

THESIS FOR THE DEGREE OF LICENTIATE OF ENGINEERING

# **Metabolic profiling of meat and fish and their impact on human metabolism**

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METABOLIC PROFILING OF MEAT AND FISH AND THE IMPACT ON THEIR  
EFFECTS ON HUMAN METABOLISM

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## **Abstract**

High intake of meat is frequently associated with increased risk of disease, while the opposite is true for fish; yet few studies have compared meat and fish in terms of both its composition of low-molecular weight (LMW) molecules and its effects on metabolite concentrations in humans. The work presented in this thesis aimed to use metabolomics to get a holistic overview of compositional differences between fish and meat, and the effects of dietary fish or meat intake on human metabolism.

To investigate the metabolite composition of meat and fish, fillets of beef, pork, chicken, cod, salmon and herring (fresh and pickled), n=6 per fish or meat type, were minced, mixed and samples of both raw and baked minced fillet taken. The metabolite profile of the samples was measured using gas chromatography-mass spectrometry (GC-MS) based- metabolomics. Metabolite profiles of fish and meat were compared, as well as the individual fish and meat types.

To investigate the effects on plasma metabolite profile of replacing chicken and pork with herring, GC-MS-metabolomics analysis of plasma samples from of a randomized crossover trial with eleven healthy obese men and women (age 24–70 years) was conducted. Subjects were randomly assigned to four weeks of herring diet or a reference diet of chicken and lean pork, five meals per week, followed by a washout and the other intervention arm.

The composition experiments revealed that the LMW composition (amino-acids, other nitrogen-containing compounds, lipids, organic acids/alcohols and sugars) of meat and fish differed less than expected, while there was wide variation within the measured meat and fish samples. Eating herring instead of chicken and lean pork led to several metabolite changes in the tricarboxylic acid cycle and urea cycle, as well as differences for several metabolites including methyl-histidine, a potential biomarker of meat intake. These results support the idea that it is not sufficient to broadly compare 'meat' and 'fish' in nutrition studies due to wide variation in metabolite composition, and that replacing lean pork and chicken with herring may have profound consequences on energy and amino acid metabolism.



## Publications

The licentiate thesis is based on the work performed in the following publications.

- I. Vincent A, Undeland I, Savolainen O, Sandberg A-S, Ross A B. **A comparative metabolomics study of commonly consumed meat and fish.** *In preparation.* 2016
- II. Vincent A, Savolainen O, Sen P, Lindqvist H, Vendelbo Lind M, Undeland I, Sandberg A-S, Ross A B. **Herring and Chicken/Pork Meals Lead to Differences in Plasma Levels of TCA Intermediates and Arginine Metabolites in Overweight and Obese Men and Women.** *Journal of Molecular Nutrition and Food Research (Pending revision).* 2016

## **Abbreviations**

DHA - docosahexaenoic acid

EPA - eicosapentaenoic acid

GC-MS – gas chromatography-mass spectrometry

HDL – high density lipoprotein

LMW- low molecular weight

LC n-3 PUFA – long chain omega-3 polyunsaturated fatty acid

OPLS-DA - orthogonal projection to latent structures-discriminant analysis

PCA – principal component analysis

TCA - tricarboxylic acid

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# 1 Introduction

Fish has long been the main source of protein in the human diet, with meat as a welcome, but expensive supplement. Improvements to farming and logistics over the past 150 years have meant that meat intake has now substantially supplanted fish as the main source of protein for many people. High intake of meat is frequently associated with increased risk of disease [1-3], yet few studies have compared meat and fish in terms of both composition and effects on human health.

Herring has potential as a sustainable alternative to other fish sources in the Nordic countries, as well as potentially being beneficial for health if consumed instead of meat. In earlier work it was hypothesised that replacing chicken and pork as protein sources with herring would reduce inflammation and improve plasma lipids in healthy obese subjects, based on high amounts of LC n-3 PUFA, vitamin D and selenium in herring compared to chicken and pork. However it was found that herring only increased HDL concentrations without any effect on markers of inflammation or other standard clinical chemistry measurements in healthy overweight subjects [4, 5].

Based on previous work that found that herring and beef led to many different post-prandial plasma metabolite responses [6], we have used metabolomics to further analyse the compositional differences between herring and other marine and terrestrial protein sources, and applied metabolomics to test if substituting chicken and pork for herring in the diet over four weeks leads to diet-induced metabolic changes in the fasting plasma metabolome. The work presented in this thesis aims to get a holistic overview of fish compared to meat for both composition and effects on human metabolism.

## 2 Objectives

Two studies were performed to investigate the significance of replacing meat with fish; the first a compositional study; the second, a metabolomics analysis of samples from a diet intervention study. This thesis is written based on the results of these two studies, which had the following objectives:

- To investigate the variation in metabolic profiles of commercially available fillets from common meats and fish available in Sweden.
- To assess the metabolic changes of replacing chicken or pork with herring as the main dietary source of protein on the human plasma metabolome.

## **3 Background**

Over recent years there has been increased attention paid to the amount of protein as a proportion of nutrient intake, and recent revisions of the Nordic Nutrition Guidelines have increased the proportion of energy that should come from protein as part of a healthy diet [7]. Often the main sources of protein in the diet, including meat, fish, dairy and soya-based proteins are compared based on amino acid, and perhaps fatty acid composition, while little attention is paid to what other compounds are present in these protein sources. While the fat component of these foods can be quite high; from 1 up to 30 %, the low molecular weight (LMW) compounds contribute to a low part of the total weight, but can be of interest for possible effects on health and as potential biomarkers that can differentiate between different foods.

### **3.1 Nutritional biomarkers**

A nutritional biomarker provides information of nutritional status in accordance to intake, it can be an ingested compound or a metabolite. There are several proposed biomarkers for fish and meat intake; DHA for fish [8]; methyl histidine and creatine for meat [9], with even carnitine suggested [10], however these biomarkers can be confounded by intake of fish oil capsules (DHA) or changes in physical activity (creatine), so there is a need to find new biomarkers that are more specific for different dietary protein sources and possibly distinguish between different types of meat and fish. Biomarkers related to intake are an important tool for improving our understanding of how diet and biology interact, as well as improving estimates of how much of specific foods we eat. The latter aspect is especially important as most subjective measures of diet (e.g. food frequency questionnaires and food diaries) are open to large amounts of error [11]. The discovery of new biomarkers of dietary intake will help to increase the precision of current and future studies on diet and how it affects health and disease [12].

### **3.2 Meat, fish, and health**

Dietary recommendations place an emphasis on choosing fish and white meat over red and processed meats for reducing disease risk. Red meat is associated with increased risk of obesity, heart disease and cancer [1-3], while fish have shown to reduce the risk of coronary heart disease and stroke [13]. However while these associations are strong, proven mechanisms behind increased risk of disease for red meat are lacking [14].

Differences in health effects of meat and fish are often associated with their fat content, particularly long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) from fish have been associated with positive cardiovascular benefits and the saturated fat content of meat associated with increased cardiovascular disease [3, 15]. As the link between saturated fat intake and cardiovascular disease has recently been questioned [16], it is crucial to broaden our understanding of how fish and meat could affect health.

### **3.3 Metabolomics**

In any organism a great number of molecular processes occur simultaneously, producing a large number of products and by-products, which are known as metabolites. The collection of these metabolites provides a chemical fingerprint, which when analysed and profiled, gives information about the subject in question. Metabolomics is an analytical tool for studying these chemical fingerprints.

#### **3.3.1 GC-MS metabolomics**

Gas chromatography mass spectrometry (GC-MS) is one of three main instrument types used for metabolomics studies. Compared to the other main instrument types, nuclear magnetic resonance and liquid chromatography coupled to mass spectrometry, GC-MS is sensitive and robust and well suited for detecting very small molecules. It has the drawback that compounds must be derivatised (chemically modified to make them volatile) before analysis, which lengthens sample preparation time and the range of molecules that can be detected.

There are hundreds of compounds in a sample, which need to be separated before detection of the  $m/z$  to avoid signal saturation. The injected sample passes first through a GC column which separates the molecules mainly on their boiling point, though this depends on the selected column, before entering the mass spectrometer. Each compound will have a recorded retention time, which can be calculated, with the use of a known alkane series, to give a retention index. The identity of the compound can then be determined by matching the retention index and matching the MS spectrum resulting from the fragmentation of the molecule. Each molecule will have an almost unique spectrum that is reproducible from instrument to instrument. This spectrum can be searched for in mass spectral libraries of thousands of molecules that will suggest potential compound matches. Combining library searching with retention index gives

good certainty for compound identification. New tools help to speed the compound identification process up, and in the studies presented here we use a MATLAB script developed by Hans Stenlund at the Swedish Metabolomics Centre in Umeå, which automatically searches GC-MS chromatograms for compounds based on retention index, mass spectra matching, and compound base peak (the largest mass fragment in a compounds mass spectra).

### **3.3.2 Applications of metabolomics in food and nutrition**

Hypothesis generating methodologies like metabolomics can help to discover the broader metabolic effects of different diets [17], and are ideally suited for bringing a wider understanding of how human metabolism responds differently to diet [6]. In the past, food composition has been analysed using specific methods such as amino acid analysis, fatty acid analysis and methods for individual or groups of vitamins. The advent of metabolomics, which aims to profile a wide range of metabolites at the same time, thus giving a wide overview of many compound classes, has provided a new tool for examining food composition. This complement to traditional quantitative methods for determining food composition is sometimes termed 'foodomics' [18]. While metabolomics is generally not fully quantitative, it facilitates the relative comparison of different food samples, which can then be followed up using quantitative methods. Similarly, metabolomics has been widely applied in nutrition science, and has been used to find novel biomarkers of food [19, 20] and determine mechanisms of action for different diets and foods [21, 22].

## **4 Methods**

### **4.1 Paper I**

#### **4.1.1 Study design and sample collection**

We aimed to compare the LMW compounds in meat and fish samples that are commercially available in the Gothenburg region of Sweden, and to compare raw and cooked meat and fish. To get a representative sample, six fillets of beef, pork, chicken, cod, salmon and herring (fresh and pickled) were randomly purchased from different supermarkets and fishmongers, so that no one meat or fish type was purchased more than once from each shop. Vegetarian protein sources were also collected, but results are not included in this paper.

#### **4.1.2 Sample preparation**

Fillets were purchased fresh, and temporarily stored at 4 °C before being processed later the same day, to mimic common household conditions. The fillets (ca 250 g) were minced and mixed on ice, whereupon two samples (ca. 55 g) were packed into individual aluminium oven dishes. One sample was baked (200 °C) until an inner temperature of 65 °C for meat or 55 °C for fish was reached. Both samples were frozen (-80 °C) and freeze-dried before pulverization in a bead shaker (1.5 min, 35 Hz). Samples were stored (- 80 °C) until extraction. Only raw samples of pickled herring were taken.

#### **4.1.3 Sample analysis GC-MS metabolomics**

The LMW compound profile of the protein-source samples was analysed using gas chromatography coupled to a tandem mass spectrometer (GC-MS/MS) [23]. Three replicates of each sample were extracted (ca. 20 mg) in methanol (90 %, 1 mL) containing stable isotope labelled internal standards. The extracts (50 µL) were dried and derivitised using oximation and silylation. Derivitised extract (1 µL) was injected into a Shimadzu TQ8030 GC-MS/MS system (Shimadzu Europa GmbH, Duisberg, Germany). The MS scanned between  $m/z$  50-750. GC-MS scan data were exported from GCSolutions software (Shimadzu Europa) as netCDF files for targeted peak detection using a Matlab script developed at the Swedish Metabolomics Centre in Umeå which matches compound retention index and MS spectra with an in-house GC-MS library. All scan data were aligned using internal standards, and normalised using overall internal standard response determined with principal components analysis (PCA) [24].

#### **4.1.4 Data analyses**

Data were scaled using unit variance normalisation and mean centring. Differences in compound content between the protein sources were investigated with PCA [25], using the SIMCA software (Umetrics AB, Umeå, Sweden). Data were further analyzed by ANOVA to determine differences in overall response between meat and fish, between the three fish sources, between the three meat sources and between the three herring preparations. Post-hoc t-tests were performed on the meat, fish and herring sub-groups. RStudio (R Studio, Boston, MA) was used for the ANOVA and t-tests. Samples were considered different if  $P < 0.01$ .

## **4.2 Paper II**

### **4.2.1 Study design**

This study was conducted as a data analyser blinded randomised crossover intervention trial and carried out in 2003. Details of the study design have been published previously [5]. Briefly, fifteen overweight and obese men and women (mean age 50.5 y, mean BMI 32.6 kg/m<sup>2</sup>, mean weight 97.5 kg) with no known chronic or serious health issues were recruited for this study. Participants were provided with frozen meals including 150 g of baked herring or 120-150 g chicken and lean pork per meal and were instructed to eat five of these meals per week for lunch or dinner during each four week intervention period. The two intervention periods were separated by a two-week washout period. Fasting blood samples used in this study were collected at baseline, week two and week four in each study period. Thirteen subjects completed the study, with two dropouts due to use of analgesic medication and inability to attend all parts of the study [5], and there was insufficient plasma for measurements for two subjects, leaving the final number of subjects analysed at 11. Samples were frozen and stored in -80°C.

### **4.2.2 Study foods**

The study foods have been previously described [5]. In brief, fresh herring, lean pork and chicken fillets were baked in the oven at 150°C until protein denaturation temperature and prepared as part of frozen ready meals within three days of catching or slaughter. Herring, lean pork or chicken fillets were made into complete meals that contained mashed potatoes, baked potato wedges, pasta or rice, with different vegetables and sauces to increase meal variety. The dishes were identical except for herring vs. chicken/lean pork between the two interventions.

### **4.2.3 Metabolomics analyses of plasma using GC-MS**

The metabolomic profile of EDTA plasma samples was analysed using gas chromatography coupled to a tandem mass spectrometer (GC-MS/MS) [26]. Briefly, plasma (100  $\mu$ L) was extracted with methanol (90 %, 900  $\mu$ L) containing ten stable isotope labelled internal standards [27]. An aliquot of the extract (200  $\mu$ L) was dried and derivitised using oxymation and silylation. Derivitised extract (1  $\mu$ L) was injected into a Shimadzu TQ8030 GC-MS/MS system (Shimadzu Europa GmbH, Duisberg, Germany).

GC-MS scan data were exported from GCSolutions software (Shimadzu Europa) as netCDF files for targeted peak detection. Peaks were aligned based on the internal standards and peaks detected and identified based on a Matlab script that identified peaks from an in house library (Swedish Metabolomics Centre, Umeå, Sweden) based on matching retention index and mass spectra. All metabolomics data were normalised using overall internal standard response determined with principal components analysis (PCA) [24].

### **4.2.4 Data analyses**

Global differences in plasma response between the meals were investigated with PCA and orthogonal partial least squares-discriminant analysis (OPLS-DA) using the SIMCA software (Umetrics AB, Umeå, Sweden) [25]. After normalization based on internal standards, data were corrected for baseline by subtracting the baseline values from the week 2 and week 4 time points, and data were subjected to unit variance (UVN) scaling and mean centring. The plasma responses to the baked herring meal were compared to the pork/chicken-based meal in an OPLS-DA analysis, where the difference between the meals was explained by the first predictive component. The OPLS model was made from all time points in the dataset, i.e. 0-4 weeks, with a 10-fold validation. Discriminating variables detected using OPLS-DA were further analysed for differences between diets using mixed model analysis and post hoc t-tests to determine differences between diets.

Mixed models were used to determine diet x time differences for individual metabolites. Meal type and time were included as fixed factors, adjusted for age and gender with subject as a random variable. Data was corrected for baseline by subtracting the baseline values from the corresponding week 2 and week 4 values. XLSTAT for Excel 2013 (Addinsoft, Paris, France) was used for all mixed model and univariate data analyses. Uncorrected P values < 0.05 are reported as of interest in line with the exploratory nature



of this work. We have reported trend results at  $P < 0.1$  if they were potentially related to significant metabolites.

#### **4.2.5 Enrichment of metabolic pathways**

To determine the overall metabolic pathways affected by the difference in diets, the significantly changed metabolites were mapped and their intensities to the human metabolic network and pathway over-representation analysis [28] was performed. A hypergeometric test estimated the relative significance of the over-represented pathways against the background KEGG pathways for *Homo sapiens* [29].

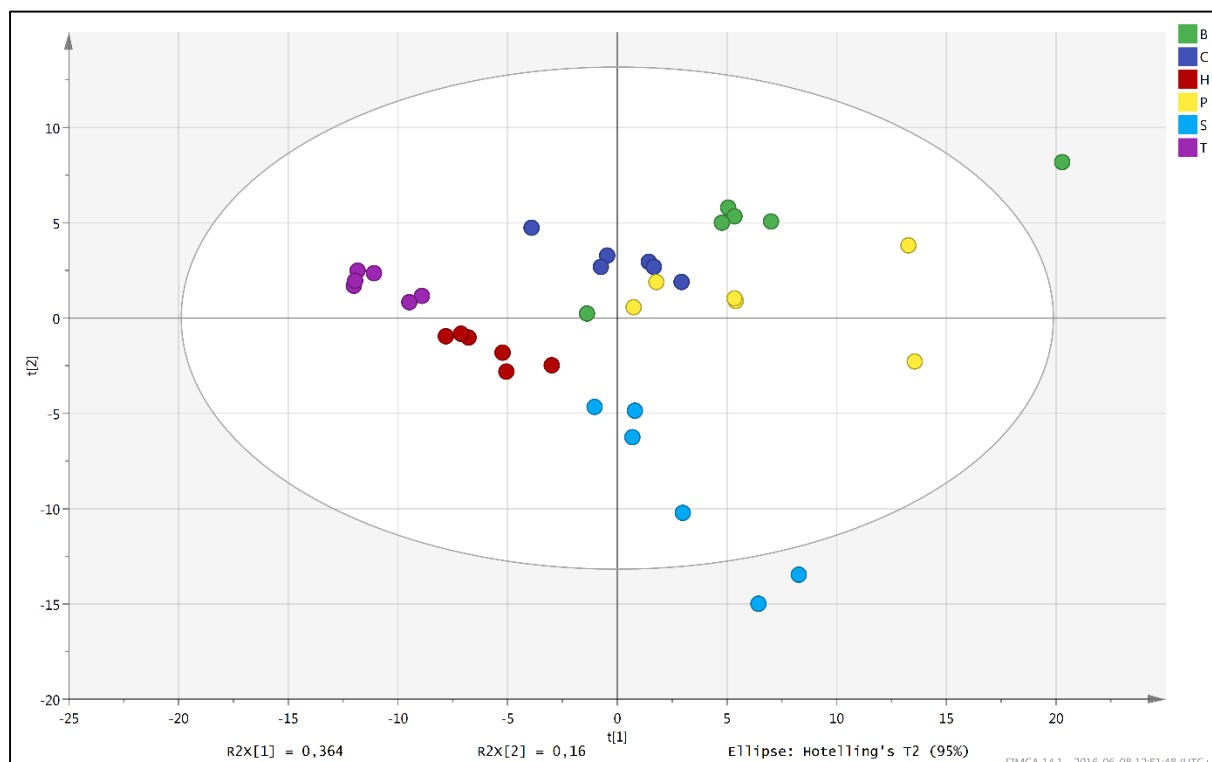
The Relative Importance of the Metabolites (RIM) involved in pathway(s) were estimated by betweenness centrality [30]. RIM is a measure of contribution of a metabolite relative to other metabolites associated with a metabolic pathway, normalized to the RIM of other metabolites involved in that pathway.

## 5 Results & Discussion

### 5.1 Paper I

#### 5.1.1 Comparison of metabolic profiles of fish and meat

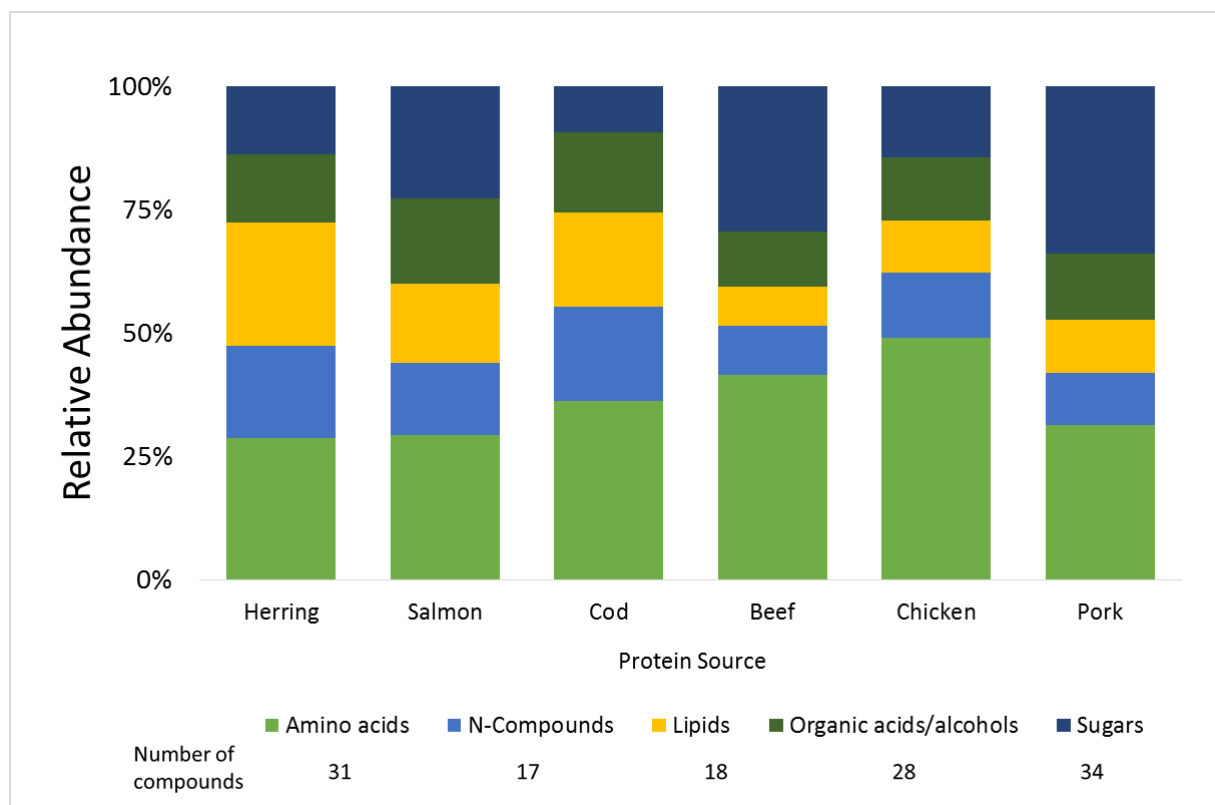
A hundred and twenty-eight compounds were detected based on combined spectral and retention time-based identification of all samples. One outlier among the beef samples was identified from the initial PCA analysis and removed (Fig 5.1.1). PCA analysis did not detect any separation between cooked and raw samples, suggesting that the controlled cooking conditions did not substantially alter overall LMW compound concentration. This was counter to our hypothesis that cooking would lead to losses of nutrients and water, which would change the overall composition [5] and/or to degradation or formation of a number of metabolites [31]. It is possible that mild cooking to a relatively low internal temperature (55 °C for fish and 65 °C for meat) meant that proteins were denatured but not significantly hydrolysed, oxidized or subjected to Maillard reactions [32, 33].



**Figure 5.1.1** PCA scores plot, coloured to fillet type, the divide between meat and fish resides mainly on component 2 ( $t[2]$ ), where as the different types of meat and fish are spread mainly along component 1 ( $t[1]$ ). B = beef, C = chicken, H = herring, P = pork, S = salmon and T = cod.

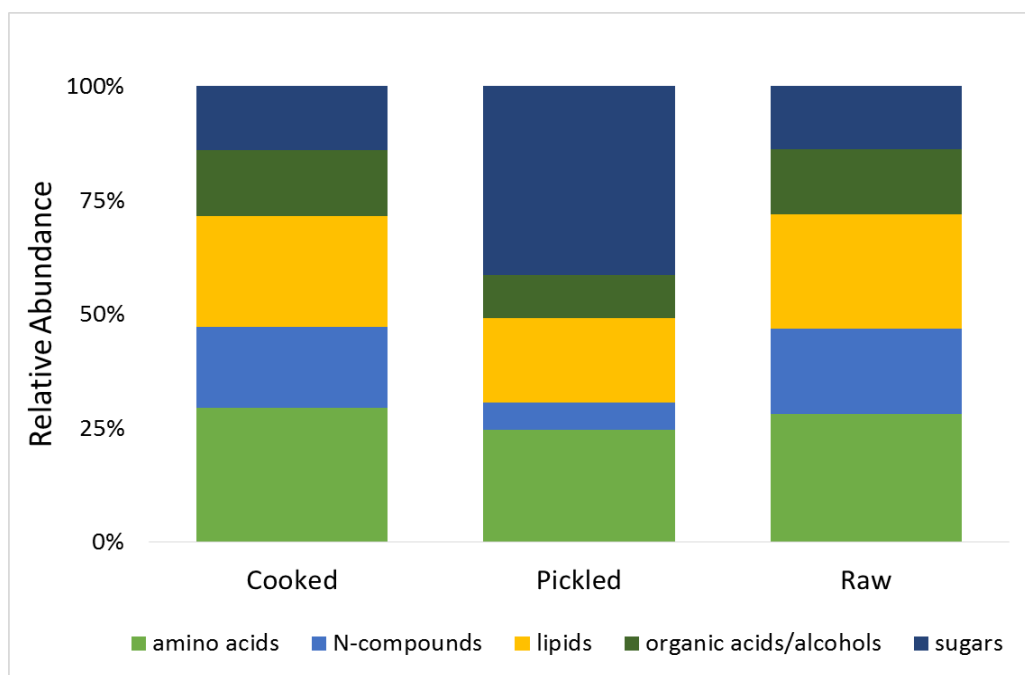
Due to the high number of compounds that were significantly different between protein sources the metabolites have been grouped into compound sub-classes; amino acids, other nitrogen-containing compounds (amines, nucleotides etc.; N-compounds), lipids

(fatty acids, sterols, glycerides etc.), organic acids/alcohols and sugars to enable a simplified overview of the results. The relative abundance of the different sub-classes in the protein sources varied between the groups, fish and meat, as well as within these groups (Fig. 5.1.2). Between fish and meat there are notable differences between lipids and N-compounds. Between the different fish and meat sources there was more variation of the amino acids and sugars; the sugars in salmon were almost double that of herring and cod, while the amino acids were higher in cod than salmon and herring; in chicken the amino acids were higher and the sugars were much lower compared to beef and pork. These differences were mainly driven by...



**Figure 5.1.2** The relative abundance of all detected compounds, given as proportions of the total recorded intensity. The bar chart shows the relative abundance for each sample. The number of compounds refers to the total number of compounds for each compound class.

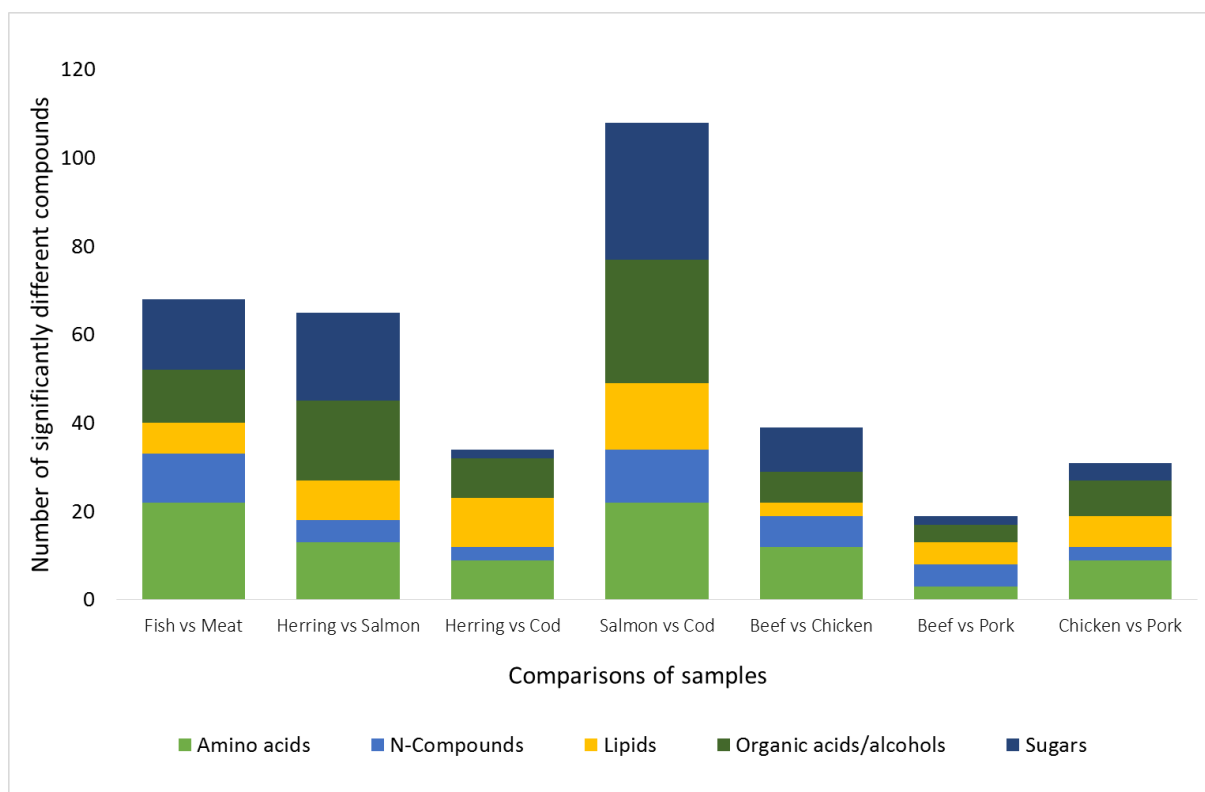
As well as comparing the fish and meat metabolite profiles, herring prepared in three different ways; raw, cooked and pickled, was compared. As with the other protein sources, there was no separation between raw and cooked herring with PCA analysis, but the pickled herring was markedly different to both the raw and cooked samples, particularly for the sugars (Fig. 5.1.3).



**Figure 5.1.3** Herring – cooked, pickled and raw. The relative abundance of all detected compounds, given as proportions of the total recorded intensity.

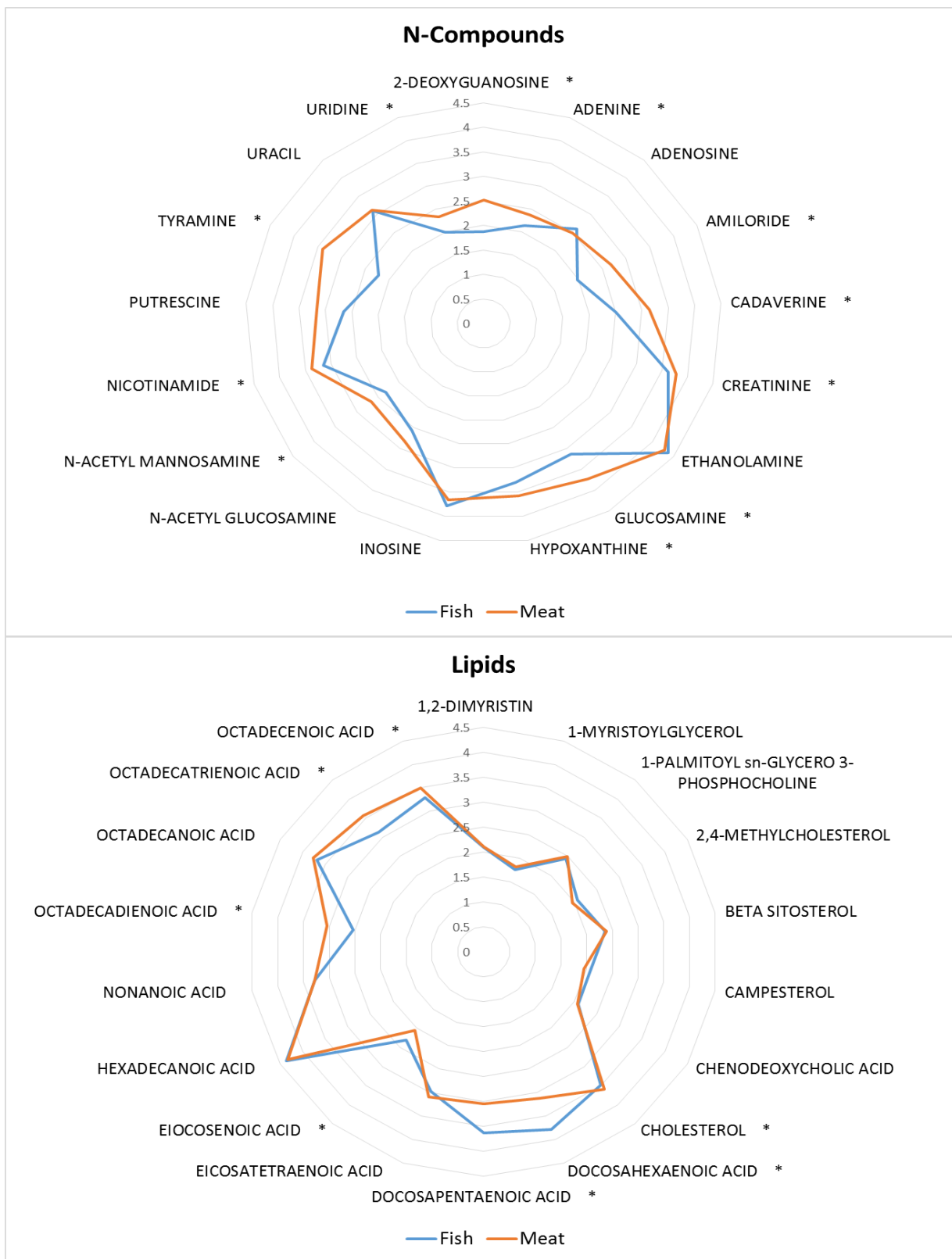
### 5.1.2 Fish vs Meat

Greater variation was observed within the fish group, than between the meat as indicated by the total number of significant compounds for each comparison (Fig. 5.1.4). Fish and meat varied predominately in amino acids and sugars (22 and 16 metabolites respectively), though the number of significant metabolites were proportional to the overall abundance of metabolites (sum of all peaks). The lipids and N-compounds (7 and 11 compounds respectively) on the other hand, had a greater variation in relation to abundance. Four of the seven lipids that showed the most variation were docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA), which were higher in fish, and octadecanoic acid (stearic acid) and octadecadienoic acid (linoleic or rumenic acid, depending on the stereochemistry) higher in meat (Fig. 5.1.5). That DHA (C22:6) and DPA (C22:5) were higher in fish was expected as was the saturated stearic acid (C18:0) in meat, as has been reported previously many times [34-36].



**Figure 5.1.4** The number of compounds in each class of compounds that differ significantly between the protein sources.

There were seven N-compounds that were markedly higher in the meat; amiloride, cadaverine, glucosamine, hypoxanthine, putrescine, tyramine and 2-deoxyguanosine. Cadaverine, tyramine and putrescine are breakdown products of lysine, tyrosine and arginine respectively, and as such could be either inherently higher in meat, or due to differences in processing time between catch/slaughter and purchase. Although both meat and seafood are purine-rich [37], the purines hypoxanthine and 2-deoxyguanosine were higher in the meat.

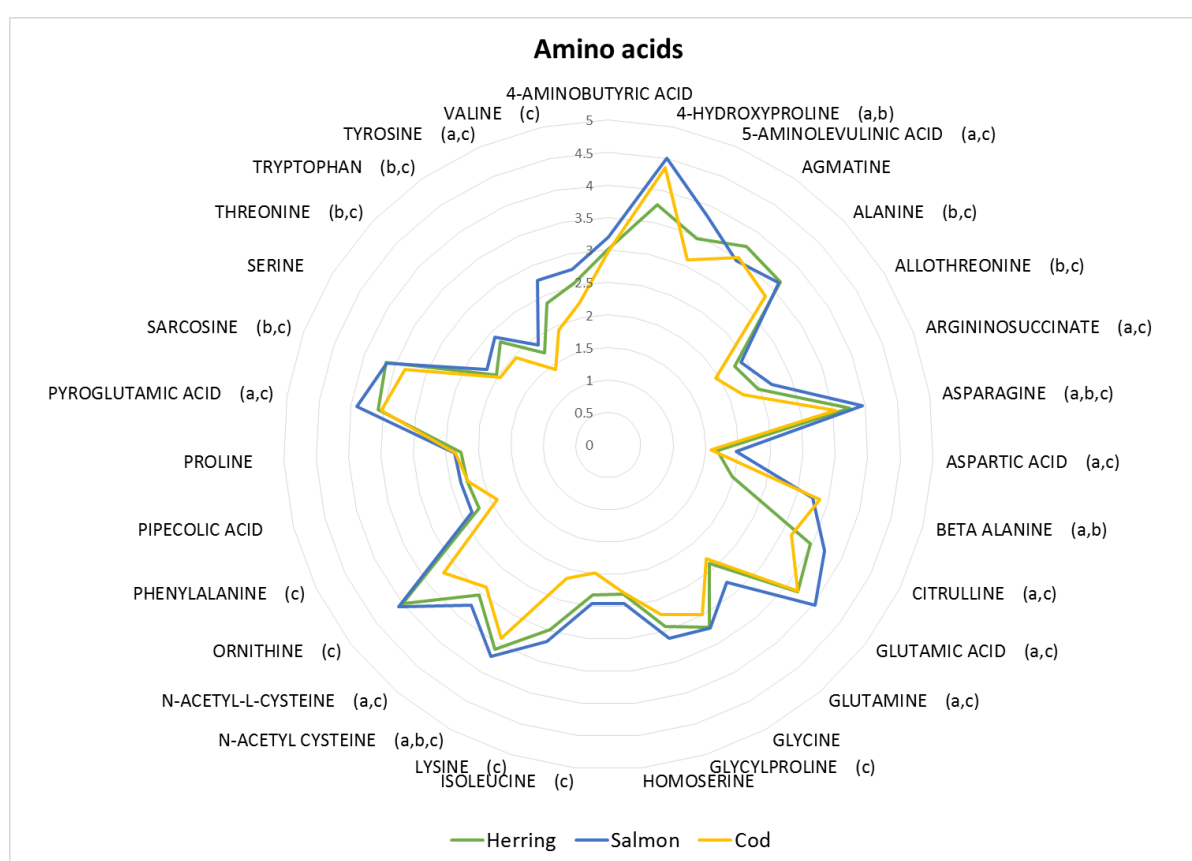


**Figure 5.1.5** The radar charts show the relative abundance of the N-compounds (top) and the lipids (bottom). The values are the log-values of the average intensities. Significant differences between the samples were tested by ANOVA. Compounds were significant at  $P < 0.01$  (\*)

### 5.1.3 Fish sub-group

The variation within the fish group is caused mainly by salmon (Fig. 5.1.4), which differs from cod and herring for the greater part in the organic acids/alcohols (28 metabolites different from cod and 18 different from herring) and sugars (31 metabolites different from cod and 20 different from herring). Herring and cod were very similar but differed in lipids. Among the other organic acids/alcohols that differed between the fish species, fumaric acid, galactitol, lactitol and mannitol were markedly higher in salmon (Fig. 5.1.6.b). The organic acid/alcohol profiles of cod and herring were very similar with the exception of glyceric acid and glutaric acid, which were higher in herring.

Of the amino acids, arginine/ornithine (the derivitisation method used converts arginine to ornithine), isoleucine, leucine and 5-aminolevulinic acid were markedly lower in cod than salmon and herring (Fig. 5.1.6.a).

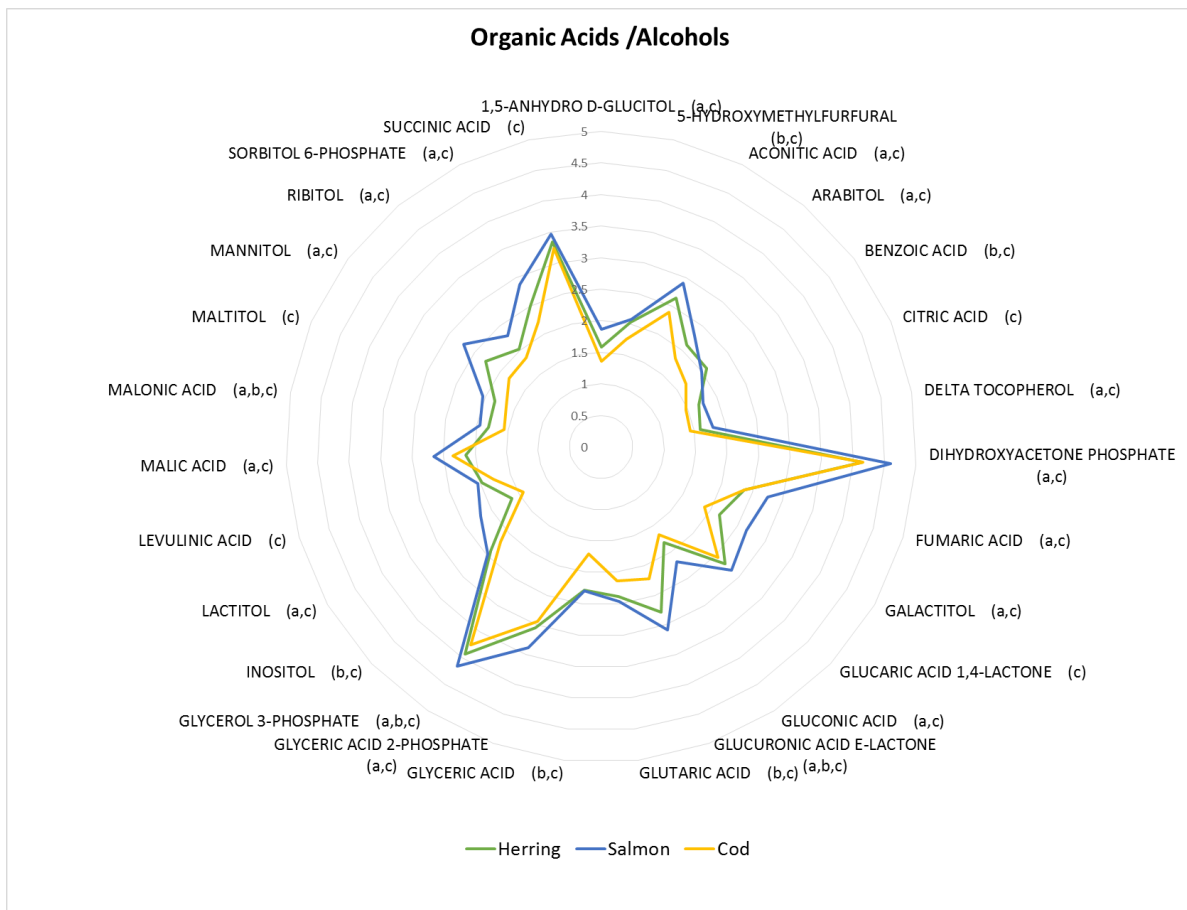


**Figure 5.1.6.a** The radar charts show the abundance of the amino acids for the cod, herring and salmon samples. The values are the log-values of the average intensities. Significant differences between the samples were tested with paired t-tests. Compounds were significant at  $P < 0.01$ .

a = herring vs salmon, b = herring vs cod, c = salmon vs cod.

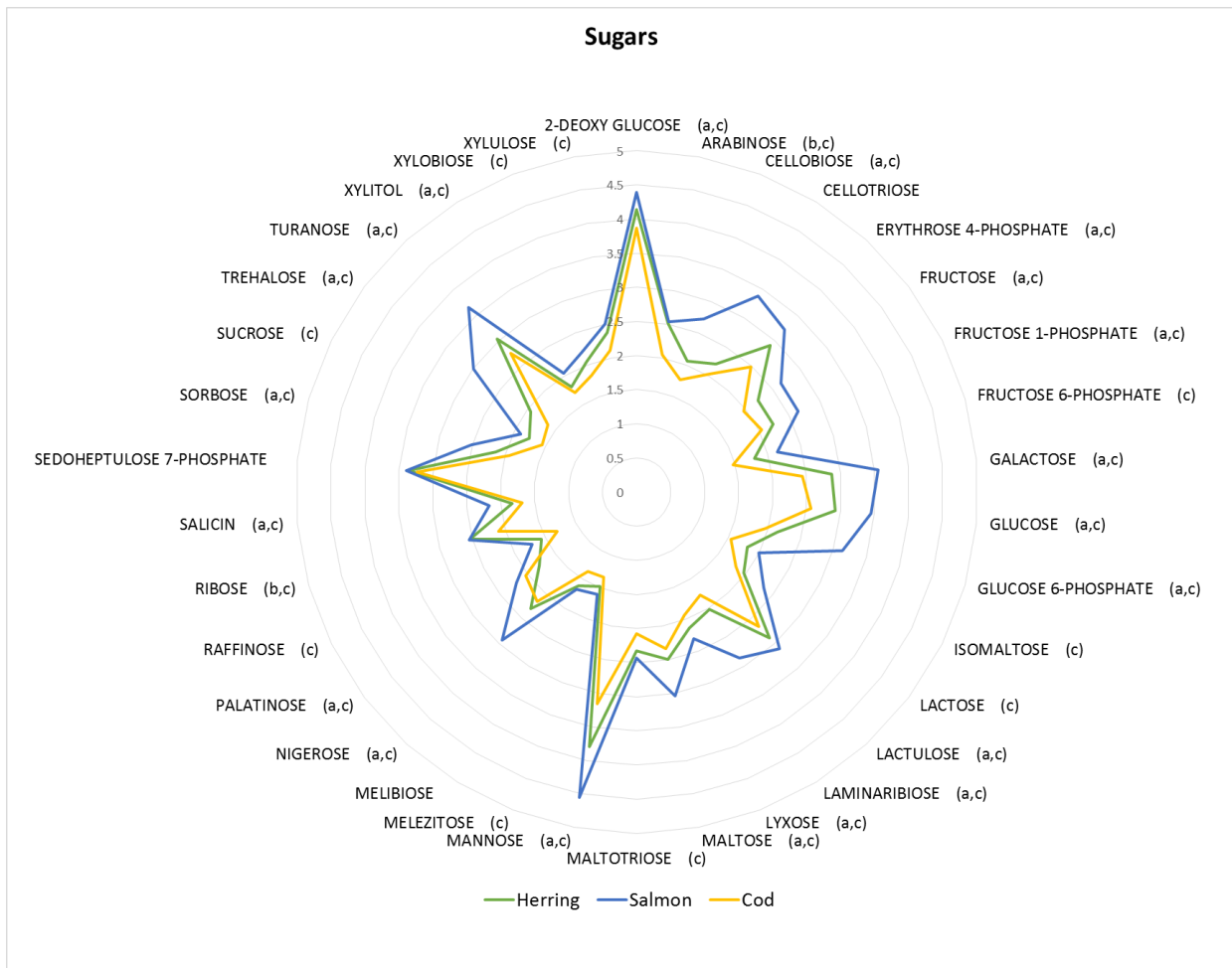
$\beta$ -alanine and 4-hydroxyproline were lower in herring compared to salmon and cod. Salmon had higher values of most LMW compounds compared to cod and herring and particularly glutamine, glutamic acid and pyroglutamic acid among the amino acids.

Salmon deviates from the other fish quite profoundly based on the sugars; for much of the profile, salmon simply has higher proportions of the sugar compounds yet follows the same pattern as cod and herring; however, the sugars cellobiose, glucose 6-phosphate, lactulose and trehalose were almost exclusive to salmon (Fig 5.1.6.c). It is important to stress here that the salmon was farmed, while herring and cod fillets were from wild fish. The relative amount of marine ingredients (fish meal and fish oil) in aquafeed has in the last decade largely decreased [38].



**Figure 5.1.6.b** The radar charts show the abundance of the organic acids/alcohols for the cod, herring and salmon samples.

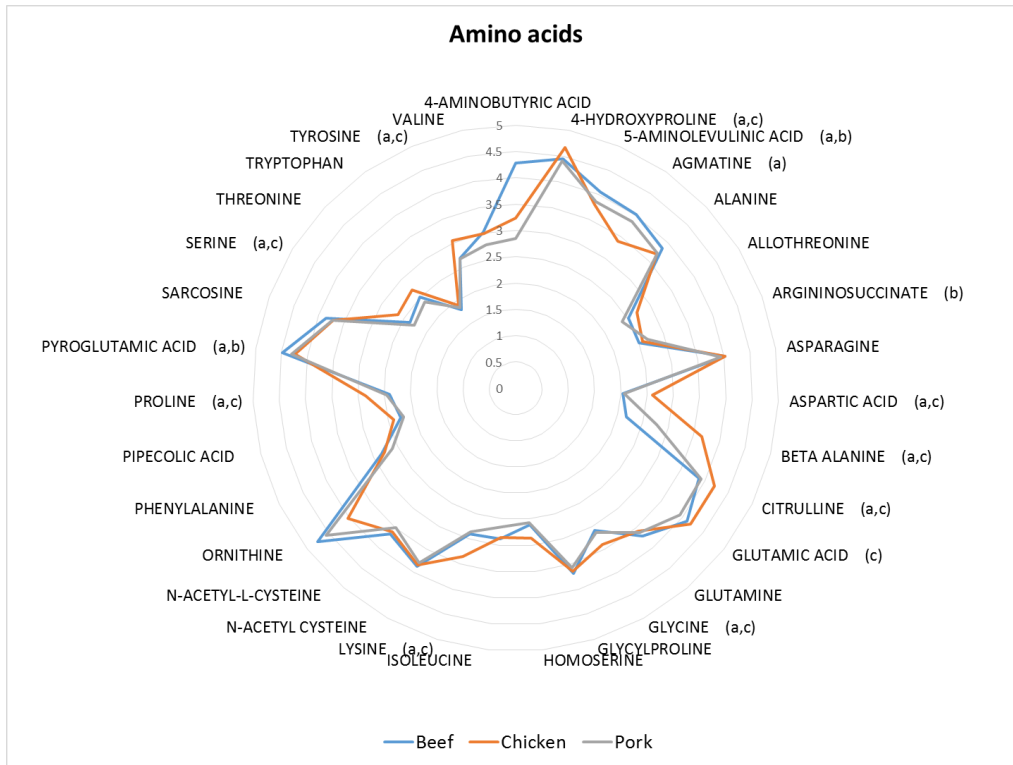




**Figure 5.1.6.c** The radar charts show the abundance of the sugars s for the cod, herring and salmon samples.

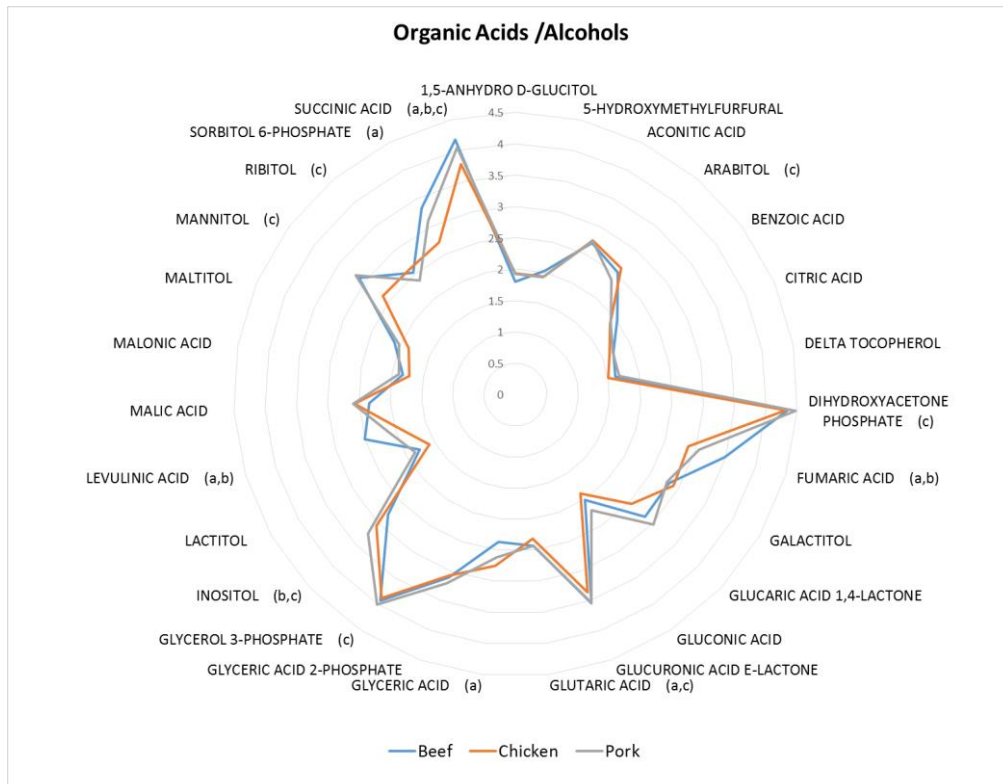
### 5.1.4 Meat Sub-group

In the meat group there were fewer significantly different compounds (Fig. 5.1.4), least of all between beef and pork. Almost half of the abundance of chicken was represented by the amino acids and it is in this compound class that chicken deviates the most from the other meats (Fig. 5.1.7.a). Aspartic acid,  $\beta$ -alanine, citrulline, 4-hydroxyproline and proline were all found to be higher in chicken than beef and pork. Beef had higher levels of fumaric acid, levulinic acid and sorbitol 6-phosphate, whereas chicken had a markedly lower value of mannitol compared to beef and pork (Fig. 5.1.7.b). The sugar profiles of pork and beef were very similar but chicken had generally much lower values. The major differences came from sorbose and galactose, as well as glucose and fructose along with their phosphate equivalents (Fig 5.1.7.c).

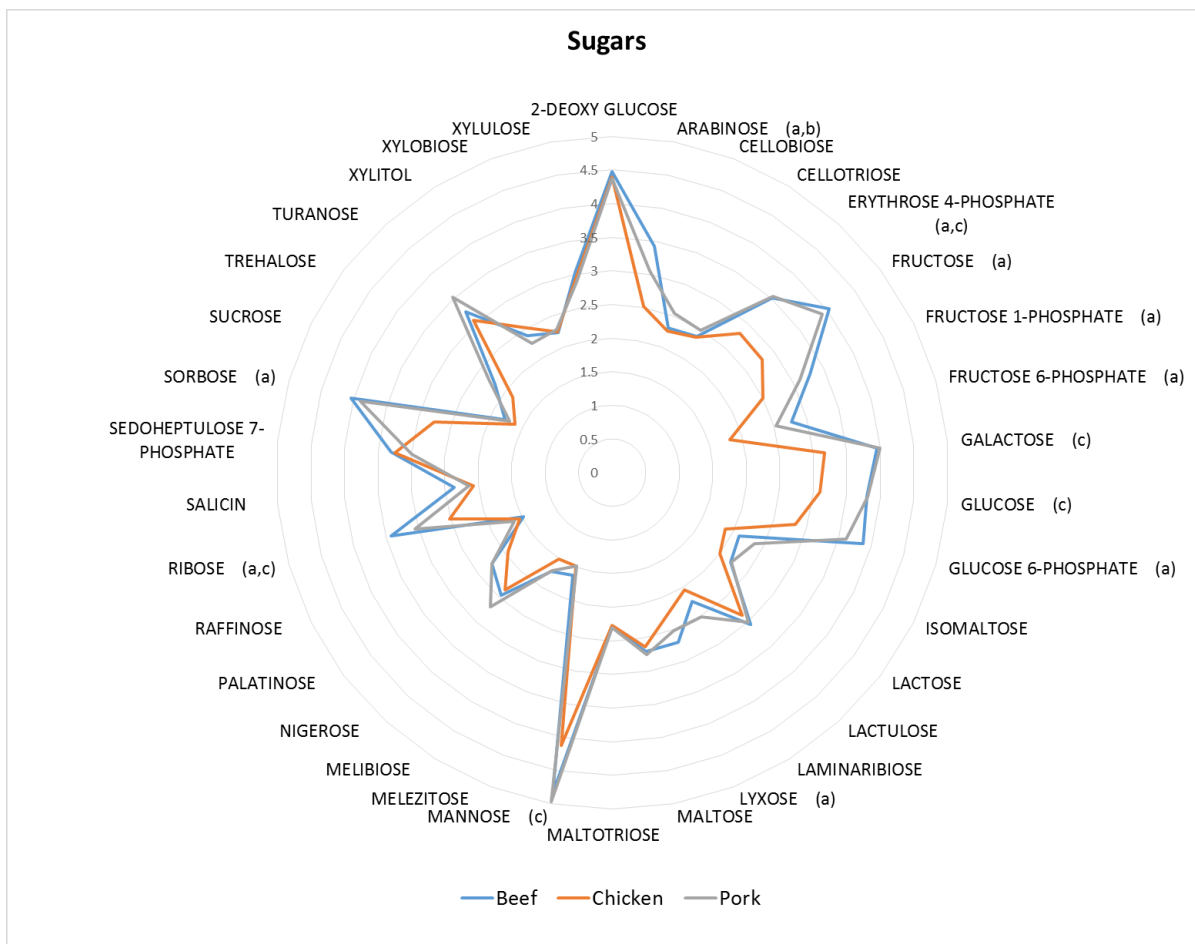


**Figure 5.1.7.a** The radar charts show the abundance of the amino acids for the beef, chicken and pork samples. The values are the log-values of the average intensities. Significant differences between the samples were tested with paired t-tests. Compounds were significant at  $P < 0.01$ .

a = beef vs chicken, b = beef vs pork, c = chicken vs pork



**Figure 5.1.7.b** The radar charts show the abundance of the organic acids/alcohols for the beef, chicken and pork samples.



**Figure 5.1.7.c** The radar charts show the abundance of the sugars for the beef, chicken and pork samples.

## 5.2 Paper II

### 5.2.1 Previous results

In the primary analysis of this study, it was found that baked herring intake increased plasma HDL compared to chicken and pork, as well as resulted in a higher plasma content of the fatty acids DHA, EPA and decreased fatty acids palmitoleic acid, oleic acid and n-6 dihomogamma-linolenic acid. No other effects were observed for other lipoproteins, fatty acids or markers of inflammation [5].

In order to understand the broad metabolic effects of replacing lean pork and chicken as the predominant dietary protein source with herring, the plasma metabolic profile was analyzed using GC-MS metabolomics. Fifteen overweight and obese women and men with a mean age of 50.5 (range 24–70) years volunteered for the study. Two subjects were discontinued from the study, one due to difficulties to attend to the study visits and the other due to regular treatment with the anti-inflammatory drug Ibuprofen. There was

insufficient plasma available for two other subjects, with a total of eleven subjects included in the metabolomics analysis

### 5.2.2 Statistical analysis

Out of 199 metabolites detected based on combined spectral and retention time-based identification, forty-six were observed to best differentiate between the two diets in the OPLS-DA model ( $R^2 = 0.47$ ,  $Q^2 = 0.59$ ). No outlier samples were found, and all time points for all subjects were kept in the analysis. From the forty-six metabolites selected by OPLS-DA analysis, eighteen metabolites were found to have significant diet x time interactions based on the follow-up mixed-model analysis.

The herring intake led to seven metabolites increasing in comparison to the chicken and lean pork intake, while eleven metabolites increased on the meat diet relative to the herring diet (Table 1). The herring diet led to increases (p value for diet x time interactions) of plasma asparagine ( $P = 0.019$ ), erythritol ( $P = 0.020$ ), ornithine ( $P = 0.005$ ), glutamine ( $P = 0.012$ ), glucosamine ( $P = 0.005$ ), shikimic acid ( $P = 0.021$ ) and p-coumaric acid ( $P = 0.037$ ). Of these only glucosamine was significant at both two weeks ( $P = 0.016$ ) and four weeks ( $P = 0.039$ ). Glutamine, ornithine and shikimic acid were found to be significant after four weeks only (Table 1).

Metabolites increased by the pork/chicken diet (p-value for diet x time interaction) were agmatine ( $P < 0.001$ ), cellobiose ( $P = 0.045$ ), citric acid ( $P = 0.027$ ), eicosanoic acid ( $P = 0.009$ ), fumaric acid ( $P = 0.028$ ), gluconic 1,4-lactone ( $P = 0.045$ ), glycolic acid ( $P = 0.005$ ), isocitric acid ( $P = 0.002$ ), oxalic acid ( $P = 0.001$ ), methyl histidine ( $P = 0.001$ ) and sorbose ( $P = 0.041$ ). Only isocitric acid and oxalic acid were significant at both two weeks ( $P = 0.023$ ,  $P = 0.025$ ) and four weeks ( $P = 0.029$ ,  $P = 0.006$ ) while citric acid and agmatine were only increased on the chicken/pork diet after four weeks (Table 1).

**Table 1** Plasma metabolites that were found to change relative to baseline after diets containing either baked herring fillets or a combination of chicken and pork fillets<sup>a</sup>

Metabolite AUC, GC-MS response	Baked herring		Chicken/Pork		MM <sup>b</sup>	P	
	2w	4w	2w	4w		2w <sup>c</sup>	4w <sup>c</sup>
Increased after intake of baked herring							
Asparagine	8 ± 5	14 ± 6	-3 ± 6	2 ± 4	0,019	0,116	0,057
Erythritol	188 ± 95	58 ± 62	-210 ± 155	-295 ± 143	0,020	0,084	0,069
Glucosamine	4 ± 3	8 ± 3	-7 ± 6	-8 ± 6	0,004	0,016	0,039
Glutamine	133 ± 152	793 ± 351	-200 ± 199	-149 ± 298	0,012	0,140	0,038
Ornithine	3 ± 217	526 ± 113	-413 ± 242	-185 ± 173	0,005	0,277	0,002
p-Coumaric acid	7 ± 4	8 ± 6	-10 ± 7	4 ± 7	0,037	0,016	0,623
Shikimic acid	-108 ± 176	503 ± 101	-431 ± 325	-217 ± 225	0,021	0,434	0,022
Increased after intake of chicken/pork							
Agmatine	-84 ± 54	-211 ± 49	13 ± 41	79 ± 46	< 0,001	0,201	0,003
Cellobiose	-76 ± 37	-26 ± 46	8 ± 15	18 ± 14	0,045	0,011	0,363
Citrate	-91 ± 240	-376 ± 216	319 ± 193	223 ± 219	0,027	0,161	0,044
Eicosanoic acid	-20 ± 7	-10 ± 7	-3 ± 6	18 ± 13	0,009	0,065	0,079
Fumarate	-25 ± 16	-15 ± 15	15 ± 8	2 ± 11	0,028	0,043	0,202
Gluconic acid 1,4-lactone	-566 ± 353	-633 ± 499	301 ± 353	645 ± 493	0,045	0,090	0,084
Glycolate	-45 ± 13	15 ± 21	34 ± 20	32 ± 20	0,005	0,002	0,569
Isocitrate	-3 ± 3	-1 ± 3	3 ± 1	5 ± 2	0,002	0,023	0,028
Methyl histidine	-33 ± 11	-13 ± 12	44 ± 15	31 ± 18	0,001	0,003	0,051
Oxalate	-12 ± 4	-14 ± 5	11 ± 7	5 ± 2	0,001	0,025	0,006
Sorbose	-6 ± 4	-8 ± 5	4 ± 3	6 ± 5	0,041	0,091	0,078

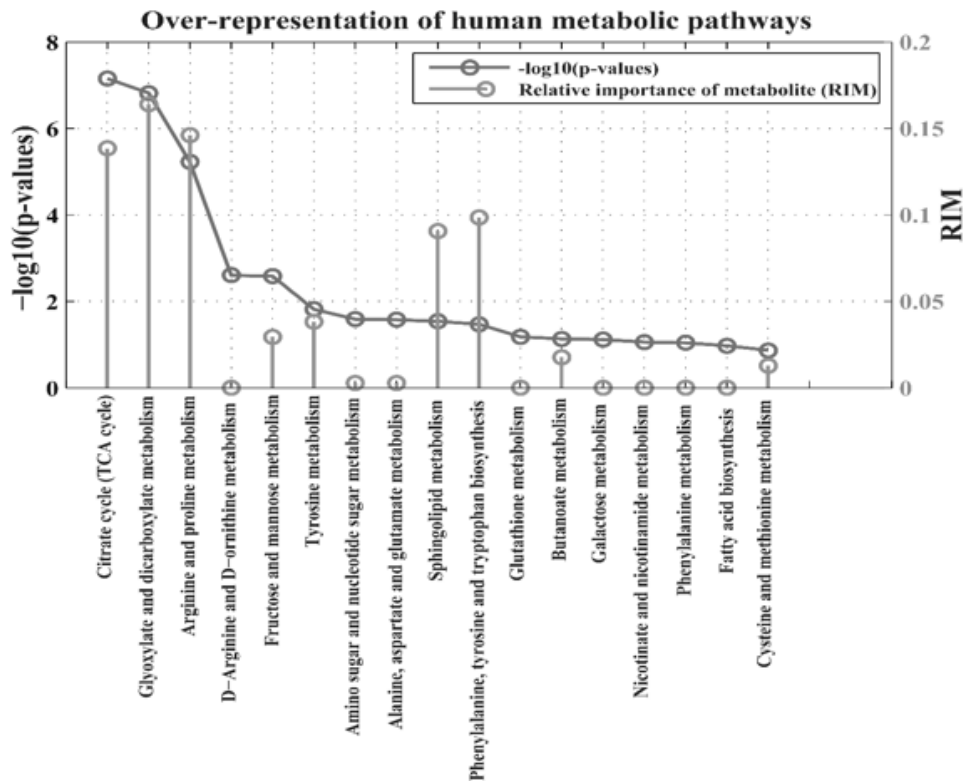
<sup>a</sup> Values are given as means ± SEMs divided by 1000, n = 11. Named metabolites are those that had a combined retention index and mass spectra matching score over 90%. The plasma response of all metabolites in the table differs for the two diets at the P value indicated in the same row.

<sup>b</sup> significance values from the mixed model (MM) analysis over all time points.

<sup>c</sup> significance values for paired t-tests at the single time point.

### 5.2.3 Pathway Over-representation

In order to understand the global effects of replacing chicken/pork with herring, we performed a pathway over-representation analysis based on mapping the significantly changed metabolites onto the human metabolic pathway map and found three pathways that were significantly affected by the intervention, namely the tricarboxylic acid cycle, glyoxylate metabolism and arginine and proline metabolism (Fig. 5.2.1). After further inspection of the metabolic changes (Fig. 5.2.2), it was clear that the reference diet altered flux through the TCA cycle, possibly via an effect on lipid metabolism, and that amino acid metabolism was affected by the herring diet, marked by the increase in several amino acids and amino-acid metabolites close to the urea cycle.



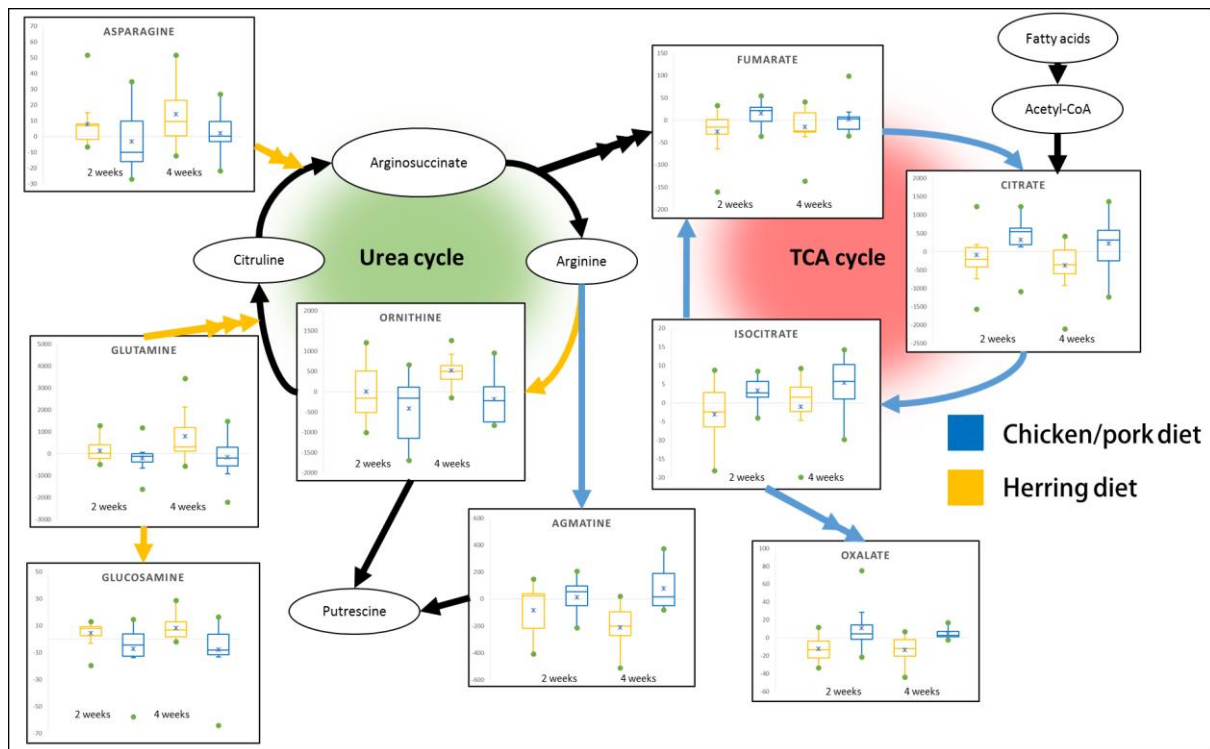
**Figure 5.2.1.** Stem plot (light grey stems) showing the cumulative relative importance of the metabolites (RIM) identified and associated with metabolic pathways with a dark grey curve denotes the significance of over-represented pathways.

## 5.2.3 Metabolomics

### 5.2.3.1 Shifts in Central Energy Metabolism

Lipid metabolism results in increased levels of acetyl-CoA in the mitochondria, which subsequently enters the TCA cycle to form citrate, a metabolite found here to be higher after the chicken/pork diet at both two and four weeks. Citrate alone would not indicate increased flux through the TCA cycle, though other TCA cycle metabolites isocitrate and fumarate were also increased while the subjects ate chicken and pork, and pathway analysis independently identified the TCA cycle as being influenced by the intervention. The increase in the activity of the TCA cycle from the reference diet suggests that there is an altered flux of acetyl-CoA either from lipid metabolism or glycolysis. The herring diet contained much higher amounts of fat compared to the reference diet, which may explain a difference in lipid metabolism and the subsequent effects on the TCA cycle intermediates. However an increased amount of fat from the herring diet might be expected to increase flux through the TCA cycle due to increased production of acetyl-CoA via  $\beta$ -oxidation compared to the reference diet, whereas the reverse was found to be true. This in part could be explained by the increase in lipid transport to the liver from the

peripheral tissues (indicated by the increase in HDL-cholesterol). Other work has found that LC n-3 PUFA such as those found in the herring diet have affected lipid metabolism, observed in the reduction of plasma lipids [39]. It has been shown that hyperlipidaemia is attenuated through activation of PPAR $\alpha$  by LC n-3 PUFA in the intestine and lipid metabolism in the liver [40, 41]. Our data add to these findings and suggest that central energy metabolism is a key regulatory node affected by switching from meat to fish.



**Figure 5.2.2.** Metabolite map of a selection of the significant molecules found in table 2. The significant metabolites are given as box plots. The yellow arrows show the pathways of the metabolites increased by the herring diet. The blue arrows show the pathways for the mixed diet and the black arrows provide the missing links.

### 5.2.3.2 Shifts in Arginine Metabolism

Both diets appear to have affected arginine metabolism but led to different endpoints; herring resulted in a higher prevalence of arginine/ornithine while the pork/chicken diet gave agmatine. It should be noted that the GC-MS method used does not allow a distinction between arginine and ornithine as arginine is converted to ornithine during the derivitisation procedure. Arginine and ornithine are closely associated metabolically; arginine is converted to ornithine after the loss of urea in the urea cycle, and can be used as a precursor for endogenous synthesis of arginine. Arginine is decarboxylated to make agmatine, suggesting that compositional differences between herring, chicken and pork may lead to a shift in flux through the urea cycle. Although elevated plasma ornithine is a

biomarker for excess arginine, the level of plasma amino acids does not correlate with amino acid intake [42]. It is also possible that the difference in arginine metabolism due to the diet is related to the differential effects on TCA-cycle metabolism – altered flux between fumarate and oxaloacetate will also likely alter flux through the urea cycle. A shift in arginine metabolism could have profound effects on overall metabolism due to its role as a substrate for nitric oxide synthesis. Altered nitric oxide synthesis in turn impact on many different physiological parameters including immune and vascular function [43], suggesting new avenues for research on dietary protein and health.

#### ***5.2.3.3 Oxalic acid and the glyoxylic pathway***

Oxalic acid was increased after both two and four weeks of the reference diet. Oxalic acid is an end-point metabolite of vitamin C, and glyoxylic acid metabolism but the intervention did not alter foods that would be likely to differ in vitamin C content, making it most likely that instead the glyoxylic acid pathway was upregulated on the reference diet. Although this pathway is not commonly observed in humans it has been shown to exist in human liver cells [44]. The glyoxylic acid pathway allows the conversion of fats to carbohydrates, and could be differentially upregulated as a result of the different fat content between the two diets [45], or alternatively related to a differential load of amino acids, as glycine, serine and 4-hydroxyproline are all precursors of glyoxylate, which is in turn converted to oxalic acid [46]. The increase in plasma oxalic acid is potentially concerning as it readily chelates calcium, and calcium oxalate crystals make up to 80 % of kidney stones [47], though it is unclear if there is a correlation between plasma and urinary oxalate [46].

#### ***5.2.3.4 Glucosamine***

The amino-monosaccharide glucosamine, a constituent of cartilage, not regularly consumed in high amounts [48, 49], was increased after consumption of herring compared to chicken and pork. Dietary intake of glucosamine has been associated with affecting joint health [50], though glucosamine supplementation has not been found to have any consistent effect on joint health [51]. The change in plasma glucosamine between the diets may be due to endogenous regulation rather than to glucosamine intake, and considering the foodomics study showed higher amounts of glucosamine in pork than herring (Paper I), and that it is intimately involved in the nutrient-sensitive hexosamine pathway [52], a shift in glucosamine metabolism is most probable.



## **5.3 Further discussion**

### **5.3.1 Foodomics**

Surprisingly, no studies have previously used metabolomics to compare the composition of meat and fish muscle. Many studies have used metabolomics to study the effects of conditioning and processing factors for meat and fish muscle quality. Here the variation in metabolic profiles of commercially available fillets from common meats and fish available in Sweden has been investigated. The profiles suggest that terrestrial meat and fish could be separated best through the profiles of non-amino-acid nitrogen-containing compounds and lipids. Interestingly there was a greater variation within meat and fish than between meat and fish. The variation in these sub-groups were predominantly in the amino-acid and sugar compound-classes. Within the fish group, salmon had an almost meat-like sugar profile. Within the meat group, beef and pork were very similar but the sugar and amino-acid profiles of chicken were markedly different.

### **5.3.2 Biomarkers of intake**

There were several compounds that were more prominent in a particular protein source compared to others. Previously we identified  $\beta$ -alanine and 4-hydroxyproline as possible biomarkers for beef intake when compared to herring [53], but here a higher level of 4-hydroxyproline was seen in chicken than beef. However there were higher levels of 4-hydroxyproline in beef than herring, with herring having the lowest levels of all species. (comparison with cod and salmon). In some cases there is no certain relationship between intake of a compound and plasma concentrations after metabolism. 4-hydroxyproline is a metabolite of proline and the most common component of collagen. Our extraction method is only designed to capture those compounds not bound in proteins, and does not necessarily reflect overall 4-hydroxyproline (free and bound up in protein). It is interesting however that  $\beta$ -alanine, a non-protein amino acid, was higher in chicken than beef and pork, it was even relatively high in salmon and cod, in fact only herring provided a lower value. It was not significant between the general comparison of meat and fish, and suggests that it may be of limited value as a biomarker.

Methyl histidine has been identified as a urinary biomarker of meat consumption [9], and from the intervention it was observed for the first time that methyl-histidine increases in

fasting plasma after a meat intake compared to herring intake. The isomers 3- and 4-methylhistidine could not be distinguished but both of which have been associated with meat intake. Finding and validating biomarkers of meat intake are of importance for nutritional epidemiology to complement dietary recall-based methods for measuring meat intake. These data support doing further work to establish whether plasma methyl histidine could be a biomarker of meat intake.

### **5.3.3 Diet and health**

As part of a nutritious diet, protein is an important part how does the source of protein affect our health? The metabolic profiles of different protein sources have been compared and metabolomics analysis comparing herring to chicken and pork has been performed to investigate this. The foodomics results indicate that the red meats and salmon were found to have high sugar contents. As to whether the sugar content would have an effect on the overall sugar consumption of a whole meal is difficult to predict but it may be worth considering when choosing a 'lean' meat.

The intervention study found a shift in energy metabolism between the two diets, which could be attributed to changes in lipid metabolism and would be supported by work comparing herring with animal protein sources in rodent models of disease (animal study refs). Recent work with a similar design comparing lean seafood with non-seafood diets found metabolic changes they ascribed to a higher rate of mitochondrial lipid metabolism after the diet of lean fish [54], which could support the findings, and although the macro-nutrient composition of herring is quite different to lean fish and other seafood, the metabolic profile of herring with cod is very similar. A remarkable finding from this work is how quickly these changes occur in a relatively low-intensity intervention (five meals/week), highlighting the importance of the choice of dietary protein source for metabolic health.

## 6 Conclusions

From the LMW composition study we found out that salmon resembled the different meat sources more than cod and herring in as much that the amounts of sugars were high. Although the meats and fish categories could be told apart from one another, there was far more variation within the individual fish and meat species. Meats could be told from fish primarily by the lipid and nitrogen-containing compound content. The composition of organic acids/alcohols and sugars could differentiate between the species. Surprisingly, cooking did not significantly affect the metabolite profile of the different muscle sources. The results suggest that it is not sufficient to broadly compare 'meat' and 'fish' in nutrition studies as the compositional differences vary widely within meat and fish.

When compared to a mixed chicken and pork-based diet, herring had a differential effect on both amino acid and central energy metabolism, especially around the conditionally essential amino acid arginine. The urinary dietary biomarker for meat intake, methyl-histidine, was observed here in the plasma to be increased after meat intake compared to herring, a finding that warrants further investigation. The findings support the hypothesis that there are metabolic effects of herring that are not related to its LC n-3 PUFA content alone.

## **7 Future Work**

The small-molecule composition results showed that there was more to animal and fish protein sources than amino acids and lipids. Future studies should place more emphasis on the considerable diversity and heterogeneity of the small molecule content of the different types of meat and fish, in addition to the amino acid and fatty acid profiles. The influence of the differences in metabolite composition on processing and health effects requires greater attention in future work on protein sources in the diet.

The intervention study was relatively short-term, and there were no differences in the major clinical outcomes, aside from expected changes to fatty acid profile. Longer-duration studies with more subjects are required to confirm our findings.

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