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URINARY BIOMARKERS OF BIOFORTIFIED BEEF IN HEALTHY WOMEN EXPLORED BY UNTARGETED METABOLOMICS

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A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Metabolism and Human Nutrition
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ABBREVIATIONS

1MH	1-methylhistidine
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3
3-HBC	3-hydroxybutyrilcarnitine
3-HOC	3-hydroxy-5-octenoylcarnitine
AGEs	Advanced Glycation End-products
AI	Adequate Intake
ALA	α-linolenic acid
AndroTGlu	Androstenedione glucuronide
BMI	Body Mass Index
Calcitriol	1,25-dihydroxyvitamin D_3
CAWG	Chemical Analysis Working Group
CEHCs	3´-Carboxychromanols
CON	Control meat group
CMBHCs	Carboxymethylbutyl Hydroxychromans
CR	Chylomicrons Remnants
DehydroTGlu	Dehydrotestosterone glucuronide
DHA	Docosahexaenoic Acid
EFSA	European Food Safety Authority
EPA	Eicosapentanoic Acid
ESI	Electrospray Ionization
EU	European Union
FA	Fatty acid
H_2Se	Hydrogen Selenide
HPLC	High performance Liquid Chromatography
HUBRO	Health Survey in Oslo
LC-MS	Liquid Chromatography–Mass Spectrometry
LDL	Low-Density Lipoprotein
Me_3Se^+	Trimethylselenonium Ion
MS/MS	Tandem Mass Spectrometry
MUFA	Monounsaturated Fatty Acids
m/z	Mass-to-charge Ratio
NCDs	Non-Communicable Diseases
Neu5Ac	N-Acetylneuraminic Acid
NEXS	Department of Nutrition, Exercise and Sports

NMBU	Norwegian University of Life Sciences
PC1	First Principal Component
PCA	Principal Component Analysis
PCBs	Polychlorinated Biphenyls
PCs	Principal Components
PGluTyr	Pyroglutamyltyrosine
PLS-DA	Partial Least Square-Discriminant Analysis
PUFA	Polyunsaturated Fatty Acids
Q1	First Quadrupole
Q2	Second Quadrupole
QC	Quality Control
RT	Retention Time
SA	Sialic acids
SDA	Stearidonic Acid
SD	Standard Deviations
SeCys	Selenocysteine
SeMet	Selenomethionine
SePhp	Selenophosphate
SFAs	Saturated Fatty Acids
SUP	Supplemented meat group
TCA	Tricarboxylic Acid Acid
TMAO	Trimethylamine-N-oxide
TOF	Time-of-Flight
VDR	Vitamin D Receptor
VIP	Variable Importance on Projection
WHO	World Health Organization
α-TOC	α-Tocopherol
δ-CEHC	2’-Carboxyethyl-6-hydroxychromane
ω-3	Omega 3
ω-6	Omega 6
SULT	Sulfotransferases
PAPS	Phosphoadenosine-5´-phosphosulfate

ABSTRACT

Background: The prevalence of overweight and non-communicable chronic diseases is rising all over the globe. The high consumption of energy dense foods on behalf of high nutrient-dense food leads to lower intake of essential vitamins and minerals, such as vitamins D, E, K, and selenium. These micronutrients are related with numerous human vital functions and their deficiency is positively associated with higher risk of chronic diseases and mortality. Bovine meat is an important source of several micronutrients, with higher bioavailability compared to other plant-based foods. Meat consumption is expected to increase worldwide, therefore the biofortification of bull's feeds can be an innovative strategy to increase population's exposure to nutrients. Metabolomics techniques are capable to explore if the supplementation will ultimately lead to a higher micronutrient's uptake in the body.

Objective: The aim of the present study was to explore the differences on urinary metabolic fingerprint of women ingesting 300g of beef a day from bulls fed concentrate supplemented with extra vitamin D, E, K, and selenium compared to the regular composite feed.

Methodology: A 32 days double-blind randomized cross-over human intervention study with two intervention periods, each for 6 days, was conducted in 35 healthy women. The participants were instructed to eat 300g of grinded beef meat as raw weight per day, either from bulls fed with regular control feed or meat supplemented with vitamin D, E, K and selenium, combined with their habitual diet. Fasting urine samples were collected in the morning before and after each intervention period and were analyzed by LC-MS untargeted metabolomics. Multivariate and univariate analysis were applied do identify discriminative features between the two interventions.

Results: A total of 7 and 6 metabolites for positive and negative mode, respectively, were selected as discriminative of the two interventions. Among these, markers of overall meat intake, as well as markers of animal feed, markers related with the participants diet and inflammation-related markers were identified as upregulated or downregulated for the supplemented intervention. No markers specifically related to the biofortification were observed.

Conclusions: Based on our methodology, the ingestion of biofortified beef did not results in a higher level of related metabolites when comparing the two interventions. Minor changes indicate that consequences of biofortification were very small. Further research is needed to understand if a higher increase of vitamin D, E, K, and selenium on animal´s feed composite can lead to different outcomes.

Keywords: beef; biofortification; biomarkers; human intervention; meat; metabolites; supplemented meat; untargeted LC-MS metabolomics; urine

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INTRODUCTION

The worldwide prevalence of overweight, obesity, and non-communicable diseases (NCDs) has increased during the last decade, becoming a growing threat to social and economic stability (1). Dietary habits and physical activity are critical modifiable risk factors in the development of metabolic disorders and cardiovascular diseases (2). Poor dietary habits consisting of high consumption of energy dense foods on behalf of high nutrient-dense foods are a widespread big concern due to a lower consumption of complex carbohydrates, healthy fats, and consequently lower intake of vitamins and minerals (3). The suboptimal intake of vitamins and minerals are accountable for about 7.3% of the global burden of disease, with higher prevalence in populations from developing countries, resulting in multiple micronutrient deficiencies among more than two billion people affected (4,5). Micronutrient malnutrition can lead to serious economic implications, being one of the major public health concerns since it can contribute to high rates of morbidity and even mortality (4).

Throughout Europe, micronutrient deficiencies are highly prevalent, for instance in vitamin D, E, and selenium (6–9).Vitamin D is essential for the functioning of several systems and body functions including immune, nervous, and cardiovascular systems and other inflammatory processes (10). Vitamin E has been found to have a strong antioxidant activity and thus plays an important role in the prevention of several inflammatory processes (11). Selenium is involved in the immune response, fertility, anti-inflammatory effects, and thyroid hormones production (12). Although there is still a need to confirm some vitamin K effects on human health, many studies show that vitamin K is very promising for the prevention of vascular calcification as well as bone, metabolic, and cardiovascular diseases (13,14). Hence, the implementation of successful policies and programme responses to avoid micronutrient deficits are imperative for the prevention of several diseases (15).

Biofortification emerged as a cost-effective method to supply micronutrients to populations that may have limited access to diverse diets or where policies and practices limit use of direct fortification (16,17). The nutritional improvement of food crops (for example, beans and lentils) through biofortification (plant breeding, transgenic techniques and agronomic practices) have been intensively studied and disclosing a good efficacy on improving the micronutrient composition of the crops (16,18,19). Biofortification in animal production refers to the supplementation of animal´s feed or alteration of their housing environment, leading to an improvement of nutritional quality of the meat (20). Biofortified meat is still an unexploited area, thus, evidence is lacking whether the improvement in the meat can lead to a higher uptake of nutrients in human metabolism.

Meat is an important source of B complex vitamins, zinc, selenium, phosphorus, iron, and high biological value protein (21). Compared to plant-based foods, it has higher bioavailability for several micronutrients (22,23). Nonetheless, emerging evidence shows its negative impacts on health, particularly higher risk for metabolic disorders (24). Fat content and other meat contaminations, such as polychlorinated biphenyls (PCBs), are a matter of concern regarding meat composition (25). Nevertheless, meat consumption has been increasing for the last decade and it´s expecting to continue to rise at a global level, becoming an essential vehicle to increase population exposure to micronutrients (26). Therefore, new actions should be adopted to overcome the gaps on meat composition and enhance the nutritional quality of beef. To my knowledge, supplementation of bulls feed with extra nutrients is an innovative strategy for which there is limited research.

To accurately investigate if the consumption of supplemented meat will lead to a higher micronutrient's uptake in the body, metabolomics techniques can be employed. Metabolomics refers to the study of circulating or excreted metabolites, which are chemical compounds originated in foods that underwent metabolism and can be measured in biological fluids such as urine and blood (27). By investigating the metabolome, the biological activity of food components and/or nutrients can be explored in an objective manner. Metabolites derived from vitamins and minerals have already been identified, for instance 3´-Carboxychromanols (CEHCs) for vitamin E, 5C-aglycone for vitamin K, trimethylselenonium ion for Selenium and 24,25-dihydroxyvitamin D3 [24,25(OH)2D3] for vitamin D (28–31). However, an untargeted metabolomics approach, which allows the measurement of thousands of metabolites at a time, may enable the identification of new such metabolites and potentially help decipher new metabolic pathways (32).

Aims

The aim of the present study is to explore the differences on urinary metabolic fingerprint of women ingesting 300g of beef a day from bulls fed concentrate supplemented with extra vitamin D, E, K and selenium compared to the regular composite feed.

We hypothesize that an increase in vitamin and mineral levels in animal feeds will lead to increased levels of related urinary metabolites in humans consuming the meat. If our hypothesis is confirmed, this will be the first study using the urinary fingerprinting to support meat biofortification efficacy in humans.

BACKGROUND

Over the past decades there has been a large body of evidence for interactive links between lifestyle behavior (diet and physical activity) and the risk of NCDs (33). Smoking, physical activity, alcohol intake and body weight management are some of the modifiable risk factors for the development of NCDs (2). Moreover, diet was considered the number one risk factor for the rising incidence of metabolic disorders such as obesity, diabetes, and cardiovascular disease, which are now a major public health problem across the world (34,35). The World Health Organization (WHO) estimates that mortality from NCDs is on the rise, being accountable for 74% of the deaths globally in 2019 (1). Economic costs of unhealthy diets and low physical activity in the European Union (EU) were calculated to be approximately 1.3 billion euros per year, thus primary prevention is considered the most cost-effective and sustainable strategy to cope with the NCDs epidemic (36).
The global increase of obesity and overweight arises from an imbalance between energy intake and expenditure which can be explained by the overconsumption of high energy density foods with low nutrition value (37,38). Another consequence of these poor dietary habits is the high prevalence of suboptimal intakes for several micronutrients, due to lower intakes of fruits, vegetables, whole grains, omega-3 rich fish, nuts and seeds, all nutrient rich foods that should be present in most of our daily meals (3,39).
Some studies have reported high prevalence of micronutrient inadequacy in Europe, particularly for vitamin D, vitamin C, selenium, iron, calcium, folate and vitamin E (6,8,40–42). These micronutrients are related to numerous human vital functions and their deficiency is positively associated with higher risk of chronic diseases, infections, mortality and morbidity (15,43). Even in moderate levels, micronutrient malnutrition can lead to detrimental effects on human function (4). Therefore, despite the worldwide problem with excess of energy intake, an adequate exposure to trace minerals and vitamins deserves also great attention in public health initiatives.

Vitamin D

Vitamin D, or calciferol, is a liposoluble pro-hormone available in two forms: vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) (44). Vitamin D_3 can be synthesized in 80-90% upon sun light exposure, which is absorbed by 7-dehydrocholesterol in the skin, transformed in previtamin D_3, and rapidly converted to vitamin D_3. Vitamin D_3 is subsequently metabolized in the liver to 25-hydroxyvitamin D_3 and in the kidney to 1,25-dihydroxyvitamin D_3 (calcitriol) - its biologically active form - which binds to the vitamin D receptor (VDR) to allow its diverse physiological functions (Figure 1)(45,46). Vitamin D major

urinary metabolites are 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] and 25-hydroxyvitamin D_3 [25$(OH)D_3$] conjugated with glucuronic acid (31). Taheri *et al.* showed an increase in 25$(OH)D_3$ after a higher intake of vitamin D through supplementation (47). During wintertime, when sun exposure is scarce, vitamin D becomes an essential nutrient, being pivotal to ensure its intake through food (fatty fish, eggs and fish liver oils) and supplementation (48).

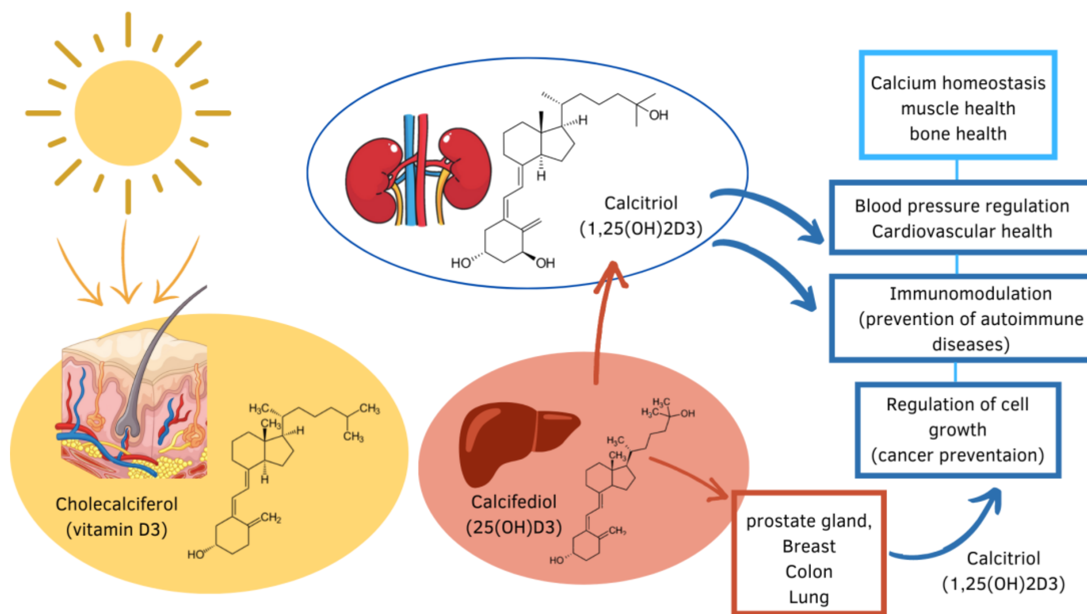


Figure 1. *Metabolism of 25(OH)D_3 to 1,25$(OH)_2D_3$ and its physiological effects. Adapted from M. Holick, 2004* (45).

Vitamin D plays an important role for many vital functions, particularly in the immune responses, muscle function, inflammation plus nervous and cardiovascular systems (10,44). Moreover, this vitamin is involved on the maintenance of calcium and phosphorus serum levels and in bone homeostasis, by acting on calcium and phosphate intestinal absorption, renal excretion, and calcium bone mobilization (49,50). In relation to the immune response, there are emerging vitamin D health relationships with epithelial barrier regeneration and maturation of immune cells. The vitamin D effect on viral and bacterial infections is also proved through a mechanism involving macrophages activation and antimicrobial peptide production by epithelial and immune cells (10).

Moreover, its deficiency has been shown to be associated to obesity, as well as cardiometabolic risk factors such as hyperglycemia, insulin resistance, dyslipidemias and hypertension (51–53). Although sun exposure represents a major environmental risk factor for skin cancer, there is evidence that vitamin D plays immunomodulatory and anti-tumor functions, and may be critical in lung cancer prevention and reduction of cancer death risk (54,55).

According to the European Food Safety Authority (EFSA), the current vitamin D recommendations for an Adequate Intake (AI) in children and adults is 15 ug/day, although the typical intakes in European countries are far below the minimal requirements with 5,5 and 3,6 μg/day in men and women, respectively, with higher intakes in the Northern countries (42,56). Across the European population, 25-hydroxyvitamin D deficiency represents a concerning prevalence of 40,4% and 13,0% for levels below 50nmol/L and 30nmol/L, correspondently (40).

Vitamin E

Vitamin E is a lipid-soluble molecule comprised of a family of tocopherols (α, β, γ, δ), and tocotrienols (α, β, γ, δ), where α-tocopherol (α-TOC) is the most biologically active form (57). Vitamin E can be found in many fat-rich foods, including nuts, for instance peanuts, almonds, sunflower seeds, sesame seeds and food oils such as soybean, corn, and peanut oil (58). Vitamin E absorption requires the presence of other lipid rich-foods and follows the same intestinal absorption, hepatic metabolism and cellular uptake processes as other fat-soluble vitamins (28). Its absorption involves non-specific transporters to α-TOC and takes place in the upper gastrointestinal tract (59).

The first step is the dissolution of the vitamin E in the lipid portion of the meal followed by absorption through emulsification into lipid droplets at both gastric and duodenal levels. Then, α-TOC is merged into chylomicrons and subsequently secreted into the bloodstream. The α-TOC transported by the chylomicrons can be used by extra-hepatic tissues while the chylomicron remnants (CR) take α-TOC to the liver (Figure 2) (60)(61).

The liver is the main responsible for tocopherols and tocotrienols metabolism and plays a key role in determining the circulating concentrations of the several tocopherols and tocotrienols and in restricting α-TOC accumulation in tissues. The tocopherol metabolism starts with an initial catabolism and side chain shortening involving oxidation of the hydrophobic side chain via cytochrome P450-catalyzed reactions (phase I metabolism), which leads mainly to α-, β-, γ-, and δ-CEHC (2'-carboxyethyl-6-hydroxychromane) and their precursors, carboxymethylbutyl hydroxychromans (CMBHCs). The phase II metabolism is characterized by glucuronidation and sulfation of these metabolites and lastly urinary excretion (62). Some conjugates of α-CEHC have already been identified in urine, such as glucuronide, sulfate, glycoside, glycine, glycine glucuronide and taurine (28). Urinary α-CEHC excretion has been shown to be correlated with α-TOC intake (63–65).

Vitamin E has been found to be very effective in the prevention and reversal of several inflammatory processes due to its antioxidant properties (11). Previous studies reported an inverse association between vitamin E supplementation and risk of cardiovascular disease, by regulation of cell properties such as signal transduction, cell proliferation and gene

expression (66). Atheroprotective effects of α-TOC, together with its metabolite α-CEHC, have received considerable attention due to their anti-inflammatory and anti-oxidative effects, for instance with inhibitory effects against oxidized low-density lipoprotein (LDL) formation (67). Moreover, vitamin E was also related with Alzheimer's disease attenuation risk, of about 26%, since oxidative stress is of major importance in Alzheimer's pathophysiology (68).

According to the EFSA, the current α-TOC recommendations for an AI in women and men are 11 and 13 mg/day, respectively (69). The average intakes in European population ranges between 7,8 and 12,5 mg/day in women and 8,2 and 16,0 mg/day in men, thus, not all population is reaching vitamin E requirements (59).

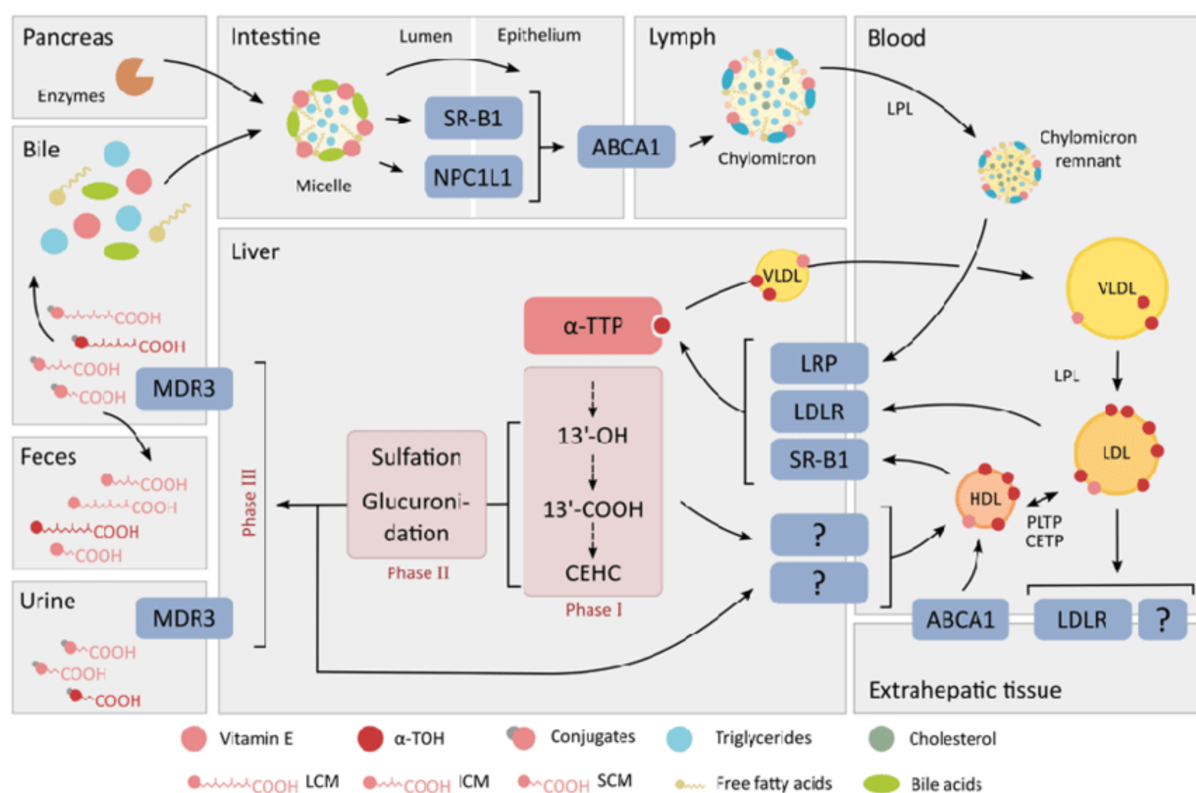


Figure 2. *Absorption, transport, and metabolism of Vitamin E. SR-B1, Scavenger receptor class B type 1; LPL, Lipoprotein lipase; NPC1L1, Niemann-Pick C1-like 1; VLDL, Very low density lipoproteins; HDL, High density lipoproteins; α-TO,: α-tocopherols; α-TTP, α-TOH transfer protein; LDL, Low density lipoproteins; LRP, LDL receptor-related proteins; LDLR, LDL receptor; 13'-OH, 13'-hydroxychromanol; 13'-COO,: 13'-carboxychromanol; CEH,: Carboxyethylhydroxychromanols; HDL, High density lipoproteins; PLTP, Phospholipid transfer protein; CETP, Cholesteryl ester transfer protein; LCM, Long-chain metabolites; ICM, Intermediate-chain metabolites; SCM, Short-chain metabolites. Figure from Schmölz L. at al., 2016* (28).

Vitamin K

Vitamin K naturally occurs in two different dietary forms: phylloquinone (vitamin K_1) and menaquinones (vitamin K_2). In plants, the only important molecular form is vitamin K_1, which has a phytyl side chain. Menaquinones are designated as MK-n, according to the number (n) of prenyl units, which can vary from MK-4 to MK-13. Apart from MK-4, the other menaquinones can be synthesized by the gut bacteria. The MK-4 and MK-7 are the most abundant menaquinone isoforms in the human diet (70). Vitamin K_1 is the main dietary source of vitamin K and is naturally present in vegetable oils, some fruits and mainly in green leafy vegetables (kale, broccoli, cabbage, spinach). Vitamin K_2 can be a product of human gut bacteria or found in fermented and animal-based foods. Menadione (vitamin K_3) is the synthetic form and a catabolism product of vitamin K_1, usually used in animal's feed (71,72).

The dietary vitamin K, mainly as phylloquinone, is absorbed chemically unchanged from the proximal intestine and follows a well-established pathway that is common for all fat-soluble vitamins, which includes: bile salt- and pancreatic-dependent solubilization, uptake of mixed micelles into the enterocytes, packaging into chylomicrons, secretion into lymph lacteals, and entry into the blood via the thoracic duct (Figure 3) (73).

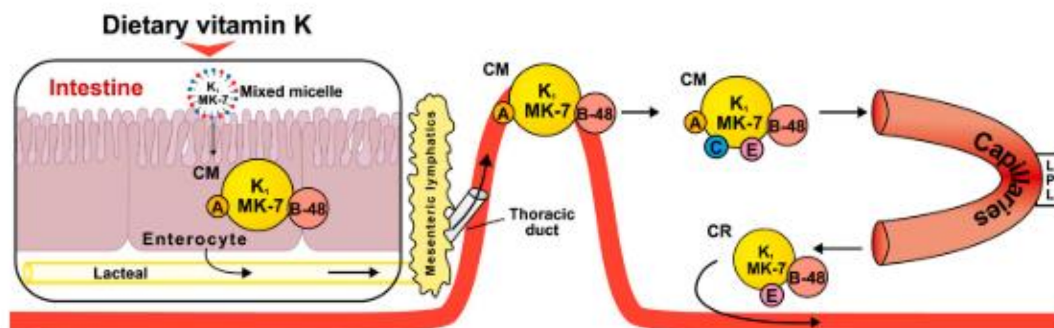


Figure 3. Intestinal absorption of dietary phylloquinone (K_1), and MK-7, in the intestinal lumen. CM, chylomicron; LPL, lipoprotein; MK, menaquinone. Image from Shearer et al., 2012 (73).

The CR are thereafter taken by the liver and the majority of vitamin K_1 is retained to assist carboxylation of clotting factors. The long chain derivates of Vitamin K_2 are redistributed through the bloodstream for extra-hepatic tissues, essentially bone and vasculature (74). In the liver, w-hydroxylation starts within the endoplasmic reticulum by a cytochrome P450 mixed function oxidase followed by oxidation of the alcohol and aldehyde dehydrogenases (Figure 4) which results in the formation of vitamin K carboxylic acid, a metabolic intermediate. ω-hydroxylation products undergoes repeated side-chain shortening via

the β-oxidation pathway until two major carboxylic acid metabolites with 7- and 5-carbon side chains are reached (phase 1 transformations) (73,75). The originated metabolites are thereafter excreted in bile and urine mainly as glucuronides (phase-2 transformations) (29). Harrington et al. demonstrated that urinary excretion of vitamin K metabolites reflects dietary phylloquinone intake (76).

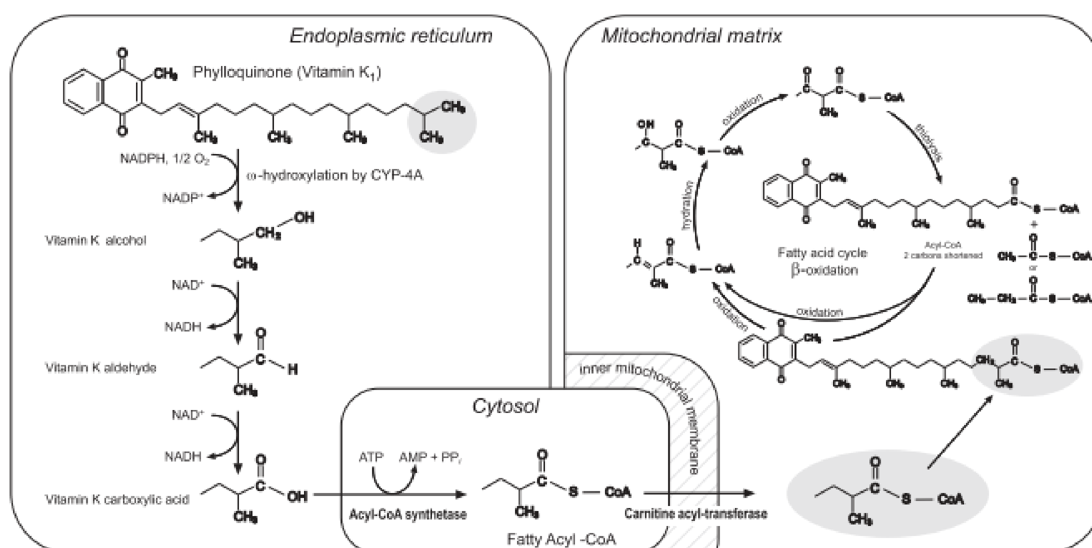


Figure 4. Vitamin K_1 metabolism. Image from Card et al., 2014 (75).

The biological functions for which vitamin K is mostly well-known are associated with blood coagulation, acting as a cofactor for a specific carboxylation reaction which transforms selective glutamate residues to γ-carboxyglutamate residues (29). Recently its role included a range of physiological processes such as regulation of bone and soft tissue calcification, inflammation, cell growth and proliferation, cognition and several oxidative processes and fertility (77).

Vitamin K deficiency in adults is generally associated with 1) antibiotics, leading to lower vitamin K–producing bacteria in the gut, 2) drug interaction, for instance to reduce cholesterol or used for obesity; orlistat being an example, 3) malabsorption disorders, such as celiac disease, cystic fibrosis, short bowel syndrome, or 4) extremely low intake of vitamin K on the diet (72). The relationship between vitamin K deficiency and bone fractures was already reported in several studies as well as its positive effects on cardiovascular system through a vitamin dependent hormone secreted in bone´s osteoblasts – osteocalcin – which acts on insulin producing cells and other specific tissues to increase glucose and fat metabolism (78). Hence, vitamin K is a promising tool for prevention and treatment of bone, metabolic and cardiovascular disorders (14). Manna *et al.* reported an emerging beneficial effect of vitamin K supplementation in insulin sensitivity and glucose tolerance improvement, preventing insulin resistance and therefore decreasing the risk of type 2 diabetes (79).

The vitamin K_1 dietary reference values for the European population are set at 70 µg/day for adults, including pregnant and lactating women. The mean intake estimated by EFSA for 9 EU countries ranged between 72 and 196 µg/day in adults (80).

Selenium

Selenium is a trace mineral with major structural functions on various enzymes particularly glutathione peroxidase, thioredoxin reductase and deiodinases (81). The organic Selenium forms are selenomethionine (SeMet), selenocysteine (SeCys) and methylated derivates. Selenate and selenite are the inorganic forms (82). The main food sources of Selenium are meat, chicken, fish, eggs, milk, cereals and protein-rich nuts, such as pistachios, walnuts and Brazil nuts (83). SeMet is present in plants, animals and food supplements whilst SeCys is found in animal-derived food. Selenite is found in supplements and selenate in plants and fish products (82).

Dietary selenium is mainly absorbed in the small intestine and subsequently transferred to the liver where is going to be metabolized and used for selenoproteins production. All the absorbed Selenium forms are converted into hydrogen selenide (H_2Se) in the enterocytes and transported through the bloodstream linked to VLDL and LDL fractions, albumin, and α-globulin. Afterwards, H_2Se is converted to selenophosphate (SePhp) and incorporated into selenoproteins as SeCys (Figure 5)(82). Selenium excretion occurs mainly through urine and feces, whereas breath, saliva and hair are minor contributors. Within the first 24h hours of ingestion, the rate of selenium excretion in urine is higher (84). Some urine metabolites already identified for selenium include trimethylselenonium ion (Me_3Se^+), selenosugars and Se-methylselenoneine (30).

Selenium is a semi metal with a role in many biological functions, such as proper functioning of the immune system, fertility, anti-inflammatory effects, and production of thyroid hormones (12,81). Potential cardiovascular benefits can be attributed to selenium for preventing oxidative modification of lipids, inhibiting platelet aggregation and inflammation reduction (85). Moreover, there is evidence of a therapeutic benefit of a selenium supplementation as an antioxidant on metabolic disorders (86). Serious health consequences have been reported has a consequence of selenium deficiency including necrotizing cardiomyopathy, peripheral myopathy, anemia, reduced muscle tune and alterations in skin appendages (hair and nails) (81).

The selenium recommended daily intake according to EFSA corresponds to 70 µg/day whilst the average selenium intakes in adults ranged from 31,0 to 65,6 µg/day (84).

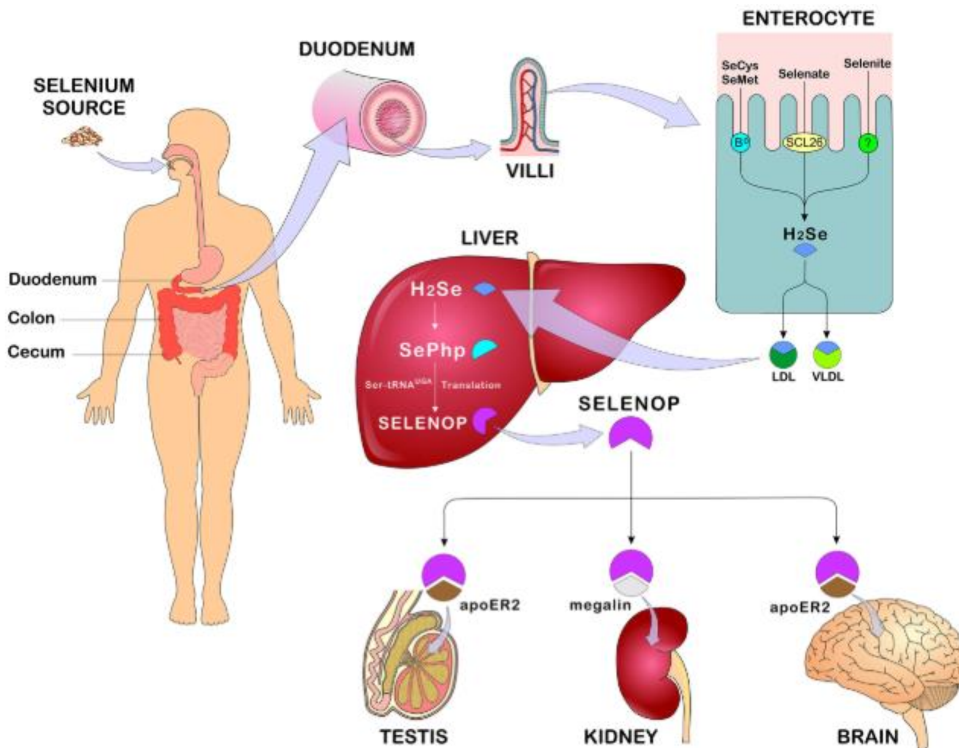


Figure 5. Selenium absorption, metabolism, and distribution. Selenium absorption occurs in the duodenum, cecum, and colon. SeMet and SeCys are absorbed by active transport in the enterocytes while selenate is absorbed by passive transport. In the enterocyte all the Selenium forms are converted to H2Se and transported to blood circulation bound mainly to LDL and VLDL. In the liver, H2Se is transformed to SePhp and integrated into selenoproteins as SeCys. SELENOP is the main form of transport for other tissues through apoE2 and megaline. Se, selenium; SeMet, selenomethionine; SeCys, selenocysteine; H2Se, hydrogen selenide; LDL, low-density lipoprotein; VLDL, Very low-density lipoprotein; SePhp, selenophosphate; SELENOP, selenoprotein P; apoE2, apolipoprotein E receptor 2. Image from Ferreira et al., 2021 (82).

Essential fatty acids

Omega-3 (ω-3) polyunsaturated fatty acids (PUFAs) include α-linolenic acid (ALA; 18:3 ω-3), stearidonic acid (SDA; 18:4 ω-3), eicosapentanoic acid (EPA; 20:5 ω-3), docosapentaenoic acid (DPA; 22:5 ω-3), and docosahexaenoic acid (DHA; 22:6 ω-3). These PUFAs are named ω-3 since their double bond is located on the third carbon atom from the methyl end of the fatty acid chain (87). The PUFAs differ from monounsaturated fatty acids (MUFAs) for having more than one double bond in the carbon chain (88). The human body is not capable to synthesize endogenously ω-3 PUFAs, as a result of the limitation of the enzyme responsible for inserting *cis* double bonds (87). Hence, ensuring an adequate intake through the diet and supplementation is essential. Oily fish are the main source of ω-3 in diet, such as herrings, sardines, pollock roe, tuna, salmon, mackerel, trout and sea brass amongst other seafoods (89). Green leafy vegetables as well as chia seeds, walnut, perilla

and flax are plant-based sources of ω-3 (90). ALA is a medium-chain ω-3 fat found in plants and grass-fed meat. ALA is partially converted to long-chain ω-3 fatty acids within the human body (91).

The digestion of ω-3 starts in the stomach by the action of gastric lipases, with the partial break down of triglycerides into diacylglycerol and fatty acids (FA), forming large emulsions of fat globules. In the intestinal lumen happens the complete absorption, where fat emulsion (FA, cholesterol and monoacylglycerols) is absorbed into enterocytes, largely by passive diffusion, through the release of bile salt and pancreatic lipases (87). Free FA are then integrated into chylomicrons and enter the circulation via the lymphatic system. The half-life of ALA, DHA and EPA are 1h, 20h and 39-67h, respectively (92). Ruan *et al.* identified 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid as a strong biomarker of fish oil intake (93).
ω-3 fatty acids are integral structural components of the phospholipid layer of cell membranes, being responsible for the fluidity of the membranes and selective permeability. Moreover, PUFAs are essential for the synthesis of lipid-related metabolites, for instance eicosanoids, prostaglandins, leukotrienes and thromboxanes, lipoxins, which play a key role in vascular physiology (87,94). Linoleic acid (ω-6) (18:2 ω-6) is also an essential fatty acid, with arachidonic acid (20:4 ω-6) as the major end product. In general, arachidonic acid gives rise to pro-inflammatory eicosanoids while ω-3 PUFAs leads to the production of anti-inflammatory eicosanoids (90). An adequate intake of ω-3, together with a low ratio of ω-6/ω-3 fatty acids, have been showing a great positive effect on several health parameters, with a protective role on inflammatory, cardiovascular and chronic diseases (90). An increase on ω-6 fatty acids ingestion and a very high omega 6/omega-3 ratio is found in today´s Western diets. Hence, ensuring an optimal intake of ω-3 is essential for the prevention of several cardiovascular diseases (95).
EFSA sets the reference values for adults on 0,5% of energy from alpha-linolenic acid and 4% from linoleic acid. The recommended intake for long-chain omega-3 PUFAs ranges from 250-500 mg/day (94).

Meat and health

Meat consumption represents an important source of energy, high-quality dietary protein as well as several vitamins (B12, B6 and niacin) and minerals (iron, selenium, zinc and phosphorus) (21). The average protein content in meat ranges between 12,3% (duck) and

34,5% (chicken), corresponding to a high digestibility protein. Moreover, meat contains a high nutritional value since it supplies all the essential amino acids (lysine, threonine, methionine, phenylalanine, tryptophan, leucine, isoleucine, valine), which can´t be produced endogenously, with no limiting amino acids (96). Several nutrients in meat, for instance vitamin D, selenium and iron have shown a higher bioavailability compared with plant-based foods (23,97,98). Furthermore, the lean component of red meat is a source of bioactive substances such as carnitine, taurine, carnosine, ubiquinone, glutathione and creatine (21).

Despite its nutritional richness, there have been mounting evidence of a positive association between meat intake, in particular processed meat, and all-cause mortality due to its energy and fat excess (24,99–102). Taking into account the current scientific evidence, high red meat consumption may be harmful with regard to risk of cardiometabolic diseases such as stroke, coronary heart disease and heart failure as well as higher risk of cancer (24,25). Some additives introduced during processing and other contaminants, including PCBs, residues of antibiotics and hormones used during production can contribute for these health outcomes. Moreover, the presence of saturated fatty acids (SFAs), advanced glycation end-products (AGEs) (produced during cooking), nitrate and nitrosamine, nitrite, phosphatidylcholine and L-carnitine have been pointed out as other potential components associated with higher risk of NCDs (25). Other suggested mechanisms for the disease-promoting effects of red meat consumption can be Trimethylamine-N-oxide (TMAO) production by the gut microbiome and the pro-oxidant effects of heme and iron present in meat (103). Figure 6 illustrates some of the potential mechanisms that can potentially explain the role of meat consumption in disease development.

Worldwide meat consumption per capita increased between 2000 and 2019 (104). Furthermore, it is estimated to rise within the next decade by 14% compared with the average between 2018 and 2020, mainly due to population growth and greater affordability (26,105). The economic upswing, particularly in developing countries, will allow for an increase on meat purchase together with higher urbanization, greater labour participation and food service expenditures (105,106). Nevertheless, per capita meat consumption may be stagnant or decrease in countries with higher incomes where environmental, ethical/animal welfare and health outcomes are a rising motivation factors for the population (105).

Considering the future perspectives of meat consumption pointing to an uprise, new strategies should be devised in order to accomplish an improvement in the nutrition profile of meat products, thus, potentially increasing population exposure to health promoting nutrients (101,105). As described above, micronutrients are required in small amounts for specific functions in our body, yet moderate deficiencies can cause serious health

consequences. Biofortification emerged as an important tool to help population meet their dietary requirements, becoming a cutting-edge approach to increase some beneficial nutrients exposure and hopefully improve overall nutritional status. Nonetheless, there is limited evidence of the effect of supplementing animal's feeds with extra vitamins and minerals on human health markers.

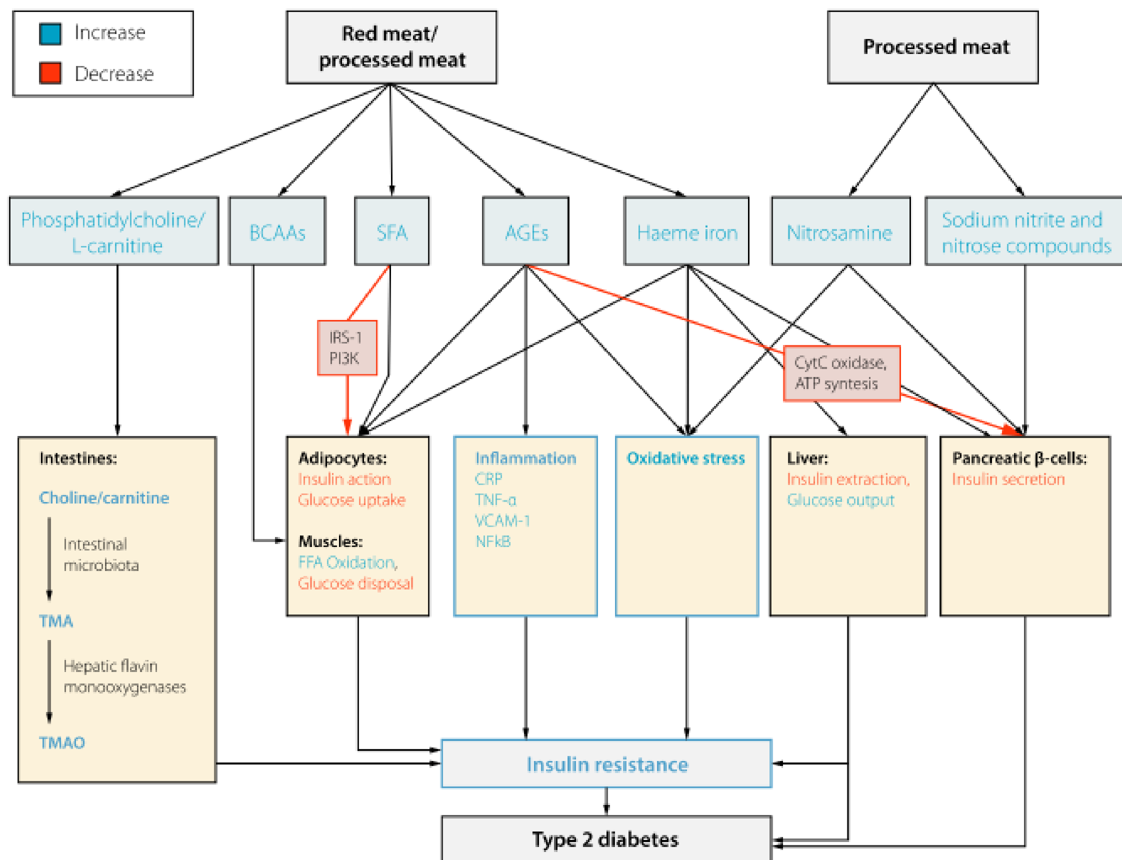


Figure 6. Possible mechanisms of red and processed meat metabolites on type 2 diabetes etiology. The higher intake of saturated fatty acids (SFA) increases intracellular fatty acyl-CoA and diacylglyceride, which leads to the decrease of insulin activation of insulin receptor substrate 1 (IRS-1)-associated phosphatidylinositol 3-kinase (PI3K) in muscle, resulting in the decreased insulin-stimulated glucose transport activity. High advanced glycation end-products (AGEs) elevates the formation of nitric oxide through the induction of expression of nitric oxide synthase and, therefore, impair the glucose-stimulated insulin secretion, by inhibition of cytochrome C oxidase and ATP synthesis. BCAA, branched amino acid; FFA, free fatty acid; CRP, C-reactive protein; TNF-a, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1; TMA, trimethylamine, TMAO, trimethylamine N-oxide; NFkB, nuclear factor kappaB. Image from Wolk A., 2017 (25).

Biofortification

Biofortification in animal production refers to the adjustment of cattle´s feed component or housing environment as part of animal husbandry. Unlike food fortification,

biofortification is not an exogenous or post-production alteration of the food, since the nutritional composition of the animal is naturally altered through agronomical practices, during animal´s growth phase (16,20). Since hypovitaminosis D is becoming a global public health priority issue, vitamin D has been receiving the most attention from scientific research, disclosing promising results in terms of vitamin D status improvement in humans after the consumption of biofortified meat (20,107,108). However, further research is needed as robust human clinical trials to confirm previous findings and explore the effect of other essential nutrients biofortification on human metabolic parameters. Reliable dietary assessment instruments are required to determine more accurately our cellular activity and physiological status (109).

Metabolomics, one of the omics techniques that is focused on measuring metabolites in human biofluids such as urine or blood, is now a key tool for the study of biomarkers of exposure. Considering that food composition databases are often unsatisfactory or unavailable, biomarkers of exposure emerged to accurately reflect the intake through any biological specimen (110). Metabolomic analysis complements biochemical information obtained from genes, transcription factors and proteins, widening the knowledge on cell biology and physiology (111,112). Metabolomics in nutrition research has also been focusing on determining metabolic variations and changes in metabolic profiles related to different dietary interventions (113). Untargeted metabolomics allows the comprehensive profiling of thousands of such metabolites, with varying molecular complexities. By applying the untargeted metabolomics techniques, we can get to know the metabolic pathway of some food constituents such as vitamins and minerals. Since urinary metabolites of these vitamins have been reported in the literature, I believe that untargeted metabolomics is the right technique to explore such data.

Liquid chromatography–mass spectrometry metabolomics pipeline

The untargeted Liquid chromatography–mass spectrometry (LC-MS) metabolomic experimental pipeline can be described by the following workflow: 1) study design, 2) sample collection, 3) LC-MS data acquisition, 4) pre-processing, 5) data pre-treatment, 6) statistical analysis and 7) identification and biological interpretation (Figure 7).

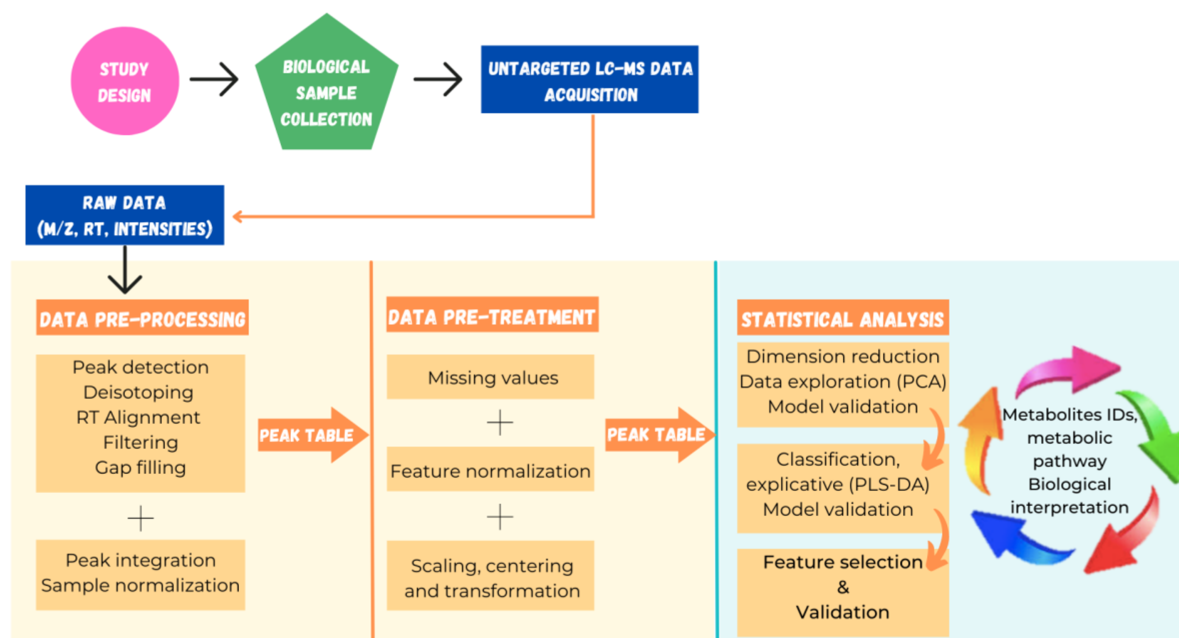


Figure 7. Metabolomics workflow. Starting with study design, followed by sample collection, data acquisition and steps from raw data to selected features and search for biological interpretation.

1. Study design

A suitable design of the study is the first step to ensure robust and trustworthy results. The study design should take into consideration the hypothesis of the study, study duration, intervention and control groups, blinding, outcome measures, inclusion criteria, statistical power, eligibility criteria, methodology for data collection, and how to ensure compliance throughout the experiment (114,115).

2. Samples characteristics, preanalytical processing and sample preparation

Urine is a biological fluid which contains over 95% water, and it consists of endogenous and exogenous metabolites, derived from metabolism in tissues and microbiota. Preparation of urine samples is easy due to low protein content, reducing the risk of analyte loss (114).
The first step is the centrifugation of the urine samples to remove human cells/bacteria or other non-cellular constituents and the supernatant is diluted based on the requirements of the analytical platform (116). Since urine volume greatly differs across urine samples, and consequently metabolites concentrations, it is necessary to normalize, either to osmolarity or urine volume. Then the samples are aliquoted usually to 1 replicate of 150-300 uL plus a pooled quality control (QC) sample containing 50-300 uL from each sample (114).

3. Data acquisition: Liquid Chromatography – Mass Spectrometry

Liquid chromatography coupled with mass spectrometry is one of the most powerful and widely used tools for metabolomics identification via both untargeted and targeted analysis. The basic principle of this analytical technique is combining the physical separation power of LC with the highly sensitive and selective mass analysis of MS based on the singular mass-to-charge ratio (m/z) of each metabolite of interest. High performance liquid chromatography (HPLC) allows separation of compounds of a wide range of polarity into individual components (117). It consists of a dynamic process where a liquid mixture, for instance, urine samples, are distributed between two phases: stationary and mobile. Figure 8 illustrates the LC-MS system workflow.

The samples containing several analytes of interest are pumped through an LC column – nonpolar stationary phase – by a polar mobile phase flowing through it with the aim of transporting the sample – a process called elution. This partition technique, named as reversed-phase, is the most widely used for compounds separation due to its high versatility, stability and large metabolite coverage (118). The chemical affinities differences between the components in the sample accordingly to their interactions between the two phases lead to different migration rates. After this specific separation, the emerging components flow out of the column by a detector at the end of the column (119).

The next step is the interface between the eluted metabolites derived from the HPLC and the MS, where is necessary to make the transition from samples at high pressure in a condensed phase in LC into a gas-phase vacuum system in MS. A chromatography- mass spectrometer system consists of 3 major components functioning under high vacuum: an ion source, a mass analyzer, and a detector (120).

The ion source on the instrument is responsible for the interface between the two different environments is the electrospray ionization (ESI) technique. The ESI is well suited to polar/ionic metabolites, with a minimal fragmentation, leading to a great quantitative analysis with good sensitivity. It generates multiple charged ions on both positive and negative mode depending on their chemical formula. The analytes containing only Hydrogen, Carbon and Oxygen are expected to be detected in negative mode whilst metabolites containing Nitrogen are expected preferably ionized in positive mode (116).

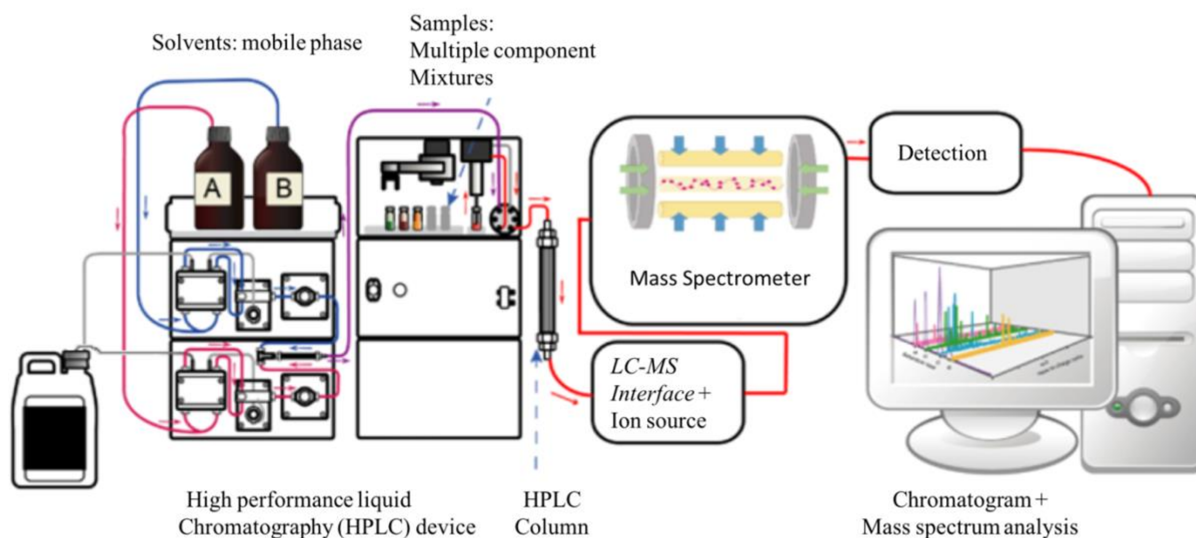


Figure 8. Liquid chromatography – mass spectrometry system workflow. Image from Norena, D., (2022) (121)

Mass analyzers can be categorized as: quadrupole, ion trap, time-of-flight (TOF), Orbitrap, and Fourier transform ion cyclotron (117). Our analysis was carried out using the Q-TOF analyzer, a "hybrid" instrument that combines quadrupole technologies with a time-of-flight mass analyzer. The main advantage of this instrument is the combination of the high compound fragmentation performance of quadrupole with the fast analysis and high mass resolution of TOF (122). In Q-TOF-MS instrument the third quadrupole has been replaced by TOF tube. The first quadrupole (Q1) runs a mass filter to select one or more ions of interest according to their m/z, and the second quadrupole (Q2) acts as a collision cell, responsible for fragmenting the ions through neutral gas molecules. Thereafter, all ions enter the flight tube where the mass separation will take place (122). TOF is a mass analyzer which separates the ions based on the time each ion takes to move from the ion source to the detector. Since all charged ions have the same kinetic energy, the ones with lower masses get higher speed and strike the detector first (120).

The untargeted metabolomics approach employs two distinct scan types for data acquisition: 1) single MS mode, which makes use of a different frequency on Q1 and Q2 to provide an accurate mass of the unfragmented parent ion (precursor) by transmit it through the quadrupole and 2) second mode (MS/MS), which uses Q1 as mass filter and then Q2 as a fragmentation step. The resulting ions or any unfragmented parent ion are transmitted to the TOF analyzer to accurately measure their mass. The detection system at the end converts the flight time of each ion into a mass signal (123).

4. Data mining

Metabolomics data, specifically untargeted metabolomic studies, generate high-dimensional and complex datasets which are naturally challenging to handle due to its high number of variables combined with a low number of samples. Despite its potential to generate a wealth of data, there are many detected variables derived from random or systematic errors unrelated to the research question. Hence, data mining, i.e., pre-processing, normalization, and data reduction are essential to overcome these biological and analytical variations and extract the most relevant information. Figure 9 illustrates LC-MS data mining workflow.

A) Data preprocessing (124)

Data pre-processing is needed to convert the raw LC-MS data into a working table i.e., peak list, where each detected ion is translated into a feature set comprising of a m/z and retention time (RT). Generally, the peak height is used to determine each feature´s intensity. The typical workflow for processing MS data comprises the steps outlined below:

a) **Raw data import:** the raw data files acquired from analyzed samples are converted to cdf format (NetCDF) and afterwards imported into a software, for instance MZmine.

b) **Peak detection:** this step aims to identify and get the information regarding the intensity of the features across distinct chromatographic runs for each sample (125) It generates a mass list with the detected ions for each scan and converts each MS spectrum to pairs of m/z and intensity values (124). Subsequently a chromatogram is built for each mass that can be detected over the scans.

c) **Chromatogram deconvolution:** helps to reduce the detection of several ions originating from shouldered or split peaks. The optimization of this parameter allows the removal of redundant peaks i.e., duplicate ions in the peak list, that are just a result of poor chromatography rather than true metabolite pattern.

d) **Deisotoping:** aims to find those peaks in a peak list which form an isotopic pattern and is responsible to keep only one isotope, either the most intense or the one with the lowest m/z.

e) **Alignment:** targets to correct for analytical errors derived from instrumental variations during data acquisition, characterized by m/z and RT shifts within samples and/or plates. Hence, alignment corrects any m/z and RT deviation by assigning an average value of m/z and RT for each feature. The purpose of peak list

alignment is to match relevant peaks across numerous samples and its ultimate result is a final peak list.

f) **Filtering:** aims to select only the main features of our samples by filtering the peak list to keep only features with a minimum number of occurrences ("Minimum peaks in a row").

g) **Gap filling:** the previous peak list contains some missing values due to low intensity, higher expected RT, or m/z shifts and/or bad peak shape. Gap filling enables to retrieve the intensity of a peak in all samples, even if it was not detected in the previous steps. This step is very helpful in decreasing the number of irrelevant peaks although awareness is needed to avoid excluding biologically significant features.

In the end of the above steps the resulting data matrix typically consisting of "metabolite features" identification where each peak is expressed by a feature set defined as the combination of RT and a m/z with the correspondent intensity (represented by the peak area) calculated as the averages for the particular feature over all runs (126). It is noteworthy to mention that pre-processing is of major importance, especially for untargeted metabolomics, considering that the features lost in this step can no longer be recollected in the subsequent steps.

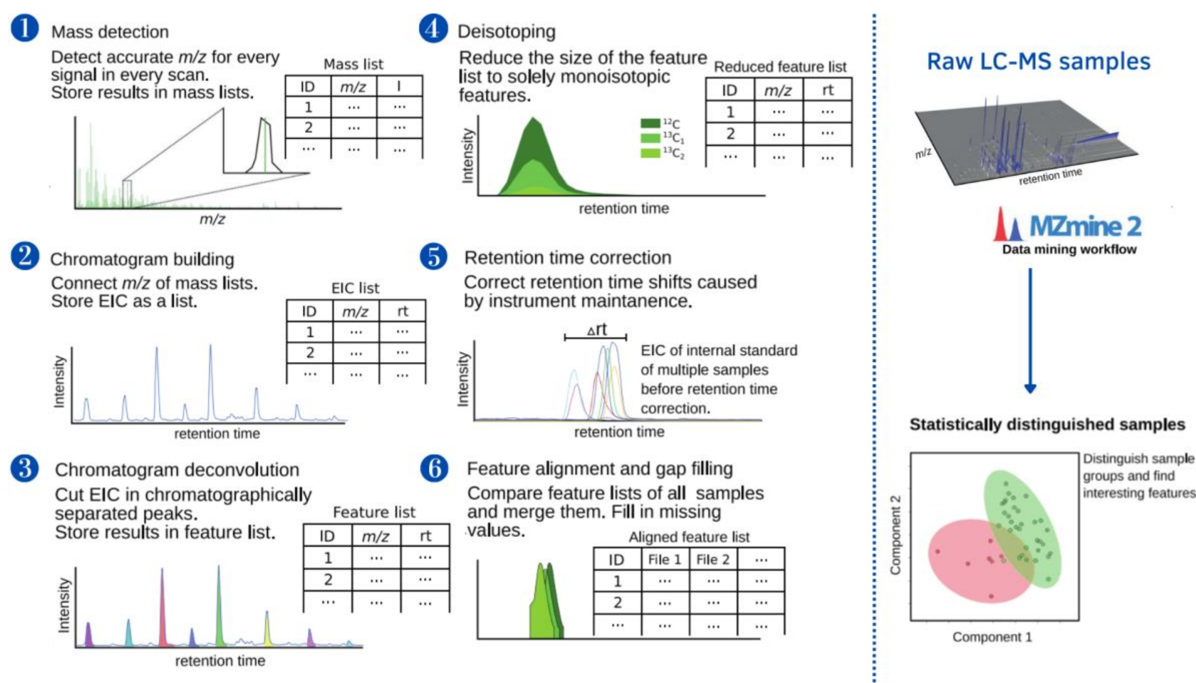


Figure 9. LC-MS data mining workflow. From raw data handling in MzMine to statistical interpretation. EIC, extracted ion chromatogram. Adapted from Korf et al., 2020 (127).

B) Normalization

Normalization within the metabolomics workflow is key to remove confounding variations attributed to experimental errors that may take place during sample preparation and/or other analytical inaccuracies that can be source of variation while preserving the relevant data from biological sources (114). It also helps to distinguish between biological variance and experimental imprecisions and improves the statistical analysis performance and interpretation (114,128).

Normalization in an early stage of the sample preparation aims to adjust the concentrations across samples and can be performed through different strategies. The most common are: 1) correction by creatinine level; 2) measurement of total solute concentration (osmolality); 3) specific gravity; 4) 24-h urine volume or 5) total ion current (129–131).

The human body strictly controls blood volume and composition, being less pivotal to normalize serum and plasma samples. Unlike plasma and serum, a normalization step is crucial to improve comparability between urine samples, since overall concentration of metabolites fluctuates according to water intake, external environment and other physiological factors (132,133).

Other strategies to remove unwanted systematic bias are data scaling and transformation. While samples normalization adjusts the variations across samples (i.e., within chromatograms), data scaling and transformation allow the comparison between metabolites of different samples (i.e., between chromatograms) (125). Scaling methods ensures that different samples can be comparable by dividing each variable in the data matrix by a factor. Transformation are conversions of the data, such as log transformation, in order to correct the dataset for non-equal variance uncertainty disparities linked to some or all metabolites under analysis (i.e, heteroscedasticity) (125,134).

C) Data Reduction

The high number of features originated from previous steps makes statistical analysis challenging unless data reduction strategies are applied. Data reduction is responsible for discarding noisy features originated in 1) data acquisition, for instance by removing peaks also present in blanks as well as peaks eluting very early or very late (initial 0.3 minutes or last 0.5 minutes of a run time) in the chromatogram; 2) data pre-processing, when is set a low noise threshold and results on including irrelevant features in the dataset, or 3) others which are not relevant for the dietary intervention study.

5. Statistical analysis

After data pre-treatment the next step is to explore the dataset. Metabolomics studies are made up of a large number of metabolites in biological material and there are multiple data analysis strategies possible to be applied depending on the study question. Typically the goal is the identification of treatment differences (supervised) or the detection of patterns (unsupervised) in large sets of pre-processed data (134–136).

In LC-MS based metabolomics studies it is possible to apply univariate or multivariate statistical analysis. Ideally, we should apply both since their combination allows for a more comprehensive detection as they provide complementary information. Univariate statistical approaches analyze one feature at a time, being a challenge at handling collinear variables. Multivariate methods make use of all features simultaneously considering the relationship between variables, in contrast to univariate methods, which focus exclusively on the mean and variance of a single variable (136).

A) Multivariate analysis

Multivariate analysis addresses the variation between all variables and all samples. Multivariate analysis is relevant for highlighting the relationship between samples and variables, explaining how they are connected and how can they contribute for the biological phenomenon (134,137). This analysis can be divided into two groups: supervised and unsupervised methods.

Principal Component Analysis (unsupervised method)

Unsupervised methods are a starting point for exploring overall data, in which there is no prior knowledge of the true classes of the samples neither any outcome (111). Principal component analysis (PCA) is a typical unsupervised method for data exploration, which is particularly useful for identification of the main patterns and outlier detection. PCA aims to summarize the information as well as possible using a limited number of variables (138). The principle consists of finding linear combinations, named principal components (PCs), that are responsible for pointing the directions of maximum variance in the multivariate space (Figure 10) (134). The result is a new orthogonal space which retains much of the information in the initial dataset while decreasing the variables number significantly (139).

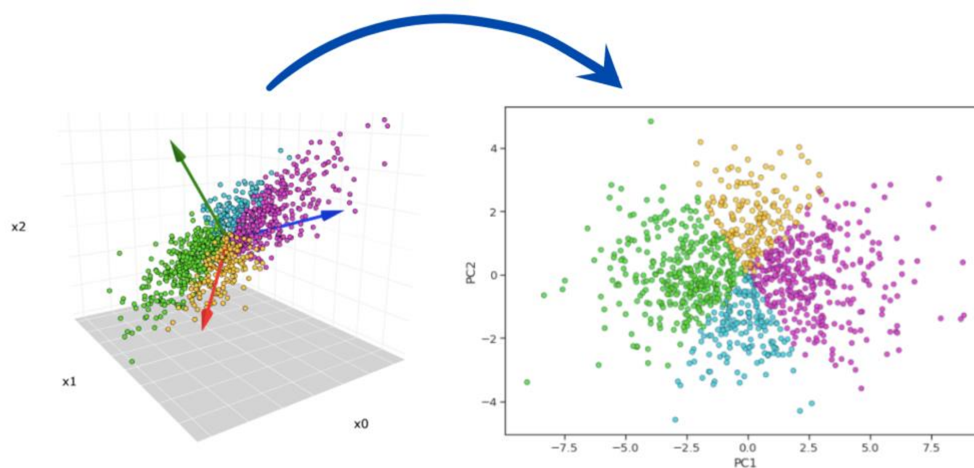


Figure 10. Example of a Principal Component analysis (PCA). On the left, the original 3-dimensional data set. The red, blue and green arrows represent the direction of the first, second and third principal components, correspondently. On the right, the scatterplot after PCA reduced from 3 dimensions to 2 dimensions. Image adapted from Cheng, 2022 (140).

The model is projected onto low-dimensional planes by the PCs that express the most variance. The first principal component (PC1) represents the direction of the space with the greatest variance in the data. The PC2 depicts the second direction of largest variance in the orthogonal subspace at PC1, and so forth. When we investigate the result of this components representation in space it becomes easier to visualize some metabolic signatures (141). PCA detects patterns through similarities and differences among the samples consequently can be used to discover samples relationships and identify possible confounding factors. Although PCA is very efficient on detecting clusters and outliers, we only use this analysis as a way to point some future analysis since its accuracy can improve when applying supervised methods (111).

Partial-least Squares Discriminatory Analysis (supervised method)

After spotting some potential discriminative features, supervised methods use information on class membership to select biomarkers of interest (111). Supervised approaches aim to identify metabolic patterns that are correlated with the phenotypic variable we are studying while downweighing other sources of variance (137). Since this method uses class membership information, it can classify and predict where each sample belongs with respect to those already classified (111). Therefore, this statistical method provides a way to filter the metabolic information which is not correlated to the classes.

PLS-DA is one of the most widely used method in metabolomics which combines dimensionality reduction with the construction of a predictive model (i.e., discriminant

analysis) (142). PLS-DA is used to measure a specific feature contribution for the discrimination of the different sample groups (137). In order to highlight the most discriminant variables between the groups and understand whether the features are up or downregulated, PLS-DA creates latent structures and variable importance plots. As for PCA, each PC is orthogonal to each-other, and they can be plotted to observe clusters (Figure 11) (141).

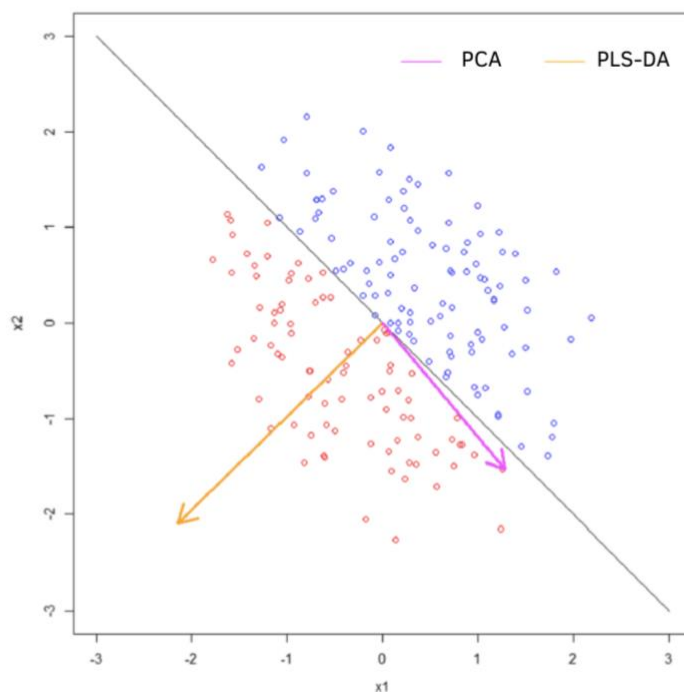


Figure 11. Comparison between the PC1 computed by PCA (in pink) and PC1 computed by PLS-DA (orange) shows a data set where PLS-DA picks the direction that most separates the labels whilst PCA points the direction that least helps separate them. Image from Ruiz-Perez et al., 2020 (143).

In metabolomics studies is very common to deal with highly collinear and noisy data, therefore applying PLS-DA modelling is a main advantage since this technique provides an easy visual interpretation of complex datasets. Furthermore, it provides several statistics such as loading weight, regression coefficient and variable importance on projection (VIP) which gives us information regarding the main variables (144).

Validation methods

Despite of these advantages, PLS-DA modelling it´s prone to *overfitting* (i.e., keeping noise rather than real information) since it is excessively trying to obtain a class separation. This problem can be solved by applying resampling techniques such as cross-validation, and then ensure a good performance of the selected discrimination model (134).

B) Univariate analysis

Univariate methods are a very common approach due to its ease of use and interpretation. The features are analyzed independently thus they are not able to recognize the presence of interactions across features. This can be a disadvantage when applied alone on the data but since multivariate methods generally mask significant variables, since all metabolites are analyzed ate the same time, it is important that univariate methods are also integrated in the data analysis. Univariate methods thus filter potentially the most significant features, by reducing the number of mzRT features considered in the multivariate model (145). Depending on the dataset and the goal of the study, several statistical methods can be applied. The most well-known and used methods when comparing two or more groups are Students t-test and ANOVA, respectively, which can give easily answers to discover statistical significance.

Nevertheless, other statistical approaches are available according to the dataset and depending on the aims of the study.

6. Metabolite identification and biological interpretation- > generating new hypotheses

Metabolite identification is a fundamental step that translates raw data into biological context. Identification aims to explore the identity of selected discriminative features obtained from data analysis. The statistical analysis and visualization tools allow the selection of the most biologically interesting features which reduces the identification workload to a reasonable minimum. The first step is to recognize the parent ion of each feature, since during ionization processes many adducts, and fragments can be generated from the same compound. Through MATLAB it is possible to group the features with high correlation and similar RT which makes it easy to spot potential adducts or fragments.

Once the parent ion has been identified, fragmentation experiments on a tandem mass spectrometry (MS/MS) at different collision energies are performed and the result is the formation of product ions ("daughter ions"). MS/MS is applied since it has a higher specific mass spectrometric detection compared with LC-MS (Figure 12). This technique will allow the elucidation of the chemical structure of the parent ion based on fragments respective molecular masses and fragmentation patterns (146). After extracting the information regarding the parent ions and its fragmentation products, a search in several databases and biological databanks, identification tools and/or software is performed for structure elucidation. The unknown markers are also investigated manually.

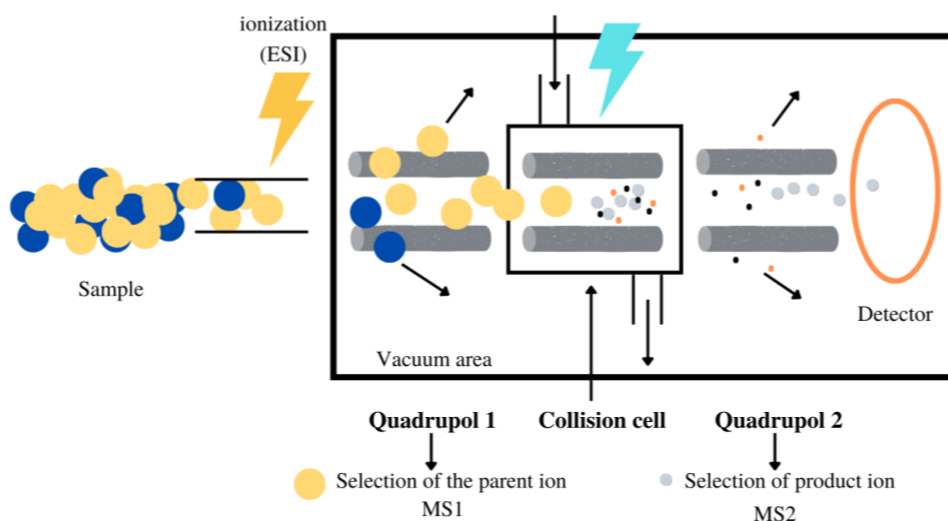


Figure 12. Tandem mass spectrometry (MS/MS). Adapted from Vogeser et al., 2007 (146).

Several online databanks, databases and spectral libraries can be used for spectra comparison, human metabolites information, and to provide hints regarding the substructures of unknown compounds. The main online databanks and databases are MassBank (http://www.massbank.jp), METLIN (http://metlin.scripps.edu/), Human Metabolome Database (HMDB), mzCloud (https://www.mzcloud.org/), FooDB (www.FooDB.ca), Chemspider http://www.chemspider.com/), and PhytoHUB (www.phytoHUB.eu) - are essential for identity annotation. They provide information about chemical structures, physicochemical properties, biological functions, and metabolites pathway. SIRIUS is also a useful software which can predict potential structures through their fragmentation spectra (147).

7. Confirmation of identification

After the identification, the candidate metabolites are assigned to level 1-4 according to the Chemical Analysis Working Group (CAWG) of the Metabolomics Standards which proposed in 2007 the minimum reported standards for metadata relative to metabolite identification (148)(149):

- **Level 1:** identified compounds. The compound is identified based on a minimum of two independent and orthogonal data (e.g., RT and mass spectrum) in relation to an authentic compound analyzed with equal experimental conditions. For instance, using an authentic standard or by matching the compound properties with an in-house spectral library.

- **Level 2:** the compound is putatively annotated - there are no chemical reference standards but is based on its physiochemical properties and/or its spectral similarity with public/commercial spectral libraries.
- **Level 3:** the compound is putatively attributed to a compound class – according to its physicochemical properties and/or its spectral similarity with public/commercial spectral libraries.
- **Level 4:** unknown compound - although unidentified or unclassified, the compound can still be differentiated and (semi-) quantified based on spectral data.

8. Biological interpretation

Once the metabolite identification is completed, the biological interpretation is the next step to investigate their corresponding metabolic pathways. Some online databases are available, such as KEGG (https://www.genome.jp/kegg/), to corroborate the metabolites identification and consequently help understanding how those markers were obtained and what´s the biological meaning for the study when comparing different dietary interventions.

METHODOLOGY

Recruitment

The recruitment was done at the University by hand-outs and announcements via internet. The inclusion criteria were healthy, young, normal-weight females. Exclusion criteria was use of medications, except oral contraceptives. The participants were instructed not to take vitamin, mineral, or omega-3 supplements from one month prior to and throughout the study. They were also asked not to eat fish during the trial period, since fish metabolites could be confounders to the analysis (150). All the participants provided written informed consent before participating, and they were free to quit the study at any time. At the end of the study the participants were given a gift-card of 50€ for a local grocery store for the participation. Participants initially also completed a questionnaire to collect information regarding their age, health parameters, diet, physical activity, tobacco habits, education and work, and the use of medicines. This survey was based on the questionnaires from the Health Survey in Oslo (HUBRO).
The trial was registered and approved by the Norwegian Regional Committees for medical and health research ethics, REK (https://helseforskning.etikkom.no/), as 2016/620. REK sør-øst 2016/620 Sunnere storfekjøtt and registered in ISRCTN registry (ID ISRCTN25014465). All procedures were performed according to the ethical standards of the Declaration of Helsinki.

Study design

The study consisted of two phases: 1) the fortification of the animal feeds, and beef meat production, and 2) the human trial.

Phase 1. The bulls: section on beef meat production

The two different meat samples were acquired from two distinct groups of bulls. This study started in July and August 2015 when twelve calves from Animal Production Experimental Farm at the Norwegian University of Life Sciences were born. The animals received a commercial concentrate and silage until they were 6-7 months old. In February 2016 the bulls with 199 (± 14) days old were randomly assigned into the dietary treatments (Figure 13). Both concentrates were similar regarding to energy, protein, starch and neutral

detergent fiber content. The supplemented concentrate was added extra vitamin K_3, Vitamin D_3, Vitamin E as RRR-alpha-tocopheryl acetate, selenium and with ω-3 fatty acids source as Rape seeds and Camelina seeds (*Camelina sativa*). The chemical content and ingredient composition of each concentrate is described in Appendix 1.

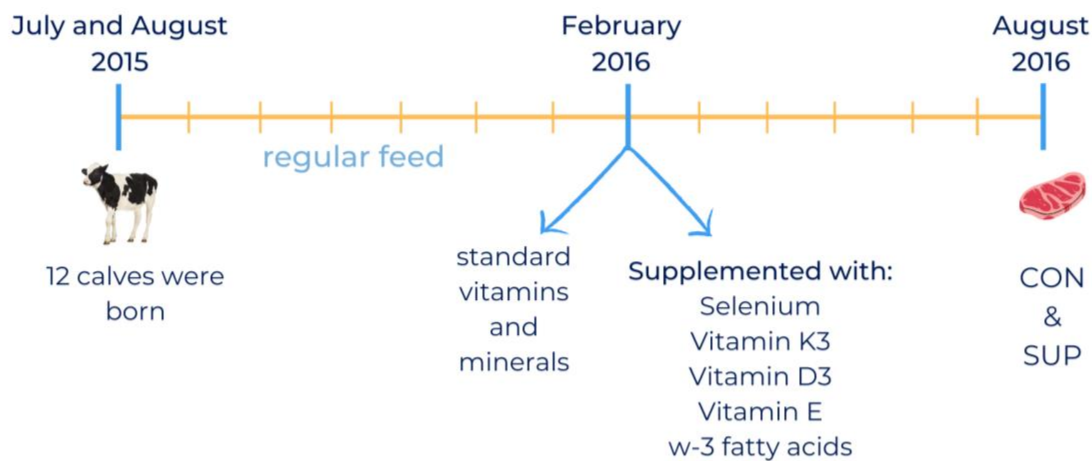


Figure 13. Beef meat production. The study started in 2015 when the bulls were born. After approximately 6 months the bulls were separated into two groups: control feed versus supplemented feed. After another 6 months the bulls were slaughter. CON, controlled meat group; SUP, supplemented meat group.

The experiment lasted for 170 days and the animals, aged 369 (± 14) days old, were transported and slaughtered in August 2016 according to approved procedures from the Norwegian Food Safety Authority. The meat from the forequarters were used for the human intervention afterwards. Table 1 shows information regarding nutrient composition in 100g meat.

This animal experiment was accomplished in accordance with Norwegian legislation controlling experiments with animals. The ARRIVE guidelines were followed throughout the trial, in accordance with the United Kingdom Animals Act 1986 and the EU Directive 2019/63/EU.

Phase 2: the human intervention trial

The human trial was a 32 days double-blind randomized cross-over human intervention study with two intervention periods, each for 6 days, where the participants were instructed to eat 300g of grinded beef meat as raw weight – 240g cooked beef - per day from the forequarters, combined with their habitual diet, except fish (Figure 14). The meat was from bulls fed with regular control feed (control group – CON), and meat supplemented with vitamin D E, K and Selenium (intervention group – SUP). This human intervention study

was conducted at the Norwegian University of Life Sciences, at a latitude of 60°N during March month when vitamin D synthesis is minimal (151).

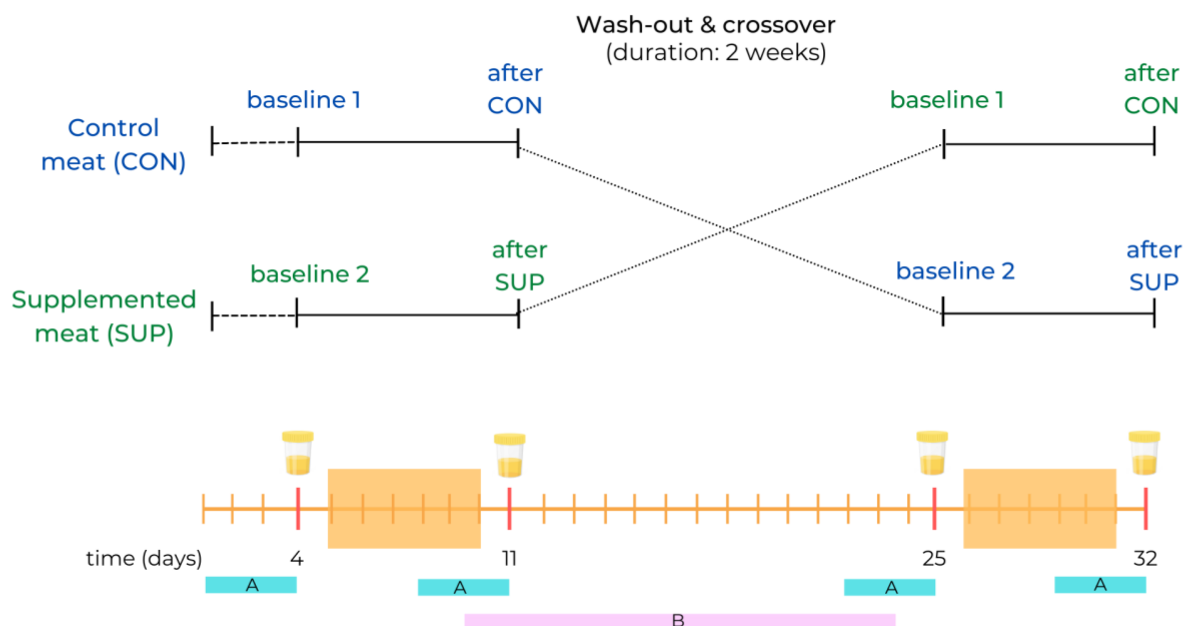


Figure 14. Timeline for the experimental design of the cross-over double-blind study. The blue bars (A) correspond to weighed dietary registration of food intake. The pink bar (B) corresponds to the washout and crossover, with habitual diet.

A) *Dietary instructions*

The dietary instructions for the two 6-day intervention periods were to distribute the meat intake throughout the day, preferably in 3 meals with balanced fat, protein, and carbohydrate contents.

The participants received recipes about how to prepare healthy meat dishes, adding preferably vegetables, but they were also free to use the meat according to their personal tastes. The volunteers were instructed to search for recipes at matprat.no (https://www.matprat.no/sok/#1/all/kj%C3%B8ttdeig/ , accessed on 28 March 2022).

Throughout the study, the participants completed four weighted registrations of their food intake, each of them for 3 days (Figure 14). The registrations occurred before and during test periods and were recorded on the official diet tool "Kostholdsplanleggeren", and the nutrient intakes were calculated using data from the Norwegian Food Composition Database (Appendix 2). Most of the participants were students studying nutrition and food science and were thereby familiar with three-day weighed dietary registration.

B) *Study procedures*

Fasting urine samples, as well as blood pressure, pulse and body weight were collected in

the morning (between 07:00 and 10:00) at four time-points. The height was measured only on the first visit using a portable stadiometer (Seca 217, Seca, Hamburg, Germany) to the nearest 0.1 cm. Body weight was measured without heavy clothes and shoes to the nearest 0.1 kg using a digital scale (Soehnle, Nassau, Germany). Body Mass Index (BMI) was the result of weight divided by the square of height.

C) Urine sample preparation for LC-MS analyses

After the urine collection, the samples were stored bellow 5°C. Subsequently 1mL of each sample was transferred into cryo-tube vials. The remaining urine from each subject, for the four timepoints, was pooled and 1 mL of the pool also transferred into a cryo-tube. Until the analysis day, the cryo-tubes were stored at -80°C.

The samples were randomized into two 96-well microtiter plates (Waters, Hedehusene, Denmark) in a way that samples from the same participant were kept on the same plate. On the analysis day, the samples were centrifuged at 3000xg for approximately 2 minutes. Thereafter 150uL of each sample was transferred to a well on the 96-well plates and diluted with 150uL solvent prepared from aqueous 5% 70:30 (v/v) acetonitril (ACN) : methanol (Optimagrade LC–MS, Fisher Scientific, US) containing an internal standard solution with 7 different Internal Standards (152). In order to make a quality control of the analytical platform it was used an external metabolite standard mixture composed by 44 compounds. In addition, to control the batch drift in the data preprocessing and analysis, a pooled sample was prepared with equal amounts of all the collected urine samples and then added to both plates.

The plates were thereafter sealed and kept at -80°C until the analysis. The day before the analysis the plates were transferred to a fridge at 4°C to be thawed and then centrifuged again (3200xg, 15 minutes) forthwith prior to analysis.

D) Data acquisition - Sample profiling by LC(ESI)-QTOF-MS

The data acquisition and all the following steps were carried out at the Department of Nutrition, Exercise and Sports (NEXS), University of Copenhagen.

An UPLC-ESI-QTOF-MS high-performance liquid chromatographic system (Acquity UPLC, Waters, Manchester, UK) coupled through an electrospray interface to a Premier quadrupole time-of-flight mass-spectrometer (QTOF Premier, Waters) was used to analyze the urine samples. The chromatographic separation was performed with an HSS T3 column (Waters) for reversed phase chromatography.

A volume of 5µL of each sample was injected into the gradient mobile phase A (0.1 % and 0.01% formic acid in Milli–Q water in QTOF Premier and VION, respectively), mobile phase B

(methanol), mobile phase C [10% 1 M ammonium acetate in methanol (v/v)] and mobile phase D (isopropanol). Cone gas flow was set to 50 and desolvation gas to 1000 L hr^{-1}. Data were acquired in continuum mode using leucine–enkephalin (100 pg mL-1 in 0.1% HCOOH in Milli–Q:ACN 50:50, v/v) as the lock–spray agent to correct the mass accuracy at every 1 minute. ESI was performed for both positive and negative acquisition modes in separate runs with capillary probe voltage set at 6eV. Centroid data was generated in real time for masses ranging between 50 and 1500 Da.

E) Data pre-processing

Data pre-processing started with the raw spectra conversion to netCDF files by DataBridge Software (Wates, Manchester, UK) and subsequent importation to MZmine 5.53 (124). The parameters were optimized separately for negative and positive ionization modes (Appendix 3).

For negative mode the parameters were: noise level in the level 15 and selected "Centroid algorithm" which allows to detect all data points above the defined noise level as m/z peaks; the chromatogram building was achieved using a minimum time span of 0.01, m/z tolerance of 0.055 and minimum height of 4.0E1; for chromatogram deconvolution it was used a threshold of 95%, minimum retention time range of 0.01 minutes, minimum relative height of 10%, minimum absolute height of 4.0E1, minimum ratio of top/peak edge of 1.3 and peak duration range of 0.01-0.2 minutes; chromatograms were deisotoped with the isotopic peak grouper algorithm with a m/z tolerance of 0.06 (or 30ppm), a RT tolerance of 0.01, selecting the monotonic shape and a maximum charge of 1; peak alignment was performed with a weight of 10 for both retention time tolerance and m/z tolerance; the duplicate peak filter was set for a m/z tolerance of 0.5 (or 600ppm) and a RT tolerance of 0.15; the peak list was gap-filled for an intensity tolerance of 50% and absolute RT tolerance of 0.15 minutes. For positive mode the parameters were identical unless for chromatogram deconvolution with a 97% for the chromatographic threshold, minimum absolute height of 6.0E1 and minimum ratio of peak/top edge of 1.5.

For quality control of data pre-processing, known meat markers such as carnosine, anserine, and creatine were searched; when these markers were missing from the final data table, the parameters were optimized again.

F) Data pre-treatment and normalization

Before performing the data analysis, data was cleaned by excluding noisy and irrelevant features and data, that is 1) features present in blanks, 2) features eluting either very early

(<0.3) or very late (>0.9), 3) isotope peaks, duplicate peaks, as well as 4) features which were not present in 80% of the samples in at least one group.
The data was then normalized to correct for intra-individual variation and difference in urine concentration by normalizing all peak areas to the same mean, sample-wise (153). Inter-individual and batch differences were then corrected by dividing each detected feature with its overall mean of its recordings across all batches.
Data pre-treatment and normalization was performed using MATLAB (The MathWorks, Inc., Massachusetts, USA).

G) Data analysis

Initially, PCA was applied to explore the data in an unsupervised way, for each timepoint, for each treatment as well as for all data together. Thereafter, a three-step approach was applied to handle data analysis: 1) multivariate analysis on baseline corrected data, 2) univariate analysis on selected features, and 3) visual inspection of significant features.
The baseline correction was performed by subtracting the intensity of each feature in the baseline sample from the after sample, subject wise, per treatment.
The next step was to apply a supervised method on baseline corrected data by developing PLS-DA models for each ionization mode separately. The aim was to identify the most discriminant features between the intervention versus control groups. The cross-validation was performed by taking one subject out at a time, resulting in 34 models built. The discriminant features were selected based on VIP scores and selectivity ratio with 1 as a cut-off value for VIP score. The features present in at least 70% of the 34 models per each ionization mode were kept.

Once the features were selected by PLS-DA, the next step was to apply parametric paired t-test, a univariate analysis, to determine if there were differences between treatments for each selected feature. Only significant markers with a p-value below the nominal value of 0.05 were considered significant and retained. The data analyses were carried out in R with in-house developed scripts.

The further selection of markers was based on the following criteria: 1) both baselines had to be comparable, 2) the presence of a statistical difference between CON and SUP and 3) the effect of the treatment had to be either non-affected for one treatment but affected for the other, or if both treatments were affected, they had to be affected in the same direction (both up- or downregulated).

H) Identification

The intensity of the most discriminant features selected through the comparison of treatments (±0.1 min RT) – positive and negative together - were correlated across all samples by Pearson's correlations and, if strongly correlated ($r>0.7$), they were grouped together as potential fragments or adducts of the same compound. Subsequently, a visual inspection of the chromatograms was performed in *MassLynx* (Waters, Manchester, UK) for each correlated feature and were only grouped the features with the same RT and chromatographic peak shape, suggesting they are fragments of the same compound. The visual inspection was conducted on the samples with the highest signal intensity for each specific feature (Figure 15).

The next step aimed the identification of the parent ions of each feature by inspecting the raw data for known common fragments and/or adducts (e.g., Na, NH4, COOH, Cl, glucuronide neutral loss, dimer formations).
Once the parent ions were spotted through a meticulous analysis of the potential adducts, as well as comparison of peak shapes, intensities and spectra visualization, LC-MS/MS fragmentation experiments were performed on the selected parent ions. The MSMS fragmentation experiments were performed in product ion scan with collision-induced dissociation energies of 14, 28 and 42 eV, using the same other parameters as for the MS full scan experiment.

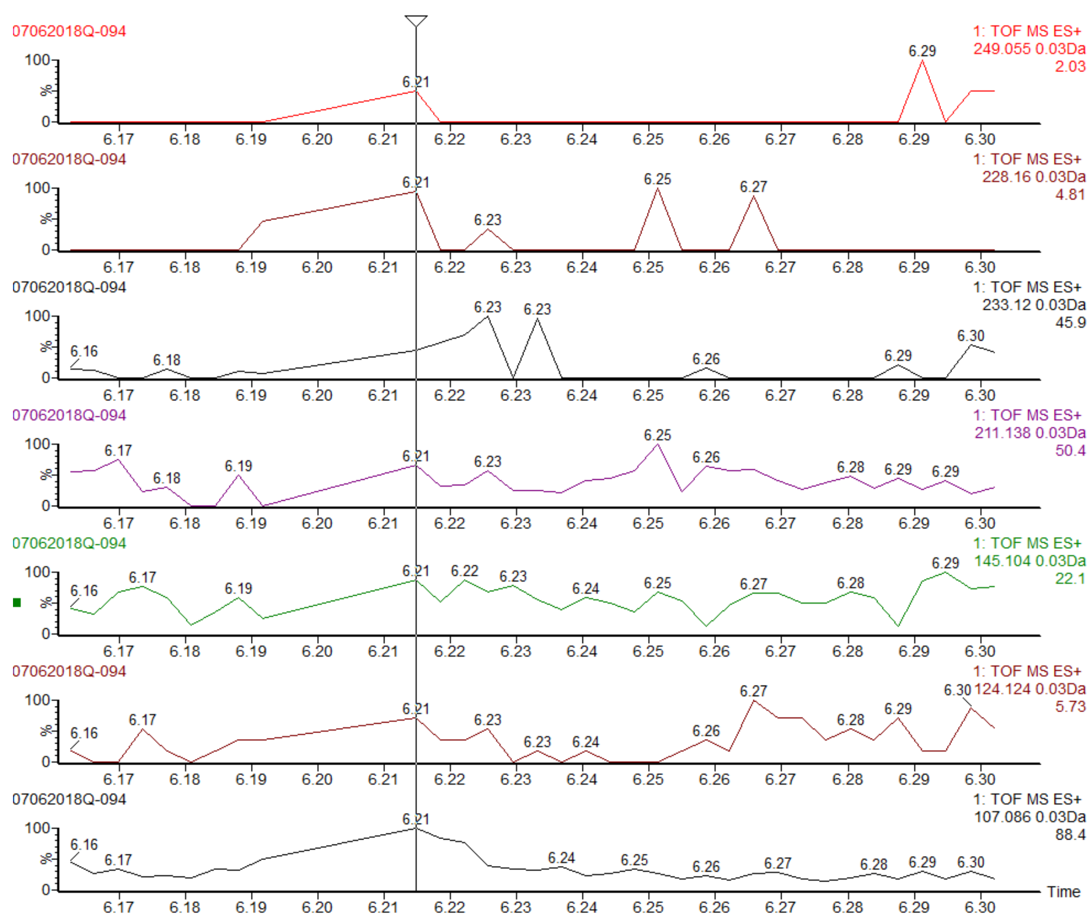


Figure 15. Visual inspection in MassLynx of potential correlated features, fragments and/or adducts. Comparison between the peak in RT 6.21 for the selected marker 107.086 with other possible correlated features.

Finally, structure elucidation was performed on different databases and software, including the Human Metabolome Database (HMDB), CFM-ID, Metlin, Sirius, KEGG, Pubchem and Phytohub. An in-house database search was also conducted to potentially match the potential biomarkers with already identified markers in previous studies.

The identified biomarkers were thereafter classified in 4 levels according to the CAWG of the Metabolomics Standards (148).

Syntheses of standards

For the confirmation of the identified compounds, the authentic standards of N-acetylneuraminic acid: (CAS 131-48-6) and Xylonic acid (CAS 526-91-0) were purchased from Sigma Aldrich (Schnelldorf, Germany), 1-methylhistidine, dehydrotestosterone, CAS 846-48-0, androstan-4,6-diene-17-Bol-3-one CAS 2484-30-2, citric acid, 1-methylhistidine and pyroglutamyltyrosine were retrieved from the chemical database at the Department of Nutrition, Exercise and Sports (University of Copenhagen).

RESULTS

Beef production outcomes

The homogenate beef meat from the intervention group contained more selenium (+26%), vitamin MK4 (+123%), vitamin D (+197%) and vitamin E (+318%) compared to the control group meat (Table 1). In relation to n-3 fatty acids, the composition in the homogenate beef meat did not differ, hence further analysis did not take it into account. (154)

Table 1. *Nutrient composition in 100g meat homogenate from the forequarter (one homogenate per group). Table from Haug et al., 2018* (151).

Nutrient			Control group	Supplemented group
Selenium	ug		10	12,6
Vitamin K	ug	K1	2,1	2
	ug	MK4	9,1	20,3
Vitamin D	ug	D3	<0,012	0,04
	ug	25-OH-D3	0,1	0,293
Vitamin E	ug	α-tocopherol	156,5	654
Crude fat	g		13,6	14,9
Fatty acids	g			
		C16:0	3,4	3,5
		C16:1 n-7	0,37	0,42
		C18:0	2,6	2,8
		C18:1 n-9	5	5,6
		C18:2 n-6	0,27	0,25
		C18:3 n-3	0,06	0,07
		C20:4 n-6	0,03	0,03
		C20:5 n-3	0,006	0,006
		C22:5 n-3	0,02	0,02
		C20:4 n-6/C20:5 n-3	5,2	4,5
		n6/n-3	3,6	2,9
		C20:5 n-3 + C22 n-3	0,023	0,025

Calcidiol in the meat from forequarters contained 0,040 ug in 100g of meat while the control meat contained <0,012 ug. The concentration of a-tocopherol in the homogenate meat was 157 and 654 ug/100g for control versus supplemented meat, respectively. The content of Vitamin K in form of K1 and MK4 n the homogenate meat was 22,3 and 11,2 ug/100g for supplemented and control meat, correspondently. The concentration of Selenium in the forequarters was 26% higher in the supplemented meats compared to the control meats (Table 1).

Human trial outcomes

Subjects

One participant did not provide a sample, therefore the samples from a total of 34 young healthy women were included in the analyses, making a total of 136 samples, four per each subject. The volunteers average age was 21,4 ± 2,0 years, varying from 19 and 29 years and BMI of 22,9 ± 2,7 kg/m^2. All the participants were students at the Norwegian University of Life Sciences (NMBU), and the majority studied *Nutrition and Food Science*, with previous knowledge on prospective food registration. The calculated nutrient intake after the 3-day food registrations, mean values, and standard deviations (SD) are presented in Appendix 2.

Data pre-processing, pre-treatment, and statistical analysis

The preprocessing of LC-MS acquired data provided a total of 5554 features in positive mode (+) and 7333 features in negative mode (-). Figure 16 illustrates MzMine visualization of a pool sample.

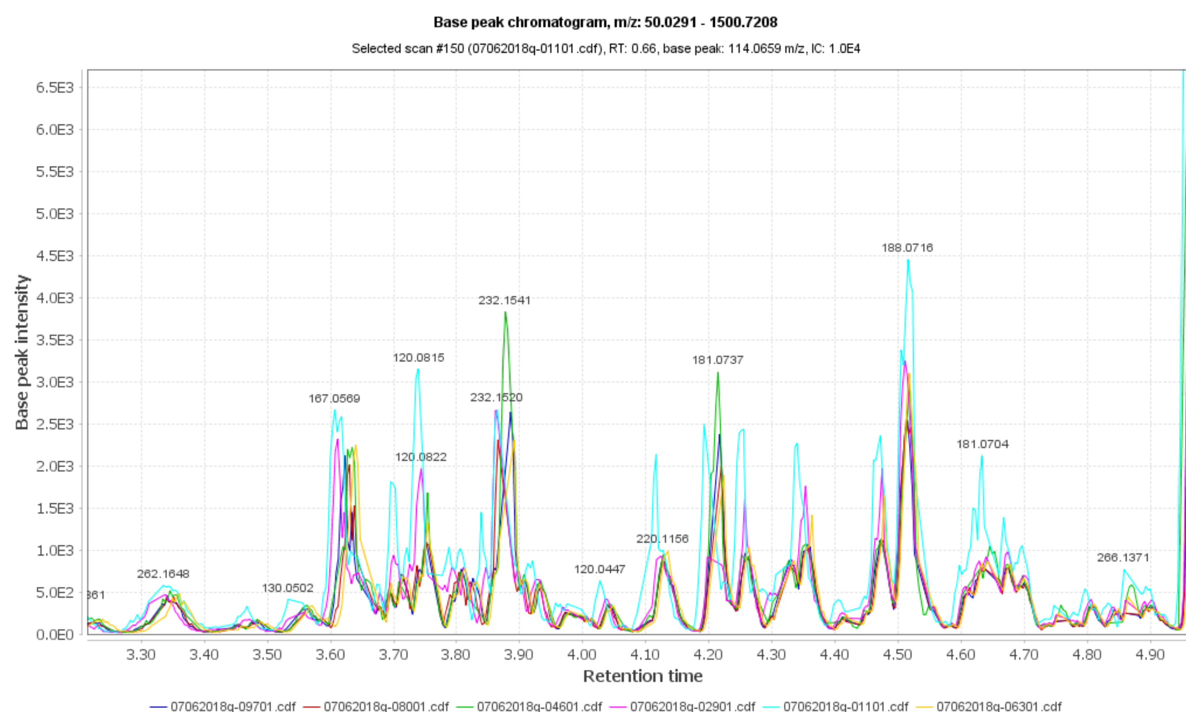


Figure 16. MzMine visualization of one pool sample (0706-011) in positive mode, in plate 1. Example of a consistent shift of 0.02-0.05 RT which will be corrected during pre-processing.

Data pre-treatment followed by multivariate analysis resulted in a total of 43 (+) and 34 (-) features selected by PLS-DA as the main features discriminating the two groups, on baseline corrected data (Figure 17).

After performing the PCA model, its visualization was achieved through scatter plots of the scores, which reflects the distribution and similarity of the samples, and scatter plots of the

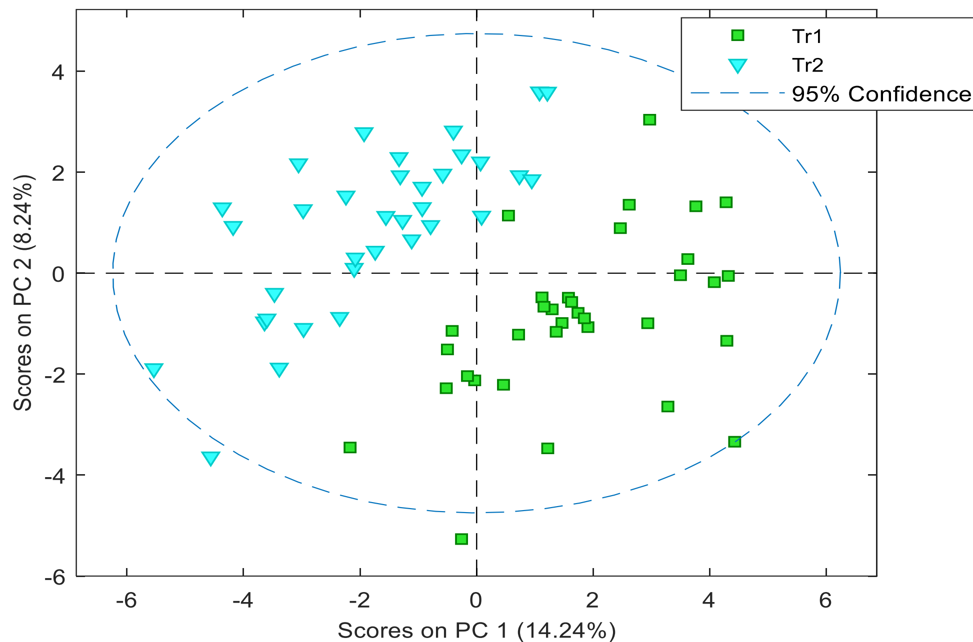


Figure 17. PCA scores plot on 43 discriminant features (68x43) for positive mode. The class was set for treatment. Tr1 supplemented meat group; Tr2 control meat group.

loadings, with variables distribution where is possible to identify which variables contribute most for each PC. In order to acknowledge which variables led to the sample's separation, the two plots were analyzed simultaneously. The scores are the coordinates of the samples while the loadings quantify the contribution of the measured variables to each component (114).

In Figure 17 we can observe the PCA scores plot on the 43 discriminant features, for positive mode. For a better interpretation, samples were colored according to the treatment (SUP versus CON groups) and plate number. Variables with similar information are grouped together, that is, they are correlated. When variables are negatively correlated, they are placed on opposite sides of the plot origin, in diagonally opposed quadrants.

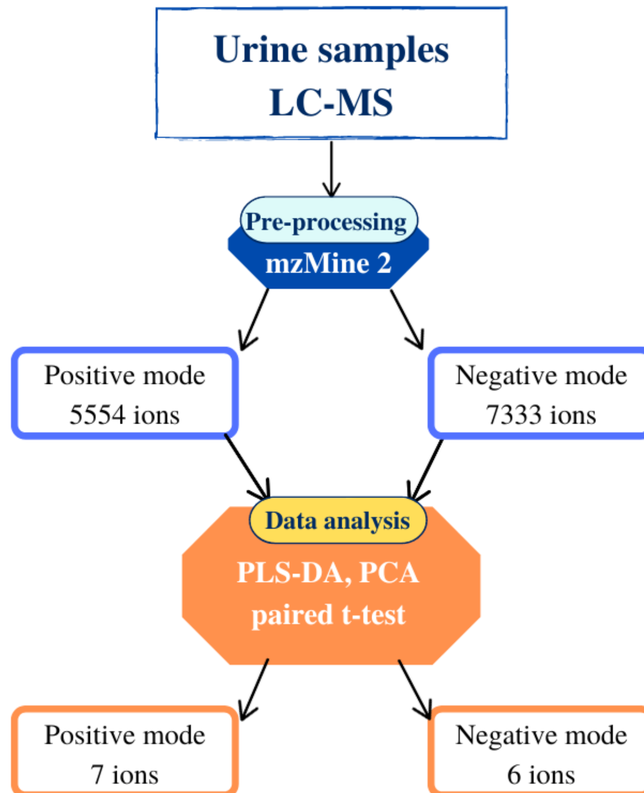


Figure 18. Flowchart from initial selected features by pre-processing to final select features after data analysis.

After univariate analysis, and visual inspection of the boxplots based on predefined criteria, a total of 7 features for positive mode and 6 features for negative mode were selected (Figure 18). An example of boxplots visual inspection is shown in Figure 19, where the 3 conditions are fulfilled: 1) comparable baselines, 2) statistical difference between SUP and CON and 3) the effect of the treatment goes on the same direction (downregulated according to the baselines).

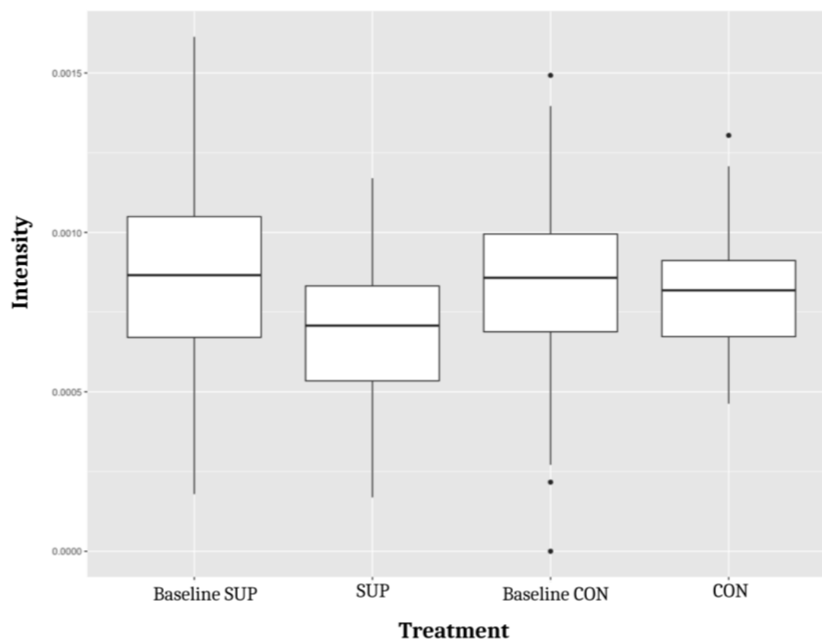


Figure 19. Boxplots for feature m/z 293.11, in positive mode. Baseline SUP, baseline before supplemented mead; SUP after supplemented meat; Baseline CON, baseline before control meat; CON, after control meat.

The heatmap was built with rearrangement of the highly correlated ions. Figure 20 shows the heatmap for both positive and negative markers. The features which are more correlated have a stronger red whilst the blue squares represent absence of correlation. As illustrated in the figure, we can identify 3 groups with strong correlation: 1) unknown [(m/z = 107,08) RT 6,21] and unknown [(m/z=121,10) RT 6,21] are strongly correlated as well as 2) 1-methylhistidine and 3-hydroxybutyrylcarnitine; and 3) citric acid, N-Acetylneuraminic acid and L-Xylonic acid.

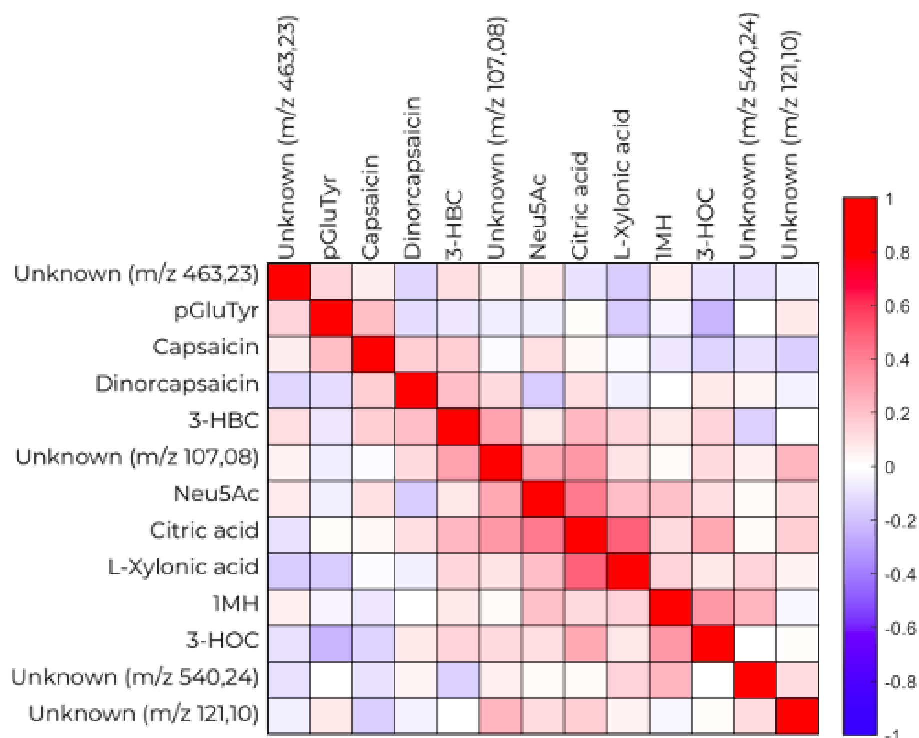


Figure 20. Heatmap, variables regrouped by similarity, for positive and negative mode, on the 13 selected markers on 136 samples, baseline before and after. PGluTyr, pyroglutamyltyrosine; 3-HBC, 3-hydroxybutyrylcarnitine, Neu5Ac, N-acetylneuraminic acid; 1MH, 1-methylhistidine; 3-HOC, 3-hydroxy-5-octenoylcarnitine.

Identification

<u>Upregulated</u>

A) Upregulated for SUP

Six out of a total of 13 markers, (+) and (-), were upregulated for the SUP group. Among the negative mode markers, one was expressed by a loss of m/z 79.95 that corresponds to a sulphate molecule. The marker was annotated as Dinorcapsaicin sulphate (m/z 356,10 [M-H]-) by crossing its fragments data with HMDB and Metlin information and Sirius structure elucidation (level II ID). The marker m/z 191,07 [M-H]-, also upregulated for SUP, was identified as citric acid, confirmed with standard (level I ID). The last negative marker upregulated for SUP was 540,24, and its identity was not confirmed, however, it has two glucuronide molecules attached suggesting it is a double glucuronide of m/z 188,136 [M-H]- parent ion. This metabolite therefore is identified at level III according to its physiochemical properties and spectral similarity with spectral libraries (level III ID) (148).

Three markers measured in positive mode showed upregulation for SUP. The marker m/z 170,09 [M+H]+ was identified as 1-methylhistidine (1MH) after confirmation with a standard (level I ID). The other two were also identified as meat biomarkers based on previous literature (level II ID) : 3-hydroxybutyrylcarnitine (3-HBC) (m/z 248,15 [M+H]+) and 3-hydroxy-5-octenoylcarnitine (3-HOC)(m/z 302,19 [M+H]+) (155,156).

Downregulated

B) Downregulated for SUP

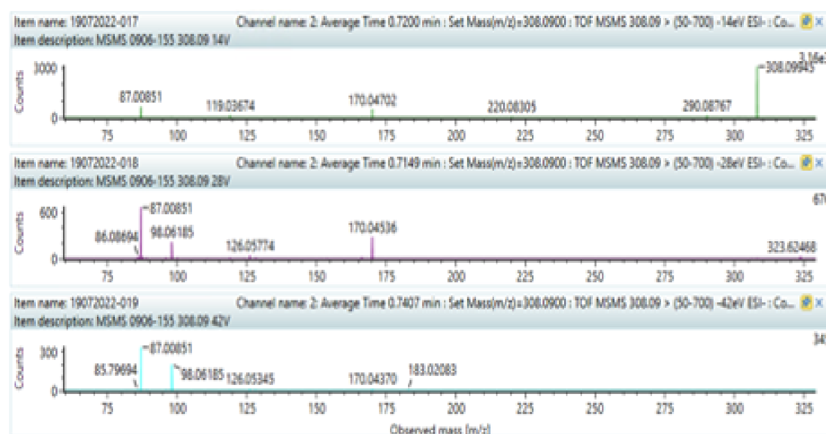
One negative marker was downregulated for SUP and was putatively annotated as Capsaicinol sulphate (m/z 400,14 [M+SO3]-) (level II).

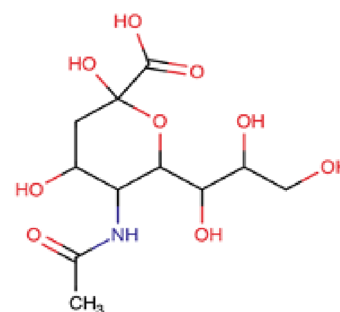
The other 2 markers downregulated for SUP were on positive mode: 293,11 [M+H]+, identified as Pyroglutamyltyrosine (PGluTyr), by crossing its fragments with spectral libraries and manual inspection of its fragments (level II); and 463,23 [M+H]+, identified as a glucuronide isomer of Androstenedione (AndroTGlu) or Dehydrotestosterone (DehydroTGlu) (level IV ID), characterized by a known loss of m/z 176,03 which resembles to a glucuronide molecule. This metabolite shares the same molecular formula as AndroTGlu and DehydroTGlu ($C_{25}H_{34}O_8$) and similar fragmentation pattern until 269.188. Afterwards, it shares 251,17, 245,11, 243,17, 241,19 and some others at lower masses, but not the main 151,11 and 133,09 of AndroGlu, which are also distinct to the main frags 121,063 and 135,11 of DehydroTGlu.

C) Downregulated for CON

Four markers were downregulated for CON. N-acetylneuraminic acid (Neu5Ac) detected as m/z 308,09 [M-H]- and confirmed with standard (level I) (Figure 21). The second negative marker was detected as L-Xylonic acid (m/z 165,03 [M-H]-).

The other 2 markers downregulated for CON, were identified as glucuronide conjugates of m/z 107,08 [M+H]+, MH $C_{15}H_{26}NO_9$) and of m/z 121,10 [M+H]+, MF $C_{16}H_{28}NO_9$), with the m/z of 364.19 (+) and 378.17 (+) respectively. Both markers were downregulated for the CON group and had common main fragments (m/z 107,086, m/z 113,020, m/z 121,100, m/z 141,018 and m/z 159,026). They are highly correlated and their fragmentation pattern, of fragments with small m/z, are similar (m/z 81,060, m/z 85,027, m/z 91,053, m/z 93,068, m/z 95,083 and m/z 105,069), indicating similar structures. However, at this stage, their identify remains unknown (level IV ID).





(a) (b)

Figure 21 - Example of structure elucidation for the level I identified markers. ***(a)*** *MSMS of N-acetylneuraminic acid [m/z 308.09 (-)] for 14ev, 28eV and 42eV;* ***(b)*** *structure elucidation explaining the MSMS fragments of N-acetylneuraminic acid. The first loss is represented by a water molecule and then three main fragments are formed: m/z 170.04, 119.03 and 87.00 (-).*

Table 2*. Overview of urinary metabolites affected by consumption of biofortified beef.*

Metabolite (level of identification)	**Molecular formula**	**Theoretical monoisotopic mass**	**Measured mass**	**Suggested ion**	**Fragments (ESI mode)**	**RT (min) QTOF**	**Differences between SUP and CON (p-value)**
Upregulated for SUP, compared with CON							
1-methylhistidine[I]	C7H11N3O2	169,08	170,09	[M+H]+ Positive mode	124,08765 (-HCOOH)[a] 109,06333 (previous, -CH3) 83,06045 (previous, -CH3) 56,05046 (previous, -HCN)	0,62	0,0153
Citric acid[I]	C6H8O7	192,02	191,017	[M-H]- Negative mode	435,95918[a] 191,02 111,01 87,01	1,14	0,017
3-hydroxybutyryl carnitine[II]	C11H21NO5	247,14	248,149[a]	[M+H]+ Positive mode	189,07368 (-C3H9N) 144,10123 (-C4H8O3)) 103,03656 (-C7H15NO2) 85,027845 (previous, -H2O)	1,37	0,037
	C15H27NO5	301,188	302,19	[M+H]+	225,11016 141,08932	4,29	0,007

3-hydroxy-5-octenoylcarnitine[II]				Positive mode	123,07881 (previous, -H2O) 99,0797 (-C9O4H17N) 57,03197 (previous, -C3H6)		
Dinorcapsaicin sulphate[II]	C16H23NO6S	357,12	356,108[a]	[M+SO3]- Negative mode	276,159 (-SO3) 191,0709 135,0444 122,0369 113,022 79,95629 [SO3]-	6,04	0,015
Unknown glucuronide[IV]			540,254[a]	[M+2Glu-H]- Negative mode	364,2189 (M+Glu]- 188,136 [M-H]- 87,00851	6,008	0,008
Downregulated for SUP, compared with CON							
Pyroglutamyltyrosine[II]	C14H16N2O5	292,105	293,119	[M+H]+ Positive mode	247,10656(-HCOOH) 182,08072 (-C5H5NO2) 136,07566 (previous, -HCOOH) 119,0499 (previous, -NH3)	4,7	0,0009
Capsaicinol sulphate[II]	C18H27NO7S	401,15	400,142[a]	[M+SO3]- Negative mode	320,18867 (-SO3) 288,12225 236,13093 219,10316 (previous, -NH3) 193,0394 175,02451 (previous, -H2O) 165,09395 122,03836 113,02525 85,02951 (previous, -CO) 79,95856 [SO3]-	5,99	0,002
Unknown glucuronide[IV]	C25H34O8	462,22	463,237[a]	[M+Glu]+ Positive mode	287,20019 [M-Glu] + 269,18684 (-H2O) 229,1576 (previous, -C3H4) 211,14408 (previous, -H2O) 205,1207 93,06986 79,05380 (previous, -CH2)	6,34	0,002

Downregulated for CON, compared with SUP							
Unknown glucuronide[IV]			107,086[a]	[M+Glu]+ Positive mode	346,187 (-H2O) 329,15804 (previous, -NH4) 153,12643 (previous, -176,031) 135,11661 (previous, -H2O) 121,1001 (previous, -CH2) 107,08535 (previous, -CH2)[a] 97,06458 73,02863	6,21	0,003
Unknown glucuronide[IV]			121,101[a]	[M+Glu]+ Positive mode	378,1738 202,14265 (-C6H8O6) 185,11517 (previous, -NH3) [M-Glu] + 167,10548 (previous, -H2O) 139,10955 (previous, -CO) 121,10006 (previous, -H2O)[a] 107,08667 (-previous, -CH2) 95,08378 (previous, -C) 77,03 [C6H5]	5,89	0,023
L-Xylonic acid [I]	C5H10O6	166,047	165,039[a]	[M-H]- Negative mode	147,02965 (-H2O) 129,01893 (-2H2O) 119,035 117,018 111,009 101,024 99,008 89,023 87,008 85,029 83,014 75,008 (previous, -C) 72,993 71,013 59,01329 (previous, -O) 57,034	0,746	0,002
N-acetylneuraminic acid [I]	C11H19NO9	309,105	308,09	[M-H]- Negative mode	406,06 [M+HSO4]- 388,09 290,08551 (previous, -H2O) 220,08116	0,739	0.034

					194,92741		
					170,04536		
					119,03396		
					96,95934 (194.927 - H2SO4)		
					87,00851[a]		
					59,01232 (previous, - CO)		

CON – control meat group. RT – time of retention. SUP - Supplemented meat group. I-IV – the four levels of identification according to Sumner *et al.* (148)

[a] Fragments correlated through data analysis

DISCUSSION

Biofortification is a promising approach to overcome the high prevalence of micronutrients deficit in Europe. Meat is a great medium for it, owing to its ubiquitous consumption pattern. In the present study we aimed to explore whether cattle biofortification could lead to a higher access high-quality and nutrient-dense meat. Our results pointed towards markers of overall meat intake (1-methylhistidine, 3-hydroxybutyrilcarnitine and 3-hydroxy-5-octenoylcarnitine), markers of animal feed (capsaicinol), markers related with the participant's diet (dinorcapsaicin, L-xylonic acid and citric acid), and potential meat-related inflammation markers (pyroglutamyltyrosine and N-acetylneuraminic acid). No markers related to the micronutrients used in biofortification process were observed.

Markers of meat intake

1-methylhistidine

1MH, also known as tau-methylhistidine or telemethylhistidine is a well-described marker of meat intake, originating from both endogenous (muscle breakdown) and exogenous sources (diet) (157,158). It is formed as a result of proteolysis of 1MH-containing proteins and peptides, as well as from the methylation of L-histidine in the N1 position of its imidazole ring (159). After its production, 1MH flows through the bloodstream and travels to the kidneys, followed by its excretion in the urine. Anserine is present in many kinds of vertebrate muscle and differs a lot between species and cuts (155). Anserine was previously identified as source of urinary 1MH, and evidence shows that more than 85% of its dietary intake in humans is excreted as 1MH (160,161). 1MH has been already identified as a marker of meat intake within the last few days after ingestion (155,162,163). Cross *et al.* reported the half-live of 1-MH to be approximately 12 hours. Thus, it is considered a short term biomarker of red meat intake(164), whereas the elimination half-life of anserine is on the order of 4 hours (161).

3-hydroxybutyrilcarnitine

3-HBC is an acylcarnitine which has as a general role to transport acyl-groups, such as organic acids and fatty acids, from the cytoplasm into the mitochondria. The transport of acyl-groups into the mitochondria is required for beta-oxidation to occur and its function is to break down the acyl-groups to produce energy (165). 3-HBC is classified as a short chain acylcarnitine therefore it belongs to the most abundant group of acylcarnitines in the body,

which comprises more than half of the acylcarnitines quantified in biofluids and tissues (165). 3-HBC was identified as a meat biomarker detected 8 hours after meat intake (156).

3-hydroxy-5-octenoylcarnitine

3-HOC is classified as a medium-chain acylcarnitine, being less abundant in the body than short-chain acylcarnitines (166). It can derive either through esterification with L-carnitine or over the peroxisomal metabolism of longer chain acylcarnitines (167). Red meats have high concentration of carnitine, especially beef and lamb. Despite some variations on individual physiological conditions (aging, some diseases, and pregnancy), carnitine shows a dose-response with intake of several meat types, hence, a higher urinary level of carnitine following the increased meat intake is in accordance with data from food science (155,162).

These 3 markers (1MH, 3-HBC, and 3-HOC) were upregulated for both intervention groups compared with the baselines, in accordance with the calculated nutrient intake, where the meat consumption was significantly higher compared with the habitual diet (Appendix 2) (168). The identified markers of overall meat intake were higher for SUP maybe due to slight differences between the meat's composition.

Markers of animal's feed

Capsaicinol sulphate

Capsaicin, the pungent ingredient of chili peppers, is a plant secondary metabolite and is likely produced by the plant as natural defense against herbivores and fungi. The genus *Capsicum*, known as chili pepper fruit, synthesizes it by addiction of a branched-chain fatty acid to vanillyamine (169). The natural sources of capsaicin besides red chili are paprika, gendot and curly chili (170).
Capsaicin has been studied extensively on the past decades, with promising results as pharmacological agent related with analgesic properties. Some authors hypothesize a beneficial role in obesity, cardiovascular diseases, metabolic syndrome, several cancers, neurogenic bladder and dermatological conditions (169) (171). Rosca *et al* also suggested a possible antimicrobial effect of capsaicin which could lead to beneficial modulation of gut microbiota (172). Nevertheless, more studies are needed to confirm these effects in humans. Capsaicin has been used as an animal feed additive, showing several biological benefits on meat quality and growth performance when added to animal feed composite (173). The potential reason for finding lower levels of capsaicin in the SUP compared with CON may

indicate a lower uptake of capsaicin in the bulls fed with the fortified diet, and consequently a lower excretion on participant's urine.

Markers of participant's diet

Citric acid

Citric acid (citrate) is an organic acid with three carboxylate groups which can be produced endogenously, working as an intermediate in the tricarboxylic acid (TCA) cycle, also known as Krebs cycle, or introduced with diet, mainly in citrus fruits (most concentrated in lemons and limes). The TCA cycle is the main energy supply to the body and an important part of aerobic respiration, and is the final common pathway for carbohydrate, lipids and protein oxidation (174). Citric acid can be a natural preservative and food stabilizer, and is usually added to foods and soft drinks (175).

Rakuša *et al.* reported that the use of antioxidants, such as citric acid, in vitamin D_3 food supplements, may be beneficial for vitamin D_3 stabilization (176). Hence, citric acid may had been added to the supplements used in the SUP and led to higher citric acid accumulation in the bull's meat. Other explanation might be the menstrual cycle fluctuations, which can also lead to metabolic changes in the energy metabolism, specifically in the citric acid cycle. Draper *et al.* reported an increase of citric acid on the periovulatory phase, possibly indicative of a catabolic state (177). The third hypothesis might be that the SUP ate higher citrus fruits on the day prior to the urine collection and thus, led to an increase of its urinary metabolites. This last explanation is in accordance with the data obtained for L-Xylonic acid, which is also a product of metabolism of citrus fruits, and was demonstrated to be decreased for the CON, compared with the SUP.

Dinorcapsaicin sulphate

Dinorcapsaicin ($C_{16}H_{23}NO_3$) is an organic compound and belongs to the class of methoxyphenols, meaning it contains a methoxy group (-O-CH_3) attached to the benzene ring of its phenol moiety (178). The current literature has limited information regarding dinorcapsaicin, with only a few studies detecting it in higher concentrations in pungent peppers, such as red bell peppers (Capsicum annuum), Italian sweet red peppers, green bell peppers, orange bell peppers, and yellow bell peppers (178–180).

As described in the methods section, the participants were instructed to follow some recipes at matprat.no. One of the first options on the website was to cook the meat was based on a Mexican cuisine, "chili con carne" (chili with meat), which includes chili as main ingredient (also chili beans, green pepper, red pepper, and chili powder/paprika).

Dinorcapsaicin showed an upregulation in SUP maybe due to a higher intake of dinorcapsaicin-rich pungent peppers in the day prior the urine collection. Our group did not have access to the detailed information of the weighted food registrations, thus it was not possible to confirm which meals the participants have had in the day prior the urine collection.

Sulphate conjugate

After the uptake of most of organic compounds in the body, biotransformation reactions occur. On the first step (phase I) the compound suffers oxidation, hydrolysis, or reduction, adding a variety of groups (hydroxyl, amino, carboxyl or thiol groups) to the molecule. This step leads to the formation of primary metabolites. The phase II uses endogenous agents to form secondary metabolites through conjugation reactions (181). If the compound enters in the body already with functional groups, they may undergo direct conjugation. The phase II is responsible for the inactivation of the original molecule and for the increase of its hydrophilicity, aiming to enhance the excretion.

A molecule with hydroxyl groups, particularly phenolic groups as dinorcapsaicin, are readily conjugated with sulfuric acid. In the presence of sulfotransferases (SULT), the sulfuric acid is activated as phosphoadenosine-5´-phosphosulfate (PAPS) and conjugates with the metabolite. Sulfate conjugates are mainly reported in negative-ion mode (182).

L-Xylonic acid

L-Xylonic acid ($C_5H_{10}O_6$), also known as L-Xylonate, is a compound which contains a saccharide unit attached to a carboxylic acid group. It belongs to the class known as sugar acids and derivates and is an extremely weak basic compound (183). Xylonic acid was already identified as one of the major product arising from the L-ascorbic acid metabolism (184).

A higher ingestion of citrus fruits in the SUP compared with CON in the day prior the urine collection could have led to higher excretion of vitamin C related metabolites. This hypothesis is in accordance with the data obtained for citric acid, which is also a product of metabolism of citrus fruits, and was demonstrated to be increased for the SUP, compared with the CON.

Markers of inflammation

N-acetylneuraminic acid

The cell surfaces of all vertebrates are ornamented with a complex collection of sugar chains, which are commonly attached to lipids and proteins. Sialic acids (SA), a family of sugar units with a 9-carbon backbone, are usually found attached to these chains (185). The most predominate SA in most mammals are *N*-acetylneuraminic acid (Neu5Ac) and its hydroxylated form, N-glycolylneuraminic acid (Neu5Gc) (186). Neu5Ac ($C_{11}H_{19}NO_9$) is an acetyl derivate of the amino sugar neuraminic acid, which is the most common form as sialic acid in many glycoproteins, glycolipids, and polysaccharides (187). Neu5Ac can be synthesized in vivo from N-acetylated D-mannosamine or D-glucosamine. Neu5Ac is widely distributed throughout human tissues and fluids, such as serum, cerebrospinal fluid, saliva, urine and human milk. Some evidence has been pointed towards an antioxidant effect of Neu5Ac, as well as a potential role on inflammation reduction (188–190). Free Neu5Ac in the urine can be a marker of sialic acid excretion from the kidney and thus, can eventually predict the metabolic rate of sialic acid intake (191). Moreover, Neu5Ac was detected in high amounts in some animal food products such as beef, pork, lamb, chicken, turkey, eggs, milk and cheese (192).

Neu5Gc in humans is incorporated in tissues from dietary sources (mainly from red meat), and not produced endogenously, due to a deficiency in the gene encoding the hydroxylase responsible for the conversion of CMP-Neu5Ac to CMP-Neu5Gc. Unlike Neu5Ac, Neu5Gc was already described as an infectious agent in beef, which has been associated with chronic inflammation and autoimmune diseases (103,193). Some authors already hypothesized that incorporation of Neu5Gc into tissues of red meat consumers could interact with inflammation-provoking antibodies and result in chronic inflammation, which may promote carcinogenesis and atherogenesis (103,186). Moreover, Alisson-Silva *et al*. proposed that by increasing the ingestion of Neu5Ac (for instance, by adding it to the meat), may prevent the Neu5Gc incorporation in human tissues, since both compete for integration in human cells (103).

According to the results obtained on this study, Neu5Ac was not upregulated for any of the treatments compared with the baselines. On the contrary, the control group showed a downregulation compared to the SUP and baselines. The potential explanations for this effect might be that 1) Neu5Ac was more retained in tissues after the CON, leading to a lower urinary excretion or 2) SA can be excreted in urine at different free forms (192); hence, an increase in the Neu5Gc in the body (from meat intake) could have led to a higher

excretion of SA in the form of Neu5Gc and decreased levels of urinary excretion in the form of Neu5Ac.
Additional studies are needed to confirm this hypothesis and to elucidate this phenomenon.

Pyroglutamyltyrosine

PGluTyr is a dipeptide containing a sequence of two alpha-amino acids joined by a peptide bond. Pyroglutamyls are cyclic dipeptides formed from glutamic acid reaction of the side-chain carboxylic acid with the alpha amine in the same glutamic acid moiety to create a 5-oxoproline ring structure. PGluTyr is obtained by a formal condensation of the carboxy group of pyroglutamic acid with the amino group of tyrosine.
Pyroglutamyl peptide is usually found in protein hydrolysates and fermented foods, hence, it may be formed due to protein hydrolysis in the body but there is little information regarding its functions (194). Some pyroglutamyls were already identified as a result of manufacturing process, since their levels raise in the blood after food intake. Moreover, Kasai *et al.* showed that pyroglutamyls can be formed spontaneously simply by heating (195). To my knowledge there are very limited published studies reporting PGluTyr in human urine, and there is reduced information about it in general.
There is one study from Kiyono *et al.* which demonstrated that PGluTyr exhibited a significant protective effect against colitis in mice via different mechanisms such as decrease of colonic myeloperoxidase activity. This study indicated an important role of PGluTyr in colonic inflammation suppression.
PGluTyr was decreased for both interventions, compared with the baselines, after the consumption of 300g of beef. This metabolite should be further investigated as a possible meat-related marker and modulator of the inflammatory response. According to the data analysis, PGluTyr was downregulated for SUP compared with CON, in accordance with blood analysis performed to the participants, where inflammatory markers (interleukine-6 and interleukine-8) were significantly increased for SUP (168). This represents an important finding that suggests the potential impact of the high consumption of beef on the inflammatory response.

This study hypothesized that a biofortified meat would reveal more urinary metabolites related with the micronutrients supplemented, compared with the CON. Our hypothesis was not validated, disclosing other sources of variation such as diet confounders, potential inflammation markers, and other intra-individual changes.
Biofortification was recently shown to be a good strategy to enhance blood levels of vitamin D and Selenium in healthy women (168). Moreover, Pfrimer *et al.* reported that milk

biofortification with vitamin E and selenium could lead to a positive effect on elderly immune response (196). Although there is a paucity of data about the effects in humans after consuming vitamins-enriched meat, current evidence clearly shows that fortification of animals' feed can lead to a further increase in meat vitamins' concentration (20). Recent findings have successfully demonstrated the ability to enhance vitamin D content in beef through a vitamin D_3 fortification of animal feeds (197). Regarding vitamin K evidence is still lacking, with only some studies related to chicken eggs biofortification, but with no evidence of whether it led to improvements in humans (198).

In the present study, despite the presence of statistical differences between the two meats in terms of micronutrient's concentration, it was not enough to reflect in the urinary fingerprint. As mentioned above on the results section, there were very slight differences between the two meats in terms of micronutrients concentrations. Thus, it would be very unlikely to find discriminative features related with the biofortification. For instance, after a search on the dataset, it was possible to detect δ-CEHC (m/z 265.146 +; RT 6,25), a very well-known excreted urinary metabolite of α-TOC. Nonetheless, its concentration did not differ compared to baselines and/or between treatment intensities, which can be the reason why the data analysis did not select it as a discriminative marker.

Other possible reasons for not finding biofortification related micronutrients were: 1) the lack of a more controlled diet, since the participants were allowed to eat whatever they wanted, thus, chances of finding other sources of micronutrients in their diet were high (source of confounder); 2) different data analysis strategies could have been applied and, therefore, maybe lead to different results, for instance similar to δ-CEHC, detected but not fulfilling the data analysis criteria; 3) the platform used for the analysis (method), 4) more interesting markers can be among the unknown (unidentified) compounds; 5) intra-individual differences may result in different measurable levels in urine for the same amount of micronutrients ingested (for instance different metabolization from gut bacteria, variations on enzyme availability, pKa, transporters and so forth); and 6) blood could have been a better biological fluid to evaluate our hypothesis, since some compounds are only seen in plasma/serum, but not in urine.

Blood samples were also collected on the phase II of the study and blood analysis were performed by the Norwegian partner-group, and published elsewhere (168). On the contrary to our outcomes, it showed that SUP was effective at enhancing blood levels of selenium and vitamin D. For that reason, plasma metabolome may have been a better way to evaluate this study hypothesis and provide a wider coverage of metabolite information. Plasma is more informative since it provides the real levels of the micronutrients in the blood, and not only their excretion, as it happens for urine. Hence, ideally our hypothesis would be better validated if we had crossed the information of both biological fluids. The

present dissertation does not include any information regarding the plasma. However, the analyses were carried out until the identification step. In the near future, together with my collaborators in NEXS, I intend to publish a paper with both urine and plasma outcomes.

To my knowledge, there are no published studies where a more precise and rigorous analysis were applied to explore whether such biofortification will have ultimately effect on the consumer. Hence, this study was pioneer on using untargeted metabolomics in the urinary fingerprint and may provide directions to upcoming studies. Additionally, inflammation markers potentially related with meat intake were described on this study and may lead to interesting findings regarding the association between meat consumption and human health. These results require further research to confirm the suggested hypotheses.

In short, future studies should increase the levels of micronutrients in the feed concentrates, above the amounts used on this study (151), standardized the participant's diets throughout the intervention and, if possible, should cross the outcomes from blood and urinary metabolome for a better biological interpretation. Biofortification is a cost-effective strategy which can enhance the nutritional quality of several foods, for instance red meat, and may contribute to a greater exposure to nutrients in targeted populations at risk. Hence, further research in the form of robust human clinical trials, assessing multiple biological fluids is required to explore biofortification efficacy.

CONCLUSION

Based on our methodology, the ingestion of biofortified beef had no effect on the increase of micronutrient's related metabolites on the urinary fingerprint. Nevertheless, further investigation Is needed to better interpret if a higher increase of vitamins D, E, K, and selenium on animal's feed composite can lead to different outcomes. Moreover, this was the first study demonstrating some phenomena related with potential meat-induced inflammation markers, which need further research for a better biological interpretation. Lastly, blood metabolome analysis may be a more reliable method to assess the effectiveness of beef biofortification since it represents the real levels of nutrient's uptake by the body.

APPENDIX

Appendix 1. Composition of the experimental feed concentrates (per kg feed). **IU**, International Unit; **g**, grams; **mg,** milligram. Table from Haug *et al.*, 2018 (154).

		Control	Supplemented
Calculated from ingredients			
Dry matter	g	870	870
Net energy lactation (NEl_{20})	MJ	6	6
Crude protein	g	149	149
AAT N_{20}	g	107	104
PBV N_{20}	g	2	1
Crude fat	g	46,4	44,7
Starch	g	314	321
NDF	g	175	175
Linoleic/a-lonolic acid		9,3	2
Calcium	g	11,4	11,2
Phosphorous	g	5,4	5,5
Magnesium	g	3,1	3,2
Sodium	g	4,8	4,9
Additions (per kg feed)			
Vit A	1000 IU	4	4
Vit B1	mg	0	0
Vit B2	mg	0	0
Bit B6	mg	0	0
Vit D3	1000 IU	1	4
Vit E as all-rac a-tocopheryl acetate	IU	30	30
Vit E as RRR-a-tocopheryl acetate	IU	0	500
Vit K3	mg	0	10
Selenium as sodium selenite	mg	0,2	0,2
Selenium as Se-yeast	mg	0	0,5
Cu	mg	15	15
Mn	mg	30	30
Zn	mg	70	70
I	mg	3,5	3,5
Co	mg	0,4	0,4

Appendix 2. Calculated nutrient intake of the 34 study participants, after a 3-day food reporting. Mean values and standard deviations (SD) are presented. The habitual diet represents the mean nutrient intakes at baseline, and the washout period, the "300g beef" represents the mean nutrient intakes when consuming SUP and CON. Table from Haug *et al.* (168).

N = 34	Habitual Diet	'300 g Beef'	*p*	SeDEK-Beef	REGULAR Beef	*p*
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
Energy, kcal	1848 ± 336	1971 ± 306	0.117	1894 ± 313	2049 ± 383	0.071
Higher satiety, % of participants	3	77	<0.001	-	-	-
Intake of beef, g	12.7 ± 2.3	312.2 ± 8.4	<0.001	-	-	-
Intake of all meat, g	84.8 ± 17.8	391.0 ± 28.8	<0.001	-	-	-
Intake of vegetables, g	221 ± 145	314 ± 176	0.020	-	-	-
Intake of cereals, g	222 ± 80	186 ± 86	0.080	-	-	-
Fat E%	36.33 ± 6.01	42.73 ± 4.55	<0.001	42.26 ± 5.2	43.53 ± 6.6	0.385
Saturated fat E%	12.53 ± 3.10	17.60 ± 2.69	<0.001	17.65 ± 3.3	17.56 ± 3.7	0.918
Trans unsaturated E%	0.08 ± 0.18	0.45 ± 0.42	<0.001	0.44 ± 0.56	0.46 ± 0.50	0.909
Monounsaturated E%	13.21 ± 3.20	15.71 ± 2.53	<0.001	14.97 ± 3.2	16.44 ± 3.2	0.062
Polyunsaturated fat E%	6.40 ± 1.89	4.56 ± 1.53	0.007	4.24 ± 1.5	4.88 ± 1.7	0.107
Carbohydrate (incl. fiber) E%	45.68 ± 7.11	35.54 ± 5.13	<0.001	36.15 ± 5.8	34.94 ± 6.7	0.429
Added sugar E%	5.65 ± 4.98	3.97 ± 3.34	0.105	4.03 ± 4.4	3.88 ± 3.7	0.881
Protein E%	18.12 ± 3.67	21.37 ± 2.30	<0.001	21.53 ± 3.0	21.21 ± 2.3	0.620
Fat, g	73.7 ± 21.1	91.5 ± 15.8	<0.001	90.0 ± 15.9	95.0 ± 21.1	0.128
Saturated fat, g	25.08 ± 8.41	37.05 ± 6.05	<0.001	36.1 ± 6.5	38.0 ± 9.2	0.333
Trans unsaturated fat, g	0.51 ± 0.33	1.07 ± 0.25	<0.001	1.01 ± 0.3	1.14 ± 0.4	0.095
Monounsaturated fat, g	26.85 ± 8.73	34.62 ± 6.27	<0.001	31.91 ± 6.0	35.92 ± 9.2	0.036
Polyunsaturated fat, g	13.39 ± 5.71	10.07 ± 3.98	0.007	9.07 ± 3.8	11.10 ± 4.8	0.055
n-3, g	2.43 ± 1.99	1.76 ± 1.82	0.151	1.63 ± 1.9	1.94 ± 1.9	0.599
n-6, g	9.51 ± 3.35	7.46 ± 2.78	0.008	6.65 ± 2.4	8.28 ± 3.5	0.032
n-6/*n*-3	5.86 ± 3.02	5.64 ± 1.80	0.722	5.29 ± 1.9	5.99 ± 2.7	0.220
Cholesterol, mg	281 ± 134	262 ± 97	0.514	249 ± 107	276 ± 123	0.341
Carbohydrate (incl. fiber) g	219 ± 47.8	185.3 ± 46.9	0.005	178 ± 51	193 ± 60	0.263
Starch, g	108.5 ± 29.7	95.3 ± 33.4	0.089	91.6 ± 35	99.0 ± 45	0.458
Mono- and disaccharides, g	76.2 ± 35.0	62.0 ± 24.3	0.057	60.8 ± 25	63.3 ± 31	0.714
Sugar, added, g	26.6 ± 25.1	18.1 ± 15.3	0.094	16.63 ± 16	19.49 ± 17	0.498
Fiber, g	26.7 ± 10.8	23.1 ± 9.8	0.149	22.33±10	23.83 ± 11	0.560
Protein, g	81.4 ± 21.0	104.9 ± 14.9	<0.001	102 ± 18	108 ± 16	0.214
Salt, g	6.07 ± 2.11	6.18 ± 2.47	0.843	6.66 ± 3.2	5.69 ± 2.7	0.175
Vitamin A, (RAE)	1227 ± 1327	634 ± 349	0.014	593 ± 436	675 ± 491	0.469
Retinol, µg	648 ± 1007	250 ± 210	0.028	217 ± 205	283 ± 383	0.379
Beta-carotene, µg	4217 ± 3562	3885 ± 3589	0.704	3858 ± 4350	3913 ± 4252	0.957
Vitamin D [a], µg	4.91 ± 3.26	5.20 ± 1.78	0.650	6.39 ± 1.7	4.01 ± 2.9	<0.001
Vitamin E, αTE	13.16 ± 4.75	11.49±3.70	0.112	11.38 ± 3.3	11.61 ± 5.0	0.821
Vitamin K (total) [b] µg	100.71 ± 83.5	157.7 ± 88.0	0.008	155.6 ± 94	159.9 ± 119	0.869
MK4, µg	10.57 ± 7.05	54.15 ± 5.63	<0.001	69.29 ± 2.8	39.00 ± 10.2	<0.001
Thiamin, mg	1.53 ± 0.46	1.26 ± 0.49	0.022	1.29 ± 0.6	1.23 ± 0.5	0.648
Riboflavin, mg	1.50 ± 0.42	1.50 ± 0.34	0.923	1.45 ± 0.4	1.55 ± 0.4	0.288
Niacin, mg	18.28 ± 6.05	22.15 ± 3.91	0.003	21.38 ± 3.8	22.92 ± 4.7	0.141
Vitamin B6, mg	1.80 ± 0.57	1.72 ± 0.49	0.527	1.64 ± 0.5	1.79 ± 0.6	0.243
Folate, µg	309 ± 127	282 ± 107	0.336	261 ± 91	302 ± 144	0.173
Vitamin B12, µg	5.16 ± 2.22	7.10 ± 1.31	<0.001	6.96 ± 1.5	7.24 ± 1.7	0.480
Vitamin C, mg	104.4 ± 67.1	119.5 ± 70.8	0.370	120.5 ± 95	118.6 ± 72	0.929
Calcium, mg	835 ± 328	737 ± 254	0.169	726 ± 357	748 ± 327	0.793
Iron, mg	10.77 ± 3.20	14.08 ± 2.90	<0.001	13.88 ± 3.0	14.28 ± 3.5	0.616
Sodium, mg	2405 ± 871	2454 ± 1016	0.833	2644 ± 1297	2263 ± 1074	0.192
Potassium, mg	3210 ± 1071	3440 ± 979	0.358	3382 ± 913	3499 ± 1242	0.662
Magnesium, mg	337 ± 115	326 ± 103	0.693	313 ± 99	340 ± 123	0.316
Zinc, mg	9.76 ± 2.16	20.55 ± 2.08	<0.001	20.11 ± 2.5	20.98 ± 2.6	0.162
Selenium, µg	49.12 ± 25.69	61.61 ± 28.54	0.023	66.82 ± 37.9	56.41 ± 12.8	0.134
Copper, mg	1.31 ± 0.53	1.30 ± 0.42	0.955	1.24 ± 0.4	1.36 ± 0.5	0.330
Phosphorous, mg	1509 ± 350	1573 ± 307	0.424	1527 ± 334	1620 ± 389	0.296
Iodine, µg	76.38 ± 34.81	55.72 ± 27.45	0.008	51.33 ± 29	60.10 ± 37	0.283

[a] The vitamin D concentration in SeDEK and REGULAR beef is the sum of analyzed values for D3 + (25(OH)D3 × 5). In the NFCD [45], only vitamin D is given. [b] Vitamin K and MK4 are not given in the NFCD (Matvaretabellen.no) and the intake was estimated using values from 'the Danish Food Composition Database [38] and from the US Food Composition databases [46]. Total K is the sum of K1 + MK4.

Appendix 3. Parameters used for every step in MZmine for pre-processing.

Batch step		Parameters
Negative mode	Raw data import	
	Mass detection	Noise level: 15
	Chromatogram builder	Min time span (min): 0.01; Min height: 4.0E1; m/z tolerance: 0.055 mz or 30 ppm
	Chromatogram deconvolution	Chromatographic threshold: 95%; Search minimum in RT range (min): 0.01; Minimum relative height: 10%; Minimum absolute height: 4.0E1; Min ratio of peak/top edge: 1.3; Peak duration range (min): 0.01-0.2
	Isotopic pattern	m/z tolerance: 0.06 or 30 ppm; Retention time tolerance: 0.01; Monotonic shape; maximum charge: 1
	Join aligner	m/z tolerance: 0.06 or 30 ppm; Absolute retention time tolerance: 0.15; Weight for both m/z tolerance and retention time tolerance: 10
	Duplicate peak filter	m/z tolerance: 0.5 or 600 ppm; RT tolerance: 0.15
	Peak list rows filter	Min peaks in a row: 5 Minimum peaks in an isotope pattern: 1; m/z range: 50-1000; RT range: 0-7; peak duration range: 0.01-0.2
	Peak finder	Intensity tolerance: 50%; m/z tolerance: 0.06 or 30 ppm; Absolute retention time tolerance: 0.15
Positive mode	Raw data import	
	Mass detection	Noise level: 15
	Chromatogram builder	Min time span (min): 0.01; Min height: 4.0E1; m/z tolerance: 0.055 mz or 30 ppm
	Chromatogram deconvolution	Chromatographic threshold: 97%; Search minimum in RT range (min): 0.01; Minimum relative height: 10%; Minimum absolute height: 6.0E1; Min ratio of peak/top edge: 1.5; Peak duration range (min): 0.01-0.2
	Isotopic pattern	m/z tolerance: 0.06 or 30 ppm; Retention time tolerance: 0.01; Monotonic shape; maximum charge: 1
	Join aligner	m/z tolerance: 0.06 or 30 ppm; Absolute retention time tolerance: 0.15; Weight for both m/z tolerance and retention time tolerance: 10
	Duplicate peak filter	m/z tolerance: 0.5 or 600 ppm; RT tolerance: 0.15
	Peak list rows filter	Min peaks in a row: 5 Minimum peaks in an isotope pattern: 1; m/z range: 50-1000; RT range: 0-7; peak duration range: 0.01-0.2
	Peak finder	Intensity tolerance: 50%; m/z tolerance: 0.06 or 30 ppm; Absolute retention time tolerance: 0.17

Appendix 4. Model diagnostics: PLS-DA model characteristics.

PLS-DA models		**Urine**
Positive mode		
Inital	Cval class error	0,44 ± 0,027
Optimised	Cval class error	0,08 ± 0,032
Selected	Number of features	72 (31 / 79)
Negative mode		
Initial	Cval class error	0,50 ± 0,031
optimised	Cval class error	0,24 ± 0,037
Selected	Number of features	77 (33 / 98)

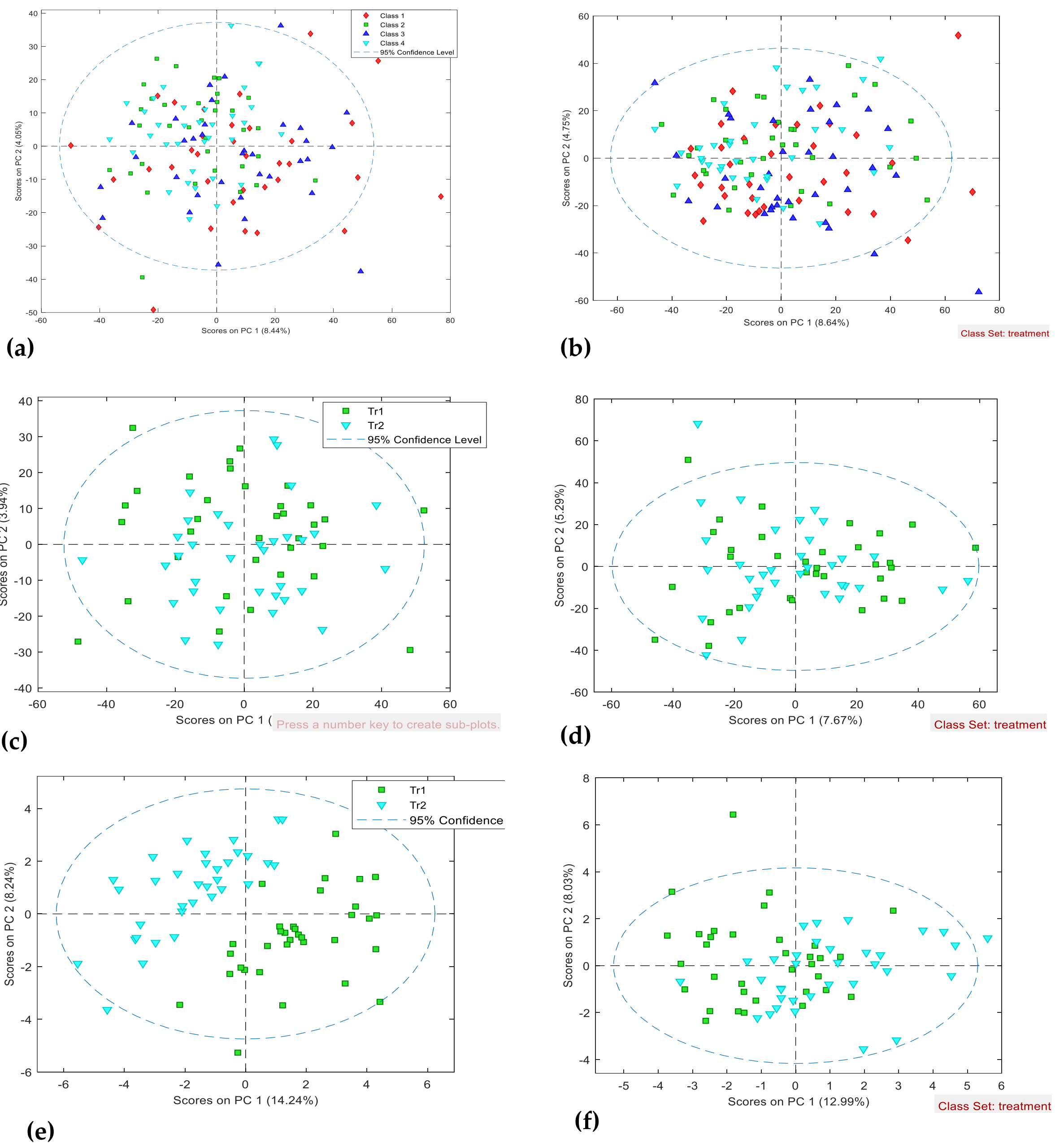
For the initial models, the mean and standard deviation of the results from all 34 models, for urine, before cross-validation and variable selection are reported. Results reported for the optimized model are based on features selected in the initial models, but after cross-validation and variable selection. *CVal class error* – cross validation error

Appendix 5. Principal component analysis (PCA) scores plot of UPLC-QTOF/MS data of control meat compared with supplemented meat.

Positive mode: (a) PCA on all 5444 features and all 136 samples (136x5554), selected markers; (c) PCA scores plot on Baseline Corrected data, all 5554 features (68x5554); (e) PCA scores plot on 43 discriminant features (68x43), non-corrected data.

Negative mode: (b) PCA on all 7333 features and all 136 samples, on selected markers; (d) PCA on all 7333 features, baseline corrected; (f) PCA on 34 discriminant features, common in 70% of the 34 models, non-corrected data.

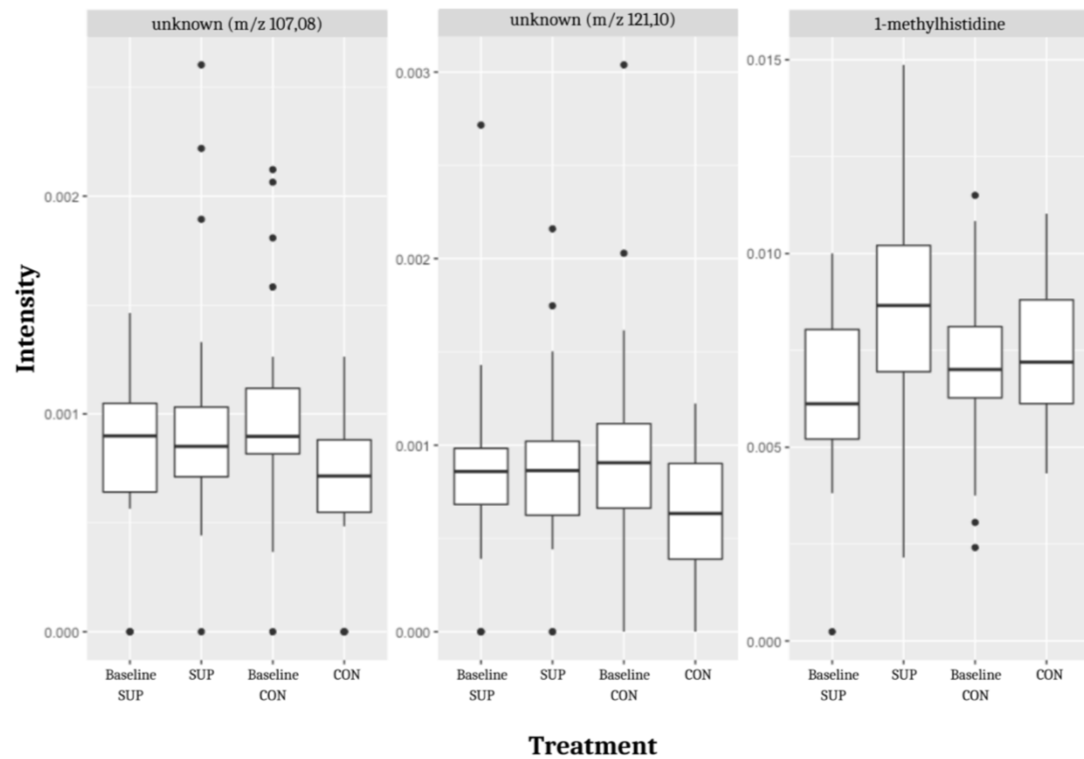
The class was set for treatment. Tr1 supplemented meat; Tr2 control meat.

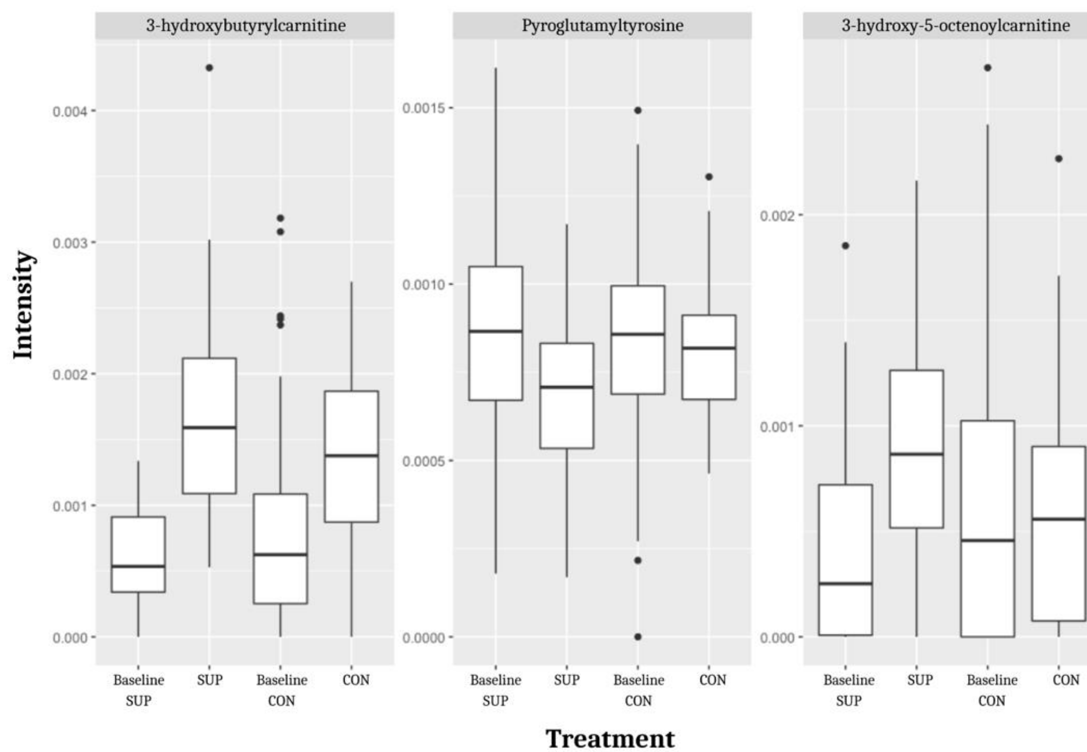


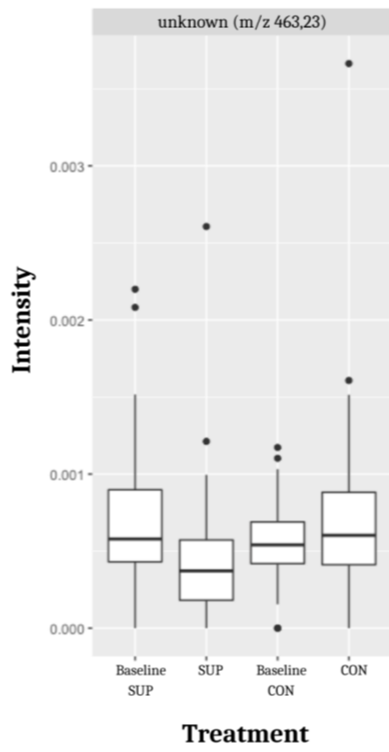
Appendix 6. Statistics of selected markers for positive and negative mode.

Positive mode					Negative mode				
Feature	RT	m/z	p-value baselines	p-value treatments	Feature	RT	m/z	p-value baselines	p-value treatments
1	4,9799	105,681	0,061309	0,118251	1	0,73931	87,0088	0,761264	0,034267
2	6,2180	107,086	0,221425	0,003192	2	0,68308	89,0231	0,660629	0,004326
3	0,62558	120,967	0,262857	0,018677	3	4,5996	123,0435	0,50973	0,21499
4	5,8932	121,101	0,241533	0,023753	4	0,65935	138,9703	0,865999	0,048382
5	0,62259	124,077	0,138323	0,015309	5	8,9034	139,0065	0,325751	0,022654
6	0,72554	127,083	0,018677	0,090746	6	4,6262	161,9882	0,85819	0,168876
7	1,2582	129,058	0,002817	0,262857	7	5,6280	165,0566	0,852741	0,221425
8	4,6430	133,085	0,298851	0,002829	8	0,74616	165,0801	0,542938	0,002183
9	6,0331	135,114	0,116627	0,083064	9	5,1464	187,5870	0,112619	0,152717
10	5,3291	154,085	0,079938	0,089613	10	5,1414	187,7401	0,852741	0,026086
11	0,94807	155,056	0,202515	0,012474	11	5,1382	189,1979	0,293203	0,027323
12	0,63196	162,063	0,010655	0,133382	12	6,8222	223,1322	0,879293	0,006141
13	0,71397	162,073	0,037427	0,052568	13	4,0050	247,0151	0,366155	0,032889
14	6,1215	171,135	0,660629	0,12497	14	4,2263	272,1422	0,796	0,007981
15	2,3793	185,091	1	0,334232	15	5,0736	326,0886	0,325751	0,015309
16	5,3685	195,124	0,143003	0,672929	16	6,0444	356,1082	0,262857	0,015309
17	5,2694	211,136	0,133382	0,022654	17	5,7759	361,1497	0,600514	0,004326
18	6,0579	223,168	0,087448	0,293203	18	6,1510	382,0968	0,761264	0,027323
19	0,64877	227,119	0,190563	0,18478	19	5,9940	400,1422	0,436471	0,002999
20	6,4128	237,170	0,030984	0,041102	20	5,8201	409,1141	0,351606	0,009574
21	0,71975	246,171	0,0137	0,707119	21	1,1858	435,9577	0,588786	0,017781
22	6,3806	247,128	0,800149	0,262857	22	1,1394	435,9579	0,520683	0,00907
23	1,3747	248,150	0,174478	0,037427	23	3,9651	445,0991	0,152717	0,010102
24	9,3238	256,301	0,097633	0,039096	24	6,7204	465,2495	0,213691	0,004591
25	6,1723	257,175	0,277755	0,042616	25	5,8080	479,2196	0,531753	0,018677
26	5,0450	265,375	0,695946	0,00907	26	0,62811	515,9113	0,255613	0,002999
27	1,4194	282,121	0,108717	0,034267	27	6,0089	540,2439	0,277755	0,008183
28	4,7074	293,119	0,722957	0,000903	28	6,7011	592,3485	0,277755	0,000369
29	0,82314	300,118	0,01383	0,032772	29	6,7446	615,3340	0,06433	0,012474
30	4,2947	303,199	0,270121	0,007358	30	6,7892	641,3495	0,325751	0,032772
31	6,7879	341,272	0,260304	0,018346	31	6,4911	731,3791	0,531753	0,037427
32	5,3373	350,089	0,054769	0,044472	32	6,4879	811,3234	0,660629	0,013137
33	6,2506	363,205	0,084244	0,108717	33	6,7236	893,5919	0,690262	0,035411
34	5,3695	389,181	0,000903	0,084244	34	6,6877	1049,567	0,604342	0,000727
35	6,5170	410,287	0,035729	0,047658					
36	0,53221	426,902	0,277755	0,016099					
37	6,3526	429,214	0,813224	0,015098					
38	5,4133	431,225	0,000523	0,879293					
39	6,3403	463,237	0,270237	0,002046					
40	6,4422	476,196	0,116627	0,101225					
41	6,2617	478,202	0,919362	0,000628					
42	6,7923	956,708	0,710331	0,406984					
43	6,8228	967,675	0,048382	0,042536					

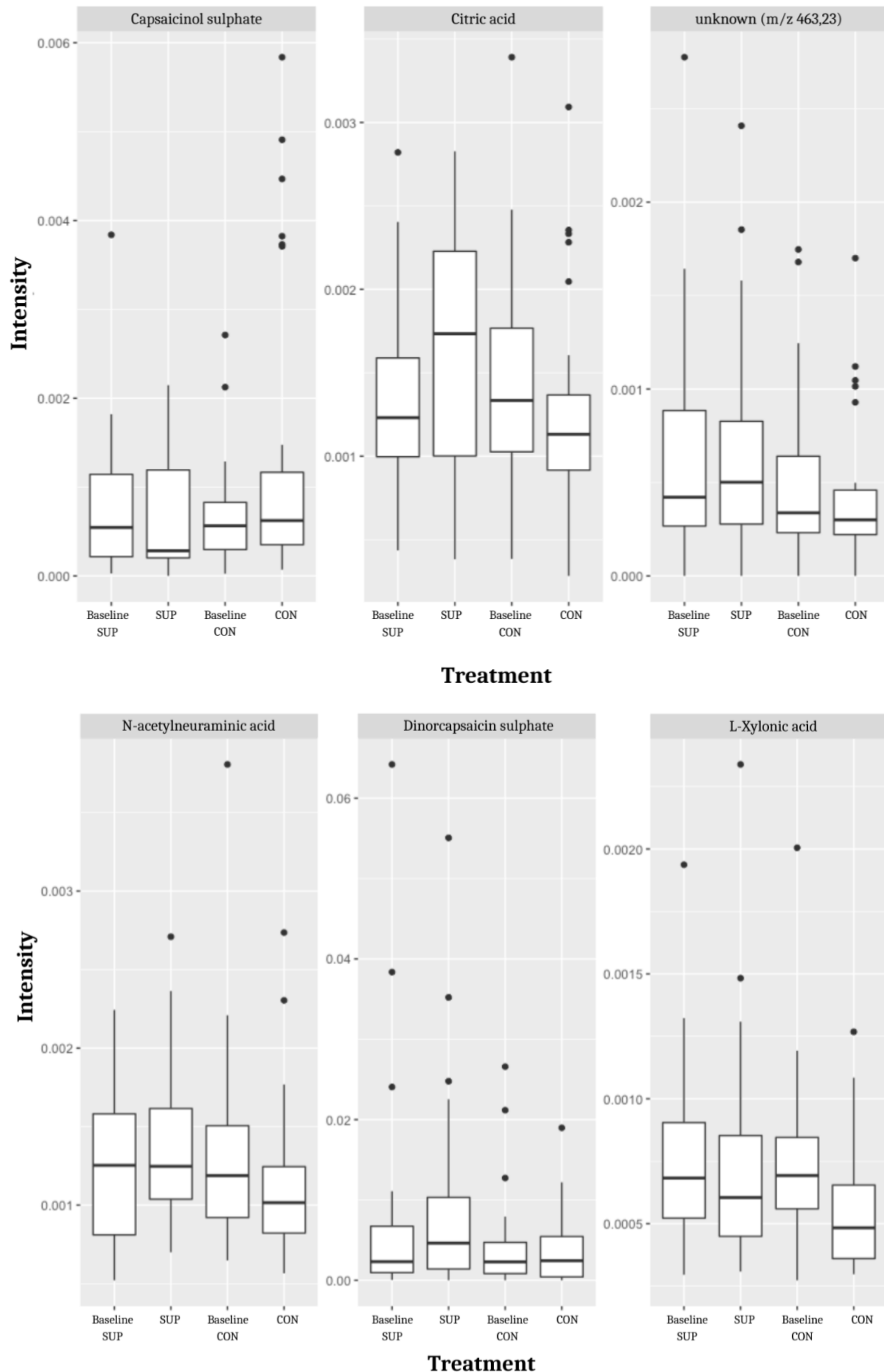
Appendix 7. Boxplots of the selected features on positive mode.






unknown (m/z 463,23)
Intensity
0.003
0.002
0.001
0.000
Baseline
SUP
SUP
Baseline
CON
CON
Treatment

Appendix 8. Boxplots of the selected features on negative mode.



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