Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and
Agricultural Sciences
Department of Food Science

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Abstract

Epidemiological studies have demonstrated several health benefits associated with whole grain intake, including prevention of cardiovascular disease, type II diabetes, colorectal cancer, obesity and mortality. Whole grain products are therefore recommended by national agencies. However, a nationwide investigation performed in 2010 revealed that the Swedish population only consumed about half of the recommended daily intake. There are various challenges associated with the estimation of whole grain intake. Common methods used for measuring consumption may differ in how comprehensive and detailed the provided information is, which may affect accuracy. Participants may also influence accuracy due to limited memory capacity, which is central in some methods. Moreover, different definitions of whole grain and whole grain products has been used in different studies. Alkylresorcinols (AR), is a group of phenolic lipids that has been suggested as biomarkers of whole grain wheat and rye intake. AR may therefore serve as an objective tool to estimate whole grain intake and to avoid problems inherent to self-reported intake. Alkylresorcinols are derivates of 1,3-dihydroxybenzene and are only found in the outer layers of wheat, rye and barley and can therefore mirror the intake of whole grain products made from these cereals. AR in plasma has been evaluated and used as biomarkers in several studies but the relative short half-life, limits their use to populations with a stable and frequent intake. Moreover, some studies have suggested differences in AR concentrations between men and women. Recently, AR were found in adipose tissue samples from humans and the adipose tissue concentrations were correlated with whole grain product intake in a pilot study. The aim of this study was to quantify alkylresorcinols in human plasma and to compare the concentration between women and men. The aim was further to correlate AR concentrations in adipose tissue, with the concentrations in plasma of Swedish women and men. The result showed no significant difference of plasma alkylresorcinols between women and men (P=0.142-0.966) which contradicted to other studies in which men had higher values. However, the adipose tissue alkylresorcinols showed a significant difference between men and women (P=0.003-0.047). A strong correlation between the plasma alkylresorcinols and adipose tissue alkylresorcinols was observed (r=0,466-0,823, P=<0.0001). The correlation for the shortest alkylresorcinol homologue (C17:0) was significantly strongest for both men, women and the sexes together (r=0.801- 0.823, p<0.0001).

Sammanfattning

Epidemiologiska studier har visat att flera kroniska sjukdomar så som hjärt- och kärlsjukdom, typ II diabetes, kolorektalcancer, fetma och dödlighet kan förebyggas genom att äta fullkorn. Intag av fullkornsprodukter rekommenderas därför av nationella myndigheter. Däremot visar en undersökning från 2010 att intaget av fullkorn i Sverige är ungefär hälften av det rekommenderade intaget. Det finns många utmaningar med att mäta fullkornsintag. Vanliga metoder som används vid estimering av fullkornsintag skiljer sig i hur omfattande och detaljerad informationen är, vilket kan påverka noggrannheten av resultatet. Då minnet hos deltagarna ibland är en central faktor för att mäta fullkornsintag kan det påverka resultaten negativt. Även de olika definitioner som förekommer av fullkorn i olika studier försvårar ett jämförbart resultat. Alkylresorcinoler (AR) är en grupp av fenoliska lipider som kan användas som biomarkörer för fullkornsintag av vete och råg. AR kan därför användas som ett objektivt verktyg för att mäta fullkornsintag och på så vis undvika problem som är associerade med självrapporterat intag. Alkylresorcinoler är derivat av 1,3-dihydroxybensen och förekommer endast i de yttre lagren hos vete och råg, vilket därför kan spegla intaget av fullkornsprodukter tillverkade av dessa sädesslag. AR har i flera studier använts som biomarkörer för fullkornsintag. De har däremot en relativt kort halveringstid, vilket begränsar dess användning till populationer där fullkornsintaget är frekvent och stabilt. Tidigare studier har visat en skillnad i AR-koncentrationen i plasma mellan män och kvinnor. I en förstudie har man nyligen hittat AR i fettväv från människor där koncentrationen korrelerades med fullkornsintaget. Målet med det här arbetet var att kvantifiera alkylresorcinoler i plasma från människor och jämföra koncentrationerna mellan kvinnor och män. Det var även att korrelera AR-koncentrationen i fettväv med koncentrationen i plasma hos svenska kvinnor och män. Resultatet visade ingen signifikant skillnad mellan kvinnor och män (P=0.142-0.966) i plasma vilket strider mot tidigare studier där män hade högre värden. Däremot fanns det en signifikant skillnad i AR-koncentrationen mellan kvinnor och män (P=0.003-0.047) i fettväv. AR i plasma korrelerade starkt till AR i fettväv (r=0,466-0,823, P=<0.0001). Korrelationen för C17:0 var signifikant starkast för både kvinnor, män och båda tillsammans (r=0.801- 0.823, p<0.0001).

Table of contents

Abbreviations

AR Alkylresorcinol(s)
AT Adipose tissue
CV Correlation of variation
CVD Cardio vascular disease
CHD Coronary heart disease
FFQ Food frequency questionnaire
GC Gas chromatography
MS Mass spectrometry
SD Standard deviation
WG Whole grain
24HDR 24 hour dietary recall

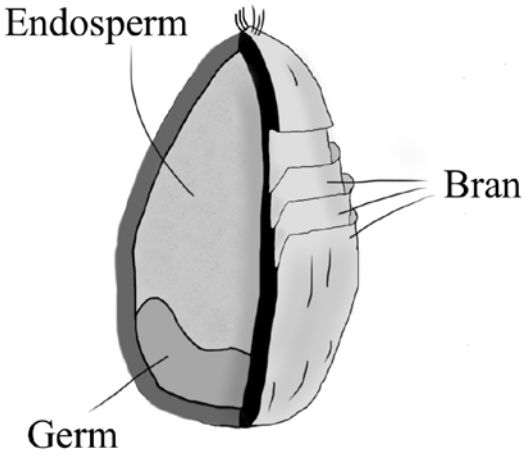
n number of participants
r correlation factor
p probability

1 Background

1.1 Whole grain and heath

Whole grain (WG) includes the bran, germ and endosperm of the cereal grain (Figure 1), as in the proportions existing in the intact kernel (Nordic Councils of Ministers 2012a). In a refined cereal product only the endosperm is retained and many vitamins, minerals and other bioactive compounds are thereby lost (Sonestedt 2013).

One of the dietary recommendations of the National Food Agency states that whole grain should be the first option of bread, cereals, pasta and rice instead of products made from refined grains. The advice is based on findings from epidemiological studies where WG has been consistently related to a reduced risk of different chronic diseases such as cardiovascular disease (CVD), type II diabetes, colorectal cancer and obesity (Livsmedelsverket 2013).



According to the nationwide eating habit investigation ”Riksmaten” performed in Sweden 2010-11 the average daily intake of whole grain was 39 g for women and 46 g for men (Amcoff et al. 2012). The recommended daily intake in Sweden is 70 g and 90 g respectively (Livsmedelsverket, 2013).

Dietary fibre and associated bioactive agents in whole grain may have a protective effect against CVD by improving endothelial function, decreasing blood pressure and serum lipids, to better the glucose and insulin metabolism and ease inflammation and oxidative stress (Tang et al. 2015). The result from a recent meta-analysis suggests a significant association with reduced risk of coronary heart disease when consuming a high amount of whole grain (Tang et al. 2015).
Other evidence also suggest that whole grain may protect against different cancers. For example, a meta-analysis suggests that high whole grain intake is associated with reduced risk of developing colorectal cancer. The relative risk was 20% lower per 90 g of whole grain consumed daily (Aune et al. 2011). The dietary fiber from the WG is fermented by the microflora in the colon. The fermentation products lower the pH in the colonic content which has shown to be a protective mechanism. Also the fecal bulking that is generated by the fiber may have a protective effect (Nordic Councils of Ministers 2012b).

Obesity is a global problem, in 2014 about 13% of the adult world population were obese (WHO 2015). Whole grains are lower in energy density compared to refined cereal products and may therefore help to prevent the problem (Nordic Councils of Ministers 2012a). A recent meta-analysis showed that a high intake of WG have a lowering effect on body fat compared to a low intake (Pol et al. 2013). Obesity also increases the risk of type-2 diabetes (Livsmedelsverket 2015). It has been shown by several studies that the risk of developing type-2 diabetes is reduced by the intake of whole grain (Jacksson et al. 2014; Chanson-Rolle et al. 2015). Intake of WG has also been associated with lower mortality (Johnsen et al. 2015). In a large Scandinavian cohort the intake of WG and mortality was investigated and the results indicated that the WG intake is an important factor in preventing early death (Johnsen et al. 2015). Also, WG has been associated with lower total CVD mortality in the US (Wu et al. 2015). Moreover, in a meta-analysis it was concluded that the intake of whole grain may reduce the risk of T2D, CVD and weight gain (Ye et al. 2012).

1.2 Methods to measure whole grain intake

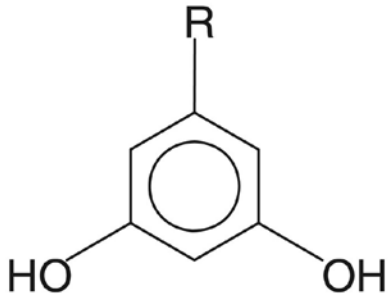
Diet investigation methods that can be applied in measuring WG intake are 24 h dietary recall (24HDR), diet diaries, food frequency questionnaires (FFQ) and diet history interviews (Wirfält & Andersson 2013). The methods differ in the aspects of how detailed and comprehensive the provided information of the diet is, and also the time dimension they reflect. The 24HDR and diet diary enable the participant to give detailed information, however they may be expensive. The diet information from the FFQ and history interviews are more comprehensive, though the methods do not provide detailed quantified data (Wirfält & Andersson 2013).

A regular problem with reporting WG intake is not only the limitation of the participants' memory capability, but also the reliability of the data reported by the individuals (Wirfält & Andersson 2013). It might be difficult for the consumer to distinguish between whole grain products from non-whole grain, for example recognize whole grain bread, since the brown colour is not a definite indicator (Lang et al. 2003). The definition of WG varies between countries and a consistent definition of a whole grain product is lacking (van der Kamp et al. 2014). Therefore it is important to declare what definition has been used to obtain accurate results (Ross et al. 2015). In certain studies the data on WG content is based on the information on the food package or in recipe, however occasionally the WG data is insufficient (Ross et al. 2015). Databases that report WG content would therefore be a useful source of information (Ross et al. 2015). To enable an analysis of the possible physiological effects of the different cereals it is important to report the individual grain types, not only the total whole grain intake (Ross et al. 2015). A problem in this aspect is the mixed-cereal products. The inconsistent reporting of WG intake may also be due to the variety of WG products and study design (Ross et al. 2015). All of these problems cause difficulties in drawing definite conclusions between WG and health (Ross et al. 2015). The use of alkylresorcinols (AR) as biomarkers may improve the accuracy in the estimation of whole grain intake. By measuring AR it is possible to avoid certain bias based on the human capacity of memory and estimation (Ross et al. 2015).

1.3 Alkyleresorcinols as biomarkers

A biomarker can work as a compound that can be measured in the body, such as plasma and urine, after consumption of a specific food, to mirror the intake of a particular nutrient. In this case Alkylresorcinols are biomarkers for whole grain intake from rye, wheat and barley (Landberg et al. 2008).

Alkyleresorcinols are 1,3- dihydroxyalkylbenzene derivates containing uneven numbers of carbons. The length of the alkyl chain varies between 15 and 27 carbons and the chain occurs with different degrees of unsaturation (Ozlem 2011). AR are only present in the outer layers of wheat, rye and barley grains, making it possible to use AR as biomarkers for whole grain intake (Ross et al. 2004). Homologues of the alkylresorcinols mentioned in this thesis are C17:0, C19:0, C21:0, C23:0 and C25:0. By calculating the ratio C17:0/C21:0 it is possible to determine which source the AR originates from. The ratio for rye is ~1.0, common wheat ~0.1 and durum wheat ~0.01 (Chen et al 2004).



Alkylresorcinol	Abbreviation	R	Molecular weight
			g/mol
5-*n*-Heptadecylresorcinol	C17:0	$C_{17}H_{35}$	348
5-*n*-Nonadecylresorcinol	C19:0	$C_{19}H_{39}$	376
5-*n*-Heneicosylresorcinol	C21:0	$C_{21}H_{43}$	404
5-*n*-Tricosylresorcinol	C23:0	$C_{23}H_{47}$	432
5-*n*-Pentacosylresorcinol	C25:0	$C_{25}H_{51}$	460

Figure 2, structure of alkylresorcinols (Landberg et al. 2008)

By using biomarkers to measure food intake it is possible to avoid errors based on memory and the human estimation of intake as they are objective and accurate measures. The bias associated with questionnaires are independent from the errors linked with biologic variables, enabling biomarkers to work as compliments for such methods (Bingham 2002). Limitations of biomarkers do however exist. With regard to association with risk factors for disease, food-group analysis from questionnaires may be more informative as there are many constituents in food that are yet unidentified or unknown. To make a health claim that is related to a certain constituent as it was the only factor is therefore unwise. It is more likely that the compound and health claim is related to a food-group or specific food (Potischman 2003).

1.4 Aim of the thesis

The aim of this study was to quantify alkylresorcinols in plasma samples and to compare the concentrations between women and men. The aim was further to correlate the plasma AR concentrations to corresponding adipose tissue AR concentrations and to evaluate gender differences in adipose tissue AR concentrations. AR-data from adipose tissue samples were kindly provided by MSc Huaxing Wu.

2 Materials and Methods

2.1 The study and the subjects

In total 240 plasma samples derived from the prospective cohorts ”Swedish mammography cohort” (SMC) (Karolinska institutet 2015a) (n=92) and ”Swedish cohort in men” (COSM) (Karolinska institutet 2015b) (n=148) were analyzed (Table 1). The individuals had provided various information about diet and lifestyle through questionnaires at baseline. Gender and energy intake was considered while the AR intake was not available for this study. For analysis, the samples were divided into 8 batches. A control sample was included for each batch in quadruplicate in order to allow estimation of the the between- and within-batch variation as coefficient of variation (CV).

Table 1. Mean age, BMI and energy intake of the subjects

	Women	Men
Age	65.7	86.9
BMI	24.2	24.9
Energy intake [kcal]	1749.2	2442.7

2.2 Analytical method

The plasma samples were analyzed according to Wierzbicka et al. (2015). Briefly, alkylresorcinol standards (C17:0, C19:0, C21:0, C23:0, C25:0) of increasing concentration were mixed with 15 µl internal standard (AR C20:0 concentration 1 µg/ml) by adding 100 µl of each standard and 2.9 ml of 2% formic acid in methanol. Each plasma sample (150 µl) and four references of 200 µl each were mixed with 15 µl of the internal standard and then vortexed. AR were then extracted with 3 ml ether by freezing the water phase in ethanol-dry ice bath, enabling to pour the organic phase into new tubes. The procedure was repeated two times more and the extracts were evaporated in 35°C heating block under a steam of nitrogen (g). One ml 100% methanol was then added to the samples. For sample cleanup solid phase extraction was done by using Oasis Max columns. The elutes were evaporated in 60 °C heating block under a steam

of nitrogen (g) together with the standards. The samples were silylated with 200 µl triflouroacetic anhydride and then vortexed and incubated at 40 °C in 30 minutes. After transferring the samples to GC-vials they were evaporated in 60 °C heating block. 20 µl undecane were then added to the vials and the samples were then analysed in GC-MS.

2.3 Statistical analysis

Differences of homologue and total plasma AR concentrations, C17:0/C21:0 ratio between women and men were evaluated by t-tests based on log-transformed values. Plasma AR concentrations were correlated with adipose tissue AR concentration data by calculating the spearman's rank correlation coefficients. Factors as age, BMI, energy intake and gender were tested as predictors of log-AR concentrations using GLM. All statistical analysis was performed with SAS and Minitab software.

3 Result

3.1 Data quality

All samples were divided and analyzed in 8 batches. The intra- and inter batch precision was estimated by the coefficient of variation (CV). The within- and between batch CV for total AR concentration in plasma was <7% (Table 2) and <9% (Table 3), respectively. Based on the results of quality control samples, data obtained was regarded as accurate (adequate precision and similar mean value as established previously for this quality control sample).

Table 2. CV of within-batch variation

Batch	1	2	3	4	5	6	7	8	Mean
CV [%]	8.3	6.1	4.0	2.4*	7.5	4.4	11.6	9.7	6.7

*CV based on three QC replicates due to loss of one replicate during extraction.

Table 3. Control sample total AR concentration and CV of between-batch variation

Batch	1	2	3	4	5	6	7	8	CV [%]
AR tot [nmol/l]	101.8	106.5	102.6	103.1	94.9	80.3	98.4	89.5	8.9

3.2 Alkylresorcinol concentrations in plasma

The AR concentrations were not normally distributed, but right –skewed. The median plasma AR homologue concentration was calculated separately for men and women (Table 4). The median total plasma AR concentration was 48 and 51 nmol/l for women and men, respectively. The corresponding mean±standard deviation (SD) was 70.0±72.6 and 61.9±46.5, respectively. Corresponding values for all individuals together was 51 nmol/l and 65.0±57.8. No significant difference was observed for plasma AR homologues between men and women (Table 4). The median AR C17:0/C21:0 ratio was 0.3 for both men and women and there was no significant association between the sexes.

Table 4. The total plasma AR- and homologue concentration (nmol/l) for men and women

	C17:0	C19:0	C21:0	C23:0	C25:0	Total	C17:0/C21:0
Women	8.2±8.4 (5.1)	22.4±26.1 (15.6)	23.8±22.1 (17.3)	7.3±7.6 (5.4)	8.4±11.6 (5.6)	70.0±72.6 (47.6)	0.3±0.2 (0.3)
Men	7.7±7.0 (6.3)	20.9±15.6 (16.9)	20.9±14.5 (18.0)	6.2±5.2 (4.8)	6.2±6.4 (4.1)	61.9±46.5 (51.4)	0.4±0.2 (0.3)
P	0.776	0.273	0.732	0.228	0.142	0.966	537

Values are reported as mean±SD and (median). P- value of the difference in AR concentration between women and men.

3.3 Alkylresorcinol concentrations in adipose tissue

The median total AT AR concentration in women was 0.9 µg/g and 1.1 µg/g in men. Corresponding values for all individuals was 1.0 µg/g. Total AR- and individual AR homologue concentration was significantly higher in men than in women (Table 5). For the adipose tissue C17:0/C21:0 ratio, no significant difference was observed for men and women (p=0.825).

Table 5. The total adipose tissue AR- and homologue concentration (ug/g) for men and women

	C17:0	C19:0	C21:0	C23:0	C25:0	Total	C17:0/C21:0
Women	0.1±0.1 (0.1)	0.3±0.3 (0.3)	0.4±0.3 (0.3)	0.1±0.1 (0.1)	0.2±0.1 (0.1)	1.0±0.8 (0.9)	0.3±0.5 (0.3)
Men	0.1±0.1 (0.1)	0.4±0.3 (0.3)	0.5±0.4 (0.4)	0.2±0.1 (0.1)	0.2±0.2 (0.1)	1.3±1.0 (1.1)	0.3±0.2 (0.3)
P	0.006	0.003	0.010	0.024	0.047	0.006	0.825

Values are reported as mean±SD and (median). P-value of the difference in AR concentration between women and men.

3.4 Correlations between plasma AR and adipose tissue AR concentrations

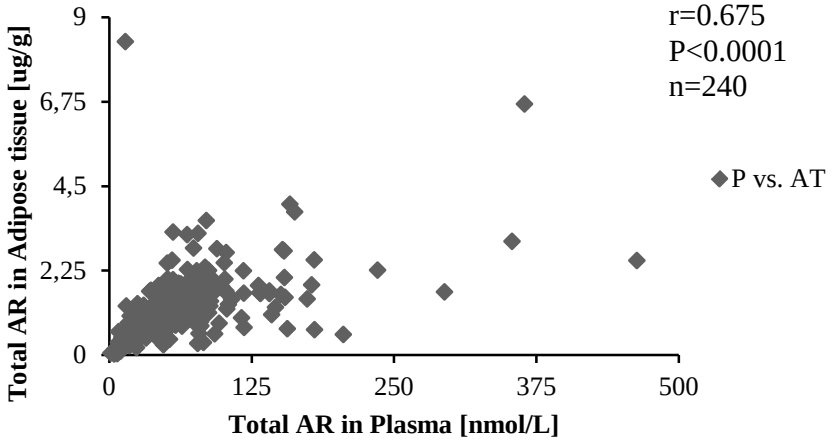
The plasma AR homologue concentrations were significantly correlated with AR homologues in adipose tissue($r=0.47-0.82$, $p<0.0001$) (Table 6). The strongest correlation was observed for C17:0. A scatter plot for women and men jointly is seen in Figure 1 ($r=0.675$, $p<0.0001$).

Table 6. Correlation between plasma AR and adipose tissue AR in men and women

	XC17:0 vs. YC17:0		XC19:0 vs. YC19:0		XC21:0 vs. YC21:0		XC23:0 vs. YC23:0		XC25:0 vs. YC25:0		Xtot vs. Ytot	
	r	P	r	P	r	P	r	P	r	P	r	P
Women	0.823	$<10^{-4}$	0.683	$<10^{-4}$	0.618	$<10^{-4}$	0.572	$<10^{-4}$	0.466	$<10^{-4}$	0.658	$<10^{-4}$
Men	0.801	$<10^{-4}$	0.703	$<10^{-4}$	0.619	$<10^{-4}$	0.598	$<10^{-4}$	0.512	$<10^{-4}$	0.683	$<10^{-4}$
Both	0.806	$<10^{-4}$	0.707	$<10^{-4}$	0.611	$<10^{-4}$	0.577	$<10^{-4}$	0.486	$<10^{-4}$	0.675	$<10^{-4}$

X= plasma Y= Adipose tissue

Figure 1. Correlation between total plasma AR and adipose tissue AR in both men and women



3.5 Factors affecting AR concentration in plasma and adipose tissue

To investigate the influence of other factors than whole grain wheat and rye intake on the AR concentration in plasma and adipose tissue; age, energy intake and sex were tested (Table 7). Only energy intake was significantly associated with AR concentration in both plasma (P= 0.004) and AT (P=0.022).

Table 7. Tested factors of which may affect AR concentration in plasma and adipose tissue

	Plasma	Adipose tissue
	p	p
Age	0.226	0.229
Energy intake	0.004	0.022
Gender	0.582	0.132*

*There was no independent gender effect

4 Discussion

To assure a sufficient quality of the data generated by the analysis, a control sample was included in each batch in quadruplicate. Precision was estimated by CV and accuracy was estimated by comparing the mean values of AR homologue concentrations to established values for that sample. The analysis was approved if the within- and between batch variation was <15%. As the CV of within-batch variation was <7% and the between-batch variation for total AR concentration was <9%, obtained data was accepted and used in further data analysis.

In this study no significant difference in plasma AR concentration between women and men was found. The result do not comply with a previous study based on the European prospective investigation into cancer and nutrition (EPIC), where plasma samples from 100 individuals were analyzed. The AR concentration was higher for all the homologues in the samples from men in that study (Montonen et al. 2010). Median plasma AR concentrations were slightly higher for men and considerably higher for women than has been shown in other free-living Swedish men and women before (Kyrø et al. 2014). This may be due to difference in whole grain intake of men and women in the different studies. Both men and women in the current population were older than in many previous studies (Kyrø et al. 2014, Landberg et al. 2013) and it is well known that men and women at higher ages consume more WG than younger people (Thane et al. 2007). The differences in intake may be smaller among elderly and the potential differences in AR metabolism by men and women suggested by Montonen et al. 2010 may be smaller among elderly than in younger individuals. The median C17:0/C21:0 was similar for both men and women which indicates that men and women consumed similar proportions of wheat and rye and that their habitual intake is a mixture of wheat and rye.

Alkylresorcinols in adipose tissue has only been reported once before in a limited number of subjects (n=20), also from the Swedish mammography cohort. The average total AR concentration in women was 0.54 ug/g (Jansson et al. 2010) compared to the 1.0 ug/g shown in this study. As the number of AT samples analyzed differ between the studies it may contribute to the different results. Another reason might be that the current validated method used is slightly different than the method used by Jansson et al. 2010. Total AR- and individual

AR homologue concentration was higher for men than women. Partly this may be due to the difference of energy intake between the sexes (Table 1), as men eat more in total it is more likely that they consume more whole grain than women. Energy intake was a significant determinant of AR concentration in both plasma and adipose tissue, and supports the idea of higher overall intakes of foods, including whole grains among men compared with women. However this was not reflected in differences in plasma AR concentrations between men and women. The median C17:0/C21:0 ratio was similar for men and women and mirrored that of plasma

It has been shown that AR has a similar metabolism as tocopherols (Ross et al. 2004) and that women metabolizes AR faster than men, which would result in lower levels of AR in the plasma per time unit (Montanen et al. 2010). However, this theory do not comply with the result in this study. Contrariwise as the men show higher AR content in adipose tissue, the AR metabolism may differ between men and women in AT.

The plasma AR correlates strongly with the AT AR for the individual homologues and total AR for women, men and jointly. However the correlation was strongest for homologue C17:0 and moderate for C25:0. The correlation indicates that the WG intake mirrors the AT AR content in a similar way as AR in plasma. This is surprising, since most compounds analyzed in adipose tissue reflect long-term intake/status due to a longer turn-over rate of adipose tissue (Arab 2003). It appears that AR in adipose tissue for some reason is more rapidly exchanged. This merits further investigation.

In conclusion, there was no significant difference in plasma AR concentration between men and women. However, a significant difference in the alkylresorcinol concentration in adipose tissue was found between men and women. Plasma AR concentrations correlated well with AR concentrations in adipose tissue in both women and men. Plasma AR and adipose tissue AR should be compared with estimated long-term whole grain wheat and rye intake in order to evaluate which matrix that would best reflect long-term intake.

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