Institute for Preventive Medicine, Nutrition, and Cancer
Folkhälsan Research Center
and Division of Clinical Chemistry
Faculty of Medicine
University of Helsinki
Finland

Cereal alkylresorcinols as dietary biomarkers – absorption and occurrence in biological membranes

Anna-Maria Linko-Parvinen

Academic Dissertation

To be publicly discussed with the permission of the Faculty of Medicine of the University of Helsinki, in the lecture room 3, Biomedicum Helsinki, on November 24th, 2006, at 12 noon.

Helsinki 2006

Supervisors:

Professor Herman Adlercreutz, MD, PhD
Institute for Preventive Medicine, Nutrition and Cancer,
Folkhälsan Research Center,
and Division of Clinical Chemistry,
University of Helsinki, Finland

José Luis Peñalvo, PhD
Institute for Preventive Medicine, Nutrition and Cancer,
Folkhälsan Research Center,
and Division of Clinical Chemistry,
University of Helsinki, Finland

Reviewers:

Professor Heikki Kallio, PhD
Department of Biochemistry and Food Chemistry,
University of Turku, Finland

Professor Antti Aro, MD, PhD
Department of Health and Functional Capacity,
National Public Health Institute,
Helsinki, Finland

Official opponent:

Professor Sari Mäkelä, MD, PhD
Functional Foods Forum,
and Department of Biochemistry and Food Chemistry,
University of Turku, Finland

ISBN 952-92-1262-3 (paperback)
ISBN 952-10-3509-9 (PDF)
http://ethesis.helsinki.fi/
Helsinki University Printing House
Helsinki 2006

Att våga är att förlora fotfästet en liten stund.
Att inte våga är att förlora sig själv.

Søren Aabye Kierkegaard (1813-1855)

To my Family

Table of contents

LIST OF ORIGINAL PUBLICATIONS 6

ABBREVIATIONS 7

ABSTRACT 8

INTRODUCTION 11

1 REVIEW OF THE LITERATURE 12

1.1 EPIDEMIOLOGICAL STUDIES OF THE ROLE OF WHOLE-GRAIN PRODUCTS ON HUMAN HEALTH AND POSSIBLE PROTECTIVE MECHANISMS 12

1.2 BIOMARKERS OF DIETARY INTAKE 15

1.3 CEREAL ALKYLRESORCINOLS 17

1.3.1 Occurrence of alkylresorcinols in foods *17*

1.3.2 Biological effects in vitro *20*

Antioxidative properties 20

Alkylresorcinols in membranes 21

Other biological effects 21

1.3.3 Animal and human studies *22*

Absorption and elimination 22

Suggested metabolic interactions of alkylresorcinols 23

Effects on growth 23

1.4 ABSORPTION AND METABOLISM OF LIPOPHILIC COMPOUNDS, WITH VITAMIN E AS AN EXAMPLE 24

2 AIMS OF THE STUDY 26

3 MATERIALS, STUDY DESIGNS, AND METHODS 27

3.1 HUMAN STUDIES 27

3.1.1 Analysis of human plasma alkylresorcinols after intake of whole-grain rye bread compared to low-fibre wheat bread (Study II) *27*

Subjects 27

Study design and diets 27

3.1.2 Incorporation of alkylresorcinols into erythrocyte membranes (Study III) *28*

3.1.3 Distribution of alkylresorcinols in human plasma, erythrocytes, and lipoproteins (Study V) *28*

Subjects 28

Study design and diets 29

3.2 ANIMAL STUDIES 29

3.2.1 Kinetics of alkylresorcinols in pig plasma (Study IV) *29*

Pigs 29

Study design and diets 30

3.3 METHODS 32

3.3.1 Solvents and reagents ... *32*
3.3.2 Alkylresorcinol reference compounds ... *32*
3.3.3 Instrumentation ... *32*
3.3.4 Isolation of lipoproteins ... *33*
3.3.5 Quantitative analysis of alkylresorcinols ... *33*
Plasma ... 33
Erythrocytes ... 34
Lipoproteins ... 34
Test breads ... 34
3.3.6 Serum enterolactone ... *34*
3.3.7 Other analytical methods ... *35*
3.3.8 Quality assurance ... *35*
3.3.9 Assessment of pharmacokinetic parameters ... *35*
3.3.10 Statistical analysis ... *36*

4 RESULTS ... 36

4.1 ANALYTICAL METHODS FOR ALKYLRESORCINOLS IN PLASMA, ERYTHROCYTES, AND LIPOPROTEINS (STUDY I, II, III, IV, AND V) ... 36
Plasma ... 36
Erythrocytes and lipoproteins ... 38
Precision ... 38
4.2 KINETICS OF ALKYLRESORCINOLS IN PIGS (STUDY IV) ... 39
4.3 PLASMA ALKYLRESORCINOL AND SERUM ENTEROLACTONE CONCENTRATIONS (STUDY II, III, IV, AND V) ... 42
4.4 ALKYLRESORCINOL OCCURRENCE IN ERYTHROCYTE CELL MEMBRANES (STUDY III AND V) 49
4.5 ALKYLRESORCINOL OCCURRENCE IN LIPOPROTEINS (STUDY V) ... 52

5 DISCUSSION ... 55

5.1 METHOD DEVELOPMENT AND ANALYSIS OF ALKYLRESORCINOLS IN PLASMA AND BIOLOGICAL MEMBRANES ... 55
5.2 ABSORPTION AND DISTRIBUTION OF ALKYLRESORCINOLS IN PLASMA ... 56
5.3 ALKYLRESORCINOLS AS BIOMARKERS OF WHOLE-GRAIN INTAKE ... 62

6 CONCLUSIONS ... 66

7 FUTURE STUDIES ... 67

8 ACKNOWLEDGEMENTS ... 68

9 REFERENCES ... 70

List of original publications

This thesis is based on the following original publications referred to in the text by their Roman numerals.

I Linko, A.-M., Parikka, K., Wähälä, K. and Adlercreutz, H. (2002) Gas chromatographic – mass spectrometric method for the determination of alkylresorcinols in human plasma. *Analytical Biochemistry* 308(2):307-313.

II Linko, A.-M., Juntunen, K.S., Mykkänen, H.M. and Adlercreutz, H. (2005) Whole-grain rye bread consumption by women correlates with plasma alkylresorcinols and increases their concentration compared with low-fibre wheat bread. *Journal of Nutrition* 135(3):580-583.

III Linko, A.-M. and Adlercreutz, H. (2005) Whole grain rye and wheat alkylresorcinols are incorporated into human erythrocyte membranes. *British Journal of Nutrition* 93 (1):11-13.

IV Linko, A.-M., Ross, A.B., Kamal-Eldin, A., Serena, A., Bjørnbak Kjaer, A.K., Jørgensen, H., Peñalvo, J.L., Adlercreutz, H., Åman P. and Bach Knudsen K.E. (2006) Kinetics of the appearance of cereal alkylresorcinols in pig plasma. *British Journal of Nutrition* 95(2):282-287.

V Linko-Parvinen, A.-M., Landberg, R., Tikkanen, M.J., Adlercreutz, H. and Peñalvo, J.L. (2006) Whole-grain alkylresorcinols are transported in human plasma lipoproteins, and their intake corresponds to plasma concentrations. *Submitted for publication.*

Abbreviations

AR	alkylresorcinol(s)
BMI	body mass index
C15:0-C25:0	alkylresorcinols with respective carbon chain lengths
CHD	coronary heart disease
CV	coefficient of variation
CVD	cardiovascular diseases
DEAE	diethylaminoethyl
DM	dry matter
EDTA	ethylenediaminotetra-acetic acid
ENL	enterolactone
GC	gas chromatography
HDL	high-density lipoprotein
LDL	low-density lipoprotein
MS	mass spectrometry
TG	triacylglycerols
VLDL	very low-density lipoprotein

Abstract

Background

Several studies link the consumption of whole-grain products to a lowered risk of chronic diseases, such as certain types of cancer, type II diabetes, and cardiovascular diseases. Cereal fibre together with the biologically active compounds associated with the dietary fibre complex have been proposed to be protective. However, the final conclusions of the exact protective mechanisms remain unclear, partly due to a lack of a suitable biomarker for the whole-grain cereals intake. The mammalian lignan enterolactone (ENL) has previously been suggested to function as a possible biomarker for whole-grain intake, but is produced in the human gut from lignans in several plant products in the diet. Alkylresorcinols (AR) are phenolic lipids abundant in the outer parts of wheat and rye grains usually with homologues of C15:0-C25:0 alkyl chains. According to *in vitro* studies they possess several biological activities and have been proposed to function as specific biomarkers of whole-grain intake. The total amount of AR and the homologue ratios are different in wheat and rye grains.

Aims

In the present work a quantified method for the analysis of AR in plasma was developed. The method was used to determine human and pig plasma AR concentrations after the intake of whole-grain wheat and rye products compared to low-fibre wheat bread diets to assess the usability of AR as biomarkers of whole-grain intake. AR plasma concentrations were compared to serum ENL concentrations. AR absorption and elimination kinetics were investigated in a pig model. AR occurrence in human erythrocyte membranes and plasma lipoproteins were determined, and the distribution of AR in blood was evaluated.

Methods

Plasma AR were analysed by gas chromatography – mass spectrometry (GC-MS) after incubation with water, extraction, and purification with ion exchange chromatography (Study I). For the analyses of erythrocyte AR, the cells were separated, washed and deproteinised, AR were extracted and the samples were purified in a similar manner as with plasma analysis. Lipoproteins were isolated by sequential ultracentrifugation. Lipoprotein AR were analysed in a similar manner as plasma, but extracted four times for better analyte recovery. Serum ENL concentrations were measured by time-resolved fluoroimmunoassay.

In a randomised, crossover dietary intervention (2x8 weeks) with intake of whole-grain rye and low-fibre wheat bread, plasma AR and serum ENL concentrations from 39 postmenopausal women were measured and compared to habitual diet (Study II). AR were analysed in human plasma and erythrocyte membranes (n=4) after a one-week intake of whole-grain products compared to a one-week intake of low-fibre products. The concentrations were compared to

those of the subjects with a two-weeks habitual diet (n=4) (Study III). Plasma, erythrocyte, and lipoprotein AR were analysed (n=8 women, 7 men, n=total of 15) after constant intake of whole-grain rye or wheat crisp bread in a 1x1 week crossover study. Before the study weeks the subjects followed a low-fibre wheat bread diet for one week (Study V). AR absorption and elimination kinetics were determined in a pig model. Four pigs were constantly fed either whole-grain rye bread with rye bran or low-fibre white wheat bread. In part 1 of the study, AR appearance in plasma from portal vein and mesenteric artery was followed for 9 h on days 5 and 7 of the feeding study. In Part 2, AR appearance was followed for 16 h after single feeding of rye bread after a 5-day low-fibre diet. The study was repeated for each pig (Study IV).

Results

AR can be quantified in plasma, erythrocytes, and lipoproteins using a GC-MS method. Fasting plasma AR concentrations (n=39) were significantly ($p < 0.001$) higher after an eight-week consumption of whole-grain rye bread (214±7.1 g/d (mean±SEM), 352±24.7 nmol/l) or after an eight-week habitual Finnish diet (88.3±8.7 nmol/l) compared to an eight-week low-fibre bread period (36.6±4.2 nmol/l). Plasma AR concentrations correlated with the intake of rye bread ($r=0.34$, $p < 0.05$). After a one-week constant intake of whole-grain rye or wheat crisp bread (97.5±0.97 and 95.5±2.1 g/d, respectively, n=15) plasma AR concentrations increased significantly (222±24.5 and 107±14.1 nmol/l, respectively) compared to the one-week low-fibre bread diets (30.1±10.7 and 25.4±3.0 nmol/l) ($p < 0.05$), but inter-individual variation in the concentrations was high, 55.1-389 nmol/l after whole-grain rye intake and 34.4-231 nmol/l after whole-grain wheat intake. No correlation to individual bread intake was seen, although mean plasma AR concentrations increased significantly with the increasing intake of AR from whole-grain wheat bread and more with whole-grain rye bread. AR homologue C17:0/C21:0 –ratio was significantly different after intake of whole-grain wheat and rye compared to low-fibre periods or habitual diet ($p < 0.05$).

ENL concentrations increased in women (n=39) with the eight-week whole-grain rye bread intake and in men (n=7) with the one-week whole-grain rye bread intake, but not with whole-grain wheat bread. With women (n=8) no increase was seen within one week with whole-grain rye or wheat crisp bread intake, although AR concentrations did increase. The changes in ENL concentrations were smaller and more variable than in AR when related to the intake of whole-grain products.

There was no difference in AR concentrations measured from pig portal vein and mesenteric artery after the intake of whole-grain rye bread. The maximum plasma AR concentration was seen at 3-4 h with single feeding. The absorption half-life was 1.20 h and apparent elimination half-life 3.93 h.

We determined AR in erythrocyte membranes, and their concentrations increased significantly with a one-week intake of whole-grain rye or wheat bread

(315±31.2 and 210±17.3 nmol/l of packed cells, respectively) compared to low-fibre bread diet (n=15). In another study (n=4) erythrocyte AR concentrations increased 231±63.7 nmol/l of packed cells with a one-week whole-grain bread intake compared to a one-week low-fibre bread diet.

AR are incorporated into VLDL, 46±2.1% of the total AR(mean±SEM, n = 15), LDL, with 20±1.3%, and HDL, with 33±1.5%, and their concentrations systematically increase with the intake of whole-grain wheat and rye bread compared to low-fibre wheat bread.

Conclusions

Plasma AR seem to be absorbed *via* the lymphatic system from the small intestine, like many other lipophilic compounds. Their apparent elimination half-life is relatively short and is similar to that of tocopherols, which have a similar chemical structure. AR are incorporated into erythrocyte membranes. It is possible that erythrocyte AR concentrations reflect a longer term intake of AR. Plasma AR concentrations increased significantly after a one- to eight-week intake of whole-grain wheat and rye bread. The concentrations were also higher after habitual Finnish diet compared to diet with low-fibre bread. Inter-individual variation after a one-week intake of the same amount of bread was high, but the mean plasma AR concentrations increased with increasing AR intake. In one study a correlation between plasma AR and bread intake was seen. AR are incorporated into plasma lipoproteins, and VLDL and HDL were the main AR carriers in human plasma.

Based on these studies, plasma AR could function as biomarkers of dietary whole-grain products. AR are exclusively found in whole-grains and are more suitable as specific biomarkers of whole-grain intake than previously suggested mammalian lignan ENL. Plasma AR C17:0/C21:0 –ratio could distinguish whether whole-grain products in the diet are mainly wheat or rye. Further and prolonged studies are needed to confirm these data and the analysis of AR in large epidemiological studies could eventually demonstrate their functionality as biological markers.

Introduction

In the face of an increase in the incidences of diet associated diseases, more attention has been paid to improve human nutrition. One of the recent recommendations is to include whole-grain products in the daily diet. Consumption of whole-grain cereals has been linked to a reduced risk of several chronic diseases, such as type II diabetes, certain cancers, and cardiovascular diseases (CVD) (Slavin *et al.* 2001, Truswell 2002). Cereal fibre and several compounds associated with it, as well as their synergistic effects, have been suggested to be protective (Lapointe *et al*. 2006, Slavin 2003, Slavin *et al*. 1999, Steinberg 1997). However, an apparent disease preventive role of whole-grains and the exact protective mechanisms have remained uncertain. A reliable biomarker for whole-grain intake is needed to verify the intake and to clarify the relation of the intake of whole-grains to disease prevention (Edge *et al*. 2005).

Alkylresorcinols (AR) are phenolic lipids abundant in the outer parts of the kernels of whole-grain wheat and rye, which are two of the main cereals consumed by humans. AR have been shown to be biologically active and possess membrane stabilizing and antioxidative properties when incorporated into biological membranes (Kozubek and Tyman 1999, Ross *et al*. 2004b). AR, being specific for whole-grain wheat and rye in the human diet, have been suggested to function as dietary biomarkers of whole-grain intake (Ross *et al*. 2004b, Ross *et al*. 2001b). Previously, concentrations and occurrence of AR *in vivo* were unknown, and therefore verifying the usability of AR as biomarkers in humans has been difficult.

In the present study, a new method for the analysis of AR in plasma was developed. The method was used to measure AR concentrations in human and pig plasma after the intake of whole-grain wheat and rye bread to determine AR usability as biomarkers of whole-grain intake. With modifications, the method was used to measure AR in human erythrocytes and plasma lipoproteins to assess AR incorporation into the membranes and their distribution in blood.

1 Review of the literature

1.1 Epidemiological studies of the role of whole-grain products on human health and possible protective mechanisms

Recently, increasing attention has been paid to human nutrition and its role in health promotion and prevention of chronic diseases (Marquart *et al*. 2003, Slavin 2004). Several epidemiological studies have linked the intake of whole-grains to a reduced risk of certain chronic diseases, such as type II diabetes, cardiovascular diseases (CVD), obesity, and certain types of cancer (Hallmans *et al*. 2003, Slavin *et al*. 2001, Truswell 2002). The exact protective mechanisms of whole-grains have remained unclear, although several biologically active compounds, such as vitamin E, folate, and phenolic compounds have been investigated (Slavin 2003, Slavin *et al*. 1999). Grain kernels are structurally divided into three main fractions: bran and aleurone layer, endosperm, and germ. They vary largely in chemical and structural composition. The germ includes lipids, proteins, minerals, and most of the vitamins, including vitamin E, while endosperm contains mainly carbohydrates and some proteins (Slavin 2004). Most of the biologically active compounds together with structural polymers in the fibre complex exist in the bran fraction of the grains (Trowell 1976). Grain processing can increase the amount of bioactive compounds or their bioavailability by releasing them from the matrix. On the other hand, in highly refined products most of the bioactive compounds are removed together with the bran (Slavin 2004).

Indigestible carbohydrates in whole-grains, most importantly insoluble fibre, increase faecal weight and decrease intestinal transit time (Bruce *et al*. 2000). The intake of dietary cereal fibre has been linked to reduced body weight by several mechanisms, such as the intake of insoluble fibre, or fermentability of fibre, and soluble fibre, or the viscosity of fibre, which both can affect energy metabolism with hormonal, colonic, and intrinsic effects (Slavin 2005). Fermented carbohydrates, and their metabolites, short-chain fatty acids, can reduce serum cholesterol concentrations and decrease the risk for colon cancer (Slavin 2004). A reduced risk for cancer has been explained also by other mechanisms, such as decreased faecal transit time, and dilution and esterification of bile acids (Korpela *et al*. 1992, Zhang *et al*. 1993). The reduced cancer risk has also been suggested to be linked to the intake of certain minerals, vitamins, such as vitamin E, and bioactive compounds, such as mammalian lignan enterolactone (ENL) (Slavin 2004 and references therein). ENL has been proposed to protect against certain diseases, such as breast, prostate, and colon cancers, and CVD (Adlercreutz 2002, Adlercreutz and Mazur 1997, Thompson 1998). ENL has been linked to a reduced risk of these diseases (Ingram *et al*. 1997, Pietinen *et al*. 2001, Vanharanta *et al*. 2003), although opposite data has been presented for breast (Kilkkinen *et al.* 2004) and prostate cancer (Kilkkinen *et al*. 2003, Stattin *et al*.

2002), and no final conclusions about its role in disease prevention has been made (Boccardo *et al.* 2006, Ganry 2005, Kilkkinen *et al.* 2006).

Whole-grains slow the digestion, tend to have a low glycaemic index, they can improve insulin sensitivity (Pereira *et al.* 2002), affect glucose and insulin metabolism, and thereafter reduce a risk of type II diabetes mellitus (Montonen *et al*. 2003, Salmeron *et al*. 1997, Slavin 2004). Therefore, whole-grains can possibly also reduce the risk of certain cancers, since high serum insulin has been linked to colon and breast cancer (Slavin 2004 and references therein). McIntosh et. al (2003) showed that rye fibre-based food, compared to wheat fibre-based food, is more effective in improving bowel health biomarkers, and it reduced faecal β-glucuronidase, postprandial insulin and plasma glucose concentrations, and increased faecal butyrate and faecal output (McIntosh *et al.* 2003). Jensen *et al*. recently correlated lower homocystein concentrations, lower insulin, C-peptide, and leptin levels to whole-grain intake (Jensen *et al.* 2006). The intake of whole-grains has been linked to reduced all-cause mortality. In the Iowa Women's Health Study, the total mortality of post-menopausal women was inversely associated with whole-grain intake but positively associated with refined grain intake (Jacobs *et al.* 1999, Jacobs *et al.* 2000). In the Norwegian County Study, the total mortality of women and men was inverse and graded across whole-grain consumption.

Pietinen *et al*. (1996) showed an inverse association between cereal fibre and CHD mortality that remained significant after adjustment to other dietary factors. They speculated that postprandial lipid response, glucose and insulin metabolism, or haemostatic factors may be the factors explaining the findings (Pietinen *et al.* 1996). Liu *et al*. suggested that the lower risk of CHD events associated with high intake of whole-grains could be explained by the intake of dietary cereal fibre, vitamin B6, folate, and vitamin E (Liu *et al.* 1999). The intake of cereal fibre has been linked to a reduced risk of CHD (Liu *et al.* 1999, Wolk *et al.* 1999) and myocardial infarction (Rimm *et al.* 1996). Interestingly, in some studies soluble fibre has shown no effect on CHD risk (Rimm *et al.* 1996). Total mortality and cardiovascular mortality have been inversely associated with whole-grain breakfast cereals compared to refined breakfast cereals (Liu *et al.* 2003). Jensen *et al*. (2004) found a link between the intake of whole-grains, and specifically bran, and a reduced risk of CHD (Jensen *et al.* 2004). They stated, however, that other factors, such as overall healthier diet and lifestyle, and total fibre from the diet can affect the results. A 23% risk reduction was seen in coronary heart disease (CHD) and cardiovascular deaths, and a 21% risk reduction in cancer death rate between the highest and the lowest whole-grain bread intake score (Jacobs *et al*. 2001). The intake of whole-grain cereals has also been linked to lower total cholesterol and LDL levels (Bruce *et al.* 2000, Jensen *et al.* 2006). Truswell (2002) reviewed several studies concerning whole-grains and CHD risk, and concluded that there was no clear association between total cereal consumption and CHD. However, Truswell reported that five separate

epidemiological cohort studies showed an inverse association of cereal fibre or whole-grains and CHD, and that the results can partly be explained by the cholesterol lowering effect of fibre, although several other mechanisms may be involved (Truswell 2002).

In addition to fibre, certain compounds, such as vitamin E (tocopherols and tocotrienols), plant sterols, fermentable carbohydrates and oligosaccharides, and their metabolic end-products, have been suggested to play a role in disease prevention (Slavin *et al.* 1999). For example, whole-grain antioxidants and many phenolic compounds have been shown to affect the risk factors of CHD (Slavin 2004), and it is thought that the synergistic effect of different compounds in the fibre complex of whole-grains is the key factor in disease prevention (Slavin 2003, Slavin 2004).

Antioxidants protect tissues against free radicals that can affect DNA, lipids, and proteins by initiating processes leading to several chronic diseases. Total antioxidant activity in whole-grains is relatively high, even higher than in most fruits and vegetables (Miller *et al.* 2000). However, difficulties in measuring the antioxidant capacity in foods have lead to contradictory results. There has also been speculation on how important the total antioxidant capacity of whole-grain products is for human health (Seal 2006), since most of the trials are performed *in vitro*. Only recently more attention has been paid to the bioavailability of the studied compounds and to the testing methods of the antioxidant capacity of whole-grain cereals (Adom and Liu 2005, Liyana-Pathirana and Shahidi 2006).

Vitamin E is regarded as the most important lipid-soluble antioxidant (Bjørneboe *et al.* 1990, Burton *et al.* 1983, Chow 1985). It can protect polyunsaturated fatty acids from oxidation and retain selenium in a reduced state and act as an intracellular antioxidant (Slavin 2004). Several antioxidants, such as soluble phenolic acids, flavonoids, tocopherols, and insoluble matrix-bound antioxidants, as well as compounds produced during food processing, can have local effects on the cells in the intestinal wall and systemic effects after absorption (Slavin 2003, Slavin 2004). However, total antioxidant capacity might not tell whether the tested compounds are actually absorbed in human subjects and whether they have biological effects *in vivo*.

LDL in its oxidised form is found in atherosclerotic plaques and plays an important role in atherosclerosis. In the arterial wall LDL can undergo oxidative modification and attract monocytes into the arterial lesion leading to the formation of macrophages and foam cells and eventually fully developed ateromas (Berliner *et al.* 1995, Steinberg 1997). LDL oxidation and thereafter cellular events involved in atherosclerosis development might be affected by dietary components, such as vitamin E (Kaul *et al.* 2001, Lapointe *et al.* 2006, Princen *et al.* 1995). Several other compounds, such as ascorbic acid, β-carotene, fatty acids, polyphenol compounds, such as flavonoids, and certain foods in general, such as fruits, olive

oil, tea, and red wine, and even whole diets, Mediterranean diet in particular, have been investigated in relation to preventing LDL oxidation (Lapointe *et al.* 2006). Most data regarding LDL susceptibility to oxidation is based on *in vitro* studies, and relating the results to human subjects is difficult since circumstances *in vivo* are remarkably different.

There are only a few published studies of the effects of whole-grain cereals on LDL oxidation. In the study by Jenkins *et al.*, the intake of wheat bran had no clear effect on LDL concentration or oxidation during a three months follow-up. (Jenkins *et al*. 2002). In another study, rye bran enriched bread intake for six weeks did not affect LDL oxidation lag-time compared to habitual diet or low-fibre wheat diet (Harder *et al*. 2004). However, ferulic acid, and other hydroxycinnamates extracted from rye have in other studies shown inhibition of LDL oxidation *in vitro* (Andreasen *et al*. 2001). So far, no conclusion regarding the diet and LDL oxidation has been made, but a synergistic effect of certain foods or compounds has been suggested. As with CVD and CHD, more controlled and dose-dependent studies with specific compounds and foods are needed to determine whether certain diets are cardio-protective (Lapointe *et al.* 2006).

1.2 Biomarkers of dietary intake

Most of the data in epidemiological studies of the effects of whole-grain intake is based on food frequency questionnaires and food diaries, which are dependent on memory and accurate and unambiguous reporting, and are prone to subjects' unconscious altering of dietary habits during reporting (Kaaks *et al*. 1997, Wild *et al*. 2001). Self-reporting has been stated to flatten the association of dietary factors with disease end-points and cause within-person errors (Jacobs *et al.* 2001). In epidemiological studies other measures of consumption, such as food consumption data or food supply data are used, but often no data of individual intake is available (Kantor *et al*. 2001). Food composition tables provide information of average food ingredients, but can cause errors in epidemiological studies. Difficulties in defining whole-grain foods and serving sizes can disturb data interpretation in epidemiological studies regarding the role of whole-grains in the diet (Kantor *et al*. 2001, Lang and Jebb 2003, Liu *et al*. 1999). A reliable biomarker of whole-grain intake would provide information on cereal consumption, and a biomarker that could distinguish between the intake of whole-grain cereals and other products considered healthy, such as fruits and vegetables, could provide conclusive data about their possible protective roles in the diet. According to Scientists from the Whole Grains and Health Summit in 2005, the long-term effects of whole-grain intake and risk of chronic diseases, as well as the compounds responsible for the health effects should be investigated, and the bioavailability of the bioactive compounds should be determined. Therefore, a biomarker of the intake of whole-grains, specific grains, or grain components is needed. (Edge *et al.* 2005).

A good biomarker should be highly sensitive and specific, minimally invasive, inexpensive, and its analysis method should be validated and standardised (Crews *et al*. 2001, Weber 2001). It should provide more accurate and objective data than obtained by traditional methods (Kaaks *et al.* 1997). Individual absorption, metabolism, excretion, and interactions in human body can affect the reliability of a biomarker. The choice of the matrix where it is analysed in, e.g. plasma, cell membranes, or tissues, as well as metabolic end-products of the measured compound, are important when determining the link between exposure and a biomarker. (Crews *et al*. 2001, Kaaks *et al*. 1997). Measurement reproducibility is affected by a compound's metabolic turn-over rate and the stability of dietary intake, as well as its redistribution and storage in the body (Kaaks *et al.* 1997). There is a lack of knowledge of absorption, distribution, and storage of many non-nutrients in the diet. Dose-dependent studies are scarce, and most of the studies of non-nutrients as biomarkers have focused on the intake of whole food, where complexity and other food components affecting absorption and distribution can affect the results (Crews *et al.* 2001). Absorption of lipophilic compounds like vitamin E, for example, is enhanced by the simultaneous intake of fat (Kaaks *et al.* 1997). If the biomarker is a metabolic end-product, its concentration is a combination of intake, absorption, and metabolism, and might not correlate solely to intake. (Wild *et al.* 2001). Short-term variation and intra-individual variation's effect on distribution, retention, and interactions within the body should be determined (Grandjean 1995).

According to Kaaks *et al*. (1997), biomarkers can be divided into those that provide an absolute quantitative and time-related measure of dietary intake, which requires knowledge on pharmacokinetics and metabolic balance between the intake and excretion of the compound, and into those that measure the concentration of a given factor in biological fluids or tissues, and provide a correlation to a dietary factor, but for which there is no time dimension, and therefore only provide a correlation to dietary intake level. Most of the biomarkers fall into the latter category and only give information whether individuals can be classified based on high or low dietary intake. In addition to a biomarker's ability to identify certain dietary factors related to incidence of diseases, the biomarker itself could be biologically active. (Kaaks *et al.* 1997). This, however, is not necessary.

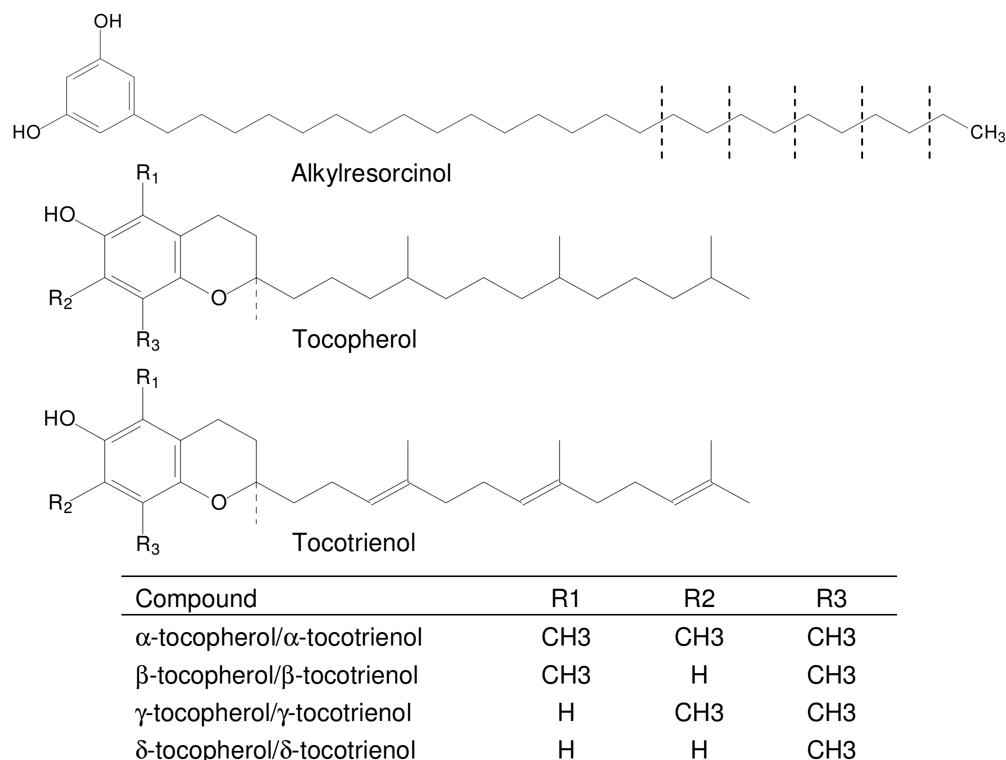
The mammalian lignan enterolactone (ENL) has been studied as a potential biomarker for whole-grain intake (Stumpf 2004). ENL, and another lignan metabolite enterodiol, are formed by intestinal microflora from plant lignans present widely in nature (Borriello *et al*. 1985, Setchell *et al*. 1981, Setchell *et al*. 1980). Enterodiol is further converted to ENL. The main sources of plant lignans in human diet are berries, fruit, vegetables, oily seeds, especially flaxseed and sesame seed, wine, and whole-grains (Mazur and Adlercreutz 1998, Milder *et al*. 2005, Peñalvo *et al*. 2005a). Whole-grains, rye in particular, have been known to contain plant lignans matairesinol and secoisolariciresinol (Nilsson *et al*. 1997). Recently, other mammalian lignan precursors have been identified in rye

(Heinonen *et al*. 2001), and in other plants (Peñalvo *et al.* 2005b, Saarinen *et al.* 2000). The intake of whole-grain cereals, spefically rye, has been linked to increased ENL concentrations, but no direct correlation to intake has been shown (Jacobs *et al*. 2002, Juntunen *et al*. 2000, Kilkkinen *et al*. 2001, Kilkkinen *et al*. 2002, Vanharanta *et al*. 2002). It is thought that since enterolactone is produced from several sources in the diet, it, together with enterodiol, would reflect a healthy diet and the intake of lignans or individual gut microflora activity instead of the intake of a certain food (Horner *et al*. 2002, Johnsen *et al*. 2004, Kilkkinen *et al*. 2002, Stumpf 2004, Stumpf *et al*. 2000a).

1.3 Cereal alkylresorcinols

1.3.1 Occurrence of alkylresorcinols in foods

Cereal 5-n-alkylresorcinols (AR) are lipophilic 1,3-dihydroxybenzene derivatives that differ mainly with their odd-numbered hydrocarbon chains attached to position 5 of the benzene ring. Their structure resembles that of tocopherols that have a chromanol ring with a phenolic group and a hydrocarbon phytyl chain with 16 carbons (Figure 1).



Compound	R1	R2	R3
α-tocopherol/α-tocotrienol	CH3	CH3	CH3
β-tocopherol/β-tocotrienol	CH3	H	CH3
γ-tocopherol/γ-tocotrienol	H	CH3	CH3
δ-tocopherol/δ-tocotrienol	H	H	CH3

Figure 1. *Chemical structures of alkylresorcinol, tocopherol, and tocotrienol, and list of tocopherol and tocotrienol side chains. The dashed lines in alkylresorcinol structure represent different alkylresorcinol homologue lengths (C15:0-C25:0).*

Due to their structure, AR are lipophilic, and their $\log_{10}$ octanol-water partition coefficients vary from 8.5 to 13.4, depending on the chain length. Log_{10} octanol-water partition coefficient is, for example, above 8 and mostly around 12 for fat-soluble vitamins (A, D, E, and K), and 1.4 and -4.0 for water-soluble ferulic acid and L-ascorbic acid, respectively (Eastwood 1999). AR are named in a similar way as fatty acids (Table 1). As an example, AR with a hydrocarbon chain with 17 carbon atoms is named as AR C17:0.

Table 1. *Nomenclature of alkylresorcinols, their molecular weights, and their homologue percentages in different cereals *(Ross et al. 2003c).*

Alkylresorcinol name	Empirical formula	Abbreviation	Molecular weight (g/mol)	Homologue%*		
				Rye	Wheat	Barley
5-n-pentadecylresorcinol, 5-pentadecyl-1,3-benzenediol (cardol, adipostatin A)	$C_{15}H_{31}$	C15:0	320	1-2	-	-
5-n-heptadecylresorcinol, 5-heptadecyl-1,3-benzenediol	$C_{17}H_{35}$	C17:0	348	23-25	4-5	3-10
5-n-nonadecylresorcinol, 5-nonadecyl-1,3-benzenediol	$C_{19}H_{39}$	C19:0	376	31-32	29-35	11-13
5-n-heneicosylresorcinol, 5-heneicosyl-1,3-benzenediol	$C_{21}H_{43}$	C21:0	404	22-25	46-51	24-27
5-n-tricosylresorcinol, 5-tricosyl-1,3-benzenediol	$C_{23}H_{47}$	C23:0	432	10-11	0-12	16-18
5-n-pentacosylresorcinol, 5-pentacosyl-1,3-benzenediol	$C_{25}H_{51}$	C23:0	460	8-10	3-4	35-44

In nature, the highest amounts of AR are found in the *Triticae* subfamily of the meadowgrass (*Poaceae*) family in rye, wheat, and triticale, and in small amounts in barley (Kozubek and Tyman 1999, Ross *et al*. 2003c), with homologues varying from C15:0 to C25:0. AR are not found in other subfamilies of meadowgrass, such as oats, rice, corn, millet, or sorghum (Mattila *et al*. 2005, Ross *et al*. 2003c). AR are located in the bran fraction, in pericarp and testa of the grain, and in aleurone layer, but only in minute amounts in the endosperm. Their relative homologue percentages are similar in all grain parts, but differ between the species (Table 1) (Ross *et al*. 2001a). In addition to cereals, certain AR homologues are found in some bacteria, fungi, and in some higher plants, but not in the edible parts of these species (Bandyopadhyay *et al*. 1985, Kozubek and

Tyman 1999, Ross *et al*. 2003c). AR are found in certain soil bacterial walls, e.g. in *Azotobacter chroococcum* and in *Pseudomonas* species, where they can affect membrane metabolism, have antioxidative properties, and provide biochemical protection for the bacteria (Bitkov *et al.* 1992, Kozubek *et al.* 1996). In plants AR might provide protection against pathogens, such as fungi, during seedling development (Deszcz and A. 2000, García *et al.* 1997, Miché *et al.* 2003).

AR homologue C17:0/C21:0 –ratio was about 0.1 in wheat based products and about 1.0 in rye based products, and it has been suggested to indicate the type of cereals in foods (Chen *et al*. 2004, Ross *et al*. 2004b). Barley contains exceptionally high amounts of C25:0 (Chen *et al*. 2004, Ross *et al*. 2003c, Zarnowski *et al*. 2002). The main AR homologues in wheat and rye are C17:0-C25:0 with saturated hydrocarbon chains, but low amounts of homologues with unsaturated hydrocarbon chains or with hydroxyl or keto-groups in the chains are present in <10% in wheat, barley, and triticale, and in ~20% in rye (Kozubek and Tyman 1995, Ross *et al*. 2003c, Seitz 1992). Structures with two resorcinol groups exist in minor amounts in grains (Suzuki *et al*. 1999).

Chen *et al*. (2004) reported AR concentrations of 227-639 μg/g DM for whole-grain wheat grains and on average 8 μg/g DM for barley grains (Chen *et al.* 2004). Ross *et al*. (2003) measured AR concentrations of 489-618 μg/g DM for wheat grains, 720-761 μg/g DM for rye grains, 439-647 μg/g DM for triticale grains, and 42-51 μg/g DM for barley grains. Zarnowski et. al (2002) stated that in barley AR exist in concentrations of 41-210 μg/g DM (Zarnowski *et al.* 2002). Wheat and rye bran have high amounts of AR, 2211-3225 μg/g DM and 2758-4108 μg/g DM respectively (Chen *et al*. 2004, Mattila *et al*. 2005). In earlier studies, extreme AR concentrations up to 5000 μg/g DM for rye grains, 900 μg/g DM for wheat grains, and 150 μg/g DM for barley grains were reported (Gasiorowski *et al*. 1996). No AR were detected in white wheat flour or bread (Mattila *et al*. 2005, Ross *et al*. 2003c).

AR concentrations in wheat and rye vary considerably depending on the cultivar and location (Chen *et al*. 2004, Ross *et al*. 2003c). For example variation of 200-1480 μg/g DM has been reported for several cultivars of *Triticum* (wheat), and concentrations of one cultivar in a single field had a range of over 500 μg/g (Ross *et al*. 2003c). Variation of different analysis methods naturally affects the results from different studies. In wheat-based bran or whole-grain products AR concentrations have been measured to vary between 142-1784 μg/g DM (wholemeal wheat bread-wheat bran based products) (Ross *et al*. 2003c), and whole-grain wheat soft bread contained 202-353 μg AR /g DM (Chen *et al.* 2004). Rye crisp bread contained 490-804 μg AR/g DM (Chen *et al.* 2004), but AR values up to 1007 μg/g DM have been reported (Ross *et al*. 2003c). Soft whole-grain rye bread was reported to contain AR 197-686 μg/g DM (Chen *et al.* 2004). AR, as well as several other bioactive compounds in the bran, seem to remain intact during food processing (Liukkonen *et al*. 2003, Ross *et al*. 2003c).

One average serving of wheat-bran product contains at most ~70 mg AR and one serving (40 g) of whole-grain wheat bread about 10 mg AR (Ross *et al*. 2003c). Extreme consumers of whole-grains in the United Kingdom were suggested to receive up to ~200 mg AR/day (Ross *et al*. 2003b). Average daily intake of AR was estimated to be 11.8 mg/d in the and 22.9 mg/d in Sweden based on food consumption data and 11.9 (the United Kingdom), 17.5 (Sweden), 18.5 (Norway), 39.8 (Finland), and 37.1 (Denmark) mg/d based on food supply data (Ross *et al*. 2005).

AR concentrations *in vivo* have been unknown and consequently the role of AR in biological systems has been unclear. AR, which mainly exist in whole-grain wheat and rye products in human diet, have been suggested to function as specific biomarkers for whole-grain wheat and rye intake (Ross *et al*. 2004b). They could provide valuable information on the reliability of whole-grain intake data for epidemiological studies regarding the link between the intake of whole-grain products and the occurrence of certain chronic diseases.

1.3.2 Biological effects *in vitro*

Antioxidative properties

AR seem to be rather weak antioxidants *per se*, as pure compounds *in vitro*. Kamal-Eldin *et al*. (2001) examined AR antioxidant activity by radical inhibition and inhibition of auto-oxidation of sunflower oil triacylglycerols, in comparison to α-tocopherol. They saw no antioxidant activity for AR C15:0 regarding radical scavenging and hydrogen donation power. They explained the lack of antioxidant activity by meta-positioned hydroxyl groups, instead of ortho- or para-positioning that would lead to enhanced antioxidative properties, at least *in vitro* (Kamal-Eldin *et al.* 2001, Zhang 1999). They also remarked that the rather high redox potentials of AR might not be sufficient to compete with the scavenging of oxidised radicals. They criticised the previous results with positive antioxidative effects of AR (Kozubek and Nienartowich 1995, Winata and Lorenz 1996), and pointed out possible 4-methyl impurity in the tested AR solution and rather high concentrations of the tested AR compounds. AR seemed to be poor antioxidants compared to vitamin E (Kamal-Eldin *et al.* 2001). AR have shown a decrease in peroxidation products when incorporated into biological membranes (Kozubek and Nienartowich 1995). AR could influence other, more potent membrane-bound antioxidants, which could explain the stated increased antioxidant activity that seemed to be related to AR chain length in some studies. AR with longer chain lengths have appeared to be more potent antioxidants. AR metabolites might also play a role in antioxidative processes. (Hladyszowski *et al*. 1998, Kozubek and Nienartowich 1995, Kozubek and Tyman 1999, Ross *et al*. 2004b).

Parikka *et al*. (2005) conducted further *in vitro* studies on AR antioxidant activity, and deduced that AR had no direct antioxidative effect, tested by ferric reduction ability of plasma (0-1000 μM) or with 2,2-diphenyl-1-picrylhydrazyl

radical assay (0-15 μM) compared to hydrophilic ferulic acid. They also examined LDL oxidation by monitoring copper-induced formation of conjugated dienes after incubating LDL with AR C15:0 and C17:0 (0, 2.5, and 25 μM). They saw a 65-min increase in lag-time with C15:0 (25 μM). Similar effect, but to a lesser extent, was seen with C17:0. They concluded that AR antioxidant activity might be based on their ability to interact with biological membranes. (Parikka *et al*. 2006).

Alkylresorcinols in membranes

AR are lipophilic and are able to form liposomal membranes themselves and incorporate into biological membranes *in vitro* (Kozubek 1987a). They can affect the liposome size, increase membrane permeability, and enhance the bilayer rigidity and stability (Gubernator *et al*. 1999, Kozubek 1987a, Kozubek and Demel 1980). When incorporated into erythrocyte membranes *in vitro*, AR caused haemolysis of erythrocytes at extracellular concentrations of 10-15 μM. Haemolytic efficacy was dependant on the degree of saturation and chain length, monounsaturated and shorter chain homologues being more effective (Kozubek 1987b, Kozubek and Demel 1980, Kozubek and Tyman 1999). They can also affect membrane mobility and bind, mainly with hydrogen bonds, to adjacent cholesterol and phospholipids in the membranes (Kozubek *et al*. 1988), and to proteins, and interact with several membrane-bound enzymes (Kozubek 1995). AR, depending on the chain length, are able to inhibit acetylcholinesterase at concentrations of 18-90 μM, and increase the activity of Ca^{2+} -dependent ATPase with concentrations up to 50 μM. The effects were explained by direct interactions with the enzymes and by indirect membrane-altering effects. (Kozubek *et al*. 1992). AR are able to entrap aqueous solutions into the membranes (Przeworska *et al*. 2001).

Other biological effects

AR may possess antimutagenic and anticarcinogenic activities (Gasiorowski *et al.* 1996). AR, as other compounds with a resorcinol moiety, can inhibit cyclo-oxygenase-2 (COX-2) promoter activity, and thereafter might provide protection in colon carcinogenesis (Mutoh *et al*. 2000), to which increased COX-2 activity has been linked (Wu *et al*. 2003). According to Parikka *et al*. (2005), C15:0 and C17:0 out of the tested C15:0-C23:0 were the most effective at 100 μM and 24 h incubation in showing an antigenotoxic effect on hydrogen peroxide-induced DNA damage in HT29 human colon cancer cells. An increased cellular capacity against oxidative damage caused by AR was suggested. Authors pointed out that the AR concentrations were several times higher than those usually found in plasma, but speculated that high enough tissue concentrations could be reached to achieve these effects *in vivo*. (Parikka *et al.* 2006). Previously anticarcinogenic properties for AR with various chain lengths and tested with several cell lines, have been described, as reviewed by Ross et. al (2004) and Kozubek and Tyman (1999). AR up to concentrations 12.5 μg/ml (~33 μM), compared to anthocyanins

up to concentrations 25 μg/ml (~56 μM) were more potent in reducing the rate and frequency of induced mutations in cultured lymphocytes, while anthocyanins were more effective in inhibiting free radical generation in human granulocytes *in vitro* (Gasiorowski *et al.* 2000a). AR together with anthocyanins exhibited immunomodulatory effects in lymphocyte cultures (Gasiorowski *et al.* 2000b). Certain AR are able to cause copper-dependent DNA cleavage and inhibit DNA polymerase β (Lytollis *et al.* 1995, Starck *et al.* 2000).

AR have shown antimicrobial properties against gram-positive bacteria and certain fungi and filarial parasites (Himejima and Kubo 1991, Kanda *et al.* 1975, Suresh and Kaleysa 1990). AR seem also to inhibit glycerol-3-phosphate dehydrogenase and prevent triacylglycerol accumulation in adipocytes (3T3-L1 cells), and they showed a 50% reduction of adipocyte triacylglycerols with concentrations of 10.7-5.0 μM for C15:0-C21:0 (Rejman and Kozubek 2004). AR have been shown to increase platelet thromboxane biosynthesis at concentrations of 0.02-2 μM, and the effect was suggested to be due to their haemolytic properties on the platelets or their free radical scavenging properties (Hengtrakul *et al.* 1991).

1.3.3 Animal and human studies

Absorption and elimination

Average ileal digestibility of AR from rye bran-enriched bread in human subjects was ~58% and seemed to be higher for shorter chain homologues, ~59% for C17:0-C21:0 and ~47% for C23:0-C25:0. Decreased uptake was suggested with higher AR intake (Ross *et al.* 2003a). With pigs the diet affected AR ileal digestibility, and recoveries, i.e. the amount not absorbed for ileal digesta were 37% after whole-grain and 40% after aleurone, and significantly lower, 21% ($p < 0.01$), after pericarp-testa based diet. These diets contained different amounts of AR. Pericarp-testa diet provided the lowest and aleurone diet the highest amounts of AR. The dose and dietary source of AR, as well as AR metabolism by intestinal microflora were suggested to affect the absorption. (Ross *et al.* 2003b).

AR distribution was studied with rats that were fed a single dose (4.6 mg/kg) of [4-^{14}C]-5-n-heneicosylresorcinol (^{14}C21:0). Most of the radioactivity was found in faeces (61%) and urine (31%), with peak amounts at 24h. Hydrolysis of the urine samples resulted in higher recovery suggesting conjugation of the compounds or their metabolites. In faeces, ^{14}C21:0 was found mostly unchanged and hydrolysis did not improve the recovery. In another experiment with rats with ^{14}C21:0, almost no radioactivity could be detected after 60h in blood, while peak amounts were seen at 7-12 h. (Ross *et al.* 2003b). AR have been detected also in rat perirenal adipose tissues with concentrations of ~2-4 μg/g of fresh tissue (Ross *et al.* 2004a).

Human urine collected after wheat bran diet contained minute amounts of intact AR (mainly AR 17:0) in conjugated forms. It was suggested that AR are metabolised by ω-oxidation, with further shortening of the alkyl chain by β-oxidation, and conjugated with glucuronide and/or sulphate (Ross *et al*. 2004b). The end metabolites in urine were determined to be 3,5-dihydroxybenzoic acid and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (Ross *et al*. 2004d). No quantisation of the compounds was reported.

Suggested metabolic interactions of alkylresorcinols

Rats fed a diet with high amounts of AR (1, 2, or 4 g/kg diet or 100-450 mg/kg body weight/day) had elevated γ-tocopherol concentrations in liver and lungs ($p < 0.01$), while plasma of γ- or α-tocopherol did not change (Ross *et al*. 2004a). The authors presented a hypothesis according to which AR inhibit tocopherol-ω-hydroxylase pathway, where γ-tocopherol is the major substrate and α-tocopherol a poor substrate (Parker *et al*. 2004), and thereafter inhibit the elimination of γ-tocopherol. AR also showed inhibition of synthesis of γ-tocopherol water-soluble hydroxychroman metabolites in Hep G3 cell cultures. The sesame seed lignan, sesamin, blocks the cytochrome P450 enzyme, tocopherol-ω-hydroxylase, and therefore strongly inhibits tocopherol ω-hydroxylation, a key step in tocopherol metabolism in hepatocytes. This was used as a positive control. The effects of AR compared to sesamin were weaker, and the authors concluded that AR, instead of direct blockage, might competitively inhibit tocopherol metabolism. Shorter chain AR showed a more pronounced effect and they might be more extensively metabolized by tocopherol-ω-hydroxylase pathway, or longer chain AR might be poorer substrates for ω-oxidation and thereafter less able to compete for the enzyme binding (Ross *et al*. 2004b). High intake of AR (4 g/kg diet) in rats caused decreased (47%, $p < 0.001$) liver cholesterol concentrations and reduced the percentage of cholesterol (35%, $p < 0.05$) in liver lipids compared to a diet with no AR (Ross *et al*. 2004a).

Effects on growth

AR were previously regarded as antinutritional components in rye. Ross *et al*. reviewed the studies carried out to examine animal growth with rye-based diet (Ross *et al*. 2004b). Two studies with high AR intake concentrations linked AR with decreased growth of rats and pigs (Sedlet *et al.* 1984, Wieringa 1967). Two other studies with similar AR concentrations showed no changes in the growth of rats, when they were fed a diet fortified with purified AR compared to a control diet with no AR (Bock *et al*. 1981, Ross *et al*. 2004a). Animal growth inhibition with high rye intake was thought to be due to rye soluble fibre fraction, mostly arabinoxylan, instead of AR (Ross *et al*. 2004b, Vinkx and Delcour 1996). No direct cytotoxicity was seen with AR (Rejman and Kozubek 2004). Rats have tolerated AR C15:0 up to 5 g/kg body weight (Suresh and Kaleysa 1990). For resorcinol LD_{50} acute oral dose has varied from 300 to 980 mg/kg (Lynch *et al*.

2002). Ross *et al*. speculated if possible tissue accumulation of AR exists that could lead to high AR concentrations in body (Ross *et al*. 2004b), but no evidence exists that this could present any health risks for humans if AR are consumed as a normal part of the diet.

1.4 Absorption and metabolism of lipophilic compounds, with vitamin E as an example

Lipophilic compounds, such as vitamin E and AR, with high $\log_{10}$ octanol-water partition coefficients are easily incorporated into oil and probably behave in a similar manner in the gastrointestinal tract (Borel 2003, Sakaeda and Hirano 1998). Such highly lipophilic food microconsituents are absorbed in duodenum *via* the lymphatic system, secreted in chylomicrons, and transported to liver. The absorption is variable, and the concentrations measured depend on dose, matrix where they are introduced into the bowel, and the method of absorption measurement. Absorption is assumed to follow the same intraluminar events as for the major dietary lipids, which are emulsification, solubilisation in micelles, diffusion across the unstirred water layer, and permeation through the enterocyte membrane. The acidic environment in the stomach can liberate the lipophilic compounds from the food matrix *(*. Dietary lipids can affect the absorption of other lipophilic compounds by facilitating the release of the compounds from the matrix by providing a hydrophobic phase, stimulating biliary secretion and micelle production, and increasing their solubility into micelles, and by inducing chylomicron synthesis and thereafter enhancing transport of the compounds from enterocytes. Other factors affecting absorption of lipophilic compounds are the structure of the compound, molecular linkages, amounts consumed, and individual metabolism (Borel 2003).

Metabolism of lipophilic tocopherols and tocotrienols (vitamin E) has been examined extensively. They are absorbed unesterified from the small intestine after incorporation into micelles in the presence of bile salts and pancreatic enzymes, incorporated into chylomicrons and secreted into lymph. Chylomicron remnants formed by lipoprotein lipase hydrolysis from triacylglycerols are taken up by the liver and thereafter secreted in VLDL. Some vitamin E from chylomicrons and VLDL is transferred to peripheral cells, e.g. to liver, skeletal muscle and adipose tissue, and to HDL, and some ends up in LDL *via* VLDL and HDL metabolism (Bjørneboe *et al.* 1990, Hacquebard and Carpentier 2005). LDL and HDL are the main vitamin E carriers (Behrens *et al*. 1982, Hall *et al*. 2005). In women, HDL contained more α-tocopherol than LDL, while the opposite was seen in men. VLDL had the greatest capacity to carry α-tocopherol (μg/mg protein) but it is present only in small amounts in fasting plasma (Behrens *et al.* 1982, Cohn *et al.* 1992). There is transfer of α-tocopherol between LDL and HDL, while VLDL and chylomicrons are poorer donors of α-tocopherol (Cohn *et al.* 1992). To get more reliable information of individual vitamin E status in human subjects and to

take into account the variations of carrier lipoprotein concentrations, it has been recommended that plasma tocopherol concentrations should be related to plasma lipid concentrations (Horwitt *et al*. 1972). Vitamin E is secreted into bile, and also metabolised into water-soluble products and secreted into urine (Birringer *et al*. 2001, Bjørneboe *et al*. 1990, Frank 2005). For vitamin E, the plasma concentrations reflect vitamin redistribution of the newly absorbed vitamin E rather than absorption, and there is a lack of dose-dependent increase in plasma concentrations. It is not known if there is a limit for vitamin E absorption (Borel 2003). Vitamin E uptake varies widely between individuals even when it is rather stable for one individual over time (Roxborough *et al*. 2000). The ratios of distribution of α-tocopherol and γ-tocopherol in plasma lipids (μmol/g) and erythrocytes (μmol/l) were 0.80 and 0.67, respectively (Lehmann *et al*. 1988). It has been suggested that erythrocyte vitamin E status might be more sensitive compared to plasma concentrations to predict individual susceptibility to oxidative stress and atherosclerosis (Simon *et al*. 1998). The molecular structure of vitamin E resembles that of AR, and it can be assumed that AR behave in a similar manner as vitamin E during absorption and transportation in plasma.

2 Aims of the study

Alkylresorcinols (AR) are phenolic lipids that exist in high amounts in the outer layers of wheat and rye grains and in processed whole-grain products. It has been suggested that AR could function as specific biomarkers for whole-grain intake in human diet. Such a biomarker is needed to get precise information of whole-grain intake and to elucidate the effects of whole-grain consumption on human health. Several biological functions, determined mainly *in vitro*, are described for AR, and the effects are mostly seen in biological membranes, where AR are incorporated *in vitro* due to their lipophilic nature. AR have attracted increasing interest as potential biomarkers and as bioactive compounds in human nutrition. In this work, AR usability as biomarkers, their occurrence in human subjects, and AR absorption in pigs were investigated.

The aims of the present study were:

1. To develop a quantitative gas chromatographic-mass spectrometric method for the determination of AR in plasma (Study I).

2. To measure AR concentrations in human plasma after the intake of high-fibre whole-grain rye bread or low-fibre wheat bread compared to the habitual Finnish diet, and to compare the concentrations to the serum ENL concentrations in postmenopausal women (Study II).

3. To determine AR incorporation into human erythrocyte membranes *ex vivo* and to measure their concentrations in the membranes after the intake of whole-grain products (Study III).

4. To evaluate AR absorption and elimination kinetics in pig plasma and to measure the absorption of AR after constant intake of whole-grain rye or whole-grain wheat bread and after a whole-grain rye bread bolus in pigs (Study IV).
5. To analyse AR concentrations in plasma, erythrocytes, and lipoproteins *ex vivo* after the intake of whole-grain rye or wheat crisp bread compared to low-fibre wheat bread, and to compare plasma AR concentrations to serum ENL concentrations (Study V).

3 Materials, study designs, and methods

3.1 Human studies

3.1.1 Analysis of human plasma alkylresorcinols after intake of whole-grain rye bread compared to low-fibre wheat bread (Study II)

Subjects

This study was part of a larger study in which the effects of fibre-rich rye bread on glucose and lipid metabolism, and on insulin metabolism were examined (Juntunen *et al*. 2003). The study comprised 39 post-menopausal healthy women 59±0.94 (mean±SEM) years old with a body mass index (BMI) of 26.8±0.5 kg/m^2. Postmenopausal women were selected because of an increased susceptibility to type II diabetes mellitus. The inclusion criteria were elevated serum total cholesterol concentration (5.0-8.5 mmol/L), non-HDL-cholesterol 3.5-6.5 mmol/L, and BMI 20-33. The subjects did not have diabetes mellitus or diseases that could affect lipid metabolism or the function of bowel and they did not use any medication (lipid lowering medication, laxatives, or corticosteroids) for such conditions. Serum follicle stimulating hormone concentrations were used to determine the postmenopausal status.

Study design and diets

The subjects were advised to keep their body weight and lifestyle habits, such as exercise, alcohol consumption, and smoking, as well as regular medication constant during the study. They were instructed not to use any cholesterol-lowering foodstuffs, probiotics, or products affecting bowel function. During the run-in period before the study, the subjects kept a 4 day food record to determine average energy intake. The study design (2x8 weeks randomized crossover intervention with an eight-week wash-out period) and the dietary advice given to the participants have been previously described together with the whole study (Juntunen *et al.* 2003).

All breads were baked in Fazer Bakeries Ltd. (Vantaa, Finland) and Vaasan & Vaasan Oy (Helsinki, Finland) bakeries. High-fibre rye bread (17% of dietary fibre) portions weighed 24.1-28.1 g and low-fibre wheat bread (2.8% of dietary fibre) portions 20.8-25.0 g. One portion of rye bread contained 206 kJ (range 174-234 kJ) energy and 4.4 g (4.1-4.6 g) of fibre, and a portion of wheat bread 241 kJ (233-249 kJ) energy and 0.6 g (0.5-0.8 g) of fibre. AR contents of the breads were not determined. Minimum bread intake was 4-5 portions of the

test bread. No maximum for bread intake was set, but subjects were advised to eat bread according to their normal cereal consumption and to keep the intake of cereal products constant. During the bread periods subjects replaced all bread and cereal products in their diet with the test bread so that the energy from bread was at least 20% of the total daily energy. Subjects were allowed to eat one additional portion of wheat or rye cereal products (sweet pastry or porridge) per day during both bread periods, and rice and pasta according to their normal eating habits. The portion was recommended to be rye-based product during the rye bread period and wheat-based product during the wheat bread period, but this was not obligatory. Apart from cereal products subjects were instructed to follow their normal eating habits during the follow-up. Dietary compliance was followed by food records. Over-night fasting blood samples were taken before and after each period, and plasma AR and serum ENL concentrations were analysed. The nutritional composition of test breads was analysed at VTT Biotechnology (Espoo, Finland). The Ethics Committee of Kuopio University Hospital, Kuopio, Finland approved the study.

3.1.2 Incorporation of alkylresorcinols into erythrocyte membranes (Study III)

This was a pilot study, in which nine volunteers participated. Four of them (Group I) avoided rye, wheat and barley products for one week and then continued for another week with their habitual Finnish diets, including whole-grain rye bread (average 3.5 pieces per d) and whole-grain wheat bread rich in AR (2 pieces per d). Four subjects (Group II) followed their normal diets with whole-grain rye (2 pieces per d) and whole-grain wheat bread (2 pieces per d) for two weeks, and one subject maintained a gluten-free diet with no rye, wheat or barley products. The breads were commercially available whole-grain wheat and rye bread. Overnight fasting blood samples were drawn after the first and the second week of the experiment and plasma and erythrocyte AR concentrations were analysed. The Ethics Committee for the Department of Medicine, Helsinki University Central Hospital, Helsinki, Finland approved the study.

3.1.3 Distribution of alkylresorcinols in human plasma, erythrocytes, and lipoproteins (Study V)

Subjects

Eight women and seven men, age 24.9±1.2 years (mean±SEM) and BMI 24.4±0.99 kg/m^2, participated in the study. The exclusion criteria were diseases or medication affecting bowel function, plasma lipoproteins, or erythrocytes, antibiotics taken within three months before the study, serious chronic illnesses, and alcohol abuse. Haemoglobin had to be above 125 g/l for women and 135 g/l for men.

Study design and diets

The study was a 2x1 week randomized crossover study with one-week wash-out periods before the study weeks. Subjects were instructed to eat 8 pieces (100 g) of whole-grain wheat (wheat diet, WD) or whole-grain rye crisp bread (rye diet, RD) per day during the test bread periods. During the wash-out periods they consumed soft low-fibre (non-wholegrain) wheat bread (LF I and LF II). Subjects were allowed to eat one portion of white low-fibre product, such as pasta, but not any additional bread or any other whole-grain cereal products other that the study breads, during the follow-up. Otherwise their dietary habits were not changed. Energy, protein, carbohydrate, fat, and fibre contents were 1300 kJ/100g DM, 8.7 g/100g DM, 64 g/100g DM, 2.1 g/100g DM, 17 g/100g DM for rye crisp bread, and 1500 kJ/100g DM, 11 g/100g DM, 66 g/100g DM, 5.9 g/100g DM, 10 g/100g DM for wheat crisp bread. Whole-grain flour content was 72% of DM for rye crisp bread and 48% of DM for wheat crisp bread. AR homologue content for whole-grain rye crisp bread was C17:0 148 μg/g DM, C19:0 275 μg/g DM, C21:0 145 μg/g DM, C23:0 69 μg/g DM, and C25:0 53 μg/g DM, total AR 690±15.8 μg/g DM and for whole-grain wheat crisp bread C17:0 14.4 μg/g DM, C19:0 138 μg/g DM, C21:0 118 μg/g DM, C23:0 26.9 μg/g DM, and C25:0 10.7 μg/g DM, total AR 308±5.1 μg/g DM. Soft low-fibre bread contained 1150 kJ/100g of energy, 10 g/100 g of protein, 47 g/100 g of carbohydrates, 4.8 g/100g of fat, and 3.8 g/100 g of fibre. It contained no whole-grain flour, and contained no AR. All breads were provided by Vaasan & Vaasan Oy (Espoo, Finland).

Over-night fasting blood samples were taken after WD, RD, LF I, and LF II, and plasma, erythrocyte, and lipoprotein AR concentrations, serum ENL concentrations, and serum α- and γ-tocopherol and retinol concentrations were analysed. Haemoglobin, blood cells, and lipoprotein fractions were measured from all samples. In addition, plasma AR and serum ENL concentrations were analysed after habitual diet before the study and one week after the study. Subjects recorded the intake of the breads and any other cereal products during the study. The Ethics Committee for the Department of Medicine, Helsinki University Central Hospital, Helsinki, Finland approved the study.

3.2 Animal studies

3.2.1 Kinetics of alkylresorcinols in pig plasma (Study IV)

Pigs

The experimental part of the study was carried out by Prof. K.E. Bach Knudsen's research group at the Department of Animal Health, Welfare and Nutrition, Danish Institute of Agricultural Sciences, Foulum, Tjele, Denmark. Four male castrated pigs from the Danish Institute of Agricultural Sciences' Swine herd,

Foulum, Denmark with an initial body weight of 44.6±2.4 kg were used in the study.

Study design and diets

The study comprised two parts, Part 1 and Part 2. Two experimental diets with soft and crisp wheat bread (WD) and soft and crisp whole-grain rye bread (RD) were used in the studies. Soft breads were produced by Nordmills Cerealia AB (Malmö, Sweden) and crisp breads by Wasa Bread AB (Filipstad, Sweden). Rye crisp bread contained whole-grain rye flour, rye bran (Wasa T2), fat, salt, and wheat crisp breads white wheat flour, purified wheat fibre (essential cellulose, Vitacel WF 600, Rettenmair and Söhne, Ellwagen-Holzmühle, Germany), sugar, salt, and dry malt as main ingredients. WD and RD were balanced with vitamins and minerals, and provided 19, 15, and 66% of energy from fat, protein, and carbohydrates, respectively. There was 119 g/kg DM of protein, 68 g/kg DM of fat, 773 g/kg DM of carbohydrates, and 230 g/kg DM of dietary fibre in WD and 127 g/kg DM of protein, 73 g/kg DM of fat, 699 g/kg of carbohydrates, and 237 g/kg DM of fibre in RD. WD did not contain any AR. RD contained AR homologues C17:0 262 μg/g DM, C19:0 372 μg/g DM, C21:0 284 μg/g DM, C23:0 134 μg/g DM, and C25:0 106 μg/g DM, with a total amount of 1158 μg AR/g DM.

Each pig was surgically fitted with two catheters, one in the portal vein and the other in the mesenteric artery. The pigs were given Streptocillin up to 4 days after the surgery. Up to one week before surgery pigs were fed a diet made of wheat, barley, and soybean (AR content 54 μg/g DM). The last week before surgery and 10 days after the surgery pigs were fed whole-grain rye diet with casein, vitamins, and minerals (873 g whole-grain rye/d, AR content 655 μg/g DM). After this the pigs were gradually introduced to experimental diets in a cross-over design (Part 1). The pigs were fed either rye (1250±15 g DM/day (mean±SEM)) or wheat (1250±24 g DM/day) bread in equal amounts three times daily (at 07.00, 15.00, and 22.00 hours) in a cross-over design. Portal and arterial samples were collected on days 5 and 7 at -30, 0, 30, and then at 60 min intervals up to 540 min. Pigs were fed again at 480 min. After this trial, the pigs were fed a semi-synthetic diet with wheat starch (707 g/kg), cellulose (80 g/kg), casein (182 g/kg), and vitamins and minerals (28 g(kg) with no AR for 4 days, deprived of food for 24 hours and then given one portion of RD (492 g DM) (Part 2). Blood samples were taken at -30, 0, 30, and then at 60 min intervals up to 960 min, after which the pigs were fed RD. The study was repeated the following week. The pigs in Part 2 with exception of one were the same as in Part 1. The experiment followed the guidelines of the Danish Ministry of Justice.

The study designs of the human and animal studies are summarised in Table 2.

Table 2. *The study designs of human (Study II, III, and V) and animal studies (Study V).*

Study	Number of subjects	Age (years)	BMI	Test breads	Bread intake	Study design	Wash-out period	Wash-out duration
II	39	59±0.94	26.8±0.5	Low-fiber wheat/ whole-grain rye bread	178±6.5/ 214±7.1 g/d	2x8 weeks	Habitual Finnish diet	8 weeks
III Group I	4	nd	nd	Whole-grain rye/ whole-grain wheat bread	nd	1x1 week	Low-fiber wheat bread diet	1 week
III Group II	4	nd	nd	Habitual Finnish diet	nd	2 weeks	-	-
V	15	24.9±1.2	24.4±0.99	Whole-grain wheat/ whole-grain rye crisp bread	95.5±2.1/ 97.5±0.97 g/d	1x1 week	Low-fiber wheat bread diet	1 week
IV Part I	4 pigs	-	-	Low-fiber wheat/ whole-grain rye diet	1250±15/ 1250±24 g DM*/d	1x1 week	Whole-grain rye diet	10 days
IV Part II	4 pigs	-	-	Whole-grain rye diet	492 g	single feeding	Semi-synthethic low-fiber wheat diet	5 days

*DM=dry matter

3.3 Methods

3.3.1 Solvents and reagents

Methanol and n-heptane were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Acetic acid, diethyl ether, sodium chloride, copper sulphate and sodium hydroxide were purchased from Merck KGaA (Darmstadt, Germany). Trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) were obtained from Pierce Chemical Co. (Rockford, IL, USA). DEAE (diethylaminoethyl) -Sephadex A-25 and Sephadex G-25 were purchased from Pharmacia Biotech AB (Uppsala, Sweden), pyridine from Romil Ltd. (Waterbeach, Cambridge, UK), and EDTA was from Sigma-Aldrich Co (St. Louis, MO, USA). Buffers were prepared at the HUSLAB, Hospital District of Helsinki and Uusimaa, Helsinki, Finland. A standard sample used in retinol and tocopherol analysis was a certified serum standard (SRM 968a, National Institute of Science and Technology, Gaithersburg, MD, USA) .

3.3.2 Alkylresorcinol reference compounds

5-*n*-Alkylresorcinols (C15:0, C17:0, C19:0, C21:0, C23:0 and the internal standard C20:0) were prepared by M.Sc. K. Parikka in Prof. K. Wähälä's group, Department of Chemistry, University of Helsinki, or purchased from ReseaChem Life Science, Burgdorf, Switzerland.

3.3.3 Instrumentation

The gas chromatography-mass spectrometry (GC-MS) instrument was a Fisons instrument MD 1000 quadrupole mass spectrometer combined with a Fisons GC 8000 gas chromatograph (Fisons Instruments, Manchester, UK). The column used was a 12 m BP-1 column from SGE (SGE International Pty. Ltd, Australia) with film thickness of 0,25 µm and internal diameter of 0,22 mm. Helium was used as the carrier gas with a flow rate of 1 ml/min. The temperature of the column was kept at 150 °C for 1 minute, then raised 40 °C/min to 230 °C and further 9 °C/min to 290 °C, where it was kept for 2 minutes, then raised 12 °C/min to 300 °C, where it was kept for 6 minutes. The temperatures of the injector and the ion source were 300 °C and 250 °C respectively. The interface temperature was 270 °C. The ionization energy was 70 eV. The splitless injection volume of the sample was 1 µl. After one minute splitless time, the split-ratio was 1:30. Plasma lipoproteins, triacylglycerols, and proteins were measured from serum by Konelab 60i (Thermo Electron Corporation, Waltham, MA, USA). Haemoglobin and blood cells were measured from blood by Sysmex KX-21 (TOA Medical Electronics Co., LTD, Kobe, Japan). Haematocrit measurement from erythrocyte suspensions was performed with Adams Autocrit Centrifuge (Clay-Adams Inc.,

New York, USA). Lipoprotein ultracentrifugation was performed with Beckman Optima LE-80K ultracentrifuge with Ti 50.4 rotor. Serum ENL was analysed with AutoDELFIA 1235 Automatic Immunoassay System (Wallac Oy, Turku, Finland).

3.3.4 Isolation of lipoproteins

VLDL (density 1.006 g/ml), LDL (density 1.019-1.063 g/ml), and total HDL (density 1.063-1.21 g/ml) were isolated from 3 ml of plasma by sequential ultracentrifugation as previously described (Havel *et al.* 1955, Höckerstedt *et al.* 2002). Samples were stored at -80 °C until further analysis. Lipoprotein fractions (sample volume 2 ml) were purified, and EDTA, unbound AR, and other small molecular weight compounds were removed by size exclusion gel filtration with Sephadex G-25 column (1x20 cm) and eluting with phosphate buffered saline (pH 7.4) (Helisten *et al*. 2001). A method by Lowry *et al*. was used to measure the protein concentrations of the collected fractions (Lowry *et al*. 1951).

3.3.5 Quantitative analysis of alkylresorcinols

Plasma

An internal standard AR C20:0 in methanol (1.8 µg/ml, 20 µl) was added into 500 µl of plasma and the samples were incubated with 500 µl of ionized water at +37 °C overnight and extracted three times with 3 ml of diethyl ether and vigorous shaking with a Vortex mixer. Organic phases, separated by freezing the water phase, were collected and evaporated to dryness, and purified with an ion-exchange chromatography with DEAE–Sephadex A-25 column (column dimensions 0.5x1.5 cm) in a free base form (Fotsis and Adlercreutz 1987). The sample was applied to the column in 2x300 µl of methanol and neutral steroids were eluted with 6 ml of methanol and discarded. Alkylresorcinols were collected by eluting the column further with 6 ml of 0.1 M acetic acid in methanol. This fraction was evaporated to dryness and th samples were derivatised with silanising agent (pyridine: HMDS:TMCS 9:3:1 (v:v:v)). Ions with m/z 464, 492, 520, 534, 548, and 576 for alkylresorcinols C15:0, C17:0, C19:0, C20:0, C21:0, and C23:0, respectively, were monitored and used for quantitative determinations. In addition an ion with m/z 604 was used for the detection of the alkylresorcinol C25:0 in plasma. An ion with m/z 268 for all alkylresorcinols was used as a confirmatory ion where necessary. Quantification of alkylresorcinols was done by relating the peak area of the analyte to the peak area of the internal standard (AR C20:0) in the sample. Standards with known amounts of alkylresorcinols were analysed in the same way. Based on the measured peak area ratio of the standard and the known amount of alkylresorcinol in the standard mixture, a linear graph was drawn. The equation for the line was used to calculate the amount of each alkylresorcinol in the sample. The method was validated for the quantification of alkylresorcinols C15:0, C17:0, C19:0, C21:0, and C23:0. The

standard graph for alkylresorcinol C23:0 was used for the quantification of the alkylresorcinol C25:0.

Erythrocytes

Erythrocytes were analysed with a modified method for the analysis of tocopherols (Bieri *et al*. 1979, Linko and Adlercreutz 2005, Simon *et al*. 2001). Erythrocytes were separated from plasma by centrifugation (3500 x g, 10 min, 10 °C). In Study III the cells were washed three times with 0.15 M NaCl in 10 mM Na-phosphate buffer, pH 7.4, and made to a 50% haematocrit suspension. Samples were stored at -20 °C until analysis. An internal AR standard C20:0 was added to 3 ml of suspension and proteins were denaturated with 5-7 ml ice-cold methanol and vigorous shaking. AR were extracted twice with 10 ml n-heptane, which was evaporated to dryness. In Study V erythrocytes were washed three times with 0.9% NaCl-solution. The washing solution was removed and 1 ml of cells was pipetted, into which 1 ml of 2 mmol/l EDTA-solution was added. The cells were stored at -80 °C. An internal standard C20:0 was added and proteins were denaturated by adding 5 ml of methanol and shaking. In both studies AR were extracted three times with 7 ml of n-heptane. After extraction, the samples were dissolved into 0.5 ml of methanol and purified with DEAE-Sephadex A-25 (column dimensions 0.5x1.5 cm) in a free base form, and analysed with the GC–MS as with the plasma method.

Lipoproteins

VLDL, LDL, and HDL AR concentrations were analysed otherwise similar to the plasma method, but they were extracted four times with 4 ml diethyl ether to achieve better extraction recovery.

Test breads

The analysis of AR in breads was performed by PhD A. Ross (Study IV) and by MSc R. Landberg (Study V) in Prof. P. Åman's research group at Department of Food Science, Swedish University of Agricultural Science, Uppsala, Sweden. AR were analysed by GC, and GC-MS where necessary (Ross *et al*. 2001a, Ross *et al*. 2003c).

3.3.6 Serum enterolactone

Serum ENL concentrations were measured by a time-resolved fluoroimmunoassay (Adlercreutz *et al.* 1998, Stumpf *et al.* 2000b). [6,7-^{3}H]-estradiol-17-glucuronide was used as an internal standard. Unconjugated ENL was extracted with diethyl ether from the samples after overnight incubation with 2 M sulphatase and 0.2 M β-glucuronidase. The samples with assay buffer, antiserum in bovine serum albumin (dilution 1:250 000), and europium-labelled

ENL derivative (dilution 1:400 000) were added on prewashed goat anti-rabbit IgG microstrips and analysed with the AutoDELFIA 1235 Automatic Immunoassay System.

3.3.7 Other analytical methods

Retinol, and α- and γ-tocopherol analysis was done at Prof. Georg Alfthan's laboratory at the Department of Epidemiology and Health Promotion, National Public Health Institute, Helsinki, Finland. Ethanol solution (50%, 800 µl) with ascorbic acid, butylated hydroxytoluene, and tocol as an internal standard for tocopherols was added to the sample (100 µl), and the samples were mixed and analytes were extracted with 1 ml hexane. An aliquot of 800 µl was evaporated to dryness under nitrogen and redissolved in 100 µl methanol. An Inertsil ODS-3 column (2.1x100 mm, 3 µm, GL Sciences, Japan), with methanol as the mobile phase (0.3 ml/min, injection volume 5 µl), was used to separate α- and γ-tocopherols. Tocopherols were detected using peak height tocol -ratios by their fluorescence at 292/324 nm. Retinol was detected in a similar way at 325 nm. Plasma standard sample traceable to a certified serum standard was used as a control for tocopherols and freshly prepared all-retinol standards in ethanol was used as a control for retinol.

3.3.8 Quality assurance

Two to three pooled control plasma samples, either pooled plasma from the Finnish Red Cross or pooled plasma from other studies, were included in every plasma analysis batch together with a blank sample of water. For erythrocytes duplicates of a control sample, which was a sample from one person in Study III and a sample pooled from one day samples from all subjects in Study V, and a blank sample were included in every batch. In lipoprotein AR measurements we used two pooled LDL-samples from other studies together with a blank sample. If the blank sample contained any AR, the whole batch was reanalysed. Plasma and erythrocyte samples were analysed as duplicates, but due to the scarcity of the sample material, lipoprotein AR were analysed as single measurements. In ENL analysis, duplicates of three control samples with different concentrations were analysed. In all analyses, if control samples exceeded±2SD of the total average value of the analysed samples, the analysis was repeated. If the coefficient of variation (CV) for duplicate analytes deviated more than 15%, the sample was reanalysed.

3.3.9 Assessment of pharmacokinetic parameters

The pharmacokinetic parameters calculated in Study III were maximum plasma concentration (C_{max}), time of peak concentration observed (t_{max}), area under the plasma concentration time curve from 0 to 8 or 15 h ($AUC_{0–8\ h}$/$AUC_{0–15\ h}$) corrected for baseline (0 h), the elimination half-life, and the absorption half-life.

The parameters were calculated with PK Solutions 2.0 Noncompartmental Pharmacokinetics Data Analysis program (Summit Research Services, Montrose, CO, USA).

3.3.10 Statistical analysis

The normality of the data was tested with Shapiro-Wilk's test. A correlation analysis was done with Spearman's rho. Non-parametric statistics (Friedman's test followed by Wilcoxon's signed rank test) were used to determine the between-group and within-group differences in the studies. When data was normally distributed, paired samples T-test and general linear model with repeated measures were used to test differences in study groups. Statistical analysis was performed with SPSS 11.0.1 (SPSS Inc., Chicago, IL, USA) or with Minitab version 11 (State College, PA, USA). Differences were considered significant at the 95% confidence level ($p < 0.05$). Results are expressed as mean±SEM, and as a sum of AR homologues C17:0-25:0, unless otherwise stated.

4 Results

4.1 Analytical methods for alkylresorcinols in plasma, erythrocytes, and lipoproteins (Study I, II, III, IV, and V)

Plasma

Analysis of plasma AR consists of sample incubation with water, extraction of the analytes, purification of the sample by ion-exchange chromatography, and analysis by gas chromatography-mass spectrometry. Incubating the samples with enzymes β-glucuronidase (0.1 U/ml) or sulphatase (1 IU/ml) did not improve the yield compared to overnight incubation with water. However, the overnight incubation gave higher yields and better precision compared to shorter incubation (3 h), but with a 36 h incubation no improvement in the results was seen. Diethyl ether, 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)) were tested as the extraction solvents. Diethyl ether gave the best extraction recovery of the analytes. We tested DEAE-Sephadex (column dimensions 0.5x3 cm) in acetate form and Sep-Pak C18 solid phase extraction (Waters Co., Milford, MA, USA) in addition to DEAE-Sephadex (column dimensions 0.5x1.5 cm) in a free base form in sample purification. Poor recovery of AR was found with Sep-Pak C18 or DEAE-Sephadex in acetate form, and the analytes were eluted in several fractions, probably due to binding of AR to the column.

AR-free plasma either from a person following a gluten-free diet or carbon-treated plasma removing AR was used when the recoveries were tested. The mean recovery for all alkylresorcinol standards was 112±10.8% (mean±SD). Limit of detection was 2.1-3.3 pg and limit of quantification 4.1-6.5 pg on column. The range of linearity was up to 230, 270, 220, 650, and 210 pg per injection and up to 22.8, 26.6, 32.2, 32.6, and 30.8 ng in a sample for AR C15:0, C17:0, C19:0, C21:0, and C23:0, respectively. Most of the analysed samples fitted to this range. If the sample AR concentration was higher, it was diluted and reanalysed. C25:0 could not be tested, since we did not have a reference compound for that compound. C23:0 was used to quantify C25:0.

A sample chromatogram of plasma AR and the respective AR standards is presented in Figure 2. With whole-grain rye diet, in front of AR homologue peaks C19:0, C21:0, C23:0, and C25:0 smaller peaks with the same base ion fragment (m/z 268) as for AR are seen. These are monounsaturated derivatives of AR. Similar peaks have also been detected in cereal samples (Ross *et al*. 2004c). Due to the lack of reference compounds and small amount of these compounds in the samples, we did not quantify these peaks.

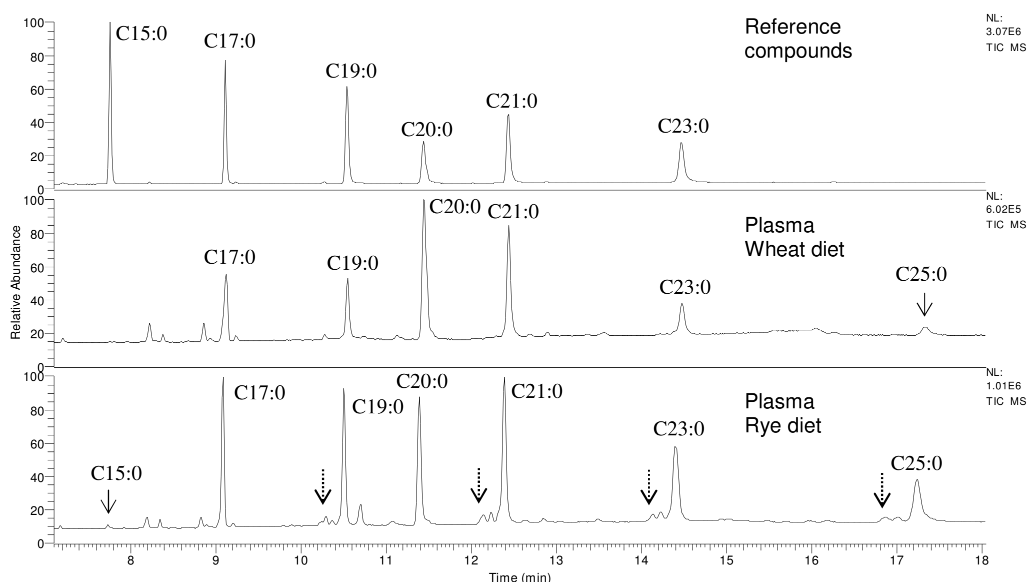


Figure 2. *Total ion current chromatogram of alkylresorcinols in a standard mixture and in a plasma sample from the same person after whole-grain wheat and rye diets. The dashed arrows point to the unsaturated derivatives of alkylresorcinols C19:0, C21:0, C23:0, and C25:0 in plasma.*

Erythrocytes and lipoproteins

At first erythrocytes were separated from blood and the cell membranes were isolated with hypotonic lysis (Clark and Switzer 1977, Hanahan and Ekholm 1974). AR concentrations were similar to those of washed whole erythrocytes indicating that AR are incorporated into the cell membranes. In Studies III and V the two different washing solutions (0.15 M NaCl (0.9%) in 10 mM Na-phosphate buffer and 0.9% NaCl-solution) did not cause any difference in the results. In Study V the washed erythrocytes were pipetted, which seemed to be more precise than preparing and measuring a 50% haematocrit solution as was done in Study III. Erythrocyte AR extraction was tested with diethyl ether and n-heptane. Diethyl ether was difficult to separate from buffer-methanol solution, and n-heptane therefore gave better analyte recoveries and was used in the analyses. After washing the cells and protein denaturation with MeOH the analysis method was similar to that of plasma analysis, except for the extraction solvent. With lipoprotein analysis the extraction with diethyl ether was performed four times to increase the yield of AR. Otherwise the method was the same as for plasma AR. The actual recoveries after addition of reference compounds could not be performed for erythrocyte or lipoprotein samples, since added AR would not have been incorporated into the erythrocyte membranes or lipoproteins, which would have affected the recoveries and would not have given data comparable to samples acquired *in vivo*.

Precision

Intra-assay CV for analysis of AR in plasma samples was <10% (n=10) during method development (Study I), and <10% in Study II and III, 10% in Study IV, and <10% in Study V. For ENL, intra- and inter-assay precision was <10% in Studies II and V. In Study V for erythrocytes, intra-assay precision was 5% (n=10). Interassay coefficients of variation are presented in Table 3. In Study V, the inter-assay CV for lipoproteins was 20%.

Table 3. *Inter-assay precision of alkylresorcinol analysis in plasma, erythrocytes, and lipoproteins.*

Study	AR (nmol/l or nmol/l packed cells)	CV (%)	Number of assays
Study I (Plasma)	344	9.6	7
Study II (Plasma)	249	15.2	7
Study III (Plasma)	172	7.0	2
Study IV (Plasma)	189	15.0	12
Study V (Plasma)	466	11.9	4
Study V (Erythrocytes)	178	10.8	8
Study V (Lipoproteins)	4	20	23

4.2 *Kinetics of alkylresorcinols in pigs (Study IV)*

Arterial and venous AR concentrations were similar (r^2=0.96, $p < 0.05$) in all measurement time points when the pigs ate rye diet on habitual basis for 5 to 7 days (Part 1), and when they were fed a single rye bread meal (Part 2). There were no clear differences in the absorption of AR homologues C17:0-C25:0 after habitual whole-grain rye bread diet (Figure 3) or wheat bread diet (data not shown) in Part 1 or after single rye bread feeding in Part 2 (Figure 4).

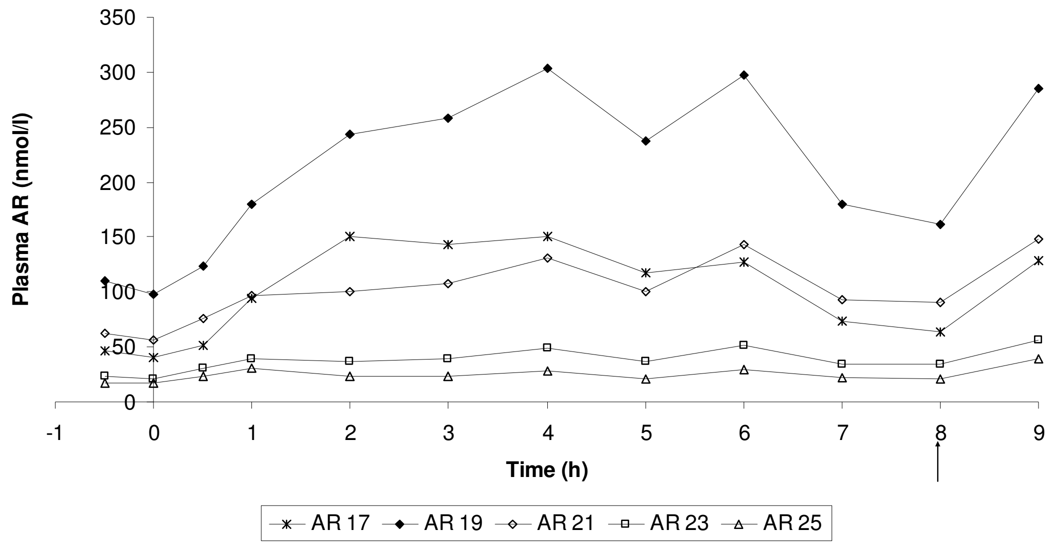


Figure 3. *Mean pig plasma alkylresorcinol (AR) homologue (C17:0-C25:0, nmol/l) concentrations after habitual rye consumption (Part 1 of Study IV, n=4) measured after morning feeding. The arrow shows the second feeding.*

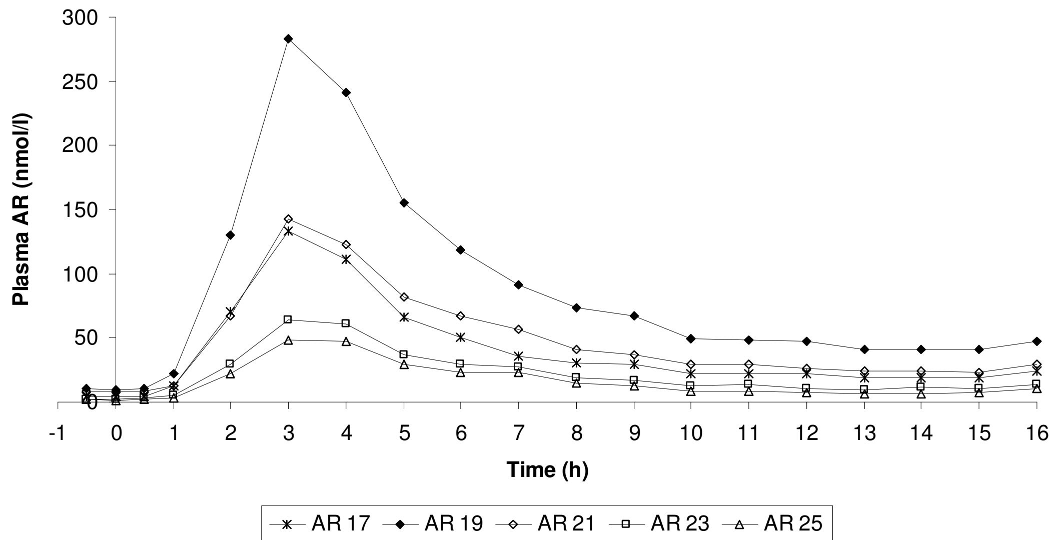


Figure 4. *Mean pig plasma alkylresorcinol (AR) homologue (C17:0-C25:0, nmol/l) concentrations after intake of a single rye bread portion (Part 2 of Study IV, n=4).*

In Part 1, the maximum AR plasma concentrations varied between the pigs, and within the pigs after habitual rye consumption in repeated studies. The maximum AR concentrations were 732 and 971, 538 and 895, 744 and 613, and 1406 and 854 nmol/l for the four pigs, respectively, in repeated studies. The t_{max} varied from 3 to 6 h. The baseline AR concentrations during habitual rye intake after a 9-hour food deprivation were 102-772 nmol/l (260±77 nmol/l), being in five out of seven cases less than 200 nmol/l.

In Part 2, the individual and between animal variations in peak plasma AR concentrations were lower. The maximum concentrations from repeated studies were 989 and 913, 500 and 701, 821 and 753, and 605 and 672 nmol/l for the four pigs respectively. The t_{max} was similar in repeated studies for all pigs (3-4 h), except for one. The individual curves for the appearance of AR in plasma in Part 2 are presented in Figure 5.

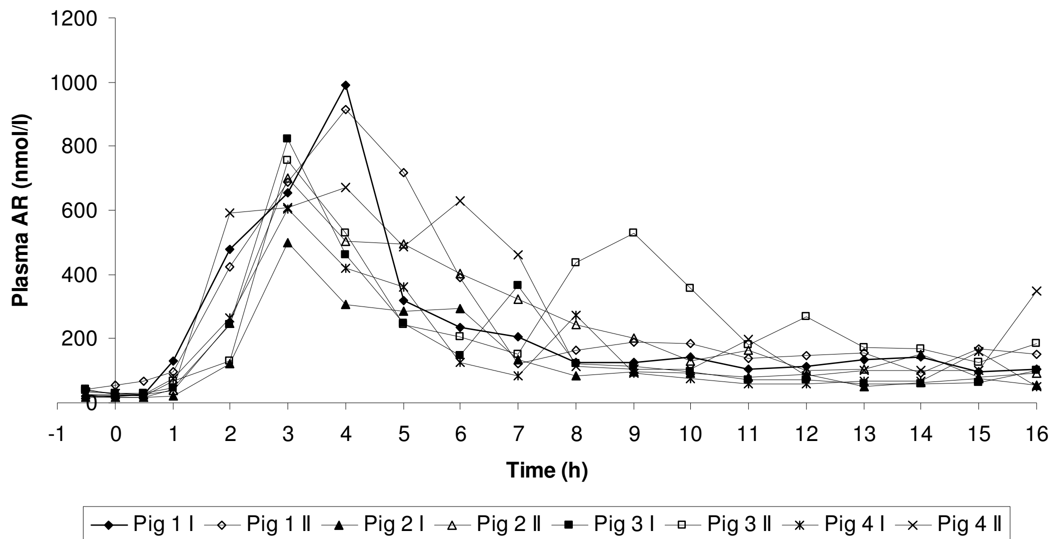


Figure 5. *Individual appearance of total alkylresorcinols (AR) in plasma (nmol/l) after single intake of rye bread in pigs with repeated feedings (Part 2 of Study IV, n=4 with repeated studies marked as I and II*).

The absorption half-life for total AR was 1.20 h, and the apparent elimination half-life 3.93 h. The absorption and elimination half-lives were similar for all homologues C17:0-25:0 (1.02-1.57 h and 3.35-3.96 h respectively for arterial and venous samples). Half-life duration was not related to AR homologue chain length. The area under the curve was slightly larger when the pigs were fed rye diet for the second time in Part 1 and 2 of the study, but the difference did not reach statistical significance. Plasma AR baseline concentrations were ~30 nmol/l after AR-free diet, although no AR were detected in the diet breads. Before the

test diet, the pigs were fed basal diet containing minute amounts of AR. After single feeding of the bread during the AR-free diet, AR concentrations in the pigs increased up to 74 nmol/l at 180 min.

4.3 Plasma alkylresorcinol and serum enterolactone concentrations (Study II, III, IV, and V)

Plasma AR concentrations after the low-fibre diets with little or no AR in human subjects (one week in Study III and V, eight weeks in Study II) and in pigs (5 days in Study IV) were low. In different studies, the variations in plasma AR were large after the habitual diet. However, within the studies with repeated periods and measurements the average plasma AR concentrations were similar. AR concentrations returned to the baseline after eight weeks with habitual diet in Study II after 39 women were fed whole-grain rye or low-fibre wheat diet for eight weeks. In Study III AR concentrations remained the same throughout the two-weeks follow-up with the habitual diet (n=4), and were remarkably lower compared to the other studies with habitual diets, being on average at the same level as the values of the other four subjects in this same study with low-fibre diet. In Study V (n=15) plasma AR concentrations in human subjects were lower after the one-week habitual diet at the end of the trial (total of four weeks) compared to the concentrations with habitual diet at the beginning of the study. In human subjects, the intake of whole-grain rye bread 214±7.1 g/d (n=39, mean±SEM) for eight weeks in Study II, and the intake of whole-grain rye crisp bread 97.5±0.97 g (n=15, 690 µg AR/g DM) for one week in Study V, increased plasma AR concentrations significantly. With pigs in Study IV, the intake of rye bread, 1250 g DM/d (1158 µg AR/g DM) for 5-7 days, raised plasma AR concentrations similarly as in human subjects. Intake of whole-grain wheat bread, 95.5±2.1 g (n=15, 308 µg AR/g DM) for one week in Study V, increased plasma AR concentrations about half of that with rye bread. See Table 4 for plasma AR concentrations measured from fasting samples after the study periods.

Table 4. *Fasting plasma alkylresorcinol (AR) concentrations (nmol/l) in human subjects in Studies II, III, V, and for pigs in Study IV after intake of whole-grain wheat and rye breads, mixed whole-grain diet, low-fibre breads, or habitual diet for different periods. Values are presented as mean±SEM (range). Two values in the same column in the same study show the results from repeated periods within that study. Means in a column without a common letter differ ($p < 0.05$, Wilcoxon signed rank test).*

	Plasma AR (nmol/l)				
	Whole-grain wheat bread diet	Whole-grain rye bread diet	Mixed whole-grain bread diet	Low-fibre wheat bread diet	Habitual diet
Study II (n=39, 8 wk)	-	352±24.7[a] (147-766)	-	36.6±4.2[b] (10.9-130)	97.7±12.0[c]/ 88.3±8.7[c] (34.1-418)/ (12.9-245)
Study III (n=4+4,1 wk)	-	-	207±54.7[a] (56.3-288)	32.4±12.2[b] (4.4-55.5)	37.8±15.4[c]/ 41.2±23.5[c] (10.8-69.0)/ (11.0-110)
Study IV (n=4 pigs, 5-7 d)	-	260±93.1[a] (140-537)	-	34.6±4.9[b] (24.2-47.7)	-
Study V (n=15, 1 wk)	107±14.1[b] (34.4-231)	222±24.5[a] (55.1-389)	-	30.1±10.7[c]/ 25.4±3.0[c] (5.5-171)/ (9.6-46.0)	127±33.9[b,d]/ 74.6±17.3[d] (7.5-434)/ (11.5-263)

Serum ENL concentrations were measured in Study II and V. They increased significantly with whole-grain rye bread intake in women (n=39) in Study II and in men (n=7) in Study V, but not in women (n=8) in Study V. The values are presented in Table 5.

Table 5. *Serum enterolactone (ENL) concentrations (nmol/l) after intake of whole-grain wheat or rye bread compared to low-fibre or habitual diet in Study II and V. Values are presented as mean±SEM (range). In Study V n=15 represents the combined data from women and men. Two values in the same column in the same study show the results form repeated periods within that study. Means in a column without a common letter differ (p < 0.05, Wilcoxon signed rank test).*

	Serum ENL (nmol/l)			
	Whole-grain wheat bread diet	Whole-grain rye bread diet	Low-fibre bread diet	Habitual diet
Study II (n=39, 8 wk)	-	$53.5±10.0^{a}$ (4.4-371)	$22.7±4.0^{b}$ (0.68-150)	$32.8±4.8^{c}$/ $27.1±3.9^{c}$ (0.08-136)/ (2.3-127)
Study V (n=15, 1 wk)	$11.4±2.8^{b}$ (0.71-41.3)	$20.6±3.1^{a}$ (1.3-38.7)	$9.7±1.4^{b}$/ $12.1±2.2^{b}$ (0.29-55.9)/ (1.6-27.5)	$16.5±3.7^{a,b}$/ $15.9±2.6^{a,b}$ (0.29-55.9)/ (0.74-37.0)
Study V Women (n=8, 1 wk)	16.2±3.0 (5.8-41.3)	22.8±3.1 (1.3-38.7)	12.9±1.1/ 17.3±2.0 (7.2-18.7)/ (3.3-27.5)	21.7±4.4/ 19.5±2.7 (3.5-55.9)/ (6.1-37.0)
Study V Men (n=7, 1 wk)	$5.9±1.8^{b}$ (0.71-21.4)	$18.0±3.2^{a}$ (1.4-35.8)	$6.1±1.0^{b}$/ $6.1±1.2^{b}$ (0.76-10.9)/ (1.6-14.6)	$10.5±2.2^{a,b}$/ $11.9±2.3^{b}$ (0.29-18.9)/ (0.74-25.4)

In human subjects, individual plasma AR concentrations after the eight-week (Study II) and one-week (Study V) periods varied widely after the intake of whole-grain breads (Figures 6 and 7). Inter-individual variation was remarkable even after daily intake of the same amount of breads in Study V (Figure 6). ENL serum concentrations also varied after eight-weeks (Study II) and one-week (Study V) whole-grain bread periods (Figures 8 and 9). In Figures 7 and 9 plasma AR and serum ENL patterns on individual basis during the follow-up are shown (Study V, n=15). With AR a clear pattern following the habitual, low-fibre, or whole-grain wheat or rye bread diets in almost all individuals during the follow-up was seen (Figure 7). Serum ENL concentrations did not change in all subjects (n=5 in Study II and n=2 men in Study V) in spite of high intake of whole-grain rye bread that is known to be rich in plant lignans from which ENL is produced. In certain cases, ENL concentrations decreased with the intake of whole-grain rye bread (n=3 in Study II and n=1 woman in Study V).

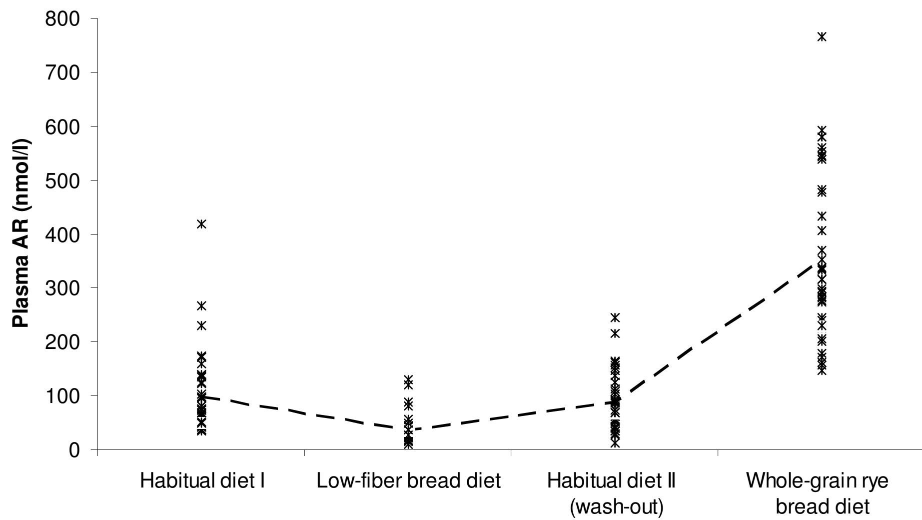


Figure 6. *Individual plasma alkylresorcinol (AR) concentrations (nmol/l) in human subjects after an eight-week habitual, low-fibre, or whole-grain rye bread diets (Study II, n=39). (--) mean of all subjects.*

Figure 7. *Individual variation of plasma alkylresorcinol (AR) concentrations (nmol/l) in human subjects (Study V, n=15) after habitual, low-fibre, and whole-grain wheat and rye bread diets (one-week periods). (--) mean of all subjects.*

Figure 8. *Individual serum enterolactone (ENL) concentrations (nmol/l) in human subjects after eight-week habitual, low-fibre, or whole-grain rye bread diets (Study II, n=39). (--) mean of all subjects.*

Figure 9. *Individual variation of serum enterolactone (ENL) concentrations (nmol/l) in human subjects (Study V, n=15) after habitual, low-fibre, whole-grain wheat and rye bread diets (one-week periods). (--) mean of all subjects.*

In Study V with human subjects (n=15) mean plasma AR concentrations increased significantly with increasing amounts of AR intake after one week AR-free low-fibre diet, whole-grain wheat bread, and rye whole-grain bread periods (0, 29, and 67 mg AR/d during LF, WD, and RD) (Figure 10). In Study II plasma AR concentrations correlated to whole-grain rye bread intake (r=0.34, $p < 0.05$).

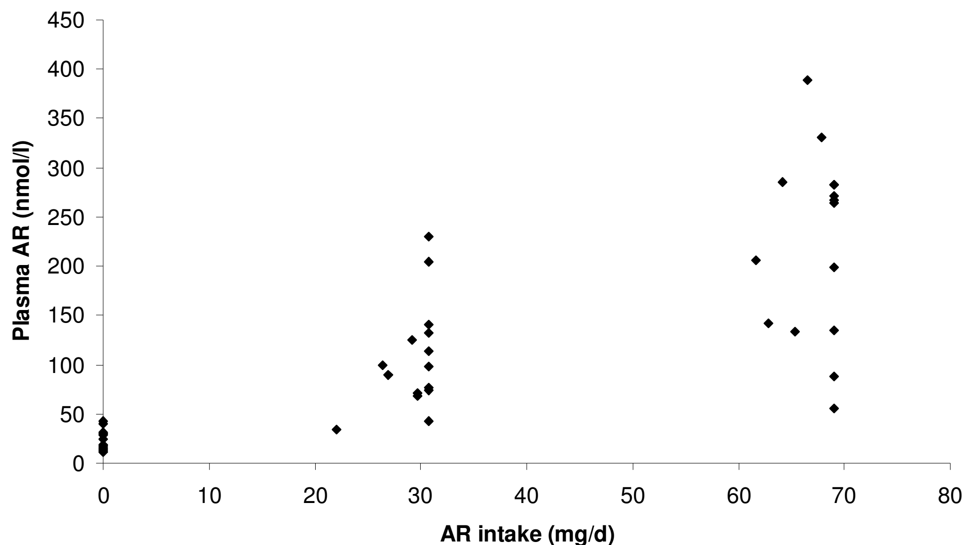


Figure 10. *Relation of mean plasma alkylresorcinol (AR) concentrations (nmol/l) to alkylresorcinol intake after intake of alkylresorcinol-free low-fibre diet (0 mg AR/d), whole-grain wheat bread (29 mg AR/d) and whole-grain rye bread (67 mg AR/d) (one-week periods) with repeated measurements (Study V, n=15).*

Plasma AR homologue C17:0/C21:0 -ratio was significantly different after a one-week intake of whole-grain wheat and rye breads in Study V compared to low-fibre diets or to habitual diet, and after an eight-week intake of whole-grain rye compared to low-fibre wheat and habitual diets in Study II (Table 6).

Table 6. *Alkylresorcinol (AR) homologue C17:0/C21:0 -ratio in plasma after intake of whole-grain wheat or rye bread, low-fibre wheat bread, and habitual diets in human subjects (Study II and V) and in pigs after constant intake and after single rye bread feeding (Study IV). Bread AR C17:0/C21:0 -ratio was measured in Study IV and in Study V. Values are presented as mean±SEM (range). Two values in the same column in the same study show the results form repeated periods within that study. Means in a column without a common letter differ ($p < 0.05$, Wilcoxon signed rank test).*

	AR C17:0/C21:0			
	Whole-grain wheat bread diet	Whole-grain rye bread diet	Low-fibre wheat bread diet	Habitual diet
Study II (n=39, 8 wk)	-	$0.84{\pm}0.04^{a}$	$0.53{\pm}0.08^{b}$	$0.74{\pm}0.06^{c}$/ $0.68{\pm}0.05^{c}$
Study IV (n=4 pigs, 1 wk)	-	$0.73{\pm}1.10^{a}$	$0.47{\pm}0.04^{b}$	-
Study IV (n=4 pigs, single feeding)	-	0.84±0.03	-	-
Study V (n=15, 1 wk)	$0.10{\pm}0.01^{b}$	$0.60{\pm}0.06^{a}$	$0.01{\pm}0.01^{c}$/ $0.05{\pm}0.03^{c}$	$0.28{\pm}0.05^{d}$/ $0.52{\pm}0.09^{a,d}$
Study IV, bread		0.92		
Study V, bread (n=3)	$0.12{\pm}0.002^{b}$	$1.02{\pm}0.01^{a}$	-	-

4.4 Alkylresorcinol occurrence in erythrocyte cell membranes (Study III and V)

We analysed erythrocyte AR in two studies (Study III and V), and showed that AR are incorporated into the cells *in vivo*. The concentrations decreased significantly during one-week low-fibre diets compared to whole-grain diets in both studies (Table 7).

Table 7. *Erythrocyte alkylresorcinol (AR) concentrations (nmol/l packed cells) in Studies III and V after one-week intake of whole-grain wheat or rye bread or mixed whole grain diet compared to habitual diet or one-week low-fibre diet. Values are presented as mean±SEM (range). Two values in the same column in the same study show the results form repeated periods within that study. Means in a column without a common letter differ ($p < 0.05$, Wilcoxon signed rank test).*

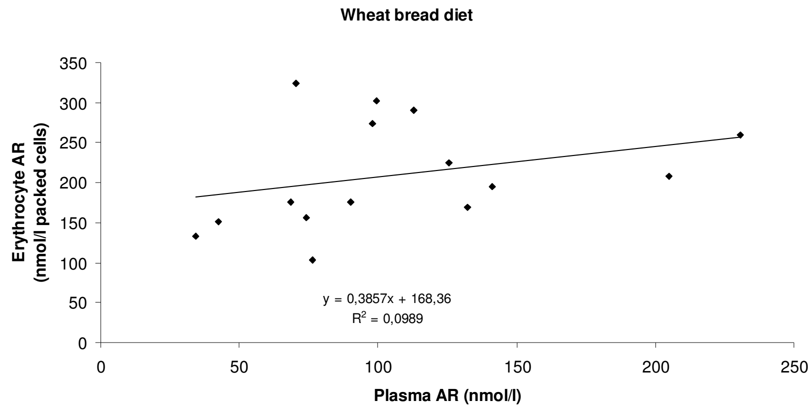
	Erythrocyte AR (nmol/l packed cells)				
	Whole-grain wheat bread diet	Whole-grain rye bread diet	Mixed whole-grain bread diet	Low-fibre bread diet	Habitual diet
Study III (n=4+4)	-	-	248±65.7[a] (136-436)	17.2±2.6[b] (13.2-24.3)	62.5±25.8[c]/ 69.6±17.7[c] (16.7-136)/ (25.8-107)
Study V (n=15)	210±17.3[a] (103-324)	315±31.2[a] (164-627)	-	50.9±6.4[c]/ 62.1±4.6[c] (23.0-111)/ (24.0-86.3)	-

In Study III plasma to erythrocyte AR ratio after the intake of whole-grain cereals (n=4+4) was 0.7±0.1. The ratio of AR in human plasma and erythrocytes was 0.7±0.1 after RD and 0.5±0.1 after WD (one-week periods), and 0.5±0.1 and 0.4±0.05 after one-week low-fibre periods (LF I and LF II) in Study V. The distribution of AR homologues C17:0-C25:0 in plasma and erythrocyte membranes after the intake of wheat and rye breads in Study V (n=15) and after mixed whole-grain diet in Study III (n=4+4) are shown in Table 8.

Table 8. *Alkylresorcinol homologue percentages in plasma and erythrocytes after one-week intake of mixed whole-grain diet (Study III, n=4+4) and whole-grain wheat and rye breads (Study V, n=15) and plasma to erythrocyte alkylresorcinol homologue ratios. Erythrocyte values marked (*) differ compared to plasma homologue ratio (paired samples test, p < 0.05).*

	C17:0	**C19:0**	**C21:0**	**C23:0**	**C25:0**
			%		
Study III					
Mixed whole-grain bread diet					
Plasma	13±2.3	34±0.75	34±2.6	14±0.18	5±1.2
Erythrocytes	5±1.5*	31±1.4*	36±2.5	16±2.2	12±0.79*
Plasma/erythrocytes	1.3±0.13	0.73±0.15	0.71±0.18	0.55±0.12	0.51±0.12
Study V					
Whole-grain wheat bread diet					
Plasma	5±0.56	23±0.77	53±1.1	15±0.74	4±0.63
Erythrocytes	3±0.27*	24±0.79	51±0.96*	16±0.67	7±0.55*
Plasma/erythrocytes	0.90±0.10	0.51±0.07	0.55±0.07	0.50±0.07	0.34±0.06
Study V					
Whole-grain rye bread diet					
Plasma	17±1.4	24±0.89	30±1.0	17±0.74	12±1.1
Erythrocytes	10±0.57*	27±0.83*	30±1.1	18±0.58	15±1.2*
Plasma/erythrocytes	1.2±0.10	0.63±0.05	0.72±0.07	0.70±0.09	0.59± 0.08

Correlations of erythrocyte AR concentrations to plasma AR concentrations with and without correction for plasma cholesterol concentrations are shown in Figures 11 and 12 after a one-week intake of whole-grain wheat and rye bread diets in human subjects in Study V (n=15). Plasma AR correlation to erythrocyte concentrations was higher, though not necessarily strong after adjustment to cholesterol.



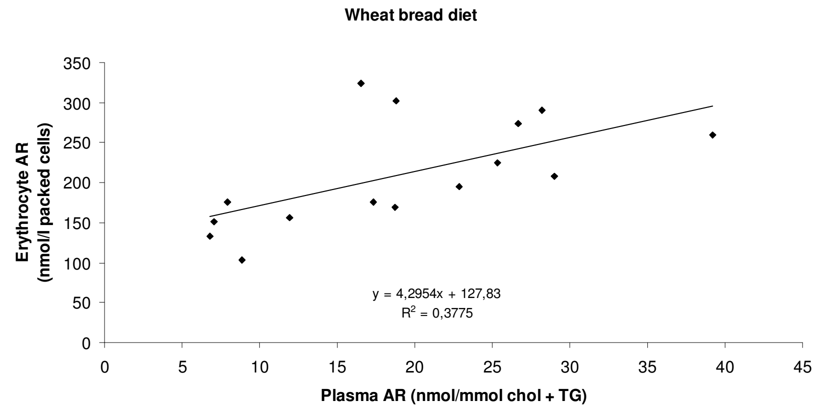
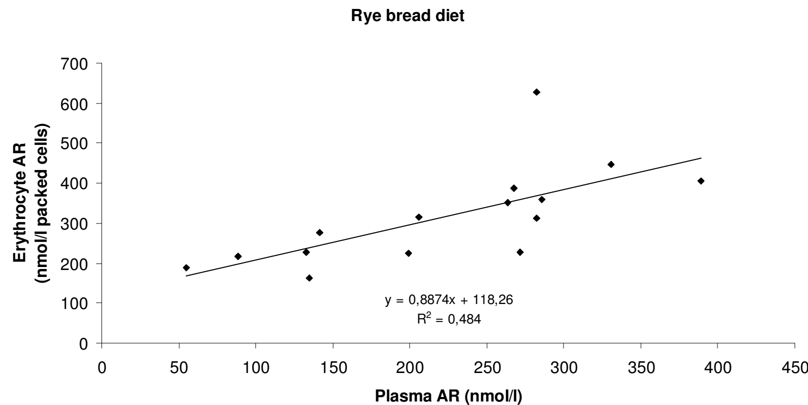


Figure 11. *Correlation of erythrocyte alkylresorcinol (AR) concentration (nmol/l packed cells) to plasma alkylresorcinol concentrations (nmol/l) and after adjustment for plasma cholesterol and triacylglycerols (nmol/mmol chol + TG) after one-week intake of whole-grain wheat in human subjects. (Study V, n=15).*



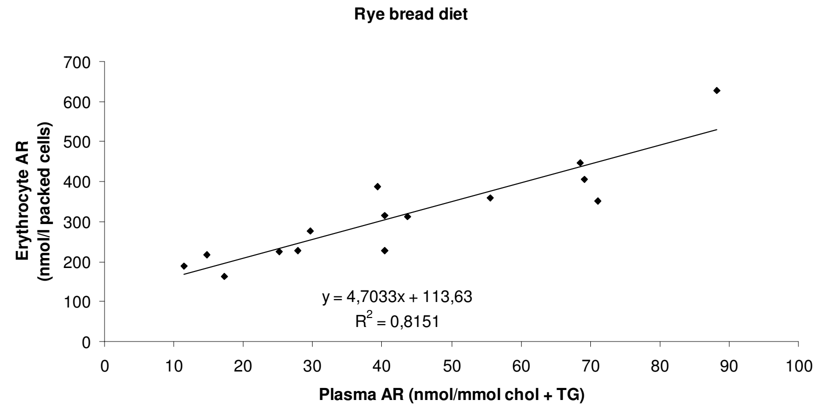


Figure 12. *Correlation of erythrocyte alkylresorcinol (AR) concentration (nmol/l packed cells) to plasma alkylresorcinol concentrations (nmol/l) and after adjustment for plasma cholesterol and triacylglycerols (nmol/mmol chol + TG) after one-week intake of whole-grain rye in human subjects. (Study V, n=15).*

In Study III the correlation (r^2) of plasma AR to erythrocyte AR concentrations was 0.530 (n=4) after one-week intake of whole-grain breads following a one-week low-fibre period, and 0.262 (n=4) after constant intake of whole-grain products for two weeks.

In human subjects erythrocyte mean corpuscular volume measured with an automatic cell counter did not change during the four-week follow-up (measured in one-week periods) in Study V, and AR incorporation into erythrocytes did not seem to change the cell size or affect haemoglobin values of the subjects.

4.5 Alkylresorcinol occurrence in lipoproteins (Study V)

We separated human fasting plasma VLDL, LDL, and total HDL with ultracentrifugation and analysed AR in these fractions. The samples were taken after one-week whole-grain rye and whole-grain wheat bread diets and after low-fibre diets. There were no differences in plasma or lipoprotein AR concentrations between women (n=8) and men (n=7), and therefore the data was combined for the analyses (n=15). The results did not differ depending on which whole-grain period was first, and the data from the two rye bread periods and that from the two wheat bread periods was combined. Lipoprotein concentrations did not change during the study.

AR plasma concentrations (nmol/l) correlated well to plasma AR concentrations adjusted for cholesterol and triacylglycerols (TG) (nmol/mmol cholesterol + TG) (r^2=0.868-0.936 after the different diets, $p < 0.001$) (Figure 13).

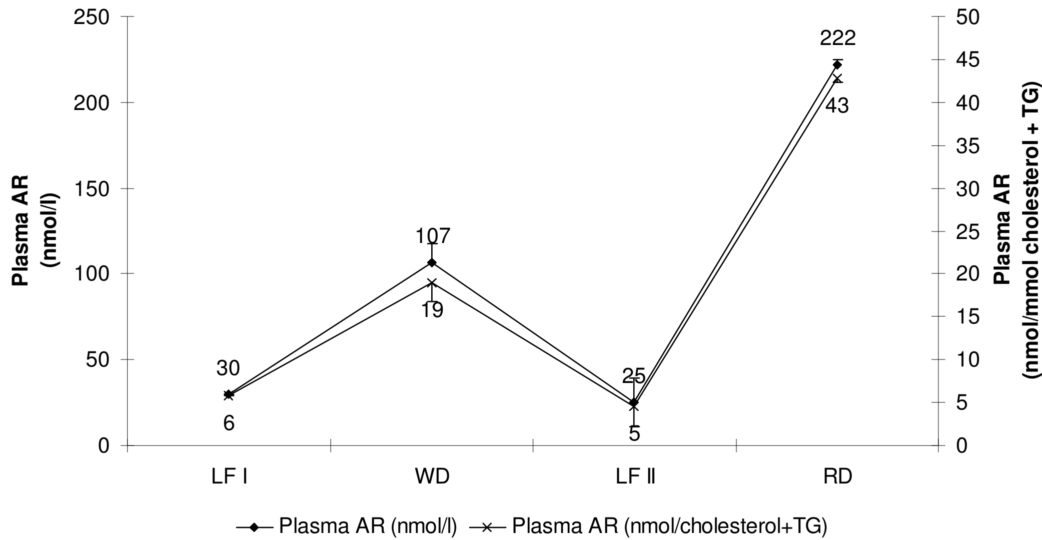


Figure 13. *Mean plasma alkylresorcinol (AR) concentrations before (nmol/l) and after adjustment for plasma cholesterol and triacylglycerols (TG) (nmol/mmol cholesterol + TG) with SEM in human subjects (Study V, n=15).*

After RD and WD 72±3.9% and 69±2.5% of plasma, AR concentrations were measured in the lipoproteins, respectively. The correlation between plasma (nmol/l) and total lipoprotein (nmol/l of plasma) AR was strong (r^2=0.839, $p < 0.05$ (RD), r^2=0.939, $p < 0.05$ (WD)). AR were quantified in all lipoprotein particles, proportionally in highest amounts in VLDL. Table 9 shows AR concentrations in total cholesterol and in lipoprotein subclasses calculated per litre of plasma.

Table 9. *Alkylresorcinol (AR) concentrations in total cholesterol and in lipoprotein classes (VLDL, LDL, and HDL) after low-fibre diets and whole-grain wheat and whole-grain rye bread diets. Values are presented as mean±SEM (range). Means in a column without a common letter differ (p < 0.05, Wilcoxon signed rank test).*

	AR (nmol/l plasma)			
	Low-fibre wheat bread diet I	Whole-grain wheat bread diet	Low-fibre wheat bread diet II	Whole-grain rye bread diet
Tot cholesterol	23.1±7.3 (6.1-119)	73.4±10.3[a] (25.7-176)	20.0±2.4 (8.6-46.2)	166±19.7[b] (55.8-329)
VLDL	11.7±6.3 (0-97.5)	35.8±8.6[a] (3.1-124)	8.7±2.0 (1.0-29.9)	69.4±14.9[b] (4.8-232)
LDL	3.9±0.54 (1.8-8.6)	16.0±2.1[a] (5.4-35.7)	4.1±0.59 (1.3-9.0)	37.2±4.2[b] (14.9-66)
HDL	7.5±1.1 (3.2-17.7)	21.7±3.0[a] (6.5-48.6)	7.2±0.68 (2.4-13.1)	59.7±10.6[b] (23.0-172)

AR homologue patterns in plasma, erythrocytes, VLDL, LDL, and HDL differed after the one-week intake of whole-grain wheat or rye in Study V. The homologue patterns also differed when measured from different matrices, although some homologues, especially C19:0, C21:0, and C23:0, existed in more similar proportions in the measured matrices. Plasma AR homologue pattern was closest to that of the breads consumed. Retinol (431-453 mg/l), α-tocopherol (8.2-8.6 mg/l) and γ-tocopherol (0.67-0.84 mg/l) concentrations did not change significantly during the follow-up (Study V).

5 Discussion

5.1 Method development and analysis of alkylresorcinols in plasma and biological membranes

Ross *et al.* reviewed the analysis methods for AR in foods and in biological samples (Ross *et al*. 2004c). Rauls and Penney analysed less lipophilic olivetol than AR in rabbit serum with HPLC-UV (Rauls and Penney 1982). AR metabolites have been analysed in urine by GC-MS after enzymatic hydrolysis and purification with thin layer chromatography (TLC), and intact AR in human and pig ileal digesta and faeces by GC-MS and GC-FID (Ross *et al*. 2003a, Ross *et al*. 2003b, Ross *et al*. 2004d). TLC purification and analysis with TLC and GC has been used for measurement of AR in rat adipose tissue and blood cells (Ross *et al*. 2004a), and HPLC-radiochemical detection has been used to detect ^{14}C-labelled AR metabolites in rat plasma, urine, and faeces (Ross *et al.* 2003b).

In the first study of this work a novel method for the analysis of AR in plasma samples was developed. AR can be measured from plasma with a relatively simple and fast quantitative GC-MS method that consists of extraction of alkylresorcinols from plasma, purification of the sample and analysis by GC-MS. During method development we noticed that enzymatic hydrolysis with sulphatase or β-glucuronidase did not increase measured plasma AR concentrations, indicating that AR are transported unconjugated in plasma. However, over-night incubation increased the yield compared to 3 h incubation, which is probably due to AR binding to plasma proteins, and liberation of them during the incubation. Later on we found that AR are incorporated into lipoproteins, which partly explains these findings. Diethyl ether was chosen as the extraction solvent due to its best extraction properties and easy evaporation. It has a dielectric constant of 4.33, which is between that of the tested petroleum ether and ethyl acetate, and it seemed to be most appropriate extraction solvent for lipophilic AR.

The GC-MS method presented here is the first quantitative method using synthethic reference compounds identical to the analytes and similarly behaving internal standard suitable for analysis of AR in biological samples in larger scale. We used synthetic internal standard AR C20:0, which does not naturally occur in plasma. Deuterium labelled or ^{13}C analogues for all AR homologues used as internal standards behaving identically to the analytes during sample pre-treatment and analysis, would have been the most accurate option and provide the best quantisation, but these were not available. AR C20:0 in the GC-MS elutes in the middle of all AR homologues, which might cause deviation of the results for longer and shorter chain AR eluting further away from C20:0. The same happens for the reference compounds used to quantify the analytes, which partly

corrects the deviation. AR C23:0 reference compound was used to quantify C25:0 due to lack of a synthetic AR C25:0. This can cause underestimation of C25:0 concentrations in the samples. The error, however, should be small according to the results we achieved when other AR homologues were calculated in a similar manner with AR reference compounds with alkyl chains two carbons shorter. High recovery in reference compounds during the method development can be explained with endogenous AR, although the blank was reduced from the values. The recoveries were measured with reference compounds and their concentrations could vary and cause deviation in the results. The same analysis method as for plasma was used for quantisation of lipoprotein AR with the exception at the extraction with diethyl ether, which was done four times to achieve better analyte recovery from fatty samples. The high CV in lipoprotein control samples is due to very low concentrations of AR in them, but these samples were the only ones available. The advantage of the plasma analysis method is fast and selective purification by ion-exchange chromatography instead of TLC. GC-MS is selective and highly sensitive and therefore only a fraction of a sample is needed.

In erythrocyte AR analysis we first isolated the cell membranes and analysed AR in those. We found out that the concentrations were the same as measured from washed, whole erythrocytes indicating that AR are incorporated into the cell membranes and that we actually measured the AR incorporated into the membranes instead of AR externally bound to the cells. We did not analyse unwashed cells, however. The analysis method was adapted from erythrocyte vitamin E analysis (Bieri *et al*. 1979, Simon *et al*. 2001). Vitamin E structure is similar to that of AR and is known to be incorporated into erythrocyte membranes. In a pilot study with human subjects (Study III) 0.15 M NaCl (0.9%) in 10 mM Na-phosphate buffer was used as a washing solution, but it was changed to 0.9% NaCl-solution that was more easily available (Study V). The two different solutions did not change the results. In Study III a 50% haematocrit solution of the washed cells was prepared and the haematocrit value, which was used in the concentration calculations, was measured. In Study V the washed cells were pipetted and the pipetting volume was used in the calculations. The precision with the latter method was better although pipetting the thick cell solution caused minor variation in the volumes.

5.2 Absorption and distribution of alkylresorcinols in plasma

AR absorption was tested in a pig model with habitual intake of AR and with a single feeding with AR-rich bread. There were no differences in the mesenteric artery and the portal vein AR concentrations during the absorption. This indicates that AR are not absorbed into the bloodstream *via* portal vein from the gut like amino acids, sugars, and short-chain fatty acids, but are absorbed *via* the lymphatic system like other lipophilic compounds. Since there were no

differences in arterial and venous AR concentrations, we were not able to calculate the actual absorption over time or dose-dependent absorption, but a response in plasma AR concentrations to AR intake was seen. With a single feeding a higher and sharper peak with more equal within and between pig concentrations of plasma AR was seen compared to prolonged AR consumption. This can be due to 24 h fasting before the feeding of the bolus of AR-rich whole-grain rye bread, and therefore accelerated gastric emptying and more effective absorption.

Time of the peak AR concentration (3-4 h) after single rye bread intake in pigs was similar to that of tocotrienols (3.3-4.4h for tocotrienols, as was the apparent elimination half-life, 3.93 h for AR (2.3-4.4 h for tocotrienols) (Schwedhelm *et al.* 2003, Yap *et al.* 2004). With human subjects a longer apparent elimination half-life, 4.8 h, was found (Landberg *et al*. 2006). This difference in the results remains to be clarified. Longer t_{max}-values (~10 h) for radiolabelled AR in a previous study with rats might indicate that in those studies AR metabolites, rather than intact, absorbed AR were measured (Ross *et al.* 2003b). The difference can also be due to heterogeneity in dietary habits or species specific factors.

In our study with pigs all AR homologues were absorbed in a similar manner. Plasma AR homologue pattern reflected that of the diet. This is in contrast to later studies with AR absorption in human subjects, where relative proportions of AR C23:0 and C25:0 increased in plasma, and C17:0 decreased over 1-5 hour period after a single intake of 190 mg of AR in rye bread (Landberg *et al.* 2006). In some pigs two peaks at 3-4 h and 6-9 h in AR plasma concentrations were seen, which could be caused by enterohepatic circulation or colonic absorption. This data is similar to that obtained with human subjects after single intake of AR, when two peaks in the absorption curves were seen, first at 2-3.2 hours, and the second at 6.6-6.8 hours (Landberg *et al.* 2006). Low within and between animal variations in AR concentrations with single rye feeding together with high inter-animal variation during habitual rye feeding might be due to individual metabolism and storage of AR in body tissues and liberation of them during constant intake. We detected AR in pig plasma (~30 nmol/l) after five to seven days controlled AR-free diet for, and after feeding the pigs with this diet, AR concentrations rose up to 74 nmol/l peaking at 3 h. This can be due to storage of AR in cell membranes or fatty tissues, and their liberation into bloodstream even with no AR intake. The slight peaking in AR concentrations could also indicate enterohepatic circulation. It is also possible that the pigs ate some AR in the diet, e.g. in the breads, although AR were not detected in the samples of the bread fed to the pigs during the AR-free diet period. This data is in contrast to studies with rats fed with radioactive AR (Ross *et al.* 2003b), where no radioactivity in blood was detected after 60 h. This might be caused by AR metabolism or differences in measurement techniques, lack of sensitivity of liquid scintillation counting, or other measurement dependent factors, or species-specific differences.

We showed that AR are incorporated into human erythrocyte membranes during the intake of whole-grain products. The ratio of AR in plasma and erythrocytes was 0.7±0.1 after whole-grain rye diet and 0.5±0.1 after whole-grain wheat diet (Study V, n=15). This ratio is similar, especially after high intake of AR from whole-grain rye bread, to that found for α- (0.80) and γ-tocopherol (0.67) when erythrocyte tocopherols were related to plasma lipids (Lehmann *et al.* 1988), and when ^{2}H-labelled α-tocopherol enrichment was tested in erythrocytes (45% enrichment) (Jeanes *et al.* 2005), suggesting that the distribution of AR in plasma and blood cells is similar to that of tocopherols. This is logical, since the AR molecular structure and thereafter e.g. the properties in distribution are similar to those for tocopherols. Based on our studies, there is no evidence of a specific transporter as for tocopherols (Hosomi *et al.* 1997). It is possible, however, that AR use, if any, the same transporters as tocopherols.

In Study III with human subjects it appeared that longer-chain AR are incorporated into erythrocytes in a higher proportion than shorter-chain AR. This is in agreement with *in vitro* studies (Kozubek and Tyman 1999), and is likely to be caused by higher hydrophobicity of long-chain AR and therefore more prone incorporation into lipophilic environment. In Study V with human subjects a similar tendency, but to lower extent, was seen with longer-chain AR. In Study V, the AR concentrations in erythrocytes during one-week low-fibre diet remained higher compared to Study III. This could be caused by more intensive whole-grain consumption and accumulation of AR in tissues and into the cell membranes during the whole-grain bread weeks in Study V, although the concentrations after the whole-grain periods were similar in both studies. *In vitro* studies have shown membrane permeability changes when AR are incorporated into them (Kozubek and Tyman 1999 and references therein). AR incorporation into erythrocytes did not change their size, measured with an automatic cell counter (Study V), indicating that no swelling or shrinkage due to permeability changes occurred *in vivo*. AR concentrations used *in vitro* have been 3.5-30 µmol/l (Kozubek 1987a, Kozubek 1987b). In our studies, the highest measured concentrations from fasting samples were 627 nmol/l packed cells. It is possible that soon after absorption higher AR concentrations in erythrocytes exist, which could have an effect on permeability properties. It is unlikely, however, that concentrations would reach the concentrations used *in vitro*.

In Study V, AR occurrence in lipoproteins VLDL, LDL, and HDL isolated from human plasma samples was examined. It was shown that AR are incorporated into all lipoprotein classes and seem to be transported exclusively in lipoproteins in human plasma in an unconjugated form. The amount of AR measured from all the lipoprotein fractions was close to that of total plasma AR related to plasma cholesterol level. Adjusting AR plasma concentrations to cholesterol gave better correlation to erythrocyte concentrations. Since AR are mainly transported in plasma lipoproteins (69-72%), it could be reasonable to adjust plasma AR concentrations to cholesterol concentrations as is

recommended for vitamin E to eliminate variations in carrier lipoprotein concentrations (Behrens *et al*. 1982, Bjørneboe *et al*. 1990).

In Study V, the AR homologue composition differed after whole-grain wheat and rye bread diets, and somewhat also in the measured matrices, plasma, erythrocytes, and lipoproteins, probably due to differences in incorporation of the homologues into the membranes. AR homologue patterns in plasma, erythrocytes, or lipoproteins did not correlate to each other when determined from different matrices. Plasma AR homologue pattern seemed to best reflect that of the bread consumed. VLDL had the greatest capacity to carry AR. However, since VLDL is present in small amounts in plasma, HDL together with VLDL seem to be the main AR carriers when AR concentrations are related to lipoprotein plasma concentrations (e.g. VLDL nmol/l plasma). LDL had AR concentrations about half of that in VLDL and HDL. This is in contrast to α-tocopherol, for which LDL and HDL are the main carriers in human fasting plasma. As for AR, VLDL had the greatest capacity to carry α-tocopherol, but contained proportionally lowest amounts of α-tocopherol (Behrens *et al.* 1982, Bjørnson *et al.* 1976). In nonfasting rats α-tocopherol was distributed between VLDL and HDL (Bjørneboe *et al*. 1986, Bjørneboe *et al*. 1990). In men LDL carried more α-tocopherol than HDL (Behrens *et al.* 1982), but this was not observed for AR in our study.

AR seem to be absorbed *via* the lymphatic system from the small intestine in chylomicrons, are most probably transported to liver, and from there to VLDL. From VLDL they can be redistributed to HDL and to LDL. HDL can possibly receive AR straight from chylomicrons, which would explain higher AR concentrations in HDL compared to LDL. This absorption pathway is comparable to that of lipophilic tocopherols, with chemical structure resembling that of AR (Bjørneboe *et al.* 1990, Frank 2005). Peripheral tissues, especially adipose and other fatty tissues, could also receive AR *via* lipoproteins. In rats, for example, AR are found in the adipose tissues (Ross *et al*. 2004a). Hypothetically, from LDL, AR could be delivered into macrophages or atherosclerotic plaques where they could have stabilizing effects, since *in vitro* AR can stabilize e.g. phospholipid bilayers (Gubernator *et al*. 1999, Kozubek *et al*. 1988, Kozubek and Tyman 1999). These theories naturally demand further research. The absorption, distribution, suggested metabolism and excretion of AR (Ross *et al*. 2004b, Ross *et al*. 2004d) are presented in Figure 14.

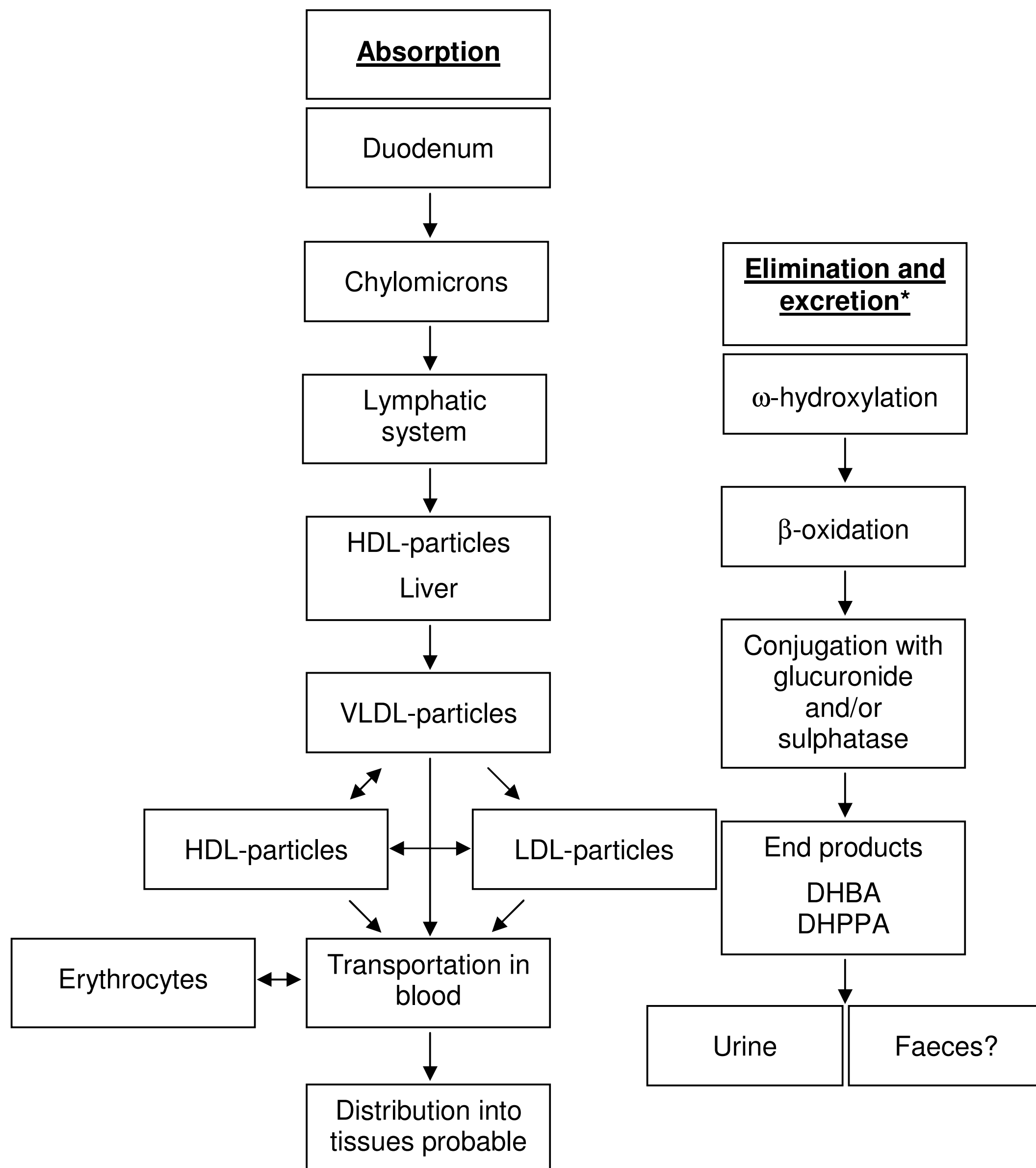


Figure 14. *Suggested absorption, metabolism, and excretion of alkylresorcinols *(Ross et al. 2004b, Ross et al. 2004d).*

Kamal-Eldin *et al*. (2001) found no changes in antioxidant activities with AR *in vitro*, but admitted that more studies of membrane stabilising and possible antioxidative effects of AR in the membranes, together with *in vivo* studies, should be performed to be able to conclude whether AR can function as antioxidants (Kamal-Eldin *et al.* 2001). Parikka *et al*. (2005) showed reduced LDL oxidation susceptibility with *in vitro* concentrations of 25 μmol/l with C15:0. Compared to our studies, the concentrations were high. It is unlikely that these concentrations could be reached *in vivo*. In our studies maximum concentrations were 766 nmol/l in plasma and 176 nmol/l of plasma in lipoproteins.

It is possible that AR cause changes in LDL and HDL membranes and therefore affect the oxidation susceptibility in this manner. AR antioxidant activity has been explained also to be due to their indirect effects on tocopherol, especially γ-tocopherol, levels (Kamal-Eldin *et al*. 2001, Ross *et al*. 2004a). Intake of AR increased γ-tocopherol concentrations in the liver in rats, and *in vitro* -studies have shown inhibitory effects of AR on γ-tocopherol metabolism (Ross *et al*. 2004a). In our study, α- and γ- tocopherol, as well a retinol, concentrations remained stable during the one-week intake of whole-grain wheat and rye bread. AR did not seem to affect the measured serum antioxidant concentrations. Cholesterol concentrations also remained unchanged during the one-week interventions. In our studies no liver or other tissue tocopherol or cholesterol concentrations were determined. It is possible that AR can cause changes in these tissues in the human body and affect the cell membrane properties, and directly or indirectly affect oxidative susceptibility.

Jenkins *et al.* (2002) saw no change in LDL oxidation susceptibility after intake of wheat bran for three months. The amount of formed conjugated dienes was measured, but no LDL oxidation resistance (lag-time) that commonly is used to measure LDL oxidation susceptibility was presented (Jenkins *et al.* 2002). Therefore the results are not directly comparable to our study. In the study by Harder *et al.* (2004) with intake of rye bran enriched bread for six weeks, no reduction of LDL oxidation lag time compared to habitual diet or wheat diet was reported. Habitual diet was used as a wash-out period, but bread intake was not restricted and the dietary intervention may not have been sufficient enough to affect LDL oxidation susceptibility, and the *in vitro* analysis of oxidation might not measure the changes caused by hydrophilic ferulic acid, which was tested as the active compound. (Harder *et al.* 2004). Jones *et al*. (2004) investigated plasma antioxidant capacity and urine antioxidant output after consumption of three daily portions (23 g) of whole-grain food for eight weeks, continued with six daily servings (23 g) of whole-grain food for another eight weeks. They reported progressively increasing antioxidant output in urine with increased whole-grain intake, but plasma antioxidant capacity did not change. There was speculation that fasting plasma samples were not good indicators of the antioxidant status (Jones *et al*. 2004, Seal 2006). Our study showed that AR are incorporated into human plasma lipoprotein particles. This differs from several other antioxidative

compounds that often are hydrophilic and stay in extracellular compartments, which can affect their efficacy on lipoprotein oxidation susceptibility.

5.3 Alkylresorcinols as biomarkers of whole-grain intake

AR concentrations increased significantly in all experiments after the intake of whole-grain products and decreased during low-fibre diets. With gluten-free and thereafter cereal-free diets no AR could be detected in human plasma. However, after low-fibre diets following habitual or increased consumption of whole-grain products, low amounts of AR could be detected in human or pig plasma. This suggests that AR are stored in tissues or cell membranes, or in lipoproteins and can be slowly liberated from there into plasma.

AR are absorbed rapidly form the small intestine, and their elimination half-life is rather short (3.93 h), according to our studies. With a single feeding of AR-rich whole-grain rye bread, pig plasma AR concentrations decreased within 16 hours, but remained nearly five times higher compared to the baseline. With constant intake of whole-grain rye in the same pigs, the AR concentrations were high after over-night fasting. A similar effect was seen in all human studies when constant intake of whole-grain rye or wheat was compared to low-fibre diets. With habitual Finnish diets AR concentrations were higher than with one- to eight-week low-fibre diets in all studies. Plasma AR concentrations appeared to further increase with all subjects with increased intake of whole-grain wheat or rye products. In Study II, a correlation between AR plasma concentrations and intake of rye bread was seen. However, in Study V with one-week constant intake of whole-grain rye or wheat, no correlation on individual basis between bread, AR, or fibre intake to fasting plasma or erythrocyte AR concentrations was seen. We did not find a dose-dependent relationship with the intake of cereal products and plasma or erythrocyte AR. When mean values of plasma AR concentrations from all subjects were evaluated, a clear and statistically significant increase in plasma AR concentrations compared to increasing AR intake from whole-grain wheat and rye breads was seen.

In Study V after the intake of the same amount of AR (mg/d) in the diet, a large inter-individual variation in plasma after whole-grain wheat bread diet (34.4-231 nmol/l) and whole-grain rye bread diet (55.1-389 nmol/l) was seen. This can be explained by differences in individual factors affecting AR absorption, such as nutritional status, other foods consumed together with the test bread, including the amount of fat in the diet, and factors affecting the transit time and function of the intestinal tract. In Study V, a large variation in erythrocyte AR concentrations especially after one-week low-fibre periods compared to Study III was found. After Study III, it was speculated whether erythrocyte AR could be used as long-term biomarkers due to more stable concentrations after low-fibre diet. Erythrocyte AR concentrations were higher compared to plasma indicating AR storage in the

membranes, which potentially could reflect long term intake. Plasma and erythrocyte AR concentrations correlated, though not necessarily strongly (r^2=0.484 with rye diet in Study V (n=15) and r^2=0.530 with mixed whole-grain diet in Study III (n=4)). The correlation was stronger after adjustment of plasma AR concentrations to plasma cholesterol and triacylglycerols (r^2=0.8151 in Study V (n=15)). Erythrocyte AR concentrations did not correlate to the intake of AR or breads from the diet. The potential usability of erythrocyte AR as long-term biomarkers should be investigated in studies with prolonged whole-grain intake and following low-fibre bread diet when AR plasma and erythrocyte concentrations are monitored.

Average serum ENL concentrations increased significantly during yhe eight-week intake of whole-grain rye in Study II in women ($p < 0.05$, n=39) and the one-week intake of whole-grain rye in Study V in men ($p < 0.05$, n=7), but not in women in Study V (n=8). When whole-grain products were removed from the diet for one week, ENL concentrations in men dropped to half, while only a slight decrease in concentrations was seen in women. Again, in men, a significant increase in ENL concentrations with one-week intake of whole-grain rye was seen. Serum ENL concentrations in men were lower compared to women, but the difference between women and men reached statistical significance ($p < 0.05$) only during the low-fibre diets. This data indicates that women receive plant lignans and then produce ENL from other sources than whole-grain products in higher amounts than men, or that intestinal microflora is remarkably different in women compared to men in this study. There were individuals in Study II and V whose ENL concentrations did not increase at all with intake of rye bread, while AR concentrations did increase and no intake of antibiotics was recorded. Since plasma AR concentrations with the same individuals increased, these individuals did consume whole-grain products. In certain cases, ENL concentrations even decreased with the intake of rye. Similar cases with minimal changes in ENL concentrations with intake of plant lignans have been seen in previous studies (Stumpf *et al.* 2000a). This could be caused by inactive intestinal microflora or other individual metabolic events that result in inefficient conversion of plant lignans to ENL. In Study II, serum ENL concentrations were higher after the eight-week habitual diet, low-fibre wheat bread diet, and whole-grain rye intake compared to Study V with one-week dietary interventions, indicating higher intake of plant lignans by individuals in Study II. In Study II, the subjects were older (59±0.94 years with BMI 26.8±0.5 kg/m^2) postmenopausal women, while in Study V women (n=8) were 27±2.1 years old with BMI 23.3±1.0 and men (n=7) were 22±0.37 years old with BMI 25.7±1.7. The subjects in Study II were recruited from Eastern Finland and in Study V from the Helsinki area. These differences in the subjects' ages and the place of residence may affect dietary habits and probably explain the differences in ENL concentrations.

These results show that ENL is not a specific biomarker of whole-grain intake. In contrast to ENL, plasma AR concentrations rose, although to different

extents, in all subjects after the intake of whole-grain products. The response of AR concentrations to the intake of whole-grain products was more constant and predictable compared to ENL. AR are absorbed as such from the digestive tract. This data shows that plasma AR compared to serum ENL could better function as specific biomarkers for whole-grain intake, although this study did not directly evaluate the usability of ENL as a dietary biomarker. ENL has been thought to reflect the intake of lignans in the diet, and generally healthy dietary habits, and enterolactone to enterodiol –ratio could potentially be used as a marker of the function of the intestinal microflora (Kilkkinen *et al*. 2001, Kilkkinen *et al*. 2002, Stumpf 2004). In addition, enterodiol measured together with ENL is thought to be a better marker of lignan intake than ENL alone, although even then the intra-individual variation remained high (Peñalvo *et al.* 2005b).

Some authors have presented hypotheses that cereal-based diet could be a cause for chronic diseases (Jönsson *et al*. 2005), although even then the main cause has been reasoned to be highly refined cereal products (Cordain *et al*. 2005). Measuring serum ENL and plasma AR together could provide information on dietary habits, and possibly distinguish a difference between the intake of fibre-rich whole-grain cereals from other fibre-rich products abundant in plant lignans, such as seeds, vegetables, and fruits. This could elucidate the possible protective role of whole-grain cereals for chronic diseases compared to other sources of dietary fibre in the human diet. A trial with controlled intake of whole-grain bread and careful recording of fruit and vegetable intake should be conducted to confirm these ideas and to compare responses in plasma AR and serum ENL concentrations to dietary factors.

Total plasma AR concentrations together with AR C17:0/C21:0 –ratio may determine the intake of whole-grain wheat and rye products. Plasma AR concentrations after habitual diet seemed to increase even after single intake of high amount of rye bread. However, C17:0/C21:0 –ratio increased only after a regular intake of rye products. Low C17:0/C21:0 –ratio together with low total plasma AR concentration showed that the subject had consumed mainly low-fibre wheat products. In certain individuals, relatively low plasma AR values did occur during habitual diets after recorded intake of rye or other whole-grain cereals (~50 nmol/l), but even then the C17:0/C21:0 -ratio was high (~0.5 or more). The C17:0/C21:0 -ratio was 0.60-0.84 after controlled whole-grain rye bread intake and 0.10-0.12 after controlled whole-grain wheat bread intake. After habitual diet the mean C17:0/C21:0 -ratio was lower (0.28±0.05 and 0.52±0.09 (a one-week period)) in Study V with 15 subjects compared to Study II with 39 subjects (0.74±0.06 and 0.68± 0.05 (an eight-week period)). After controlled low-fibre intake with 39 women in Study II (an eight-week period) and in four pigs in Study IV (5-7 days), the ratio was relatively high (0.53 and 0.47), compared to Study V, in which the ratio was 0.01-0.05. Subjects in Study II were allowed to eat additional cereal products, and were followed for longer period than in Study V, which can affect dietary compliance. Also species-specific differences can explain

the different results between human subjects and the pigs. In all studies a constant intake of rye bread significantly increased the AR C17:0/C21:0 -ratio. On average, plasma AR concentrations about 35 nmol/l seem to reflect low-fibre based diet, and concentrations above 200 nmol/l indicate a high intake of whole-grain rye-based products in the diet. We suggest that AR C17:0/C21:0 -ratio together with the total AR concentration in plasma could be used as an indicator of the intake of whole-grain cereals and more specifically the type of cereals, i.e. the higher the ratio, the more whole-grain and especially rye based products have been included in the diet. This data supports the usability of total plasma AR concentration together with the homologue C17:0/C21:0 –ratio as specific biomarkers of the intake of whole-grain products in the diet.

6 Conclusions

The present work aimed to determine the usability of cereal alkylresorcinols (AR) as biomarkers of the intake of whole-grain products in the human diet. The aim was to analyse AR in human plasma, determine their absorption and responses in plasma concentrations after the intake of whole-grain wheat or rye products, and to analyse their occurrence in certain biological membranes. A pig model was used to determine AR absorption and elimination kinetics. The results of the study can be summarised as follows:

I AR can be determined and quantified with the use of analogous reference compounds in human plasma, erythrocyte membranes, and lipoproteins by GC-MS.

II AR are absorbed *via* lymphatic system from the small intestine, and are transported in plasma mainly in lipoproteins, VLDL and HDL being the main carriers. The absorption and transportation of AR resembles that of the structurally similar tocopherols. Relating plasma AR concentrations to cholesterol and triacylglycerols in plasma might provide more accurate estimation of the individual AR status by eliminating the effects in variation of carrier lipoprotein concentrations. Some results suggest that enterohepatic circulation is possible, but the exact mechanism and the nature of the possible compounds in bile are uncertain.

III Plasma AR concentrations increase systematically, although not necessarily dose-dependently on individual basis, after the intake of whole-grain wheat or rye products. Mean plasma AR concentrations (nmol/l) significantly increased with increasing intake of AR from the diet (mg/d). The increase in plasma AR concentrations after whole-grain intake is more regular and predictable than that of enterolactone (ENL). Plasma AR concentrations gave better estimation of the intake of whole-grain products in the diet compared to ENL.

IV AR C17:0/C21:0 -ratio was higher after whole-grain wheat and rye intake compared to low-fibre diets. The total AR concentration in plasma together with homologue C17:0/C21:0 -ratio could be used as specific dietary biomarkers to determine the amount and the type of whole-grain products in the diet providing a valuable tool in epidemiological studies when the role of the diet for human health is evaluated. Plasma AR and serum ENL together could be used as dietary biomarkers to show the origin of the main source of dietary fibre, whether it is mainly from whole-grain cereals or other plant origin foods.

7 Future studies

The usability of AR as biomarkers should be confirmed in a larger and prolonged study with controlled and variable intake of whole-grains. AR concentrations in plasma and erythrocytes should be followed with habitual diet, continuous intake of whole-grain products, and recorded intake (e.g. food diaries) of whole-grain cereals. Follow-up should be continued after a change to low-fibre diet to follow the decrease of AR concentrations in plasma and erythrocytes membranes. For this, at best, a faster analysis method suitable for analysis of multiple samples should be developed. Such a method could be a high performance liquid chromatography method that requires less sample pre-treatment than GC-MS or time-resolved fluoroimmunoassay suitable for the analysis of several samples at the same time with relative ease. Possible AR metabolites in urine should be determined after intake of whole-grain cereals to better understand AR metabolism in humans. Also, LDL oxidation and possible effects of AR on the oxidation susceptibility after prolonged intake of whole-grains should be followed.

8 Acknowledgements

This work was carried out during years 2001-2006 in the laboratory of Professor Herman Adlercreutz at the Institute for Preventive Medicine, Nutrition, and cancer, Folkhälsan Research Center, and at the Division of Clinical Chemistry, University of Helsinki. I thank Docent Per-Henrik Groop, former Research Director of the Folhälsan Research Center, for supplying the research facilities and making it possible to continue and carry out my research work. Financial support was granted by Sigrid Jusélius Foundation, Finska Läkaresällskapet, Maud Kuistila Memorial Foundation, Ida Montin Foundation, The Finnish Medical Society Duodecim, all of which I gratefully acknowledge.

I am grateful that my supervisor, Professor Herman Adlercreutz, gave me a possibility as a young student to work as member of his team. I have been privileged to be able to work with him in a research field where he has been a pioneer and has led the way through the decades. I have been given the freedom to work and write manuscripts independently, and yet Professor Adlercreutz has never lost faith in my work. I admire your enthusiasm in science.

Special thanks are to my other supervisor PhD José L. Peñalvo, who has always helped me with any difficulties I have met in my work as well as in life in general. I am grateful for his friendship with many lively conversations full of laughter.

I was fortunate to have Professor Matti J. Tikkanen as our collaborator during the last part of this work. I want to thank him for letting me do part of this work in his laboratory, and for his invaluable help and comments especially on the last manuscript.

I want to express my warmest thanks to the official referees of my thesis, Professor Antti Aro and Professor Heikki Kallio, who, in spite of their other duties, found time to review this thesis and give valuable comments on it.

It has been wonderful to have two other researchers currently working with alkylresorcinols. I want to give special thanks to PhD Alastair Ross, a collaborator and a great friend, who has always been optimistic with our research. I thank MSc Rikard Landberg for collaboration and precious ideas and comments on the results we have achieved.

I want to thank Professor Per Åman, PhD Afaf Kamal-Eldin, Professor Knud-Erik Bach Knudsen, and Professor Kristiina Wähälä for collaboration and kind help during these years.

A research group would not be worthy of praise without its members who create the spirit of the group. I want to thank my colleagues Satu-Maarit Heinonen, Pia Fyhrquist, and Katariina Stumpf for inspiring conversations and relaxing laughs during sometimes hard days. The work in laboratory would not have been possible without excellent assistance from Anja Koskela, who has solved many peculiar problems we have encountered, Adile Samaletdin, Inga Wiik, Ritva Takkinen, and Aila Heikkinen. They all provided tremendous help throughout my work and truly create the base for our research group. I also wish to thank Terhi Hakala and Kirsti Räsänen for excellent technical assistance and Aila Koponen for always helping me with the literature search. Marjatta Valkama is thanked for invaluable help in technical matters. Marja Parvinen is warmly thanked for carefully reviewing the language of this thesis.

I express my gratitude to my whole loving family. Especially my mother Linnéa deserves my special thanks for helping me in all matters of life. I thank my mother and father, Juhani, for showing the way in life. It has been an honour to follow your footsteps in science. I also want to thank all my relatives, who with their own work have created a strong base to become a scientist. I thank my sister Laura for being there and turning my head into other things than work whenever needed. I thank Matti for always trusting me, and my grandparents for their love and interest in my work.

I owe my special thanks to my beloved Tero. Thank you for standing by me at all times.

Helsinki, November 2006

Anna-Maria Linko-Parvinen

9 References

Adlercreutz, H. Phyto-oestrogens and cancer. The Lancet Oncology 3: 364-373 (2002).

Adlercreutz, H. & Mazur, W. Phyto-oestrogens and western diseases. Ann. Med. 29: 95-120 (1997).

Adlercreutz, H., Wang, G. J., Lapcik, O., Hampl, R., Wähälä, K., Mäkelä, T., Lusa, K., Talme, M. & Mikola, H. Time-resolved fluoroimmunoassay for plasma enterolactone. Anal. Biochem. 265: 208-215 (1998).

Adom, K. K. & Liu, R. H. Rapid peroxyl radical scavenging capacity (PSC) assay for assessing both hydrophilic and lipophilic antioxidants. J. Agric. Food Chem. 53: 6572-6580 (2005).

Andreasen, M. F., Landbo, A. K., Christensen, L. P., Hansen, A. & Meyer, A. S. Antioxidant effects of phenolic rye (Secale cereale L.) extracts, monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low-density lipoproteins. J. Agric. Food Chem. 49: 4090-4096 (2001).

Bandyopadhyay, C., Gholap, A. S. & Mamdapur, V. R. Characterization of alkenylresorcinol in mango (*Mangifera indica* L.). J. Agric. Food Chem. 33: 377-379 (1985).

Behrens, W. A., Thompson, J. N. & Madère, R. Distribution of α-tocopherol in human plasma lipoproteins. Am. J. Clin. Nutr. 35: 691-696 (1982).

Berliner, J. A., Navab, M., Fogelman, A. M., Frank, J. S., Demer, L. L., Edwards, P. A., Watson, A. D. & Lusis, A. J. Atherosclerosis: basic mechanisms: oxidation, inflammation, and genetics. Circulation 91: 2488-2496 (1995).

Bieri, J. G., Tolliver, T. J. & Caliguani, B. S. Simultaneous determination of α-tocopherol and retinol in plasma and red cells by high performance liquid chromatography. Am. J. Clin. Nutr. 32: 2143-2149 (1979).

Birringer, M., Drogan, D. & Brigelius-Flohe, R. Tocopherols are metabolized in HepG2 cells by side chain omega-oxidation and consecutive beta-oxidation. Free Radical Biology & Medicine 31: 226-232 (2001).

Bitkov, V. V., Nenashev, V. A., Pridachina, N. N. & Batrakov, S. G. Membrane-structuring properties of bacterial long-chain alkylresorcinols. Biochim. Biophys. Acta 1108: 224-232 (1992).

Bjørneboe, A., Bjørneboe, G. E., Bodd, E., Hagen, B. F., Kveseth, N. & Drevon, C. A. Transport and distribution of α-tocopherol in lymph, serum and liver cells in rats. Biochim. Biophys. Acta 889: 310-315 (1986).

Bjørneboe, A., Bjørneboe, G. E. & Drevon, C. A. Absorption, transport and distribution of vitamin E. J. Nutr. 120: 233-242 (1990).

Bjørnson, L. K., Kayden, H. J., Miller, E. & Moshell, A. N. The transport of α-tocopherol and β-carotene in human blood. J. Lipid Res. 17: 343-352 (1976).

Boccardo, F., Puntoni, M., Guglielmini, P. & Rubagotti, A. Enterolactone as a risk factor for breast cancer: a review of the published evidence. Clin. Chim. Acta 365: 58-67 (2006).

Bock, H. D., Flamme, W. & Kesting, S. Preliminary experiments with rats on alkylresorcinols in rye. Nahrung. 25: 805-810 (1981).

Borel, P. Factors affecting intestinal absorption of highly lipophilic food microconstituents (fat-soluble vitamins, carotenoids and phytosterols). Clin. Chem. Lab. Med. 41: 979-994 (2003).

Borriello, S. P., Setchell, K. D. R., Axelsson, M. & Lawson, A. M. Production and metabolism of lignans by the human faecal flora. J. Appl. Bacteriol. 58: 37-43 (1985).

Bruce, B., Spiller, G. A., Klevay, L. M. & Gallagher, S. K. A diet high in whole and unrefined foods favorably alters lipids, antioxidant defenses, and colon function. J. Am. Coll. Nutr. 19: 61-67 (2000).

Burton, G. W., Joyce, A. & Ingold, K. U. Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? Arch. Biochem. Biophys. 221: 281-290 (1983).

Chen, Y., Ross, A. B., Åman, P. & Kamal-Eldin, A. Alkylresorcinols as markers of whole-grain wheat and rye in cereal products. J. Agric. Food Chem. 52: 8242-8246 (2004).

Chow, C. K. Vitamin E and blood. World Rev Nutr Diet. 45: 133-166 (1985).

Clark, J. M. & Switzer, R. L. (1977) Isolation and characterization oferythrocyte membraens. In: Experimental Biochemistry. (Clark, J. M., ed.), pp. 191-194. Freeman, W.H. and Company, San Francisco.

Cohn, W., Gross, P., Grun H, Loechleiter, F., Muller, D. P. & Zulauf, M. Tocopherol transport and absorption. Proc. Nutr. Soc. 51: 179-188 (1992).

Cordain, L., Eaton, S. B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B. A., O'Keefe, J. H. & Brand-Miller, J. Origins and evolution of the Western diet: health implications for the 21st century. Am. J. Clin. Nutr. 81: 341-354 (2005).

Crews, H., Alink, G., Andersen, R., Braesco, V., Holst, B., Maiani, G., Ovesen, L., Scotter, M., Solfrizzo, M., van den Berg, R., Verhagen, H. & Williamson, G. A critical assessment of some biomarker approaches linked with dietary intake. Br. J. Nutr. 86: 5-35 (2001).

Deszcz, L. & A., K. Higher cardol homologs (5-alkylresorcinols) in rye seedlings. Biochim. Biophys. Acta 1483: 241-250 (2000).

Eastwood, M. A. Interaction of dietary antioxidants in vivo: how fruit and vegetables prevent disease? QJM 92: 527-530 (1999).

Edge, M. S., Jones, J. M. & Marquart, L. A new life for whole grains. J. Am. Diet. Assoc. 105: 1856-1860 (2005).

Fotsis, T. & Adlercreutz, H. The multicomponent analysis of estrogens in urine by ion exhange chromatography and GC-MS-I. quantitation of estrogens after initial hydrolysis of conjugates. Journal of Steroid Biochemistry 28: 203-213 (1987).

Frank, J. Beyond vitamin E supplementation: an alternative strategy to improve vitamin E status. J. Plant Physiol. 162: 834-843 (2005).

Ganry, O. Phytoestrogens and prostate cancer risk. Prev. Med. 41: 1-6 (2005).

García, S., García, C., Heinzen, H. & Moyna, P. Chemical basis of the resistance of barley seeds to pathogenic fungi. Phytochemistry 44: 415-418 (1997).

Gasiorowski, K., Brokos, B., Kozubek, A. & Oszmianski, J. The antimutagenic activity of the two plant-derived compounds. A comparative cytogenetic study. Cellular & Molecular Biology Letters 5: 171-190 (2000a).

Gasiorowski, K., Brokos, B. & Tabaka, H. Evaluation of the immunomodulatory activity of four compounds exerting antimutagenic effects on human lymphocytes in vitro. Cellular & Molecular Biology Letters 5: 469-481 (2000b).

Gasiorowski, K., Szyba, K., Brokos, B. & Kozubek, A. Antimutagenic activity of alkylresorcinols from cereal grains. Cancer Letters 106: 109-115 (1996).

Grandjean, P. Biomarkers in epidemiology. Clinical Biochemistry 41: 1800-1803 (1995).

Gubernator, J., Stasiuk, M. & Kozubek, A. Dual effect of alkylresorcinols, natural amphiphilic compunds, upon liposomal permeability. Biochim. Biophys. Acta 1418: 253-260 (1999).

Hacquebard, M. & Carpentier, Y. A. Vitamin E: absorption, plasma transport and cell uptake. Current Opinion in Clinical Nutrition and Metabolic Care 8: 133-138 (2005).

Hall, W. L., Jeanes, Y. M. & Lodge, J. K. Hyperlipidemic subjects have reduced uptake of newly absorbed vitamin E into their plasma lipoproteins, erythrocytes, platelets, and lymphocytes, as studied by deuterium-labeled alpha-tocopherol biokinetics. J. Nutr. 135: 58-63 (2005).

Hallmans, G., Zhang, J.-X., Lundin, E., Stattin, P., Johansson, A., Johansson, I., Hultén, K., Winkvist, A., Lenner, P., Åman, P. & Adlercreutz, H. Rye, lignans and human health. Proc. Nutr. Soc. 62: 193-199 (2003).

Hanahan, D. J. & Ekholm, J. E. The preparation of red cell ghosts (membranes). Methods Enzymol. 31: 168-172 (1974).

Harder, H., Tetens, I., Let, M. B. & Meyer, A. S. Rye bran bread intake elevates urinary excretion of ferulic acid in humans, but does not affect the susceptibility of LDL to oxidation ex vivo. Eur. J. Nutr. 43: 230-236 (2004).

Havel, R. J., Eder, H. A. & Bragdon, J. H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. 34: 1345–1353 (1955).

Heinonen, S., Nurmi, T., Liukkonen, K., Poutanen, K., Wähälä, K., Deyama, T., Nishibe, S. & Adlercreutz, H. In vitro metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. J. Agric. Food Chem. 49: 3178-3186 (2001).

Helisten, H., Höckerstedt, A., Wähälä, K., Tiitinen, A., Adlercreutz, H., Jauhiainen, M. & Tikkanen, M. J. Accumulation of high-density lipoprotein-derived estradiol-17beta fatty acid esters in low-density lipoprotein particles. J. Clin. Endocrinol. Metab. 86: 1297-1300 (2001).

Hengtrakul, P., Mathias, M. & Lorenz, K. Effects of cereal alkylresorcinols on human platelet thromboxane production. The Journal of Nutritional Biochemistry 2: 20-24 (1991).

Himejima, M. & Kubo, I. Antibacterial agents from the cashew *Anacardium occidentale* (Anacardiaceae) nut shell oil. J. Agric. Food Chem. 39: 418-421 (1991).

Hladyszowski, J., Zubik, L. & Kozubek, A. Quantum mechanical and experimental oxidation studies of pentadecylresorcinol, olivetol, orcinol and resorcinol. Free radical research 28: 359-368 (1998).

Horner, N. K., Kristal, A. R., Prunty, J., Skor, H. E., Potter, J. D. & Lampe, J. W. Dietary Determinants of Plasma Enterolactone. Cancer Epidemiol. Biomarkers Prev. 11: 121-126 (2002).

Horwitt, M. K., Harvey, C. C., Dahm, C. H. J. & Searcy, M. T. Relationship between tocopherol and serum lipid levels for determination of nutritional adequacy. Ann. N. Y. Acad. Sci. 203: 223-236 (1972).

Hosomi, A., Arita, M., Sato, Y., LKiyose, C., Ueda, T., Igarashi, O., Arai, H. & Inoue, K. Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. FEBS Lett. 409: 105-108 (1997).

Höckerstedt, A., Tikkanen, M. J. & Jauhiainen, M. LCAT facilitates transacylation of 17 beta-estradiol in the presence of HDL3 subfraction. J. Lipid Res. 43: 392-397 (2002).

Ingram, D., Sanders, K., Kolybaba, M. & Lopez, D. Case-control study of phyto-oestrogens and breast cancer. Lancet 350: 990-994 (1997).

Jacobs, D. R. J., Meyer, H. E. & Solvoll, K. Reduced mortality among whole grain bread eaters in men and women in the Norwegian County Study. European Journal of Clinical Nutrition 55: 137-143 (2001).

Jacobs, D. R. J., Meyer, K. A., Kushi, L. H. & Folsom, A. R. Is whole grain intake associated with reduced total and cause-specific death rates in older women? The Iowa Women's Health Study. Am. J. Public Health 89: 322-329 (1999).

Jacobs, D. R. J., Pereira, M. A., Meyer, K. A. & Kushi, L. H. Fiber from whole grains, but not refined grains, is inversely associated with all-cause mortality in older women: the Iowa women's health study. J. Am. Coll. Nutr. 19: 326S-330S (2000).

Jacobs, D. R. J., Pereira, M. A., Stumpf, K., Pins, J. J. & Adlercreutz, H. Whole grain food intake elevates serum enterolactone. Br. J. Nutr. 88: 111-116 (2002).

Jeanes, Y. M., Hall, W. L. & Lodge, J. K. Comparative (2)H-labelled alpha-tocopherol biokinetics in plasma, lipoproteins, erythrocytes, platelets and lymphocytes in normolipidaemic males. Br. J. Nutr. 94: 92-99 (2005).

Jenkins, D. J., Kendall, C. W., Augustin, L. S., Martini, M. C., Axelsen, M., Faulkner, D., Vidgen, E., Parker, T., Lau, H., Connelly, P. W., Teitel, J., Singer, W., Vandenbroucke, A. C., Leiter, L. A. & Josse, R. G. Effect of wheat bran on glycemic control and risk factors for cardiovascular disease in type 2 diabetes. Diabetes Care 25: 1522-1528 (2002).

Jensen, M. K., Koh-Banerjee, P., Franz, M., Sampson, L., Gronbaek, M. & Rimm, E. B. Whole grains, bran, and germ in relation to homocysteine and markers of glycemic control, lipids, and inflammation 1. Am. J. Clin. Nutr. 83: 275-283 (2006).

Jensen, M. K., Koh-Banerjee, P., Hu, F. B., Franz, M., Sampson, L., Grønbæk, M. & Rimm, E. B. Intakes of whole grains, bran, and germ and the risk of coronary heart disease in men. Am. J. Clin. Nutr. 80: 1492-1499 (2004).

Johnsen, N. F., Hausner, H., Olsen, A., Tetens, I., Christensen, J., K.E., K., K., O. & A., T. Intake of whole grains and vegetables determines the plasma enterolactone concentration of Danish women. J. Nutr. 134: 2691-2697 (2004).

Jones, A. R., Hepples, M. L., Coulson, J., Kuznesof, S., Richardson, D. P. & Seal, C. J. Increased wholegrain food consumption and indicators of adiposity: preliminary results from the CHEW-IT study. Proc. Nutr. Soc. 63: 70A (2004).

Juntunen, K. S., Laaksonen, D. E., Poutanen, K. S., Niskanen, L. K. & Mykkänen, H. M. High-fiber rye bread and insulin secretion and sensitivity in healthy postmenopausal women. Am. J. Clin. Nutr. 77: 385-391 (2003).

Juntunen, K. S., Mazur, W., Liukkonen, K. H., Uehara, M., Poutanen, K. S., Adlercreutz, H. C. T. & Mykkänen, H. M. Consumption of wholemeal rye bread increases serum concentrations and urinary excretion of enterolactone compared with consumption of white wheat bread in healthy Finnish men and women. Br. J. Nutr. 84: 839-846 (2000).

Jönsson, T., Olsson, S., Ahrén, B., Bøg-Hansen, T. C., Dole, A. & S., L. Agrarian diet and diseases of affluence - Do evolutionary novel dietary lectins cause leptin resistance? BMC Endocrine Disorders 5 (2005).

Kaaks, R., Riboli, E. & Sinha, R. (1997) Biochemical markers of dietary intake. In: Application of Biomarkers in Cancer Epidemiology (Toniolo, P., Boffetta, P., Shuker, D. E. G., Rothman, N., Hulka, B. & Pearce, N., eds.), pp. 103-126. IARC Scientific Publications.

Kamal-Eldin, A., Pouru, A., Eliasson, C. & Åman, P. Alkylresorcinols as antioxidants: hydrogen donation and peroxyl radical-scavenging effects. J. Sci. Food Agric. 81: 353-356 (2001).

Kanda, N., Ishizaki, N., Inoue, N., Oshima, M. & Handa, A. DB-2073, a new alkylresorcinol antibiotic I. taxonomy, isolation and characterization. The Journal of Antibiotics XXVIII: 935-942 (1975).

Kantor, L. S., Variyam, J. N., Allshouse, J. E., Putnam, J. J. & Lin, B.-H. Choose a Variety of Grains Daily, Especially Whole Grains: A Challenge for Consumers. J. Nutr. 131: 473S-486S (2001).

Kaul, N., Devaraj, S. & Jialal, I. Alpha-tocopherol and atherosclerosis. Exp. Biol. Med. 226: 5-21 (2001).

Kilkkinen, A., Erlund, I., Virtanen, M. J., Alfthan, G., Ariniemi, K. & Virtamo, J. Serum Enterolactone Concentration and the Risk of Coronary Heart Disease in a Case-Cohort Study of Finnish Male Smokers. Am. J. Epidemiol. (2006).

Kilkkinen, A., Stumpf, K., Pietinen, P., Valsta, L. M., Tapanainen, H. & Adlercreutz, H. Determinants of serum enterolactone concentration. Am. J. Clin. Nutr. 73: 1094-1100 (2001).

Kilkkinen, A., Valsta, L. M., Virtamo, J., Stumpf, K., Adlercreutz, H. & P., P. Intake of lignans is associated with serum enterolactone concentration in Finnish men and women. J. Nutr. 133: 1830-1833 (2002).

Kilkkinen, A., Virtamo, J., Vartiainen, E., Sankila, R., Virtanen, M. J., Adlercreutz, H. & P., P. Serum enterolactone concentration is not associated with breast cancer risk in a nested case-control study. Int. J. Cancer 108: 277-280 (2004).

Kilkkinen, A., Virtamo, J., Virtanen, M. J., Adlercreutz, H., Albanes, D. & Pietinen, P. Serum enterolactone concentration is not associated with prostate cancer risk in a nested case-control study. Cancer Epidemiol. Biomarkers Prev. 12: 1209-1212 (2003).

Korpela, J. T., Korpela, R. & Adlercreutz, H. Fecal bile acid metabolic pattern after administration of different types of bread. Gastroenterology 103: 1246-1253 (1992).

Kozubek, A. The effect of 5-(n-alk(en)yl)resorcinols on membranes. I. Characterization of the permeability increase induced by 5-(n-heptadecenyl)resorcinol. Acta Biochim. Pol. 34: 357-367 (1987a).

Kozubek, A. The effect of 5-(n-alk(en)yl)resorcinols on membranes. II. Dependence of the aliphatic chain length and unsaturation. Acta Biochim. Pol. 34: 387-394 (1987b).

Kozubek, A. Interaction of alkylresorcinols with proteins. Acta Biochim. Pol. 42: 241-246 (1995).

Kozubek, A. & Demel, R. A. Permeability changes of erythrocytes and liposomes by 5-(n-alk(en)yl)resorcinols from rye. Biochim. Biophys. Acta 603: 220-227 (1980).

Kozubek, A., Jezierski, A. & Sikorski, A. F. The effect of nonadec(en)ylresorcinol on the fluidity of liposome and erythrocyte membranes. Biochim. Biophys. Acta 944: 465-472 (1988).

Kozubek, A. & Nienartowich, B. Cereal grain resorcinolic lipids inhibit H2O2-induced peroxidation of biological membranes. Acta Biochim. Pol. 42: 309-315 (1995).

Kozubek, A., Nietubyc, M. & Sikorski, A. F. Modulation of the activities of membrane enzymes by cereal grain resorcinolic lipids. Z. Naturforsch. 47: 41-46 (1992).

Kozubek, A., Pietr, S. & Czerwonka, A. Alkylresorcinols are abundant lipid components in different strains of Azotobacter choococcum and Pseudomonas spp. Journal of Bacteriology 178: 4027-4030 (1996).

Kozubek, A. & Tyman, J. H. P. Cereal grain resorcinolic lipids: mono and dienoic homologues are present in rye grains. Chem. Phys. Lipids 78: 29-35 (1995).

Kozubek, A. & Tyman, J. H. P. Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. Chem. Rev. 99: 1-25 (1999).

Landberg, R., Linko-Parvinen, A.-M., Kamal-Eldin, A., Vessby, B., Adlercreutz, H. & Åman, P. Human plasma kinetics and relative bioavailability of alkylresorcinols after intake of rye bran. In press. (2006).

Lang, R. & Jebb, S. A. Who consumes whole grains, and how much? Proc. Nutr. Soc. 62: 123-127 (2003).

Lapointe, A., Couillard, C. & Lemieux, S. Effects of dietary factors on oxidation of low-density lipoprotein particles. The Journal of Nutritional Biochemistry (2006).

Lehmann, J., Rao, D. D., Canary, J. J. & Judd, J. T. Vitamin E and relationships among tocopherols in human plasma, platelets, lymphocytes, and red blood cells. Am. J. Clin. Nutr. 47: 470-474 (1988).

Linko, A.-M. & Adlercreutz, H. Whole grain rye and wheat alkylresorcinols are incorporated into human erythrocyte membranes. Br. J. Nutr. 93: 11-13 (2005).

Liu, S., Stampfer, M. J., Hu, F. B., Giovannucci, E., Rimm, E., Manson, J. E., Hennekens, C. H. & Willett, W. C. Whole-grain consumption and risk of coronary heart disease: results from the Nurses' Health Study. Am. J. Clin. Nutr. 70: 412-419 (1999).

Liu, S., Willett, W. C., Manson, J. E., Hu, F. B., Rosner, B. & Colditz, G. Relation between changes in intakes of dietary fiber and grain products and changes in weight and development of obesity among middle-aged women. Am. J. Clin. Nutr. 78: 920-927 (2003).

Liukkonen, K.-H., Katina, K., Wilhelmsson, A., Myllymäki, O., Lampi, A.-M., Kariluoto, S., Piironen, V., Heinonen, S.-M., Nurmi, T., Adlercreutz, H., Peltoketo, A., Pihlava, J.-M., Hietaniemi, V. & Poutanen, K. Process-

induced changes on bioactive compounds in wholegrain rye. Proc. Nutr. Soc. 62: 1-6 (2003).

Liyana-Pathirana, C. M. & Shahidi, F. Importance of insoluble-bound phenolics to antioxidant properties of wheat. J. Agric. Food Chem. 54: 1256-1264 (2006).

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275 (1951).

Lynch, B. S., Delzell, E. S. & Bechtel, D. H. Toxicology review and risk assessment of resorcinol: thyroid effects. Regul. Toxicol. Pharmacol. 36: 198-210 (2002).

Lytollis, W., Scannell, R. T., An, H., Murty, V. S., Reddy, K. S., Barr, J. R. & Hecht, S. M. 5-Alkylresorcinols from Hakea trifurcata, that cleave DNA. J. Am. Chem. Soc. 117: 12683-12690 (1995).

Marquart, L., Wiemer, K. L., Jones, J. M. & Jacob, B. Whole grains health claims in the USA and other efforts to increase whole-grain consumption. Proc. Nutr. Soc. 62: 151-160 (2003).

Mattila, P., Pihlava, J.-M. & Hellström, J. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. J. Agric. Food Chem. 53: 8290-8295 (2005).

Mazur, W. & Adlercreutz, H. Naturally occurring oestrogens in food. Pure & Appl. Chem. 70: 1759-1776 (1998).

McIntosh, G. H., Noakes, M., Royle, P. J. & Foster, P. R. Whole-grain rye and wheat foods and markers of bowel health in overweight middle-aged men. Am. J. Clin. Nutr. 77: 967-974 (2003).

Miché, L., Belkin, S., Rozen, R. & Balandreau, J. Rice seedling whole exudates and extracted alkylresorcinols induce stress-response in *Escherichia coli* biosensors. Environmental Microbiology 5: 403 (2003).

Milder, I. E., Arts, I. C., van de Putte, B., Venema, D. P. & Hollman, P. C. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br. J. Nutr. 93: 393-402 (2005).

Miller, H. E., Rigelhof, F., Marquart, L., Prakash, A. & Kanter, M. Antioxidant content of whole grain breakfast cereals, fruits and vegetables. J. Am. Coll. Nutr. 19: 312S-319S (2000).

Montonen, J., Knekt, P., Järvinen, R., Aromaa, A. & Reunanen, A. Whole-grain and fiber intake and the incidence of type 2 diabetes. Am. J. Clin. Nutr. 77: 622-629 (2003).

Mutoh, M., Takahashi, M., Fukuda, K., Matsushima-Hibiya, Y., Mutoh, H., Sugimura, T. & Wakabayashi, K. Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure. Carcinogenesis 21: 959-963 (2000).

Nilsson, M., Åman, P., Härkönen, H., Hallmans, G., Bach Knudsen, K. E., Mazur, W. & Adlercreutz, H. Content of nutrients and lignans in roller milled fractions of rye. J. Sci. Food Agric. 73: 143-148 (1997).

Parikka, K., Rowland, I. R., Welch, R. W. & Wähälä, K. In Vitro Antioxidant Activity and Antigenotoxicity of 5-n-Alkylresorcinols. J. Agric. Food Chem. (2006).

Parker, R. S., Sontag, T. J., Swanson, J. E. & McCormick, C. C. Discovery, characterization, and significance of the cytochrome P450 ω-hydroxylase pathway of vitamin E catabolism. Ann. N. Y. Acad. Sci. 1031: 13-21 (2004).

Peñalvo, J. L., Haajanen, K. M., Botting, N. & Adlercreutz, H. Quantification of lignans in food using isotope dilution gas chromatography/mass spectrometry. J. Agric. Food Chem. 53: 9342-9347 (2005a).

Peñalvo, J. L., Heinonen, S.-M., Aura, A.-M. & Adlercreutz, H. Dietary sesamin is converted to enterolactone in humans. J. Nutr. 135: 1056-1062 (2005b).

Pereira, M. A., Jacobs, D. R. J., Pins, J. J., Raatz, S. K., Gross, M. D., Slavin, J. L. & Seaquist, E. R. Effect of whole grains on insulin sensitivity in overweight hyperinsulinemic adults. Am. J. Clin. Nutr. 75: 848-855 (2002).

Pietinen, P., Rimm, E. B., Korhonen, P., Hartman, A. M., Willett, W. C., Albanes, D. & Virtamo, J. Intake of dietary fiber and risk of coronary heart disease in a cohort of Finnish men: the alpha-tocopherol, beta-carotene cancer prevention study. Circulation 94: 2720-2727 (1996).

Pietinen, P., Stumpf, K., Männistö, S., Kataja, V., Uusitupa, M. & Adlercreutz, H. Serum enterolactone and risk of breast cancer: a case-control study in eastern Finland. Cancer Epidemiol. Biomarkers Prev. 10: 339-344 (2001).

Princen, H. M., van Duyvenvoorde, W., Buytenhek, R., van der Laarse, A., van Poppel, G., Gevers Leuven, J. A. & van Hinsbergh, V. Supplementation with low doses of vitamin E protects LDL from lipid peroxidation in men and women. Arterioscler. Thromb. Vasc. Biol. 15: 325-333 (1995).

Przeworska, E., Guernator, J. & Kozubek, A. Formation of liposomes by resorcinolic lipids, single-chain phenolic amphiphiles from Anacardium occidentale L. Biochim. Biophys. Acta 1513: 75-81 (2001).

Rauls, D. & Penney, L. L. Analysis of olivetol in rabbit serum by high-performance liquid chromatography. J. Chromatogr. 234: 500-502 (1982).

Rejman, J. & Kozubek, A. Inhibitory effect of natural phenolic lipids upon NAD-dependent dehydrogenases and on triglyceride accumulation in 3T3-L1 cells in culture. J. Agric. Food Chem. 52: 246-250 (2004).

Rimm, E. B., Ascherio, A., Giovannucci, E., Spiegelman, D., Stampfer, M. J. & Willett, W. C. Vegetable, fruit, and cereal fiber intake and risk of coronary heart disease among men. JAMA-Journal of the American Medical Association 275: 447-451 (1996).

Ross, A. B., Becker, W., Chen, Y., Kamal-Eldin, A. & Åman, P. Intake of alkylresorcinols from wheat and rye in the United Kingdom and Sweden. Br. J. Nutr. 94: 496-499 (2005).

Ross, A. B., Chen, Y., Frank, J., Swanson, J. E., Parker, R. S., Kozubek, A., Lundh, T., Vessby, B., Åman, P. & Kamal-Eldin, A. Cereal alkylresorcinols elevate γ-tocopherol levels in rats and inhibit γ-tocopherol metabolism in vitro. J. Nutr. 134: 506-510 (2004a).

Ross, A. B., Kamal-Eldin, A., Jung, C., Shepherd, M. J. & Åman, P. Gas chromatographic analysis of alkylresorcinols in rye (*Secale cereale* L) grains. J. Sci. Food Agric. 81: 1405-1411 (2001a).

Ross, A. B., Kamal-Eldin, A., Lundin, E. A., Zhang, J. X., Hallmans, G. & Åman, P. Cereal alkylresorcinols are absorbed by humans. J. Nutr. 133: 2222-2224 (2003a).

Ross, A. B., Kamal-Eldin, A. & Åman, P. Dietary alkylresorcinols: Absorption, bioactivities, and possible use as biomarkers of whole-grain wheat- and rye-rich foods. Nutr. Rev. 62: 81-95 (2004b).

Ross, A. B., Kamal-Eldin, A., Åman, P., Lundin, E., Zhang, J.-X. & Hallmans, G. (2001b) Alkylresorcinols are absorbed by humans. VTT symposium 213, Whole grain and human health, International symposium, Haikko Manor, Finland.

Ross, A. B., Shepherd, M. J., Bach Knudsen, K. E., GlitsØ, L. V., Bowey, E., Phillips, J., Rowland, I., Guo, Z.-X., Massy, D. J. R., Åman, P. & Kamal-Eldin, A. Absorption of dietary alkylresorcinols in ileal-cannulated pigs and rats. Br. J. Nutr. 90: 787-794 (2003b).

Ross, A. B., Shepherd, M. J., Schüpphaus, M., Sinclair, V., Alfaro, B., Kamal-Eldin, A. & Åman, P. Alkylresorcinols in Cereals and Cereal Products. J. Agric. Food Chem. 51: 4111-4118 (2003c).

Ross, A. B., Åman, P., Andersson, R. & Kamal-Eldin, A. Chromatographic analysis of alkylresorcinols and their metabolites. J. Chromatogr. A 1054: 157-164 (2004c).

Ross, A. B., Åman, P. & Kamal-Eldin, A. Identification of cereal alkylresorcinol metabolites in human urine-potential biomarkers of wholegrain wheat and rye intake. Journal of Chromatography B 809: 125-130 (2004d).

Roxborough, H. E., Burton, G. W. & Kelly, F. J. Inter- and intra-individual variation in plasma and red blood cell vitamin E after supplementation. Free Radical Research 33: 437-445 (2000).

Saarinen, N. M., Warri, A., Mäkelä, S. I., Eckerman, C., Reunanen, M., Ahotupa, M., Salmi, S. M., Franke, A. A., Kangas, L. & Santti, R. Hydroxymatairesinol, a novel enterolactone precursor with antitumor properties from coniferous tree (Picea abies). Nutrition and cancer 36: 207-216 (2000).

Sakaeda, T. & Hirano, K. O/W lipid emulsions for parental drug delivery. III. Lipophilicity necessary for incorporation in oil particles even after intravenous injection. J. Drug Target. 6: 119-127 (1998).

Salmeron, J., Manson, J. A. E., Stampfer, M. J., Colditz, G. A., Wing, A. L. & Willett, W. C. Dietary fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women. The journal of the American medical association 277: 472-477 (1997).

Schwedhelm, E., Maas, R., Troost, R. & Böger, R. H. Clinical pharmacokinetics of antioxidants and their importance on systemic oxidative stress. Clin. Pharmacokinet. 42: 437-459 (2003).

Seal, C. J. Whole grains and CVD risk. Proc. Nutr. Soc. 65: 24-34 (2006).

Sedlet, K., Mathais, M. & Lorenz, K. Growth-depressing effects of 5-n-pentadecylresorcinol: a model for cereal alkylresorcinols. Cereal Chem. 61: 239-241 (1984).

Seitz, L. M. Identification of 5-(2-oxoalkyl)resorcinols and 5-(2-oxoalkenyl)resorcinols in wheat and rye grains. J. Agric. Food Chem. 40: 1541-1546 (1992).

Setchell, K. D. R., Lawson, A. M., Borriello, S. P., Harkness, R., Gordon, H., Morgan, D. M., Kirk, D. N., Adlercreutz, H., Anderson, L. C. & Axelson, M. Lignan formation in man-microbal involvement and possible roles in relation to cancer. The Lancet 2: 4-7 (1981).

Setchell, K. D. R., Lawson, A. M., Mitchell, F. L., Adlercreutz, H., Kirk, D. N. & Axelson, M. Lignans in man and in animal species. Nature 288: 740-742 (1980).

Simon, E., Gariepy, J., Cogn, A., Moatti, N., Simon, A. & Paul, J.-L. Erythrocyte, but not plasma, vitamin E concentration is associated with carotid intima-

media thickening in asymptomatic men at risk for cardiovascular disease. Atherosclerosis 159: 193-200 (2001).

Simon, E., Paul, J.-L., Atger, V., Simon, A. & Moatti, N. Erythrocyte antioxidant status in asymptomatic hypercholesterolemic men. Atherosclerosis 138: 375-381 (1998).

Slavin, J. Why whole grains are protective: biological mechanisms. Proc. Nutr. Soc. 62: 129-134 (2003).

Slavin, J. Whole grains and human health. Nutrition Research Reviews 17: 99-110 (2004).

Slavin, J. L. Dietary fiber and body weight. Nutrition 21: 411-418 (2005).

Slavin, J. L., Jacobs, D., Marquart, L. & Wiemer, K. The role of whole grains in disease prevention. J. Am. Diet. Assoc. 101: 780-785 (2001).

Slavin, J. L., Martini, M. C., Jacobs Jr, D. R. & Marquart, L. Plausible mechanisms for the protectiveness of whole grains. Am. J. Clin. Nutr. 70: 459S-463S (1999).

Starck, S., Deng, J.-Z. & Hecht, S. Naturally occuring alkylresorcinols that mediate DNA damage and inhibit its repair. Biochemistry 39: 2413-2419 (2000).

Stattin, P., Adlercreutz, H., Tenkanen, L., Jellum, E., Lumme, S., Hallmans, G., Harvei, S., Teppo, L., Stumpf, K., Luostarinen, T., Lehtinen, M., Dillner, J. & Hakama, M. Circulating enterolactone and prostate cancer risk: a Nordic nested case-control study. Int. J. Cancer 99: 124-129 (2002).

Steinberg, D. Lewis A. Conner Memorial Lecture: Oxidative Modification of LDL and Atherogenesis. Circulation 95: 1062-1071 (1997).

Stumpf, K. (2004) Serum enterolactone as a biological marker and in breast cancer: from laboratory to epidemiological studies. In: Medical Faculty, p. 78. University of Helsinki, Helsinki.

Stumpf, K., Pietinen, P., Puska, P. & Adlercreutz, H. Changes in serum enterolactone, genistein, and daidzein in a dietary intervention study in Finland. Cancer Epidemiol. Biomarkers Prev. 9: 1369-1372 (2000a).

Stumpf, K., Uehara, M., Nurmi, T. & Adlercreutz, H. Changes in the time-resolved fluoroimmunoassay of plasma enterolactone. Anal. Biochem. 284: 153-157 (2000b).

Suresh, M. & Kaleysa, R. R. Cardol: the antifilarial principle from *Anacardium occidentale*. Curr. Sci. 59: 477-479 (1990).

Suzuki, Y., Esumi, Y. & Yamaguchi, I. Structures os 5-alkylresorcinol-related analogues in rye. Phytochemistry 52: 281-289 (1999).

Thompson, L. U. Experimental studies on lignans and cancer. Bailliere's Clinical Endocrinology and Metabolism 12: 691-705 (1998).

Trowell, H. Definition of dietary fiber and hypotheses that it is a protective factor in certain diseases. Am. J. Clin. Nutr. 29: 417-427 (1976).

Truswell, A. S. Cereal grains and coronary heart disease. European Journal of Clinical Nutrition 56: 1-14 (2002).

Vanharanta, M., Mursu, J., Nurmi, T., Voutilainen, S., Rissanen, T. H., Salonen, R., Adlercreutz, H. & Salonen, J. T. Phloem fortification in rye bread elevates serum enterolactone level. Eur. J. Nutr. 56: 952-957 (2002).

Vanharanta, M., Voutilainen, S., Rissanen, T. H., Adlercreutz, H. & Salonen, J. T. Risk of cardiovascular disease-related and all-cause death according to serum concentrations of enterolactone: Kuopio Ischaemic Heart Disease Risk Factor Study. Arch. Intern. Med. 163: 1099-1104 (2003).

Weber, P. Role of biomarkers in nutritional science and industry - a comment. Br. J. Nutr. 86: 93-95 (2001).

Wieringa, G. W. On the occurrence of growth inhibiting substances in rye (Publication No 156). Wageningen, The Netherlands: Institute of Storage and Processing of Agricultural Produce. (1967).

Wild, C. P., Andersson, C., O'Brien, N. M., Wilson, L. & Woods, J. A. A critical evaluation of the application of biomarkers in epidemiological studies on diet and health. Br. J. Nutr. 86: 37-53 (2001).

Winata, A. & Lorenz, K. Antioxidant potential of 5-n-pentadecylresorcinol. Journal of Food Processing and Preservation 20: 417-429 (1996).

Vinkx, C. J. A. & Delcour, J. A. Rye (Secale cerealeL.) arabinoxylans: A critical review. Journal of Cereal Science 24: 1-14 (1996).

Wolk, A., Manson, J. E., Stampfer, M. J., Colditz, G. A., Hu, F. B., Speizer, F. E., Hennekens, C. H. & Willett, W. C. Long-term intake of dietary fiber and decreased risk of coronary heart disease among women. The journal of the American medical association 281: 1998-2004 (1999).

Wu, A. W., Gu, J., Ji, J. F., Li, Z. F. & Xu, G. W. Role of COX-2 in carcinogenesis of colorectal cancer and its relationship with tumor biological characteristics and patients' prognosis. World Journal of Gastoenterology 9: 1990-1994 (2003).

Yap, S. P., Yuen, K. H. & Wong, J. W. Pharmacokinetics and bioavailability of α-, γ- and δ-tocotrienols under different food status. Pharmaceutical Press 53: 67-71 (2004).

Zarnowski, R., Yoshikatsu, S., Yamaguchi, I. & Pietr, S. J. Alkylresorcinols in barley (*Hordeum vulgare* L. *distichon*) grains. Z. Naturforsch 57c: 57-62 (2002).

Zhang, H.-Y. Theoretical methods used in elucidating activity differences of phenolic antioxidants. J. Am. Oil Chem. Soc. 76: 745-748 (1999).

Zhang, J. X., Hallmans, G., Adlercreutz, H., Åman, P., Westerlund, E., Lundin, E. & Stenling, R. Effects of oat and rye fractions on biliary and faecal bile acid profiles in Syrian golden hamsters (Mesocricetus auratus). Br. J. Nutr. 70: 525-536 (1993).