New Alkylresorcinol Metabolites

Identification, Quantification and Evaluation as Dietary Biomarkers

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Abstract

Alkylresorcinols (AR) are amphiphilic phenolic lipids that are extensively metabolized in the liver and form metabolites that can be detected in plasma and urine. Two AR metabolites, 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-propanoic acid (DHPPA), have been evaluated as urinary biomarkers of whole grain wheat and rye intake. AR metabolite concentrations are currently measured by chromatographic techniques but for samples from large epidemiological studies, immunoassay techniques are preferred due to rapid and cheap analysis. The aim of this thesis was to develop high-throughput analytical methods for the analysis of alkylresorcinol metabolites and to evaluate recently discovered putative metabolites 3,5-dihydroxycinnamic acid (DHCA), 5-(3,5-dihydroxyphenyl) pentanoic acid (DHPPTA), 3,5-dihydroxycinnamic acid amide (DHCA-amide) and 2-(3,5-dihydroxybenzamido) acetic acid (DHBA-glycine) as biomarkers of whole grain wheat and rye intake.

Polyclonal antibodies against DHBA and DHPPA were produced and characterized in terms of cross-reactivity, assay sensitivity, precision and accuracy. A developed ELISA method was used for analysis of urine samples and results were compared with GC-MS. A lack of agreement was found. Several putative AR metabolites were identified by LC-MS/MS, including DHPPTA, DHCA, DHBA-glycine and DHCA-amide. These compounds showed high cross reactivity in ELISA and likely explains the lack of agreement between the two methods.

The medium-term reproducibility and relative validity of DHCA, DHPPTA and DHBA-glycine were found to be moderate to excellent in 24h urine collections. Moreover, the reproducibility was good for these metabolites also in spot-urine samples. Age and sex were found to be important determinants besides whole grain intake. The results suggest that determination of AR metabolites in 24h urine and single or duplicate spot urine samples appear to be suitable medium to long-term biomarkers of whole grain wheat and rye intake. These findings need to be confirmed in other populations before using these biomarkers in epidemiological studies.

Keywords: Alkylresorcinol, ELISA, antibodies, dietary biomarker, AR metabolites, whole grain

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Dedication

To my grandfather

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Wierzbicka, R., Eyer, L, Landberg, R., Kamal-Eldin, A. & Franek, M. (2014). Development of antibodies for determination of alkylresorcinol metabolites in human urine and elucidation of ELISA cross-reactivity. *Journal of Immunological Methods* 413, 12-24.

II Wierzbicka, R., Wu, H., Franek, M., Kamal-Eldin, A. & Landberg, R. (2015). Determination of alkylresorcinols and their metabolites in biological samples by gas chromatography-mass spectrometry. *Journal of Chromatography B*, 1000, 120-9.

III Wierzbicka, R., Zamaratskaia, G., Kamal-Eldin, A. & Landberg, R. Novel urinary alkylresorcinol metabolites as biomarkers of whole grain intake in free-living Swedish adults (submitted).

IV Landberg, R., Wierzbicka, R., Shi, L., Nybacka, S., Kamal-Eldin, A., Hedblad, B., Lindroos, A.K., Winkvist, A. & Bertéus Forslund. H. New alkylresorcinol metabolites in spot urine as biomarkers of whole grain wheat and rye intake in a Swedish middle-aged population (manuscript).

The contribution of Roksana Wierzbicka to the papers included in this thesis was as follows:

I Participated in planning the experiments, performed the laboratory analyses, evaluated the results and had the main responsibility for writing and revising the manuscript.

II Participated in planning the experiments, performed the laboratory analyses of urinary AR metabolites, evaluated the results and had shared main responsibility for writing and revising the manuscript, with Huaxing Wu.

III Participated in planning the experiments and performed the laboratory analyses, data analysis and manuscript writing.

IV Participated in planning the experiments, performed the laboratory analyses, and participated in manuscript writing.

Abbreviations

3DWFR	Three-day weighed food records
ADHBA	4-amino-3,5-dihydroxybenzoic acid
AP	Alkaline phosphatase
AR	Alkylresorcinols
DHBA	3,5-dihydroxybenzoic acid
DHBA-glycine	2-(3,5-dihydroxybenzamido)acetic acid
DHCA	3,5-dihydroxycinnamic acid
DHCA-amide	3,5-dihydroxycinnamic acid amide
DHPAA	3,5-dihydroxyphenyl acetic acid
DHPPA	3-(3,5-dihydroxyphenyl)-propanoic acid
DHPPTA	5-(3,5-dihydroxyphenyl) pentanoic acid
ELISA	Enzyme-linked immunosorbent assay
WG	Whole grain

1 Background

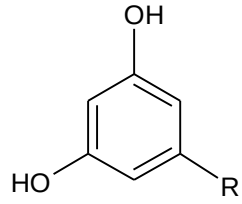
It is widely accepted that diet, along with other lifestyle factors, has a major impact on human health (Millen *et al.*, 2016; Mozaffarian, 2016; Anand *et al.*, 2015; Ross *et al.*, 2015; Conlon & Bird, 2014; Linko *et al.*, 2005a). It has been estimated that diet and lifestyle factors explain about 80% of the deaths from cardiovascular disease world-wide and over one-third of cancers (Celis-Morales *et al.*, 2016). Cereal grains constitute the primary source of energy and protein intake world-wide and have therefore played an important role of the human diet for thousands of years (Spiller, 2002). Today, most cereals are consumed as refined grains, but refined grains only started to dominate cereal consumption around 100 years ago (Slavin, 2007). From a health perspective, grains should preferably be consumed whole. Epidemiological studies have consistently shown inverse associations between whole grain cereal intake and the risk of developing obesity (Kirwan *et al.*, 2016; Pol *et al.*, 2013), type 2 diabetes (Chanson-Rolle *et al.*, 2015; Aune *et al.*, 2013), cardiovascular disease (Wei *et al.*, 2016; Tang *et al.*, 2015), and some cancers (Aune *et al.*, 2016; Meija *et al.*, 2015).

Whole grain refers to "intact, ground, cracked or flaked caryopsis, whose principal anatomical components – the starchy endosperm, germ and bran – are present in the same relative proportions as they exist in the intact caryopsis" (definition by AACC, 1999). In contrast, refined grains have the bran and germ removed during the milling process (Fardet, 2010). Therefore, the nutritional value of refined grains is considerably lower than that of whole grain cereals, which are rich in minerals, vitamins, dietary fibre and many bioactive compounds (Andersson *et al.*, 2014). The most common whole grain cereals consumed world-wide are wheat, rice and maize, followed by oats, rye, barley, triticale, millet and sorghum (Fardet, 2010).

Assessing whole grain food intake is complicated due to the different definitions of whole grain used, large variations in whole grain content in cereal foods, lack of food composition data and because consumers have difficulty distinguishing whole grain foods from other products (Ross *et al.*, 2015; Lang & Jebb, 2003). Moreover, most studies relying on self-reported intake data, such as 24-h recalls or food frequency questionnaires (FFQ) (Kaaks & Ferrari, 2006; Kaaks *et al.*, 2002), are known to suffer from large systematic and random measurement errors (Tasevska *et al.*, 2011; Kaaks *et al.*, 2002). Thus, there is a need for dietary biomarkers that can provide an objective measure of intake and complement self-reported methods in assessment of intake of whole grain foods (Holen *et al.*, 2016b). Using dietary biomarkers in large-scale epidemiological studies in relation to disease endpoints requires large sample sets to be analysed. Therefore rapid, accurate, sensitive and low-cost methods are highly warranted.

1.1 Alkylresorcinols and their occurrence

Alkylresorcinols (AR), are amphiphilic 1,3-dihydroxy-5-alkyl phenolic lipids, have been proposed as specific dietary biomarkers of whole grain wheat and rye intake (Landberg *et al.*, 2014; Ross, 2012). Two AR metabolites 1,3-dihydroxy-benzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) have been quantified in urine in free form and as a mixture of aglycones and glucuronide and sulphate conjugates(Ross *et al.*, 2004c) In a recent study, 5-(3,5-dihydroxyphenyl) pentanoic acid (DHPPTA) and 2-(3,5-dihydroxybenzamido)acetic acid (DHBA–glycine) were suggested as metabolites from ARs (Zhu *et al.*, 2014).



Alkylresorcinols	**Alkylresorcinol metabolites**
5-n-heptadecyl resorcinol, R=C17:0	**DHBA,** R= COOH
5-n-nonadecyl resorcinol, R=C19:0	**DHPPA,** R=CH_2CH_2COOH
5-n-heneicosyl resorcinol, R=C21:0	**DHPPTA**, R=$(CH_2)_4COOH$
5-n-tricosyl resorcinol, R=C23:0	**DHBA-glycine,** R=$CONHCH_2COOH$
5-n-pentacosyl resorcinol, R=C25:0	

Figure 1. (Left) structure of common alkylresorcinols present in wheat and rye grains and (right) four putative urinary metabolites in humans.

Alkylresorcinols have been found in bacteria, fungi, algae, mosses, higher plants and animals (Haliclonidae) (Kozubek & Tyman, 1999). Among plants commonly consumed by humans, odd-numbered AR have been found in high amounts in grain of rye (*Secale cereale*), wheat (*Triticum aestivum*) and the rye-wheat hybrid triticale, and in low amounts in barley (*Hordeum vulgare*) and millet (Andersson *et al.*, 2008; Chen *et al.*, 2004; Zarnowski & Suzuki, 2004; Ross *et al.*, 2003c; Kozubek & Tyman, 1999).

Alkylresorcinols are found mainly in the outer part of the grain (Ross *et al.*, 2003c) or, more precisely, in the cuticulum of the testa/inner cuticulum of the pericarp (Landberg *et al.*, 2008b), but not in large amounts in refined grains or in other food products (Chen *et al.*, 2004). Alkylresorcinol homologues with odd alkyl chains of 17-25 carbon atoms are the most common AR compounds in cereal grains, though unsaturated derivatives such as 5-alkenyl-, 5-oxoalky- and 5-hydroxyalkylresorcinols are also found, particularly in rye (Kozubek & Tyman, 1999). In addition, even-numbered AR homologues (C18:0, C22:0 and C24:0) have recently been identified in quinoa (*Chenopodium quinoa*) (Ross, 2014).

The source of whole grain in food products can be distinguished by determination of the AR C17:0/C21:0 ratio, which is 1.0 in whole grain rye, 0.1 in whole grain wheat and 0.01 in whole grain durum wheat (Landberg *et al.*, 2006a; Chen *et al.*, 2004). The AR content in rye is 268-1444 µg/g dry matter (DM) and in wheat 220-943 µg/g (DM), while the content in refined rye flour is 40-280 µg/g DM and in refined wheat flour at most 50 µg/g DM (Landberg *et al.*, 2014). Average daily intake ranges from 12 mg in the United Kingdom to nearly 40 mg in Finland (Ross, 2012).

1.2 Alkylresorcinol absorption, distribution, metabolism and excretion (ADME)

1.2.1 Alkylresorcinols absorption

Animal and human studies have shown that AR are absorbed to a great extent in the small intestine and metabolised before excretion in urine (Ross *et al.*, 2004c). The apparent absorption of AR is 60-80%, as determined in ileostomised humans (Ross *et al.*, 2003a) and cannulated pigs and rats (Ross *et al.*, 2003b). Alkylresorcinol absorption varies between individuals and animal models (Landberg *et al.*, 2014) depending on the intake level, but not with intake frequency. It appears that the absorption differs slightly between different AR homologues, with shorter alkyl-chain homologues being absorbed to a greater extent that those with a longer alkyl chain (Landberg *et al.*, 2009a; Ross *et al.*, 2004c).

The cellular mechanism(s) of AR absorption in the small intestine is still unknown. Similarly to tocopherols, AR may be absorbed by passive diffusion and to some extent actively by scavenger receptor class B (SR-BI) (Rigotti, 2007). The majority of ingested AR are absorbed in the small intestine and transported in chylomicrons via the lymphatic system to the liver, where they are mainly incorporated into very-low-density lipoprotein (VLDL) or high- density lipoprotein (HDL), as also reported for tocopherols (Linko-Parvinen *et al.*, 2007). In a complementary mechanism, AR could possibly be directly transported from chylomicrons to HDL or VLDL during lipolysis (Linko-Parvinen *et al.*, 2007). Incorporation into chylomicrons and direct outflow to HDL have been suggested for tocopherols, with availability of fat as a decisive factor (Anwar *et al.*, 2006).

1.2.2 Alkylresorcinol distribution

The amphiphilic structure of AR makes them easy to incorporate into biological membranes, where they can modify functions and properties (Kozubek, 1989). It has been observed that long-chain AR are incorporated more easily into membranes and diffuse faster across membranes than short chain AR (Linko & Adlercreutz, 2005; Kozubek, 1989). Moreover, studies in humans and rats have confirmed that AR are incorporated into erythrocyte membranes (Linko & Adlercreutz, 2005; Ross *et al.*, 2003b). More recently, AR have been shown to accumulate in adipose tissue in rats and humans (Wu *et al.*, 2015; Jansson *et al.*, 2010; Ross *et al.*, 2004b), while whole grain intake

has been shown to be correlated with AR content in adipose tissue in analyses under controlled conditions (Wu *et al.*, 2015). The distribution of AR in other organs or tissues is unknown.

1.2.3 Alkylresorcinol metabolism

Alkylresorcinol metabolism shares similarities with tocopherol metabolism, which consists of two phases: an oxidative step (phase 1) and a conjugation reactions (phase 2) (Marklund., 2012; Ross *et al.*, 2004a). AR metabolites: 1,3-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) have been detected and quantified as a mixture of aglycones and glucuronide and sulphate conjugates in urine (Zhu *et al.*, 2016; Hanhineva *et al.*, 2015; Marklund *et al.*, 2010; Ross *et al.*, 2004a). More recently, 5-(3,5-dihydroxyphenyl) pentatonic acid (DHPPTA) and 2-(3,5-dihydroxybenzamido) acetic acid (DHBA-glycine) have also been described as urinary metabolites derived from AR (Zhu *et al.*, 2014). Moreover, an untargeted urine metabolite profiling study has identified a sulphated conjugate of 3,5-dihydroxycinnamic acid (DHCA) after a whole grain rye bread intervention and suggests to be a metabolite from AR (Bondia-Pons *et al.*, 2013).

Phase I metabolism

The first step in AR metabolism is the introduction of a hydroxyl (-OH) group at the end of the alkyl chain through omega (ω) oxidation (Ross *et al.*, 2004c). This process is catalysed by cytochrome P450 isoenzyme CYP4F2 (Marklund *et al.*, 2013b; Ross *et al.*, 2004c) and is probably located in the endoplasmic reticulum and cytoplasm (Mustacich *et al.*, 2010). The ω-hydroxyl group is then oxidised to a carboxylic acid. The carboxylic acid is further activated to an acyl-CoA ester that undergoes β-oxidation. During beta β-oxidation, the alkyl chain is gradually shortened, by two carbons at a time, to DHPPA and DHBA as final products (Ross *et al.*, 2004c) (Figure 2).

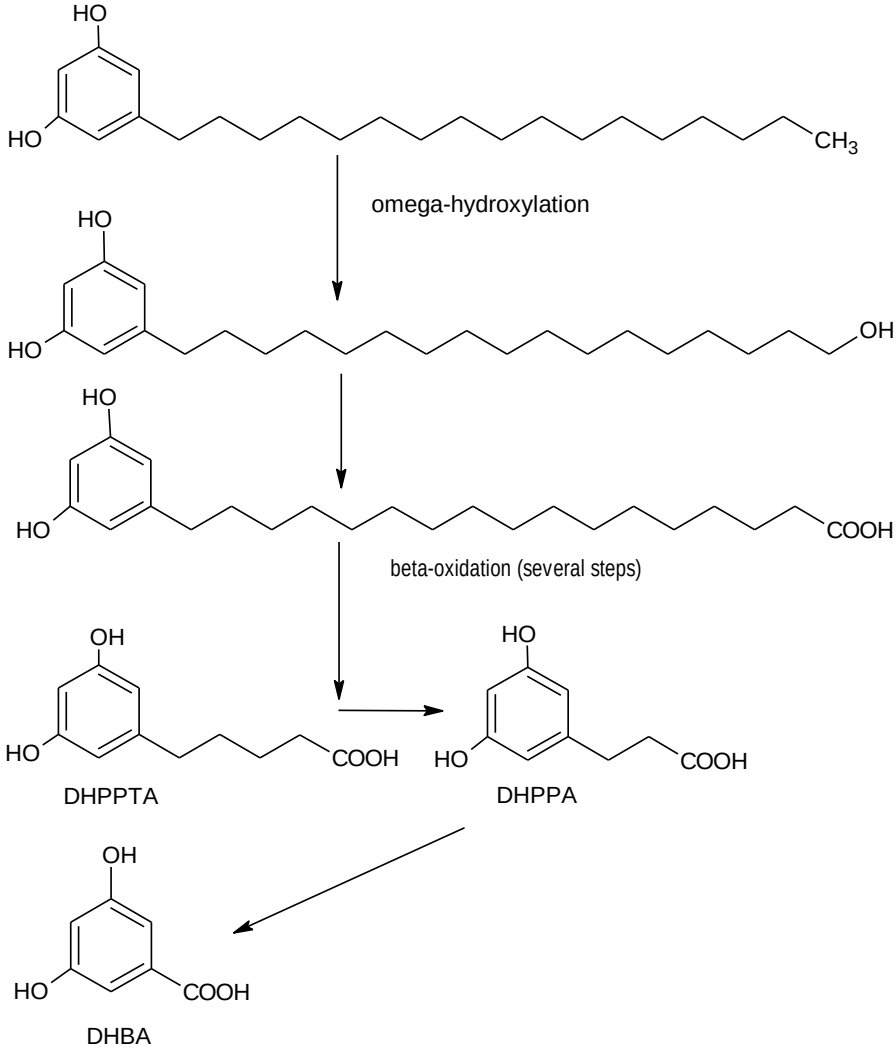


Figure 2. Suggested metabolic pathway of alkylresorcinols (AR), represented by AR C17:0 degraded to 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-propanoic acid) (DHPPA) (Ross *et al.*, 2004c), and to 5-(3,5-dihydroxyphenyl) pentanoic acid (DHPPTA), recently presented as an AR metabolite (Zhu *et al.*, 2014).

Phase II metabolism

The end products of β-oxidation are converted to water-soluble AR metabolites that are partially conjugated by phase II metabolism before urinary excretion (Ross *et al.*, 2004a)**.** In phase II, excretion properties are improved by conjugation of a polar group to xenobiotics or endogenous compounds. Glucuronidation, sulfation and amino acid conjugation are the main reactions

in phase II metabolism (Knights *et al.*, 2011; Zamek-Gliszczynski *et al.*, 2006). Glucuronidation involves conjugation of endogenous compounds, xenobiotics or their metabolites with a glucuronic acid (Zamek-Gliszczynski *et al*., 2006). This reaction is catalysed by uridine 5′-diphosphate (UDP)-glucuronosyltransferase enzymes, which transfer glucuronic acid from a co-substrate, UDP-glucuronic acid, to the substrate (Kutsuno *et al*., 2013). Based on evolutionary diversity, UGT1 and UGT2 are two subfamilies of the UGT enzyme. In mammals such as humans, mice and rats, UGT1 is present. The UGT enzymes are found in the endoplasmic reticulum of different tissues, with liver as a major site for glucuronide conjugation (Katoh *et al*., 2007). Some of the glucuronide conjugates can be deconjugated in acidic environments, since the glucuronidation process is reversible. *In vivo*, deconjugation reactions are mediated by β-glucuronidase (Zamek-Gliszczynski *et al*., 2006).

Sulphation is a conjugation with a sulpho-group catalysed by sulphotransferase enzyme(s). Sulphotransferase applies 3'-phosphoadenosine-5'-phosphosulphate (PAPS) as a co-substrate to covalently attach sulphonic acid to hydroxyl or amine groups of the substrate (Strott, 2002). Sulphotransferase is available mainly in the liver, but can also be found in the intestine, kidney and other tissues. In most cases, addition of the sulphone moiety to the compound increases the solubility and decreases the biological activity (Gamage *et al*., 2006).

Less is known about amino acid conjugation than about glucuronidation and sulphation reactions. Amino acid conjugation is limited to a number of carboxylic acids: aryloxyacetic, arylacetic, aromatic and heteroaromatic acid (Knights *et al.*, 2011; Pacifici *et al.*, 1991). Glycine is the most common amino acid utilised as an aglycone acceptor in amino acid conjugation in humans. Unlike the other conjugation reactions, amino acid conjugation comprises two enzyme systems that operate in two steps:

Activation of the carboxylic acid moiety with adenosine triphosphate (ATP) and formation of reactive xenobiotic CoA thioester intermediate mediated by ATP-dependent acid:CoA synthetase (ACSM).

Transfer of the activated acyl group to the amino group of glycine. This reaction is catalysed by the acyl-CoA:amino acid *N*-acyltransferase (Knights *et al.*, 2007; Pacifici *et al.*, 1991).

Glycine conjugation is readily saturable and metabolism of some carboxylic acids is therefore dose-dependent. Relatively few drugs and chemicals are activated to acyl-Co A thioester and their alkyl- or alkxyl- chain position plays an important role in the conjugation process. It has been observed for benzoic acids that the para- or meta- chain position exhibits the highest activity when ortho-situated benzoic acid is not able to conjugate to acyl-CoA esters (Knights

et al., 2011). Moreover, *in vivo* studies have shown that human liver and kidney homogenates from newborns have a limited capacity to form glycine conjugates of benzoic and p-amino-benzoic acid and that the possibility for glycine conjugation decreases with age (Knights *et al.*, 2011). As mentioned, DHBA-glycine has recently been detected and described as an AR urinary metabolite (Zhu *et al.*, 2014).

Recent studies using three *in vitro* platforms: human cytochrome P450 4F2 (CYP4F2), human liver S9 and HepG2 incubated with C19:0 cells, have confirmed suggested metabolic pathways whereby AR are transformed to phenolic lipids via CYP4F2-mediated ω-oxidation and subsequent β-oxidation (Marklund *et al.*, 2013b). To date, no conjugated AR have been found in plasma, which indicates that ω-oxidation is placed before the conjugation reaction in the AR metabolic pathway or that AR phase II metabolism is not sufficient to excrete the conjugated metabolites to the circulatory system (Landberg *et al.*, 2014). However, small amounts of intact AR have been detected in urine after β-glucuronidase and sulphatase deconjugation. This indicates that phase I and phase II metabolism of AR may occur in parallel, as has been observed for tocopherols, or that AR homologues may undergo a conjugation reaction (Hashiguchi *et al.*, 2011). Both DHBA and DHPPA have been detected in plasma and urine in free form as a product and as a mixture of aglycones or conjugates (Marklund *et al.*, 2012; Koskela *et al.*, 2008). Moreover, it could be speculated that DHBA-glycine is formed from DHBA through the amino acid conjugation performed in phase II metabolism.

1.2.4 Alkylresorcinol excretion

The AR metabolites DHBA and DHPPA are mainly eliminated from the body by urinary excretion (Ross *et al.*, 2004a). In a recent study, DHBA and DHPPA were recovered in ileostomy effluent from four ileostomised subjects consuming rye bran and it was found that 20-25% of ingested AR appeared as two main AR metabolites in the ileostomy effluent (mostly as DHPPA) (Marklund *et al.*, 2014b). This suggests that biliary excretion is an important route of AR metabolite elimination (Landberg *et al.*, 2014; Marklund *et al.*, 2014b). However, it is unknown to what extent other metabolites are excreted through this route. The quantitative recovery of ingested AR excreted as metabolites in urine is higher at lower AR intake (90%). It may be explained by a change in the elimination route and/or decreased absorption at high doses (Landberg *et al.*, 2009d; Landberg *et al.*, 2006). This is supported by a previous finding of AR metabolites undergoing enterophatic circulation at high intake (Soderholm *et al.*, 2009).

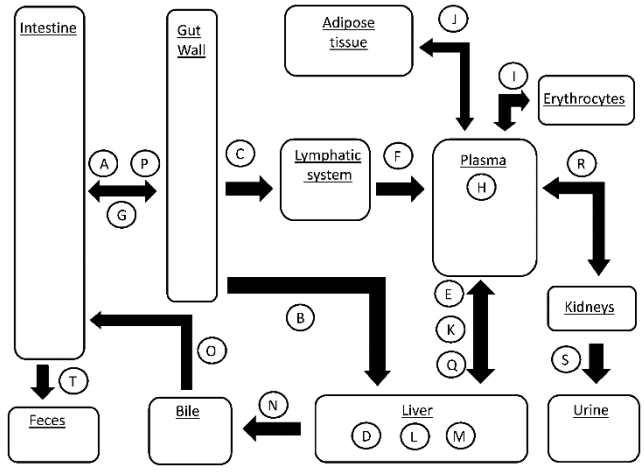


Figure 3. Suggested alkylresorcinol (AR) metabolic pathway in humans (Marklund *et al.*, 2014b): Following uptake from the intestine to the gut wall (A), AR are transferred to the circulatory system through the portal vein (B) or lymphatic pathway (C). Portal absorption delivers AR to the liver (D), where a proportion is metabolised to 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-propanoic acid (DHPPA) before entering the circulatory system (E). Lymphatic absorption of AR is slower, but AR absorbed through this pathway are not subjected to hepatic metabolism (F). Alkylresorcinols may be metabolised in the gut wall and excreted to the intestinal lumen (G). In the circulatory system, AR are mostly present in lipoprotein particles (H) and erythrocytes (I), but can be reversibly transferred to *e.g.* adipose tissue (J) or liver (K). In the liver, AR can be reassembled to different lipoproteins (L) or undergo phase I and II metabolism (M). Metabolites such as DHPPA and possibly conjugated AR are excreted via the bile (N) to the intestine (O), where they are available for reabsorption (P). After formation in the liver, DHBA and DHPPA are secreted (Q) into plasma and can be rapidly eliminated by the kidneys (R) to the urine (S). If not reabsorbed, intact AR and their metabolites excreted from the gut wall and bile eventually appear in faeces (T). The figure was re-produced by permission from Journal of Nutrion

1.3 Alkylresorcinols and their metabolites as dietary biomarkers

1.3.1 Dietary biomarkers

Dietary biomarkers (DB) are biochemical, functional or clinical indicators that reflect intake or status of certain foods or nutrients (Combs *et al.*, 2013). Dietary biomarkers may provide an objective measure of intake of a specific compound, nutrient, food or diet and have the potential to provide a more accurate intake assessment, which could improve chronic disease risk prediction in epidemiological studies (Aubertin-Leheudre *et al.*, 2010a; Bingham *et al.*, 2008). Dietary biomarkers can be classified into three different categories, concentration, recovery and prediction, depending on their relationship with intake (Tasevska *et al.*, 2006; *Kaaks et al.*, 2002).

Concentration biomarkers are based on measurement of a nutrient or compound concentration at a given point in time. They are correlated to intake, but do not offer a quantitative measurement as recovery biomarkers, since factors other than intake also affect their concentration. Variations in digestion, absorption and distribution in body compartments, excretion and endogenous synthesis and metabolism affect the concentration of these biomarkers (Freedman *et al.*, 2010a; Kaaks *et al.*, 2002). Serum fatty acids, plasma vitamin C and carotenoids as markers for fat, fruit and vegetable intake, respectively, are examples of classical concentration biomarkers (Bingham *et al.*, 2008; Wolk *et al.*, 2001; Kaaks *et al.*, 1997).

Recovery biomarkers are based on a quantitative relationship between intake and output in a specific period and offer an absolute measurement of a subject's intake (Kaaks *et al.*, 2002). Examples of recovery biomarkers include: double-labelled water as a marker for energy intake (Livingstone & Black, 2003) and 24-h urinary excretion of nitrogen and potassium as a marker for protein (Bingham, 2003) and potassium intake (Tasevska *et al.*, 2006), respectively. Recovery biomarkers can be useful in validation studies as a reference method, since they can be quantitatively measured and compared with conventional dietary assessment methods on the same scale (Kaaks *et al.*, 1997). They can also be used to study the measurement error structure of conventional intake assessment tools and for regression calibration studies (Spiegelman *et al.*, 2005). However, biomarkers are not without limitations. Only a very limited number of such biomarkers exist to reflect a few dietary factors and they typically reflect intake over short time periods (Willett., 2012).

Prediction biomarkers, unlike recovery biomarkers, only account for a small proportion of the intake recovered, but the correlation to intake is relatively strong, time-related and sensitive in a dose-response manner and the impact of non-dietary determinants is smaller than for concentration biomarkers. In

general, prediction biomarkers are characterised by high reproducibility, high correlation with intake and high predictive potential (Tasevska *et al.*, 2011; Tasevska *et al.*, 2005). Therefore, they can be classified as an intermediate between recovery and concentration biomarkers.

1.3.2 Biomarker validity and reproducibility

Validity and reproducibility are two critical features in assessment of dietary biomarkers. Validity can be reflected by the correlation between biomarker measurement and true intake. Despite the inherent weaknesses of any dietary assessment method, food records have often been used as a surrogate of 'true' intake and correlation of the biomarker to surrogate measurements has been taken to indicate relative validity (Eysteinsdottir *et al.*, 2012). Validation of a biomarker is a necessary stage in obtaining high-quality research data (Hunter *et al.*, 2010). A valid biomarker has been defined as "a biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiological, toxicological, pharmacological, or clinical significance of the test results"(Hunter *et al.*, 2010).

Validation is the process of "assessing the biomarker and its measurement performance characteristics, and determining the range of conditions under which the biomarker will give reproducible and accurate data" (Hunter *et al.*, 2010). Validation of any biomarker should be performed based on the analytical, biological and utilisation steps (Odriozola & Corrales, 2015a).

Reproducibility reflect the stability of the biomarker over time, based on the correlation between repeated measurements within the same individual, and depends on the elimination half-life of the biomarker and the intake frequency (van Dam & Hu, 2008; Kaaks *et al.*, 1997). Reproducibility is also dependent on the intake range in the population and is assessed by the intra-class correlation coefficient (ICC), defined as the ratio of between-participant variance to total variance (Vineis, 1997). The ICC ranges in value between 0.0 and 1.0, with ICC being equal to 1.0 if there is exact agreement between repeated measures within the same individual (Landberg *et al.*, 2014). The reproducibility can be considered good when ICC is >0.4-0.5 (Sun *et al.*, 2017). A long-term biomarker exhibits high ICC over months or even a year (Kaaks, 1997). Stable and regular intake may compensate for a short half-life of a biomarker (van Dam & Hu, 2008).

When developing a potential dietary biomarker, factors that may not be important in conventional assessment methods, but which may affect biomarker measurement, need to be taken into consideration and evaluation.

Such factors include genetic variability, lifestyle/physiological factors (*e.g.* smoking), dietary factors (*e.g.* nutrient-nutrient interaction), analytical methodology and biological sample handling (Hedrick *et al.*, 2012). Therefore, various stakeholders such as academia, the food industry, regulation agencies and the health sector should be involved in biomarker development and validation, in order to improve understanding of the connection between dietary biomarkers and endpoints (Odriozola & Corrales, 2015a; Jenab *et al.*, 2009).

1.3.3 Application of dietary biomarkers in food and health studies

Dietary biomarkers can be used in observational studies to examine the relationship between diet and diseases (Ross *et al.*, 2015) and in intervention studies to check compliance (Holen *et al.*, 2016a; Mayne *et al.*, 2016; Marklund *et al.*, 2014a). When a biomarker is used as a measure of exposure/intake in epidemiological studies, it should preferably reflect the intake during the time window in which the disease under study develops. Typically, it takes years to develop a chronic disease and thus high long-term reproducibility is highly desirable for most dietary biomarkers in epidemiological studies (Landberg *et al.*, 2014).

When using dietary biomarkers to measure dietary compliance in dietary intervention studies (McKeown *et al.*, 2016; Marklund *et al.*, 2014a), they can be used in secondary analyses to study the effect after removing non-compliant subjects and to improve motivation through participants knowing that their records can be cross-checked by biomarker measurements on biological samples (Ross, 2012). Recent studies have confirmed the potential for using dietary biomarkers in dietary intervention studies (Kristensen *et al.*, 2017; Marklund *et al.*, 2014a; Landberg *et al.*, 2009b).

Moreover, dietary biomarkers can potentially be used to validate dietary assessment methods and to reflect dietary intake when self-reports are lacking or when food composition tables are inadequate (Hedrick *et al.*, 2012). A combination of dietary biomarkers and self-reported methods could, under certain circumstances, be used to improve the precision in diet-disease association studies (Freedman *et al.*, 2010b).

A few biomarkers of whole grain and dietary fibre have been suggested. One of the first was the mammalian lignin enterolactone (ENL). Enterolactone is produced by gut microbiota from its precursors, *i.e.* plant lignans, and has been proposed as a biomarker of whole grain intake (Peterson *et al.*, 2010; Adlercreutz, 2007). However, since ENL precursors are also found in large amounts in other foods, such as coffee, and because of the great impact of antibiotics and non-dietary determinants, it has been suggested to be at best a

biomarker of a healthy lifestyle (Seibold *et al.*, 2014; Adlercreutz, 2007; Linko-Parvinen *et al.*, 2007; Horner *et al.*, 2002; Kilkkinen *et al.*, 2001). More recently, AR and their metabolites DHBA and DHPPA have been proposed and evaluated as specific dietary biomarkers of whole grain wheat and rye intake (Landberg *et al.*, 2014; Ross *et al.*, 2012a; Ross *et al.*, 2004a).

Untargeted metabolomics has recently proven to be a powerful approach in the discovery of novel putative biomarkers of whole grain foods (Sang, 2016); (Zhu *et al.*, 2016; Hanhineva *et al.*, 2015; Odriozola & Corrales, 2015b). In a recent study, several specific biomarkers of whole grain rye were suggested (Hanhineva *et al.*, 2015). However, any novel biomarker or biomarker panel needs to be validated before implementation and so far validation has only been reported for AR and their metabolites.

1.3.4 Alkylresorcinols as dietary biomarkers

Alkylresorcinol molecules are stable and remain intact throughout food processing (Chen *et al.*, 2004). They are also specific to the outer parts of wheat and rye grains, are absorbed by humans and are measurable in human samples. Moreover, recent studies on reproducibility and relative validity of AR and their metabolites demonstrated that they can be used as biomarkers of whole grain wheat and rye intake (Marklund *et al.*, 2013a; Andersson *et al.*, 2011a). However, AR metabolites have limitations as biomarkers in epidemiology due to the modest reproducibility in plasma and spot urine samples and lack of 24 h urine samples in large observational studies (Marklund., 2012).

A number of studies have been undertaken to evaluate plasma AR homologues and plasma and urinary AR metabolites as dietary biomarkers of whole grain wheat and rye intake (McKeown *et al.*, 2016; Kyro *et al.*, 2014; Zhu *et al.*, 2014; Landberg *et al.*, 2013; Ross *et al.*, 2012a; Aubertin-Leheudre *et al.*, 2010a). Most of these studies have been conducted in the Nordic countries, where consumption of whole grain, especially rye, is high and frequent (Landberg *et al.*, 2012). Some studies have also evaluated AR as biomarkers in populations where consumption of whole grain products is lower and mainly consists of wheat products (McKeown *et al.*, 2016; Landberg *et al.*, 2012).

Total plasma AR homologues are generally well correlated to whole grain and cereal fibre intake under controlled intervention conditions and under free-living conditions (Landberg *et al.*, 2013; Ross *et al.*, 2012a; Landberg *et al.*, 2011; Landberg *et al.*, 2009d; Linko *et al.*, 2005a). Moreover, the source of whole grains in food products can be distinguished by determination of the AR C17:0/C21:0 ratio. Hence AR homologues in plasma can be used as

biomarkers to trace the source of whole grain intake (rye/wheat)(Landberg *et al.*, 2008a). This opens up new possibilities to examine the health effects of different kinds of grain.

In human studies, the half-life of AR in plasma is estimated to be around 5 h and the maximum AR concentration is >3000 nmol/L (Landberg *et al.*, 2006). Consequently, AR reflect short to medium-term intake. On the other hand, AR have slow absorption (t_{max} = 6.7 h) and therefore if the whole grain is consumed more than once per day, fluctuations in plasma AR concentrations are dampened and under such conditions plasma AR concentration can be considered a long-term biomarker (Landberg *et al.*, 2014). Consequently, in the Nordic population with reported high and frequent whole grain intake, fasting AR plasma concentration shows moderate to high reproducibility (ICC=0.47-0.9) under controlled and regular whole grain consumption, as well as under free-living conditions (Landberg *et al.*, 2013; Landberg *et al.*, 2009b). Based on this, AR concentrations in plasma can be classified as specific concentration biomarkers of whole grain wheat and rye intake (Kyro *et al.*, 2014; Landberg *et al.*, 2008a). In fact, they have been successfully used as such in relation to disease endpoints in epidemiological studies and as a measure of compliance in whole grain intervention studies (Biskup *et al.*, 2016; Kirwan *et al.*, 2016; Knudsen *et al.*, 2014; Kyro *et al.*, 2014; Marklund *et al.*, 2014a). Recently, AR in adipose tissue has been suggested as a long-term biomarker of whole grain intake (Wu *et al.*, 2015; Jansson *et al.*, 2010).

Moreover, it has been suggested that DHBA and DHPPA AR metabolites in plasma have longer elimination half-life (10 h for DHBA and 16 h for DHPPA) than intact AR and might therefore be better long-term biomarkers than intact AR (Soderholm *et al.*, 2009). However, the half-life of the metabolite can also reflect its formation half-life from intact AR. Moreover, non-dietary determinants may affect metabolite concentrations and this can affect the reproducibility negatively. Hence, metabolites may be less useful than intact AR (Montonen *et al.*, 2012).

Alkylresorcinol metabolites in urine as biomarkers

The ease of urine sample collection and the minimal interference in participants' daily life makes urine a suitable matrix for exposure biomarker assessment. Urine provides a cumulative biomarker measurement (Marklund *et al.*, 2013a). The use of 24-h urine samples avoids the extraneous variability in biomarkers due to very short-term changes in exposure and the timing of sample collection. A spot urine sample, like a plasma sample, provides a snapshot of the biomarker concentration. Adjustment for differences in dilution, using creatinine, is generally necessary and improves the precision,

despite this measurement also being subject to within-person variability and dependent on individuals' meat intake (Sun *et al.*, 2017). Typically, 24-h urine samples are not available in large epidemiological studies, but spot urine samples may be available to a greater extent. It is therefore desirable to evaluate whether biomarker measurements in such samples are useful for independent intake assessment.

The AR metabolites DHBA and DHPPA in urine have been proposed and evaluated as biomarkers of whole grain rye and wheat intake (McKeown *et al.*, 2016; Landberg *et al.*, 2012; Aubertin-Leheudre *et al.*, 2010a). The apparent half-life of DHBA and DHPPA has been found to be longer than that of intact AR: 10-12 in urine (Soderholm *et al.*, 2011), indicating that AR metabolites can be used as long-term biomarkers.

In a recent study feeding human subjects whole grain wheat breads containing 61 mg AR, DHPPTA and DHBA-glycine in free and conjugated form were identified as new AR metabolites in human urine (n=12) and their half-life was estimated to be 11.1 and 16.6 h, respectively (Zhu *et al.*, 2014). Both DHBA and DHPPA in urine are reported to be well correlated with intake of whole grains and cereal fibre (Marklund *et al.*, 2013a; Aubertin-Leheudre *et al.*, 2010a; Aubertin-Leheudre *et al.*, 2008). Moreover, urinary excretion of AR metabolites is reported to increase with elevated AR intake, although the proportion of ingested AR recovered in urine as DHBA and DHPPA decreases with increased intake (Landberg *et al.*, 2009d). Excretion of AR metabolites in urine samples shows moderate medium-term reproducibility when assessed in 24-h collection for DHBA (ICC = 0.46-0.51) and lower in creatinine (CR)-adjusted morning urine for DHPPA (ICC = 0.32-0.37) (Marklund *et al.*, 2013a). These results are comparable to values reported for fasting plasma AR as medium-term biomarkers (Landberg *et al.*, 2012; Andersson *et al.*, 2011b), indicating that AR metabolites can also be used as short/medium term biomarkers of whole grain intake.

In summary, the results obtained to date show that AR concentrations in plasma and their metabolites in 24-urine collections and perhaps also in spot urine samples are valid and reproducible biomarkers of whole grain wheat and rye intake in populations with a regular and high whole grain intake. However, there is a need for more long-term biomarkers to be used in populations with less frequent intake.

1.4 Chromatographic analysis of alkylresorcinols and their metabolites

To date, several analytical methods have been developed and applied for the quantification of AR and AR metabolites in biological matrices such as plasma, erythrocyte, adipose tissue and urine (Landberg *et al.*, 2012; Ross *et al.*, 2012a; Jansson *et al.*, 2010; Linko-Parvinen *et al.*, 2007). Chromatographic methods with different detection means, such as GC-MS (Marklund *et al.*, 2010), high performance liquid chromatography- coulometric electrode array detection (HPLC-CEAD) (Koskela *et al.*, 2008; Koskela *et al.*, 2007), ultra-performance liquid chromatography-quadrupole time of flight/mass spectrometry (UPLC-QTOF/MS) (Bondia-Pons *et al.*, 2013) and liquid chromatography-mass spectrometry (LC-MS) (Zhu *et al.*, 2014), have been proven to be useful for analysis of AR and their metabolites because they appear at low concentrations in biological samples (nmol-µmol/L range) (Andersson *et al.*, 2014). In previous studies, AR homologues in plasma have mostly been analysed by GC-MS (Landberg *et al.*, 2009c; Linko *et al.*, 2002). More recently, a high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and utilised for analysis of plasma AR homologues (Ross *et al.*, 2016; Ross *et al.*, 2010). Good agreement between GC-MS and LC-MS/MS up to 150 nmol/L has been shown, but care is needed when comparing data where plasma AR concentrations are expected to be high (Ross *et al.*, 2016).

Due to a lack of synthetic reference standards for conjugated AR metabolites, various conjugated AR metabolites have been hydrolysed with β-glucuronidase and sulphatase activity before GC-MS analysis (Marklund *et al.*, 2010). The AR metabolites are then extracted, purified by solid phase extraction (SPE) and silylated prior to quantification (Marklund *et al.*, 2010). In a later study, (Marklund *et al.*, 2011) compared GC-MS and HPLC-CEAD by analysing 114 urine samples from free-living Swedish people consuming their habitual diet and concluded that both these methods work similarly and can be used interchangeably. However, they also concluded that the analytical run time of HPLC-CEAD is longer than that of GC-MS and that purification of deconjugated DHBA metabolites is a necessary step due to the interference of the co-eluting compounds when using HPLC-CEAD ((Marklund *et al.*, 2011). Moreover, in a recent study using an LC-MS approach, DHPPTA and DHBA-glycine were identified as AR metabolites present at low concentrations in urine (Zhu *et al.*, 2014). However, no validation was performed for the method used for detection and quantification of DHPPTA and DHBA-glycine in that study. Moreover, DHCA-sulphate has been identified in urine from 20 Finnish women and men consuming whole grain rye in a study using UPLC-

QTOF/MS(Bondia-Pons *et al.*, 2013), but could only be quantified on a relative basis as no quantitative method is currently available.

In general, the potential methods for analysis of AR and AR metabolites reported to date have been evaluated with regard to assessment of precision, linear range and limit of detection, recovery after spiking and quantification (Andersson *et al.*, 2014). They have since been successfully applied for rapid, sensitive and robust determination of AR and their metabolites in biological samples. However, rapid and cheap high-throughput methods suitable for analysis of thousands of samples are currently lacking.

1.5 Immunoassays

In order to allow rapid and accurate determination of the concentrations of AR and their metabolites as biomarkers in large datasets in epidemiological investigations, rapid, accurate and sensitive analytical methods are essential. Immuno-based techniques may provide such possibilities.

In general, immunoassays are fast, sensitive methods which allow large numbers of samples to be analysed simultaneously and therefore they are widely used in clinical chemistry (Kristensen *et al.*, 2016; Weber *et al.*, 1990), endocrinology (Chanson *et al.*, 2016; M, 1980), environmental science (Ranganathan *et al.*, 2015; Ahn *et al.*, 2011; Deng *et al.*, 2002) and food analysis (Liu *et al.*, 2015; Vass *et al.*, 2008; Franek, 2005; Lee *et al.*, 2004). Enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent immunoassay (FIA) are conventional immunoassay methods that use different labels for antigen (analyte) detection (Centi & Mascini, 2012).

Immunoassays methods are based on the antibodies ability to bind to the specific structure of a molecule (Wild, 2005). The specificity and affinity of antibodies, when incorporated into a testing format, determine the analytical characteristics of the established detection system. Antibody properties are a crucial factor in the development of immunochemical methods (Franek, 2005). Therefore, optimal haptens, *i.e.* low-molecular weight compounds corresponding to the analyte of interest, need to be identified and evaluated in order to obtain analyte-specific antibodies.

A hapten molecule is a small molecule which is not able to elicit the immune response in the animal by itself and therefore usually needs to be derivatised and conjugated to a larger molecule, such as a protein. The product of the conjugation reaction is an immunogen, which is able to elicit antibody formation by the animal's immune system (Eyer & Franek, 2012).

Antibody production is based on two major steps:

1) Activated hapten is covalently bound (conjugated) to a carrier protein to produce the immunogen.
2) Immunisation of animals by the immunogen. The way in which the hapten binds to a protein determines the character of the antibody specificity (Franek, 2005). It is commonly found in immunochemistry that the position of a chemical bridge spacer in the hapten structure plays a key role in antibody specificity (Franek, 1987).

Antibodies for conventional immunoassays are usually polyclonal or monoclonal (Eyer, 2012; Cernoch *et al.*, 2011). Polyclonal antibodies are produced by immunisation of rabbits, goats, sheep or pigs (Cernoch *et al.*, 2011; Hanly *et al.*, 1995). This method is hampered by the fact that is not possible to produce the antibody with identical specificity even in two animals of the same species. Monoclonal antibodies, on the other hand, are produced by hybridoma technology (Hanack *et al.*, 2016), but suffer from low predictability of the results (Franek, 2005). However, they can be produced in unlimited amounts while retaining the same properties.

Antibodies incorporated into an immunoanalytical system can be characterised in terms of cross-reactivity and assay sensitivity by one of the most common immunoassays, ELISA. It is currently the method of choice for analysing a large series of samples, *e.g.* blood and urine samples, for intervention and epidemiological studies, due to its simple, quick and relatively inexpensive assay performance (Cernoch *et al.*, 2011; Vass *et al.*, 2008; Cespedes *et al.*, 2006; Franek *et al.*, 2006). To date, no ELISA method is available for AR, but several assays have been developed for nonylphenol, an analogous phenolic lipid with similarities to AR (Estévez *et al.*, 2006; Zeravik *et al.*, 2004; Franek *et al.*, 2001).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA approach is used to detect a specific antibody or antigen (analyte) in a sample using an enzymatic label. Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are the enzymes most commonly used in ELISA systems (Cernoch *et al.*, 2011; Vass *et al.*, 2008; Al-Dujaili, 2006;). The ELISA method can be set up in variety of formats depending on nature of the analyte, required sensitivity, cost and availability of antigen and antibody(Centi & Mascini, 2012). Characterisation of antibodies can be carried out by competitive direct and indirect ELISA formats for detection of small molecules

(Figure 4), whereas assays for large molecules such as proteins are based on non-competitive formats such as Sandwich ELISA.

In direct competitive ELISA, a specific antibody is immobilised on the solid phase (microtitre plate). After incubation, non-bound antibodies are washed away and free analyte and labelled tracer are added. The concentration of tracer is constant and free analyte is added as a standard or as an unknown sample. There is then competition for the binding site of the antibody between free analyte and labelled tracer. The amount of analyte is inversely proportional to the absorbance intensity of the tracer measured by spectrophotometer (Figure 4a). The concentration of analyte is calculated from a calibration curve.

In indirect competitive ELISA, a solid phase is coated with an immunogen and the specific antibodies and free analyte are added in the second step where the conjugated analyte on solid phase and free analyte compete for the antibody binding sites (Figure 4b). After incubation and washing, secondary-labelled antibody able to bind to the specific primary antibody is added in order to detect the reaction signal that results from competition between conjugated analyte and free analyte. A lower signal is caused by higher analyte concentration (Figure 4b). The amount of analyte in the samples is proportional to the signal developed.

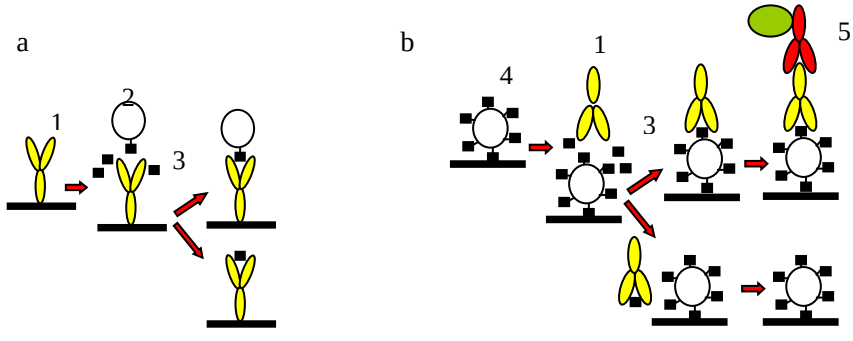


Figure 4. Schematic diagram of a) direct competitive enzyme-linked immunosorbent assay (ELISA) and b) indirect competitive ELISA, where 1 is specific antibody, 2 is tracer, 3 is free analyte, 4 is coated immunogen and 5 is secondary (detection) antibody labelled with enzyme.

2 Aims of the thesis

The overall aim of this thesis was to develop high-throughput analytical methods for the analysis of alkylresorcinol metabolites and to evaluate recently discovered metabolites as biomarkers of whole grain wheat and rye intake. Specific objectives of the work performed in Papers I-IV were to:

1. Develop and characterise polyclonal and monoclonal antibodies against two main AR metabolites, DHBA and DHPPA, and evaluate their use for development of a competitive ELISA system **(I).**

2. Confirm the presence of the recently suggested AR metabolites DHPPTA, DHCA, DHCA-amide and DHBA-glycine and to evaluate their cross-reactivity in ELISA methods **(I).**

3. Develop an optimised GC-MS method for rapid, sensitive and robust determination of all currently reported AR metabolites in urine **(II).**

4. Evaluate new AR metabolites as biomarkers of whole grain rye and wheat intake by assessing their medium-term reproducibility and relative validity in 24-h urine excretion among Swedish men and women known to consume whole grain wheat and rye regularly **(III);** and determine their reproducibility in creatinine-adjusted spot urine in an independent population of middle-aged Swedish men and women **(IV).**

3 Materials and methods

3.1 References compounds and enzymes

Reference compounds and enzymes used in Papers I-IV are listed in Table 1.

Table 1. *Reference compounds and enzymes used in Papers I-IV*

Trivial name	Supplier	Purity	Paper/s
Alkylresorcinols			
C17:0, C19:0, C21:0, C23:0, C25:0	Reseachem LifeScience Burgdorf, Switzerland	>95%	I-II
Metabolites			
DHBA[a]			I-IV
DHPPA[b]	Reseachem LifeScience Burgdorf, Switzerland	>95%	I-IV
DHPAA[c]			I-II
DHPPTA[d]			I-IV
DHBA-glycine[e]			I-IV
DHCA[1f]		>98%	I-IV
DHCA-amide[2g]	T.Vontor, Hradec Kralove, Czech Republic	*	I-IV
Internal standards			
Syringic acid	Sigma Chemicals, St.Louis, USA	>98%	I-IV
C20:0, C22:0, C24:0, C26:0 and their mixture	Reseachem LifeScience Burgdorf, Switzerland	>95%	II

Table 1 (contd.)

Trivial name	Supplier	Purity	Study
Enzymes			
Type H-1 β-glucuronidase/sulphatase from *Helix pomatia*	Sigma-Chemicals, St. Louis, USA	.	I-IV
HRP[h]	Sigma-Aldrich Chemie, Steinheim, Germany	-	I
AP[i]	New England BioLab, United Kingdom	-	I
Other compounds:			
ADHBA[3j]	T.Vontor, Hradec Kralove, Czech Republic	-	I
BSA[k]		-	I
OV[l]	Sigma-Aldrich Chemie, Steinheim, Germany	-	I
TG[m]			*I*

*The product contains 78% DHCA-amide, 17% DHCA, 5% unidentified compounds

[1, 2, 3] were synthesised by Ing. Vontor Hradec Kralove, Czech Republic (purity >98% and *, determined by NMR analysis) *78% DHCA-amide, 17% DHCA, 5% unidentified compounds)

[a]1,3-dihydroxy-benzoic acid; [b]3-(3,5-dihydroxyphenyl)-propanoic acid, [c]3,5-dihydroxyphenyl acetic acid; [d] 5-(3,5-dihydroxyphenyl)pentanoic acid, [e]2-(3,5-dihydroxybenzamido)acetic acid, [f]3.5-dihydroxycinnamic acid and [g]3,5-dihydroxycinnamic acid amide, [h]horseradish peroxidase, [i]alkaline phosphatase, [j]4-amino-3,5-dihydroxybenzoic acid, [k]bovine serum albumin, [l] ovalbumin, [m]porcine thyroglobulin.

3.2 Urine sample collection and preparation

The urine samples used for experimental work in Paper **I** and for biomarker validation and reproducibility assessment in 24-h urine collections were obtained from healthy, free-living Swedish men and women enrolled in a previous biomarker study (Marklund *et al.*, 2013a). Urine samples from the DDIET-study that have been previously analysed and presented (Nowotny *et al.*, 2015) were used for GC-MS method optimisation and validation in Paper **II**. The spot urine samples used to estimate biomarker reproducibility in Paper **III** were from individuals within the pilot study for the Swedish CArdioPulmonary bioImage Study (SCAPIS) (Nybacka *et al.*, 2016).

Sample preparation in Paper **I,** including the steps prior to direct and indirect ELISA and LC-MS/MS, were performed up to liquid-liquid extraction according to a previously reported method (Marklund *et al.*, 2010). Samples in Papers **III** and **IV** were analysed according to a method described in Paper **II**, referred to as the method developed by (Wierzbicka *et al.*, 2015).

An overview of the methods used for preparation of urine samples prior to ELISA, LC-MS/MS and GC-MS analysis is presented in Figure 5**.** In brief, all urine samples used were hydrolysed to release conjugated AR metabolites and the deconjugated compounds were extracted in liquid-liquid extraction. The pooled organic extracts were evaporated to dryness under nitrogen at 60 ºC. Sample extracts for immunoassay analysis were dissolved in buffer and used directly for direct/indirect ELISA (**I**). Sample extracts for LC-MS/MS analysis were dissolved in 150 µL water-methanol (70/30 v/v) solution, sonicated and transferred to HPLC vials (**I**). Sample extracts for GC-MS were purified on SPE columns, followed by deriviatisation (**II-IV**).

To evaluate the most suitable hydrolysis procedure for all reported AR metabolites in Paper **II,** different enzyme concentrations (12.2, 25, 40, 50 and 70 mU) were tested. Moreover, to determine a suitable derivatisation procedure, a standard sample (100 µL) was mixed with four different ratios of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (70:30; 80:20; 90:10; 99:1) and incubated for 60 min, 90 min or 120 min. The final protocol for analysis of urine samples prior to AR metabolite determination in Papers **I-IV** is presented in Figure 5.

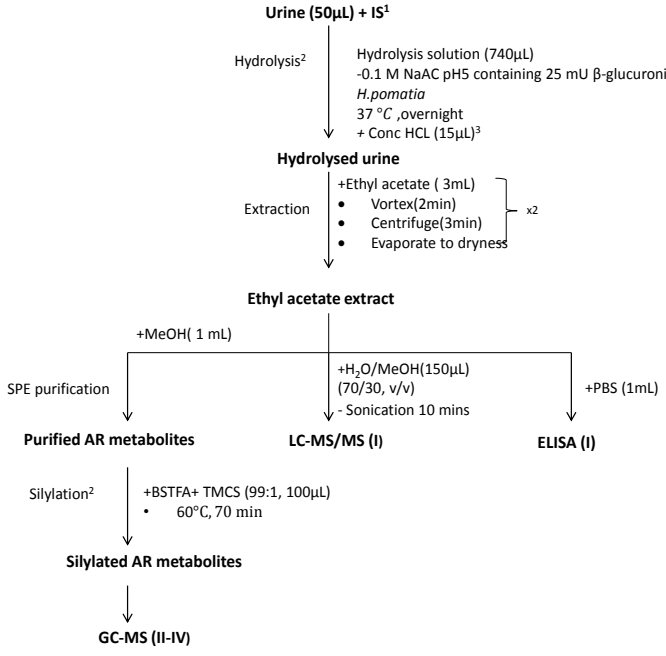


Figure 5. Schematic diagram of urine sample preparation prior to ELISA, LC-MS/MS (I) and GC-MS/MS (II-IV). [1]Syringic acid used as internal standard (IS) for LC-MS/MS and GC/MS analysis. [2]Step evaluated in Paper II. [3]Termination of hydrolysis and pH adjustment by addition of concentrated HCl (15 µL) in LC-MS/MS and GC-MS analysis.

3.3 Alkylresorcinol metabolite determination by ELISA

In order to develop an ELISA method for AR metabolite determination in human urine samples, antibodies with good analytical properties raised against DHBA and DHPPA compounds are needed. Development and characterisation of polyclonal antibodies against DHBA and DHPPA and the establishment of competitive direct and indirect ELISA, including optimisation and evaluation, was performed in Paper **I**.

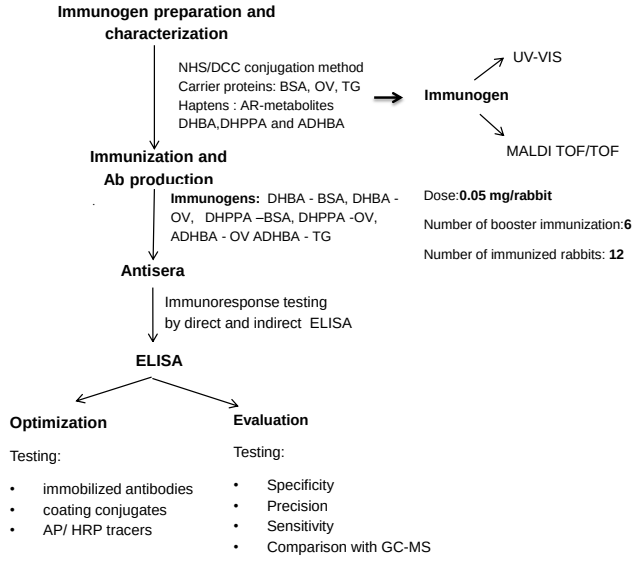


Figure 6. Schematic diagram showing development and characterisation of polyclonal antibodies against DHBA and DHPPA in work reported in Paper I.

3.3.1 Immunogen preparation and characterisation

In total, six carboxylic acid-coupled hapten-protein conjugates were prepared by coupling DHPPA and DHBA to bovine serum albumin (BSA), ovalbumin (OV) and porcine thyroglobulin (TG). A derivative of DHBA with an -NH_2 group (ADHBA) was synthesised and coupled to BSA, OV and TG through an azo-coupling reaction. Moreover, different hapten-enzyme conjugates were produced by conjugation of DHBA, DHPPA and DHPAA to horseradish peroxidase (HRP) and alkaline phosphatase (AP) enzymes using the N-hydroxysuccinimide/dicyclohexylcarbodiimide (NHS/DCC) method **(I).**

In general, preparation of hapten-protein conjugates and hapten-enzyme conjugates was based on two major steps:

1) Hapten molecule (DHBA, DHPPA), NHS and DCC were dissolved in dimethylformamide (DMF) buffer and the mixture was stirred overnight at room temperature in order to allow hapten activation.

2) Conjugation reaction between the moiety of activated hapten and the amino group of the protein/enzyme molecule. The conjugates obtained were dialysed, purified on Sephadex G-25, lyophilised and stored at -20 °C **(I).**

The immunogens obtained were subsequently characterised by ultraviolet (UV) absorption spectra and matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF) mass spectra, in order to confirm and determine the results of the conjugation reaction and to assess the hapten density (Figure 6). Mass spectrometric analysis was carried out using the Ultraflex III MALDI-TOF/TOF mass spectrometer operated in linear positive ion detection mode, as previously described (Cernoch *et al.*, 2011). The average number of hapten groups conjugated to BSA was calculated from the increase in molecular weight after conjugation of the respective hapten with BSA **(I).**

3.3.2 Immunisation and antibody production

Alkylresorcinol metabolites (DHBA and DHPPA) and ADHBA-protein conjugates were used for animal immunisation in order to elicit the animal immune response and to produce the polyclonal antibody **(I)**.

The immunogens prepared (DHBA-OV, DHBA-BSA, DHPPA-BSA, DHPPA-OV ADHBA-OV and ADHBA-TG) were used for rabbit immunisation. Each of the immunogens was injected into two rabbits and at one-month intervals six booster injections were administered to all 12 rabbits. Both indirect and direct ELISA were used for measuring the antibody titre to monitor the immune response, in tests performed 10-14 days after booster immunisation. The rabbits were exsanguinated 10 days after the last immunisation and the antiserum collected was stored at -20 ºC until use (Figure 6).

3.3.3 ELISA for AR metabolites

Characterisation of polyclonal antibodies raised against the above-mentioned AR metabolites by competitive direct and indirect ELISA was optimised by testing different combinations and dilutions of immobilised antibodies, coating conjugates and alkaline phosphatase and peroxidase tracers (Figure 6) **(I).**

Direct ELISA

Antibody diluted with coating buffer (200 µL) was added to wells of microtitre plates and incubated at 4 ºC overnight. Unbound components were removed by washing three times in $PBST_{20}$ buffer. Standard or sample (100 µL) and enzyme conjugate diluted in assay buffer (100 µL) were then added to the

wells. After 1 hour of incubation, unbound components were removed by washing three times. Finally, colour signal was developed as follows:

a) ***HRP conjugate***

A 200 µL aliquot of enzyme substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2 was added to each well and the enzymatic reaction was stopped by addition of 100 µL of 1M H_2SO_4. Absorbance was measured at 450 nm.

b) ***AP conjugate***

A 200 µL aliquot of enzyme substrate (p-nitrophenyl phosphate) in diethanolamine buffer (DEM) was added to wells and the plates were incubated at 37 ºC for 60 minutes. Absorbance was measured at 405 nm.

Indirect ELISA

Hapten-protein conjugate was added to wells of microtitre plates and incubated overnight at 4 ºC. Unbound components were removed by washing the plate three times with $PBST_{20}$ buffer and then 1% casein was added to the wells to reduce non-specific binding. The plates were washed three times, dried overnight at room temperature and stored at room temperature. The coating procedure and stabilisation by 1% casein were carried out for a large number of microtitre plates at the same time, in order to maintain standard performance conditions for a large number of samples. The standard or the sample in PBS (100 µL) and antibody in PBS (100 µL) were added to the wells. The plates were shaken and then incubated (1 h at 4 ºC). After incubation, the ELISA plates were washed three times. The detection antibody conjugate swine immunoglobulin against rabbit immunoglobulin labelled with HRP (SWAR-HRP) was added to each well and they were incubated again (1h, 4 ºC). The washing step was repeated. Enzyme substrate, chromogen and H_2O_2 were added and after 15 min the enzymatic reaction was stopped by adding 100 µL of 1M H_2SO_4. The absorbance was measured at 450 nm.

3.3.4 ELISA evaluation

All antibodies raised against DHBA and DHPPA metabolites were fully characterised in terms of specificity and assay sensitivity before the final selection **(I).** The ELISA developed was tested and evaluated for precision and accuracy using urine samples. Established ELISA was used to detect the concentration of AR metabolites in 120 urine samples from free-living

individuals and the results were compared against data obtained previously using GC-MS (Marklund *et al.*, 2013a)**.**

Assay specificity was determined by testing for cross-reactivity among standard analytes and related compounds. Percentage of cross-reactivity (CR) was calculated by the equation:

IC_{50} *of standard analyte/*IC_{50} *of respective cross-reactant x 100*

where IC_{50} is the concentration of standard or competitor resulting in a 50% reduction of enzyme conjugate binding.

The assay was optimised in the microtitre plate format to achieve maximum sensitivity. Imprecision of the ELISA method was estimated by replicate determination of AR metabolites in urine samples with low, medium and high concentrations of AR metabolites, measured in the same assay (intra-assay) or in different assays (inter-assay) over a one-week period. Intra-assay and inter-assay coefficient of variation (CV) was calculated.

Analytical recovery was evaluated using spiked DHBA and DHPPA samples and percentage recovery was calculated according to the equation:

(AR metabolite conc. measured x100)/(Measured blank + theoretical spiked added AR metabolite conc.)

Limit of detection (LOD) was evaluated as:

$LOD = mean\ A_0 + 3 \times SD$

where A_0 is the mean value of absorbance for *n* zero standards and SD is standard deviation of the mean absorbance.

3.3.5 Identification and structural elucidation of cross-reactants by LC-MS/MS

In order to investigate potential reasons for differences in results between GC-MS and ELISA, an LC-MS/MS method was developed and employed in Paper **I** to identify potential cross-reacting compounds in human urine by identifying suspicious peaks in chromatograms obtained from samples showing the poorest agreement between the two methods. The LC-MS/MS spectra were acquired using different high-energy collision dissolution modes. A structure search using the Chem Spider database and Mass Frontier software, version 7.0 was conducted for interpretation of the MS/MS spectra. Reference standards for suspected interfering compounds (DHPPTA and DHBA-glycine) were purchased commercially, while DHCA and DHCA-amide were synthesised in-house to compare standard data with those obtained in urine samples. A specific ELISA test was employed to confirm isomeric identity of the resorcinol moiety.

3.4 Determination of AR metabolites by GC-MS

A previously reported GC-MS method for determination of DHBA and DHPPA (Marklund *et al.*, 2010) was modified in Paper **II** for rapid, sensitive, robust and simultaneous determination of all currently reported AR metabolites in urine and putative biomarkers identified in Paper **I**. The method described in Paper **II** was used for analysis of urinary AR metabolites in Papers **III** and **IV.**

The procedure comprises enzymatic hydrolysis of conjugated AR metabolites, extraction of the deconjugated compounds by liquid-liquid extraction, purification on SPE columns and derivatisation of AR metabolites as described in section 3.2 (*Urine sample preparation*). The derivatised urine samples in Papers **II-IV** were analysed according to the GC-MS protocol described in Paper **II**, which is referred to as the method developed by Wierzbicka *et al.* (2015). In brief, a 1.5 µL sample was injected into a GC-MS and quantified in electron impact-selected ion monitoring (EI-SIM) using molecular ions and at least one confirmatory compound specific ion. The AR metabolites DHBA, DHPPA, DHPPTA, DHCA, DHBA-glycine and DHCA-amide in the urine samples were identified by comparing GC retention times with retention times for synthetic standards. Molecular ions were used for AR metabolite and internal standard quantification.

GC-MS method evaluation

The GC-MS method used for determination of all reported AR metabolites was evaluated and is described in Paper **II**.

In brief, an eight-point standard calibration curve was used to establish calibration linearity by an internal standard method. Specificity was evaluated as non-interference at retention time of the respective analyte from endogenous matrix components and internal standard using the proposed sample preparation procedure and GC-MS conditions. The theoretical limit of detection (LOD) and limit of quantification (LOQ) were determined as the concentration where signal to noise ratio (S/N) was 3 and 10, respectively, based on S/N determined for an analyte with low endogenous concentration in a sample. Precision was estimated by replicate determinations (n=3) for analytes in urine samples at low, medium and high concentrations, measured within the same assay (intra-assay) or in different assays performed over a one-week period (inter-assay). The recovery was evaluated by spiking quality control urine samples with three concentrations covering expected low, medium and high analyte concentrations in authentic samples. Four replicate samples were prepared at each spiking level. Moreover, to ensure method

stability, triplicate samples of two different quality control urines were included in each batch **(III** and **IV).**

3.5 Statistical analysis

In Paper **II**, differences between the silylation reagents were evaluated using general linear model (GML) with BSTFA/TMCS ratios and incubation times entered as fixed factors. Linear regression was applied to examine the stability of the AR metabolites by entering time (days) as a continuous variable and sample concentration (n=4 levels) as a fixed factor. Minitab 16 (State College, PA, USA) was used for statistical analysis and $P<0.05$ was considered statistically significant.

In Paper **III,** differences in intake and urinary excretion between occasions were evaluated by paired t-test. In paper **IV** paired t-test was used to evaluate differences in creatinine-adjusted metabolite concentrations in spot-urine samples between occasions. Spearman´s rank correlation coefficient (r_s) was calculated to examine the relationship between AR metabolites in urine and AR homologues in plasma. Moreover, to investigate how the individual AR metabolites in urine were related to each other, Spearman's rank correlations coefficient was calculated for the two occasions. The medium-term reproducibility of urinary AR metabolites excretion was assessed by calculating their ICC and 95% confidence interval (CI) (**III and IV**). The relative validity of urinary AR metabolites was assessed by calculating Spearman's rank correlation coefficient for whole grain intake, AR intake and AR metabolite urinary excretion on the same occasion, different occasions and mean of occasions. To assess the recovery across different intakes (**III**), a test for trends was conducted by GLM with log-transformed metabolite recovery as dependent variable and quartile median AR intake as independent variable. In addition, multiple linear regression models were used in order to evaluate associations between reported whole grain intake (independent variable) and log-transformed creatinine adjusted urinary AR metabolites (dependent variable) adjusted for confounding factors. Confounding factors were selected after analyzing Spearman's rank correlation coefficients between urinary AR metabolites and previously suggested confounding factors such as sex, age and BMI. Confounders were included if they were significantly correlated with urinary AR metabolit cocentrations (**IV**). All statistical analyses were performed using SAS v.9.1 (SAS Institute) and $P<0.05$ was considered statistically significant.

4 Results and discussion

4.1 Quantification of Alkylresorcinol metabolites in urine by ELISA

When this PhD project started, DHBA and DHPPA were the only known urinary metabolites of AR.

In Paper **I**, polyclonal antibodies against DHBA and DHPPA were developed, incorporated into enzyme-linked immunosorbent assay (ELISA) formats and characterised in terms of assay sensitivity, cross-reactivity, precision and accuracy. The optimised ELISA method was used for determination of AR metabolites in 120 urine samples taken from intervention and/or cross-sectional studies previously analysed by GC-MS. At this point, the other metabolites (DHPPTA, DHCA, DHCA-amide, DHBA-glycine) were found to contribute to ELISA.

4.1.1 Immunogen preparation and characterisation

Immunogens were successfully prepared by conjugation of the carboxyl group in DHBA and DHPPA metabolites with amino groups in BSA and OV protein. In this case, the 3,5 dihydroxyphenyl moiety was considered an immunodominant determinant. Hence, antibodies with high specificity towards that area were expected. Moreover, 4-amino-3,5-dihydroxybenzoic acid (ADHBA) was successfully conjugated to OV and TG proteins, in order to obtain antibodies towards the carboxyphenyl moiety. In this case, the carboxyl group was situated in an immunodominant position and thus high specificity towards this side of the ADHBA molecule was anticipated (Figure 7).

The absorption spectra of DHPPA-BSA and DHPPA-OV conjugates showed symmetrical peaks at 280 nm, whereas surprisingly, the conjugates having DHBA hapten linked to the same carrier proteins exhibited spectrum shifted to longer wavelengths (285-350 nm). The spectral change in the DHBA hapten was ascribed to the electron-withdrawing effect of the carbonyl function

in the peptide bond (-CONH-) resulting in hydroxyl ionisation in the resorcinol moiety. On the other hand, the longer aliphatic chain CH2-CH2-CONH- in DHPPA-protein conjugate contributed to neutral ionisation state of the hydroxyl groups through the electron-donating effect. Hapten density rate for the DHBA-, DHPPA- and DHBA-azo-BSA was 39.8, 26.9 and 1.5 (mol/mol), respectively. Hapten density for OV conjugates was not measured because MALDI-TOF/TOF mass analysis did not provide a suitable spectral characteristic.

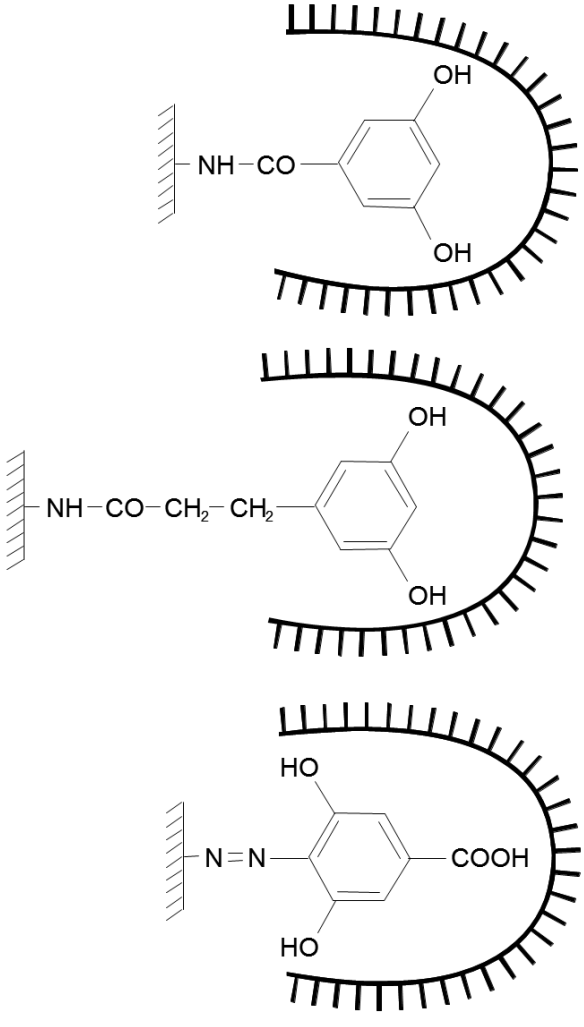


Figure 7. Recognition strategy schematically depicted for DHBA, DHPPA and 4-Amino-3,5-dihydroxybenzoic acid (ADHBA) hapten protein conjugates. In the hapten design, the upper DHBA, DHPPA structures are bound to carrier protein via a carboxyl group which is a natural part of the molecules. Aliphatic chains of the molecules serve here as a spacer by which the

haptens are linked to carrier proteins. According to immunochemical theory, the areas of the hapten removed from the spacer mostly have a high degree of specificity (3,5-dihydroxyphenyl moiety), while the vicinity of the spacer is typified by low specificity (cross-reactivity). Hapten with a short spacer azo group between two hydroxy groups was designed to achieve high specificity towards the opposite side of the ADHBA molecule (carboxyphenyl moiety). Hatched curves represent antigen binding site of the antibody.

4.1.2 Antibody production and assay optimisation

Polyclonal antibodies raised against DHBA and DHPPA exhibited a titre higher than 1/2000 and showed promising binding characteristics in the indirect ELISA. Therefore, the indirect format was initially used for antibody characterisation. However, it was not possible to achieve a stable and robust assay, even though great efforts were made by testing various blocking agents, coating conditions and incubation times and temperatures. Based on these observations, a direct ELISA system was further investigated. This format proved to be more stable and less sensitive to temperature changes and to other unpredictable factors. Direct ELISA was used for antibody characterisation and subsequently for DHBA and DHPPA quantification in human urine samples.

The ELISA employing alkaline phosphatase (AP) conjugates exhibited good calibration curves, whereas peroxidase conjugates were surprisingly not recognised by the corresponding antibodies.

The DHBA-azo-immunogens with low hapten density produced antibodies with strong binding to immunogen and negligible analyte displacement, and hence they were excluded from further evaluations. Table 2 summarises results of the optimised assays for DHPPA and DHBA quantification.

Table 2. *Optimised ELISA systems for DHBA and DHPPA determination developed in Paper I*

Direct ELISA [a]				
Coating antibody	*Antibody dilution*	*AP conjugate*	*AP conjugate dilution*	*IC_{50} (µmol/L)*
Anti DHPPA-OV	1:500	DHPPA-AP	1:500	4.93
Anti DHPPA-BSA	1:10 000	DHPPA-AP	1:5000	1.80
Anti DHBA-OV	1:5000	DHBA- AP	1:5000	1.76
Indirect ELISA				
Antibody	*Antibody dilution*	*Coating conjugate*	*Conjugate dilution*[a]	*IC_{50} (µmol/L)*
Anti DHPPA-OV	1:20 000	DHPPA-BSA	1:10 000	1.30
Anti DHPPA-BSA	1:10 000	DHBA-OV	1:20 000	8.33
Anti DHBA-OV	1:50 000	DHBA-BSA	1:50 000	5.42

[a]Concentration of coating conjugate prior to dilution was 0.2 mg/mL.

*Each antibody was obtained from one rabbit.

[b]IC_{50} was determined from duplicate wells in multiple experiments (n=6) over a period of 8 weeks

4.1.3 Evaluation of ELISA and method comparison

At the start of the study, the specificity of the antibodies in optimised assays was tested by measuring cross-reactivity among DHPPA or DHBA as standard analytes and for chemically related compounds, including AR homologues. The antibody raised against DHPPA showed relatively low cross-reactivity with 3,5-dihydroxyphenyl acetic acid (DHPAA) (12 and 20%) in both direct and indirect formats and negligible reactivity with DHBA and intact ARs. Because DHPAA is not a product of AR metabolism and dietary precursors of DHPAA are minor, it is unlikely that it will cause interferences in the ELISA for DHPPA in body fluids. In addition, negligible cross-reactivity between DHPPA and DHBA can be associated with ionisation state of the resorcinol OH- groups, as deduced from the absorption spectra. It can be postulated that the antibody primarily distinguished various degrees of ionisation of hydroxyl groups in AR, whereas chemical differences in the site of conjugation (-COOH versus CH2-CH2-COOH) were not recognised by the antibody.

Direct ELISA based on the antibody against DHBA showed 19% cross-reactivity with DHPPA, whereas cross-reactivity with other structurally related compounds tested was below 2%. As expected, the cross-reactivity with ferulic acid in all reagent combinations was negligible.

Standard curves constructed for metabolites were in the range 0.005-55 µmol/L and were suitable for quantification of DHBA and DHPPA in urine samples after 20-fold dilution in order to reduce potential matrix interferences.

The direct ELISA developed was evaluated in terms of assay sensitivity, precision and recovery. The results obtained were acceptable for determination of both metabolites and are presented in Table 3.

Table 3. Performance analytical parameters of the ELISA method described in Paper I

.Metabolite	LOD[1] [µmol/L]	IntraCV[2] [%]	InterCV[2] [%]	Recovery [%]
DHBA	0.09	1.3-9.4	3.5-20	102[a]
DHPPA	0.06	0.7-7.2	5.1-11.5	104[b]

[1] Determined in phosphate buffer saline (PBS) by direct ELISA.

[2]Samples (n=3) having low, medium and high concentrations of both metabolites were measured, each sample was prepared in triplicate.

[a]Mean recovery of the concentrations tested (0.64, 3.24, 12.97 µmol/L).

[b]Mean recovery of all concentration tested (0.54, 2.74, 10.97 µmol/L).

In the method comparison, AR metabolite concentrations in urine measured by ELISA were observed to be three- to five-fold higher by GC-MS (r^2=0.09 and r^2=0.31 for DHBA and DHPPA, respectively). Poor agreement between those methods (Figure 8) was probably due to presence of other and unknown compounds such as newly discovered DHPPTA, DHBA-glycine and sulphated conjugate of DHCA (Zhu *et al.*, 2014; Bondia-Pons *et al.*, 2013).

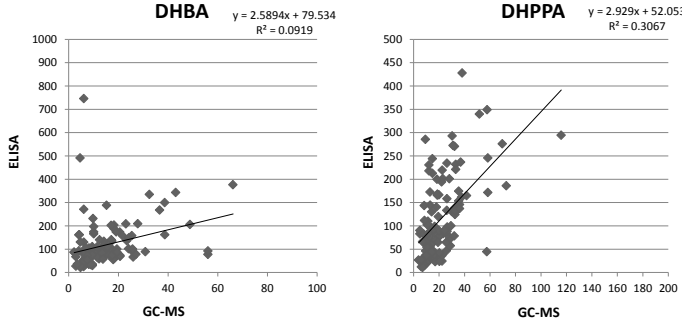


Figure 8. Correlation between concentrations of (left) 3,5-dihydroxybenzoic acid (DHBA) and (right) 3-(3,5-dihydroxyphenyl)-propanoic acid (DHPPA) determined in 111 urine samples by direct ELISA and GC-MS.

4.1.4 Further testing of ELISA cross-reactivity of identified resorcinolic compounds

Two putative alkylresorcinol-derived metabolites, DHCA and DHCA-amide, identified by LC-MS/MS in Paper I and DHPPTA and DHBA-glycine (Figure 9), recently reported as AR metabolites (Zhu *et al.*, 2014), showed high cross-reactivity in the antibodies developed against DHBA and DHPPA (Table 4). The chemical identities of the new compounds were confirmed in Paper I by comparing retention times and spectra with those of reference standards.

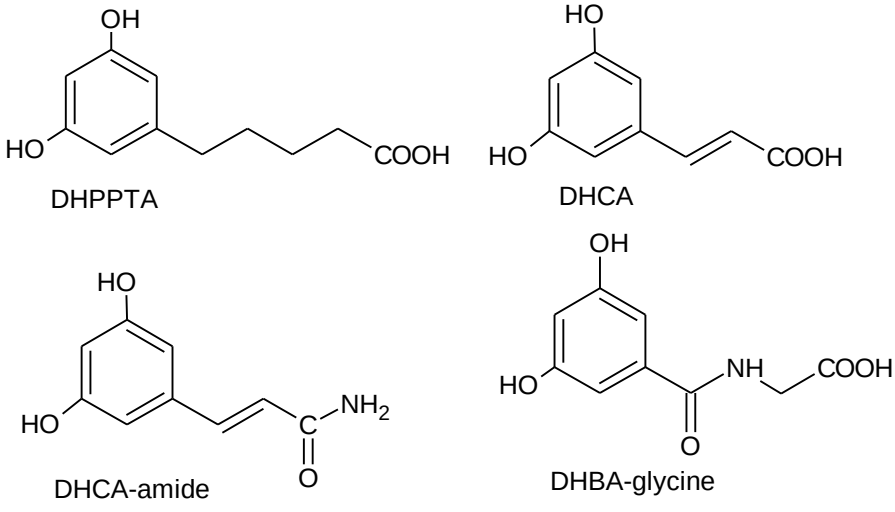


Figure9. Chemical structure of new putative AR metabolites: DHPPTA, 5-(3,5-dihydroxyphenyl) pentanoic acid; DHCA, 3,5-dihydroxycinnamic acid, DHCA-amide, 3,5-dihydroxycinnamic acid amide, DHBA-glycine, 2-(3,5-dihydroxybenzamido)acetic acid.

The 3,5-DHCA molecule, as well as 3,5-DHCA-amide and DHBA-glycine, have a planar structure due to a conjugated π electron system as the DHBA hapten attaches to the immunogen via a peptide linkage. Therefore these three compounds exhibited a strong ELISA response with antibodies against DHBA (cross-reactivity was 115, 420 and 5000%, respectively) (Table 4).

Moreover, the structure of isomeric 3,4-DHCA did not elicit any measurable response to the compound in the ELISA test, thus showing that the antibody can specifically recognise only the 3,5-dihydroxyphenyl (resorcinol) moiety in the molecules. On the other hand, the peptide bond in DHPPA-protein conjugates does not hinder rotation of the resorcinol group and thus does not result in a planar system. As expected, antibodies raised against DHPPA showed very low cross-reactivity with DHBA-glycine, which has a planar structure, as well as DHBA, DHCA and DHCA-amide. Moreover, cross-reactivity of the prepared antisera was associated with ionisation state of the resorcinol OH- groups deduced from the absorption spectra, as mentioned above. One can speculate that the antibodies are able to distinguish various ionisation degrees of hydroxyl groups in the AR structures.

Table 4. *Cross-reactivity among selected compounds in direct ELISA*

Compound	Reagent combination	
	Anti DHPPA-BSA DHPAA-AP	Anti DHBA-OV DHBA-AP
	Cross-reactivity (%)*	
DHCA	12	115
DHCA-amide	60	420
DHPPTA	26	11
DHBA-glycine	1	5000

*Number of experiments performed n = 3

4.2 Quantification of DHBBA, DHPPA and new alkylresorcinol metabolites in urine by GC-MS

A modified method based on Marklund *et al*. (2010) was developed in Paper **II** to determine AR metabolites (DHBA, DHPPA) along with two novel (DHPPTA and DHBA-glycine) and two putative AR metabolites (DHCA and DHCA-amide).

Chromatographic separation for all recently reported AR metabolites was obtained within 7 min (Figure 10), which is 9 min shorter than with existing methods (Marklund *et al.*, 2010).

The method described did not show any apparent interference for AR metabolites or for the internal standard when analysed in blank and spiked urine samples for detected AR metabolites. The optimised GC-MS conditions were applied to a large number of samples. Sensitivity of the method is highly dependent on the instrument conditions and therefore maintenance of the established GC-MS conditions was performed every 200 injections. The limit of quantification, recovery and precision of the GC-MS method developed were satisfactory (Table 5). Moreover, statistical evaluation of the effect of a four-day time period and concentration interaction revealed no significant influence on the stability of AR metabolite concentrations.

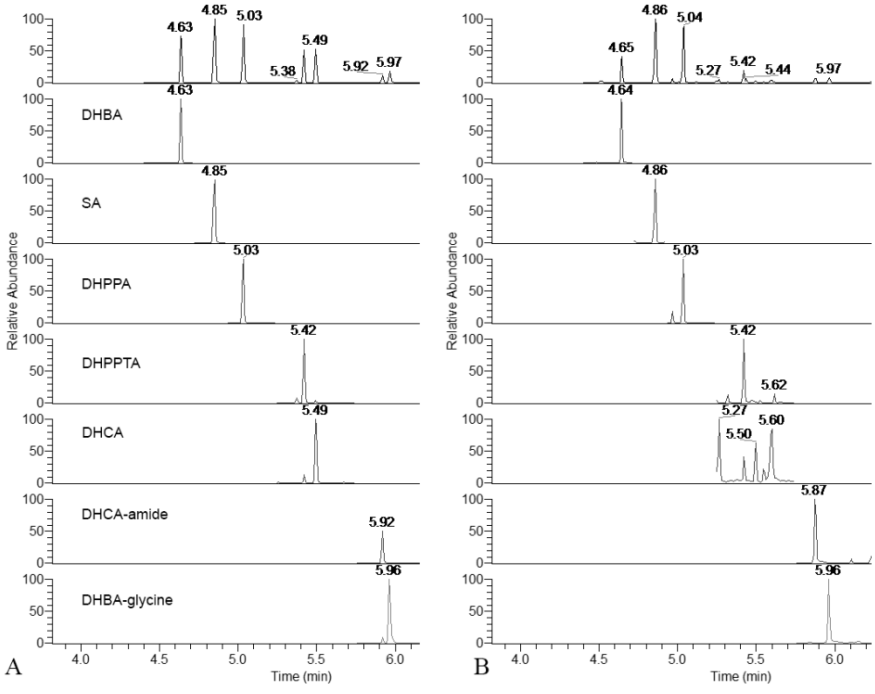


Figure 10. Total ion chromatogram (TIC) and selected ion monitoring (SIM) chromatograms for molecular ions from DHBA, SA, DHPPA, DHPPTA, DHCA, DHCA-amide and DHBA-glycine in (left) standard solution and (right) a blank urine sample treated according to the experimental protocol.

The most suitable enzyme concentration for simultaneous hydrolysis of DHBA, DHPPA, DHPPTA, DHBA-glycine, DHCA and DHCA-amide was optimised and was found to be 25 mU β-glucuronidase from *H. pomatia*. Detected concentrations of DHPPTA, DHCA and DHCA-amide were much higher after the deconjugation procedure compared with the procedure with no deconjugation step. These results confirm that DHPPTA, DHCA and DHCA-amide are present in urine in the free form or as a conjugates, as reported previously for DHBA and DHPPA (Marklund *et al.*, 2010(Koskela, 2007 #410). Moreover, the deconjugation step did not affect DHBA-glycine, which shows that this molecule is not sulphated or glucuronidated.

In order to obtain the highest efficiency of the silylation procedure, several conditions, *i.e.* effects of BSTFA/TMCS ratio and time of incubation for DHPPTA, DHCA, DHCA-amide and DHBA-glycine, were systematically evaluated. The results showed that all AR metabolites were similarly derivatised under all conditions tested. In the final protocol, a BSTFA/TMCS ratio of 99:1 was used (Figure 5), since a 70:30 ratio could cause

contamination of the GC-MS and problems with the syringe injection. Moreover, 70 min, which was 10 min longer than in the previous method, was a sufficient time for derivatisation of all AR metabolites. When several sample batches need to be analysed, the derivatisation step should preferably be conducted at the same time for all batches to lower the inter-batch variation.

Table 5. *Parameters for GC-MS method validation described in Paper II*

AR metabolite	Parameters			
	LOD[1]	Recovery[2]	IntraCV[3]	InterCV[4]
	$\mu molL^{-1}$	%	%	%
DHBA	0.3	108	8.9	13.1
DHPPA	1.5	90	2.9	9.3
DHCA	0.1	97	4.8	10.8
DHPPTA	0.1	80	8.8	4.3
DHCA-amide	0.4	75	9.0	6.7
DHBA-glycine	0.1	88	7.4	11.4

[1]Limit of detection was determined as the concentration where signal to noise ratio (S/N) was 3.
[2]The recovery was evaluated by spiking quality control urine samples with three contents covering expected low, medium and high analyte concentrations in authentic samples.
[3]Intraassay CV (mean) was determined from the intrassay CV of three occasions from batch 1.
[4]Interassay CV (mean) was determined from the interassay CV from three occasions.

4.3 Evaluation of the new alkylresorcinol metabolites as dietary biomarkers

The new suggested AR metabolites DHPPTA, DHCA, DHBA-glycine and DHCA-amide, quantified according to the method described in Paper **II,** were evaluated as biomarkers of whole grain rye and wheat intake by assessing the medium-term reproducibility and relative validity in 24-h urine samples from a Swedish free-living population (Paper **III)** and by determination of their reproducibility in spot urine samples after adjustment of creatinine and correlation with intake in a population of middle-aged Swedish men and women (Paper **IV**).

4.3.1 Excretion and reproducibility of Alkylresorcinol metabolites

Paper III

Urinary excretion of AR metabolites did not differ significantly between the first and second occasion ($P>0.05$) except for DHCA-amide, for which excretion was higher on the second occasion ($P<0.05$). DHBA and DHPPA urinary excretion was similar to that in a previous study based on the same participants (Marklund *et al.*, 2013a) and comparable to that in a study on Finnish women (Aubertin-Leheudre *et al*., 2010). The highest urinary excretion among confirmed and putative AR metabolites was found for DHCA-amide, followed by DHPPA, DHBA, DHCA, DHBA-glycine and DHPPTA. The concentration of DHCA-amide was considerably higher than that of the other AR metabolites and the recovery of excreted DHCA-amide exceeded 891 % of the ingested AR dose. This strongly suggests the presence of additional dietary precursors along with AR. The proportion of the individual metabolite in total urinary AR metabolite excretion (sum of DHPPA, DHBA, DHCA, DHBA-glycine and DHPPTA) was: DHPPA 42%, DHBA 33%, DHCA 13%, DHBA-glycine 9% and DHPPTA 2% (Figure 11).

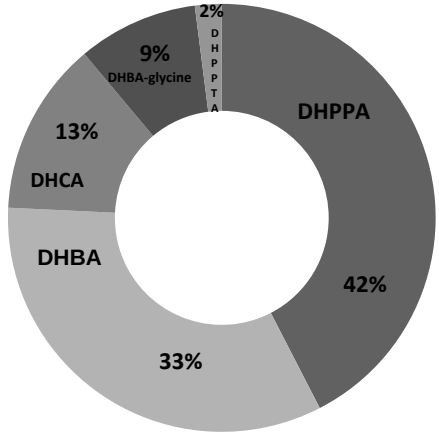


Figure 21. Proportion of the individual alkylresorcinol (AR) metabolites in total 24-h urinary AR metabolite excretion obtained in Paper III.

The different metabolites were well correlated with each other: DHBA r= 0.43-0.78, DHPPA r=0.34-0.78, DHCA r=0.50-0.81, DHPPTA r=0.25-0.81, and DHBA-glycine r=0.34-0.69. DHCA-amide correlated only with DHBA-glycine (r=0.69) and DHPPTA (r=0.25). Moreover, modest to strong correlations between different putative and novel AR metabolites and AR homologues in plasma were observed. This is indirect evidence that they are all metabolites of intact AR homologues (Landberg et al., 2009; Ross *et al*., 2004).

The reproducibility for DHCA, DHPPTA and DHBA-glycine estimated in Paper III was good to excellent and appeared somewhat higher than that of DHPPA and DHBA. The highest reproducibility was shown by DHCA and DHPPTA (ICC = 0.63, 95% CI = 0.49-0.75 for both). This can be due to longer half-life of these components, as indicated in a previous study (Zhu *et al.*, 2014).

The reproducibility of DHBA and DHPPA in 24-h urine samples was similar to that previously reported for DHBA and DHPPA (Marklund *et al.*, 2013a) and fasting plasma AR concentration (Andersson *et al.*, 2011b), but higher than for fasting plasma AR metabolites (Montonen *et al.*, 2012).

In comparison, medium-term reproducibility of all currently reported AR metabolites investigated in this thesis was similar to or higher than that

observed for the other biomarkers used in epidemiological studies, such as plasma carotenoids, vitamin D analytes and enterolactone (Kotsopoulos *et al.*, 2010).

Paper IV

In Paper **IV,** AR metabolite concentrations were measured in a second batch of spot urine samples and adjusted for creatinine concentration. The highest concentrations were found for DHBA and DHPPA, followed by DHCA, DHBA-glycine and DHPPTA. The DHBA and DHPPA concentrations measured in Paper IV appeared to be in the same range as in Paper III and double those found in spot urine samples from US women (Landberg *et al.*, 2012). The relative proportion of the AR metabolites was 33%, 33%, 16%, 14% and 4% for DHBA, DHPPA, DHCA, DHBA-glycine and DHPPTA, respectively (Figure 12), which is comparable to that found in Paper III.

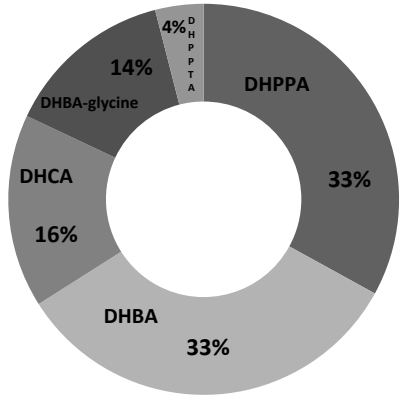


Figure 32. Proportion of the individual alkylresorcinol (AR) metabolites in total AR metabolite excretion measured in spot urine samples obtained in Paper IV.

The results showed that creatinine-adjusted AR metabolites in spot urine were highly correlated with each other ($r=0.46$-0.83, $P<0.001$). The only exception was DHPPTA, which was not correlated with DHPPA ($r=0.17$, $P>0.05$) and

modestly correlated with DHBA and DHBA-glycine. The strongest correlation was found for DHCA (r=0.68-0.81). The significant correlation between the recently suggested AR metabolites (DHCA, DHBA-glycine and DHPPTA) and established AR metabolites (DHBA and DHPPA), along with the presence of their free and/or conjugated forms in urine after controlled whole grain wheat and rye intake (Zhu *et al.*, 2014; Bondia-Pons *et al.*, 2013), strongly suggests they are AR metabolites. However, the structure of the DHCA molecule suggests that biochemically, this compound cannot be formed through the established hepatic AR metabolism pathway.

The reproducibility of AR metabolites in spot urine determined in Paper IV was similar to that previously reported for DHBA and DHPPA in 24-h excretions (Marklund *et al.*, 2013a) or higher than that shown for DHBA and DHPPA in creatinine-adjusted spot urine (Marklund *et al.*, 2013a; Landberg *et al.*, 2012). Moreover, the reproducibility of the new AR metabolites (DHCA, DHBA-glycine and DHPPTA) in spot urine samples taken two weeks apart was compared with the reproducibility of the same metabolites in two 24-h urine collections taken 2-3 months apart in a population with a wide intake range in Paper III. The results were of the same magnitude (or slightly higher for the new metabolites), probably because of smaller fluctuations resulting from shorter time interval between samplings.

4.3.2 Relative validity and determinats beyond intake

Paper III

The 24-h urine excretion of DHBA, DHPPA, DHCA, DHPPTA was significantly correlated to self-reported intake of total whole grain, whole grain rye and whole grain rye + wheat, irrespective of when intake and excretion were measured. DHBA-glycine was only correlated to whole grain rye and whole grain rye+ wheat intake, where the intake was measured on the first occasion. The poor correlation between DHBA-glycine and other AR metabolites suggests that this metabolite may have other, more important, precursors than AR. It could also be the case that DHBA-glycine is more extensively excreted through bile and therefore urinary DHBA-glycine shows lower correlations with other urinary AR metabolites and AR intake.

Spearman's rank correlation coefficient calculated for whole grain rye intake on the first occasion and metabolite excretion was highest for the new putative AR metabolite DHCA (r_s = 0.61-0.66), followed by DHPPA (r_s=0.56-0.66) and DHBA (r_s=0.54-0.67). These results were similar to those in studies where whole grain intake was estimated by weighed food records (Marklund *et*

al., 2013a; Aubertin-Leheudre *et al.*, 2010b) and higher than in studies where biomarker measurement was correlated with long-term intake assessment methods, *e.g* FFQ (Landberg *et al.*, 2012). Correlations between whole grain wheat intake and urinary metabolites were weak and in most cases absent. The good correlation between whole grain rye and weak correlation between whole grain wheat was probably because rye was the main source of whole grain intake, with unstable and low wheat intake, in the population studied (Paper **III**). Further studies based on participants with higher and more stable wheat intake might provide a better correlation between AR urinary excretion and whole grain wheat intake.

In general, AR metabolites were not correlated with whole grain oats, barley, maize or rice. Any slight correlations observed can be due to the minor dietary source of DHBA and DHPPA detected in the non-AR-containing cereals (Landberg *et al.*, 2009a).

The strong correlation observed between AR metabolite excretion and AR intake was probably due to the fact that rye contains high amount of AR and was the main source of whole grain in Paper **III.**

Recovery of ingested AR excreted as metabolites in urine was higher in participants with lower AR intake. This pattern has also been observed in other studies (Landberg *et al.*, 2009d). It can be explained by a change in the elimination route and decreased absorption with high doses.

Paper IV

The results of Paper IV showed that AR metabolites were correlated with intake. However when investigating their independent association with whole grain intake, only DHBA, DHCA and DHBA-glycine were associated. This is because sex and age were independently associated and found to be possible confounders of the intake in the Spearman correlation analyses. The sex-related differences in AR metabolism were also suggested by Marklund *et al*(Marklund *et al.*, 2012) and have been observed for γ-tocopherols (Frank *et al.*, 2008). The results in Paper IV support the idea that sex is a strong independent predictor and should be taken into account when applying urinary AR metabolites as biomarkers.

The results of Papers III and IV suggest that DHCA and DHPPTA measured in a single 24-h urine sample and one or several spot urine samples may be used as a medium to long-term biomarker of whole grain wheat and rye intake. However, this should be confirmed in populations with lower whole grain intake before testing the applicability of these metabolites as biomarkers in large epidemiological studies.

4.3.3 General discussion

At the start of this PhD project, the only known AR metabolites were DHBA and DHPPA. Previous studies have suggested that these metabolites have a longer half-life than intact AR (Soderholm *et al.*, 2009) and that their 24-h excretion correlates well with estimated intake in a dose-dependent manner (Landberg *et al.*, 2009d). Based on this, they have potential as dietary biomarkers. Therefore polyclonal antibodies against these metabolites were developed and optimised in this thesis in order to obtain a simple and inexpensive method for analysing a large number of samples for epidemiological studies. Paper I showed that antibodies developed against DHBA and DHPPA exhibited high cross-reactivity. When investigating this further, it was found that identified compounds with structural similarities (DHCA, DHCA-amide, DHPPTA and DHBA-glycine, identified as potential AR metabolites by others) showed high cross-reactivity (Zhu *et al.*, 2014; Bondia-Pons *et al.*, 2013). Antibodies against DHBA exhibited high cross-reactivity against DHCA, DHCA-amide and DHBA-glycine (115, 420 and 5000 %, respectively). Slight cross-reactivity was also observed for antibodies against DHBA metabolites with DHPPA. Current knowledge considering the presence of the new putative AR metabolites in urine, their structure and their role as cross-reactants in the assays developed in Paper I probably explains the lack of agreement between ELISA and GC-MS results. It remains to be determined whether ELISA can be applied for quantification of AR metabolites in urine samples after taking the new knowledge about cross-reacting compounds into account. Purification or fractionation of sample extracts could potentially be applied, and/or correction models taking differences in cross-relativity of different metabolites into account. Establishment of a valid GC-MS method (Paper II) allows accurate determination of different metabolites and could thus provide valid data for development of correction models for ELISA and for use in evaluation of the new metabolites as biomarkers.

Among the new metabolites identified (Papers III and IV), DHCA was identified as a likely AR metabolite based on its correlation with other AR metabolites and with plasma AR concentrations and the intake of whole grain wheat and rye (Paper III). DHCA has also been found in urine as a sulphate conjugate after controlled rye intake (Bondia-Pons *et al.*, 2013). However, the double bond between carbon 2 and 3 in the propionic acid part of the molecule suggests that this compound cannot be formed through the established hepatic AR metabolism pathway (Ross *et al.*, 2004c). It remains to be determined how this metabolite is formed. Moreover, DHCA-amide was present in high concentrations in urine in Papers III and IV, but it was generally poorly

correlated with other AR metabolites, plasma AR concentration and whole grain wheat and rye intake, and was found in higher amounts in urinary 24-h excretion than the corresponding AR ingested. Thus, it appears that this molecule is derived from other sources than AR, or at least has major determinants other than AR. Based on this, it should not be further evaluated as a biomarker of whole grain wheat and rye intake.

Papers III and IV showed that the new putative AR metabolites in 24-h urine collections and spot urine samples had good to excellent reproducibility and that they were in general as well or better correlated to intake than existing biomarkers. An exception was DHBA-glycine, where a weak correlation with whole grain intake and no correlation with AR intake was observed (Paper III). This can be due to the fact that this metabolite can have other precursor than AR, or that it may be affected by non-dietary determinants or can be more readily excreted in bile. However, when considering using DHCA, DHPPTA and DHBA-glycine as biomarkers of whole grain intake, future studies should be performed in populations with lower whole grain intake before the biomarkers are tested in relation to endpoints in epidemiological studies. This is particularly important since Paper IV indicated that age and sex influenced the validity of AR metabolites in spot urine samples and therefore should be taken into account when using urinary AR metabolites as biomarkers. Other determinants may also play a role in different populations and should therefore be evaluated.

In conclusion, the results presented in Papers III and IV support the suggestion that new AR metabolites, particularly DHCA, appear promising as a biomarker of whole grain wheat and rye intake in populations where these cereals are consumed frequently and regularly. It remains to be determined whether these new putative biomarkers can be used in combination with traditional dietary assessment methods to improve intake estimations and reduce sample requirements (Freedman *et al.*, 2010). Moreover, as already mentioned, AR metabolites together with plasma AR can be used to measure dietary compliance in dietary intervention studies and especially to monitor compliance within individuals over treatment periods.

5 Main findings

- Lack of agreement between ELISA and GC-MS was observed, due to the presence of as yet unknown metabolites which cause a high degree of cross-reactivity with the antibodies developed (Paper I)
- Cross-reactivity is likely caused by the presence of DHPPTA, DHCA, DHBA-glycine and DHCA-amide and possibly by additional yet unknown compounds (Paper I).
- A GC-MS method for rapid, sensitive and robust determination of all currently reported AR metabolites in urine was developed (Paper II) and applied in biomarker evaluation studies (Papers III and IV).
- The medium-term reproducibility of DHCA, DHPPTA and DHBA-glycine in 24-h urine samples was found to be moderate to excellent. DHCA and DHPPTA metabolites correlated well with self-reported whole grain rye and wheat intake. This suggests that measurement of DHCA and DHPPTA excretion in a single 24-h urine sample may adequately reflect medium to long-term whole grain wheat and rye intake and thus they have potential as biomarkers in populations with similar intake patterns (Paper III).
- The reproducibility of DHCA, DHPTTA and DHBA-glycine in single-spot urine samples after creatinine adjustment determined in an independent population of middle-aged Swedish men and women over a period of 2 weeks was good. The results suggested that AR metabolites determined in a single or duplicate spot urine samples could be useful biomarkers of whole grain intake (Paper IV).

6 Future research

- Studies to evaluate the performance of the assay developed after different purification or fractionation steps could be undertaken. Correction models based on relative metabolite abundance and cross-reactivity could also be tested.
- More effort could be focused on identification and validation of other possible compounds as cross-reactants in urine, in order to utilise the ELISA method for quantification of AR metabolites in urine samples.
- Animal feeding and cell studies with pure AR homologues need to be performed, in order to confirm DHCA and DHBA-glycine as AR metabolites. Moreover, alternative intake sources of these compounds should be identified.
- To confirm the usefulness of new AR metabolites (particularly DHCA) in 24-h and spot urine collections as dietary biomarkers of whole grain wheat and rye, further studies in populations with lower intake are needed.
- Human pharmacokinetic studies should be performed in order to determine the half-life of new putative AR metabolites (DHCA, DHPPTA, DHCA-glycine and DHCA-amide) as a possible long-term biomarker of whole grain intake.
- Alkylresorcinols or other compounds in adipose tissue could be evaluated as more long-term biomarkers of whole grain wheat and rye intake.

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