

Alkylresorcinol Metabolites

Candidate Biomarkers for Whole Grain Wheat and Rye
Intake

Matti Marklund

*Faculty of Natural Resources and Agricultural Sciences
Department of Food Science
Uppsala*

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Abstract

Alkylresorcinols (AR), a group of phenolic lipids present in the outer parts of wheat and rye, have been evaluated as biomarkers for the intake of whole grain (WG) products of these cereals. AR may be utilized to facilitate the investigation of diet-disease associations in epidemiological studies. In the body, AR are metabolized to two phenolic acids, DHBA and DHPPA, which can be detected in plasma and urine, either as such or as more polar conjugates. The aim of this thesis was to evaluate AR metabolites DHBA and DHPPA as biomarkers for the intake of WG wheat and rye.

A gas chromatographic – mass spectrometric method was established that allowed the reliable quantification of AR metabolites in urine samples. This method was comparable to a previously established HPLC-CEAD method. Plasma pharmacokinetics of AR and their metabolites were compared in rats administered intact AR orally or AR metabolites intravenously. The elimination was faster for AR metabolites than for intact AR and the elimination half-lives increased linearly with the number of carbon atoms in the side chain. The formation of metabolites did not differ between rats given shorter or longer AR homologues, indicating that the different homologues were absorbed to a similar extent. Several anthropometric and dietary factors were identified by multivariate statistical tools as potential determinants of urinary AR metabolites and were hypothesized to influence the formation and excretion of AR metabolites at different stages during AR elimination. The reproducibility and relative validity of urinary AR metabolites as biomarkers for the intake of whole grain wheat and rye were assessed in Swedish men and women consuming their habitual diet. AR metabolites were well correlated to self-reported intakes of WG and cereal fibre. The reproducibility of urinary AR metabolites over 2-3 months was dependent on the stability of intake and was higher in 24-h urine collections compared to spot samples.

Due to the scarcity of 24-h urine collections in large observational studies and the modest reproducibility in plasma and spot urine samples, the utility of single AR metabolite measurements alone to reflect long-term intakes in epidemiological studies is limited. However, other applications of AR metabolites as biomarkers might prove useful in future studies (e.g., as a compliance check in intervention studies or as reference methods in validations of other dietary assessment tools).

Keywords: Alkylresorcinol, biomarker, DHBA, DHPPA, metabolites, rye, wheat, whole grain

Author's address: Matti Marklund, SLU, Department of Food Science,
P.O. Box 7051, 750 07 Uppsala, Sweden
E-mail: Matti.Marklund@slu.se

Dedication

To family and friends.

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List of Publications

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

- I Marklund M, Landberg R, Åman P, Kamal-Eldin A. (2010). Determination of alkylresorcinol metabolites in human urine by gas chromatography-mass spectrometry. *Journal of Chromatography B* 878(11-12), 888-894.
- II Marklund M, Landberg R, Åman P, Kamal-Eldin A. (2011). Comparison of gas chromatography-mass spectrometry and high-performance liquid chromatography with coulometric electrode array detection for determination of alkylresorcinol metabolites in human urine. *Journal of Chromatography B* 879(9-10), 647-651.
- III Marklund M, Strömberg E, Hooker A, Hammarlund-Udenaes M, Åman P, Landberg R, Kamal-Eldin A. Plasma pharmacokinetics of alkylresorcinols and their metabolites in rats (manuscript).
- IV Marklund M, Landberg R, Andersson R, Åman P, Kamal-Eldin A. Alkylresorcinol metabolism is affected by factors other than intake of whole grain wheat and rye (submitted).
- V Marklund M, Landberg R, Andersson A, Åman P, Kamal-Eldin A. (2012). Alkylresorcinol metabolites in urine correlate with intake of whole grains and cereal fibre in free-living Swedish adults. *British Journal of Nutrition* (accepted for publication).

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The contribution of Matti Marklund to the papers included in this thesis was as follows:

- I Participated in planning experiments and evaluation of results, performed laboratory and statistical analyses, and was responsible for writing and revising the manuscript.
- II Participated in planning experiments and evaluation of results, performed laboratory and statistical analyses, and was responsible for writing and revising the manuscript.
- III Participated in planning the study and evaluation of results. Was responsible for laboratory and statistical analyses. Had the major responsibility for manuscript preparation.
- IV Participated in planning experiments and evaluation of results, supervised laboratory analyses, and performed statistical analyses. Was responsible for writing and revising the manuscript.
- V Participated in the planning of the study. Conducted the clinical study, performed laboratory and statistical analyses. Had the major responsibility for writing and revising the manuscript.

Abbreviations

3DWFR	Three-day weighed food record
AR	Alkylresorcinol(s)
CI	Confidence interval
DB	Dietary biomarker(s)
DHBA	3,5-dihydroxybenzoic acid
DHPAA	3,5-dihydroxyphenyl acetic acid
DHPPA	3-(3,5-dihydroxyphenyl)-1-propanoic acid
FFQ	Food frequency questionnaire
GC-MS	Gas chromatography-mass spectrometry
HDL	High-density lipoprotein
HPLC-CEAD	High performance liquid chromatography-coulometric electrode array detection
ICC	Intra-class correlation coefficient(s)
PLS	Partial least squares
$t_{1/2}$	Elimination half-life
WG	Whole grain
r_s	Spearman's rank correlation coefficient(s)

1 Background

A high intake of whole grain (WG) has been associated with decreased risk of chronic diseases (such as coronary heart disease, diabetes, obesity, and some cancers) in several observational studies (McKeown *et al.*, 2012; Mellen *et al.*, 2008; Priebe *et al.*, 2008; Jensen *et al.*, 2006; Montonen *et al.*, 2003). Although the direct mechanisms are largely unknown, the health promoting effects of WG consumption are considered to be mainly attributable to the abundance of dietary fibre and phytochemicals in cereals (Fardet, 2010).

Defined by AACC International (formerly known as the American Association of Cereal Chemists), "whole grains shall consist of the 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". The main cereals consumed as whole grain worldwide are wheat, rice and maize, followed by oats, rye, barley, triticale, millet, and sorghum (Fardet, 2010). Among Nordic populations, rye is the main source of whole grains in Denmark, Finland, and Sweden, while wheat dominates in Norway (Kyro *et al.*, 2011).

Evaluations of the relationship between diet and disease might be hampered due to large diversities between studies in the estimation of WG intake (e.g., WG definitions used, cereals included as WG and inclusion threshold for WG content in products to be considered as WG product) (Thane *et al.*, 2005; Jensen *et al.*, 2004; Jacobs *et al.*, 1998).

The compliance and accuracy of intake estimation largely affect the assessment of treatment effects in intervention studies and the evaluation of diet-disease associations in observational studies respectively. The traditional methods of intake estimation are based on self-reports and an example is the widely used food frequency questionnaire (FFQ). It offers a cheap estimation of long-term intake (Willett, 1998), although the accuracy has been questioned (Schatzkin & Kipnis, 2004; Bingham *et al.*, 2003). Other estimation methods,

e.g., food records and recalls, are more expensive and repeated measurements are often needed to estimate long-term intake (Willett, 1998).

The traditional dietary assessment methods suffer from systematic and random under and over estimation, due to memory and motivation, which together with true intake variation contribute to measurement errors (Fraser, 2003). To some extent, these errors might be accounted for by calibration to 'reference' methods, e.g., weighed food records (Willett, 1998). However, the crucial assumption for such calibration, that the errors of the two methods are uncorrelated, is in many cases doubtful (Subar *et al.*, 2003).

In addition to the aforementioned measurement errors and calibration problems, the estimation of WG intake is subjected to additional obstacles. Firstly, consumers may encounter difficulties in identifying whole grain products and determining the whole grain content in such foods (Kantor *et al.*, 2001). Secondly, differences between studies in terms of WG definition, cereals included as WG, and units used for quantification, complicate study comparisons (Frølich & Åman, 2010; Jensen *et al.*, 2004; Lang & Jebb, 2003). Finally, the limited number of WG related questions in FFQ in combination with a large variation of WG content among cereal products is likely to affect the precision of WG intake estimation (Fraser *et al.*, 2005).

1.1 Dietary biomarkers

Dietary biomarkers (DB) are compounds that can be used to reflect the intake of certain foods or nutrients. DB represent a less subjective measurement of intake than traditional dietary assessment methods, as they are generally not subjected to human perception and opinions (Marshall, 2003). Consequently, the measurement errors of biomarkers are likely to be independent of those of traditional methods.

Depending on whether the DB correlate to intake or provide quantitative measurements of intake, they can be classified in different categories (Kaaks *et al.*, 2002). *Recovery biomarkers* are based on precise and quantitative knowledge of the physiological balance between intake and output and offer an absolute measurement of a subject's intake (Kaaks *et al.*, 2002). Examples of recovery biomarkers are 24-h excretion of nitrogen, doubly labelled water, and potassium as biomarkers for intakes of protein, energy, and potassium, respectively (Tasevska *et al.*, 2006; Bingham, 2003; Livingstone & Black, 2003). *Concentration biomarkers* are correlated to intake but do not offer a quantitative measurement of intake, since they may exhibit diverse quantitative relationships with intake between and within individuals (Kaaks *et al.*, 2002). Biomarkers of this category can be used to rank individuals and examples

include serum fatty acids and plasma vitamin C and carotenoids as biomarkers for intakes of fat and fruit/vegetables, respectively (Bingham *et al.*, 2008; Jansen *et al.*, 2004; Wolk *et al.*, 2001). Finally, a third category of dietary biomarkers, *prediction biomarkers*, has been suggested (Tasevska *et al.*, 2005). Compared to recovery biomarkers, only a small proportion of the intake can be quantified as a biomarker, though the relationship with intake is fairly constant between and within individuals. Urinary excretions of sucrose and fructose during 24-h were proposed and implemented as prediction biomarkers for sugar intake (Tasevska *et al.*, 2011; Tasevska *et al.*, 2005).

Two key features of DB are their reproducibility and validity. A valid biomarker measures what it is intended to do (Kaaks *et al.*, 1997) and validation can be reflected by the correlation between biomarker measurement and the true intake. Repeated weighed food records may be used as surrogates for 'true' intake and the correlation between biomarker and surrogate indicates the relative validity of the biomarker, which is subjected to measurement errors in both the biomarker and the surrogate method, although the measurement errors of the surrogate are assumed to be random.

Reproducibility indicates the correlation between repeated measurements within the same individual (Kaaks *et al.*, 1997) and is often assessed by calculation of the intra-class correlation coefficient (ICC). The ICC is a measure of the proportion of total variation that is due to variation between individuals. A high ICC indicates a proportionally small variance over time within the same individual compared to the variance between individuals and thus a high reproducibility between measurements. A low reproducibility may be accounted for by repeated measurements and the reproducibility is dependent on the stability of intake (van Dam & Hu, 2008).

The requirements of the DB are dependent on its purpose. As a surrogate measurement of intake, DB can be used in observational studies to investigate the associations between diet and disease (Bingham *et al.*, 2008; Key *et al.*, 2007). To evaluate diet-disease relationship, intake usually needs to be assessed over several years, as the time for diseases to develop is typically long. Hence the biomarker should preferably reflect long-term intake. For this purpose, good reproducibility of the biomarker is crucial, since a reproducible biomarker reduces the number of measurements needed to estimate the true long-term biomarker level. Recently, the combined use of biomarkers and self-reports has been proposed as an advantageous tool in the assessment of diet-disease associations (Freedman *et al.*, 2010a).

Alternatively, DB can be used to assess compliance in dietary intervention studies (Baldrick *et al.*, 2011). Compliance biomarkers are not only useful as alternative control tools of compliance but could also act as additional

motivating factors if subjects are aware of biomarker measurements (Ross, 2012).

Since the measurement errors of dietary biomarkers can be considered as independent compared to those of traditional assessment methods, biomarkers may be utilized as ‘reference’ methods for the validation of traditional dietary assessment methods (Fraser *et al.*, 2005; Kaaks *et al.*, 1997). However, the independence of measurement errors between different methods is a crucial assumption in these validations and in most cases it is questionable how well this assumption is fulfilled if two methods based on similar methodology (e.g., food records and FFQ) are utilized in validation.

1.2 Alkylresorcinols

A group of phenolic lipids, alkylresorcinols (AR), have been extensively evaluated as biomarkers for the intake of whole grain and bran products of wheat and rye (**Figure 1**). This thesis focuses on the evaluation of AR metabolites as novel biomarkers for WG wheat and rye intake to be used as a complement or replacement to intact AR.

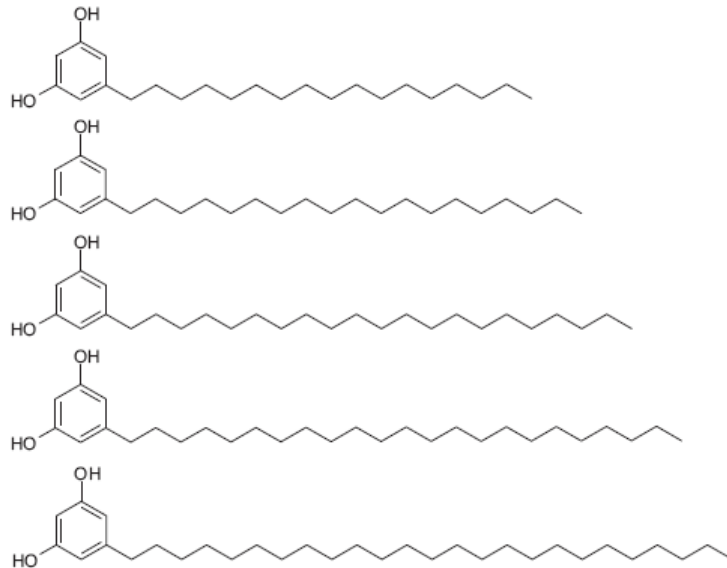


Figure 1. The five major cereal alkylresorcinol homologues have saturated odd-numbered alkyl chains with 17-25 carbon atoms. The trivial names of the homologues are based on the degree of saturation and the length of the alkyl chains (C17:0-25:0, top to bottom).

1.2.1 Occurrence

Alkylresorcinols are 1,3-dihydroxy-5-n-alk(en)ylbenzene derivatives found in several families of plants, mosses, algae, fungi, and bacteria (Kozubek & Tyman, 1999). Due to their antimicrobial activities observed *in vitro*, AR have been suggested to be involved in the plants' defence system (Stasiuk & Kozubek, 2010; Kozubek & Tyman, 1999).

Table 1. Content of AR in cereal grains, bran, and WG products of rye and wheat.¹

	Range ($\mu\text{g/g}^2$)	Reference
<i>Rye</i>		
Grains ³	568-1441	(Shewry <i>et al.</i> , 2010; Ross & Kochhar, 2009; Andersson <i>et al.</i> , 2008a; Kulawinek <i>et al.</i> , 2008; Landberg <i>et al.</i> , 2008; Ross <i>et al.</i> , 2001)
Bran	1266-4108	(Ross & Kochhar, 2009; Kulawinek <i>et al.</i> , 2008; Mattila <i>et al.</i> , 2005; Chen <i>et al.</i> , 2004)
WG bread	197-707	(Ross & Kochhar, 2009; Kulawinek <i>et al.</i> , 2008; Mattila <i>et al.</i> , 2005; Chen <i>et al.</i> , 2004; Ross <i>et al.</i> , 2003c)
Crisp bread	490-1007	(Chen <i>et al.</i> , 2004; Ross <i>et al.</i> , 2003c)
<i>Wheat</i> ⁴		
Grains ³	138-1429	(Ross & Kochhar, 2009; Andersson <i>et al.</i> , 2008a; Kulawinek <i>et al.</i> , 2008; Chen <i>et al.</i> , 2004; Ross <i>et al.</i> , 2003c; Hengtrakul <i>et al.</i> , 1990)
Bran	2211-2672	(Kulawinek <i>et al.</i> , 2008; Chen <i>et al.</i> , 2004; Ross <i>et al.</i> , 2003c)
WG bread	142-608	(Ross & Kochhar, 2009; Kulawinek <i>et al.</i> , 2008; Chen <i>et al.</i> , 2004; Ross <i>et al.</i> , 2003c)
White bread	26-47	(Ross & Kochhar, 2009)
Crisp bread	58-420	(Chen <i>et al.</i> , 2004; Ross <i>et al.</i> , 2003c)
<i>Durum wheat</i>		
Grains ³	54-751	(Andersson <i>et al.</i> , 2008a; Landberg <i>et al.</i> , 2006a; Ross <i>et al.</i> , 2003c)
WG Pasta	40-435	(Ross & Kochhar, 2009; Landberg <i>et al.</i> , 2006a; Chen <i>et al.</i> , 2004)

¹AR, Alkylresorcinol; WG, whole grain ²Dry matter. ³Includes grains and whole gra meal. ⁴Includes grains and WG meal. ⁵The upper limit of the range was obtained by analyzing 125 samples of common wheat grown in different locations.

Among commonly consumed plants, AR are only abundantly present in wheat (*Triticum* Spp.) and rye (*Secale cereale*) (**Table 1**). Although triticale contains AR (Gohil *et al.*, 1988; Radcliffe *et al.*, 1981) and small amounts of AR (<210 $\mu\text{g/g}$) are present in barley (Andersson *et al.*, 2008b; Chen *et al.*, 2004; Zarnowski & Suzuki, 2004; Ross *et al.*, 2003c; Zarnowski *et al.*, 2002), the contribution to dietary AR is negligible due to the limited consumption of these

cereals. In addition, very small content of AR has been reported in other food materials (e.g., maize, garden peas, mango, and animal fat) (Jansson *et al.*, 2010; Knoedler *et al.*, 2009; Gembeh *et al.*, 2001; Kozubek & Tyman, 1999). Although the content of AR in cereals is highly heritable, it is also affected by the environment (Andersson *et al.*, 2010).

Cereal alkylresorcinols are predominantly present as saturated derivatives with odd-numbered alkyl chains ranging from 17-25 carbon atoms (Ross *et al.*, 2003a) (Figure 1). However, unsaturated, keto, and hydroxy derivatives have also been found, especially in rye (Knodler *et al.*, 2007; Ross *et al.*, 2004a; Kozubek & Tyman, 1999; Seitz, 1992). The trivial names of the different AR derivatives indicate, similar to fatty acids, the chain length and degree of saturation (e.g., C17:0).

AR in the cereal kernel are exclusively located in the outer parts (Ross *et al.*, 2001; Tluscik, 1978), more precisely in the outer cuticulum of testa/inner cuticulum of pericarp (Landberg *et al.*, 2008). Therefore, bran and whole grain products of wheat and rye contain AR (Table 1), while they are absent or found in very small concentrations in refined products (Ross & Kochhar, 2009; Mattila *et al.*, 2005; Chen *et al.*, 2004).

1.2.2 Absorption

The suggested main route of AR absorption consists of uptake at the small intestine and incorporation into chylomicrons, which are transported via the lymphatic system from enterocytes to the systemic circulation (Linko-Parvinen *et al.*, 2007). Direct high-density lipoprotein (HDL) efflux from enterocytes to the portal vein has been observed for tocopherols *in vitro* and might be a complementary absorption pathway for AR (Anwar *et al.*, 2006). The fraction absorbed in this way would potentially be exposed to hepatic metabolism before entering the systemic circulation.

The apparent absorption of AR in humans has been investigated by measuring the content of AR in ileal effluent from ileostomy-operated patients and was determined to about 60% (Ross *et al.*, 2003a). There were, however, large differences between patients (45-71%) and homologues with shorter-chains were seemingly absorbed to a larger extent than the longer-chained homologues (i.e., disappeared to a greater extent). The apparent absorption in pigs was similar to humans (Ross *et al.*, 2003b). Absorption might be lower in rats, which were observed to fecally excrete 61% of the given dose, mainly as unchanged AR (Ross *et al.*, 2003b). However, this percentage might underestimate absorption, since it accounts for unabsorbed AR and possibly also absorbed AR excreted in bile.

1.2.3 Distribution

Absorbed AR in plasma are mainly (70-80%) found in lipoproteins and among them, HDL and very-low-density lipoprotein are the main carriers of AR (Linko-Parvinen *et al.*, 2007). In contrast, no AR are present in the plasma water (Linko-Parvinen *et al.*, 2007). It has been suggested that AR, after entering the systemic circulation from lymph, are transported in chylomicrons to the liver where they are redistributed to very-low-density lipoprotein and HDL. These lipoprotein particles are subsequently secreted back to the circulation.

The amphiphilic nature of AR enables them to be incorporated in biological membranes (Stasiuk & Kozubek, 2010). A substantial proportion of AR in blood are located in erythrocytes and concentrations in erythrocytes correlate well with plasma concentrations (Linko-Parvinen *et al.*, 2007; Linko & Adlercreutz, 2005). Longer AR homologues are more easily incorporated into the erythrocyte membranes compared to shorter homologues (Linko & Adlercreutz, 2005).

In addition, AR were observed in adipose tissue from free-living individuals (Jansson *et al.*, 2010) and habitual intake might lead to accumulation in other tissues.

1.2.4 Metabolism

Since intact AR have not yet been found in bile and only minute amounts have been quantified in urine (Ross *et al.*, 2004b), an extensive metabolism of AR is responsible for their rather fast elimination from plasma, with elimination half-life ($t_{1/2}$) of about five hours (Landberg *et al.*, 2006b). The metabolism of AR is hypothesized to share similarities with tocopherol metabolism. In fact, AR were found to competitively inhibit γ -tocopherol metabolism *in vitro* and AR increased hepatic γ -tocopherol levels in rats (Ross *et al.*, 2004c). Like tocopherols, the biotransformation of AR includes both oxidative metabolism (phase I) and conjugation reactions (phase II).

Phase I metabolism

During phase I metabolism, polar functional groups are introduced or unmasked in lipophilic compounds to enhance excretion or allow conjugation reactions (Gibson & Skett, 2001). Phase I reactions include oxidation, reduction, and hydrolysis, which are mediated by several enzyme families (Gibson & Skett, 2001; Timbrell, 1999). The suggested phase I metabolism of AR shares similarities with the metabolism of other phenolic lipids e.g., tocopherols (Sontag & Parker, 2002; Birringer *et al.*, 2001), Vitamin K (Shearer & Barkhan, 1973), and nonylphenol (Thibaut *et al.*, 2000).

Phase I metabolism of tocopherols is initiated by ω -oxidation, which is mediated by Cytochrome P450 isoenzyme CYP4F2 (Sontag & Parker, 2007; Sontag & Parker, 2002). This microsomal enzyme is expressed in liver and kidney (Hirani *et al.*, 2008) and has diverse functions, e.g., Vitamin K₁ oxidase (McDonald *et al.*, 2009). The carboxylated intermediary tocopherol metabolites resulting from the ω -oxidation is activated to an acyl-CoA ester and the activated tocopherol metabolite undergoes subsequent β -oxidation (Birringer *et al.*, 2001). Through β -oxidation, side chains are gradually shortened, finally leading to the formation of carboxymethylbutyl-hydroxychroman and carboxyethyl-hydroxychroman end-products (Birringer *et al.*, 2001).

Since AR inhibit tocopherol oxidation, it is likely that ω -oxidation of AR is mediated by CYP4F2 (Ross *et al.*, 2004c). As the shorter-chained homologue C15:0 is more effective in inhibiting tocopherol ω -oxidation compared to a mixture of homologues, it is possible that CYP4F2 prefers to hydroxylate short-chained homologues (Ross *et al.*, 2004c). However, the weaker inhibition caused by the AR mixture could partly reflect analytical artifacts caused by solubility issues in the *in vitro* system.

The major metabolites of AR, 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA), are two phenolic acids first discovered in human urine after consumption of WG wheat (Ross *et al.*, 2004b). Their formation represents, together with the competitive inhibition of tocopherol ω -oxidation, the strongest evidence of the proposed pathway for AR metabolism (**Figure 2**). Similar to their tocopherol equivalents (carboxyethyl-hydroxychromans and carboxymethylbutyl-hydroxychromans), the smaller AR metabolite (DHBA) is the theoretical result of an additional β -oxidation cycle of its precursor (DHPPA).

Phase II metabolism

During phase II metabolism, polar groups are conjugated to xenobiotics or endogenous compounds to increase excreatability. Like phase I metabolism, specific enzymes catalyze the conjugation reactions and several factors can influence the activity of these enzymes, e.g., anthropometry, genes, diet, and life style (Harris & Waring, 2008; Williams *et al.*, 2002; Liston *et al.*, 2001).

Glucuronidation and sulfation are the two major phase II metabolic pathways during which AR, their end-products, and possible intermediate metabolites could be conjugated. During glucuronidation a sugar moiety is covalently bound to endogenous compounds, xenobiotics, or their metabolites (Zamek-Gliszczyński *et al.*, 2006).

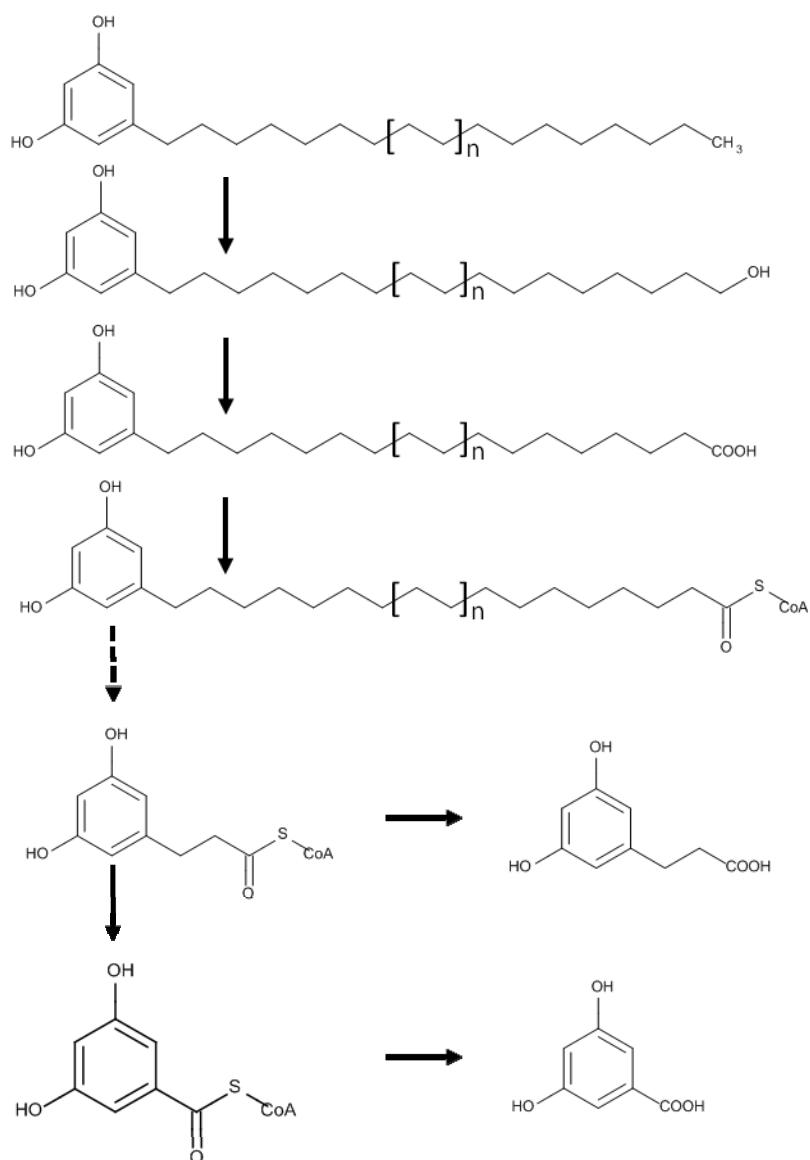


Figure 2. Suggested metabolic pathway of the major cereal AR ($n=1-5$). ω -oxidation of AR results in carboxylated alkylchains, which are activated and subsequently shortened by several cycles of β -oxidation. The end-products of β -oxidation are hydrolysed to the two major AR metabolites, DHBA and DHPPA. The dashed arrow indicates several cycles of β -oxidation. In addition to this phase I metabolism, conjugation with glucuronic and sulfonic acids increases the hydrophilicity and thereby simplifies excretion.

The process is catalyzed by UDP-glucuronyltransferases which use uridine diphosphate glucuronic acid as co-substrate and different isoforms of the enzyme are found in the endoplasmic reticulum of different tissues, although the liver is the major site for glucuronidation (de Leon, 2003; McGurk *et al.*, 1998). In addition to glucuronidation, other glycosylations with different co-substrates may contribute to phase II metabolism. For example, the major metabolite of γ -tocopherol in mouse urine was identified as γ -carboxyethyl-hydroxychroman β -D-glucoside (Cho *et al.*, 2009).

Sulfation of xenobiotics is catalyzed by several human cytosolic sulfotransferases with diverse substrate specificity (Hashiguchi *et al.*, 2011; Coughtrie *et al.*, 1998). Several sulfotransferases were shown to exert diverse activities towards different tocopherols and tocopherol metabolites (Hashiguchi *et al.*, 2011). Sulfotransferases uses 3'-phosphoadenosine-5'-phosphosulfate as a co-substrate to covalently bind sulfonic acid to hydroxyl or amine groups of the substrate (Strott, 2002). Due to the fast depletion of co-substrate, sulfation is considered a low capacity phase II reaction (Zamek-Gliszczynski *et al.*, 2006).

Although the phrasing might be misleading, phase II metabolism does not necessarily need to be preceded by phase I metabolism. Intact AR were found in very small amounts in human urine, however only after incubation with deconjugating enzymes β -glucuronidase and sulfatase. This indicates that conjugation reactions can occur on intact AR and that phase I and phase II metabolism of AR might occur in parallel, similar to what has been proposed for tocopherols (Hashiguchi *et al.*, 2011; Freiser & Jiang, 2009; Jiang *et al.*, 2007). Both UDP-glucuronyltransferase and cytochrome P450 isoenzymes (e.g., CYP4F) are microsomal proteins and might use AR as substrates (Sanders *et al.*, 2008; Nyquist & Morre, 1971).

Although no conjugated AR have been observed in plasma, AR metabolites are extensively found as conjugates in plasma (Koskela *et al.*, 2008). It is possible that AR metabolites function as better substrates for conjugating enzymes than their precursors (Hashiguchi *et al.*, 2011) or that conjugated AR are metabolized to conjugated DHBA and DHPPA within the metabolizing cells before secretion to plasma.

1.2.5 Excretion

As end-products of AR metabolism, 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) are eliminated from the body by excretion (Ross *et al.*, 2004b). The elimination of AR metabolites has been suggested to be slower than AR elimination (Soderholm *et al.*, 2011; Soderholm *et al.*, 2009; Landberg *et al.*, 2006b). If true, this would indicate

that AR metabolite concentrations are less affected by the time between intake and measurement; an advantageous feature of a dietary biomarker. However, estimations of AR metabolite elimination were conducted by measuring concentrations in plasma and urine after consumption of rye bread and not by administration of pure DHBA and DHPPA (Soderholm *et al.*, 2011; Soderholm *et al.*, 2009). Consequently, the half-lives reported, defined as the time required for a 50% reduction of maximum concentration (or excretion rate), may not exclusively reflect the elimination of AR metabolites, but also AR absorption and metabolism. Hepatically formed AR metabolites could theoretically exit the body in faeces (*via* bile) or urine and thereby complete the route of AR absorption, distribution, metabolism and excretion (**Figure 3**).

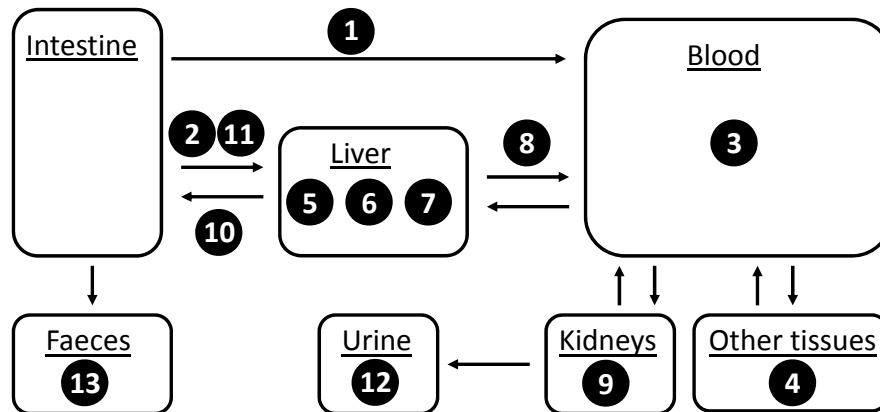


Figure 3. The suggested fate of ingested alkylresorcinols. For more detailed information, see text. In brief, ingested alkylresorcinols are absorbed in the small intestine and transported in chylomicrons via the lymphatic system (1) and/or via direct HDL transfer from the intestine through the portal vein and liver to the systemic circulation (2). Absorbed alkylresorcinols are carried in plasma lipoproteins and erythrocytes (3). From the systemic circulation, AR can be distributed to other body compartments, e.g. adipose tissue (4). In the liver, alkylresorcinols can be reassembled in lipoproteins (5) and secreted back to the circulation or metabolized to two phenolic acids by CYP450 mediated ω -oxidation and several cycles of β -oxidation (6). In addition, phase II metabolism results in the formation of conjugated metabolites (7). The metabolites can be secreted to the systemic circulation (8) and subsequently renally excreted (9). Biliary secretion (10) represents another excretion route of AR metabolites, which can theoretically be reabsorbed in the intestine (11) to complete enterohepatic circulation. Ingested AR appear eventually, as such or as metabolites, in urine (12) and faeces (13).

Biliary excretion

So far, no studies have reported biliary excretion of AR or their two major metabolites, DHBA and DHPPA. However, DHBA and DHPPA were recently quantified in ileostomy effluent from four subjects consuming rye bran and 20-25% of the ingested dose was present as the two AR metabolites,

predominantly as DHPPA (unpublished data). In addition, DHPPA has been identified in bile from pigs (Rikard Landberg, personal communication, March 14, 2012). This indicates the important role of biliary excretion in AR elimination. Consumption of sesame seeds increased the biliary excretion of intact γ -tocopherol in rats, probably by inhibiting the ω -hydroxylating enzyme (Yamashita *et al.*, 2000). In a similar way, it is possible that the inhibition of metabolizing enzymes could induce or increase biliary excretion of intact AR. The plasma concentration profile of AR metabolites after a single dose of AR indicates that biliary excreted AR metabolites could be absorbed in the intestine and thereby they would be subjected to enterohepatic circulation, which might be repeated (Soderholm *et al.*, 2009). This would prolong the time that AR metabolites reside within the body. Finally, it is possible that biliary excreted AR metabolites are further degraded by intestinal microbiota before arriving in faeces (Griffiths & Smith, 1972).

Urinary excretion

When rats were given a single radiolabelled dose of AR, 31% of the radioactivity was recovered in urine (Ross *et al.*, 2003b). The urinary excretion of intact AR is very minor and the fraction of ingested AR to finish up in urine is due to the excretion of DHBA and DHPPA (Ross *et al.*, 2004b). Of the two metabolites, DHPPA is more abundant in urine and both metabolites are present as free aglycones and conjugates (Landberg *et al.*, 2009a; Koskela *et al.*, 2007).

The proportion of ingested AR that is excreted as DHBA or DHPPA in urine decreases with an increasing dose (Landberg *et al.*, 2009a). This phenomenon might be attributable to a decreased absorption at higher intake levels. This was observed in pigs fed breads containing different amounts of AR (Ross *et al.*, 2003b). However, the difference in apparent absorption could also be due to differences in experimental breads regarding fibre content and botanical fractions used. A dose-dependent shift in elimination has been proposed as an alternative explanation of the decreased urinary recovery (Landberg *et al.*, 2009a). According to this, the proportion of AR metabolites excreted in urine would decrease at higher intakes of AR, while other, alternative elimination routes would become more predominant. The increased biliary elimination of tocopherols at higher intakes supports this idea (Kiyose *et al.*, 2001). Furthermore, since AR-containing foods are rich in dietary fibre, a high AR intake is accompanied by a high fibre intake. High fibre diets might decrease deconjugation of biliary excreted AR metabolites and thereby suppress enterohepatic circulation (Adlercreutz, 2010). This would decrease the amounts of AR metabolites available for renal excretion. Finally, DHBA

and DHPPA from other potential sources might, to a small extent, overestimate the urinary recovery, especially at low AR intakes.

1.3 Analysis of AR metabolites

Several analytical methods have been utilized to quantify AR concentration in plasma, such as gas chromatography-mass spectrometry (GC-MS) (Landberg *et al.*, 2009b; Linko *et al.*, 2002), gas chromatography/tandem mass spectrometry (Nagy *et al.*, 2008), and liquid chromatography/tandem mass spectrometry (Ross *et al.*, 2010). So far, the quantification of AR metabolites has only been performed by high performance liquid chromatography-coulometric electrode array detection (HPLC-CEAD) (Koskela *et al.*, 2008; Koskela *et al.*, 2007).

The presence of conjugated DHBA and DHPPA in plasma and urine indicates the necessity of deconjugation prior to quantification (Koskela *et al.*, 2008; Koskela *et al.*, 2007). Deconjugation is commonly performed by incubation with hydrolysing enzyme preparations, which can target specific conjugates or deconjugate several different conjugates, e.g., β -glucuronidase from *Helix pomatia* does not only deconjugate glucuronides and glucosides (Cho *et al.*, 2009) but also possesses sulfatase activity.

Quantifications of urinary AR metabolites by HPLC-CEAD have been preceded by sample pre-treatments of diverse degree (Soderholm *et al.*, 2011; Aubertin-Leheudre *et al.*, 2010b; Aubertin-Leheudre *et al.*, 2010c; Aubertin-Leheudre *et al.*, 2008; Guyman *et al.*, 2008; Koskela *et al.*, 2007); from direct urine analysis to deconjugation and subsequent sample clean-up (including liquid-liquid and solid phase extractions). Plasma DHBA and DHPPA have been quantified after deconjugation, acidification and repeated liquid-liquid extraction (Aubertin-Leheudre *et al.*, 2010a; Aubertin-Leheudre *et al.*, 2010b; Aubertin-Leheudre *et al.*, 2010c; Soderholm *et al.*, 2009; Koskela *et al.*, 2008). Deconjugations of plasma and urinary AR metabolites have been performed by simultaneous incubation with two separate enzymes (β -glucuronidase and sulfatase). Both methods have utilized syringic acid as the internal standard.

Although the sample preparation in some cases is quick, the analytical run time of HPLC-CEAD is rather extensive (Weckwerth, 2007) and when analyzing AR metabolites, the time between two injections exceeds one hour. This is considerably longer than the run-time of the GC-MS methods used for the quantification of intact AR (Landberg *et al.*, 2009b; Linko *et al.*, 2002), which is less than 30 minutes. The tedious analysis limits the sample throughput and hinders quantification of large sample sets. In addition, gas chromatography offers a greater number of theoretical plates than liquid

chromatography (Moldoveanu & David, 2002), which can be beneficial for peak separation. In fact, the interference of co-eluting compounds has prevented the quantification of urinary DHBA under some conditions (Guyman *et al.*, 2008).

1.4 AR metabolites as biomarkers

1.4.1 Specificity

Even though urinary excretion of DHBA and DHPPA is strongly related to the intake of WG rye and wheat, small amounts of dietary AR in refined cereal products and bran-enriched foods might contribute to AR metabolite concentration in plasma and urine (Ross & Kochhar, 2009). Nevertheless, low concentrations (<7.1 $\mu\text{mol/L}$) of both AR metabolites have been detected in urine from celiac patients, with diets assumingly free from AR (Koskela *et al.*, 2007). This could be due to patients not complying with a diet excluding wheat, rye and barley or to analytical artefacts e.g., co-elution of interfering compounds (Guyman *et al.*, 2008). However, there might be alternative sources of DHBA and DHPPA, not related to AR.

Table 2. *Reported content of DHBA in different food and beverages.*

Sample	Concentration	Reference
Peanuts ¹	4-16 $\mu\text{g/g}$	(Reddy <i>et al.</i> , 1977)
Beer	0.21 \pm 31 mg/L	(Floridi <i>et al.</i> , 2003)
Wort	0.35 \pm 60 mg/L	(Floridi <i>et al.</i> , 2003)
Green tea	trace	(Gruz <i>et al.</i> , 2008)
Chickpeas	n/a ²	(Khadem & Marles, 2010)
Raspberries	n/a ²	(Khadem & Marles, 2010)
Rhubarb	n/a ²	(Medynska & Smolarz, 2005; Smolarz <i>et al.</i> , 2005)

¹Occurrence in peanuts is uncertain since the only reference reporting it classifies α -resorcylic acid as a monohydroxybenzoic acid. ²No amounts or concentrations were reported.

Although phenolic acids are abundant in nature, reported identifications of derivatives with two hydroxyl groups in meta positions to the carboxylic side chain are scarce (Ross *et al.*, 2004b). However, DHBA (or alternatively α -resorcylic acid) has been described as a constituent in different foods, food materials, and beverages (**Table 2**). In addition to the foods and beverages listed in Table 2, DHBA has been found in Horseweed (*Conyza canadensis*), *Rosa roxburghii*, and *Rhizobium* sp. isolated from *Sesbania procumbens* (Sridevi *et al.*, 2008; van Rensburg *et al.*, 2005; Mukhtar *et al.*, 2002).

On the contrary, DHPPA has not been found in foods or beverages but has been reported as a metabolite of sinapic acid and flavonoids in rats and rabbits (**Table 3**). The amounts of DHPPA reported are generally small and the degradation of the precursors to DHPPA is thought to be mainly attributable to intestinal microbiota (Takagaki & Nanjo, 2010; Griffiths & Smith, 1972; Griffiths, 1970). Additionally, olivetol (a short-chain alkylresorcinol) has been found to be microbially degraded to DHPPA (McClanahan & Robertson, 1984). Since the intake of olivetol is very limited, it will probably not contribute to DHPPA concentrations in plasma and urine from individuals consuming their habitual diet.

Table 3. *Identification of DHPPA in animals after consumption of sinapic acid and flavanoids*

Precursor	Specie	Sample	Amount	Reference
Sinapic acid	Rabbit	Urine	Major metabolite	(Griffiths, 1970)
	Rat	Urine	Not reported	
	Rat	Intestinal microflora	Not reported	
Tricetin	Rat	Urine	Small amounts	(Griffiths & Smith, 1972)
	Rat	Intestinal microflora	Not reported	
Tricin	Rat	Urine	3.1% of dose	(Griffiths & Smith, 1972)
	Rat	Intestinal microflora	Small amounts	
5,7 – Dihydroxy-3',4',5'-trimethoxyflavone	Rat	Urine	Traces	(Griffiths & Smith, 1972)
Epigallocatechin gallate	Rat	Intestinal microflora	Minor amounts	(Takagaki & Nanjo, 2010)

1.4.2 Reproducibility and validity

Few studies have assessed the reproducibility of plasma and urinary AR metabolites in free-living subjects so far (Landberg *et al.*, 2012b; Montonen *et al.*, 2012). The reproducibility of plasma metabolites was evaluated by analyzing plasma samples obtained from 100 German participants on two separate occasions, 4 months apart (Montonen *et al.*, 2012). The calculated ICC values of DHBA and DHPPA indicated substantial within-person variability of AR metabolites in plasma. Similar results were observed after the quantification of AR metabolite concentration in spot urine samples obtained from American women on two occasions, 1-3 years apart (Landberg *et al.*, 2012b). In both studies, DHPPA showed higher reproducibility than DHBA.

Several studies report good responsiveness of AR metabolites in plasma and urine to intakes of AR, WG rye and wheat, rye and cereal fibre (Aubertin-Leheudre *et al.*, 2010c; Landberg *et al.*, 2009a; Aubertin-Leheudre *et al.*, 2008; Guyman *et al.*, 2008). In a Swedish dose-response study, increases in AR intake by 100% and 300% caused 24-h AR metabolite excretion to rise by 62% and 149% respectively (Landberg *et al.*, 2009a). Correspondingly, fasting plasma concentrations of AR increased by 42% and 207% respectively. Among American participants, consumers of WG wheat and rye excreted 72% more DHPPA than non-consumers (Guyman *et al.*, 2008). Additionally, a serving increase in WG wheat and rye increased 12-h urinary DHPPA excretion by 94% after adjustment for BMI and intakes of energy and fibre. In another study, American female health professionals' intakes of WG and cereal fibre (assessed by FFQ) were generally correlated to AR metabolite concentration in spot samples (Landberg *et al.*, 2012b). Self-reported rye intake among Finnish women was correlated to 24-h urinary excretion ($P<0.001$) and fasting plasma concentration ($P<0.05$) of AR metabolites (Aubertin-Leheudre *et al.*, 2010c). Similarly, the intake of cereal fibre in the same group of women was correlated to AR metabolites in plasma and urine ($P<0.001$) (Aubertin-Leheudre *et al.*, 2008).

2 Aims of this thesis

The aim of this thesis is to evaluate the applicability of AR metabolites as biomarkers for the intake of whole grain wheat and rye. In order to achieve this, several objectives were determined and are listed below.

1. To develop a gas-chromatographic mass-spectrometry method for the quantification of AR metabolites in urine (**I**).
2. To compare the developed gas-chromatographic mass-spectrometry method with a previously published high-performance liquid-chromatographic coulometric electrode array detector method (**II**).
3. To evaluate AR and metabolite pharmacokinetics in rats and compare metabolite formation of different AR homologues (**III**).
4. To identify potential determinants of AR metabolite excretion and conjugation pattern in urine from free-living individuals and to evaluate the relative validity of different urinary conjugates of DHBA and DHPPA (**IV**).
5. To assess the relative validity and medium-term reproducibility of urinary AR metabolites in free-living individuals consuming their habitual diet (**V**).

3 Materials and methods

3.1 Reference compounds and enzymes

The reference compounds and derivatizing reagents used in studies I-V are listed in Table 4.

Table 4. Reference compounds and derivatization reagents used in the different studies.

Trivial name	Supplier	Purity	Study
<i>Alkylresorcinols</i>			
C17:0 ¹	Reseachem Lifescience, Burgdorf, Switzerland.	>95%	III
C25:0 ²	Reseachem Lifescience, Burgdorf, Switzerland.	>95%	III
<i>Metabolites</i>			
DHBA ³	Sigma-Aldrich Chemie, Steinheim, Germany.	>97%	I-V
DHPPA ⁴	Isosep, Tullinge, Sweden.	>95%	I-V
<i>Internal standards</i>			
C20:0 ⁵	Reseachem Lifescience, Burgdorf, Switzerland.	>95%	III
DHPAA ⁶	Apin Chemicals, Abingdon, UK.	>98.5%	I
Syringic acid	Sigma Chemicals, St. Louis, MI, USA.	>98%	I-V
<i>Derivatizing reagents</i>			
BSTFA ⁷	Thermo Scientific, Rockford, IL, USA.	-	I-II, IV-V
MSTFA ⁸	Sigma Chemicals, St. Louis, MI, USA	-	I
QSM ⁹	Pyridine (Merck, Darmstadt, Germany), hexamethyldisilazane (Supelco, Bellefonte, PA, USA), and trimethylchlorosilane (Supelco, Bellefonte, PA, USA)	-	I, III

¹5-n-Heptadecylresorcinol. ²5-n-Pentacosylresorcinol. ³3,5-dihydroxybenzoic acid. ⁴3-(3,5-dihydroxyphenyl)propanoic acid. ⁵5-n-Eicosylresorcinol. ⁶3,5-dihydroxyphenylacetic acid. ⁷N,O-bis-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane. ⁸N-methyl-N-(trimethylsilyl)trifluoroacetamide. ⁹Quick silylation mixture (self-prepared).

3.2 Chromatographic analysis of AR and their metabolites

The methods and instrumentation for the analysis of AR in plasma and their metabolites in plasma and urine are listed in **Table 5**. In study **I**, a gas chromatography – mass spectrometry (GC-MS) method was developed for the analysis of AR metabolites in urine (Table 5). The parts of the method developed within this thesis (indicated as bold in Table 5) are addressed in separate sections below. Apart from the optimization of sample deconjugation, the method development involved the evaluation of different internal standards and derivatization procedures. The GC-MS method developed in paper **I** was used to quantify urinary AR metabolites in studies **II**, **IV**, and **V**. In addition, high performance liquid chromatography - coulometric electrode array detection (HPLC-CEAD) was utilized to quantify urinary DHBA and DHPPA in paper **II** and the quantified concentrations were compared to those determined by GC-MS. Plasma AR and their metabolites in paper **III** were analyzed by a modification of a previously published GC-MS method for the quantification of human plasma AR (Landberg *et al.*, 2009b) and the method described in paper **I**. In the same study, the basal rat diet was analyzed for AR content by a previously published gas chromatography method (Ross *et al.*, 2001). Control samples were included in each analytical batch to evaluate the precision of the analyses. In papers **I**, **II**, and **V** the control samples were individual 24-h collections from subjects consuming different amounts of AR and thereby had a diverse AR metabolite concentration. The control samples in paper **IV** were pooled urine of 24-h collections from 24 individuals consuming their habitual diet. Pooled fasting plasma from six individuals was used as a control sample in paper **III**.

3.3 Deconjugation

In order to quantify the total concentration of AR metabolites or to determine the amounts of different conjugates, urine and plasma samples were incubated with deconjugating enzymes prior to quantification. In the first paper, urine was incubated with different concentrations of a crude mixture of *Helix pomatia* enzymes, containing β -glucuronidase and sulfatase activities, and a pure β -glucuronidase to optimize deconjugation. In the same study, urine samples from nine individuals participating in an intervention study and consuming equal amounts of AR were incubated with optimized concentrations of the two different enzyme solutions and with a pure buffer (without enzymes).

Table 5. *Methods and instrumentation for the quantification of AR and their metabolites in biological samples.*

Study	II	I-II, IV-V	III
Method	HPLC-CEAD ¹	GC-MS ²	GC-MS ²
Analyte	AR metabolites	AR metabolites	AR and their metabolites
Matrix	Urine	Urine	Plasma
Sample volume	50 µl	50 µl	50 µl
Internal standard³	Syringic acid	Syringic acid	C20:0 ⁴ Syringic acid ⁵
Incubation volume ^{6,3}	750 µl	750 µl	265 µl
Post-incubation acidification	15 µl HCl	15 µl HCl	100 µl CH ₃ COOH
Liquid-liquid phase extraction	2× 3 ml ethyl acetate	2× 3 ml ethyl acetate	3× 3 ml diethyl ether
Solid-phase extraction ²	Mixed mode (anion exchange/reversed phase)	Mixed mode (anion exchange/reversed phase)	Mixed mode (anion exchange/reversed phase)
Derivatization³	-	Silylation 1 h with 100µl BSTFA ⁷ at 60°C.	Silylation 1 h with 50µl QSM ⁸ at 60°C.
Injection volume	10 µl ⁹	1.5 µl	1.5 µl
Separation	Gradient elution delivered by LC-10AD pumps (Shimadzu Corporation, Japan).	Temperature gradient in Trace Ultra GC (Thermo Fischer Scientific, USA).	Temperature gradient in Trace Ultra GC (Thermo Fischer Scientific, USA).
Column	Inertsil ODS-3 (3× 150 mm)	BP-5 (30 m×0.25 µm×0.25 mm).	BP-5 (30 m×0.25 µm×0.25 mm).
Detector	CEAD ¹ (ESA Biosciences)	Mass: Trace DSQ II (Thermo Fischer Scientific, USA).	Mass: Trace DSQ II (Thermo Fischer Scientific, USA).
Detection	Retention times and peak area ratios	Molecular ions	Molecular ions
Quantification	Area ratio of analyte and internal standard. Multi-point calibration curves.	Area ratio of analyte and internal standard. Multi-point calibration curves..	Area ratio of analyte and internal standard. Multi-point calibration curves.

¹High performance liquid chromatography - coulometric electrode array detection. ²Gas chromatography-mass spectrometry. ³Step evaluated in study I. ⁴For quantification of AR. ⁵For quantification of AR metabolites. ⁶Samples were incubated with a sodium acetate buffer (pH 5) containing deconjugating enzymes. ⁷N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane. ⁸Quick silylation mixture. ⁹One third of extracts were evaporated to complete dryness after solid-phase extraction and reconstituted in 50 µl MeOH and 100 µl mobile phase.

The quantified concentrations were compared to assess the abundance of free and conjugated AR metabolites in urine. Similarly, in paper **IV** urine samples from 51 individuals consuming their habitual diets were incubated with and without pure β -glucuronidase or sulfatase in order to assess the amounts and proportions of free and conjugated urinary AR metabolites. The enzymes used to deconjugate AR metabolites are listed in **Table 6**.

Table 6. Enzymes¹ used for deconjugation of conjugated AR metabolites.

Enzyme	Type	Origin	Activity	Study
Mixture	H-1	<i>Helix pomatia</i>	β -Glucuronidase Sulfatase	I-V
β -Glucuronidase	B-1	Bovine liver	β -Glucuronidase	I, IV
Sulfatase	V	<i>Patella vulgata</i>	Sulfatase	IV

¹All enzymes were purchased from Sigma Chemicals (St. Louis, MI, USA).

3.4 Study design and samples

3.4.1 Human studies

Samples and subjects of the different studies are listed in **Table 7**. Ninety one volunteers (70 women and 21 men) in the Uppsala area were recruited to a study with the aim of investigating the reproducibility of plasma AR and urinary AR metabolites and their relative validity as biomarkers for the intake of whole grain rye and wheat. The participants, aged 20-70 with no diagnosed or perceived gastro-intestinal disease or symptoms, were instructed to adhere to their habitual diet and to complete a three-day weighed food record (3DWFR) on two occasions, approximately 2-3 months apart. On the last day of each recording, the participants performed a 24-h urine collection and collected 25 ml of morning urine in a separate tube. Volumes of 24-h collections were recorded and aliquots of 50 ml were kept together with morning urine samples at -80°C until analysis. Spot samples of morning urine were analyzed for creatinine to enable adjustment for diuresis. The 24-h urine collections were used in papers **I, II, IV, and V**, while the morning urine samples were only analyzed in paper **V**.

Apart from the 24-h urine collections screened for endogenous excretion of the two internal standard candidates, syringic acid and 3,5-dihydroxyphenyl acetic acid (DHPAA), urine samples from nine individuals, with equal AR intakes, participating in an intervention study were analyzed in paper **I** to determine the abundance of conjugated AR metabolites in urine.

Table 7. Human urine samples analyzed in the different studies.

Study	Female (n)	Male (n)	Inclusion criteria	Samples analyzed (n)	Sample selection
I	17	3	General ² .	40	Random
I ¹	5	4	Not available	10	Similar amounts men and women with equal AR intake
II	43	16	General ² .	114	Random
IV	34	18	General ² . Complete 24-h urine collection and 3DWFR ³ on the first occasion. FIL ⁴ \geq 1.08 if 6DWFR ⁵ were available and FIL ⁴ \geq 1.00 if only 3DWFR ³ were available. BMI < 30.	52	All men available and the first women recruited
V	50	16	General. Complete 24-h urine collection, morning urine spot sample, and 3DWFR ³ on both occasions. FIL ⁴ \geq 1.08.	132	All samples ⁶

¹Urine from 9 individuals with equal AR intakes participating in an intervention study (Landberg *et al.*, 2009a) were analyzed after incubation with different enzymes. ²Recruited participants were 20-70 years old and were not diagnosed with or perceived to have gastric-intestinal disease or symptoms. ³Three-day weighed food records. ⁴Food intake level (FIL=daily energy intake/basal metabolic rate). ⁵Six-day weighed food records. ⁶24-h collections and spot samples of morning urine.

3.4.2 Rat study

In paper **III**, 7-8 week-old male Sprague-Dawley rats were acclimatized (10 days) before they were put on an AR-free diet based on corn starch. After two weeks on the diet, catheters were surgically attached to the femoral artery and vein. On the day following surgery, rats were infused during 30 min with saline solutions containing DHBA or DHPPA at three different concentrations. Blood samples were preferably drawn through an artery catheter approximately 0, 15, 28, 60, 90, 210, and 390 mins. after infusion start. Another group of 7-8 week-old male Sprague Dawley rats were acclimatized for 17 days before kept on the AR-free diet for two weeks. These rats were not subjected to surgery and were administrated with corn oil supplemented with three different amounts of AR homologues C17:0 or C25:0 by gavage. Similar to the intravenous infused AR metabolites, twelve rats per substance were divided equally on the three dose levels. Blood was drawn 0, 3, 6, 12, 24, and 36 hours after gavage. Plasma was immediately separated from blood and kept at -80°C until analysis.

3.5 Pharmacokinetics

Pharmacokinetic parameters were non-compartmentally calculated from plasma concentration curves in paper **III** using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA). In addition, population pharmacokinetic models of the individual substances (C17:0, C25:0, DHBA, and DHPPA) were developed using NONMEM 7.2 (Icon, Leopardstown, Ireland). One, two, and three-compartment models were evaluated and different residual error models were tested. Model diagnostics were performed with Xpose 4 (Jonsson & Karlsson, 1999) and model evaluation was done in PSN (Lindbom *et al.*, 2005; Lindbom *et al.*, 2004).

3.6 Statistical analysis

All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA), unless otherwise stated.

3.6.1 Method development

Differences between derivatization reagents were evaluated using a mixed linear model with derivatization reagent, analyte, and post-derivatization procedure as fixed factors and replicate as random factor. In addition, least-squares means were calculated for derivatization reagents and *P*-values were Bonferroni-corrected. Temperature dependence of quick silylation mixture incubation was also evaluated by a mixed linear model with temperature and analyte as fixed factors and replicate as random factor. Finally, least-means squares were calculated for temperatures.

3.6.2 Method comparison

The agreement between quantifications by GC-MS and HPLC-CEAD was evaluated by the graphical investigation of difference-plots (Bland & Altman, 1986) and by weighted Deming regression analysis (Linnet, 1990). Difference-plots are useful tools to display differences between methods, but do not provide statistical tools for inference of the differences. The weighted Deming regression analysis is suitable if measurement errors are proportional and the procedure accounts for errors in both methods (Linnet, 1993). In this case, samples were not analyzed in replicates and equality of variance between the two methods was assumed. Analyse-it version 2.22 (Analyse-it Software Ltd., Leeds, UK) and Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA) were used for weighted Deming regression analysis and difference-plots.

3.6.3 Comparison of pharmacokinetic parameters

In paper **III**, differences in body weight and calculated pharmacokinetic variables between AR and their metabolites were evaluated by mixed linear models with substance and dose level as fixed factors. Least-squares means were calculated for substances.

3.6.4 Conjugated urinary AR metabolites

A general linear model was used in paper **I** to compare the relative excretion of conjugated urinary AR metabolites between metabolites and sexes. In this model, metabolite and sex were fixed factors while subject was set as a random factor. In paper **IV**, the differences in conjugation between AR metabolites were evaluated by paired t-tests. Paired t-tests were also used to estimate the presence of conjugates other than sulfoconjugates or glucuronides. Differences between the sexes in the excretion of free and conjugated urinary AR metabolites were evaluated by t-tests.

3.6.5 Possible determinants of urinary AR metabolites

In paper **IV**, possible dietary or anthropometric determinants of urinary AR metabolites were identified by multivariate data analysis. Dietary factors (intake of WG and macro and micronutrients) estimated by 3DWFR, were energy-adjusted by the nutrient density method (Willett & Stampfer, 1998). Principal component analyses were separately performed on intake and excretion to observe potential clusters of individuals and relationships between dietary components or excretion. In order to find possible determinants of urinary AR metabolites partial least squares (PLS) regression was used. The initial PLS models included numerous dietary and anthropometric factors and the regression analysis was performed in a step-wise manner where low-impact parameters were removed until models with minimum root-mean-square error of predictors were obtained. Findings from PLS analyses were confirmed by step-wise regression analyses using general linear models. Unscrambler 10.1 (CAMO, Oslo, Norway) was utilized for principal component and PLS analyses.

3.6.6 Proportion of dose recovered in urine

The recovery was defined as the amount of ingested AR that was excreted as DHBA and DHPPA in urine (paper **V**). AR intake was estimated as the mean daily intake of two 3DWFR and excretion was calculated as the mean excretion in two 24-h urine collections. A general linear model was utilized to calculate P for trend, where the log-transformed metabolite recovery was

dependent variable and the quartile median AR intake was independent variable.

3.6.7 Reproducibility

The medium-term (2-3 months) reproducibility of urinary AR metabolite excretion was assessed by calculating the intra-class correlation coefficient (ICC). In paper **V**, ICC were calculated for AR metabolites in 24-h urine collections and morning urine spot samples, as well as for self-reported intakes of macronutrients, WG, and AR. The ICC and their 95% CI were calculated by a freely available SAS macro (Hertzmark & Spiegelman, 2010).

3.6.8 Relative validity

The relative validity of urinary AR metabolites as biomarkers for the intake of WG wheat and rye was assessed by correlating self-reported intakes among free-living participants with their excretion of AR metabolites. In paper **IV**, the relative validities of 24-h urinary excretions of free, conjugated, and total AR metabolites as biomarkers of WG intake were evaluated by calculating Spearman's rank correlation coefficients (r_s) between self-reported intake, estimated by a 3DWFR, and AR metabolite excretion. Similarly, r_s between self-reported WG and fibre intakes and urinary AR metabolites (24-h excretion and creatinine adjusted metabolite concentration in morning spot samples) were calculated in paper **V**. In this study, r_s were calculated between self-reported intake and AR metabolites in urine on the same occasion and 2-3 months apart. Additionally, r_s of intake and excretion were calculated between separate occasions and their mean. The observed r_s were separately adjusted for intra-individual variance in intake and metabolite excretion. Finally, r_s were calculated between 24-h excretions and creatinine-adjusted AR metabolite concentrations in spot samples of morning urine.

4 Results and discussion

4.1 Quantification of AR metabolites

AR in plasma have been analyzed by GC-MS (Landberg *et al.*, 2009b; Linko *et al.*, 2002), gas chromatography/tandem mass spectrometry (Nagy *et al.*, 2008), and liquid chromatography/tandem mass spectrometry (Ross *et al.*, 2010). Although AR metabolites were first identified using thin layer chromatography and GC-MS (Ross *et al.*, 2004b), HPLC-CEAD have been utilized for their quantification in urine and plasma (Koskela *et al.*, 2008; Koskela *et al.*, 2007). Analysis with GC-MS has several advantages (e.g. specificity, relatively short run-time and high number of theoretical plates, which allows better separation) and the equipment is more commonly available in laboratories than HPLC-CEAD systems. Therefore, one aim of this thesis was to develop a GC-MS method for AR metabolites (**I**) and to compare it with HPLC-CEAD (**II**).

4.1.1 GC-MS method development

In the HPLC-CEAD method, syringic acid was used as the internal standard for the quantification of AR metabolites (Koskela *et al.*, 2007). Syringic acid is a phenolic acid and thus shares some structural similarities with DHBA and DHPPA, which is an important feature of internal standards. Another potential internal standard for the quantification could be 3,5-dihydroxyphenyl acetic acid (DHPAA), the structural intermediate to DHBA and DHPPA. However, both candidate compounds have been identified in biological samples; syringic acid is present in a number of cereal products (Mattila *et al.*, 2005) and small concentrations have been found in urine after berry-rich meals (Nurmi *et al.*, 2009), while DHPAA was present in rat urine after oral administration of myrecetin and related compounds (Griffiths & Smith, 1972). In paper **I**, endogenous concentrations of syringic acid and DHPPA in urine from free-living individuals were found to be $\leq 3.5 \mu\text{mol/L}$ and $\leq 4.4 \mu\text{mol/L}$,

respectively. The upper limit of detector linearity was higher for syringic acid (0.225 nmol/injection) than DHPAA (0.150 nmol/injection), which allows a higher amount of syringic acid to be used as the internal standard. In the final protocol, syringic acid was selected as the internal standard due to a narrower range of endogenous concentration in combination with a higher upper limit of detector linearity. The amount of syringic acid added to each sample (15 nmol) was approximately 100 times higher than the endogenous amounts in screened samples, though well within the range of detector linearity.

Derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide yielded larger peak areas ($P < 0.02$) than silylation with N-methyl-N-(trimethylsilyl)trifluoroacetamide or quick silylation mixture. The injection of silylated reference compounds re-dissolved in hexane yielded in peak areas 1-10% of the peak areas resulting from injection directly after derivatization. Thus, injection directly after derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide was selected in the final protocol.

The presence of AR metabolites conjugated to sulfonic and/or glucuronic acid in urine has been shown in previous studies (Koskela *et al.*, 2007). In order to quantify the total amounts of DHBA and DHPPA, urine samples were incubated with deconjugating enzymes that catalyze the hydrolysis of conjugates, thus creating homogeneity of AR metabolites. The deconjugation procedure was optimized in paper I, where urine was incubated with different concentrations of pure β -glucuronidase (which catalyzes hydrolysis of glucuronides) and a crude mixture of Roman snail (*H. pomatia*). The mixture possesses β -glucuronidase, β -glucosidase, and sulfatase activities and thereby enables deconjugation of glucuronides, glucosides, sulfoconjugates, and sulfoglucuronides. After incubation with both pure enzyme and enzyme mixture, quantified AR metabolite concentrations increased with increased enzyme concentrations up to 0.5 mU β -glucuronidase per μ l urine. No increase in DHBA and DHPPA was observed at higher enzyme concentrations. In the final protocol the enzyme mixture was selected as it can deconjugate a wider range of possible conjugates and since incubation with the enzyme mixture in fact resulted in higher AR metabolite concentrations compared to incubation with pure enzyme.

The limit of quantification, accuracy and precision of the developed GC-MS method were satisfactory (**Table 8**) and detection was not considerably affected by co-eluting compounds (**Figure 4**).

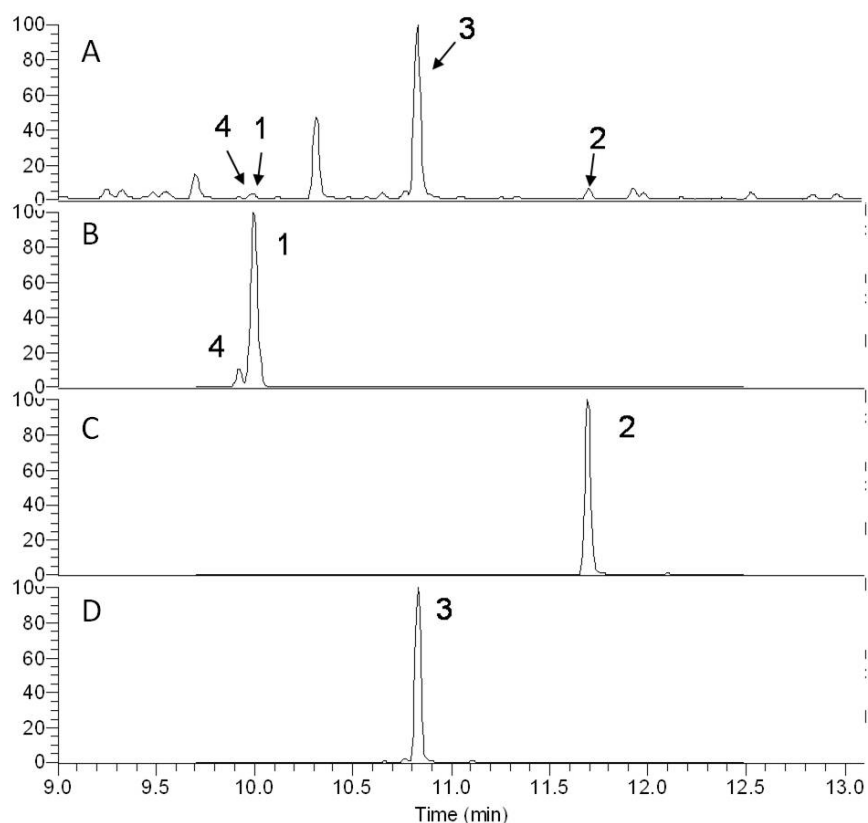


Figure 4. Chromatograms of urine analyzed for AR metabolites by gas chromatography – mass spectrometry. (A) Total ion count (TIC: m/z 50-600) (B) m/z 370; (C) m/z 398 and (D) m/z 342. Peaks: (1) DHBA, (2) DHPPA, (3) syringic acid and (4) 3,4-dihydroxybenzoic acid.

Table 8. Performance of the GC-MS method described in study I.

Parameter	DHBA	DHPPA
Limit of quantification [$\mu\text{mol/L}$] ¹	<0.02 ²	0.1
Accuracy [%] ³	94	93
Intraassay CV [%] ⁴	4.9±1.4	7.6±1.2
Interassay CV [%] ⁵	5.7±2.5	9.3±4.1

¹The limit of quantification was estimated as the concentration resulting in a signal-to-noise ratio of 10:1. ²The signal-to-noise ratio at the lowest tested concentration, 0.019 $\mu\text{mol/L}$, was 194:1. ³Determined as the slope of the linear regression between the measured and theoretical concentrations of spiked urine. ⁴Intraassay CV (mean±SD) was determined from the intraassay CV of three occasions. ⁵Interassay CV (mean±SD) was determined as the standard deviation of mean concentrations from three occasions divided by the mean concentration of all occasions.

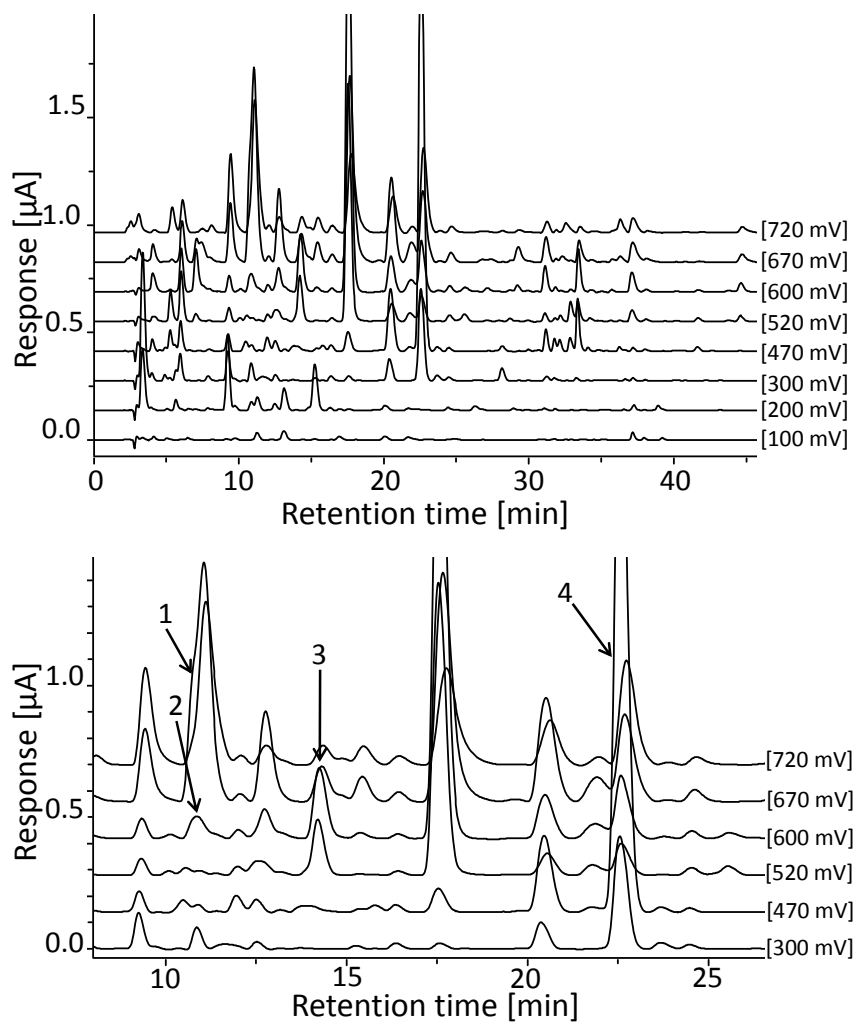


Figure 5. HPLC-CEAD chromatogram of a deconjugated and extracted human urine sample. A: Whole chromatogram with all eight channels, B: Detailed part of A with six channels covering the elution of DHBA, DHPPA and syringic acid. Due to co-eluting compounds, detection of DHBA on the dominant channel (670 mV) (1) was impaired and therefore quantification was performed using the peak area on the channel immediately before the dominant channel (600 mV) (2). No apparent interference due to co-elution was observed for DHPPA (3) and syringic acid (4).

4.1.2 HPLC-CEAD

The quantification of DHBA by HPLC-CEAD suffered from problems due to the co-elution of interfering compounds. These interferences were worse when samples were analyzed directly after deconjugation, and could be suppressed by sample extraction and purification (by solid phase extraction) although not

completely (**Figure 5**). Similar obstacles with co-eluting compounds have been reported previously (Guyman *et al.*, 2008), which indicates the need for sample purification prior to analysis.

4.1.3 Method comparison

In paper **II**, the GC-MS method developed in paper **I** was compared to a previously published HPLC-CEAD method (Koskela *et al.*, 2007). The same protocol was followed for sample pre-treatment (e.g. internal standard, enzymatic deconjugation, and two-step extraction), with differences being only in chromatography and detection. To allow comparisons of results from studies applying different quantification methods, deconjugated and extracted urine samples were analyzed by GC-MS and HPLC-CEAD to detect differences between methods.

Graphical inspection of the difference-plot and weighted Deming regression analysis showed that urine samples analyzed by HPLC-CEAD resulted constantly in higher concentrations of DHBA (0.9 $\mu\text{mol/L}$) than when analyzed by GC-MS (**Figure 6**). DHPPA concentrations quantified by the two methods differed also, but in this case the difference was both additive (intercept: 1.6 $\mu\text{mol/L}$ higher concentration by GC-MS) and proportional (slope: 10% lower concentration by GC-MS). Although these differences were significant, their practical implications are probably negligible in most cases. The additive differences were <10% than the median concentrations in this study and the proportional difference in DHPPA concentrations was of the same magnitude as the analytical imprecision reported for the two published methods. However, caution should be advised when comparing results from studies analyzed by different methods if studies are performed in populations where the intake of AR-containing foods is minor.

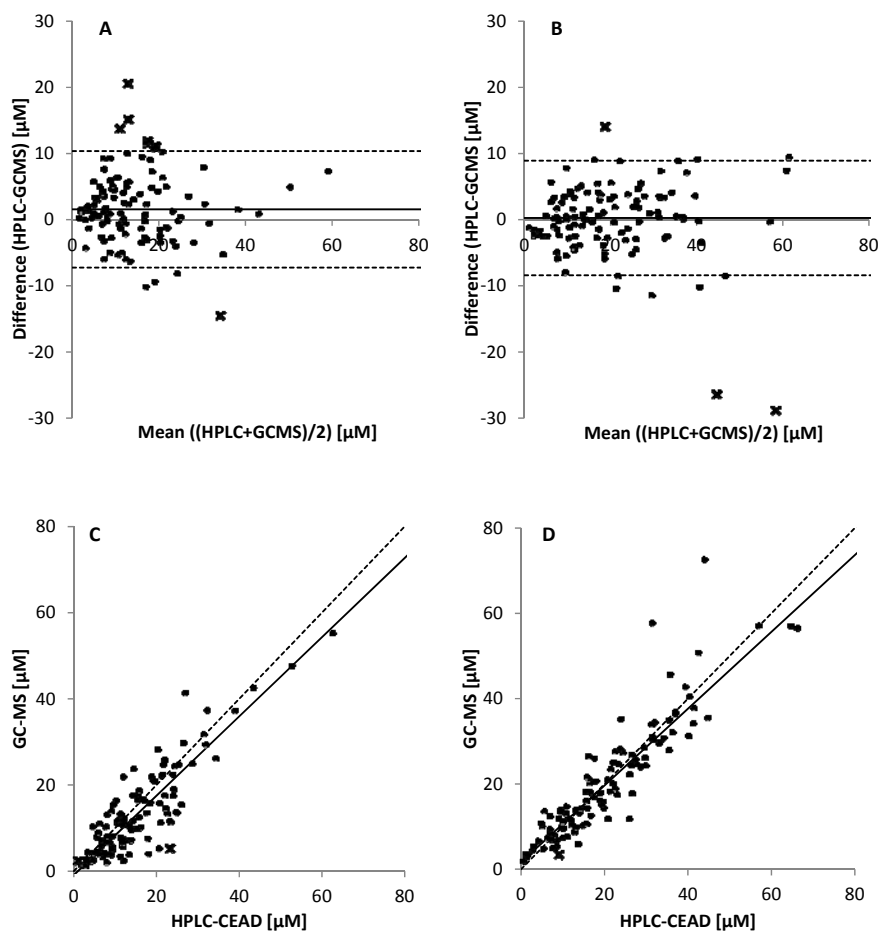


Figure 6. Difference-plots and weighted Deming regressions of alkylresorcinol metabolites in human urine. In the difference-plots of DHBA (A) and DHPPA (B), the differences between the two methods (GC-MS values subtracted from HPLC values) were plotted against the mean concentrations shown by the two methods. The mean differences are shown (solid lines), as are the limits of agreement (broken lines), corresponding to mean ± 2 SD. Concentrations quantified by GC-MS and HPLC-CEAD are plotted against each other (dots) for DHBA (C) and DHPPA (D) and the weighted Deming regressions (DHBA: $y = -0.93 + 0.92x$ and DHPPA: $y = 1.64 + 0.90x$) are indicated (unbroken lines), as are the identity lines (broken lines). Outliers excluded from the models are shown as crosses.

4.2 Formation and elimination of AR metabolites

4.2.1 Absorption, distribution and elimination in rats

A 1-compartment pharmacokinetic model with two absorption compartments has previously been used to adequately predict plasma AR concentrations in

humans (Landberg *et al.*, 2009a). Unlike humans, rats (III) and pigs (Linko *et al.*, 2006) do not display two maxima of plasma concentration, which indicates interspecies differences in absorption. However, these differences could also be influenced by the different matrices used for dosing.

In paper III, plasma concentrations of orally distributed C17:0 and C25:0 were fitted to 1-compartment models. The formation of AR metabolites did not differ between rats orally fed AR homologues C17:0 or C25:0, which shows that the different homologues were absorbed to a similar degree. However, the formation of metabolites during the first 6 hours was greater in rats given C17:0 than in rats consuming C25:0 ($P < 0.05$). This was due to a faster elimination of C17:0 ($t_{1/2} \approx 6$ h) than C25:0 ($t_{1/2} \approx 11$ h) ($P < 0.05$).

The faster elimination of C17:0 might be due to a greater activity of metabolizing enzymes toward shorter AR homologues as been previously proposed (Ross *et al.*, 2004c) or a greater ability of longer homologues to be incorporated into biological membranes (Linko & Adlercreutz, 2005). The major metabolite of orally administered AR was DHPPA and no difference in the metabolite proportions was observed between rats given C17:0 and C25:0. Plasma concentrations of intravenously administered AR metabolites were best described by 2-compartment models, where AR metabolites were rapidly distributed from the central compartment. The elimination half-lives of DHBA and DHPPA (76 mins and 110 mins respectively) were significantly shorter than intact AR and the half-lives increased linearly with the length of the side chain (P for trend < 0.001) (Figure 7). The faster elimination of AR metabolites compared to intact AR is probably due to the greater urinary excretability of metabolites, whereas intact AR are eliminated mainly by metabolism (Ross *et al.*, 2004b; Ross *et al.*, 2003b).

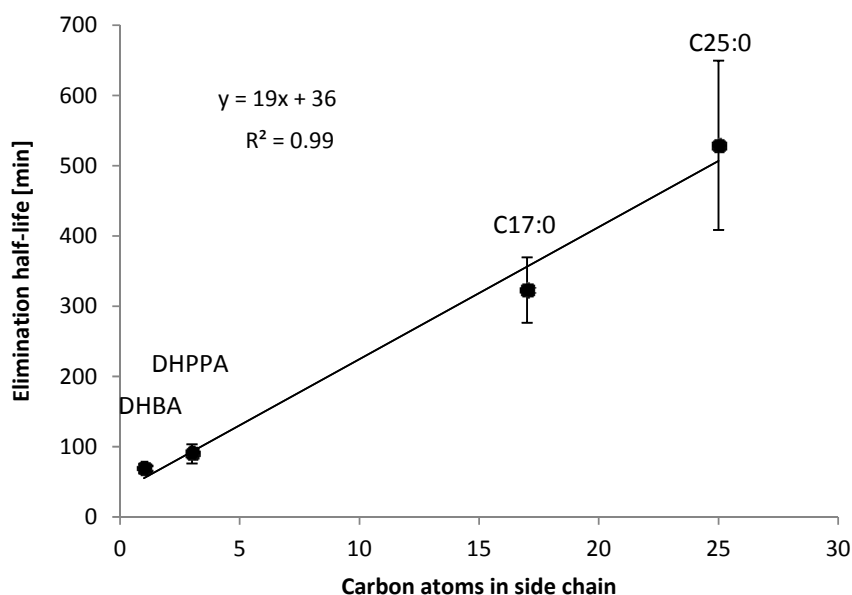


Figure 7. Elimination half-lives of AR (C17:0 and C25:0) and their metabolites (DHBA and DHPPA) in rat plasma. The elimination half-lives increased linearly with the number of carbon atoms in the side chain ($P < 0.001$). The equation of the linear regression between half-life and chain length is indicated in the figure. Error bars represent standard errors of estimates.

4.2.2 Conjugated AR metabolites in human urine

Unlike DHBA and DHPPA, AR have not yet been identified in plasma as conjugates, possibly due to an inability of hepatocytes to excrete conjugated alkylphenols to plasma (Daidoji *et al.*, 2003). The very small amounts of conjugated AR identified in human urine could be the result of renal conjugation and subsequent urinary excretion (Ross *et al.*, 2004b).

A major part of urinary AR metabolites are present as conjugates resulting from phase II metabolism of AR and/or their metabolites (Koskela *et al.*, 2007; Ross *et al.*, 2004b) (papers **I** and **IV**). Glucuronides were found to be the most abundant type of conjugates (papers **I** and **IV**). Unlike sulfotransferases, UDP-glucuronyltransferases are able to conjugate carboxylic groups, thus the number of potential forms of AR metabolite glucuronides is greater than the number of potential forms of sulfoconjugates (Sakaguchi *et al.*, 2004; Strott, 2002). In addition, the subcellular localization of the conjugating enzymes might affect the proportions of DHBA and DHPPA. If conjugation is not exclusive subsequent to terminal β -oxidation, the amphiphilic AR and possible intermediary metabolites might be less subjected to cytosolic sulfotransferases

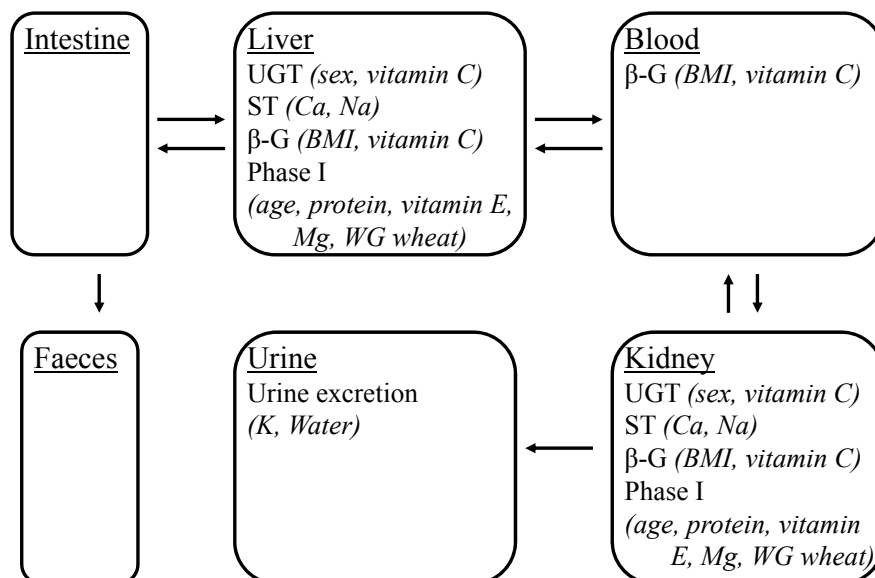


Figure 8. Potential determinants of AR metabolites in urine that were suggested to affect AR metabolite formation and elimination in different ways: conjugation catalyzed by UDP-glucuronyltransferases (UGT) and sulfotransferases (ST), endogenous deconjugation by β-glucuronidase (β-G), Phase I metabolism of AR (Phase I), reabsorption of biliary excreted AR metabolites (Reabsorption), and urine flow. Potential determinants are listed in italics after their suggested sites of effects.

than to microsomal UDP-glucuronyltransferase (Chapman *et al.*, 2004; Nyquist & Morre, 1971).

Compared to DHBA, a greater percentage of DHPPA was glucuronidated ($P < 0.01$), while the proportions of other conjugates were similar between the two AR metabolites. Incubation with the enzyme mixture resulted in higher quantified DHPPA concentrations than summarized concentrations of free, glucuronidated and sulfoconjugated DHPPA, which indicated the presence of DHPPA conjugated with both glucuronic and sulfonic acid (i.e., sulfoglucuronides). Similarly, sulfoglucuronides were identified as urinary metabolites of rats intravenously injected with resorcinol, although glucuronides were the major metabolites (Kim & Matthews, 1987). The presence of sulfoglucuronidated DHPPA and the lack of similar conjugates of DHBA could be due to diverse activities of conjugating enzymes toward the two phenolic acids, comparable to what has been observed *in vitro* for different tocopherol metabolites (Hashiguchi *et al.*, 2011).

Table 9. Potential determinants of AR metabolites in urine and their hypothesized role of action

Determinant	Suggested influence	Reference
Age	CYP ¹ enzyme activities decrease with age	(Bebia <i>et al.</i> , 2004; Schmucker, 2001)
BMI	β-glucuronidase activity increases with BMI	(Maruti <i>et al.</i> , 2010)
Sex	Higher activity of UGT ² among men	(Tanaka, 1999)
Water	Increases urinary excretion rate of glucuronides	
Whole grain wheat	Wheat AR are poorer substrates for CYP ¹ enzymes compared to rye AR.	(Ross <i>et al.</i> , 2004c)
Protein	Increases phase I metabolism	(Boullata & Armenti, 2010)
Vitamin C	Decreases UGT ² activity	(Liu <i>et al.</i> , 2000; Sikic <i>et al.</i> , 1977)
	Vitamin C rich foods increase β-glucuronidase activity	(Maruti <i>et al.</i> , 2008)
	Flavonoids from vitamin C rich foods inhibit UGT	(Williams <i>et al.</i> , 2002)
Vitamin E	Competes for the same phase I enzymes as AR ³	(Ross <i>et al.</i> , 2004c)
	Increases expression of metabolizing enzymes	(Mustacich <i>et al.</i> , 2009)
Calcium	Increases expression of sulfotransferases	(Higashi <i>et al.</i> , 2004)
Magnesium	Less effective β-oxidation in subjects with low intake	(Hisanaga <i>et al.</i> , 2004)
Potassium	Increases urine flow	(Murai <i>et al.</i> , 2010; Bannink <i>et al.</i> , 1999)
Sodium	Increases co-substrate abundance for sulfotransferases	(Lucke <i>et al.</i> , 1981)

¹Cytochrome P450. ²UDP-glucuronyltransferase. ³Alkylresorcinol.

The proportion of urinary AR metabolites conjugated with glucuronic acid was higher ($P < 0.001$) in males ($n=18$) compared to females ($n=34$), while no difference in conjugation between the sexes was observed in the limited number of subjects (5 women and 4 men) in paper I. The potential determinants were suggested to act in several different organs involved in the elimination of AR (**Figure 8** and **Table 9**). A possible explanation of the observed sex difference might be a higher activity of the glucuronidating enzyme UDP-glucuronyltransferase in men compared to women (Tanaka, 1999) or a faster phase I metabolism among women (Frank *et al.*, 2008). Apart from the sex difference, PLS analyses identified a number of potential determinants of AR metabolites in urine. Some determinants were hypothesized to affect conjugating and deconjugating enzymes (BMI, sex, vitamin C, calcium, and sodium), while others may have influenced phase I metabolism (age, protein, vitamin C, magnesium, and WG wheat) and thus

altered the substrate availability for phase II enzymes. In addition, other variables extracted from the PLS analyses were theorized to affect the urine flow (potassium and water).

Together with other factors, such as the genetic polymorphism of Cytochrome P450 enzymes affecting tocopherol metabolism (Bardowell *et al.*, 2010), the determinants listed in Table 9 might influence the elimination of AR and their metabolites. Hence, they could alter the relationship between AR intake and metabolite concentrations in plasma or urine and thereby have an impact on the performance of AR metabolites as biomarkers for the intake of WG wheat and rye.

4.2.3 Proportion of dose recovered in urine

The recovery of urinary AR metabolites is a measure of the proportion of ingested AR that terminates as metabolites in urine. The recovery decreased with increasing intake in free-living Swedes: participants in the quartile with the lowest AR intake (median: 34 $\mu\text{mol/d}$) had the highest recovery (mean: 92%), while for participants in the highest intake quartile (median: 148 $\mu\text{mol/d}$) the mean recovery was only 53% (**Figure 9**). These numbers might be over-estimations of recovery as minor AR derivatives (e.g., unsaturated homologues), not included in the calculation of AR intake, possibly share catabolic pathways with the major homologues and therefore could be precursors of parts of the urinary AR metabolites. Since rye contains substantial amounts of the minor homologues (Ross *et al.*, 2004a) and rye was the major source of AR in paper V, it is likely that considerable amounts of the minor derivatives were consumed and subsequently metabolized to DHBA and DHPPA, which were excreted in urine. Previously, higher amounts of ingested AR have resulted in lower proportions recovered as metabolites in urine (Landberg *et al.*, 2009a). In the same study AR concentrations in fasting plasma increased by 42% and 207% when AR intake was increased by 100% and 300% respectively. This indicates a dose-dependent absorption which might be reflected by decreasing urinary recovery. Supporting this, the apparent absorption in pigs decreased with increased dose, though this could also be due to effects of experimental breads in terms of fibre content and botanical fractions used (Ross *et al.*, 2003b). On the other hand, decreasing urinary recovery might also be due to a dose-dependent shift in elimination, from urinary excretion at low doses to biliary at higher doses (Landberg *et al.*, 2009a). This phenomenon has been observed for tocopherols (Kiyose *et al.*, 2001; Yamashita *et al.*, 2000).

In addition, the high fibre content of AR-rich foods dilutes the intestine content and thereby reduces the concentration of deconjugating enzymes

(Adlercreutz, 2010). This might decrease the proposed enterohepatic circulation of AR metabolites (Soderholm *et al.*, 2011) and consequently reduce the proportion available for urinary excretion. Finally, intakes of other, potential, non-AR related sources of DHBA and DHPPA might cause over-estimation in the proportion of ingested AR recovered as DHBA and DHPPA in urine at low AR intakes.

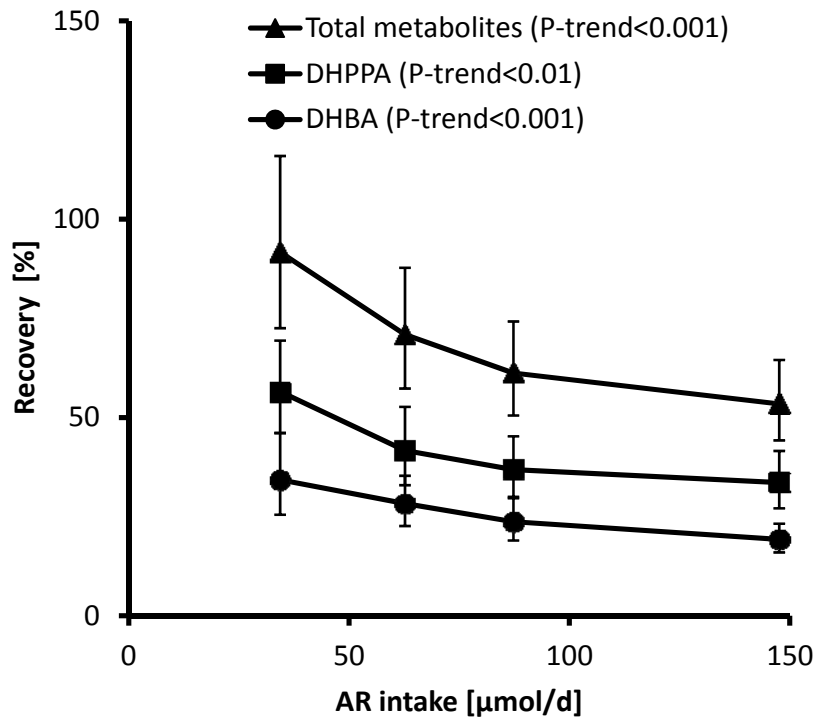


Figure 9. Recovery of daily alkylresorcinol (AR) intake as 24-h urinary excretion of AR metabolites: 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-propanoic acid (DHPPA), and their total. Participants that consumed AR and provided 24-h urine collections plus acceptable three-day weighed food records on both occasions were included ($n = 62$) and divided into quartiles based on AR intake. The quartile recoveries (geometric means) were plotted against median intake per quartile. Error bars represent 95% CI of mean recoveries.

4.3 AR metabolites as biomarkers

4.3.1 Reproducibility

Reproducibility is a crucial parameter of a dietary biomarker and indicates the stability of the biomarker within a subject over a period of time. In the case of

urinary AR metabolites, reproducibility is a measure of how stable the excretion of DHBA and DHPPA is within a subject over a period of time.

The reproducibility of 24-h excretion of AR metabolites over 2-3 months (ICC = 0.48; 95% CI: 0.30-0.66) was moderate (paper **V**) and similar to the reproducibility of AR intake and concentration in fasting plasma from the same participants (Andersson *et al.*, 2011). Plasma AR concentration in German women also showed a similar reproducibility over four months (Montonen *et al.*, 2010). In comparison to 24-h excretions, AR metabolite concentration in spot samples of morning urine had lower reproducibility (ICC=0.36; 95% CI: 0.19-0.58), probably due to the shorter excretion interval. The slightly higher ICC values of DHPPA compared to DHBA in both 24-h urine collections and spot samples of morning urine probably originates from a more diverse DHPPA excretion between participants as indicated by wider 95% confidence intervals. The same phenomenon, with lower reproducibility of DHBA in comparison to DHPPA, has been observed in spot urine samples from American women (Landberg *et al.*, 2012b) and in plasma from German individuals (Montonen *et al.*, 2012). The reproducibility of DHPPA in spot urine samples was only slightly lower in the American subjects, in spite of great diversities between the studies in regard to WG consumption and time period evaluated. However, the reproducibility of DHBA was considerably higher in the Swedish subjects, which could originate from a higher, more stable, and (between individuals) more diverse AR intake among the Swedish participants. It could also be partly attributable to the shorter time period evaluated. Additionally, unstable intakes of other potential precursors of urinary DHBA could attenuate reproducibility, especially if intake of AR-containing foods is low. In fact, the total consumption of peanuts, a potential source of DHBA (Khadem & Marles, 2010; Reddy *et al.*, 1977), increased about 10% in the US in the period between urine collections (American Peanut Shellers Association, 2011). An increased intake of a potential DHBA source (e.g., peanuts) over the time assessed might be partly responsible for the poor reproducibility of urinary DHBA observed in the American women.

Reproducibilities of AR intake and AR, DHBA, DHPPA concentrations or amounts in biological samples from different populations over diverse time periods are listed in **Table 10**. The rather fast elimination of AR and their metabolites implies that reproducibility over longer time periods is dependent on the stability of intake (van Dam & Hu, 2008).

Table 10. *Reproducibility of alkylresorcinols and their metabolites in subjects consuming habitual diets.*

	Sample			Subjects			ICC ² (95% CI)	Reference
	Type	Replicates	Period	Country	N ¹	Females		
<i>Alkylresorcinols</i>	3DWFR	2	2-3 mo	Sweden	72	76%	0.46 (0.26, 0.66)	(Andersson <i>et al.</i> , 2011)
	Fasting plasma	2	1-3 y	Sweden	36	100%	0.73 (0.56, 0.85)	(Landberg <i>et al.</i> , 2012a)
	Fasting plasma	2	4 mo	Germany	100	50%	0.42 (0.25, 0.72)	(Montonen <i>et al.</i> , 2010)
	Fasting plasma	2	2-3 mo	Sweden	51	80%	0.47 (0.27, 0.67)	(Andersson <i>et al.</i> , 2011)
	Fasting plasma	3	3 d	Sweden	18	61%	0.60 (0.36, 0.80)	(Andersson <i>et al.</i> , 2011)
	Non-fasting plasma	3	3 d	Sweden	18	61%	0.18 (0.03, 0.62)	(Andersson <i>et al.</i> , 2011)
<i>DHBA</i>	Spot urine ³	2	1-3 y	USA	104	100%	0.17 (0.05, 0.43)	(Landberg <i>et al.</i> , 2012b)
	24h urine	2	2-3 mo	Sweden	66	76%	0.46 (0.28, 0.65)	Paper V
	Spot urine ³	2	2-3 mo	Sweden	66	76%	0.32 (0.15, 0.56)	Paper V
	24h urine	3	3 d	Sweden	18	61%	0.63 (0.39, 0.81) ⁴	(Marklund <i>et al.</i> , unpublished data)
	Spot urine ³	3	3 d	Sweden	16	69%	0.67 (0.43, 0.84) ⁴	(Marklund <i>et al.</i> , unpublished data)
	Plasma	2	4 mo	Germany	100	50%	0.23 (-0.05, 0.48)	(Montonen <i>et al.</i> , 2012)
<i>DHPPA</i>	Spot urine ³	2	1-3 y	USA	104	100%	0.31 (0.17, 0.51)	(Landberg <i>et al.</i> , 2012b)
	24h urine	2	2-3 mo	Sweden	66	76%	0.51 (0.34, 0.68)	Paper V
	Spot urine ³	2	2-3 mo	Sweden	66	76%	0.37 (0.19, 0.59)	Paper V
	24h urine	3	3 d	Sweden	18	61%	0.68 (0.46, 0.85) ⁴	(Marklund <i>et al.</i> , unpublished data)
	Spot urine ³	3	3 d	Sweden	16	69%	0.65 (0.40, 0.83) ⁴	(Marklund <i>et al.</i> , unpublished data)
	Plasma	2	4 mo	Germany	100	50%	0.33 (0.06–0.56)	(Montonen <i>et al.</i> , 2012)

¹Number of subjects. ²3-day weighted food record; DHBA, 3,5-dihydroxybenzoic acid; DHPPA, 3-(3,5-dihydroxyphenyl)-propanoic acid; ICC, intra-class correlation coefficient

³Spot samples of morning urine. ⁴Evaluated at two occasions and only the lowest value is shown here.

In populations where the intake of AR is quantitatively and temporally stable within individuals and covers a wide range between individuals, the reproducibility of AR and their metabolites in biological samples can be expected to be high. Spot samples, unlike 24-h collections, are not only affected by the stability of intake between days, but also sensitive to the duration between consumption and collection within the day.

Plasma AR concentrations are logically more stable under intervention conditions where intake is controlled than under free-living conditions and the high reproducibility indicates that the number of measurements needed to evaluate compliance is very low (Landberg *et al.*, 2009c). The reproducibility of AR metabolites in intervention studies has not yet been assessed.

4.3.2 Relative validity

The 24-h excretion of DHBA and DHPPA was significantly correlated to self-reported intakes of WG wheat and rye, WG rye and cereal fibre, irrespective of when intake and excretion were measured (**Figure 10**). Metabolite concentrations in spot samples of morning urine were also correlated to the same intake parameters, although only consistently correlated when intake and excretion were measured concurrently. The total intake of WG was in general only correlated to urinary AR metabolites when measured on the same occasion. Although correlations were weaker compared to cereal fibre, the intake of total fibre was correlated to 24-h excretion independently of the time of measurement and correlated to morning urine metabolites when intake and urine were measured concurrently.

The relative validity of individual conjugates and free aglycones of urinary AR metabolites were assessed in paper IV. In general, correlation coefficients of WG intake and individual free aglycones or conjugates appeared somewhat lower than coefficients of WG intake and sums of conjugates and free aglycones. However, the observed correlations were of the same magnitude for the individual conjugates.

Rye, the richest source of AR, was the main component of WG intake and a major contributor to the intake of cereal fibre in paper V. Since the excretion of AR metabolites is well correlated to the intake of AR, the excretion of AR metabolites reflected the intake of fibre and WG, especially WG rye.

Significant correlations between the intake of WG wheat and urinary metabolites were weak and in most cases absent. The intake of WG wheat was low, unstable and not correlated to that of WG rye, hence excretion was unable to reflect WG wheat intake. It is possible that the correlation is stronger in populations where rye intake is low and a stable intake of wheat constitutes a more predominant part of WG intake (Guyman *et al.*, 2008).

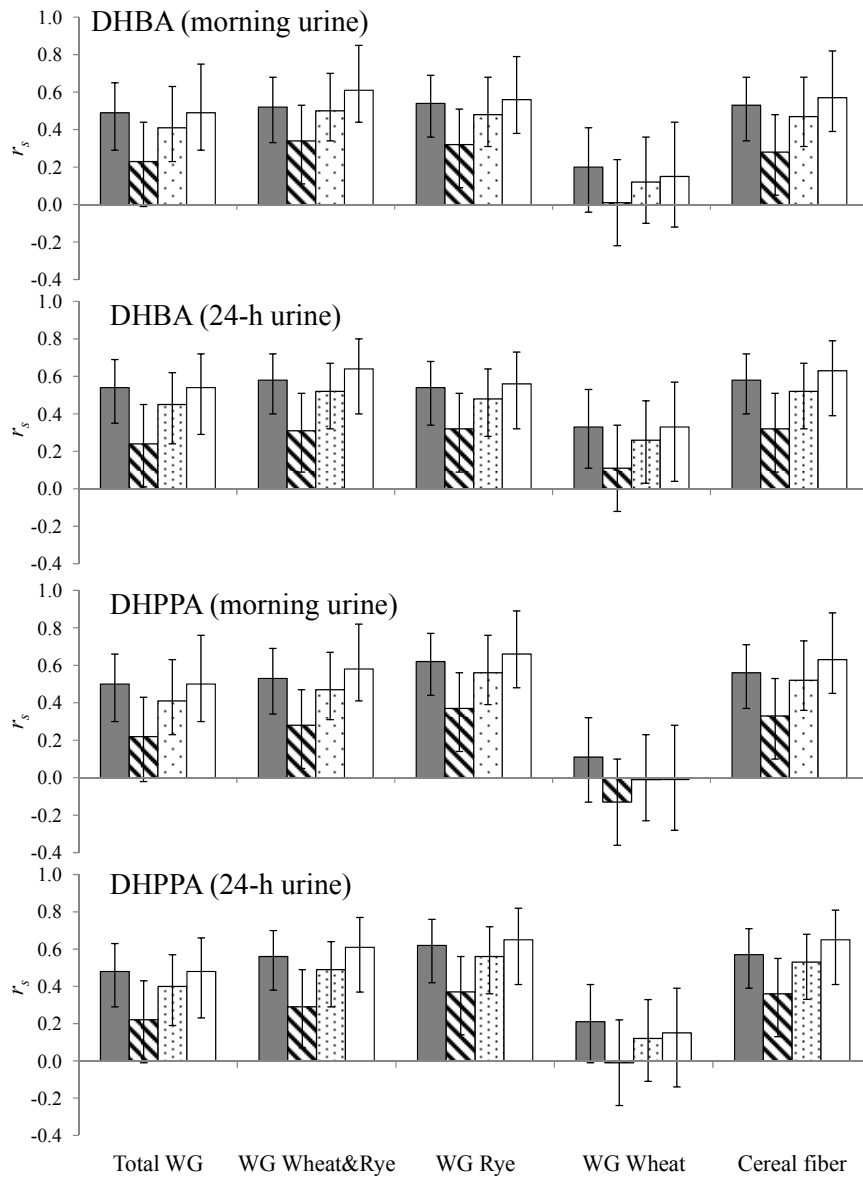


Figure 10. Relative validity of urinary alkylresorcinol metabolites estimated by calculation of Spearman's rank correlation coefficient (r_s) between self-reported intakes of whole grain (WG) and cereal fibre (estimated by 3DWFR) and urinary DHBA and DHPPA. Correlation coefficients were calculated between AR metabolite excretion and concurrent intake (grey), intake 2-3 months later (striped), mean intake on two occasions (dotted), and mean intake adjusted for intra-individual variance (white). Error bars indicate 95% confidence intervals of r_s .

Although AR metabolites in spot samples of morning urine correlated well with 24-h excretion, the lower reproducibility of AR metabolites in spot samples of morning urine resulted in weaker correlations to intake compared to 24-h metabolite excretions.

As expected, correlations were stronger when intake and excretion were measured concurrently compared to when measured separately 2-3 months apart. With the moderate reproducibility of the intake in paper **V**, it is likely that a single 3DWFR would be insufficient to estimate longer-term intake.

The r_s calculated between mean WG and fibre intakes and metabolite excretion (morning urine and 24-h) on the different occasions were significant. In order to provide a better estimate of the correlation between the 'true' intake (estimated by a currently non-available golden standard method) and metabolite excretion, adjusting the intake estimates for intra-individual variance resulted in increased r_s , although 95% CI increased similarly as a consequence of the limited number of subjects and the instability of intake.

This indicates, however, that both urine sample types reflect the medium-term (2-3 months) intake of fibre (total and cereal) and WG (total, rye, and rye+wheat) among the 66 participants in paper **V**. Due to the similar reproducibility of intake and 24-h excretion, corrected r_s between intake and 24-h excretion were alike when adjusting for intake or biomarker variabilities. As a consequence of the lower reproducibility in morning urine, adjusting r_s between intake and morning urine metabolite concentration for variances in biomarker measurement resulted in greater coefficients compared to when adjusting for intake variability.

The relative validity of AR and their metabolites among free-living subjects has been assessed in different studies (**Table 11**). The methods for estimating intake differ between the studies and the highest correlations are found when intake estimation is as detailed as possible (i.e. weighed food records). Since AR metabolites mainly reflect short-term intake, correlations between long-term intake assessment methods (e.g., FFQ) and biomarker measurements are weak unless the intake is stable over the period assessed. The cumulative nature of urine collections reduces the temporal impact of intake during the day, and thus offers greater possibilities for reflecting daily intake than measurements in plasma, which is highly time-dependent.

In previous studies and in paper **V**, the self-reported intake of cereal fibre was correlated with AR metabolites in plasma and/or urine (Landberg *et al.*, 2012b; Aubertin-Leheudre *et al.*, 2010a; Aubertin-Leheudre *et al.*, 2008). This suggests that AR metabolites might be useful to reflect cereal fibre in populations where WG rye and wheat are major contributors of cereal fibre.

Table 11. Correlations between self-reported intake and AR, DHBA, and DHPPA in biological samples from free-living subjects.

	Sample	Subjects		Intake		Correlation ¹	Reference	
		Country	N	Females	estimation			Parameter
Alkylresorcinol	Plasma	Switzerland	29	61%	3DWFR ²	Total WG	0.57 ^c	(Ross <i>et al.</i> , 2009)
	Plasma	Switzerland	29	61%	WG FFQ ²	Total WG	0.55 ^c	(Ross <i>et al.</i> , 2009)
	Plasma	Sweden	51	80%	3DWFR	WG wheat & rye	0.53 ^{c,3}	(Andersson <i>et al.</i> , 2011)
	Plasma ⁴	Denmark	360	100%	FFQ	Rye bread	0.25 ^c	(Landberg <i>et al.</i> , 2011)
DHBA ²	24-h urine ⁵	Finland	60	100%	5WDFR ²	Rye	0.52 ^c	(Aubertin-Leheudre <i>et al.</i> , 2010c)
	24-h urine	Sweden	66	76%	3DWFR ²	WG wheat & rye	0.58 ^{c,6}	Paper V
	Spot urine	Sweden	66	76%	3DWFR ²	WG wheat & rye	0.48 ^{c,6}	Paper V
	Plasma ⁷	Finland	60	100%	5WDFR ²	Rye	0.32 ^a	(Aubertin-Leheudre <i>et al.</i> , 2010c)
	Spot urine	USA	95	100%	FFQ	WG	0.16	(Landberg <i>et al.</i> , 2012b)
DHPPA ²	24-h urine ⁵	Finland	60	100%	5WDFR ²	Rye	0.44 ^c	(Aubertin-Leheudre <i>et al.</i> , 2010c)
	24-h urine	Sweden	66	76%	3DWFR ²	WG wheat & rye	0.56 ^{c,6}	Paper V
	Spot urine	Sweden	66	76%	3DWFR ²	WG wheat & rye	0.52 ^{c,6}	Paper V
	Plasma ⁷	Finland	60	100%	5WDFR ²	Rye	0.39 ^b	(Aubertin-Leheudre <i>et al.</i> , 2010c)
	Spot urine	USA	95	100%	FFQ	WG	0.16	(Landberg <i>et al.</i> , 2012b)
						0.37 ^c		

¹ Significant levels of correlations are indicated by superscript letters: ^aP<0.05, ^bP<0.01, ^cP<0.001. ²3DWFR, 3-day weighed food record; 5WDFR, 5-day weighed food record; DHBA, 3,5-dihydroxybenzoic acid; DHPPA, 3-(3,5-dihydroxyphenyl)-propanoic acid; WG, whole grain; WG FFQ, Whole grain food frequency questionnaire. ³Correlation between mean of two 3DWFR and two fasting plasma samples collected 2-3 months apart. ⁴Non-fasting plasma samples. ⁵Three consecutive 24-h collections were pooled before analysis. ⁶Evaluated on two occasions (2-3 months apart) and only the lowest value is shown here. ⁷Three consecutive plasma samples were pooled before analysis. ⁸Estimated on two occasions about 4 years apart.

4.3.3 Potential use as biomarkers

In epidemiology

One aim of this thesis was to evaluate AR metabolites as biomarkers of whole grain wheat and rye to be used in observational studies. Akin to AR, a limiting factor for this purpose is the relatively low reproducibility of the metabolites observed (Table 10), which indicates that a single plasma or urine sample in many populations will be insufficient to estimate the ‘true’ biomarker concentration/excretion, which is related to habitual intake of WG rye and wheat. As for AR previously discussed, the reproducibility of AR metabolites is dependent on the intake stability of AR-containing foods, since the formation and elimination of DHBA and DHPPA are rather quick (Paper **III**) (Soderholm *et al.*, 2011; Soderholm *et al.*, 2009). A high reproducibility of AR metabolites is possible in populations with a stable WG intake, and would reduce the number of measurements required for a reliable estimation of ‘true’ long-term biomarker status. However, the reproducibility of WG intake under free-living conditions has never been reported to be more than moderate (Landberg *et al.*, 2012b; Andersson *et al.*, 2011). If urinary AR metabolites in single samples are to be used to reflect long-term intakes of WG or fibre, variance components of excretion should be reliably addressed in the population of interest in order to allow adjustment of risk estimates for intra-individual variations.

Urine samples offer a cumulative measurement of AR metabolites, in contrast to plasma samples which are more time-dependent. This, in combination with simple and unassisted sampling, promotes the use of urinary AR metabolites for the estimation of whole grain intake. Unfortunately, the sample type where AR metabolites display the highest reproducibility and validity (i.e., >12-h urine collections) is probably the least available in large observational studies.

In addition, prospective cohort studies commonly offer non-fasting plasma samples. The reproducibility and validity of AR metabolites in non-fasting samples have so far not been assessed, although studies on AR suggest that this sample type would show lower reproducibility and validity compared to fasting samples (Andersson *et al.*, 2011; Landberg *et al.*, 2011).

The combined plasma concentrations of AR and their metabolites might possess better reproducibility and validity than what they do individually, though this has not yet been evaluated. In order to investigate this, the method utilized in paper **III** should be optimized and validated, to enable the reliable and simultaneous quantification of AR and their metabolites in plasma.

Recently, an alternative use of dietary biomarkers has been proposed, where biomarkers have been used in combination with traditional dietary assessment methods to improve intake estimations and to reduce sample requirements (Freedman *et al.*, 2010a; Freedman *et al.*, 2010b). This possible use of AR metabolites should be evaluated.

In intervention studies

Another potential use of AR metabolites as biomarkers of AR-containing foods is to assess compliance during dietary intervention studies. Previously plasma AR have been used for this purpose in one study, while similar applications of AR metabolites are still absent (Ross *et al.*, 2011). Urinary AR metabolites might be advantageous to plasma AR as they may reflect AR intake better (Paper V)(Andersson *et al.*, 2011) and can be obtained by non-invasive sampling, which does not necessitate the involvement of medical professionals.

One promising application of AR metabolites as biomarkers of compliance could be to monitor individual variations over the treatment periods. This has been proposed for plasma AR concentrations, which remained very stable during a 6-week rye intervention study (Landberg *et al.*, 2009c).

However, in order to estimate compliance from a single biomarker measurement, threshold values need to be established (Ross, 2012). Apparently, AR metabolites in plasma and urine can reflect AR intake at group level (Aubertin-Leheudre *et al.*, 2010c; Landberg *et al.*, 2009a). However, the large variations between and within individuals would be a hindrance in determining the compliance of single participants. In addition, AR metabolites have been identified in urine from celiac patients and there is some debate about whether they may be produced from foods other than WG wheat and rye. Therefore, more knowledge of alternative sources of DHPPA and DHBA is needed in order to evaluate the potential use of AR metabolites as DB.

The reproducibility of plasma concentration and urinary excretion of AR metabolites under interventional conditions needs to be assessed before establishing them as biomarkers of compliance. Obviously, if they are to be used in intervention studies focusing on WG, the intake of bran needs to be controlled and *vice versa*.

Validation of dietary assessment methods

Apart from the aforementioned biomarker applications listed above AR metabolites could potentially be used to validate traditional dietary assessments methods, since the errors of the biomarker are likely to be independent from the errors of the traditional methods (e.g. FFQ). Several models utilizing biomarkers have been proposed for this purpose (Fraser *et al.*, 2005;

Spiegelman *et al.*, 2005; Subar *et al.*, 2003; Kaaks *et al.*, 1997). However, the independence of errors between the different estimation instruments used in these validations may still be questioned (Rosner *et al.*, 2008). For example, both food records and FFQ are based on self-reports, thus their measurement errors are not likely to be independent. Similarly, two biomarkers measured in urine would also share errors to some degree.

In conclusion, since 24-h urine collections are scarcely available in epidemiological studies, the present body of evidence shows limited potential for single measurements of AR metabolites as a surrogate measurement of WG wheat and rye intake in large observational studies. This is mainly due to the inability of single samples to reflect long-term habitual WG intake. It is possible that repeated measurements could successfully reflect long-term intake. However, if AR metabolites are to be used as biomarkers in epidemiology it is strongly advised that variance components in the population of interest should be estimated to allow adjustments for intra-individual variation. Intake estimation based on combinations of biomarkers and traditional dietary assessment methods might offer a promising use of AR metabolites. As a cumulative measurement, urinary AR metabolites are likely to be advantageous compared to their plasma counterparts in terms of reproducibility, though sample availability might favour the use of plasma samples. Plasma and urinary AR metabolites might be useful as biomarkers of compliance in intervention studies, especially to monitor compliance within individuals over treatment periods. However, more information is needed on alternative sources of DHBA and DHPPA (unrelated to AR intake) and the normal range of AR metabolites in urine or plasma from subjects excluding WG and bran products of wheat and rye from their diets. Finally, urinary AR metabolites might be useful to validate traditional dietary assessment methods. Work on testing of the validity of plasma AR metabolites as biomarkers of wholegrain wheat and rye intake remains to be conducted.

5 Main findings

- A precise and selective GC-MS method for the quantification of urinary AR metabolites was developed (**I**) and was adequately utilized to analyze a large number of samples.
- A comparison of urinary AR metabolite concentrations quantified by GC-MS and HPLC-CEAD resulted in small, but significant, differences (**II**). However, these differences can be considered as negligible in most cases.
- The elimination of AR metabolites in rats was faster compared to their precursors and elimination half-lives increased with side chain length (**III**). There was no difference in the total amount of AR metabolites formed after ingestion of C17:0 and C25:0, indicating that both AR homologues were absorbed to a similar degree.
- A substantial proportion of urinary AR metabolites (especially DHPPA) was found as conjugates, and glucuronides were the main conjugates (**I**, **IV**). Several potential determinants for urinary AR metabolites were identified and included both anthropometric and dietary factors, which might affect AR metabolism and thus the performance of AR metabolites as biomarkers (**IV**).
- The reproducibility of AR metabolites in 24-h urine from free-living subjects was comparable to AR intake and plasma concentration, while it was lower in spot samples of morning urine (**V**).
- The relative validity of urinary AR metabolites as biomarkers of WG wheat and rye was good ($r > 0.5$) when assessed in both 24-h collections and spot samples (**V**).

6 Future research

- Faster and cheaper methods for the quantification of urinary AR metabolites (e.g., immunological and/or spectroscopic) are required in order to allow screening of large numbers of samples.
- Instead of using AR and their metabolites individually, their sum might be advantageous as biomarkers for the intake of WG rye and wheat. However, further method development is needed for the reliable simultaneous quantification of AR and their metabolites in plasma.
- The identification of AR metabolizing enzymes is desirable as it would increase the knowledge of AR metabolite formation and could aid the evaluation of genetic impact on biomarker performance. In addition it could help explain the actual mechanisms of the potential determinants of urinary AR metabolites.
- Concentrations of AR metabolites in plasma and urine from individuals avoiding dietary AR should be quantified and alternative sources of DHBA and DHPPA should be identified.
- The reproducibility of AR metabolites should be assessed under interventional conditions in order to evaluate their suitability as compliance biomarkers.
- The potential use of AR metabolite measurements in combination with traditional dietary assessment methods for intake estimation should be evaluated.

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