

# Alkylresorcinols as Biomarkers of Whole Grain Wheat and Rye Intake

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### Abstract

Dietary biomarkers are objective measures of food or nutrient intake and can be related to endpoints in epidemiological studies, used in the validation of dietary assessment instruments and used to check of compliance during intervention studies. Alkylresorcinols (AR), phenolic lipids present exclusively in the outer parts of wheat and rye grains, have been suggested as biomarkers of whole grain wheat and rye intake.

The overall aim with this thesis was to evaluate AR as specific biomarkers of whole grain wheat and rye intake. This was conducted by developing a rapid GC-MS method for the analysis of AR in plasma and by studying AR pharmacokinetics, dose-response, reproducibility and relative validity in human intervention studies under controlled intake conditions. Factors affecting plasma AR concentrations were investigated in free-living Danish women.

The method developed proved suitable for the analysis of relatively small sample volumes (50- 200 $\mu$ L). The results showed that AR in fasting plasma samples can be used as short-term concentration biomarkers, reflecting the intake range normally found in the Nordic countries in a dose-dependent manner. One or two repeated measurements of AR were found to adequately describe a subject's average plasma AR concentration at regular and constant intake. In free-living Danish women, rye bread was identified as the major factor affecting plasma AR concentration and there was no evidence of non-dietary factors or other foods having an effect. In conclusion, our results support that AR can be used as biomarkers in intervention studies on whole grain wheat and rye and probably also in epidemiological endpoint- and validation studies.

*Keywords:* Alkylresorcinols, AR, biomarker, whole grain, wheat, rye

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*Acti labores jucundi*  
(Marcus Tullius Cicero)

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## List of Publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I** Landberg, R., Åman P. & Kamal-Eldin, A. (2008). A rapid GC-MS method for quantification of alkylresorcinols in human plasma. *Analytical Biochemistry* 385, 7-12.
- II** Landberg, R. Linko, A.M, Kamal-Eldin, A., Vessby, B., Adlercreutz, H. & Åman, P. (2006). Human plasma kinetics and relative bioavailability of alkylresorcinols after intake of rye bran. *Journal of Nutrition* 136, 2760-2765.
- III** Landberg, R., Åman, P., Friberg, L., Vessby, B., Adlercreutz, H. & Kamal-Eldin, A. (2009). Dose-response of whole grain biomarkers: Alkylresorcinols in human plasma and their metabolites in urine in relation to intake. *American Journal of Clinical Nutrition* 89, 290-296.
- IV** Landberg, R., Kamal-Eldin, A., Andersson, A., Vessby, B. & Åman, P. (2008). Alkylresorcinols as biomarkers of whole-grain wheat and rye intake: Plasma concentration and intake estimated from dietary records. *American Journal of Clinical Nutrition* 87, 832-838.
- V** Landberg, R., Kamal-Eldin, A., Zhang, J-X., Andersson, S-O., Johansson, J-E., Hallmans, G. & Åman, P. Reproducibility of plasma alkylresorcinols during a 6-weeks rye intervention study on men with prostate cancer. *Submitted*.

**VI** Landberg, R., Kamal-Eldin, A., Åman, P., Christensen, J., Tjønneland, A., Overvad, K. & Olsen, A. Intake of rye bread is an important determinant of plasma alkylresorcinol concentration in Danish postmenopausal women. *Manuscript*.

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*Author's main contribution to the publications*

- I:** Planned experiments, performed laboratory analyses, evaluated results and had main responsibility for writing and revising the paper.
- II:** Planned the study in collaboration with supervisors. Conducted the clinical study, performed plasma sample analyses under supervision of A-ML and HA, performed data analysis, and had main responsibility for writing and revising the paper.
- III:** Planned the study in collaboration with supervisors, conducted the clinical study, performed the statistical analysis (together with a consultant), took the initiative for PK-modelling, and had main responsibility for writing and revising the paper.
- IV:** Analysed samples, performed statistical analysis (together with a consultant) and had main responsibility for writing and revising the paper.
- V:** Performed laboratory analyses (with the assistance of a student), performed statistical analysis (together with consultants), evaluated results and had main responsibility for writing and revising the paper.
- VI:** Performed the AR analysis (with the assistance of a student), performed the statistical analysis and data evaluation together with JC and AO, and had main responsibility for writing the manuscript.

## Abbreviations

ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
AR	Alkylresorcinol(s)
CEAD	Coulometric Electrode Array Detector
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
DEAE	Diethylaminoethyl
EPIC	European Prospective Investigation into Cancer and nutrition
FDA	Food and Drug Administration
GC	Gas Chromatography
GC-MS	Gas Chromatography- Mass Spectrometry
GLM	General Linear Model
HDL	High-Density Lipoprotein
HMDS	Hexamethyldisilazane
HPLC	High Performance Liquid Chromatography
ICC	Intra-Correlation Coefficient
LDL	Low-Density Lipoprotein
OR	Odds Ratio
PK	Pharmacokinetics
QSM	Quick Silylation Mixture
RCT	Randomised Clinical Trail
SNF	Swedish Nutrition Foundation
SPE	Solid Phase Extraction
TMCS	Trimethylchlorosilane
USDA	U.S Department of Agriculture
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization



# 1 Background

## 1.1 Whole grain and human health

Cereals have been an important part of the human diet since the advent of agriculture about 10 000 years ago and during the majority of that time they have been consumed as whole grain (Spiller, 2002). It is only within the last hundred years that most people have consumed refined grains (Slavin, 2005a). The positive health effects of whole grain were recognised early and during the past two hundred years physicians and scientists have recommended whole grain to prevent constipation. In the early 1970s, the ‘fibre hypothesis’ was proposed by Burkitt and colleagues (Burkitt *et al.*, 1974). It was suggested that non-refined foods, such as whole grains, fruits and vegetables, which provide dietary fibre along with other constituents, have a protective effect against ‘Western’ diseases such as coronary heart disease and colon cancer. Today, there is a general long-standing view among major governmental, scientific and non-profit organisations, mainly based on epidemiological findings, that the Western population would benefit from increased consumption of whole grain foods as a way of reducing the risk of developing chronic diseases (Cleveland *et al.*, 2000; Marquart *et al.*, 2005; National Food Institute, 2008). As a result, whole grain intake, as a part of a healthy diet, has been directly (USA) or indirectly (Nordic countries and UK) promoted in the dietary guidelines in a number of countries and by WHO (Becker *et al.*, 2004; Food Standards Agency, 2008; U.S Department of Health and Human Services & U.S. Department of Agriculture, 2005; World Health Organization, 2003).

The cereal grain is the fruit, called caryopsis, of plants belonging to the grass family (*Poaceae* or *Gramineae*) (Hoseney, 1994). Whole grain refers to ‘intact, ground, cracked or flaked caryopsis, whose principal anatomical components- the starchy endosperm, germ and bran (Figure 1)- are present in the same relative proportions as they exist in the intact caryopsis’ (definition by AACCC, 1999). This definition is also used by U.S. Food and Drug Administration (FDA)(2006). Cereals typically included in whole grain foods in the Western world are wheat, oats, rye, rice, barley and corn but also sorghum, and millet may be included as well as buckwheat, amaranth and wild rice, although not all of them are true cereals in the botanic sense (Cleveland *et al.*, 2000; Jacobs & Steffen, 2003; Slavin, 2005b; Swedish Nutrition Foundation, 2004; Thane *et al.*, 2007). There is currently no general, world-wide accepted definition of whole grain products, but typically they contain a certain proportion of intact, flaked or broken kernels, coarsely ground kernels or flour of whole grain. Different definitions have been used across studies, both in respect to the cereals that are included and to the proportion of whole grain in the food. Many of the studies on whole grain and disease have used the definition by Jacobs *et al.* (1998) which is a broad definition, but other more strict definitions have been used as well (Table 1).

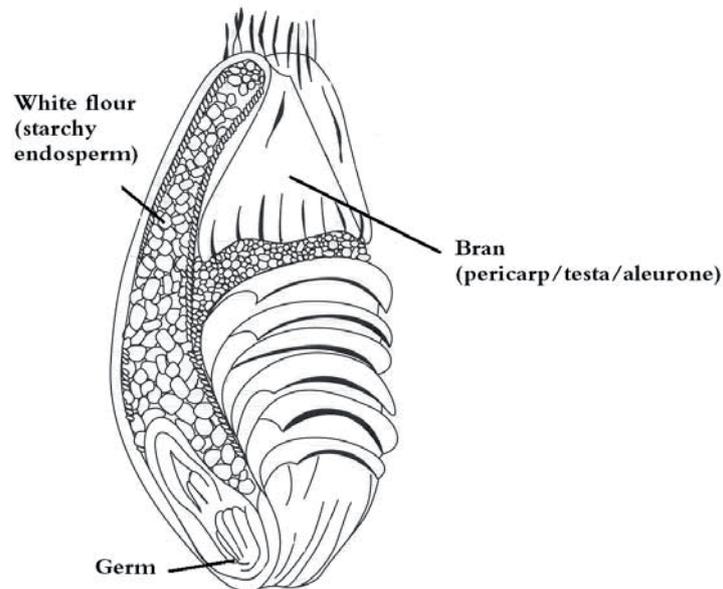


Figure 1. A principle sketch of a wheat caryopsis modified from Marquart *et al.* (2005).

Table 1. Selected definitions of whole grain product definitions

Reference	Cereals included	Constituents included	Proportion of product weight (%)
U.S. Food and Drug Administration (FDA) (2006)	Amaranth, barley, buckwheat, corn, millet, rice, rye, oats, sorghum, triticale, wheat and wild rice	According to AACC definition (see text )	≥51% whole grain ingredients by weight/reference amount customarily consumed (RACC) per day
Swedish Nutrition Foundation (2004)	Wheat, oats, barley and rye	Intact or ground whole grain seed kernels ( <i>i.e.</i> cereal grains where all components contained in the grain seed, along with the seed shell, are included)	Flour, grains and flakes must be 100% whole grain and the whole grain ingredient must exceed ≥50% weight of product dry matter
Thane <i>et al.</i> (2007)	Wheat, oats, rice, rye, barley, and corn (popcorn)	According to AACC definition (see text)	≥ 10%
Jacobs <i>et al.</i> (1998)	Wheat, rye, oats, barley and rice	Dark bread, brown rice, popcorn, wheat germ, cooked oatmeal, bulgur, couscous, breakfast cereals, bran	≥ 25% whole grain
Jensen <i>et al.</i> (2004)	Wheat, oats, rice, barley, rye, buckwheat, corn (popcorn), amaranth, psyllium	According to AACC definition (see text )	Amount whole grain in product (g) was counted
National Food Institute (2008)	Barley, corn, wheat, rye, oats, sorghum, millet and rice	According to definition similar to AACC (see text)	≥51% whole grain on product dry weight basis

Whole grain is a rich source of dietary fibre and a number of bioactive components, including minerals, vitamins, tocopherols, phytosterols, lignans and cinnamic acids. Together, these are sometimes referred to as the dietary fibre complex, which has been suggested to account for the positive health effects (Jacobs *et al.*, 1995; Jacobs & Steffen, 2003; Jensen *et al.*, 2006; Slavin *et al.*, 1997).

Many of the bioactive compounds are localised in the bran and germ which together corresponds to approximately 20% of the total kernel weight (Jacobs & Steffen, 2003). Evidence of the health benefits is mainly derived from epidemiological studies which is showing the associations and not necessarily the causal link between whole grain intake and health (Potischman & Weed, 1999). The underlying mechanisms for the protective effects are to a large extent unknown and the results from epidemiological studies still remain to be confirmed by randomised clinical trials (RCTs) (Andersson *et al.*, 2007; Kelly *et al.*, 2007; Priebe *et al.*, 2008).

#### **1.1.1 Epidemiological evidence for health benefits of whole grain**

A number of epidemiological studies have been reported where whole grain intake has been related to a relatively consistent inverse risk of several chronic diseases such as cardiovascular diseases (including coronary heart disease (CHD), ischemic heart disease (IHD) and ischaemic stroke) and type 2 diabetes (Kelly *et al.*, 2007; Mellen *et al.*, 2006; Priebe *et al.*, 2008). A meta-analysis based on seven cohort studies showed a 21 % risk reduction for CHD when comparing 2.5 whole grain servings/d with 0.2 servings/d (OR: 0.79, [95%-CI 0.73-0.85]) (Mellen *et al.*, 2006). Many of the studies were conducted in the USA, where the whole grain intake is low (Cleveland *et al.*, 2000) and mainly consists of wheat (65-75%) (Slavin, 2005a), but some were conducted in the Nordic countries, where the whole grain intake is higher (Montonen *et al.*, 2003). However, the protective effect reported has been of the same magnitude, suggesting that whole grains of different species have similar effects and that even a small intake of whole grain is associated with the desirable protective effect (Jacobs & Steffen, 2003; Jensen *et al.*, 2004; Koh-Banerjee *et al.*, 2004). Whole grain intake and cereal fibre have been consistently linked to a decreased risk of developing type 2 diabetes, with 10 out of 11 prospective cohort studies showing a significantly reduced risk in a recent Cochrane review (Priebe *et al.*, 2008). High whole grain intake and cereal fibre intake were associated with a 27-30% and 28-37% decreased risk of developing diabetes type 2, respectively. Whole grain intake has also been associated with a significantly lower BMI in most observational studies (Harland & Garton, 2007), but it is not clear whether this effect is independent of cereal fibre and a healthy life-style (National Food Institute, 2008). Several studies in different cohorts have investigated the relationship between whole grain intake and different cancer diseases and the results have been pointed in different directions (Larsson *et al.*, 2005; Lucenteforte *et al.*, 2008; McCullough *et al.*, 2001; Schatzkin *et al.*, 2007).

### 1.1.2 Intervention studies

Only a limited number of intervention studies on whole-grain and disease have been published. Most of these studies have been short-term studies on risk markers or intermediate endpoints of CVD and diabetes. A Cochrane analysis of 10 small RCTs (most of them studying oats) showed a small but significant reduction in serum total cholesterol and LDL cholesterol ( $-0.20$  mmol/L [95% CI  $-0.31$ ;  $-0.10$ ] and  $-0.18$  mmol/L [95% CI  $-0.28$ ;  $-0.09$ ], respectively) and in risk factors for CVD and type 2 diabetes (Kelly *et al.*, 2007). Preliminary results from two large non-blinded RCTs conducted in the UK show no effect of whole grain intake on CVD risk markers. In the 'WholeHeart study', 300 non-whole grain consumers were assigned to either 16 weeks with 3 portions/d or 8 weeks with 3 portions/d + 8 weeks with 6 portions/d. The primary response variable was LDL cholesterol, where no effect was observed (Professor Chris Seal, pers. Comm. 2008). In another study, 221 subjects were randomly allocated to three groups, where they were advised to consume 3 portions/d of refined grain, whole grain wheat or whole grain wheat plus whole grain oats for 12 weeks. Preliminary results showed no effect on blood biomarkers, but a significant reduction in systolic and diastolic blood pressure in the two whole grain groups compared with the refined grain group (Dr Frank Thies, pers. Comm. 2008). No RCTs have been published where whole grain intake has been related to type 2 diabetes endpoints (Priebe *et al.*, 2008). However, small, short-term intervention studies have evaluated the effect of whole grain intake on insulin sensitivity, which is related to type 2 diabetes (Andersson *et al.*, 2007; Pereira *et al.*, 2002). The results have been conflicting, and there is a need for long-term RCTs, using suitable intermediate endpoints, to establish whether whole grain intake protects against type 2 diabetes (Priebe *et al.*, 2008). No long-term RCTs have been reported where the effect of whole grain products on body weight have been investigated (National Food Institute, 2008).

## 1.2 Whole grain intake estimation methods and their limitations

In epidemiological studies, there is a desire for cheap but accurate methods for estimation of habitual dietary intake during the etiologically relevant time period (usually a time period of years) (White *et al.*, 1998). The assessment is often carried out using traditional dietary assessment methods such as food frequency questionnaires (FFQs), diet histories, 24-h recalls, and sometimes weighed food records (Willett, 1998). Those methods are either prospective or retrospective. The prospective methods often involve checklists or repeated recordings on different days. The retrospective methods are usually based on one or repeated 24-h recalls, diet history reports or FFQs. Diet histories and FFQs are designed to provide data on average intake over longer time periods, by asking about the usual frequency of food consumption (Willett, 1998). Traditional dietary assessment methods generally suffer from different types of errors (due to memory, motivation, *etc.*), which may affect their ability to accurately capture intake (Fraser, 2003). Some of these errors can be accounted for by calibration against 'reference' methods such as repeated weighed food records (Willett & Lenart, 1998). However, the assumption underlying such comparisons, that the errors in the two methods are not correlated, have been questioned in recent years, and biomarkers may instead be useful as reference methods (Subar *et al.*, 2003).

Apart from the general problems associated with the above-mentioned dietary assessment methods there are a number of obstacles to accurate assessment of whole grain intake that may affect evaluation of the health effects. Many of the studies have estimated whole grain intake as number of servings and have used different definitions of whole grain to calculate the number of daily whole grain servings (Jansen *et al.*, 2004; Lang *et al.*, 2003). The use of different definitions gives a large quantitative variation in actual whole grain content in a serving, but also a qualitative difference in the composition of nutrients and bioactive compounds, which might have implications for evaluation of the role of whole grain in the aetiology of chronic disease, since different components have different levels of activity. Thane *et al.* (2007) reported a 18-27% difference in whole grain intake by using the FDA definition compared with the less strict definition by Jacobs *et al.* (1998)(Table 1). In addition no uniform definition of serving size has been used across studies (Lang & Jebb, 2003) and different cereals have been included in different definitions (Table 1).

Different definitions may potentially lead to misclassification, which is likely to attenuate the effect of whole grain disease associations (Koh-Banerjee *et al.*, 2004). To get a more precise estimate of whole grain intake, the g/d content has been calculated in several recent studies (Jensen *et al.*, 2004; Koh-Banerjee *et al.*, 2004; National Food Institute, 2008; Thane *et al.*, 2005; 2007). In two studies, a cut-off limit of  $\geq 10\%$  whole grain content of product fresh weight was still used (Thane *et al.*, 2005; 2007). For accurate determination of g/d intake of whole grain, databases reflecting products used in the studied population are needed in order to avoid biased estimates. Large variation in whole grain content in different products used to build a certain product category may obscure the precision in estimated whole grain intake (Fraser, 2003). This can to some degree be overcome by using 'open-end' questions where subjects can report the brand of product used (Jensen *et al.*, 2004).

Irrespective of whether whole grain intake is estimated as number of servings per day, calculated on products passing certain cut-off levels, or estimated as g/d based on a strict whole grain definition, measurement errors are likely, due to difficulties for consumers in distinguishing whole grain products among other products (Kantor *et al.*, 2001). In addition, dietary assessment methods are very often not designed to assess whole grain intake and usually contain only a limited number of questions which can be used to rank subjects according to their whole grain intake. As a consequence, misclassification is likely.

A biomarker of intake could thus be used to overcome some of the obstacles related to traditional dietary assessment methods, and hence replace estimates of intake derived from those, or be used for studying the measurement errors in the traditional dietary assessment methods, *i.e.* be used for validation purposes (Beaton *et al.*, 1997; Bingham, 2002). A few biomarkers of whole grain intake have been suggested and are discussed in the following sections.

## 1.3 Dietary biomarkers

### 1.3.1 Concepts and definitions

Biological markers or biomarkers have been defined in different ways (Figure 2). Biomarkers can provide a link between diet and health and have the potential to aid the understanding of this connection by reflecting the exposure and/or the disease or health effect (Branca *et al.*, 2001). However, a biomarker is not a diagnostic test, but rather an indication of an early change that is related to disease risk or health outcome (Grandjean, 1995).

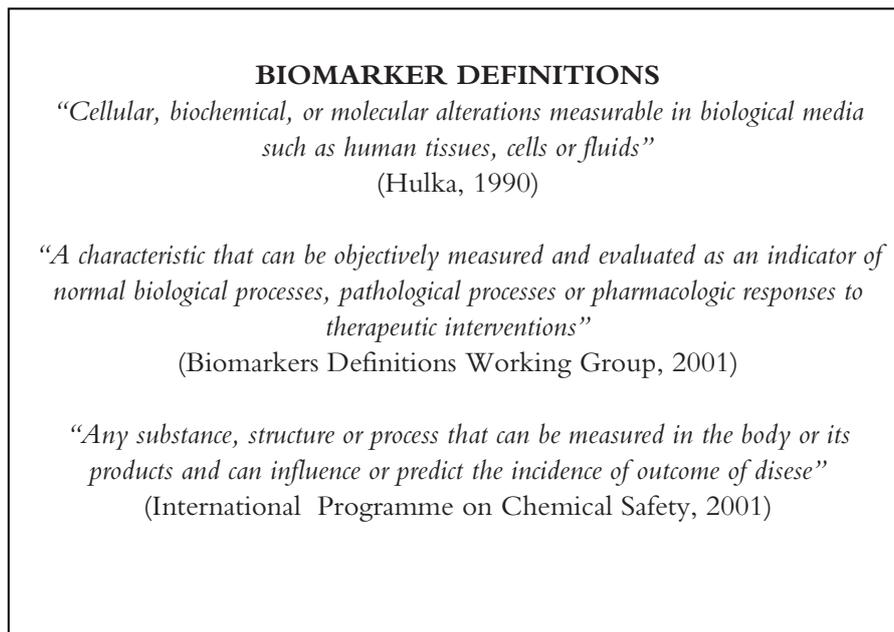


Figure 2. Selected definitions of biomarkers.

Biomarkers can be broadly classified into three different types, those of *exposure*, *effect* and *susceptibility* (Figure 3) (Branca *et al.*, 2001; Committee on Biological Markers of the National Research Council, 1987; Grandjean, 1995). In the discussion of biomarkers in the remainder of this thesis, the concept of biomarkers is restricted to dealing with those reflecting dietary exposure.

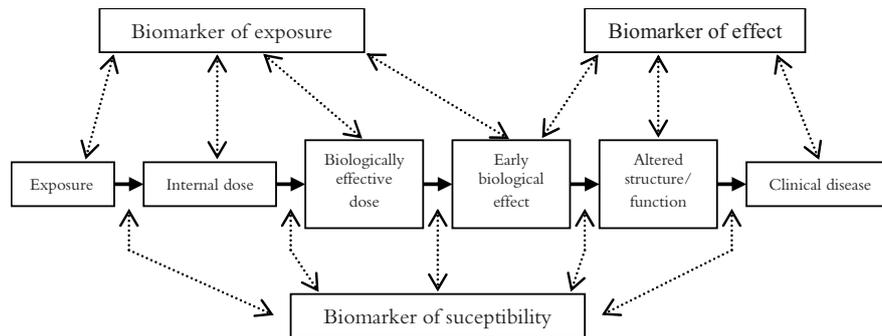


Figure 3. Biomarkers of exposure, effect and susceptibility and their relationships to events along the exposure-clinical disease axis. Adapted from Bearer (2001).

### Exposure

A biomarker of exposure may be an exogenous compound or a metabolite of such a compound that is related to exposure. Such a marker may be used to reflect dietary exposure and hence provide a link between dietary exposure and disease, especially in situations where traditional dietary assessment methods for different reasons provide poor intake estimates (Bingham, 2002; Hunter, 1998; Kaaks *et al.*, 1997; Verhagen *et al.*, 2004).

### Effect

A biomarker of effect may be an indicator of an endogenous component of the biological system, a measure of the functional capacity of the system, or an altered state of the system reflecting the disease or health endpoint (Branca *et al.*, 2001; Committee on Biological Markers of the National Research Council, 1987). In such a situation the biomarker could be a surrogate endpoint or outcome measure that is a laboratory substitute for a clinically meaningful result with a direct link to the causal pathway linking disease to outcome (Fox & Growdon, 2004).

### Susceptibility

A biomarker of susceptibility is an indicator of the susceptibility of a biological system to the challenge of exposure to a xenobiotic compound (Committee on Biological Markers of the National Research Council, 1987). Genetic alterations such as mutations, gene amplifications or recombination in genes directly or indirectly involved in the initiation or progression of disease can be such biomarkers (Branca *et al.*, 2001).

### 1.3.2 The use of dietary biomarkers

There are a great number of compounds that have been suggested and used as biomarkers of food and/or nutrient intake (some examples given in Tables 2 and 3). Biomarkers of dietary intake may serve different purposes in different types of studies. They may be used for validation of traditional dietary assessment instruments, as surrogate measures of intake or as being integrated measures of nutritional status for a nutrient (Potischman & Freudenheim, 2003). The requirements differ depending on the purpose. In intervention studies, biomarkers might be used as qualitative or quantitative measures of compliance. For example, fatty acid profiles in serum or adipose tissue can be used as indicators of short-term or long-term compliance to diets rich in certain fatty acids (Wolk *et al.*, 1998). The excretion of para-amino benzoic acid in 24-h urine collections can be used as a quantitative measure of the completeness of urine collection (Bingham & Cummings, 1986). In prospective cohort studies, biomarkers could be useful as independent measures of intakes in the study of association between diet and disease risk, or as independent tools to confirm such associations derived using traditional dietary instruments (Kaaks *et al.*, 1997). A biomarker might provide a more accurate estimate, or provide a more accurate ranking of intake than estimates derived from traditional dietary assessment methods, especially in situations where accurate food composition data are lacking (Kaaks *et al.*, 1997).

The *objectiveness* is a key feature of the biomarker, since it is independent of subjects' recognition capacity, memory and motivation (Marshall, 2003). This feature has made some biomarkers useful for validation of other dietary assessment methods (Bingham, 2003; Kaaks *et al.*, 1997; Kaaks, 1997; Livingstone & Black, 2003). This is because random errors in the biomarker (any variation that is uncorrelated to the individuals' 'true' habitual intake) can be assumed to be statistically independent of subjects' response in traditional dietary assessment (Kaaks *et al.*, 1997). Many biomarkers of food/nutrient exposure have not been thoroughly evaluated and are not fully accepted by the scientific community (Verhagen *et al.*, 2004).

A thorough understanding of the nature of the biomarker is necessary, and it will to a large extent determines its use (Marshall, 2003). As discussed in section 1.3.4, dietary biomarkers can be sorted into different classes depending on whether they provide an intake correlate or a direct quantitative measure of intake (Kaaks *et al.*, 2002; Tasevska *et al.*, 2005).

Table 2. *Examples of biomarkers of food intake*

Intake	Biomarker	Sample	Classification	Time of reflection	Evaluation	Reference
Dairy fat	Fatty acids (C15:0 and C17:0, CLA)	Serum, adipose tissue	Concentration	short- long term	Correlated to FFQ and food records	Baylin <i>et al.</i> (2002) Brevik <i>et al.</i> (2005a) Fremann <i>et al.</i> (2002) Wolk <i>et al.</i> (1998) Wolk <i>et al.</i> (2001)
Marine fat	Fatty acids (C20:5n-3 and C22:6n-3)	Serum phospholipids	Concentration	Short-medium term	Correlated to FFQ and food records	Hjartaker <i>et al.</i> (1997) Kobayashi <i>et al.</i> (2001) Marckmann <i>et al.</i> (1995)
Fruit and vegetables	Folate, $\alpha$ -carotene, $\beta$ -carotene, $\beta$ -cryptoxanthin, lutein, vitamin C	Plasma, serum, adipose tissue	Concentration	Short- long term	Correlated to FFQ and food records	Block <i>et al.</i> (2001) Brevik <i>et al.</i> (2005b) Campbell <i>et al.</i> (1994) Drewnowski <i>et al.</i> (1997) Jansen <i>et al.</i> (2004) Resnicow <i>et al.</i> (2000)
Allium vegetables	S-allyl-mercaptopuric acid (ALMA)	24-h urine	Concentration	Short-medium term	Controlled diet <sup>1</sup>	Van Kappel <i>et al.</i> (2001) Verhagen <i>et al.</i> (2001)

<sup>1</sup>Subjects were assessing their habitual diet by food records before the study. When the study started, experimental foods were given to subjects. The exact amount consumed was noted by weighing foods before and after administration.

Table 3. *Examples of biomarkers of nutrients and bioactive compounds*

Intake	Biomarker	Sample	Classification	Time of reflection	Evaluation	Reference
Protein	Nitrogen	24-h urine	Recovery	Short-term	Strictly controlled <sup>1</sup>	Bingham & Cummings (1985)
Potassium	Potassium	24-h urine, faeces <sup>3</sup>	Recovery	Short-term	Strictly controlled <sup>1</sup>	Schatzkin <i>et al.</i> (2003) Tasveska <i>et al.</i> (2006)
Sugar (fructose and sucrose)	Sucrose, fructose	24-h urine, spot urine	Predictive or concentration	Short-term	Controlled, FFQ	Bingham <i>et al.</i> (2007) Luceri <i>et al.</i> (1996)
Thiamin (B <sub>1</sub> )	Thiamin	24-h urine	Concentration	Short-term	Controlled <sup>1</sup>	Tasveska <i>et al.</i> (2005) Tasveska <i>et al.</i> (2008)
Carotenoids	Retinol, $\alpha$ -, $\beta$ -carotene, $\beta$ -cryptoxanthin, lutein, lycopene, zeaxanthin	Serum, adipose tissue	Concentration	Short-long term	FFQ and food records	El-Sohemy <i>et al.</i> (2002) Kardinaal <i>et al.</i> (1995) McNaughton <i>et al.</i> (2004)
Polyphenols <sup>2</sup>	ENL, END, daidzein, genistein, quercetin, hesperetin, kaempferol, catechin	24-h urine, spot urine, serum, plasma	Concentration	Short-medium term	Controlled, FFQ, 24-h recalls and food records	de Vries <i>et al.</i> (1998) Jaceldo-Siegl <i>et al.</i> (2008) Mennen <i>et al.</i> (2006) Noroozi <i>et al.</i> (2000) Ritchie <i>et al.</i> (2004) Verkasalo <i>et al.</i> (2001)
Selenium	Selenium	Toe-nails	Concentration	Long-term	FFQ, food collection	Longnecker <i>et al.</i> (1996)

<sup>1</sup>Subjects' reported habitual diet was given to subjects under controlled conditions. Exact amount consumed was noted by weighing foods before and after administration. <sup>2</sup>The group of polyphenols includes lignans, phenolic acids, flavanoids and stilbenes. <sup>3</sup>Faeces were collected in order to assess complete recovery.

### 1.3.3 Dietary biomarkers of whole grain intake

There is currently no generally accepted biomarker of whole grain intake. The mammalian lignan enterolactone (ENL) has been evaluated as one such biomarker (Stumpf, 2004). ENL and enterodiol (END) are mammalian lignans formed from plant lignans by the microflora in the large intestine (Adlercreutz, 2007; Setchell et al., 1980). Whole grain, especially from rye, is rich in precursors of ENL and the concentration of ENL in plasma, serum and urine increases with whole grain intake (Jacobs *et al.*, 2002; Juntunen *et al.*, 2000; Kilkkinen *et al.*, 2001). However, since there are several other sources of ENL precursors in the diet and since the formation of ENL is dependent on the gut microflora as well as other non-dietary determinants, ENL may rather reflect plant intake or a healthy life style in general (Johnsen *et al.*, 2004; Kilkkinen *et al.*, 2001; Linko-Parvinen *et al.*, 2007; Sonestedt *et al.*, 2007). At the international symposium “Whole Grain and Human Health” in Helsinki, Finland, in 2001, the group led by Professor Per Åman (Uppsala, Sweden) suggested that AR could be used as a biomarker of whole grain wheat and rye, due to their almost unique presence in the outer parts of these cereals (Ross *et al.*, 2001b). A method for the analysis of AR in plasma was developed and initial biomarker evaluation studies were conducted by a Finnish research group led by Professor Herman Adlercreutz (Linko-Parvinen, 2006; Linko-Parvinen *et al.*, 2007; Linko *et al.*, 2005; Linko & Adlercreutz, 2005; Linko *et al.*, 2002).

#### 1.3.4 Classification of dietary biomarkers

##### *Recovery biomarkers*

Recovery biomarkers quantitatively reflect the balance between intake and output (Bingham, 2002; Kaaks *et al.*, 1997; Livingstone & Black, 2003; Tasveska *et al.*, 2008). There are currently only a few biomarkers that can be allocated to this category. Examples of such biomarkers are 24-h urinary nitrogen excretion as a marker of protein intake (Bingham, 2003), doubly labelled water for energy intake (Livingstone & Black, 2003) and 24-h urinary potassium excretion for potassium intake (Tasevska *et al.*, 2006). Recovery biomarkers have a particular role for validation of other dietary methods, since they can be quantitatively measured and compared with traditional dietary assessment methods on the same scale (Kaaks *et al.*, 1997).

##### *Concentration biomarkers*

A concentration biomarker is a measure of a nutrient or a compound concentration at a given point in time, and concentration biomarkers lack the time dimension, *i.e.* they are measured without any time units (Kaaks *et al.*, 2002; Kaaks *et al.*, 1997). In contrast to recovery biomarkers, the quantitative relationship between a concentration and intake, could differ between and within individuals and populations depending on the presence and impact of determinants other than intake (Kaaks *et al.*, 2002; Kaaks *et al.*, 1997). Concentration biomarkers can be used as tools for ranking individuals according to dietary exposure rather than providing a reference method used to determine scaling factors for calibration of dietary questionnaires since a concentration biomarker can not be translated into an absolute estimate of intake (Kaaks *et al.*, 2002; 1997). However, recent research has indicated that they may also play a role in calibration studies (Fraser *et al.*, 2005; Fraser & Yan, 2007).

##### *Predictive biomarkers*

This category of biomarker was suggested by Tasveska *et al.* (2005). A predictive biomarker shows less absolute recovery than a recovery biomarker, but can be used for prediction of intake. The utility of this class of biomarkers for validation studies needs to be further investigated (Tasevska *et al.*, 2005; 2008). Sucrose and fructose in 24-h urine samples were suggested to belong to this group (Tasevska *et al.*, 2005).

### 1.3.5 Biomarker validity and reproducibility

The *validity* of a biomarker can be defined as ‘the lack of systematic measurement error when comparing the actual observation with a standard, that is a reference method which represents the truth’ (Vineis & Gallo, 2007). Kaaks *et al.* (1997) simply stated that the validity means that a ‘marker actually measures that it is intended to’ and also pointed out that most biomarkers cannot be translated into a measure of intake on an absolute scale, but only in most cases at the best provide a correlate to intake that can be used to roughly classify individuals according to high and low intakes. Maruvada & Srivastava (2004) defined the validity of an exposure biomarker as ‘the relationship between the biomarker and the actual exposure’. A biomarker might be valid at group level (providing a valid mean), but not necessarily at individual level (ranking individuals). This might be due to poor precision or different biases across subjects (Livingstone & Black, 2003).

The *reproducibility* of a biomarker describes the degree of correlation between samplings within the same individual on independent occasions (Kaaks *et al.*, 1997). Several authors use the terms *reliability* and reproducibility interchangeably (Al-Delaimy *et al.*, 2008; Sonestedt *et al.*, 2007; White, 1997; Zeleniuch-Jacquotte *et al.*, 1998), but Kaaks *et al.* suggested the term ‘reliable’ to describe a biomarker that is both valid and has a high reproducibility. A low reproducibility might to some extent be compensated for by analysing several samples from the same individual and calculating an average, or by pooling samples taken on different occasions. A low validity, on the other hand (when random errors between replicate measurements are highly correlated), cannot be compensated for by using several measures (Kaaks *et al.*, 1997). The reproducibility of a biomarker is largely determined by the stability of individuals’ intake of the food/nutrient of interest and the elimination half-life of the biomarker (Kaaks *et al.*, 1997). A short-half life might be compensated by a stable and continuous intake, as has been shown to be the case for vitamin C in plasma (Bingham *et al.*, 1995).

Reproducibility is commonly measured by the *intra-class correlation coefficient* (ICC), defined as:

$$\text{ICC} = \frac{\sigma_B^2}{\sigma_B^2 + \sigma_W^2},$$

where  $\sigma_B^2$  is the between-subject variation and  $\sigma_W^2$  the within-subject variation (Vineis, 1997). ICC values range between 0-1.0 and ICC is equal to 1.0 if there is exact agreement between repeated measures within the same individual ( $\sigma_W^2 = 0$ ). This is different to a Pearson or Spearman correlation coefficient, which takes the value 1.0 when one measure is perfectly linearly related to the others, not necessarily when these agree exactly (Vineis, 1997). The ICC compares the impact of the between-subject variability relative to the total variation and may therefore vary somewhat in different populations (Tworoger & Hankinson, 2006). A larger within-subject variation can be tolerated if the between-subject variation is also large (Vineis, 1997). The degree of reproducibility has implications for the power of a study and it should ideally be as high as possible ( $r \geq 0.6-0.7$ ) (Kaaks *et al.*, 1997; Marshall, 2003). Day-to day reproducibility is likely to be higher than more long-term measurements (Kaaks *et al.*, 1997; Marshall, 2003). Estimated reproducibility can be used to correct for measurement error caused by ‘time-to time’ variation when comparing a single biomarker measurement with intake assessed by methods reflecting long-term intake, using the formula:

$$r_{true} = r_{observed} \times \sqrt{1 + \frac{\lambda}{k}}$$

where  $r_{true}$  is the corrected correlation coefficient,  $r_{observed}$  is the observed correlation coefficient,  $\lambda$  is the within- to between-individual variance ratio for the dietary measurement, and  $k$  is the number of replicated measurements per individual. This correction, called de-attenuation, can also be applied to situations where a single biomarker measurement is related to disease risk (Rosner & Willett, 1988; Willett, 1998).

Validity and reproducibility are independent, meaning that a perfectly reproducible measurement might be consistently wrong, *i.e.* far from the ‘truth’ (low validity) (Kaaks *et al.*, 1997; Vineis & Gallo, 2007). In the converse situation, a biomarker might be unbiased, but measured with a low reproducibility, which will then give a broad and flat distribution around the true value (Vineis, 1997). A biomarker should ideally be both valid and reproducible, *i.e.* accurate, or what Kaaks *et al.* (1997) called *reliable*.

### 1.3.6 Desirable features of a dietary biomarker

The usefulness of a dietary biomarker for use in epidemiological and for intervention studies is dependent on a number of factors affecting its reproducibility and validity. These factors might differ slightly between different biomarkers and few biomarkers have been fully characterised. Despite this, the use of biomarkers in epidemiology should be encouraged, since it will assist in the elucidation of these aspects (Grandjean, 1995). Such general features are summarised below:

#### *Absorption*

The biomarker should ideally be absorbed to a consistent, preferably high, extent, with a low variation between and within individuals (Potischman, 2003). However, a number of factors can affect absorption and hence distort the relationship between intake and biological measures (Potischman, 2003). Examples of such factors are differences in transit time and gastric emptying, food matrix and whether the biomarker is delivered as a bolus or on several occasions (Rowland & Tozer, 1995).

#### *Specificity*

Ideally, the biomarker should be specific to what it is intended to reflect (Marshall, 2003). However, factors other than intake which affect the biomarker is need to be identified and controlled for statistically (Kilkinen *et al.*, 2001; Vineis, 1997). These factors could be endogenous or exogenous and a comprehensive understanding of the fate of the biomarker in the human body, may be of help in identifying these factors (Maruvada & Srivastava, 2004; van Kappel *et al.*, 2001). A biomarker might be sufficiently specific in one population but not in another (Peeters *et al.*, 2007).

#### *Sensitivity*

The biomarker concentration needs to be sensitive to intake, meaning that even a small change in intake should be possible to detect. The sensitivity is in turn dependent on a number of factors, such as bioavailability and homeostatic regulation mechanisms (Hunter, 1998; Marshall, 2003; Spencer *et al.*, 2008).

### *Temporal relationship with intake*

It is crucial to know the time-frame during which a biomarker reflects intake and to have an insight into its pharmacokinetics (Schulte & Talaska, 1995; Verhagen *et al.*, 2004). The elimination half-life needs to be known, since it will largely determine whether the biomarker is able to reflect daily, weekly, monthly or yearly intake (Marshall, 2003). A biomarker of exposure, which is related to disease endpoint, should ideally reflect the mean exposure over the etiologically relevant time period (White *et al.*, 1998). For chronic diseases such as cancer and CVD, this time period is long and thus biomarkers reflecting long-term exposure are highly warranted (Potischman, 2003). The degree of long-term reflection is not only dependent on the biomarker half-life but also on the regularity and stability of intake (Kaaks *et al.*, 1997; van Dam & Hu, 2008).

### *Stability*

In order to reflect intake of a certain food, the biomarker needs to be stable in the food and withstand processing/cooking in order to avoid confounding the food exposure estimate (Wild *et al.*, 2001). Biological specimens used for biomarker assessment need to be appropriately handled in order to avoid introducing errors inherent to the stability of the marker. Caution is needed regarding choice of anticoagulant, preservatives, freezing-thawing cycles and storage temperatures (Tworoger & Hankinson, 2006).

#### **1.3.7 Measurement error and its implications for biomarkers**

Measurement error is a measure of the deviation from trueness (Blanck *et al.*, 2003). For example, in the case of a concentration biomarker, the measurement error in the biomarker can be defined as the difference between an individual's measured biomarker concentration and the true biomarker concentration, where the true biomarker concentration is the theoretical concentration given without any laboratory or other sources of error (White, 1997). If the biomarker concentration fluctuates, the 'true' concentration is the mean over the time period assessed (White, 1997). All methods of measurement include an error and this measurement error can be divided into 1) random errors or 2) systematic errors (bias) (Beaton, 1994).

An error is considered random if it is without any predictable direction. The random error may be a function of noise in estimation of true intake, errors in the analysis of food composition, or true day-to day-variation in food intake (Tarasuk & Brooker, 1997). The random error affects the precision in

classifying subjects according to their habitual intake and a large random error, due to methodological or biological reasons, generally leads to attenuation bias, *i.e.* biases risk estimates toward null and lessens the likelihood of detecting a significant association between diet and disease (Tarasuk & Brooker, 1997; White *et al.*, 1998).

Biomarker measurement errors originate from a number of sources and can be divided into pre-analytical and analytical measurement errors (Blanck *et al.*, 2003; Tworoger & Hankinson, 2006; White, 1997). Biomarker measurement errors can be *differential* or *non-differential*, which severely affects the usefulness of the biomarker in different study designs. In a case-control study, for example, a differential error may arise if the biomarker concentration is affected by any process which is involved in the development of the disease and not only by exposure (White, 1997).

Differential measurement error can bias disease risk estimates in both directions (White, 1997), whereas non-differential measurement errors only cause attenuated risk estimates, since non-differential misclassification is a function of precision of the biomarker measurement and not the bias. The lack of precision will lead to flattened distributions which will cause more overlap and hence less distinction between the distributions (biomarker measurements of the cases and controls) (White, 1997).

Tools to estimate true dietary intake without any measurement error are limited. Hence, the ability to accurately estimate the measurement error in a calibration study and use it for correction of risk estimates is negatively affected (Thiebaut *et al.*, 2007). A biomarker has the potential to provide an unbiased estimate of actual intake and to be without errors that correlate to those of the self-reported assessment method (Prentice *et al.*, 2004). To better understand dietary measurement errors, new 'recovery biomarkers' and methods to combine 'concentration biomarkers' which do not fulfill the requirements for reference measurements but do correlate with intake with dietary assessment methods, are warranted (Prentice *et al.*, 2002; 2004). A good biomarker should preferably have a measurement error variance that is rather small (< 50%) relative to the actual consumption (Prentice *et al.*, 2004).

### 1.3.8 Biomarker validation steps

Both traditional dietary assessment instruments and biomarkers need to be validated (Andersen *et al.*, 2005; Slotnick & Nriagu, 2004). There are different strategies to evaluate a dietary biomarker, which may involve controlled animal experiments, geographical studies where intake and biomarker correlations are studied in observed populations, and controlled experiments which allow time integration of dose-response relationships (van't Veer *et al.*, 1993). When a biomarker has been independently validated and is known to reflect intake, the study design can be reversed, and dietary assessment methods can be validated against the biomarker (Hunter, 1998). If the biomarker is to be used for validation of the accuracy in other dietary assessment methods, studies under controlled conditions are needed to assess the predictability of the biomarker (Bingham, 2002). Only a few biomarkers of dietary intake have been evaluated in this way (Bingham, 2002).

Biomarker validation requires several steps (Figure 4), where sensitivity to intake, as well as determinants other than intake and temporal reflection of the biomarker need to be investigated (Hunter, 1998). The biomarker validation process is an iterative procedure resulting in a degree of validity, which may not be satisfactory until the biomarker is implemented (Slotnick & Nriagu, 2004).

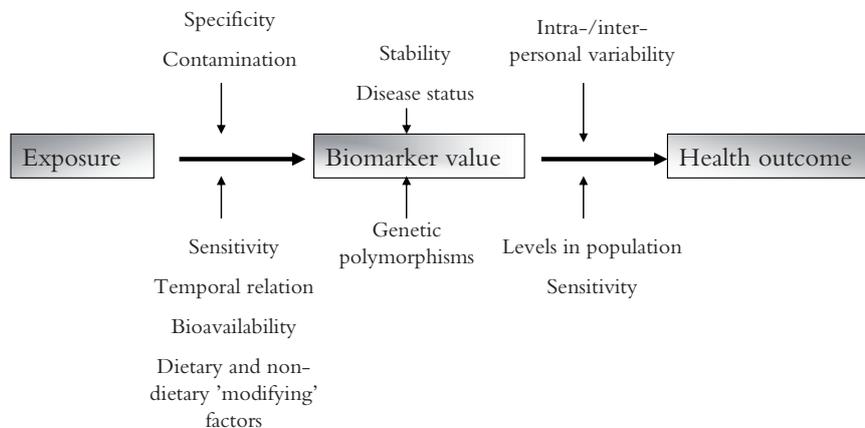


Figure 4. Different aspects affecting the exposure-biomarker-health outcome relationship and that need to be included in the biomarker validation process. Model modified from Slotnick & Nriagu (2004).

New biomarkers should be evaluated in small, well-defined, controlled studies before larger population studies are addressed, and a combination of evaluation approaches may be needed in order to validate the usefulness of dietary biomarkers (Maruvada & Srivastava, 2004).

The main aim in biomarker validation is to characterise biomarker variability and evaluate its ranking capacity (Kaaks et al., 1997; Vineis, 1997). The variability is due to biological differences (including within- and between-subject variation), measurement error (including laboratory errors), and random error (unexplained variance) forming the total variability within the group, which is the weighed sum of the different components (inversely correlated to the number of subjects, number of measurements per subject, the analytical replicate, respectively) (Vineis & Perera, 2007). In order to enable appropriate adjustment for variability, Vineis & Perera (2007) suggested that each variance component needs to be accurately estimated by:

1. Determining the variability in laboratory measurements used
2. Collecting information on sampling conditions that might affect the results
3. Taking repeated samples whenever possible to allow for determination of the short, medium and long-term variation in the biomarker within subjects
4. Collecting relevant subject information that might explain inter-subject variation

Evaluation of the ranking capacity of a biomarker as a measure of dietary intake has hitherto been conducted by simply correlating the biomarker with intake estimated by traditional dietary instruments. Validation derived from correlation to another dietary instrument is regarded as the 'upper-limit of the validity' since the biomarker is compared with an imperfect method and since some of the errors in the biomarker are correlated over time (Kaaks *et al.*, 2002). In a 'perfect' validation study, the biomarker would be compared with a method with no measurement errors, but such a method does not exist (White, 1997). Kaaks (1997) has recommended the method of triads for validation studies when data from a questionnaire, a biomarker and a 'reference method' (usually food records) are available. This method can be used to estimate the validity coefficient of each method, *i.e.* the correlation between observed intake (or a biomarker measurement correlated to intake) and the true intake. The assumption is that each method is linearly correlated to the same true intake and that the errors of the different methods are independent (Kaaks, 1997). Because the latter

assumption may not always hold and violation will lead to overestimation of validity coefficients, the estimated validity coefficients should be regarded as the 'upper limit' of the true validity coefficients.

### 1.3.9 Using a biomarker as a surrogate for exposure

Biomarkers can be used to measure exposure to certain foods or nutrients/non-nutrients in epidemiological studies either alone or as a complement to traditional dietary instruments. In the EPIC-Norfolk cohort, Vitamin C was used as a biomarker of fruits and vegetables and was inversely associated with IHD mortality (Bingham *et al.*, 2008). The estimated protective effect was about the same as observed when using 7- day food diaries, whereas an FFQ failed to demonstrate any association (Bingham *et al.*, 2008). Different phytoestrogens, mainly ENL, have been used as biomarkers of phytoestrogen intake and related to different cancer endpoints, since dietary assessment of phytoestrogen intake, especially lignans, has been hampered due to lack of database data and inconclusive results (Grace *et al.*, 2004; Ward *et al.*, 2008). In another EPIC study, plasma carotenoids, retinol and tocopherols were analysed as measures of exposure to these nutrients in relation to prostate cancer (Key *et al.*, 2007). Overall, none of the nutrients was significantly associated with a risk of prostate cancer, although the reliability of the biomarker over a period of 1-2 years as well as up to 14 years was demonstrated to be high ICC = 0.6-0.8 and 0.3-0.5, respectively (Comstock *et al.*, 2001; van Kappel *et al.*, 2001).

### 1.3.10 Using a biomarker for validation and calibration studies

#### *Validation studies*

Validation of a dietary instrument is the evaluation of whether the instrument measurement truly reflects what it is supposed to measure, and the validity is affected by the strength of the association between the measurement and 'true' intake and the specificity, *i.e.* that the measurement only reflects the variable of interest (Kaaks & Ferrari, 2006; Ocke & Kaaks, 1997). One way of validating dietary instruments is to use biomarkers, which may serve as reference for comparison of two (or more) other dietary methods (Bogers *et al.*, 2003; van't Veer *et al.*, 1993) in validation studies. The aim of a validation study is generally to estimate the validity coefficient, *i.e.* the correlation between questionnaire data and true habitual intake in subjects (Ocke & Kaaks, 1997). This is usually performed by comparing questionnaire measurements to multiple food records or 24-h recalls, corrected for attenuation caused by random errors in the reference methods

(Ocke & Kaaks, 1997). The correlation coefficient is then taken as the main criterion to evaluate whether questionnaire measures habitual diet with sufficient accuracy (Kaaks *et al.*, 1995; Walker & Blettner, 1985). The fundamental advantage of using a biomarker in validation studies is that the biomarker has errors that are very likely to be independent of those obtained by traditional dietary instruments (Willett & Lenart, 1998). Correlated errors between dietary assessment methods will lead to overestimation of the validity coefficients, whereas correlated errors within the dietary reference method will lead to underestimation (Ocke & Kaaks, 1997). Without additional information, it is impossible to predict which bias will predominate, under- or overestimation), and the validity determined is only 'relative' (Ocke & Kaaks, 1997).

#### *Calibration studies*

Calibration, refers to determination of the relationship between two measurement scales (Kaaks *et al.*, 2002). There is one scale for true dietary intake and another for measured dietary intake. Calibration in nutritional epidemiology means that values from one method are quantitatively related to values from a superior, standard method, *i.e.* calculating a calibration factor to be used to adjust relative risks derived from different questionnaires so that the estimated become unbiased (Kaaks *et al.*, 2002). In contrast to a validation study, where the true between-subject variation is to be estimated as well as the variation among subjects by comparing a questionnaire to replicated dietary records or dietary record and biomarker, a calibration study uses a single measure of a 'reference method' such as weighed food record, 24-h dietary recall or a biomarker (Willett & Lenart, 1998). The procedure is to regress the more accurate method (which at a group level represents an unbiased estimate of true intake) on the questionnaire data (Willett & Lenart, 1998). A calibration study enables 'rescaling' of results obtained across different studies and study populations where different questionnaires have been used, and hence enables comparisons. Biomarkers can be used in calibration studies, and slopes of applied regression lines between biomarker measurement and questionnaire at group level can be compared between different populations where the same questionnaire has been used. The design of such a study has to be determined based on the between- and within- subject variation in the biomarker, the costs of laboratory analysis and the precision of laboratory measurements (Willett & Lenart, 1998).

#### 1.4 Alkylresorcinols as biomarkers of whole grain wheat and rye intake

Alkylresorcinols (1,3-dihydroxy-alkylbenzene derivatives) are non-isoprenoid phenolic lipids present in several families of higher plants, algae, mosses, fungi and bacteria (Kozubek & Tyman, 1999). There is a large structural diversity, which is dependent on the source (Knödler *et al.*, 2008; Kozubek & Tyman, 1999; Ross *et al.*, 2004d).

In wheat, rye and barley grains, AR form one of the major groups of phenolic compounds (Ross *et al.*, 2004c). The most common AR compounds in cereal grains are the 5-n-alkyl-derivatives with odd alkyl chain-length commonly in the range of 17–25 carbon atoms, but different derivatives including 5-alkenyl-, 5-oxoalkyl-, and 5-hydroxyalkylresorcinols are also present to a minor extent (Figure 5). The highest proportion of unsaturated derivatives is found in rye, which contains about 20% of AR compounds other than 5-n-alkyl-derivatives (mainly 5-alkenylresorcinol) (Knödler *et al.*, 2008; Ross *et al.*, 2004c).

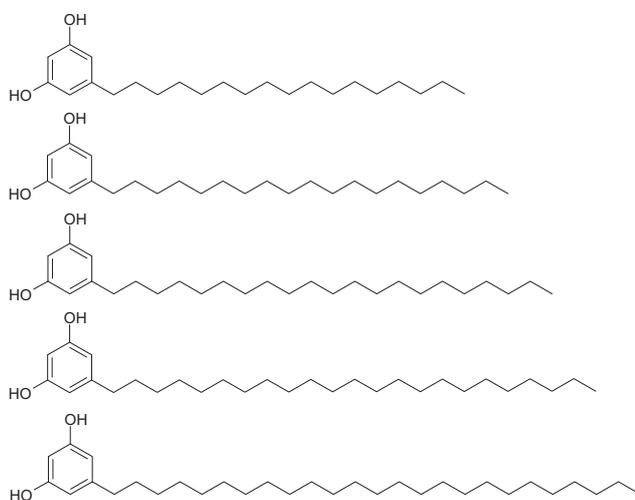


Figure 5. The major 5-n-alkylresorcinols found in cereal grains. The common homologues have odd alkyl chains in the range of C17:0–C25:0 (from top to the bottom).

### 1.4.1 Alkylresorcinol occurrence

#### *Alkylresorcinols in cereal grains*

Among plants commonly used for human consumption, AR are present in high amounts in rye (*Secale cereale*), common wheat (*Triticum aestivum*), einkorn wheat (*Triticum monococcum*), emmer wheat (*Triticum dicoccon*), spelt wheat (*Triticum spelta*) and durum wheat (*Triticum durum*) (Andersson *et al.*, 2008a; Chen *et al.*, 2004; Kulawinek *et al.*, 2008; Landberg *et al.*, 2005; Nyström *et al.*, 2008) and in lower amounts in barley (*Hordeum vulgare*) (Andersson *et al.*, 2008b; Zarnowski *et al.*, 2002) (Table 4). Small amounts (<5µg/g) have also been reported in maize (*Zea mays*) (Gembeh *et al.*, 2001) and in garden peas (*Pisum sativum*) (not a cereal grain) (Kozubek & Tyman, 1999). The content varies within and between species but the relative homologue distribution is rather constant within species (Table 4) (Chen *et al.*, 2004; Ross *et al.*, 2003b).

Table 4. Alkylresorcinol content range and C17:0/C21:0 ratio reported in cereal grains commonly used for human consumption

Cereal	Range (µg/g DM)	AR C17:0/C21:0 ratio	References
Rye ( <i>Secale cereale</i> )	568-1231	0.80-1.30	(Chen <i>et al.</i> , 2004; Kulawinek <i>et al.</i> , 2008; Mattila <i>et al.</i> , 2005; Nyström <i>et al.</i> , 2008; Ross <i>et al.</i> , 2001a)
Common wheat ( <i>Triticum aestivum</i> )	300-943	0.09-0.24	(Andersson <i>et al.</i> , 2008a; Chen <i>et al.</i> , 2004; Hengtrakul <i>et al.</i> , 1990; Mattila <i>et al.</i> , 2005)
Durum wheat ( <i>Triticum durum</i> )	194-687	0.01-0.02	(Andersson <i>et al.</i> , 2008a; Ross <i>et al.</i> , 2003b)
Einkorn wheat ( <i>Triticum monococcum</i> )	545-654	0.04	(Andersson <i>et al.</i> , 2008a; Ross <i>et al.</i> , 2003b)
Emmer wheat ( <i>Triticum dicoccon</i> )	531-784	0.05	(Andersson <i>et al.</i> , 2008a; Ross <i>et al.</i> , 2003b)
Spelt wheat ( <i>Triticum spelta</i> )	490-741	0.09-0.14	(Andersson <i>et al.</i> , 2008a; Ross <i>et al.</i> , 2003b)
Barley ( <i>Hordeum vulgare</i> )	8-210	0.05-0.46	(Andersson <i>et al.</i> , 2008b; Chen <i>et al.</i> , 2004; Zarnowski <i>et al.</i> , 2002)

The AR C17:0/C21:0 ratio has been suggested as a tool to distinguish between different cereals (Chen *et al.*, 2004) since the ratio is  $\approx 1.0$  for rye,  $\approx 0.1$  for common wheat and  $\approx 0.01$  for durum wheat (Andersson *et al.*, 2008a; Chen *et al.*, 2004; Landberg *et al.*, 2005) (Table 4).

In the cereal kernel, AR are located in the outer parts of the kernel (Ross *et al.*, 2001a; Ross *et al.*, 2004c; Tluscik, 1978). It was recently shown that  $> 99\%$  of the AR content is present in the outer cuticle of testa/inner cuticle of pericarp in hand-dissected grain samples (Figure 6) (Landberg *et al.*, 2008b).

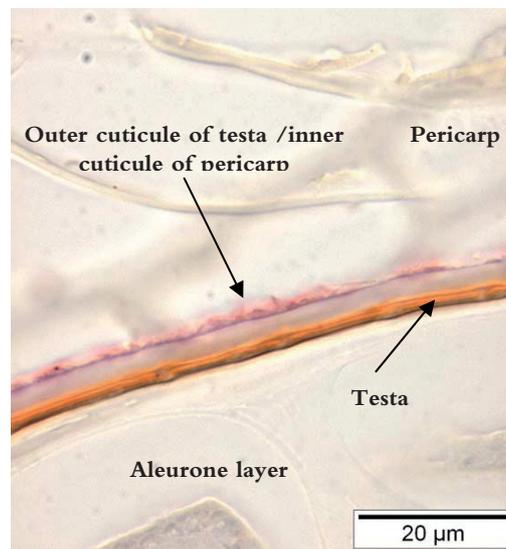


Figure 6. Light microscopy of a transversal cryo-section of a rye grain kernel stained by Fast Blue B. Alkylresorcinols appear violet.

#### *Alkylresorcinols in cereal food products*

After milling, the bran fraction contains higher amounts of AR, than whole meal while no or very small amounts of AR can be detected in germ and refined flour (Table 5) (Kulawinek *et al.*, 2008; Landberg *et al.*, 2008b; Mattila *et al.*, 2005; Ross *et al.*, 2003b).

Table 5. Alkylresorcinol content and AR C17:0/C21:0 ratio determined by different analytical methods in different technological cereal fractions and food samples

Product	Total AR content <sup>1</sup> (µg/g DM)	AR C17:0/C21:0 <sup>2</sup>	Samples (n)	Analytical method	Reference
Rye bran	2466-4100	0.9	8	Colorimetry, GC, HPLC	(Chen <i>et al.</i> , 2004; Kulawinek <i>et al.</i> , 2008; Mattila <i>et al.</i> , 2005; Ross <i>et al.</i> , 2003b)
WG rye (meal)	888-927	0.9	5	HPLC, GC	(Chen <i>et al.</i> , 2004; Kulawinek <i>et al.</i> , 2008; Mattila <i>et al.</i> , 2005; Ross <i>et al.</i> , 2003b)
Refined rye	44-286	0.9	6	HPLC, GC	(Chen <i>et al.</i> , 2004; Kulawinek <i>et al.</i> , 2008)
WG rye bread	197-686	0.9-1.1	11	Colorimetry, HPLC, GC	(Chen <i>et al.</i> , 2004; Kulawinek <i>et al.</i> , 2008; Mattila <i>et al.</i> , 2005)
Crisp bread (rye)	490-1007	0.9-1.1	16	GC	(Chen <i>et al.</i> , 2004; Ross <i>et al.</i> , 2003b)
Breakfast cereals (rye)	721	-	1	HPLC	(Kulawinek <i>et al.</i> , 2008)
Wheat bran	2670-3225	0.1-0.3	3	GC, HPLC	(Mattila <i>et al.</i> , 2005; Ross <i>et al.</i> , 2003b)
WG wheat	269-339	0.08-0.1	3	Colorimetry, GC	(Chen <i>et al.</i> , 2004; Kulawinek <i>et al.</i> , 2008)
WG wheat bread	140-497	0.1-0.2	11	Colorimetry, GC	(Chen <i>et al.</i> , 2004; Kulawinek <i>et al.</i> , 2008; Ross <i>et al.</i> , 2003b)
Wheat crisp bread	58	-	4	GC	(Chen <i>et al.</i> , 2004; Ross <i>et al.</i> , 2003b)
Breakfast cereals (wheat)	131-1784	0.08-0.27	5	HPLC	(Chen <i>et al.</i> , 2004; Kulawinek <i>et al.</i> , 2008)
WG pasta	215-270	0.01-0.03	5	GC	(Landberg <i>et al.</i> , 2005)
Wheat flour (refined)	ND-44	-	3	GC, HPLC	(Mattila <i>et al.</i> , 2005; Ross <i>et al.</i> , 2003b)
Refined wheat bread	ND-23	-	8	Colorimetry, GC, HPLC	(Chen <i>et al.</i> , 2004; Kulawinek <i>et al.</i> , 2008; Mattila <i>et al.</i> , 2005)
Oat bran	ND	-	-	HPLC, GC	(Mattila <i>et al.</i> , 2005; Ross <i>et al.</i> , 2003b)

<sup>1</sup>Total AR content is the sum of C17:0-C25:0 (as determined by GC), the sum of alkyl- and alkenyl-derivatives of C17:0-C25:0 (as determined by HPLC) and the sum of all 1,3-dihydroxy alkylbenzene derivatives present (as determined by colorimetry). <sup>2</sup>Determined by GC.

There is a large variation in AR content among different food products, determined by the milling extraction rate and the natural variation in content in wheat and rye (Table 5). Earlier methods investigating AR content in cereal food products suggested that AR were partly degraded during processing due to the lower contents detected in foods compared with raw ingredients (Al-Ruqaie & Lorenz, 1992; Verdeal & Lorenz, 1976; Winata & Lorenz, 1997). However, it was later demonstrated that this is due to entrapment of AR in the sample matrix during hydrothermal processing, probably as starch-lipid complexes, rather than degradation (Ross *et al.*, 2003b). Chen *et al.* (2004) showed that AR homologues were extracted with quantitative yield by using hot-propanol:water (3:1, v/v) for the analysis of foods and the corresponding ingredients. Studies where solvent extraction at room temperature has been used are likely to underestimate AR content.

#### 1.4.2 Analysis of alkylresorcinols in cereals and foods

A number of different analytical techniques for qualitative and quantitative analysis of AR in cereal grains and products have been presented over the years, as reviewed by Kozubek & Tyman (1999) and Ross *et al.* (2004d). Some commonly used quantitative techniques are presented in Table 6. It is important to bear in mind that results from different analytical methods may differ, and results obtained from older methods in particular need to be evaluated with some caution (Ross *et al.*, 2004d). HPLC methods may overestimate AR C17:0/C21:0 ratio, since alkylresorcinol homologues may co-elute with the following alkenylresorcinol homologue, *e.g.* C17:0 co-elutes with C19:1 (Heiniö *et al.*, 2007; Mattila *et al.*, 2005; Seitz, 1992).

Table 6. Quantitative methods used for the analysis of alkylresorcinols in cereal grains and food products.

Assay	AR quantified	Extraction	Detection	Evaluated <sup>1</sup>	Reference
Fluorometry	Total AR	Acetone	Fluorescence ( $\lambda = 350, 470$ )	No	(Evans <i>et al.</i> , 1973; Verdeal & Lorenz, 1976; Wieringa, 1967)
Colorimetry <sup>2</sup>	Total AR	Acetone, ethanol, methanol	Visible absorption ( $\lambda = 520$ nm) after reaction with Fast Blue B	Yes	(Andersson <i>et al.</i> , 2008a; Gadjia <i>et al.</i> , 2008; Landberg <i>et al.</i> , 2008a; Tluscik <i>et al.</i> , 1981)
HPLC	Total AR, individual AR homologues <sup>3</sup>	Methanol, ethanol, acetone	UV/diode array-detection ( $\lambda = 520$ nm)	No	(Francisco <i>et al.</i> , 2005; Mattila <i>et al.</i> , 2005; Mullin <i>et al.</i> , 1992)
GC	Total AR, individual AR homologues <sup>3</sup>	Ethyl acetate, acetone	Flame ionisation detector (FID)	Yes	(Gohil <i>et al.</i> , 1988; Landberg <i>et al.</i> , 2008a; Mullin <i>et al.</i> , 1992; Ross <i>et al.</i> , 2001a)

<sup>1</sup>Evaluation of method including at least the following steps: determination of limit of detection/quantification (LOD/LOQ), determination of within- and between day variability (precision of the method), testing applicability > 10 different samples. <sup>2</sup> AR reacting with azo-compound to form an azo-complex which can be detected spectrophotometrically. <sup>3</sup>HPLC methods published have not shown complete separation between alkyl- and alkenylresorcinols.

### 1.4.3 Alkylresorcinols absorption, distribution and elimination

#### *Absorption*

Absorption has been defined as the process by which unchanged drug or xenobiotic/bioactive compounds proceeds from the site of administration to the site of measurement within the body (Rowland & Tozer, 1995). The site of measurement in humans is often the blood. Like compounds with similar lipophilicity, AR are absorbed in the upper GI-tract and incorporated into chylomicrons, which are distributed to the circulation via the lymphatic system (Linko-Parvinen *et al.*, 2007; Ross *et al.*, 2003c). Linko-Parvinen *et al.* (2007) showed that 70-80% of the total AR content in plasma occurred in total lipoprotein fractions and that no AR were found in the plasma water fraction, suggesting that AR, like tocopherols, are incorporated into chylomicrons and transported to the liver and thereafter incorporated into VLDL or HDL. The highest concentration of AR among the isolated lipoproteins was found in HDL, which was suggested to be due to direct transfer of AR from chylomicrons and VLDL to HDL during lipolysis (Linko-Parvinen *et al.*, 2007). AR, may be partly secreted directly from epithelial cells into portal venous circulation by HDL efflux, which has been demonstrated for tocopherols (Rigotti, 2007).

Alkylresorcinol ileal digestibility has been estimated as a crude measurement of absorption in one study on pigs and one study on humans (Ross *et al.*, 2003a; Ross *et al.*, 2003c). In the pig study, 20 pigs were fed four different diets; whole grain rye, fractions of pericarp-testa, enriched aleurone and starchy endosperm (Ross *et al.*, 2003c). The daily AR intake was different for the different diets and ileal recovery varied according to the diet (which also represented different daily AR intake levels) and was on average  $37\pm 7\%$ ,  $21\pm 7\%$ ,  $40\pm 5\%$  for whole grain rye, pericarp-testa and enriched aleurone respectively. No AR were detected in pigs fed the starchy endosperm. It was suggested that 60-79% of the ingested AR dose was absorbed and that the amount absorbed was dependent on the matrix (or the dose). No clear difference in absorption for different AR homologues was observed (Ross *et al.*, 2003c). In the human study, 10 ileostomy operated subjects were given a high fibre rye diet rich in AR (147 mg/d) and a low fibre wheat-based diet (no AR) in two meal frequencies (nibbling and ordinary). All subjects had both diets and both frequencies (cross-over design). The ileal recovery was about  $60\pm 7\%$ , regardless of meal frequency, with no obvious difference between different homologues (Ross *et al.*, 2003c).

### *Distribution*

Distribution has been referred to as reversible transfer to and from the site of measurement (usually the blood) (Rowland & Tozer, 1995). Due to the amphiphilic nature of the AR molecules they are easily incorporated into biological membranes (Kozubek, 1995; Kozubek & Demel, 1980; Kozubek & Demel, 1981; Ross *et al.*, 2004b). Studies on rats and humans have shown that AR are distributed into erythrocyte membranes and into the adipose tissue of rats (Linko & Adlercreutz, 2005; Ross *et al.*, 2004b). The levels in erythrocyte membranes are correlated to plasma concentrations in fasting blood samples and follow changes in intake in the same way as plasma concentration. Longer AR homologues are more readily incorporated into the membranes than shorter homologues (Linko & Adlercreutz, 2005). The kinetics and the nature of the transfer processes are still unknown.

### *Metabolism*

Metabolism can be defined as the conversion of one chemical species to another (Rowland & Tozer, 1995). Metabolism is one way of facilitating the elimination of a xenobiotic compound, often by making it more polar in order to enable urinary excretion. Ross *et al.* (2003c) fed a single dose of [4-<sup>14</sup>C]-labelled AR C21:0 to rats and found most of the radioactivity in faeces (61%) and in urine (31%). The radioactivity detected in urine was not due to free or conjugated AR, whereas 90% of the radioactivity in faeces was from the intact C21:0 (Ross *et al.*, 2003c). The same research group later identified two compounds in urine (3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-propanoic acid (DHPPA)) as main AR metabolites (Ross *et al.*, 2004c; 2004d; 2004e) and suggested a similar route of AR elimination as for tocopherols (Birringer *et al.*, 2001). In this route, AR undergo cytochrome P450-dependent  $\omega$ -oxidation, followed by  $\beta$ -oxidation of the alkyl chain forming the two endproducts, DHBA and DHPPA (Figure 7). A certain proportion of the metabolites are found as glucuronide and sulphate conjugates in urine (Koskela *et al.*, 2007). Later studies have confirmed that the two main metabolites are derived from AR and a quantitative analytical method for their determination in urine and plasma has been developed (Koskela *et al.*, 2007).

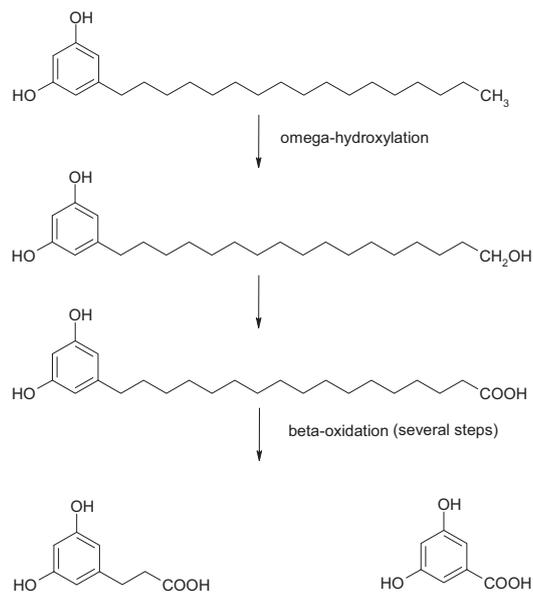


Figure 7. Pathway of alkylresorcinol metabolism suggested by Ross *et al.* (2004c) exemplified by AR C17:0 degraded to 3-(3,5-dihydroxyphenyl)-propanoic acid (DHPPA) (lower left) and 3,5-dihydroxybenzoic acid (DHBA) (lower right).

#### Excretion

Excretion can be defined as the irreversible loss of an unchanged compound or its metabolite (Rowland & Tozer, 1995). The two major AR metabolites DHBA and DHPPA are transported via the blood to the kidney, where they are excreted in urine in free and conjugated forms (Koskela *et al.*, 2007; 2008; Ross *et al.*, 2004e). In addition a small fraction of intact AR (predominantly C17:0) has been detected in urine after deconjugation (Ross *et al.*, 2004e). In plasma, 10–30% of DHBA and 60–90% of DHPPA were found to be conjugated in a study on 64 Finnish women (Koskela *et al.*, 2008), whereas almost all AR metabolites were found unconjugated in urine when analysing the same sample with and without a deconjugation step (Koskela *et al.*, 2007). The discrepancy might be due to a rather high variation between subjects and to the fact that samples from only 15 subjects were analysed in the study on urine. The amount of AR recovered as urinary metabolites after a single dose of AR in rats was about 33% after collection for 140 h (Ross *et al.*, 2003c). However, no studies on recovery of AR metabolites in human urine collections have been reported, nor any study on the pharmacokinetics of AR metabolites.

## 2 Aims of the thesis

The overall objective of this thesis was to evaluate the features and applicability of AR as specific biomarkers of whole grain wheat and rye intake. This was achieved by the following specific aims:

- 1) To develop a sensitive and repeatable GC-MS method for quantification of AR in small volume plasma samples. **(I)**
- 2) To investigate the pharmacokinetics of AR in humans after a single dose. **(II)**
- 3) To assess dose-response of plasma AR concentration and AR metabolite excretion in urine under controlled conditions. **(III)**
- 4) To compare the estimated AR intake, derived from weighed food records, with plasma AR concentration in subjects participating in a whole grain intervention study. **(IV)**
- 5) To determine the reproducibility of plasma AR concentration in subjects under controlled, constant intake conditions with low and high AR intake. **(V)**
- 6) To investigate the variation in plasma AR concentration in samples from a free-living population and to find determinants of plasma AR concentration. **(VI)**



## 3 Materials and methods

### 3.1 Reference compounds

Alkylresorcinols, AR metabolites and internal standards used in the different studies are listed in Table 7.

Table 7. Reference compounds used in the different studies<sup>1</sup>.

Trivial name	Supplier	Purity	Study
<i>Alkylresorcinols</i>			
C17:0	Reseachem Lifescience Burgdorf, Switzerland	>95% by NMR and LC-MS	<b>I,III-VI</b>
C19:0			<b>I,III-VI</b>
C21:0			<b>I,III-VI</b>
C23:0			<b>I,III-VI</b>
C25:0			<b>I,III-VI</b>
<i>Metabolites</i>			
DHBA <sup>2</sup>	Sigma-Aldrich St. Louis, USA	>98 % by unknown method	<b>III</b>
DHPPA <sup>3</sup>	Isosep, Tullinge, Sweden	>95% by NMR	<b>III</b>
<i>Internal standards</i>			
C20:0	Reseachem Lifescience Burgdorf, Switzerland	>95% by NMR and LC-MS	<b>II-VI</b>
Methyl behenate (C22:0 fatty acid methyl ester)	Larodan Fine Chemicals Malmö, Sweden	>99% by unknown method	<b>I-V</b>

<sup>1</sup> Alkylresorcinol standards used in **II** were provided by Professor Kristiina Wähälä, University of Helsinki (>95 % purity, determined by unknown method). <sup>2</sup> 3,5-dihydroxybenzoic acid.

<sup>3</sup> 3-(3,5-dihydroxyphenyl)-1-propanoic acid

### 3.2 Instrumentation

Instruments used for determination of AR in foods and plasma and AR metabolites in urine are summarised in Table 8. Details of temperature and solvent gradients *etc.* can be found in **I-VI**.

Table 8. *Instruments and columns used for determination of alkylresorcinols (AR) and their metabolites in the different studies.*

Instrument	Analyte	Matrix	Column	Study
Agilent 6890 GC <sup>1</sup> (Agilent Technologies, USA)	AR	Foods	HP-5 30m×0.25µm×0.25mm	<b>I-V</b>
GC 8000 coupled to a MD 1000 quadrupole mass spectrometer (Fisons Instruments, UK)	AR	Plasma	BP-1 12m×0.25µm×0.22mm	<b>II</b>
Trace Ultra GC coupled to a Trace DSQ mass-spectrometer (Thermo Fischer Scientific, USA)	AR	Plasma	HP-5 30×0.25µm×0.25mm	<b>III, IV</b>
Trace Ultra GC coupled to a Trace DSQ II mass-spectrometer (Thermo Fischer Scientific, USA)	AR	Plasma	TR-5 15×0.25µm×0.25mm	<b>V, VI</b>
HPLC-CEAD <sup>2</sup> (ESA Biosciences, USA)	AR metabolites	Urine	Intersil ODS-3 3×150mm	<b>III</b>

<sup>1</sup>GC, gas-chromatograph. <sup>2</sup>HPLC-CEAD, high performance liquid chromatography-coulometric electrode array detector.

### 3.3 Quantitative analysis of alkylresorcinols and their metabolites

#### *Foods*

The analysis of AR in foods (**II-III, and V**) was performed after slight modification of the method described by Ross *et al* (2003b). In brief, 0.5 milled sample mixed with internal standard (methyl behenate) was extracted by a mixture of hot 1-propanol:water (3:1, v/v) in a boiling water bath. Extracts were combined and an aliquot taken, which was dried and dissolved in ethyl acetate and injected into the GC without derivatisation. AR homologues were quantified using a relative response factor of 0.9 for all AR homologues compared with the internal standard.

#### *Plasma*

Two different analytical methods were used for the determination of AR in plasma (Table 9). Samples in **II-III** were analysed according to a method described by Linko *et al.* (2002) and samples in **V-VI** were analysed according to the method described in **I**, referred to as the method developed by Landberg *et al.* (**I**).

#### *Urine*

The two AR metabolites DHBA and DHPPA were analysed according to protocol A in the method described by Koskela *et al.* (2007) (**III**). In brief, urine was mixed with syringic acid (internal standard) and deconjugated overnight with  $\beta$ -glucuronidase and sulphatase enzymes. An aliquot was mixed with methanol and HPLC mobile phase before injection into the HPLC-CEAD system.

Table 9. The two different methods used for analysis of AR plasma concentration (**I-VI**)

Step	Linko <i>et al.</i> (2002)	Landberg <i>et al.</i> ( <b>I</b> )
Preparation	500 µL plasma + IS <sup>1</sup> (C20:0, 1.8µg/mL, 20µL) + 500 µL de-ionised water	50-200 µL plasma + IS (C20:0, 1.0 µg/mL, 15 µL)
Incubation	Overnight at 37°C	-
Extraction	Sample is vortexed (2 min) with 3mL diethyl ether. The aqueous phase is frozen in a dry-ice-ethanol bath. Organic phase is poured off. Procedure is repeated 3 times and extracts are combined	Sample is vortexed (2 min) with 4mL diethyl ether. The aqueous phase is frozen in a dry-ice-ethanol bath. Organic phase is poured off. Procedure is repeated 3 times and extracts are combined
Preparation for purification	Sample is evaporated to dryness under a nitrogen stream and dissolved in 300µL MeOH	Sample is evaporated to dryness under a nitrogen stream and dissolved in 1 mL MeOH
Sample Purification	Sample is purified on DEAE-Sephadex A-25 gel in free base form according to Fotsis & Adlercreutz (1987)	Sample is purified on a 3 cc/60 mg Oasis® MAX SPE cartridges
Gel preparation	See note 2	-
Conditioning	-	1 mL 0.1 M NaOH in 70% MeOH
Sample application	Sample (in 300 µL MeOH) is loaded and tube rinsed with 300 µL MeOH	1 mL sample (1.0 mL/min)
Washing	Neutral lipids are eluted by 6 mL MeOH	Neutral lipids are eluted by 2 mL MeOH
AR elution	AR are eluted with 6 mL 0.1 M acetic acid in MeOH	AR are eluted with 3 mL 2% acetic acid in MeOH
Derivatisation	Evaporation to dryness under a nitrogen-stream. Silylation by 100 µL QSM <sup>3</sup> for 30 min at room temperature	Evaporation to dryness under a nitrogen-stream. Silylation by 150 µL QSM for 30 min at room temperature
GC-MS analysis	1 µL sample is injected into a GC-MS. AR homologues are quantified on their molecular ions or the base ion. Multipoint standard curves (n=6-8) are prepared for each homologue and for each batch	1.5 µL sample is injected into a GC-MS. AR homologues are quantified on their molecular ions or the base ion. Multipoint standard curves (n=6-8) are prepared for each homologues and for each batch

<sup>1</sup>IS, internal standard (C20:0). <sup>2</sup>50 g DEAE-Cl<sup>-</sup> is subsequently washed with 500 mL of 20%, 50% and 100% EtOH. Ethanol is decanted and gel is resuspended in MeOH. Gel can be stored for 1 year at 8 °C. Before use, DEAE-Cl<sup>-</sup> is converted to DEAE-OH<sup>-</sup> by washing sequentially with 10 bed volumes of 0.1 NaOH in 70% MeOH, 70% MeOH, 100% MeOH. Columns are manually prepared by adding gel into Pasteur pipettes to a final height of 1.5 cm. Sample needs to be applied immediately. <sup>3</sup> QSM, quick silylation mixture (pyridine:HMDS:TMCS, 9:3:1, v/v/v)

### 3.4 Quality assurance and method comparison

#### *Foods*

A milled whole grain rye sample was included in every batch (n=2-3) throughout all studies. A batch was re-analyzed if the within-batch variation estimated as the coefficient of variation (CV) for total AR content exceeded 10%, or if the mean for the control samples in the batch analysed exceeded  $\pm 2$  SD of the mean determined for >10 batches. The within-day CV was usually <5% and between-day CV <10% for all individual AR homologues and for their sum.

#### *Plasma*

A control sample was included in every batch (n=3-5) along with a blank (water or silylation reagent). If the blank contained AR, the entire batch was re-analysed. In **II**, pooled plasma from the Finnish Red Cross study was used as the control sample (Linko *et al.*, 2002), while in **I**, **III-VI** a fasting plasma sample from a person with high AR intake was used. The batch was re-analysed if the CV for total AR content in the control sample exceeded 15% or if the mean of the control sample within a batch exceeded  $\pm 2$  SD of the mean of >8 determinations. The within-batch CV was <10% and the between-batch CV <11% for total AR determined when evaluating five replicate samples analysed in 20 batches (**V** and **VI**).

The two methods for determination of AR in plasma used in the studies were compared by analysing 10 selected samples from **II** and **III** to represent a wide sample concentration range (50-500 nmol/L). Samples were analysed in duplicates according to the procedures described in Table 9, and mean values were used for investigation of method agreement.

In addition, a small method comparison study was conducted, since samples in **II** were analysed at the Finnish laboratory and the rest at the laboratory in Uppsala. Plasma samples from **II-IV** were pooled in order to obtain three samples containing low, medium and high AR concentrations. Samples were blindly analysed in triplicate on one day (high and low concentration) and on three different days (medium concentration). The laboratory in Finland was unable to quantify the sample with low AR concentration at the time of the study, and hence this sample was omitted from the comparison.

### 3.5 Samples and study design

Samples used for method development in **I** were taken from samples reported in **III** and pooled in order to obtain three levels corresponding to a low, medium and high AR concentration. Samples from **VI** were used to compare base and molecular ion quantification. Studies reported in **II-V** were controlled intervention studies, whereas samples in **VI** were taken from a case-cohort study. A summary of study designs and subject characteristics is presented in Table 10.

Table 10. Design of human studies (II, III, IV, V and VI)

Study	Subjects (n) <sup>1</sup>	Type of study	Study design	Age (years)	BMI (kg/m <sup>2</sup> )	Diet	AR intake	Wash-out period
II	6 (SWE)	Intervention (PK)	Single dose	26±1.1	-	120 g rye bran flakes	190 mg (495 µmol)	1 wk whole grain/bran free diet
III	17 (SWE)	Intervention (dose-response)	3×1 wk cross-over	30±10	23.0±3.3	22.5 g/d or 45 g/d or 90 g/d of rye bran flakes (same as in II). The daily intake was divided between three occasions per day, and time of intake recorded	33mg (85 µmol) or 66 mg/d (170 µmol/d) or 131 mg/d (342 µmol/d)	1 wk of whole grain/bran free diet before each dose.
IV	30 (SWE)	Intervention (mixed WG)	2×6 wk cross over	59±5	28.3±2.0	112 g/d whole grain or refined grain, given as bread, crisp bread, muesli, pasta and rice	65±12 mg (WG) 6.8±1.9 mg (RF)	6-8 wk of habitual diets before each treatment
V	17 (SWE)	Intervention (Rye WG/bran)	2×6 wk cross-over	74±5	27.5±5	485 g/d of intervention diet including bread, crisp bread, muesli and porridge	620 mg/d (RD) 8.1 mg/d (RFWD)	2 wk of habitual diets before each treatment
VI	362 (DAN)	Free-living	Case-cohort	57±4	26±5	Habitual diet	Not estimated	Not applicable

<sup>1</sup>Number of subjects recruited, SWE, Swedish; DAN, Danish. <sup>2</sup>M, men; W, women; PK, pharmacokinetic study; WG, whole grain; RF, refined; RD, rye whole grain/bran diet; RFWD, refined wheat diet. <sup>3</sup>AR, intakes reported to correspond to the administered dose (I and III), estimated from 3-day weighed food records (II and IV) and advised intake (IV)

### 3.6 Assessment of pharmacokinetics

#### *Study II*

The absorption phase in six volunteers after a single dose of 190 mg AR was evaluated visually and by the method of residuals to determine whether absorption followed first-order kinetics (Rowland & Tozer, 1995). A one-compartment model was used, since visual inspection of log-transformed plasma concentration curves showed no clear distribution phase. All pharmacokinetic parameters were calculated manually from plasma concentration data.

#### *Study III*

A population pharmacokinetic model was built by using non-linear mixed-effect modelling on data from **I** (Ette & Williams, 2004a; 2004b). One- and two-compartment models were evaluated (**II**). In the final model, one central compartment was used to describe the distribution and absorption was assumed to occur from two different compartment, with different lag-times, and the relative bioavailability from these two compartments was estimated. A baseline concentration was included in the model (since low AR concentration was detected despite no whole grain intake). The model developed was tested by comparing model-predicted plasma AR concentrations with those measured at recorded time points in **III**. The agreement between observed and predicted concentrations was investigated with a Bland-Altman plot (Bland & Altman, 1999). NONMEM VI with the first-order conditional estimation method was used for model building and simulation (Ette & Williams, 2004a; 2004b). Xpose 4 (<http://xpose.sourceforge.net>) was used for model diagnostics.

### 3.7 Statistical analysis

#### *Comparison between laboratories and methods for AR analysis*

Differences between the two laboratories were tested using ANOVA with laboratory and day as fixed factors and sample as a random factor. Differences were considered significant at  $P < 0.05$ . Minitab version 14.0 (Minitab Inc, State College, PA, USA) was used for the statistical analysis. A Bland-Altman plot was used to compare agreement between the methods developed by Linko et al. (2002) and Landberg *et al.* (I) (Bland & Altman, 1986; Bland & Altman, 1999).

#### *Study I-VI*

Statistical methods and approaches used in **I-VI** are summarized in Table 11. In all studies, normality was checked by the Shapiro-Wilk test and variables which departed from normality ( $P < 0.05$ ) were  $\log_e$ -transformed before statistical analysis. All correlation coefficients are Spearman's rank correlation coefficients unless otherwise stated. P-values  $< 0.05$  were regarded as statistically significant, except for evaluation of potential carry-over effects (**IV-V**) where  $P < 0.10$  was used as the limit. In **III** and **IV**, P-values were Bonferroni corrected, in order to adjust for type 1 error. In **I-II** Minitab 14.0 (Minitab Inc, State College, USA) was used for all statistical analysis, whereas mainly SAS version 9.1 (SAS Institute Inc., Cary, N.C, USA) was used in **study III-VI**.

Table 11. *Statistical methods/procedures and models used in the different studies.*

Method/ procedure	Response variable	Model <sup>1</sup>	Study
Factorial design	Total AR concentration	Concentration level <sup>2</sup> , water incubation, sample volume	<b>I</b>
ANOVA	Total AR conc.	Deproteination	
	AR homologue recovery (%)	Homologue, conc. level, homologue×conc. level	
ANOVA <sup>3</sup>	PK-parameters <sup>4</sup>	AR homologue	<b>II</b>
Linear regression	Relative homologue composition	Time	
	Relative bioavailability	AR homologue	
Mixed linear model (Proc mixed) <sup>5</sup>	AR conc. C17:0/C21:0 ratio	Period, dose, <i>subject</i> , [AR concentration before treatment] <sup>1</sup>	<b>III</b>
	Metabolite excretion	Period, dose, <i>subject</i>	
	Metabolite recovery	AR intake, <i>subject</i>	
ANOVA <sup>6</sup> (Proc GLM)	Plasma AR, C17:0/C21:0 ratio	Sequence, <i>subject</i> within-sequence, period, treatment	<b>IV</b>
ANCOVA (Proc GLM)	Plasma AR	AR intake, <i>subject</i>	<b>IV</b>
ANOVA <sup>6</sup> (Proc GLM)	Plasma AR, C17:0/C21:0 ratio	Sequence, <i>subject</i> within-sequence, period, treatment	<b>V</b>
Mixed linear model (Proc Mixed)	Plasma AR (ICC)	<i>Subject</i>	<b>V</b>
ANCOVA (Proc GLM)	Plasma AR	Dietary <sup>7</sup> , and non dietary <sup>8</sup> factors	<b>VI</b>

<sup>1</sup>Random factors in italics and covariates are within brackets. <sup>2</sup>Sample concentration levels tested were low, medium and high. <sup>3</sup>Tukey's pair-wise comparison was used as *post hoc* test.

<sup>4</sup>PK-parameters tested were  $t_{1/2}$  and  $t_{max}$ . <sup>5</sup>Bonferroni corrected t-tests were used as *post hoc* tests.

<sup>6</sup>Carry-over effect was evaluated at the  $P < 0.10$ -level, by comparing treatment sequence, with subject nested within-sequence as the error term. <sup>7</sup>Dietary fibre, rye bread, white bread, crisp bread, other bread, beer, carcass animal fat, total energy intake.

<sup>8</sup>Cholesterol (non-fasting), BMI, and time-since last meal

## 4 Results and discussion

### 4.1 Analysis of AR in plasma (I)

The original method for analysis of AR in plasma developed by Linko *et al.* (2002) was used in **II-IV**. In this method, a rather large sample volume (0.5 mL) was used and samples were incubated over night with de-ionised water in order to release AR from proteins (Linko *et al.*, 2002). Sample purification was performed using ion-exchange chromatography on columns manually prepared in the laboratory. For analysis of samples from epidemiological studies, a large number of samples usually need to be analysed and only small sample volumes are typically available. Therefore, a new, faster method that required less sample volume was developed (**I**). A small method comparison study was undertaken to ensure comparability between the method of Linko *et al.* (2002) and that of Landberg *et al.* (**I**).

Diethyl ether was the most suitable solvent for AR extraction, giving the highest recoveries and least co-extracted ballast substances when compared with pure chloroform and mixtures of chloroform and methanol (**I**). Linko *et al.* (2002) came to the same conclusion when comparing diethyl ether with petroleum ether, ethyl acetate, n-hexane and mixtures of n-hexane:ethyl acetate (1:3 and 4:1 (v:v)), n-hexane:diethyl ether (1:1 and 1:3 (v:v)) and diethyl ether:ethyl acetate (1:1 and 4:1 (v:v)) (Linko-Parvinen, 2006). In contrast to what was found by Linko *et al.* (2002), water incubation did not affect the AR yield ( $P=0.72$ ) or precision and was therefore omitted. Altering protein conformation by changing the pH did not affect AR yield or variation, nor did ethanol precipitation ( $P=0.10$ ).

A significant interaction ( $P=0.04$ ) was observed between sample volume and AR concentration, with somewhat higher concentrations when using 200  $\mu\text{L}$  plasma compared with using 500  $\mu\text{L}$  was observed. However, the effect was small and probably not of practical importance. For population- based non-fasting samples, a sample volume of 200  $\mu\text{L}$  was found to be suitable. However, sample volume down to 50  $\mu\text{L}$  can be used when the sample concentration is  $>80$  nmol/L.

The major development in the method was the use of a SPE column for sample clean-up. A mixed mode reversed phase, strong ion exchange sorbent was selected (Oasis® MAX) and different conditioning, sample application, washing and elution solvents and volumes were tested. AR homologue recoveries, estimated from spiking experiments using the procedure developed (Table 8) were 84-92% for the different homologues and  $88\pm 7$  % for total AR ( $n=6$ ) (**I**). No discrimination caused by the method (extraction and sample purification) of any AR homologue relative to C20:0 (internal standard) was found when comparing slopes of standard curves prepared with and without sample matrix ( $P=0.09$ ). This shows that analyte losses during sample extraction and purification are equal for different AR homologues and for the internal standard.

The limit of detection (LOD) was in the range of 0.08-0.36 and the limit of quantification (LOQ) in the range 0.33-1.20 pg/injection for the different AR homologues. The lowest LOD and LOQ were found for C19:0 and C21:0. Overall, LOD and LOQ were up to ten times lower with the newly developed method (**I**) compared with the values found with the Linko *et al.* (2002) method. This might be due to less background noise obtained by the purification method used in the present method and/or to a higher sensitivity of the instrument used. Instrument sensitivity is also greatly affected by the number of samples analysed before cleaning the ion source.

AR were quantified by GC-MS using the respective molecular ions  $m/z$  492 (C17:0), 520 (C19:0), 534 (C20:0), 547 (C23:0), 576 (C23:0) and 604 (C25:0), with lower LOD and LOQ compared with when the base ion  $m/z$  268 was used. However, in some samples interference with unknown co-eluting peaks might occur, and hence the base ion could be used for quantification as well. Both methods of quantifying AR showed satisfactory agreement for total AR concentration (**I**).

### Method comparison studies

The results by the Swedish and Finnish laboratories were comparable (Figure 8) and no statistical difference attributed to laboratory ( $P=0.16$ ) was observed suggesting that results obtained in **II-IV** are analytically comparable.

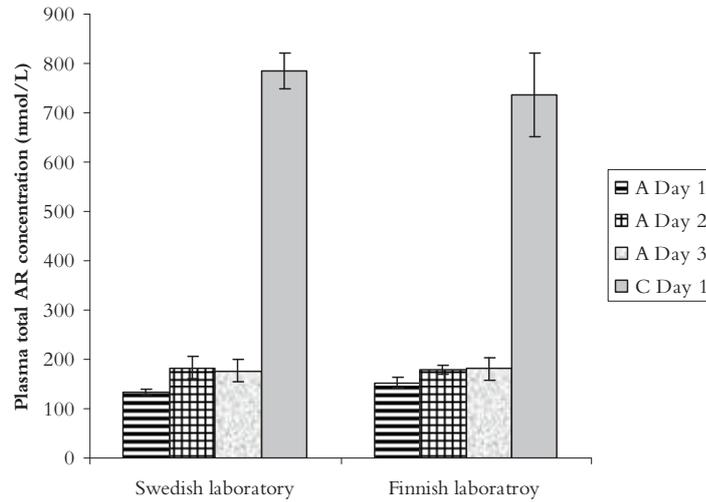


Figure 8. Mean plasma total alkylresorcinol (AR) concentration ( $n=3$ ) analysed on three different days at two different laboratories. Error bars represent standard deviation. Sample A= medium concentration, C = high concentration (analysed on day 1)

There was also a satisfactory agreement between the method by Linko *et al.* (2002) and the method developed here (**I**) when the results of 10 samples analysed by both methods on the same occasion in the laboratory in Uppsala were compared (Figure 9). Method difference was independent of sample concentration ( $P=0.312$ ). The difference between the methods was on average 10 nmol/L (on average higher values for the method by Linko *et al.* (2002)).

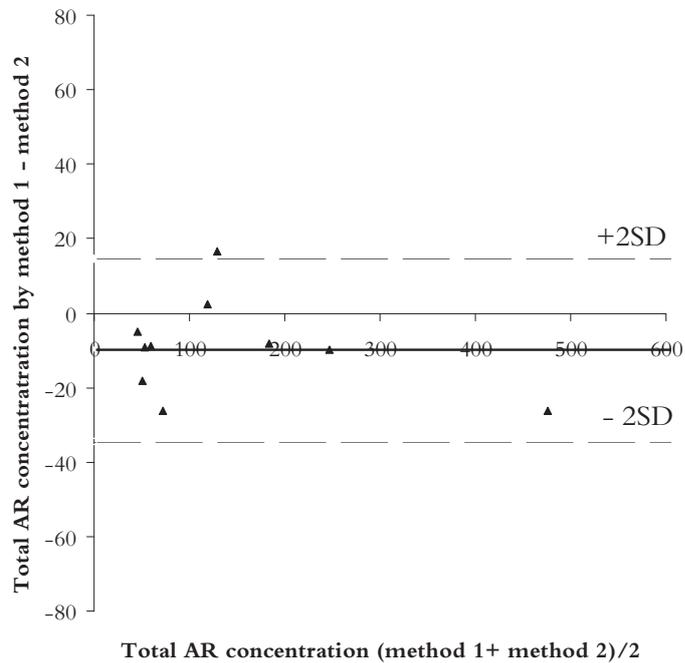


Figure 9. Bland-Altman plot showing the agreement between the methods by Landberg *et al.* (I) (method 1) and by Linko *et al.* (2002) (method 2). The bold line shows the average difference between the two methods and the dotted lines show the limits of agreement ( $-10 \pm 25$  nmol/L) within which 95% of the observations are found

## 4.2 AR pharmacokinetics (II, III)

Pharmacokinetics involve studies on the time-course of a drug/xenobiotic or a bioactive compound in the body, which is dependent on kinetic processes of absorption, distribution, metabolism and excretion (Rowland & Tozer, 1995). Primary parameters related to these processes determine the bioavailability and elimination half-life. No universally accepted term describing the corresponding events of nutrients and xenobiotics is available and terms such as ‘biokinetics’ or ‘kinetics’ have been used (Erlund, 2002). The term ‘biomarker kinetics’ has been suggested to be an analogous term to pharmacokinetics as regard biomarkers (Verhagen *et al.*, 2004). In this thesis, the term pharmacokinetics is used to describe dynamic processes related to the absorption, distribution, metabolism and elimination (ADME) of AR.

One study on the pharmacokinetics of AR has been published previously, using plasma samples from pigs after a single dose and after a habitual diet rich in AR (Linko *et al.*, 2006). A number of studies in both humans and animals have been conducted where absorption, distribution and elimination of AR have been studied without evaluating the pharmacokinetics (Linko-Parvinen *et al.*, 2007; Linko & Adlercreutz, 2005; Ross *et al.*, 2003a; Ross *et al.*, 2003c).

Plasma AR concentration time profiles showed two absorption maxima in human subjects ( $t_{\max 1}$ =2.4-3.4 h and  $t_{\max 2}$ =6.4-5.5 h, for the different homologues) (Figure 10). This is in contrast to what was found for pigs, which showed only one absorption maximum after a single dose at  $t_{\max}$  = 3-4 h (Linko *et al.*, 2006). The two absorption peaks in humans may be due to different physical absorption sites, effects of gastric emptying, second meal-effects on digestibility, different pools or due to different absorption mechanisms (Charman *et al.*, 1993; Deming & Erdman, 1999; Zhou, 2003). Enterohepatic circulation is unlikely to explain the two peaks, since the second peak is larger than the first. AR elimination half-life ( $t_{1/2}$ ) was estimated at 4.4-5.5 h for the different homologues with no statistically significant difference attributed to homologue, and was somewhat longer than that for pigs (Linko *et al.*, 2006). The longer elimination half-life observed for humans compared with pigs may be due to differences in body composition, hepatic blood flow, enzymes, *etc.* between pigs and humans (Lin, 1998).

A pharmacokinetic model was fitted to the single dose-data (**III**) (Figures 10 and 11). The best fitted model to explain the biphasic nature of the plasma concentration time profile had two absorption compartments with different bioavailability, lag-times and absorption rate constants (Figure 11).

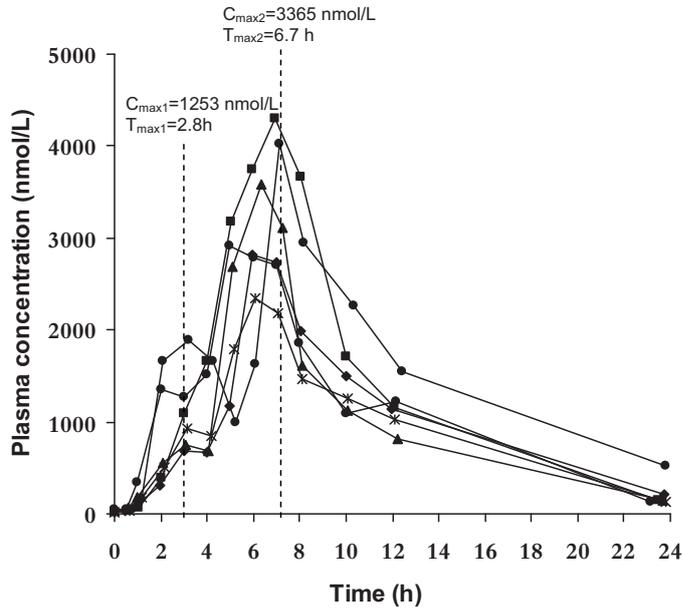


Figure 10. Total AR concentration in plasma in six subjects after a single intake of rye bran flakes with the total AR content of 190 mg. The two  $C_{max}$  and  $t_{max}$  indicated are mean values of all subjects

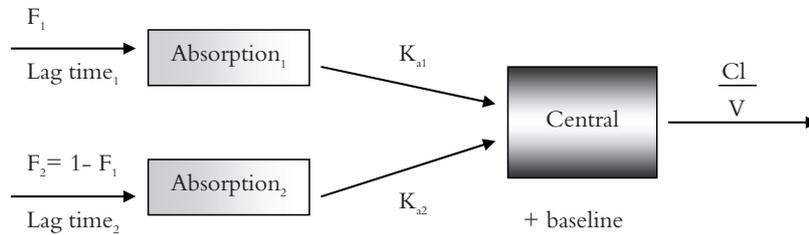


Figure 11. Pharmacokinetic model of alkylresorcinols.  $F_1$  = relative proportion of the dose absorbed from the first absorption compartment;  $F_2$  = relative proportion of the dose absorbed from the second compartment; lag-time<sub>1</sub> = lag-time for start of absorption from first absorption compartment; lag-time<sub>2</sub> = lag time for start of absorption from the second absorption-compartment;  $K_{a1}$  = absorption rate constant from the first absorption compartment;  $K_{a2}$  = absorption rate constant from second absorption compartment; Central = central compartment; baseline = baseline AR concentration (nmol/L);  $CL/V$  = clearance/volume of distribution

The model developed was used to predict plasma AR concentrations in single samples taken at the end of intervention periods of **III**. The prediction error of the model (difference between simulated and observed value) was rather small in the light of the large variation between subjects that was observed in **III** and since single dose data from only six individuals were used to build the model (Figure 12).

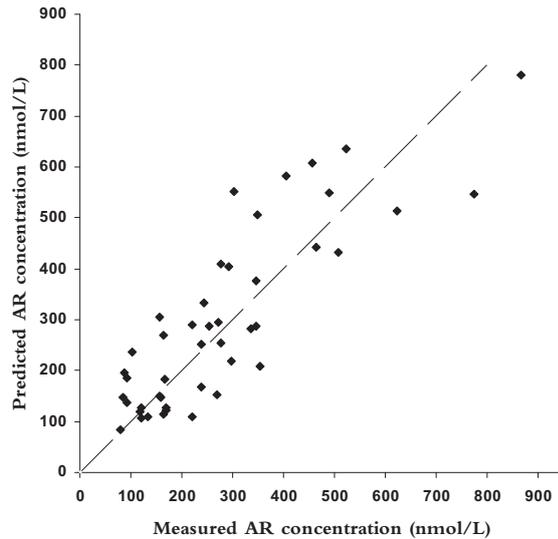


Figure 12. Comparison between measured and predicted plasma total AR concentration (nmol/L) (**III**). The dotted line is the unity line. Model was built on data from **II**

Even after a week-long wash-out period, which theoretically should be enough to clear AR from the systematic circulation, AR were still detectable at low levels (25- 68 nmol/L) (**II-V**). This has also been found in earlier studies on humans and pigs as well (Linko-Parvinen *et al.*, 2007; Linko *et al.*, 2006; Linko *et al.*, 2005; Linko & Adlercreutz, 2005; Linko *et al.*, 2002). In the PK-model, this AR concentration is referred to as 'base-line'. It has been shown that AR are incorporated into erythrocyte membranes and into adipose tissue of rats (Linko & Adlercreutz, 2005; Ross *et al.*, 2004b) and the 'base-line' level probably reflects AR liberated mainly from the slow equilibrating adipose tissue pool. In addition, a low AR intake can be expected when consuming refined grain products, since some of those products have been shown to contain some AR (**IV, VI**). The presence of AR in refined products is likely to be due to a high extraction rate in the milling process, since >99 % of AR are located in the bran fraction and no

AR have been found in the starchy endosperm (Landberg *et al.*, 2008b; Tluscik, 1978).

#### *Elimination*

The major fraction of the bioavailable AR is eliminated through hepatic metabolism and subsequent urinary excretion of free and conjugated DHBA and DHPPA, as suggested by Ross *et al.* (2004e). In the present study, the recovery of AR metabolites DHBA and DHPPA from ingested AR doses decreased with dose from  $\approx 90\%$  to about 45% (**III**). The reason for the difference in recovery is not clear, since results from different studies contradict each other. It may be due to a shift in AR elimination route at high doses or to 24-h being too short a time-period to completely recover high AR doses. A shift in elimination might be a consequence of biliary excretion of intact AR and/or metabolites at higher doses, a route which is known for  $\alpha$ -, $\gamma$ -, and  $\delta$ -tocopherol and their carboxy-methyl-hydroxychroman metabolites ( $\alpha$ -, $\gamma$ -, and  $\delta$ -CEHC) (Kiyose *et al.*, 2001; Swanson *et al.*, 1999; Traber & Kayden, 1989). The lower recovery with increased dose might also be due to the presence of hitherto unidentified AR metabolites, most likely longer metabolites derived by fewer  $\beta$ -oxidation cycles. Such tocopherol metabolites have been found to be excreted *in vivo* (Birringer *et al.*, 2001). Another explanation could be that AR absorption is dose-dependent and decreases with increased dose. Such an effect was observed in a pig study where Ross *et al.* (2003c) fed four different fractions of rye (whole grain, pericarp+testa, aleurone or starchy endosperm) containing different amounts of AR. However, such an explanation is counteracted by the rather linear increase (see Figure 15 below) in plasma AR concentration observed across doses (**III**), which suggests that absorption is independent of dose.

#### *Bioavailability*

The relative bioavailability differed between AR homologues and increased with increased alkyl side-chain length (Figure 13). Homologue C25:0 was about five times as bioavailable as C17:0 and the relative homologue composition in plasma changed during the first 8 h after intake, with a significant decrease in C17:0 and a corresponding significant increase in homologues C23:0 and C25:0 ( $P < 0.05$ ) (Figure 14). Homologues C19:0 and C21:0 did not change significantly during the corresponding time period (**II**).

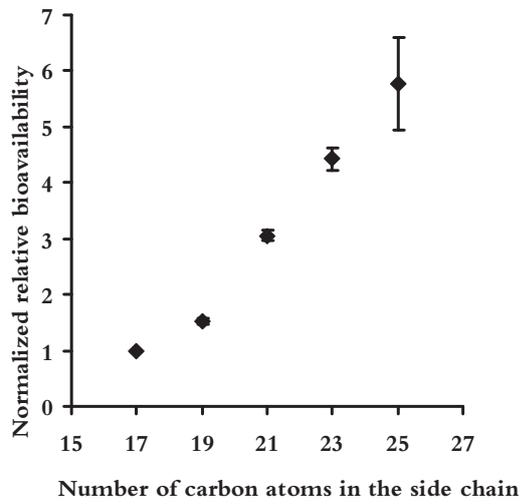


Figure 13. Dependence of the number of odd carbon atoms in the AR side chain on normalized relative bioavailability (defined as  $AUC_{AR}/dose_{AR} \times dose_{C17:0}/AUC_{C17:0}$ )

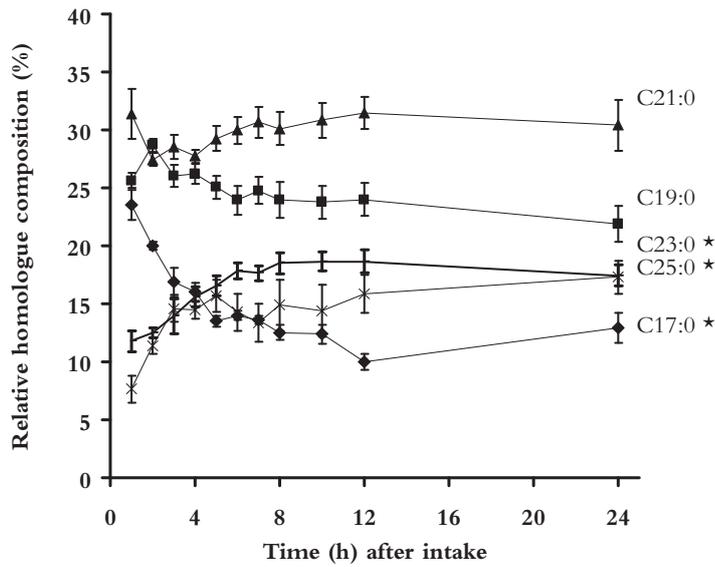


Figure 14. Relative plasma AR homologue composition after a single dose of rye bran flakes (corresponding to 190 mg total AR). Values are mean $\pm$ SEM. Asterisks indicate a statistically significant trend ( $P<0.05$ ) during 1-8 h

Theoretically, the higher bioavailability of longer AR homologues as well as the relative increase in C23:0 and C25:0 and subsequent decrease in C17:0 over time (Figure 14) may be due to higher absorption of longer AR homologues. However, in a human ileostomy study, the tendency was the opposite, with small but significantly lower absorption for C23:0 and C25:0 compared with the shorter homologues (Ross *et al.*, 2001b). Another explanation for the difference in bioavailability might be a higher affinity of shorter AR homologues to the  $\omega$ -hydroxylase (enzymes involved in AR metabolism) in combination with a more extensive distribution of longer AR homologues into other compartments from where they are liberated back to plasma. Linko & Adlercreutz (2005) showed that longer AR homologues were more readily incorporated into erythrocyte membranes and we have found in an *in vitro* system that AR in RBC is transferred into plasma and equilibrium is reached within 3 days (unpublished data).

### 4.3 Effect of intervention on AR plasma concentration (III-VI)

#### 4.3.1 Plasma AR dose-response under controlled conditions

Plasma AR concentration showed a rapid response to changes in AR intake in all studies, which can be expected due to the short half-life of 4-5 h and it increased significantly with increased intake in all studies (II-IV). The increase appeared to be linear with increased daily AR intake when given in the range 33-131 mg/d for all AR homologues (Figure 15, Table 12). This range well covers the consumption range found in the Nordic countries (Ross *et al.*, 2004a).

In two out of three intervention studies, AR were present at low concentrations ( $\approx$ 25 nmol/L) after periods when no foods known to contain them were allowed (during wash-out and run-in period) (Table 13). In addition, plasma from subjects with coeliac disease has been shown to contain no, or very low, AR concentrations (<7 nmol/L) (Linko-Parvinen, 2006) and (I). The high AR concentration observed at wash-out in III can not easily be explained, but may be due to several reasons, including liberation of AR after a high habitual AR intake, samples taken early in the morning (short fasting period), and/or unintentional intake of products containing AR. Most subjects included in III were young, highly educated and with a healthy lifestyle, factors which are associated with high habitual whole grain intake (Harland & Garton, 2007).

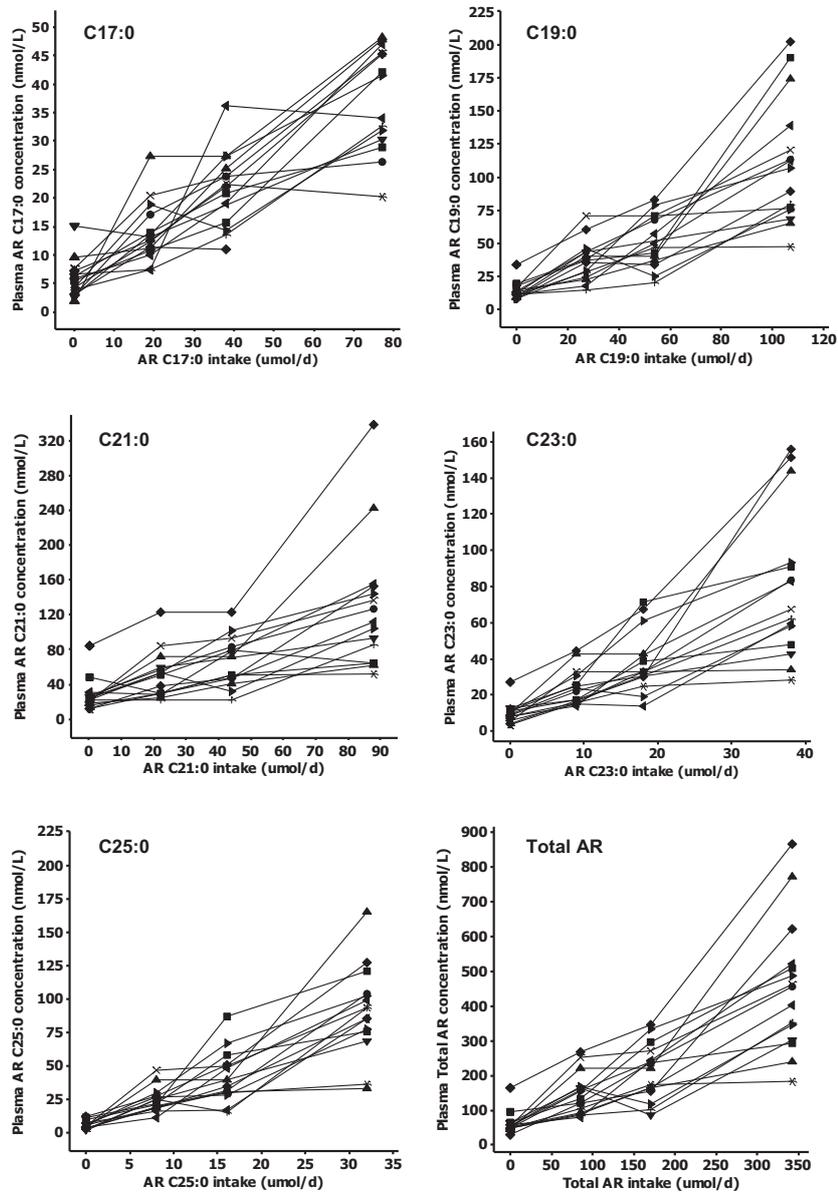


Figure 15. Plasma alkylresorcinol (AR) homologues (C17:0–C25:0) and total concentration (nmol/L) after daily intake of 33, 66 and 132 mg of total AR by 15 subjects. At dose 0, an average from run-in and wash-out periods was calculated. Statistically significant differences were found between all doses for all homologues ( $P=0.001-0.034$ ), except for homologues C21:0 and C23:0 between dose 1 and 2 ( $P=0.110$  and  $P=0.097$ , respectively) (III).

Table 12. Estimated total alkylresorcinol (AR) intake and plasma total AR concentration in different intervention studies

Study	AR intake		Plasma total AR concentration (nmol/L)	Study
	Advised (mg/d)	Estimated (mg/d)		
Whole grain intervention (n=28) <sup>1</sup>	-	6.8±1.9 <sup>2</sup>	59±57	<b>II</b>
		65.0±12.2 <sup>3</sup>	202±107	
Dose-response intervention (n=15) <sup>1</sup>	33	-	148±60	<b>III</b>
	66	-	210±81	
	131	-	455±189	
Rye whole grain/bran intervention (n=17) <sup>1</sup>	8.1	8.2±2.0 <sup>2</sup>	991±794	<b>V</b>
	620	556±160 <sup>4</sup>	75±92	

<sup>1</sup>Subjects included in the statistical analysis. <sup>2</sup>Estimated by 3 day weighed food records during refined grain treatment. <sup>3</sup>Estimated by 3 day weighed food records during whole grain treatment. <sup>4</sup>Estimated by 4 day weighed food records during rye whole grain/bran treatment

Table 13. Plasma total AR concentration after washout periods in different studies

Study	Wash-out period (d)	Plasma total AR concentration (nmol/L)	Study
RB/WB <sup>1</sup> intervention study (n=15) <sup>2</sup>	Run in 1w	23±7 (6-171)	Linko-Parvinen <i>et al.</i> (2007)
	Wash-out 1w	25±3 (10-46)	
Kinetic study (n=6) <sup>2</sup>	Run in 1w	25±9 (5-27)	<b>II</b>
Dose response intervention, (n=15) <sup>2</sup>	Run in 1w	68±33 (23-157)	<b>III</b>
	Wash-out I, 1w	60±37 (35-178)	
	Wash-out II, 1w	65±33 (18-158)	

<sup>1</sup> RB, whole grain rye bread; WB, whole grain wheat bread. <sup>2</sup>Subjects included in the statistical analysis

The fasting plasma AR concentration after 6–8 weeks of habitual diet varied considerably reflecting differences in AR intake between subjects, different times since last AR-containing meal, different absorption and elimination capacities and analytical variation. The average habitual fasting total AR concentration was about 100 nmol/L in subjects participating in the intervention studies and both the magnitude of variation and the mean values were similar those found in comparable interventions in Finnish studies (Table 14).

Table 14. *Habitual plasma total AR concentration reported from different studies. Values are reported as Mean±SD (range).*

Study	Plasma total AR <sup>1</sup> concentration (nmol/L)	Study
Dose response intervention (n=15) <sup>2</sup> (SWE)	117±69 (46-253)	<b>III</b>
Whole grain intervention (n=28) <sup>2</sup> (SWE)	102±70 <sup>3</sup> (21-251) 104±77 <sup>4</sup> (23-340)	<b>IV</b>
Rye whole grain/bran intervention (n=17) (SWE)	91±67 <sup>5</sup> 140±73 <sup>6</sup>	<b>V</b>
Whole grain intervention, (n=39) <sup>2</sup> (FIN)	97±75 (34-418) 88±54 (13-245)	(Linko <i>et al.</i> , 2005)
Whole grain intervention (n=15) <sup>2</sup> (FIN)	127±212 (8-434)	(Linko-Parvinen <i>et al.</i> , 2007)
Case-cohort (n=362) (DAN)	115±112 (7-847)	<b>VI</b>

<sup>1</sup>All samples were fasting samples except for those in **VI**. <sup>2</sup>Subjects included in the statistical analysis. <sup>3</sup>Values taken before whole grain intervention. <sup>4</sup>Values taken before refined grain intervention. <sup>5</sup>Values taken before whole grain/bran rye intervention. <sup>6</sup>Values taken before refined wheat intervention. A significant carry-over effect was detected (P<0.10)

In **IV**, the relative validity of plasma AR concentration as a biomarker of whole grain of wheat and rye was evaluated by comparing plasma total AR concentration and estimated AR intake derived from 3-day weighed food records. The Spearman-Rank correlation coefficient was 0.58 when data from all study periods were combined. The increase in plasma AR determined by intake was highly significant (p<0.0001, as evaluated by ANCOVA model). In this study, no significant correlations were found during treatments, due to the small variation in intake between individuals resulting from the study design and good compliance. Correlation coefficients were 0.33 and 0.40 for periods with habitual diet period, before the respective treatment. The degree of correlation between estimated AR intake and plasma AR concentration was recently confirmed in a study on 33 free-living Swiss subjects, where the correlation between AR intake estimated from 3-day weighed food records and plasma AR concentration was r= 0.57 (P<0.0001) (Dr. Alastair Ross, pers. Comm.. 2008).

When mean values, at group level, for the different treatments were compared across studies, a clear linear dose-response up to 131 mg AR/d was found (Figure 16). However, beyond this point, the shape of the plasma AR dose-response relationship is difficult to evaluate due to the large variation and since the only data used were from 17 men with extremely high AR intake ( $\approx 550$  mg/d) (V).

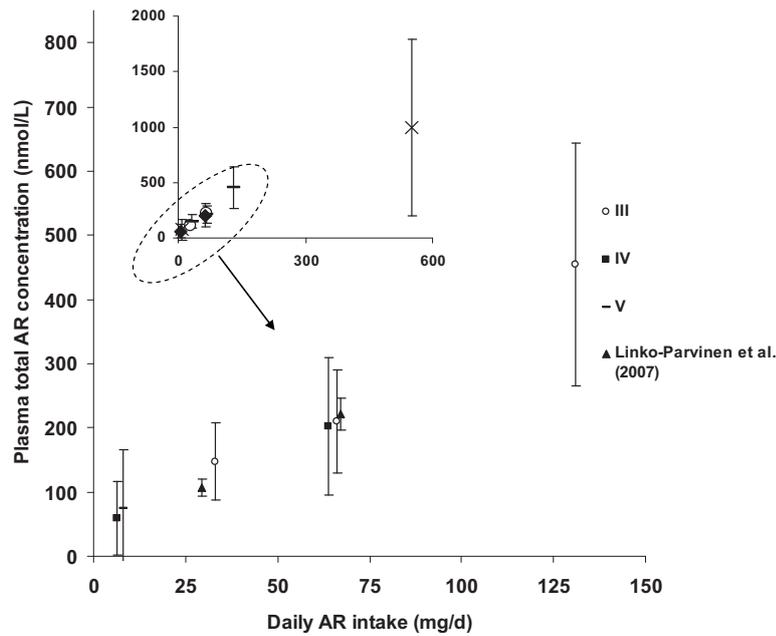


Figure 16. Plasma total AR concentration (mean $\pm$ SD) after estimated or advised AR intake in different intervention studies. The insert also covers the high intake during the rye whole grain/bran intervention period of V

#### 4.3.2 Plasma AR reproducibility at constant intake (V)

Reproducibility (largely determined by frequency of intake and the biomarker half-life) is a fundamental criterion of a biomarker, since it will affect the ability of a single sample to reflect intake over a certain time period (Kaaks *et al.*, 1997; Sonestedt *et al.*, 2007). In **V**, the reproducibility in plasma AR concentration was assessed in men with prostate cancer undertaking an intervention with constant high ( $\approx 550$ mg/d) or low ( $\approx 7.7$  mg/d) AR intake during two 6-wk periods.

Table 15. Intra-correlation coefficient<sup>1</sup> (ICC) determined for the first and the second intervention period, (95% CI), n=17

AR homologue	First intervention period	Second intervention period
C17:0	0.94 (0.88-1.0)	0.89 (0.81-0.97)
C19:0	0.89 (0.79-0.99)	0.90 (0.82-0.98)
C21:0	0.86 (0.74-0.98)	0.80 (0.66-0.94)
C23:0	0.89 (0.81-0.97)	0.85 (0.73-0.97)
C25:0	0.92 (0.86-0.98)	0.88 (0.78-0.98)
Total AR	0.90 (0.82-0.98)	0.88 (0.78-0.98)

<sup>1</sup>Intra-class correlation coefficient defined as  $(\sigma_b^2) / ((\sigma_w^2) + (\sigma_b^2))$ , where  $\sigma_b^2$  is the between-subject variance and  $\sigma_w^2$  is the within-subject variance. Variance components were estimated by a mixed linear model in SAS® v 9.1 (SAS Institute Cary, NC, USA)

ICC was  $>0.85$  with narrow 95% CI for all AR homologues. As a consequence of high ICC, 1-2 samples are needed in order to accurately assess individuals' average plasma AR concentration during a 6-week period with constant AR intake (Table 16). A high ICC is due to either low within-subject variation and/or high between-subject variation (Vineis & Gallo, 2007). The within-subject variance expressed as the coefficient of variation (CV) was 11-18% and the between-subject variance was estimated to 27-69% depending on AR homologue and intervention period. The results show that plasma AR concentration is stable during conditions where the intake is regular ( $>3$  times per day) and that 1-2 samples are sufficient to estimate the average AR concentration with an acceptable precision at a high confidence level (Table 16). It is likely; however, that plasma AR reproducibility is lower in a free-living population, where intake is more irregular.

Table 16. *Estimated number of replicate samples needed from an individual to reflect the mean plasma total AR steady-state concentration corresponding to a certain intake during a 6-wk intervention period<sup>1</sup>*

D <sup>2</sup> -level	First intervention period		Second intervention period	
	CI <sup>3</sup> (%)			
	80	95	80	95
5	8 (7.7;8.3)	19 (18.7;19.3)	5 (4.7;5.3)	12 (11.7;12.3)
10	2 (1.5;2.5)	5 (4.5;5.5)	2 (1.6;2.4)	3 (2.5;3.5)
20	1 (0.3;1.7)	2 (1.2;2.8)	1 (0.4;1.6)	1 (0.08;1.9)
30	1 (0.3;1.7)	1 (-0.1;2.2)	1 (0.4;1.6)	1 (0.08;1.9)
40	1 (0.3;1.7)	1 (-0.1;2.2)	1 (0.4;1.6)	1 (0.08;1.9)
50	1 (0.3;1.7)	1 (-0.1;2.2)	1 (0.4;1.6)	1 (0.08;1.9)
75	1 (0.3;1.7)	1 (-0.1;2.2)	1 (0.4;1.6)	1 (0.08;1.9)
90	1 (0.3;1.7)	1 (-0.1;2.2)	1 (0.4;1.6)	1 (0.08;1.9)

<sup>1</sup>Values are reported as point estimates (n=17) with the respective confidence intervals in brackets. <sup>2</sup>Precision (%) with which a subject's mean plasma total AR steady state level is to be estimated. <sup>3</sup>Confidence interval for the estimated number of samples needed to reflect the basal AR level with precision D

#### 4.4 Plasma AR concentration in free-living subjects (VI)

In **VI**, potential plasma AR determinants were investigated by analysing samples and FFQs from 360 post-postmenopausal Danish women from the prospective 'Danish Diet, Cancer and Health Study'. The frequency distribution of total AR concentration in non-fasting plasma sample was right skewed with a large variation (Figure 17). The shape of the distribution, with lower values being the most abundant, is similar to distributions of other biomarkers such as phytoestrogens in plasma and serum, plasma carotenoids and vitamin C (Grace *et al.*, 2004; Jansen *et al.*, 2004; Kilkkinen *et al.*, 2001; Kompauer *et al.*, 2006; Nierenberg *et al.*, 1991; Peeters *et al.*, 2007). The mean plasma AR concentration was 115±112 and the range was 7-847 nmol/L (**VI**). These levels are approximately twice the fasting total AR concentration estimated for 57 free-living Finnish subjects (65±35 nmol/L). The variation is most likely caused by differences in fasting status, since cereal fibre intake was almost exactly the same in the two studies (≈10g/d) and since rye bread is the main source of cereal fibre for both Danes and Finns.

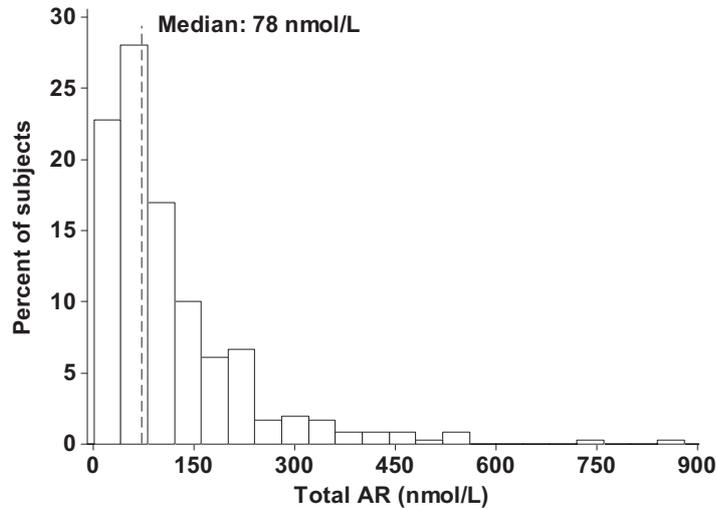


Figure 17. Frequency distribution of plasma total AR concentration in free-living Danish women (n= 360) (VI)

Habitual total AR concentration in fasting plasma samples was higher in subjects from the intervention studies than in free-living subjects, where the concentration was  $65.5 \pm 35$  nmol/L (VI). The difference is most likely due to over-all higher consumption of whole grain/bran among subjects volunteering for the intervention studies.

In VI, both endometrial cancer cases (n=182) and randomly selected controls (n=178) were used since there was no difference between cases and controls regarding AR plasma levels. Both dietary factors (identified in the FFQ), likely to contain AR and non-dietary factors (such as BMI, time since last meal and total serum cholesterol) were investigated. Plasma AR concentration from a single sample was significantly correlated to rye bread intake (Spearman's  $r=0.25$   $P<0.0001$ ). The 'true' correlation coefficient is probably higher, since substantial attenuation is expected for the observed relationship to intake due to using non-fasting plasma sample and a single plasma sample (Rosner & Willett, 1988; White, 1997). When investigated by regression models, rye bread intake was identified as the only significant food determinant of plasma AR concentration and an increase in rye bread intake of 100 g/d was associated with an increase in plasma total AR concentration of 87 % (46-139, 95% CI) in the final model, where only foods likely to contain AR were included as factors (Table 17).

Table 17. Estimated increment (%) in plasma total AR concentration corresponding to a 100 g/d increase in intake of food items, 10 g/d increase in fibre component and 1 unit increase in non-dietary variables. Potential determinants were identified among food items, fibre components and additional dietary factors evaluated by full and final models. Only estimates for significant ( $P < 0.05$ ) determinants are reported

Possible determinants evaluated	Full model <sup>1</sup>	Final model <sup>2</sup>
<i>Food items</i>		
Rye bread	94 (-0.1; 153)	87 (46;139)
Crisp bread	NS <sup>3</sup>	NS
White bread	NS	NS
Other bread	NS	NS
R <sup>2</sup>	0.120	0.120
<i>Fibre components</i>		
Total dietary fibre	22 (1; 49)	14 (0.3; 30) <sup>4</sup>
Cereal fibre	65 (19; 128)	37 (9; 74)
Non-cereal fibre	NS	NS
R <sup>2</sup>	0.023	0.019
<i>Additional dietary factors tested</i>		
Beer intake	NS	NS
Fat derived from animal carcasses	NS	NS
R <sup>2</sup>	-	-

<sup>1</sup>Model included all dietary factors and adjustment for BMI, serum cholesterol (non-fasting), total energy intake, time since last meal. <sup>2</sup>Model included all dietary factors but excluded non-dietary factors. <sup>3</sup>NS, not statistically significant ( $P > 0.05$ ). <sup>4</sup>Univariate estimate.

As expected, rye bread was the main determinant of the plasma AR concentration, since it is one of the richest sources of AR and since it accounts for 51% of total bread intake in the present study and about 65% of the total cereal intake in the Danish population (National Food Institute, 2008). At a nutrient level, both total dietary fibre and cereal fibre intake were significantly associated with an increase of plasma AR concentration 22% (1-49, 95% CI) and 65% (19-128, 95% CI) respectively. AR were not associated with other dietary fibre sources. The results are in agreement with findings by Aubertin-Leheudre *et al.* (2008), who recently showed plasma AR to be correlated to cereal fibre intake in free-living Finnish subjects (Pearson  $r = 0.28-0.42$  for the different AR homologues,  $P < 0.05$ ).

About 12% of the total variation in plasma AR concentration was explained by the model for food items and about 2% for fibre components. The amount of explained variation is rather low and is likely to be due to measurement error in both the FFQ and in the biomarker. Errors in FFQ are likely to be due to a limited number of questions related to whole grain intake and due to a large variation in whole grain content in different products whereas errors in plasma AR concentration are likely to be due to using non-fasting samples and due to the fact that the biomarker is likely to reflect short-term intake, whereas the FFQ reflects more long-term intake.

#### 4.5 Plasma AR C17:0/C21:0 ratio- What does it show?

The relative AR homologue profiles are rather stable within and between different cereal species, which results in C17:0/C21:0 ratios of about 1.0 for rye, 0.1 for wheat and 0.01 for durum wheat (Andersson *et al.*, 2008a; 2008b; Chen *et al.*, 2004; Landberg *et al.*, 2005; Ross *et al.*, 2003b). It has been suggested that the plasma C17:0/C21:0 can be used to reflect the source of whole grain intake (Linko-Parvinen, 2006). Results from **II** showed that the relative abundance of C17:0 decreased in plasma over time whereas a corresponding increase was observed for C23:0 and C25:0. Hence the C17:0/C25:0 ratio decreased from about 0.8 to about 0.4 during the first 8 h after intake, and then remained stable until 24 h. These results suggest that there are differences in AR pharmacokinetics attributable to homologue length. However, the ratio was stabilised after 8 hours, but lower than that found in rye grain.

Results from intervention studies, show that plasma AR C17:0/C21:0 ratio is changed according to intake of cereals from different sources, but that the ratio generally is lower than that found in the diet for rye intake and higher or equal to that found in wheat in the intervention studies (Table 18). This is probably explained by the lower bioavailability of C17:0 compared with C21:0 and by the fact that at low AR intake, a very small intake of rye will largely influence C17:0/C21:0 ratio.

In free-living Danish women sampled in the period 1995-1997, the plasma AR C17:0/C21:0 ratios indicated mixed consumption of wheat and rye products (**VI**). The ratio was even higher among free-living Finnish subjects, and was in the same magnitude as observed for whole grain intake dominated by rye during intervention (Table 18). Both Finnish and Danish people consume large amounts of rye products (which are often whole

grain). In Finland the rye bread intake is about 70 g/d (2007) and in Denmark it is somewhat lower, 58-65 g/d depending on year of survey (1995-2004) (National Food Institute, 2008; National Public Health Institute, 2007). The observed difference in plasma AR C17:0/C21:0 ratio is therefore likely to reflect differences in rye intake between the countries (Table 18).

Table 18. *The AR C17:0/C21:0 intake and plasma ratios in different intervention studies. Values are mean±SD*

	Dietary AR C17:0/C21:0 ratio	Plasma AR C17:0/C21:0 ratio	Reference
<i>Intervention treatments</i>			
Whole grain (6w, n=28)	0.36 <sup>1</sup>	0.30±0.23	<b>II</b>
Refined grain (6w, n=28)	0.33 <sup>2</sup>	0.34±0.23	<b>II</b>
Low dose of rye bran (1w, n=15)	0.80	0.35±0.15	<b>III</b>
Medium dose of rye bran (1w, n=15)	0.80	0.45±0.31	<b>III</b>
High dose of rye bran (1w, n=15)	0.80	0.64±0.52	<b>III</b>
Rye whole grain/bran (6w, n=17)	1.06 <sup>3</sup>	0.65±0.24	<b>V</b>
Refined wheat (6w, n=17)	0.15 <sup>3</sup>	0.27±0.22	<b>V</b>
High fibre rye bread (8w, n=39)	?	0.84±0.25	Linko <i>et al.</i> (2005)
Low fibre wheat bread (8w, n=39)	?	0.53±0.50	
Whole grain rye (1w, n=15)	1.02	0.60±0.23	Linko-Parvinen <i>et al.</i> (2007)
Whole grain wheat (1w, n=15)	0.12	0.10±0.38	Linko-Parvinen <i>et al.</i> (2007)
<i>Free-living subjects</i>			
Uncontrolled Danish diet, (n=360)	?	0.40±0.23	<b>VI</b>
Uncontrolled Finnish diet, (n=65)	?	0.62±0.22	Aubertin- Leheudre <i>et al.</i> (2008)

<sup>1</sup>Ratio for advised whole grain diet for 6-week whole grain treatment. <sup>2</sup>Ratio for advised refined grain diet for 6-week refined grain treatment. <sup>3</sup>Intake ratio was estimated from 4-day weighed food records

## 5 General discussion

The validation of a new biomarker has been described as a long, costly and iterative process, where a range of expertise is needed (Bingham, 2002; Hunter, 1998; Maruvada & Srivastava, 2004; Schulte & Talaska, 1995). The validation of AR as biomarkers of whole grain wheat and rye may be summarised as in Figure 18. Each level of the model is discussed below.

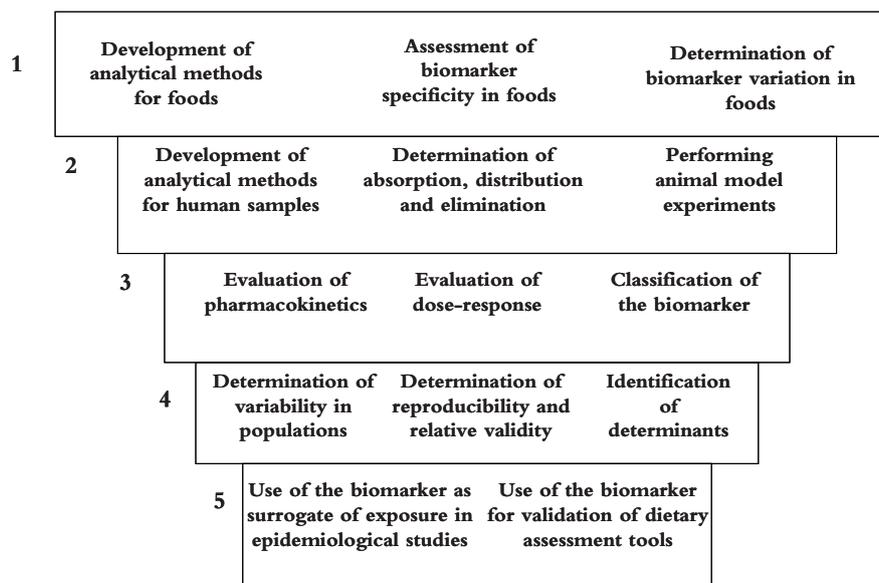


Figure 16. A top-down model with five levels for the evaluation of AR as biomarkers of whole grain wheat and rye intake. This thesis mainly covered levels 2,3 and 4 in the validation model described

#### *Level 1*

AR have been shown to be specific for whole grain/bran of wheat and rye among commonly consumed foods and are found only at very low contents in refined cereal products (Chen *et al.*, 2004; Landberg *et al.*, 2008b; Mattila *et al.*, 2005; Ross *et al.*, 2003b; Ross *et al.*, 2001a). AR in foods are stable throughout food processing (Chen *et al.*, 2004). To facilitate evaluation of AR as biomarkers, food composition data need to be collected for the most commonly consumed whole grain/bran foods. Several analytical techniques are available for determination of AR content in food products (listed in Table 6), but systematic method validation is often lacking.

#### *Level 2*

A rapid and sensitive method for the analysis of AR in rather small plasma volumes (50-200  $\mu\text{L}$ ) is available (**I**). AR share similar absorption and elimination routes as tocopherols, as evidenced by the two major metabolites DHBA and DHPPA (Ross *et al.*, 2004e). In the blood, AR are associated with lipoprotein fractions and also distributed to the membranes of erythrocytes (Linko-Parvinen *et al.*, 2007; Linko & Adlercreutz, 2005). However, the magnitude of distribution and the mechanisms are unknown. In this thesis, recovery (of ingested dose) of AR metabolites in 24 h-urine was shown to decrease with increased dose (**III**). The reason for this needs to be studied before AR metabolites can be classified and used as biomarkers of whole grain wheat and rye intake.

#### *Level 3*

Plasma AR have a short elimination half-life (4-5 h) and can be classified as a short-term concentration biomarker (**II-III**). How well plasma AR concentration can reflect more long-term intake in free-living individuals is unknown and needs to be evaluated in different populations. AR liberated from slowly equilibrating body pools may reflect more long-term intake.

#### *Level 4*

Under intervention conditions, a rather strong correlation was found between plasma AR concentration and estimated intake (**IV**) and a high reproducibility at regular intake was observed (**V**). The validity and reproducibility of AR as a biomarker in free-living individuals is unknown. In **VI**, factors potentially affecting AR concentration were identified and tested. Among these factors, rye bread was the major determinant, although a rather small proportion of the total variance was explained.

*Level 5*

No endpoint study has so far been reported in the literature, nor any study where AR have been used to validate other dietary instruments.

## 6 Main findings

- A rapid GC-MS method for determination of AR in relatively small plasma sample volumes (50-200  $\mu$ L) was developed and evaluated (**I**).
- AR showed two plasma concentration peaks after a single dose and the highest concentration occurred after about 7 hours and was  $>2000$  nmol/L in all subjects (**II**). The apparent elimination half-life was  $\approx 4.5$ h and hence, plasma AR concentration can be defined as a short-term concentration biomarker. A one-compartment pharmacokinetic model with two absorption compartments adequately described the single-dose AR pharmacokinetics (**III**).
- Fasting plasma AR concentration increased linearly with increased intake in human subjects with controlled intake of 33-131 mg total AR per day, which covers the whole grain intake range likely to be found among most people in Sweden (**III**). The relative validity of plasma AR concentration compared with intake estimated by 3-day weighed food records was high ( $r=0.58$ ) when the intake range was broad (**IV**).
- The fasting plasma AR concentration reproducibility was high ( $ICC > 0.85$ ) during a 6-week intervention with constant intake. Generally, one fasting plasma sample can be used to assess subjects' average concentration over a 6-week period with high accuracy and statistical precision (**V**). Hence, AR can be used to assess compliance in whole grain wheat and rye intervention studies.
- Non-fasting plasma AR concentration was mainly explained by rye bread intake among tested factors identified from a FFQ in free-living Danish subjects (**VI**).

## 7 Future research

- A large number of samples from free-living subjects must be analysed if AR are to be used as a surrogate of whole grain intake wheat and rye in epidemiological endpoint or validation studies. Hence, a faster and cheaper method requiring less sample material for determination of AR in plasma samples needs to be developed and validated.
- It remains to be determined whether a specific AR homologue (or the sum) should be used as the biomarker.
- In this thesis, the main focus was to evaluate plasma AR as biomarkers. However, other biological samples can also be used and should be evaluated for more long-term reflection.
- Long-term reproducibility in free-living subjects from different populations needs to be estimated. Ideally, the reproducibility should be determined in a sub-group of the same population where endpoint-studies are conducted, because this would allow correction for attenuation bias.
- A validation study where plasma AR is evaluated against other dietary assessment methods in free-living subjects is warranted to assess its validity coefficient (correlation to true intake).
- Since many prospective cohort studies use non-fasting samples, the feasibility of using non-fasting samples compared with fasting samples needs to be evaluated.

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