Metabolic signatures related to diet in human urine and blood specimens
– Review of an emerging methodology in health studies

Magnus Lindelöf
Metabolic signatures related to diet in human urine and blood specimens

Magnus Lindelöf

Supervisor: Dr Rikard Landberg, Swedish University of Agriculture, Department of Food Science
Examiner: Dr Lena Dimberg, Swedish University of Agriculture, Department of Food Science

Credits: 15 hp hec
Level: G2E
Course title: Självständigt arbete i livsmedelsvetenskap
Course code: EX0669
Programme/education: Agronom- livsmedel

Place of publication: Uppsala
Year of publication: 2012
Title of series: no: Publikation/Sveriges lantbruksuniversitet, Institutionen för livsmedelsvetenskap: 345
Online publication: http://stud.epsilon.slu.se

Key Words: Metabolomics, food, health, humans, nutrition, diet, blood, urine, lifestyle
Abstract

Metabolomics is an emerging methodology which has lately been used in nutrition and health research. There is large interest to develop metabolomics-based methods for reflection of dietary exposure in epidemiological studies and standardized intervention studies. The aim of this BSc-thesis was to investigate the possibilities if metabolomics analyses of urine and blood specimens could be used to reflect differences in subject characteristics, lifestyles, and dietary patterns in free-living humans. Empirical data were collected using database search for articles where dietary metabolomics experiment were conducted and reported in human subjects, along with reference search in the selected articles. Metabolites from food, endogenous response to exposure, and gut microbial activity were found responsible for metabolic signatures of dietary intake in both blood and urine specimens. Gender, age and diurnal variation were also found responsible for unique metabolic signatures, characterized to some extent by likely lifestyle factors such as diet. Subject characteristics, lifestyle, and time of sampling are suggested to play important roles when assessing a metabolic signature of dietary patterns. Other factors to consider is for how long the dietary effect will last, usage of proper statistical analysis, and choice of analytical platform.

To conclude, food seems to have a rather large influence on human urine and blood metabolic phenotype and identification of metabolic signatures of dietary patterns, using metabolomics, is likely to be achieved.
Table of Contents

1 Introduction ......................................... 6

2 Methods ............................................. 7

3 Metabolomics; definitions and techniques ..... 8

4 Assessment of diet and lifestyle through their metabolic signatures .................. 10
   4.1 Gender, age, BMI and diurnal variations ...................................................... 10
      4.1.1 Gender .................................................................................................. 10
      4.1.2 Age ....................................................................................................... 14
      4.1.3 BMI ...................................................................................................... 15
      4.1.4 Diurnal and circadian variation .............................................................. 16
      4.1.5 Inter- and intra-individual variation ...................................................... 17
   4.2 Foods and dietary patterns .......................................................... 18
      4.2.1 Foods rich in bioactive compounds ...................................................... 18
      4.2.2 Foods of animal origin ......................................................................... 22
      4.2.3 Cereal based foods ............................................................................... 22
      4.2.4 Macronutrients .................................................................................... 23
      4.2.5 Supplements and fortifications .............................................................. 23
      4.2.6 Food groups and diets ......................................................................... 23

5 Discussion ............................................ 25

References ................................................ 29

Acknowledgment .................................... 33
1 Introduction

Metabolomics is an emerging field and one of the latest areas adopting this method is nutrition and health research. Several studies, most on small groups, investigating dietary effects and searching for biomarkers in human blood and urine specimens have been carried out over the last ten years. Now, researchers are preparing for the next step and are planning to apply this knowledge to dietary surveys and prospective cohorts in order to find relations between diet and health (Primrose et al., 2011). To find those relations, reliable and robust metabolic signatures of dietary exposures are necessary.

The aim of this BSc-thesis was to investigate the possibility of using metabolomics analyses of urine and blood specimens to reflect differences in subject characteristics, lifestyles, and dietary patterns in free-living humans. If so, it could be further evaluated as a tool for objective measurements of these traits in future epidemiologic and intervention studies.
2 Methods

To assess the aim of this thesis, research papers investigating metabolic signatures associated with gender, age and BMI as well as foods and dietary components were reviewed. To fully understand if a metabolic signature is a response to a certain lifestyle, the relative contribution of inter- and intra-individual variation as well as diurnal variation of metabolites in blood and urine were also discussed. The empirical material in this review was mainly collected using database search at: http://www.ncbi.nlm.nih.gov/pubmed/. The review was delimited to human dietary metabolomics. Studies reporting experiments on animals, statistical and analytical methods, review articles, changes in metabolites related to medical diagnose or illness, medical and toxicology studies were excluded. To embrace material relevant to the aims and meeting the criteria, the search string “Metabolomic* AND (health OR lifestyle OR phenotype OR population OR nutrition OR food)” was used without any restriction for publication year. The filtering for human studies was used. Full search information is attached in Appendix A. In total 585 articles were found and from those 30 met the criteria and were included in the present work. References in the selected studies were investigated and additionally 17 articles were found to be within the criteria. A compilation of the selected articles summarizing study design and duration, scope of analysis and metabolites of importance, analyzed bio fluid, analytical platform, and statistical tools used are presented in Appendix B.
Metabolomics refers to the quantitatively and comprehensive study of metabolites in an organism (Dettmer and Hammock, 2004). The term metabonomics is also frequently used, but the two terms are nowadays considered denoting the same technique (Madsen et al., 2010). Metabolomics can be described as a snapshot of all small metabolites constituting an organism’s phenotype and is the latest of the “omics”- sciences, preceded by genomics, transcriptomics and proteomics. In humans, blood and urine samples are commonly the target for this holistic type of analysis. In contrast to the other “omics”, metabolomics provides broader information of the phenotype, with metabolites reflecting both genetic setup and response, and exposure to environmental factors.

The metabolomics approach is described as a hypothesis generating technique rather than a technique for testing hypothesis (Kell, 2004). This description is especially valid for untargeted approaches, aiming to detect as many metabolites in a single analysis as possible in a specimen, in contrast to targeted approaches, that focus on a number of metabolites of interest e.g. compounds related to a certain pathway and are more suited for explanation of theories (Patti et al., 2012). The scope of this article was to investigate if it is possible, using metabolomics, to generate new hypotheses exploring links between metabolic signatures (specific concentration pattern of a set of metabolites) and dietary exposure and thus the untargeted approach was of special interest.

Nuclear magnetic resonance (NMR), here always referring to $^1$H-NMR, and mass spectrometry (MS), coupled to various ionization sources, are the main technological platforms for metabolomics information collection in bio fluids. Gas chromatography (GC) -MS is suitable for targeted purposes, but are limited to small, volatile and thermally stable compounds or derivatives and thus liquid chromatography (LC) is more commonly used in metabolomics due to its ability to separate most metabolites (Issaq et al., 2009). MS is a more sensitive method, but
NMR has the advantages of high reproducibility and no need for separation or derivatization (Malet-Martino and Holzgrabe, 2011). Capillary electrophoresis coupled to UV-detector (CE-UV) have also been used as a fast and inexpensive fingerprinting tool for scanning metabolites in a sample with small possibilities for identification (Balderas et al., 2010).

The vast amount of information generated in an untargeted analysis needs to be interpreted in a rational manner and multivariate statistics is a cornerstone in metabolomics (Madsen et al., 2010). Often data pretreatment, such as orthogonal signal correction (OSC), is used and this step can enhance correlation patterns in data, but if used neglectful it will have the opposite effect (Rajalahti and Kvalheim, 2011). Principal components analysis (PCA) is performed to unsupervised identify patterns of interest and possible outliers disturbing interpretation of data (Wold et al., 1987). In order to further reduce dimensionality of the material and investigate covariance between response and data partial least squares (PLS) regression is used (Rajalahti and Kvalheim, 2011). These two methods are widely used in metabolomics studies with variants such as orthogonal-PLS-discriminant analysis (O-PLS-DA), which is a supervised analysis to assign membership of defined classes.
4 Assessment of diet and lifestyle through their metabolic signatures

4.1 Gender, age, BMI and diurnal variations

To reflect dietary exposures in a metabolic signature it is important to avoid bias and confounding factors, as well as unwanted variation that might overshadow less pronounced signals from the diet of interest. Gender, age, BMI and diurnal variation can be such factors obstructing a correct interpretation or valid result.

4.1.1 Gender

Men and women exhibit differences in genetic setup, lifestyle and other ways and the phenotypic response to these differences have been of interest in several metabolomics studies.

Metabolic signatures in blood

Plasma or serum signatures of males and females have been explored in a couple of studies (Bertram et al., 2009; Kochhar et al., 2006; Lawton et al., 2008; Mittelstrass et al., 2011). These studies have shown that men and women can be differentiated by using a metabolomics approach in observational studies and profiles showing increased lipid metabolism in females and metabolites suggesting a higher protein turnover in males have been shown to explain differences. In one of the studies, Bertram et al. (2009) used an untargeted approach and analyzed plasma samples from 75 teenagers, using NMR and PLS- discriminant analysis (DA) they found females to have higher levels of high-density lipoproteins (HDL), unsaturated lipids and choline, while males had higher levels of proline, and the branched amino acids, leucine, isoleucine and valine, as well as glucose. In another study, Lawton et al. (2008) used a targeted MS approach on blood samples from
a cohort of 269 North Americans. They showed levels of lipid metabolism associated nonanedioate, myristate, palmitoleate, glycerol and β-hydroxybutyrate to be higher in females, together with orthophosphate, α-tocopherol, and creatine. In males they found amino acid metabolism related compounds in general to be higher, such as methionine, tryptophan, creatinine, and 4-methyl-2-oxopentanoate. Energy metabolism products, with citrate, cis-aconitate and malate as most notable, were also in general found to be higher in males, so was dehydroepiandrosterone sulfate. Moreover, combined data from NMR-spectra and single plasma and urine specimens in 150 healthy adults ended up in the same conclusion (Kochhar et al., 2006). Results from these studies are confirmed in a population study by Mittelstrass et al., (2011) who have used a large quantity of MS serum data from Cooperative Health Research in the Region of Augsburg (KORA) cohort. The authors also suggest that differences in metabolome between males and females were related to differences in metabolic pathways rather than differences in random metabolites derived from environment (including the diet). This suggestion is based on connection of metabolites such as sphingomyelins, for females, and acylcarnitines, for males, to gender by linear regression analysis. Correlation analysis connected the analyzed metabolites pair wise into a network with strongly correlated or independent metabolite clusters, interpreted as different pathways, after correction for gender, age and BMI. The identified gender markers were found to be parts of different clusters and hence regarded as representatives of different pathways.

Single metabolites that are measured may lead to hypothesis of differences in metabolic pathways. A number of metabolites have been identified to discriminate between men and women (Table 1). NMR-spectra from blood plasma analyzed with PLS-DA showed that higher levels of HDL and choline from membrane lipids were associated with females in Scandinavian teenagers (Bertram et al., 2009) and in subjects of different ages (Kochhar et al., 2006). In addition Kochhar et al. (2006) attributes higher concentrations of LDL, unsaturated lipids and total lipoprotein to females and higher levels of VLDL to males. With their models based on NMR-data, the two above mentioned studies also suggested higher levels of amino acids, in particular the branched amino acids, in males to contribute to gender separation.

In a targeted MS approach, Mittelstrass et al., (2011) used linear regression analysis to evaluate gender effects of metabolites identified from PLS. The branched amino acids were also here found to be higher in males. Higher levels of creatinine in blood are also associated with male metabolic signature. Kochhar et
al. (2006) and Lawton et al. (2008) relate this change to a higher amino acid metabolism. Although high amino acid concentrations in blood have been attributed to men, it seems to be glycine and serine that are indicative of a female metabolic signature (Mittelstrass et al., 2011).

The above mentioned raised choline blood levels associated with a female metabolic signature are refined by Mittelstrass et al. (2011) who found phosphatidylcholine acyl-acyl (PC aa) and phosphatidylcholine acyl-alkyl (PC ae) species to be higher in females and in addition also found lysophosphatidylcholine acyl (LysoPC a) species to be higher in males using a targeted MS based approach. This might call for more careful handling of signals from choline moieties if they are to assign gender separation. Elevated serum lysoPCs have also been reported as marker of a high insulin response diet in a targeted MS based 12-week study and may therefore have underlying mechanisms that go beyond gender (Lankinen et al., 2010).

Table 1. Metabolites associated with gender metabolic signatures.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Matrix</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Bertram et al., 2009)</td>
<td>Blood plasma</td>
<td>HDL, unsaturated fatty acids, choline</td>
<td>Valine, leucine, isoleucine, proline,</td>
</tr>
<tr>
<td>(Lawton et al., 2008)</td>
<td>Blood plasma</td>
<td>Orthophosphate, α-tocopherol, creatine and lipid metabolism biochemicals</td>
<td>Creatinine, dehydroepiandrosteron sulfate</td>
</tr>
<tr>
<td>(Kochhar et al., 2006)</td>
<td>Blood plasma</td>
<td>HDL, LDL, choline, unsaturated lipids, lipoproteins</td>
<td>VLDL, creatine/creatinine, valine, isoleucine</td>
</tr>
<tr>
<td>(Mittelstrass et al., 2011)</td>
<td>Blood serum</td>
<td>Serine, glycine, Sphingomyelins, phosphatidyl cholines (diacyl and acyl-acyl species)</td>
<td>Acetylcarnitines, ornithine, arginine, glutamine, C6-sugars, amino acids, phosphatidylcholines (lyso species)</td>
</tr>
<tr>
<td>(Kochhar et al., 2006)</td>
<td>Urine</td>
<td>Citrate</td>
<td>Taurine, creatine/creatinine</td>
</tr>
<tr>
<td>(Simpson et al., 2007)</td>
<td>Urine</td>
<td>Citrate, fumarate, creatine</td>
<td>Carnitine, acetylcarnitine, acetone, creatinine</td>
</tr>
<tr>
<td>(van Velzen et al., 2008)</td>
<td>Urine</td>
<td>Citrate, glycine</td>
<td></td>
</tr>
<tr>
<td>(Wang et al., 2005)</td>
<td>Urine</td>
<td>Citrate, glycine</td>
<td>Creatinine</td>
</tr>
<tr>
<td>(Psichogios et al., 2008)</td>
<td>Urine</td>
<td>Citrate, glycine, creatine</td>
<td>Creatinine, taurine, TMAO</td>
</tr>
</tbody>
</table>

**Metabolic signatures in urine**

Both higher levels of citrate and glycine in urine samples, interpreted from PCA loading plots of NMR-spectra, contribute to female metabolic signature in two
smaller intervention studies (van Velzen et al., 2008; Wang et al., 2005). Wang et al. (2005) collected 30 spot urine samples from 7 men and 7 women after a 6 wk intervention with chamomile tea extract. van Velzen et al. (2008) connected citrate and glycine to females in a placebo controlled cross-over trial investigating metabolic signature of red wine and grape juice extracts. This association was found after separating inter-individual and intra-individual variation in 24 hour urine, collected at end of each two-week intervention, and subjecting the inter-individual variation to PCA. Psihogios et al. (2008) on the other hand did not find a gender separation in urinary NMR-spectra with PCA, nor with PLS-DA, in a cross-sectional study of 122 Greeks. However, after OSC filtering prior to PLS-DA they also identified citrate and glycine as contributors to a female metabolic signature.

Kochhar et al. (2006) also describes citrate as a contributor to female metabolic signature, but glycine levels are only described as a significant contributor to gender separation in older people. Besides that, subtle raise of fumarate in females have been discovered in urine samples from four non-consecutive days in a six months observational study, using a targeted approach, but not when the same samples were subjected to untargeted binning procedures (Slupsky et al., 2007). This approach can be of interest to use if an untargeted model indicates separation of classes such as genders, but more power is required to establish a significant metabolic signature.

A contributor to a male metabolic signature in urine, found by most metabolomic studies with focus on the effects of gender on biofluid metabolites, was creatinine (Psihogios et al., 2008; Wang et al., 2005). Slupsky et al., (2007) also reported that differences in male urinary creatinine concentrations, together with acetylcarnitine, contributed to the separation, but to lesser extent compared to acetone and carnitine. No distinction between creatine and its end product creatinine was made by Kochhar et al., (2006). They suggested this metabolite pair to contribute to a male metabolic signature. This is in contrast to other findings that suggest higher levels of creatine in urine to be associated with a female metabolic signature (Psihogios et al., 2008; Slupsky et al., 2007). High 3-hydroxy butyrate taurine concentration in male urine was another metabolite that was associated with a male metabolic signature (Kochhar et al., 2006). A similar conclusion was made by Psihogios et al. (2008) who also have found raised levels of trimethylamine oxide (TMAO) in males to be a discriminatory factor. TMAO are a metabolite linked to fish consumption (Holmes et al., 2008; Lenz et al., 2004; Lloyd et al., 2011b). This observation can reflect that lifestyle differences between males and females can give substantial contribution to a gender specific metabolic signature.
4.1.2 Age

Research has also been made into how age may contribute to specific metabolic profile of urine and blood specimens. In a small study, with focus on intra-individual diurnal variation in plasma NMR-spectra, Park et al. (2009) found that a model with three Principal Components (PCs) separated two distinct age groups (22-45 and 75-83 years of age). These three PCs accounted for 77% of the total variation in the urine metabolome and subject characteristics. In urine, separation into age classes are also reported using less distinct grouping (Slupsky et al., 2007). With 40 years of age as cut point, they found an increase of trigonelline and lover concentrations of creatinine, lactate, acetylcarnintine, carnitine, alanine, 3-hydroxysovalerate and cis-aconitate to be associated with an age dependent metabolic signature using their targeted approach. Intuitively, a clear distinction between age-groups will increase the possibilities to find an age specific metabolic signature and Psihogios et al., (2008) have observed age clustering using a single cut point, but division into more distinct groups (<35 and >50 years of age) yielded a clear class separation.

Another possible way to discover age-dependent metabolic signatures could be to include gender. Kochhar et al., (2006), who despite distinct age grouping (18-29 and >46 years of age), only found higher creatine/creatinine to be independently associated with higher age in PLS-DA treated NMR-spectra of urine samples. On the other hand they found that changes in levels of lipoproteins, unsaturated lipids and citrate in plasma, and urine levels of dimethylamine and citrate were associated with age in male subjects whereas for female subjects, changes in plasma alanine, tyrosine, isoleucine, and valine together with changes in urine glycine were associated with age. Using analysis of co-variance (ANCOVA) on MS derived blood plasma data from 269 North Americans Lawton et al. (2008) also found gender specific parts of a possible age metabolic signature with α-tocopherol, L-kynurenine, and glycerol-3-phosphate in blood as contributing metabolites. Considering these results, changes of metabolic signature with age seems to be different in males and females.

In addition Lawton et al. (2008) found that age was associated with (51-65 years of age, compared to 20-35 and 36-50 years old) increased blood concentrations of amino acids, aconitate, isocitrate, malate, ornithine, degradation products of purine metabolism, and metabolites related to uric acid metabolism. They suggest that this metabolic signature reflect increases in muscle degradation and inflammatory complaints, but since no other factors than race and gender were controlled their conclusions are very conservative.
Lawton et al., (2008) identified about 100 plasma metabolites that were associated with age when analyzing plasma samples from 269 subjects with LC-MS and the large number of metabolites was suggested to be helpful in the seeking for pathway changes due to age. This screening of metabolites was similar to targeted studies like D’Adamo et al. (2010) who focused on amino acids in urine specimens using NMR and showed significant differences over ages with a U-shaped distribution of most amino acid levels with peaks in young and elderly people and adults having the lowest levels.

When looking at the contribution of age to overall metabolome variation Psigios et al. (2008) stated that gender have more profound effects on the urinary profile compared to age. In contrast Lawton et al. (2008) have found far more plasma metabolites associated with age than those associated with gender. Using a more targeted approach Altmaier et al. (2011) showed that age not substantially affected the association between certain serum lipids and reported consumption of food groups by subjects in the KORA cohort.

4.1.3 BMI
Variation in Body Mass index (BMI) will occur in any population and is due to differences in physical activity level, basal-metabolic rate and thermogenesis. Irrespective of to what extent BMI is explained by life-style or genetic predisposition it is an important measure to roughly estimate overweight or obesity. An interesting question is if variation in relative body mass is reflected by a metabolic signature. Partial separation of adjacent BMI classes (18.9-24.9 kg/m² and 25-32.6 kg/m²) with PCA on blood plasma NMR-spectra were achieved in a small study (Park et al., 2009). In contrast PCA of plasma NMR-data from Scandinavian teenagers did not separate any of the three BMI groups (<20 kg/m², 20-25 kg/m² and >25 kg/m²) (Bertram et al., 2009). BMI was reported to have little impact on dietary metabolic signatures in blood serum in a targeted quantitative analysis with focus on lipids (Altmaier et al., 2011). The use of separated BMI-classes and PLS-DA may enhance the possibilities to correlate BMI and metabolome and Kochhar et al., (2006) achieved a separation between lean and overweight (<21 kg/m² and >25 kg/m²) in both urine and plasma using PLS-DA. Many of the metabolites in that study that were associated with BMI were also dependent on gender, as for those related to protein turnover, where both lean males and overweight females were associated with a higher rate of protein turnover. Dimethylamine in urine and citrate, choline, and creatine/creatinine in plasma were on the other hand
independent of gender and studies have been conducted to elucidate if BMI adjusted for gender would give two distinct metabolic signatures.

4.1.4 Diurnal and circadian variation

In contrast to gender, age and BMI, diurnal variation is more easily studied as an intra-individual effect. The intra-individual variation can be important to understand in studies with urine samples collected at different times. In a small intervention with standardized meals, 24 hour urine from two separate days were analyzed and some separation of first void, before, and afternoon samples was achieved using PCA on urine NMR spectra (Lenz et al., 2003). The authors stated that time of sampling appear to play an important role in clinical studies where metabolomics is used. On the other hand it is unclear if their model can distinguish if this separation is an effect of their intervention or circadian variation. In a larger observational study with 60 participants, PLS-DA of targeted NMR spectra from morning and afternoon spot urine indicated some differentiation between the two time points, but with considerable overlap (Slupsky et al., 2007). Dimethylamine and 1-methylnicotinamide were found to be highly significant contributors to this separation whereas creatinine, mannitol, xylose and acetone were less significant. The observed overlap might be explained by the great inter-individual variation compared to intra-individual variation observed by Lenz et al. (2003). Circadian changes of metabolic signature in urine are also proposed by Llorach et al. (2009) in a small intervention study. Using PLS-DA with orthogonal signal correction (OSC) on LC-MS data they observed separation of baseline samples and accumulated samples 6, 12 and 24 hours after intervention. This indicated different and independent metabolic signatures over time for both cocoa intervention and a cocoa free control meal. These observations were used for separation of changes in metabolic signature over time caused by their intervention and other factors contributing to the diurnal variation. Separation tendencies have also been observed between evening and morning spot urine samples from 30 individuals subjected to a one day standardized diet with creatinine as major contributing metabolite (Walsh et al., 2006). The occurrence of a diurnal pattern in urine seems doubtless, but more research is needed to investigate the relative contribution of lifestyle and circadian rhythm.

Diurnal variation of metabolic profile has also been explored in blood plasma. In 10 persons under standardized diet, NMR spectra from 24 hourly blood samples, averaged for each time point, were subjected to PCA and revealed specific patterns for morning, afternoon, and night (Park et al., 2009). The afternoon class
was the most inconsistent in duration and phasing between individuals and this was suggested to depend on absorption and postprandial differences between the subjects. Another interesting finding was that the fasting samples drawn at 6:30 and 7:30 were classified as “night” while fasting samples from 8:30 and 9:30 were classified as “morning”. This was suggested to originate from circadian variation, arousal, or physical activities and make ground for consideration of sampling time for fasting morning urine. As sampling in clinical studies often is made in the morning the delicate differences that are analyzed in metabolomics may be even harder to recognize if they are interfered with variation in metabolites caused by the transition between a “night” and “morning” metabolic signature.

Diurnal variation reflects both circadian rhythm and response to lifestyle and diet and it seems possible to identify time dependent metabolic signatures in a homogenous group, but if circadian variations were filtered from the diurnal variation it would be more likely that such metabolic signature would be valid for broader groups, as in an epidemiological studies. To summarize metabolic profile of both blood and urine seems to be a subject of variation over different times of the day. Inter-individual variation seems to overshadow intra-individual changes and more advanced studies filtering inter-individual variation can be useful in order to identify relevant diurnal changes in metabolic signature.

4.1.5 Inter- and intra-individual variation

Targeted analysis of differences in fasting serum between two samples over 4-months in 100 persons showed high inter-individual variation and low intra-individual variation in hexoses, amino acids, saturated short- and medium acylcarnitines and most sphingomyelins and glycerophospholipids (Floegel et al., 2011). Their conclusion was that these metabolites are suitable for epidemiological assessment with single samples. This was explained by the fact that low intra-individual variation indicates a reliable marker less dependent of time of sampling and that high inter-individual variation will increase the expected explanatory power of the metabolite.

Using an untargeted NMR approach, PCA of plasma data from 12 subjects on standardized diet showed little variation between two samples collected two week apart, but nevertheless was the intra-individual variation less than the inter-individual variation (Lenz et al., 2003). In the same study the observed inter-individual variation in urine NMR spectra was considerable between the two sampling days, while the intra-individual variation was lower. Walsh et al. (2006) reported the same relation for intra- and inter-individual variation in urine from 30
subjects with a diet free of choice, and moreover, when applying a standardized diet the inter-individual variation was reduced. This was supported by Favé et al. (2009) who attempted to develop a standardized metabolomics protocol to investigate dietary exposures in urine with ESI-MS approach. They found that standardized meals produced highly consistent results with clear grouping of overnight and first void urine after standardized dinner and postprandial (1.5 -4.5 hours) samples after standardized breakfast in three separate studies. Using a smaller number of the participants, the intra-individual variation in overnight and fasting urine was found to be rather low. Small numbers of this type of samples are thus suggested to function as a baseline standard when assessing effects of dietary exposure. In contrast to Lenz et al. (2003), analysis of plasma indicated considerable variation both between and within persons on a diet free of choice and, interestingly, after standardized diet, no reduction of the inter-individual variation was observed (Walsh et al., 2006).

4.2 Foods and dietary patterns

What we eat will eventually, to various extent, end up in our bloodstream and then mainly be excreted in one way or another through urine or bile. Dietary metabolites in urine, with energy metabolism products and dicarboxylic acids as important co-contributors, have shown to be powerful enough to separate different subjects as belonging to different populations (Holmes et al., 2008).

4.2.1 Foods rich in bioactive compounds

Plant derived foods are commonly rich in bioactive compounds, phytochemicals, and their acute impact on the urinary metabolic signature made it possible to distinguish between a low phytochemical diet and a standardized phytochemical diet in 21 subjects (Walsh et al., 2007). PLS-DA of NMR spectra indicated hippurate, a product known to be derived from gut microbial degradation of phenols, as positively correlated to the standardized phytochemical-rich diet and creatinine and methyl histidine as markers of the low phytochemical diet.

*Cocoa and chocolate*

Several urinary markers of single dose cocoa consumption have been found to be elevated over at least 24 hours in a small LC-MS based study (Llorach et al., 2009). Using O-PLS-DA they discovered many theobromine metabolites from cocoa together with a time dependent excretion pattern with host cocoa epicate-
chins metabolites, vanillic acid, tyrosine and the niacin metabolites hydroxynicotinic acid and trigonelline as dominating after 6 hours. While after 12 hours, microbial products of cocoa epicatechin such as phenylvaleric and phenylvaleronacetone derivatives were most apparent. The flavor metabolites 3, 5-diethyl-2-methylpyrazine, hydroxyacetophenone, and diketopiperazines were also associated with cocoa consumption. The time of excretion associations have been further refined in the same dataset by subjecting it to a two-way hierarchical cluster analysis (HCA) after filtering about 90% of MS data (Llorach-Asunción et al., 2010). Daily consumption of 40 g dark chocolate showed a changed urinary metabolic signature after one week, with increased significance after two weeks, in a 30 person cohort exploring high and low anxiety trait individuals (Martin et al., 2009). The accumulated effect over time with increased 4-hydroxyphenylacetate and decreased phenylacetylglutamine and p-cresol is suggested to reflect changes in gut microbial metabolism of active chocolate compounds. In subjects with reported high anxiety, changes of endogenous metabolism were also observed with decrease in catecholamines, corticosterone and cortisol urinary levels.

**Grape juice and wine**

A major part of ingested polyphenols from a wine and grape juice extract were suggested to appear as an increase in 24 hour urine hippuric acid excretion in an NMR based ML-PLS-DA model (van Dorsten et al., 2010). In the same cross-over study this increase was not observed in NMR analysis after grape juice extract consumption only and the hippuric acid origin was therefore suggested to originate from catechins and procyanidin di- and trimers in the wine extract. With a GC-MS and ML-PLS-DA approach targeted at polyphenols in the same study identified hippuric acid, 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid and pyrogallol as stronger markers of wine and grape juice intake. Homovanillic acid, 4-hydroxyphenylacetic acid, dihydroferulic acid, and phenylacetylglutamine were more associated with grape juice only, whereas 3- and 4-hippuric acid, syringic acid, 3-hydroxyphenylacetic acid, 4-hydromandelic acid, and vanilmandelic acid were considered markers of both treatments. In addition, to these grape and wine related polyphenols also endogenous response as elevated levels of some citric acid cycle intermediates were indicated.

**Nuts**

A nut-associated metabolic signature in urine from individuals with metabolic syndrome subjected to 12 weeks nut consumption have been described with O-
PLS of MS data (Tulipani et al., 2011). PCA identified phase II metabolites of medium-chain PUFA and dodecanedioic acid as parts of the metabolic signature related to fatty acid metabolism of unsaturated fats from the nuts. Urolithin A derivatives and pyrogallol sulfate from microbial and phase II metabolism of nut ellagitannins or gallotannins and derivatives from phase II metabolism of p-coumaryl were also associated together with hydroxyindoleacetate acid and N-acetylserotonin sulfate from nut serotonin intake.

**Tea**

Hippuric acid and 1,3-dihydroxyphenyl-2-sulfate have been identified with PCA and PLS-DA of NMR data as urinary markers of both black and green tea consumption in small experiments with rather high doses of tea after a low polyphenol run in period (Daykin et al., 2005; van Dorsten et al., 2006; van Velzen et al., 2009). Both metabolites are suggested to originate from tea polyphenols metabolized by gut microbial. Changes in hippuric acid were detected, but were not regarded as significant when comparing green tea recovery drink and mineral water (Miccheli et al., 2009). This finding can be explained by the delayed hippuric acid excretion due to its gut microbial origin (Daykin et al., 2005) with a considerable inter-individual variation in excretion kinetics (van Velzen et al., 2009). Green tea has also been associated with several unidentified aromatic NMR signals in urine and both black and green tea are responsible for changes in endogenous metabolites involved in amino acid and energy metabolism, with significantly differences between the two types of tea (van Dorsten et al., 2006).

**Coffee**

Dietary record of coffee consumption in 231 subjects from KORA cohort was associated with lipid-targeted MS data of serum using Kendall’s test (Altmaier et al., 2009). Their results showed two sphingomyelin classes, both containing a hydroxyl group and with and carboxyl group in one of them, positively associated with habitual coffee intake. They suggested the increase of the metabolites to be a cofunction of the observed increase in cholesterol with increased coffee intake. Decrease in long- and medium-chain acylcarnitines was also observed with coffee intake and was suggested to be a result of endogenous response to niacin from coffee decreasing triglycerides and free fatty acids in plasma. Acute coffee intake has been associated with urinary dihydrocaffeic acid-3-O-sulfate and feruloglycine from coffee chlorogenic acids, and those metabolites are reported as sensitive markers using targeted MS approach (Stalmach et al., 2009).
**Other foods rich in bioactive compounds**

Hippurate has been reported as marker of chamomile tea intake, which is rich in polyphenolic phytochemicals, together with increased glycine and decreased creatinine in urine (Wang et al., 2005). A soy-diet rich in isoflavones resulted in plasma NMR spectra indicating increased gluconeogenesis and lipid metabolism in 5 subjects (Solanky et al., 2003). Whether this response was induced by isoflavone bioactivity or absorbed lipids and carbohydrates is unclear. In a 20 person intervention S-methyl-L-cysteine sulphoxide and three other possibly structurally related compounds were found in urine NMR spectra after a diet rich in cruciferous vegetables using O-PLS-DA (Edmands et al., 2011). S-methyl-L-cysteine sulphoxide (SMCSO) is present in both cruciferous vegetables and *allium*, but the pattern of four possible SMSCO metabolites found, was suggested to support a metabolic signature specific for cruciferous vegetables. Proline betaine was found to highly contribute to a metabolic signature of habitual citrus consumers using PC linear discriminant analysis (PC-LDA) on urine MS data (Lloyd et al., 2011a). This was further confirmed in a 14-months study and with targeted analysis additionally derivatives and phase II products of proline betaine were associated with citrus consumption.

Postprandial effects of broccoli or raspberry as substituents of milk and corn flakes in a standard breakfast in 24 persons were investigated with flow infusion electrospray (FIE)-MS and GC-MS of urine and PC-LDA showed different metabolic signatures compared to the cereal and also a partial separation from each other (Lloyd et al., 2011b). Identification with Fourier transform ion cyclotron resonance (FT-ICR-MS) indicated two phase II metabolites, caffeoyl sulfate and methyl-epicatechin sulfate, from raspberry polyphenols to characterize that dietary component. Broccoli was characterized by ascorbate and some novel derivatives of it were suggested to serve as markers of vitamin C intake. Some orange derived metabolites from standard breakfast juice intake were acting as confounders as they were associated with both broccoli and raspberry intake, moreover, whole grain breakfast cereal was also compared, but without a significantly differing metabolic signature.

Effects on urinary metabolic signature of antioxidant supplementation, rosemary extract and vitamin E, and low ω-6/ω-3 ratio in meat products served during one year to diabetic children was examined using CE-UV and O-PLS-DA and found to yield a clearly different metabolic signature between treated and non treated objects (Balderas et al., 2010).
4.2.2 Foods of animal origin

Urinary metabolic signatures in 8-year old boys, provided with equal amount of protein from either skimmed milk or low-fat meat for seven days in addition to their normal diet, were investigated using NMR and PLS-DA in a parallel study (Bertram et al., 2007). The milk diet was associated with higher urinary hippurate and minor changes in serum lipid profile, suggested to originate from milk short-chain fatty acids. Effects of the meat diet were only detected in urine as increased excretion of creatine, histidine, and urea. With a crossover design, a high-meat diet was also studied and assigned a urinary metabolic signature different to a vegetarian diet, also higher in fiber, using O-PLS-DA on NMR spectra (Stella et al., 2006). Creatine, carnitine and acetylcarnitine, originating from meat rather than endogenous production, were associated with high-meat diet. Also elevated TMAO levels were observed and were suggested to originate from enteric bacteria transformation of L-carnitine from diet. The same results regarding creatine and acetylcarnitine were also found by Holmes et al. (2008) in urine from both East Asian and western populations with high animal protein diet studied in the large INTERMAP epidemiological study. In contrast to Stella et al. (2006) Bertram et al. (2007) did only found increased TMAO in a single subject that was identified as having a fish-rich diet.  \(N\)-acetyl-5-hydroxytryptamine was identified by Stella et al. (2006) as an endogenous response metabolite to the increased intake of large-molecule amino acid with meat and in addition higher levels of taurine, 1- and 3-methylhistidine were also associated with high-meat diet. The vegetarian diet was characterized by increased \(p\)-hydroxyphenylacetate from microbial metabolism of dietary tyrosine and decreased \(N^6,N^6,N^6\)-trimethyllysine explained as an effect of increased need for endogenous L-carnitine synthesis from \(N^6,N^6,N^6\)-trimethyllysine with a vegetarian diet. Conversely higher \(N^6,N^6,N^6\)-trimethyllysine in urine was reported as a marker of a high meat protein diet in East Asian populations (Holmes et al., 2008).

Both TMAO and 1-methylhistidine, together with anserine, have also been reported as urinary markers of acute intake of smoked salmon using an MS approach (Lloyd et al., 2011b). The 1-methylhistidine derived from intake of fish anserine is suggested to function as marker for intake of meat or fish in a diet.

4.2.3 Cereal based foods

In a GCxGC-MS based study, targeted at amino acid metabolism, postprandial plasma metabolic signature after single dose low fiber rye bread or white bread showed significant changes in 26 metabolites after 30 minutes for both breads,
while after 60 minutes only phenylalanine, methionine (higher in rye bread) and picolinic acid (higher in white bread) differed from baseline (Bondia-Pons et al., 2011). In a six-week intervention with crossover design, individuals with prostate cancer subjected to a whole grain rye and rye bran diet showed a significantly different NMR spectra in plasma, compared to a high fiber wheat diet (Moazzami et al., 2011). The metabolic signature of rye diet was mainly characterized by catabolism related 3-hydroxybutyric acid and acetone, betaine and N,N-dimethylglycine related to betaine content of bran and wholegrain and dimethyl sulfone as a suggested product of gut microbial metabolism.

4.2.4 Macronutrients

Post-exercise plasma metabolic signatures in 24 men after intake of a recovery drink with low carbohydrate and high protein content was found to be dominated of amino acids, compared to water, low- and high-carbohydrate drinks (Chorell et al., 2009). Decrease in 3-methylhistidine and an increase in pseudouridine was also indicated, and both markers were suggested to reflect altered endogenous metabolism with 3-methylhistidine indicating decrease in myofibrillar breakdown and pseudouridine reflecting increased anabolism. Separation of SAFA, MUFA and PUFA intake, using both untargeted LC-MS by dilution of urine, and targeted approach with liquid-liquid extraction of lipophilic fractions of urine, was achieved using PCA (Legido-Quigley et al., 2010).

4.2.5 Supplements and fortifications

In a parallel intervention with 96 females, wheat flour fortified with folic acid, vitamin B1, vitamin B2, ferric sodium edetate and zinc oxide induced a serum metabolic signature in the treated group substantially different to the control detected with PLS-DA of MS data (Jiang et al., 2011). Effects on endogenous metabolism were found with increased fructose-6-phosphate, sphingosine 1-phosphate and docosahexanoic acid, interpreted as increased anti-oxidative functions in the treated group.

4.2.6 Food groups and diets

Cluster analysis of food groups divided data from 160 person’s food record into 3 clusters (O’Sullivan et al., 2011). Two of these diet clusters were connected to significantly different urine NMR spectra using PLS-DA. One, which was characterized by relatively higher energy contribution from whole-meal bread, whole milk, fish, and, ice-creams and desserts and less from low-energy beverages, indi-
cated higher urinary levels of glycine, phenylacetylglutamine and acetoacetate, and one, considered as the more unhealthy resembling a traditional Irish diet, was characterized by higher contribution from white bread, sugars and preserves, butter and spreads, red meat and meat products, and alcohol and less from vegetables, indicated by higher urinary TMAO, O-acetylcarnitine, and \( \text{nm}-\text{dimethylglycine.} \) Phenylacetylglutamine, a possible product of vegetable phenylacetic acid, in the first cluster was found to be associated with the reported higher vegetable intake of that group and O-acetylcarnitine in the second group was connected to the higher red meat intake of that group.
5 Discussion

To assess a characterizing metabolic signature to a lifestyle factor including diet, requires a strict control of other factors in order to sort out metabolite signals that are specific for the diet and that are not biased by gender, age, other foods than those of interest, or all other variables that contribute to the metabolic phenotype of a biofluid. Several markers and metabolic signatures related to food intake have been discovered using metabolomics, with metabolites from ingested food, endogenous response to exposure, and gut microbial activity in both blood and urine to various extents. This covers all the important contributors to a dietary phenotype and speaks in favor for assessment of dietary metabolic signatures. On the other hand many of them are found by small intervention studies and sometimes with rather high doses used, like polyphenolic derived signatures in wine extract (van Dorsten et al., 2010) and tea (Daykin et al., 2005; Van Dorsten et al., 2006; van Velzen et al., 2009). Such signatures may be difficult to reproduce and detect in untargeted studies assessing habitual consumption.

When exploring dietary metabolic signatures, proper specificity and sensitivity of the analysis model is another important issue to address. Parts of this issue are visualized by metabolites associated with urinary metabolic signatures specific for diets, which are also part of other signatures. Hippuric acid (diet polyphenols/older persons/morning), creatinine (meat/men/older people/morning), carnitine/acetylcarnitine (meat/older persons/men), TMAO (fish/meat/men/older persons), 1-methylhistidine (meat/fish/low phytochemical diet) and taurine (men/meat/older people) are such metabolites. This stresses the problem of the multifactorial nature of metabolomics, and a holistic approach requires holistic interpretations. As an example, TMAO has been found to be a distinctive marker of fish intake, but on the other hand it has also been associated with being a male and for being old in a Greek population. The subjects in the Greek study were advised to avoid fish 24 hours prior to sampling however, no dietary record was
kept. A more thorough study design may bring clarity to the question if TMAO is a marker of age in general or a marker of lifestyle (diet) common among older persons in Greece (Psilogios et al., 2008). In addition the same ambiguity goes for high TMAO associated with Greek males. One fact that point towards TMAO as a fish marker rather than a gender marker is the better possibilities for gender correction than dietary patterns in a dietary study. Other confounding associations may be found between men and meat, with creatinine, taurine and carnitine/acetylcarnitine as mutual markers. Regardless of the possible confounders and varying results, changes of amino acid levels with age appear to be a relevant factor for metabolic signatures. To further improve sensitivity in interventions, standardized diet to create a reliable baseline has proved to be effective and is highly recommended.

Apart from the risk of false causality, specificity of markers and metabolic signatures has to be considered. Hippuric acid is an apparent example found to be associated with tea (Daykin et al., 2005; Van Dorsten et al., 2006; van Velzen et al., 2009), chamomile (Wang et al., 2005), grape and wine (van Dorsten et al., 2010), and phytochemical diet (Walsh et al., 2007). This uniformity is due to colon microbial degradation of various dietary polyphenols resulting in hippuric acid (Rechner et al., 2002). Thus, hippuric acid is a marker of polyphenol intake in general, and furthermore, it also points out the role of gut microbial in urine phenotype.

Irrespective of casual factors, it also seems possible to separate males and females in a population based on metabolic signatures in both blood and urine specimens and thus, gender should be regarded in all cases when exploring lifestyle and dietary patterns with metabolomics. Blood samples are characterized to some extent by differences in lipids and amino acids, but focus on metabolic pathways rather than compound classes seem important to identify gender metabolic signatures. Urinary samples tend to exhibit gender classes with citrate and glycine as most commonly associated with females and creatinine as most frequently associated with males (Kochhar et al., 2006; Psilogios et al., 2008; Slupsky et al., 2007; Wang et al., 2005; van Velzen et al., 2008). Although a pattern may be distinguished it is certainly not clear cut and some researcher have not found any class separation between genders (Lenz et al., 2004). All gender based analyses reviewed here were using untargeted approaches to separate gender urinary metabolome except Slupsky et al. 2007 whom in addition used a targeted approach which indicated the same major metabolites and besides that also yielded acetone and fumarate as discriminating metabolites.
All of the reviewed studies on BMI involved people in the range of a rather normal BMI. Introduction of an extreme class separating obese (BMI >30 kg/m²) subjects from lean and overweight might enhance the possibilities to find a specific metabolic signature correlated to BMI. Also, the more vague effects of BMI on metabolomic profile compared to gender and age may reflect the diffuseness of BMI as a measure of body composition as it does not take into account the relative contribution of fat, muscle or water to the body mass. To conclude, metabolic signature of BMI can be considered doubtful, unless obese persons are assessed.

In epidemiological studies, habitual consumption and long term effects of diets are of interest. This requires metabolites that are excreted slowly from the body, or that alters metabolic pathways or gut microbiota activity, preferably in combination of regular and frequent consumption of their food precursors. Although most studies reviewed here explored or found more or less acute effects of diet, changes in endogenous metabolism have been observed in blood with increased catabolism after whole-grain diet (Moazzami et al., 2011). In urine, altered microbial metabolism with accumulated changes in 4-hydroxyphenylacetate, phenylacetylglutamine and p-cresol after a period of chocolate intake (Martin et al., 2009) as well as increased endogenous L-carnitine synthesis in vegetarians has been suggested (Stella et al., 2006). Gut microbial metabolites are also important as markers of habitual food consumption as they originate from colon and hence, are absorbed later. When single samples are used in epidemiological studies, markers that exhibit high inter-individual variation, to ensure response, and low intra-individual, to strengthen prediction, is preferable. This feature is valid for many dietary metabolomics experiments.

Time of sampling is important, not only optimized sampling time in relation to dietary intake, but also have fasting urine samples in a time span of a couple of hours showed markedly different metabolic signatures (Park et al., 2009). This finding is interesting and further investigations are recommended. Choice of platform can also be of interest, where NMR serves the holistic idea of metabolomics best with retained sample integrity, but on the other hand the higher sensitivity of MS is also desirable. For instance, considering blood metabolic signature related to genders, coverage of lipids in general and PC and lipoprotein species in particular together with amino acids such as leucine, isoleucine, valine, glycine and serine can be of interest, and coverage of all these metabolites in a single analysis is desirable.

Proper statistical data treatment is also required to discover the subtle patterns responsible of a metabolic signature. The differences due to choice of statistical
method can be exemplified with Psihogios et al., (2008), who did not achieve separation of gender with urinary data subjected to PCA nor PLS-DA, but when using pretreatment with OSC prior to PLS-DA they found higher levels of citrate and glycine as contributors to a female metabolic signature. On the other hand, great care should be taken to avoid loss of important information or create models that are over fitted.

As mentioned earlier in the discussion Slupsky et al., (2007), using a targeted model, observed metabolites contributing to a gender specific metabolic signature that were not detected using a untargeted model. This may suggest that although targeted approaches leave out a lot of information they can contribute to interpretation of more holistic analyses by highlighting single metabolites or groups of metabolites that can be associated with a trait or exogenous factors. However, to fully benefit the advantages of a metabolomics view, untargeted analysis should precede targeted analysis to generate hypotheses that can be tested with the targeted analyses.

Conclusions

Food seems to have a rather large influence on human urine and blood metabolic phenotype and identification of metabolic signatures of dietary patterns, using metabolomics, is likely to be achieved. Gender and age classes tend to exhibit different metabolic signatures in both matrixes as well, but some of the discriminating metabolites can be suspected to reflect different lifestyles and diet, which probably will vary with cultures and populations. The following issues should be considered when assessing a metabolic signature attributed to lifestyle of diet:

1. Choice of matrix (blood or urine) should be decided based on the aims of the study and which effects that is of interest.
2. Elucidations whether the metabolic profile reflect acute intake/effects or long-term intake/effects?
3. Standardized sampling time to ensure reproducibility and increase chances to detect a relevant metabolic signature.
4. Careful design of the study and thorough examination of previous work is required to avoid confounding by other lifestyle or dietary factors.
5. Proper statistical treatment of data.
6. For interventions, standardized diets for comparable baseline samples are preferable to get a reliable intra-individual response.
References


of urine samples obtained from healthy British and Swedish subjects. *Journal of Pharmaceutical and Biomedical Analysis* 36(4), 841–849.


Acknowledgment

Many thanks to my supervisor Rikard Landberg for great support during the writing of this thesis and thanks to the Department of Food Science at Swedish University of Agricultural Sciences for providing the opportunity to write the thesis in connection to current research.