets based on whole grain wheat and refined wheat. This may explain the lower concentration of linoleic acid derived oxylipins in plasma after RB and wheat aleurone consumption. The diets with contrasting DF composition may therefore influence the digestibility/excretion of fatty acids to a different extent, which can result in blood lipid-lowering effect for the diets where the interaction between fatty acids and DF is highest.

Other oxylipins which were major markers discriminating between RB and WB in study II, were oxylipins derived from arachidonic acid, 5-HETE and LTB₄, which were elevated after RB consumption, Table 1 (paper III). Since these oxylipins were not detected in study I, their presence in plasma in study II can be related to the differences between the experimental diets in these two studies. The pigs in study II were fed diets artificially high in cholesterol, and therefore the presence of these metabolites in plasma may indicate the development of chronic inflammation, as LTB₄ is considered an important pro-inflammatory lipid mediators (19) (20). Because significantly lower total cholesterol and LDL cholesterol concentration was measured by Lærke et al., (2) in plasma of pigs fed RB, their elevated level in plasma of pigs fed RB in our study was not expected. However, it
has to be emphasized that plasma samples analyzed in this study were taken at the end (after 9.5 weeks) of the experiment and no samples were analyzed, which could represent the beginning of the experiment. To be able to draw a conclusion it is important to know whether these metabolites were present from the start or their concentration rose gradually during the experimental period and therefore could be linked to the development of inflammation.

**The interaction between metabolism of cholesterol and bile acids**

High DF and polyphenol rich diet can influence the metabolism of cholesterol and bile acids in several ways. One of the mechanisms is associated with inhibition of cholesterol and bile acids uptake. For example in an *in vitro* model it was found that grape seed and red wine polyphenol extracts inhibited cellular cholesterol uptake (83). Several studies have reported that bile acids bind to soluble and insoluble DF and lignin, thereby leading to increased fecal excretion. Excretion of bile acids results in decreased entero-hepatic recycling of bile acids and thus increased de novo bile acids synthesis from cholesterol. This was previously reported as a mechanism for hypocholesterolemic effects of DF (4) (3) (81). Another mechanism is related to the production of bile acids in the liver. Lærke et al., (2) reported that the expression of the gene, CYP7A1, encoding cholesterol 7α-hydroxylase, the enzyme which converts cholesterol to bile acids, was significantly lower in pigs fed RB diet compered to WB. Moreover, total cholesterol and HDL cholesterol were measured to be higher after WB intake (Study II). Multivariate data analyses of plasma samples have shown that metabolite, 7α-hydroxy-3oxo-4-cholestenoic acid was elevated after WB consumption compared to RB (paper III). And since cholesterol is a precursor for 7α-hydroxy-3oxo-4-cholestenoic acid, intermediate in the synthesis of bile acids, its high concentration after WB intake is in agreement with the lower expression of CYP7A1 after RB reported by Lærke et al., (2). Fecal excretion of bile acids was not measured in the present study, but it can be expected to be higher based on finding in the literature (4) although it has to be confirmed by the future experiments.

Multivariate data analyses have also showed that bile acids discriminated between mesenteric artery and portal vein (paper I). The higher concentration of conjugated bile acids in the portal vein compared to artery is in agreement with their recirculation in entero-hepatic circulation, while unconjugated bile acids spill-over into the systemic circulation and can be excreted into urine via glucuronization in the liver. This was confirmed by the analyses of urine in paper III, where no conjugated bile acids were detected.
Use of multi-compartmental metabolomics

Multi-compartmental metabolomics was performed in study II on the biofluids, plasma and urine as well as the experimental breads used in this study. The data was used for alignments, with purpose to better understand the metabolic responses to a given intervention. The goal of multi-compartmental alignment is to get an overview of metabolites in different compartments and to track metabolites that are similar and/or different. Similarities or differences can point towards the absence or presence of metabolic conversions and help to identify their pathways. Out of 103 identified metabolites in this study, one metabolite was detected in all three compartments, 16 metabolites were similar between bread and plasma, 3 was similar between plasma and urine and 2 was similar between bread and urine, Figure 12. The metabolite that was detected in all three compartments, plasma, urine and the breads fed to the pigs, was 9,12,13-TriHOME. In urine this metabolite was detected conjugated with glucuronic acid. This result shows that a proportion of

9,12,13-TriHOME is absorbed in the gastrointestinal tract and undergo conjugation in the liver or kidney before its excretion into urine. Since 9,12,13-TriHOME was the only oxylipin of the linoleic acid cascade detected in urine, it may indicate that the higher number of hydroxyl groups facilitates its excretion into urine and possibly diminish its involvement in OX-LDL (in contrast to 13- and 9-HODE). Moreover, 9,12,13-TriHOME is also known for its high anti-fungal properties, and is used as effective defense compound by cereals such as wheat, rye and rice (84) (12). Whether it can possess any anti-microbial activity along the digestive tract or after its absorption, is not known. Other compounds, which were similar between urine and the breads, were azelaic acid and hydroxysebacic acid. These acids could pass the gastrointestinal tract and were excreted in urine.

Figure 12. Out of 50, 37 and 39 identified metabolites in bread, plasma and urine respectively, one metabolite was detected in all three compartments, 16 metabolites were similar between plasma and breads, 3 was similar between plasma and urine and 2 was similar between bread and urine.
without metabolic conversion. Our results are in agreement with the literature reported on azelaic and sebacic acids, which reports that a proportion (> 50 % for azelaic acid and 12 % for sebasic acid) of these acids is lost in urine after oral administration (85). Both acids are also characterized by rapid plasma and renal clearance (85). Their absence in plasma in our study is therefore due to their rapid excretion in urine, so the plasma concentration of these compounds remained low.

Metabolites that were similar between plasma and urine were bile acids, which were excreted conjugated with glucuronic acid. The majority of metabolites detected in urine were conjugated with glucuronic acid and few were conjugated with sulfate. It shows that conjugation promotes excretion of many compounds, which else would be too lipophilic to be excreted in urine.

The alignment between breads and plasma metabolic profiles showed that 16 metabolites were similar; the metabolites such as PUFA, lysophosphatidylcholines and oxylipins, Figure 13. However, there was also dissimilarity in the oxylipin profiles of plasma and breads. Many more isomers of oxylipins were detected in the breads compared to plasma, which can indicate the presence of plant specific enzymes. Metabolite masses such as 297, 313 and 327 were restricted to the breads and which could be tentatively identified as isomeric HOMEs, DiHOMEs and DiHODEs. Other oxylipins, eicosanoids, were only restricted to plasma, which is in agreement with arachidonic acid being a substrate for LOX activity in mammals (Appendix B).

The biggest dissimilarity between bread and plasma versus urine profiles was the detection of many medium chained, di- and monocarboxylic fatty acids containing hydroxyl and methyl groups (Appendix C) (paper III). Since these fatty acids were not detected in the breads consumed by pigs, they may have endogenous source, and originate from microbial bioconversion in the gastrointestinal tract or what is more likely they may be a result of ω- and/or β-oxidation in the liver. The literature on dicarboxylic acids indicates that they are formed through an ω-oxidation pathway, which may increase substantially due to fat-feeding (85). When β-oxidation is saturated by the excessive delivery of fatty acids to the liver, the alternative fatty acid oxidation pathway is activated. Under this condition dicaboxylic acids are produced and excreted in urine (86). Finding of dicarboxylic acid in urine of pigs can be therefore another indication, in addition to 5-HETE and LTB₄, in plasma, that pigs were overloaded with high fat diet.

In general, multi-compartmental metabolomics showed to be useful for tracking compounds in different body compartments and therefore better understand their metabolic involvement and identify their pathways.
Figure 13. Heat map alignment of the RB and WB breads and plasma (after consumption of RB and WB). Metabolite's m/z / RT and the name of identified metabolites are listed in the heat map. The scale map for heat intensity is shown below. Abbreviations/systematic names: 13-oxo-9,11-octadecadienoic acid (13-OxoODE), 9-oxo-10,12-octadecadienoic acid (9-OxoODE), 13-hydroxy-9,11-octadecadienoic acid (13-HODE), 9-hydroxy-10,12-octadecadienoic acid (9-HODE), 7-hydroxy-4,8-octadecadienoic acid (7-HODE), 8-hydroxy-5,9-octadecadienoic acid (8-HODE), 11-hydroxy-7,9-octadecadienoic acid (11-HODE), 8-hydroxy-6-octadecenoic acid (8-HOME), 9-hydroxy-7-octadecenoic acid (9-HOME), 9-hydroxy-10-octadecenoic acid (9-HOME), 8-hydroxy-9-octadecenoic acid (8-HOME), 9-hydroperoxy-10,12-octadecadienoic acid (9-HpODE), 13-hydroperoxy-9,11-octadecadienoic acid (13-HpODE), 9,10-dihydroxy-12-octadecenoic acid (9,10-DiHOME), 12,13-dihydroxy-9-octadecenoic acid (12,13-DiHOME), 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE), 11-hydroxy-5,8,11,14-eicosatetraenoic acid (11-HETE), 8-hydroxy-5,9,11,13-eicosatetraenoic acid (8-HETE), 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 9,12,13-trihydroxy-10,15-octadecadienoic acid (9,12,13-TriHODE), 9,12,13-trihydroxy-5,10-octadecadienoic acid (9,12,13-TriHODE), 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-TriHOME) and 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-TriHOME), 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid (5,15-DiHETE), 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (LTB4), 5,11-dihydroxy-6,8,12,14-eicosatetraenoic acid (5,11-DiHETE)
Use of LC-MS for detection and identification of metabolites

Identified compounds in this study can be categorized into three classes: fatty acids, bile acids and phenolics. Because negative ionization mode during full mass scan produced abundant \([M-H]\) ions, it was used for performance of tandem mass spectrometry for the compound identification. Since identification is important for biomarker elucidation several methods were used to confirm the identity of the marker; accurate masses, databases, authentic standards and smart formula (DataAnalyses from Bruker Daltonics). Due to the differences in the chemical structure of the identified metabolites, the pattern of their MS/MS spectra varied substantially. In the case of oxylipins MS/MS spectra generated unique and characteristic spectra that shared some common features such as the loss of water \([M-H_2O]\), which corresponded to the loss of hydroxyl group in the molecule (Appendix A and B). Moreover, fragment ions that were indicative of the position of hydroxyl group were observed. Charge-driven allylic fragmentation led to formation of specific oxylipins fragments, which made the isomeric identification and structural elucidation of these compounds possible. Similar observation was reported by MacMillan and Murphy (87), when analyzing lipid hydroperoxides by negative ion electrospray MS. In addition to the unique and characteristic spectra they had high ionization efficiency. Because of unique fragmentation oxylipins are also good candidates for targeted metabolomics analyses. However, MS/MS analyses failed to distinct enantiomers of oxylipins, as it was reported by Lee et al., (67), which therefore require development of chromatographic method if identification and quantification of racemic mixtures have to be performed.

The product-ion spectra of conjugated and unconjugated bile acids produced abundant \([M-H]\) and \([2M-H]\) ions. However, unconjugated bile acids detected in this study did not generated specific fragments during MS/MS analyses, where only low intensity ions corresponding to the loss of water were observed. Their identification was mainly performed based on the retention time of authentic standards (paper II). The insensitivity to fragmentation comes from the chemical structure of unconjugated bile acids, which is a steroid ring system of three cyclohexane and one cyclopentane fused together. This problem was also reported by Tagliacozzi et al., (88) while developing a simple and rapid method for quantification of bile acids in serum samples. They reported that the lack of any exploitable fragment reduce the specificity, which can be achieved by a unique neutral loss pattern. Therefore well-developed chromatographic separation becomes mandatory to discriminate among isomeric forms. In our study, in the case of UDCA and HDCA, the chromatographic method used, failed to separate these two bile acids, and therefore their identity
was not possible to specify (paper II). The fragmentation of glycine conjugated bile acids showed high intensity ions, which corresponded to neutral losses of \([\text{M-H-CO}_2^-\text{]}\), \([\text{M-H-H}_2\text{CO}_2^-\text{]}\) and/or \([\text{M-H-CO}_2^-\text{-H}_2\text{O}^-\text{]}\) and in the case of unsaturated bile acid the ion \([\text{M-H-C}_3\text{H}_4\text{NO}_3^-\text{]}\) was also observed (paper II). The intensity of these ions depended on the location of the ring hydroxyl groups, and therefore their fragmentation pattern may offer some degree of isomer differentiation. Since MS/MS analyses showed only some degree to the isomeric specify, the identity of conjugated bile acids was also confirmed by the retention time comparisons with authentic standards.

During MS scan, phenolic acids showed high degree of decomposition, which was however stabilized when compounds were conjugated with glycine, glucuronic acid or sulfate. In general, MS/MS spectra on phenolics were characterized by the loss of \(\text{CO}_2\), but unique fragments such as the loss of methyl moiety for ferulic acid was also observed. Since phenolic acids produce specific fragmentation patterns, they are well characterized compounds for quantitative measurements (paper I).

Using LC-MS/MS in this study it was possible to identify more than a hundred of different metabolites, and many more compounds was possible to classify. Moreover, novel metabolites were detected and their structure was proposed. However, identification using LC-MS/MS has some degree of complexity and requires investment of time.

**Use of a pig as a model for human subjects**

Pigs have been used as a model for human subjects in many years due to the physiological and anatomical similarity of digestive tract and metabolism between pig and man (53). This similarity has laid a foundation for the use of the pig in many human nutritional studies (53). In this study, we used plasma and urine samples from two studies where the pigs were fed humanized diets, breads with contrasting DF composition. The metabolites, which were identified by LC-MS metabolomics in this study, have been also reported in the studies with human subjects (89) (24) (44). According to the literature the bile acid composition varies among different animal species. Bile acids vary with respect to conjugation with glycine or taurine, degree of hydroxylation and position of hydroxyl group (90). It has been reported that the major bile acids identified in humans are glycine conjugated dihydroxylated and trihydroxylated bile acids and that is in contrast to chickens, rats, dogs and sheep whose bile acids are almost entirely taurine conjugated (89) (90). In our study, we have identified mainly glycine conjugated dihydroxylated and trihydroxylated bile acids in the plasma of pig (paper II), which is in agreement with identified bile acids in humans. Another
similarity between man and pig can be related to the detection of 5-HETE and LTB₄, which are well known pro-inflammatory mediators in humans (20). Detection of these pro-inflammatory markers in plasma of pigs after a cholesterol rich diets indicates that 13-HODE and 9-HODE may have similar physiological effects in pigs as it was observed in humans (24) (91). In this respect pig can potentially be a good in vivo model to study these highly bioactive compounds and to elucidate their biological effects. There is also a great similarity in colonic fermentation of phenolic acids between pigs and humans; for both species fermentation of phenolic acids results in formation of hippuric acid and hippuric acid derivatives (41) (44). The low bioavailability of ferulic acid also seems to be similar in the two species (47). However, the main hippuric acid derivative reported in human studies, was hydroxyhippuric acid, whereas we identified methylhippuric acid as being the main hippuric acid derivative in pigs. This may indicate that in humans, hippuric acid undergoes hydroxylation, whereas in pigs it is mainly methylated. The position at which hydroxylation take place in humans, was mainly identified at C3 (44) (41). We have identified 2-methylhippuric acid, which shows that methylation takes place at C2. This minor difference indicates that dissimilarities can occur, and that care has to be taken when using pig as a model in biomarker elucidation. In general, the results of this study are in agreement with the fact that pig has many similarities to man and can be considered a good model for human subjects.
Conclusions and perspectives

Using a LC-MS metabolomics approach we succeeded in detection of dietary effects on both the plasma and urine metabolic profiles. LC-MS/MS provided important information on the identity of the detected metabolites, which could be categorized into three major classes: fatty acids, bile acids and phenolics. Their reduced or elevated levels in plasma and urine of pigs could be related to both the diets consumed and to their possible health effects, such as it was discussed in the case of 5-HETE, LTB₄, 13-HODE and 9-HODE and hippuric acids. The metabolic responses of plasma and urine also pointed towards the endogenous relationship between the presence of antioxidants and inhibition of oxidation. Therefore, the results of the present study highlight the importance of consumption of non-refined carbohydrates and enrichment of bread with fractions rich in DF and bioactive components, such as aleurone. The presence of germ in whole grain products may contribute to elevated level of oxylipins when consumed. However, our result on this subject is not clear, and therefore requires more knowledge on subcellular localization of PUFAs and PUFA oxidative enzymes and their behavior during milling, baking and storage.

The establishment of physiological level of oxylipins in the blood and tissue, at which they may have health deteriorating effect, is another important aspect to consider. TriHOMEs, which are one of the major products of the linoleic acid cascade, are known for their bitter taste. We have detected these oxylipins in plasma and urine as well as in flour and breads. However, the physiological effects of TriHOME are not known at present and to the best of our knowledge our study is the first to report it excretion into urine. Controversial result on the physiological effects of 13- and 9-HODE suggest that there is a demand for controlled in vivo experiments and in this perspective the present study show that the pig can be considered as an excellent model for studying the physiological effects of oxylipins, which could be further linked to human subjects. Moreover, LC-MS/MS of oxylipins provided unique and characteristic spectra for their isomeric identification, and therefore can be considered as an excellent method for their identification.

Our results on the absorption of phenolic acids showed that phenolic acids have low bioavailability in plasma, which is due to their high bioconversion to other phenolic acids such as hippuric acid. Since hippuric acid is one the main phenolic acids formed as a result of metabolism in the colon and liver from other dietary phenolic compounds, the establishment of its contribution to the antioxidative effect may have important future perspectives.
Our results on the presence of oxidation products in flour and breads can be related to the aspect concerning the aging of flour and bread due to storage conditions and handling of flour and bread in the daily life of people. Since oxidation of PUFAs is not a matter of month, but of days or hours, depending on the conditions, development of methods for prevention of lipid oxidation in cereals may have important future perspectives. In living plants, PUFAs are relatively stable due to the presence of naturally occurring antioxidants and enzymes, which effectively prevent lipid oxidation. Even though both wheat and rye contain natural antioxidants such as ferulic acid, the major part is bound to the plant matrix and requires enzymatic activity to be released. Therefore addition of natural antioxidants to cereal products can be a solution in the means of increasing shelf life during storage and to reduce wastage and nutritional losses by inhibiting or delaying oxidation.

In conclusion, explorative LC-MS approach provided useful and novel information on the metabolic responses in plasma and urine of pigs after consumption of diets with contrasting DF composition.
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Appendix A

Oxylipins of linoleic acid cascade

13-HODE
13-hydroxy-9,11-octadecadienoic acid

9-HODE
9-hydroxy-10,12-octadecadienoic acid

12,13-epoxy-9-hydroxy octadecenoic acid

9,10-epoxy-13-hydroxy octadecenoic acid
**9,12,13-TriHOME**
9,12,13-trihydroxy-10-octadecenoic acid

**9,10,13-TriHOME**
9,10,13-trihydroxy-11-octadecenoic acid

**9,10-DiHOME**
9,10-dihydroxy-12-octadecenoic acid

**12,13-DiHOME**
12,13-dihydroxy-9-octadecenoic acid
Other oxylipins in wheat and rye diets

9-OxoODE
9-oxo-10,12-octadecadienoic acid

7-HODE
7-hydroxy-4,8-octadecadienoic acid

8-HODE
8-hydroxy-5,9-octadecadienoic acid

11-HODE
11-hydroxy-7,9-octadecadienoic acid
**9,12,13-TriHODE**

9,12,13-trihydroxy-10,15-octadecadienoic acid

**9,12,13-TriHODE**

9,12,13-trihydroxy-5,10-octadecadienoic acid
### Appendix B

**Eicosanoids detected in plasma of pigs**

<table>
<thead>
<tr>
<th>m/z</th>
<th>Intens. [%]</th>
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<tbody>
<tr>
<td>149.0966</td>
<td>150</td>
</tr>
<tr>
<td>167.1071</td>
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<tr>
<td>275.2328</td>
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<tr>
<td>301.2197</td>
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<td>319.2254</td>
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**15-HETE**

15-hydroxy-5,8,11,13-eicosatetraenoic acid

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<td>149.1302</td>
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<td>163.1267</td>
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<td>203.1766</td>
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**11-HETE**

11-hydroxy-5,8,11,14-eicosatetraenoic acid

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**8-HETE**

8-hydroxy-5,9,11,14-eicosatetraenoic acid

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<td>155.0714</td>
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<td>179.1106</td>
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<td>203.1804</td>
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**12-HETE**

12-hydroxy-5,8,10,14-eicosatetraenoic acid

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<td>164.1575</td>
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<td>177.1659</td>
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<td>203.1932</td>
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**5-HETE**

5-hydroxy-6,8,11,14-eicosatetraenoic acid

<table>
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<th>Intens. [%]</th>
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<td>229.1964</td>
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141
5,15-DiHETE
5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid

LTB4
5-12-dihydroxy-6,8,10,14-eicosatetraenoic acid

5,11-DiHETE
5,11-dihydroxy-6,8,12,14-eicosatetraenoic acid

LTB4
5-12-dihydroxy-6,8,10,14-eicosatetraenoic acid
Appendix C

Medium chained, di- and monocarboxylic acids (un)conjugated with glucuronic acid detected in urine

Hydroxy-decenoic acid

Dodecanedioic acid

Hydroxy-tetradeconoic acid

Hydroxy-tetradecanoic acid
9,12-dihydroxy-tetradecanedioic acid

4-tetradecenedioic acid or 2-methyl-9-tridecenedioic acid