Application Of Metabolomics For The Comparison Of Postprandial Responses Between Rye And Wheat Products In Human.

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Master of Science, 2012 (45 hp)
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Abstract

Background: Previous studies have shown lower postprandial insulin response following rye intervention compared to wheat. This observation is known as the “rye factor” but the mechanism is not well understood.

Objective: The main aim of the study was to use an NMR based metabolomic approach to determine the potential metabolites that could reveal some discrepancies in postprandial response after wheat and rye consumption which would explain the rye factor.

Methods: The refined wheat bread and endosperm rye bread were served to nineteen postmenopausal women aged 61±1 year with normal glucose tolerance. Fasting as well as postprandial blood samples were collected and H^1NMR based metabolomics analysis was performed. PCA and OPLS–DA were used to compare the data using the software SIMCA.

Result Significant postprandial decrease in the branched chain Amino Acids (AAs) (leucine, isoleucine and valine), other AAs (phenylalanine, lysine proline, glutamine) and dimethyl sulfon was observed after intake of rye bread compared to wheat.

Conclusion: The change in the amino acid metabolism could be one of the probable reasons for decrease in insulin response following consumption of rye bread compare to wheat.

Abbreviation

AAs	Amino Acids
CI	Confidence Interval
CSF	Cerebrospinal fluid
CVD	Cardio Vascular Disease
FSIGTT	Frequently Sampled Intravenous Glucose Tolerance Test
GC	Gas Chromatography
GDH	Glutamate Dehydrogenase
GIP	Glucose dependent Insulinotropic hormone
H^1NMR	Proton Nuclear Magnetic Resonance
HPLC	High performance liquid Chromatography
LC	Liquid Chromatography
LC FTMS	Liquid chromatography – Fourier transform mass spectrometry
mRNA	Messenger RNA
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
OPLS-DA	Orthogonal partial least squares- Discriminant Analysis
PCA	Principal Component Analysis
SCFA	Short chain fatty acids
TCA	Tri Carboxylic Acid
TSP	Sodium-3-trimethylsilyl2,2,3,3,-tetradeuteriopropionate
VIP	Variance Importance plot

1. Introduction

1.1 Whole Grain

The most common source of dietary carbohydrates are cereal products like wheat, rye, oat, rice, barley and corn. These cereals were consumed as a whole grain in ancient times but in recent days they are also consumed as refined products. Many epidemiological studies suggest that the consumption of whole grains helps to prevent the development of many chronic diseases like diabetes, CVD (Cardiovascular Disease) and cancer. (Slavin *et a*l, 2003; Fardet *et a*l, 2006; Cleveland E.L and et al, 2000) Bioactive compounds like vitamins, carotenoids, polyphenols, minerals and alkylresorcinols, which are believed to have antioxidant and anti-carcinogenic properties, are found in whole grain and thus considered as protective food. Whole grains comprise of 3 layers: bran, germ and endosperm (fig 1-1) with endosperm covering around 80% of the whole grain while the percentage of bran and germ may vary in different grains (Slavin *et al*, 1999; Fardet. A, 2010) Nutrients are distributed in all parts of the grain but distribution is not even and it is more concentrated in the outer parts. The refining process separates the starchy endosperm from bran and germ fraction resulting in reduction of nutrients that have protective functions.

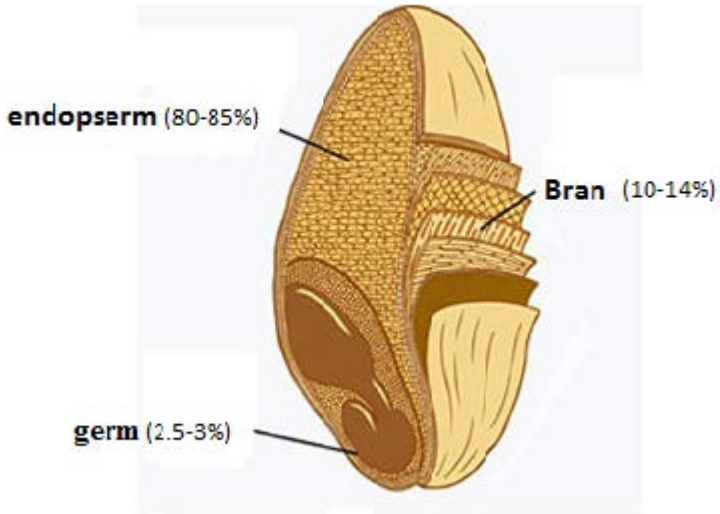

Fig 1-1 Whole Grain (http://blog.fooducate.com)

The three fractions of whole grain wheat (bran, germ and endosperm). The bran that covers the outer layer which protects the seed and contains fibres, vitamins and minerals. Endosperm provides energy to the seed and contains carbohydrates and proteins. Germ provides the nourishment to the seed and contains vitamins, minerals, antioxidant and phytochemicals.

The fibres present in whole grains help in laxation (insoluble fibres) and control of blood glucose and cholesterol (soluble fibres). The fermentation of fibres results in the production of short chain fatty acids (SCFA) (Scheppach *et al*, 1995; Slavin *et al*., 2003), which help to develop healthy colonic micro flora and lower the risk of cancer (Lia *et al*.,1995).The SCFA produced are believed to lower the blood glucose concentration by lowering gluconeogenesis and enhancing glycolysis (Roberfroid *et al*., 1998, Verbruggle *et al*, 2009). The other mechanism for glucose regulation by soluble fibres can be explained by the reduction in the gastric emptying time which ultimately reduces the intestinal glucose absorption (Wood *et al*, 2007). However, intact grain structure was found to be a more important factor than dietary fibre to control physiological response in glucose metabolism. (Fardet *et al*, 2006, Juntunen *et al.*, 2003). Further, soluble fibres also increase excretion of bile acid and thus promote synthesis of bile acids from cholesterol thereby reducing the risk of hypercholesterolemia (Lia *et al*, 1995).

1.2 Wheat and Rye

Wheat is the most widely consumed and produced cereal worldwide. In Europe, especially in the Northern and Eastern part, rye is consumed as one of the major cereals after wheat; though globally it is the least produced cereal that accounts for only 0.5% of total cereal (Bushuk *et al*, 2001). In Nordic countries, rye has been consumed since the ancient period and still it is being consumed with highest use in Denmark but lowest in Norway. However, the consumption of rye in the world is just 1kg/capita in comparison to Europe which is 6.1kg/capita in average. The macronutrient content in rye is similar to that of other cereals but it has higher fibre content of around 15-17% in comparison to wheat, which is just 10-13 % (Åman *et al.*, 1997). Many studies suggest that the higher fibre content of rye acts as an satiety enhancing factor in rye that can also protect from obesity.(Wanders et al, 2011) Further, rye is a good source of many minerals like manganese, iron, copper, zinc, selenium, magnesium and fluoride and also possesses anti-oxidative compounds like phenols, tannins and ferulic acid. Thus, the consumption of rye foods for prolong periods has shown to provide protection against many diseases like heart disease, type II diabetes, bowel cancer, etc (McIntosh *et al*, 2003).

Different studies have been conducted to better understand the health and physiological benefits of rye products. In a study by Juntunen et al, postmenopausal women were given either rye or wheat bread and then the plasma level of glucose, insulin and GIP (Glucose dependent insulinotropic hormone) were determined in fasting as wells as postprandial blood samples. It was observed that the consumption of rye products resulted in lower postprandial insulin response than white wheat bread without any alteration in the glucose level. The effect caused by the "rye factor" which is still not well understood (Juntunen *et a*l, 2003, Leinonen *et a*l,1999). The data so far suggests that after consumption of rye, lower amount of insulin is sufficient for post prandial regulation of glucose level (Juntunen *et a*l., 2003). However, further studies should be conducted to understand the mechanism behind the rye factor and the change in insulin response. Another research was conducted to determine the long term effects of rye and wheat consumption on insulin resistance and secretion. In this study, subjects were randomly assigned either rye bread or wheat bread for 8 weeks and there was 8 weeks wash out period between the bread interventions The FSIGTT (Frequently Sampled Intravenous Glucose Tolerance Test) was performed at the end of the interventions. A glucose dose of 330 mg/kg was given intravenously and blood samples were taken at different time points. The results showed an increase in the acute insulin response following intake of rye compared to wheat bread. Exactly the opposite effect was observed when the short term effects of rye and wheat were studied. The lower postprandial responses after rye bread for a prolonged time could protect the β-cells from exhaustion and moreover enhance β-cell function which would lead to higher insulin response after FSIGTT .

It is believed that lowered postprandial insulin for a prolonged period protects against metabolic disease like diabetes and obesity (Pons *et al.*, 2011, Ludwig *et al*, 2002) Previously, the higher fibre content in rye was considered to be the reason for the lower insulin response. But research by Juntunen and *et a*l in 2003, elucidated that the insulin response was independent of the fibre content, since rye with different fibre content showed similar insulin response. The same study showed a markedly increased insulin response after wheat bread intake. The structural difference between the wheat and rye bread could explain the decrease in insulin response. However, the detailed mechanism behind this effect is not well understood yet.

To understand physiological mechanism for the increase in insulin response after consumption of wheat bread compared to rye, metabolic studies should be conducted.

Metabolic studies could better apprise about the absorption, digestion and its effect in the body that can probably help to provide a holistic approach of the living system. Therefore, we performed a metabolomic study following rye and wheat intervention.

1.3 Omics Science

The omics studies genomics, Proteomics and Metabolomics aim to integrate DNA, RNA, protein and metabolites to better understand the complex biochemical networks of a living system (Dixon *et al*., 2006) The central dogma of molecular biology states that the information flows from DNA to mRNA which is then translated into protein, that performs various roles in the cells including the metabolism of small molecules giving rise to the metabolites (Veenstra, 2012) which is illiustrated in the fig. 1-2

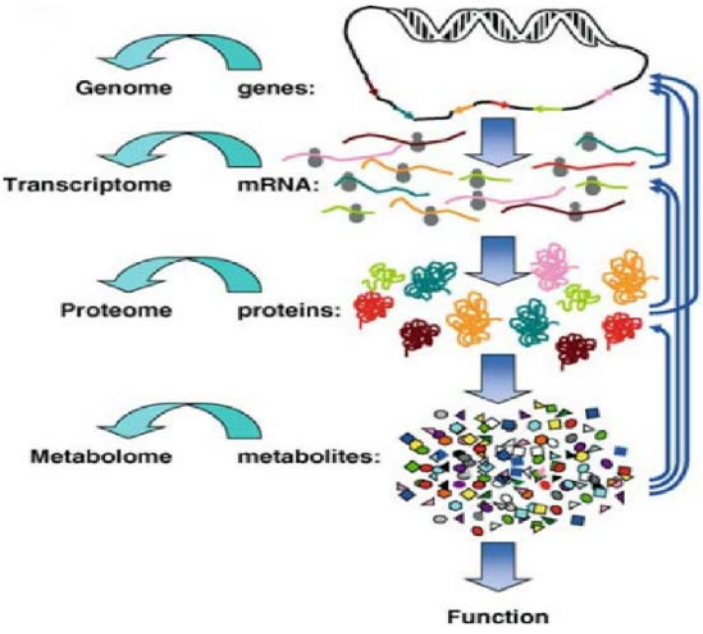

Fig 1-2 Schematic representation of Omics hierarchy

Figure shows the flow of information in living system from genes to proteins and finally to the metabolites
Adopted from Goodacre, 2005

Genomics deal with the complete genome analysis and gene expression. However, expression of a gene doesn't guarantee its translation into protein because the mRNA can undergo many posttranscriptional modifications. Further, genomics doesn't cover information about mutations, deletions or epigenetic changes. So proteomics in this case provides more information about the functional activities of a cell. Proteomics deals with the detailed study of protein structure, function, identification, analysis and its interaction. Proteomics can give much more information that is unrevealed by

genomics like posttranslational modification, stabilization of protein, protein interactions and activity (Chung *etal*., 2006). However, proteomics lacks to give detailed information about activity of the proteins, for *e.g*. some proteins are functional only after phosphorylation and some proteins can be inactive if they are mislocalized. Further, by the alternative splicing many transcripts can give more than one protein and also the interaction of proteins with RNA or other proteins makes the case more complex. Therefore, proteomics is less quantitative in many cases and quite complex to give the detailed information of the cell. Further, both the omics science cannot exactly reflect the current cellular status of the cell. The problem is to some extent solved by the emerging science **Metabolomics**. Metabolomics, which is less complex, more quantitative yet reflects the current functional status of an organism and reveals the phenotype (Vaidyanathan, 2005; Ryals., 2004). However, metabolomics is not an alternative to genomics and proteomics, but integration of all the omics science can give more detailed overview of living systems that can be used to monitor the health status of an organism (Veenstra, 2012)

1.4 Metabolomics

Metabolomics, which is interchangeably used as metabonomics, global metabolic profiling (Schnackenberg, *et al* 2007; Imaizumi *et al*, 2012) is an emerging omics science and expansion of traditional biochemistry which helps in the quantification of different metabolites of living systems. Metabolites are the products of metabolism that include metabolic intermediates, AAs, nucleotides, vitamins, ketone bodies, hormones, signalling molecules etc. that reflect the phenotype of an organism. Different approaches are used to study the metabolites.Traditionally, specific metabolites were targeted; either the end product of enzymatic pathway or metabolites derived from drug degradation, which is called **metabolite targeting** (Carraro *et al*, 2009) Further, sets of metabolites were targeted that belong to specific pathway which is called **metabolic profiling** (Go *et al*, 2010; Carraro *et al*, 2009) Any change in the metobolite profiles and their distribution reveals the biochemical state of the biologcial systems. But due to the progression in the analytical technique; comprehensive methodology is also in practice, which is the non targeted metabolite analysis called **metabolic fingerprinting.** Metabolic fingerprinting helps to compare metabolic patterns of different pathophysiologcial status and can be useful in determination of biomarker for specific disease. In this approach, unknown

metabolites can turn to characterise new pathophysiological hypothesis. (Baraldi. E and *et al*, 2009)

1.4.1 Human Metabolome

Metabolome refers to a broad range of small molecules metabolites (molecular weight <1500Da) in a cell (Psychogios N, 2011), but also includes exogenous molecules that are modified by diet, medication or environmental exposure (Robertson *et al*, 2010). The metabolites expressed in any tissue or cell are the subset of the whole Metabolome. It can be explained in terms of sets, which are illustrated in the fig. 1-3, where A represents the total human Metabolome, B is the Metabolome of an individual and C and D represents different tissues within the individual. So the Metabolome can differ between individuals and also within different organs of an individual. Thus, the metabolic profile of the liver for example is different from kidney or heart. The concentration of metabolites in a specific organ can reflect the metabolic state of that particular organ; any physiological change like disease or stress can alter the gene expression. In addition, exposure to toxic compounds or drugs can cause imbalance in metabolite concentration, which can be of diagnostic value.

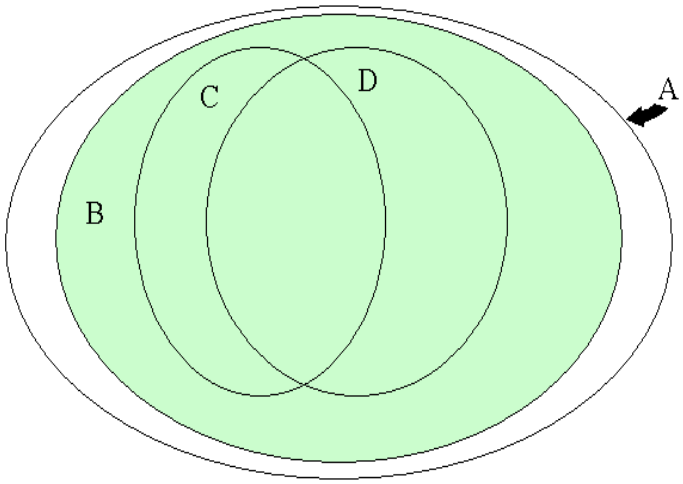

Fig 1-3 Human metabolome shown in a set

Here A represents total human metabolome, B is metabolome of an individual and C and D represents different tissues within an individual
Reprinted from Metabolic profiling (p. 317) by Harrigan .G and et al., Kluwer, 2003, USA, academic publishers group.

1.4.2 Application of Metabolomics

The main advantage of metabolomics is to anlayse a broad range of metabolites of interest simultaneously which can reduce time as well as the cost of the test. (Hunter,

2009) It gives a clear picture of current metabolic state of an organism by systemic analysis of the metabolites and thus helps to determine the functional status of an organism (Wood *et al*, 2007). Metabolomics study can be observed in many scientific areas including plant science, drug discovery, and toxicology, food science, diagnostic and functional genomics. Further, it is also applied in nutritional genomics and has been widely used to understand the metabolic response due to dietary intervention (Hunter, 2009; Veenstra, 2012).

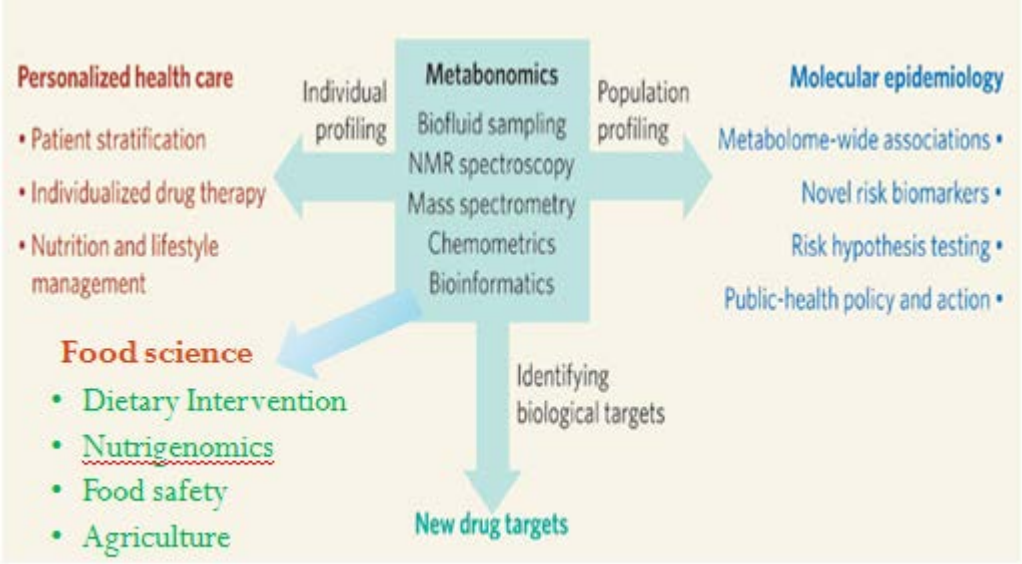

Fig 1-3 Application of Metabolomics

Applications of metabolomics in different fields from pharmacology to food science and from individual profiling to population profiling.
Adopted from Nicholson. K. J and etal , 2008

1.4.2.1 Agriculture, Food and nutrition

Metabolomics technolgies have a wide range of applications in plant science. Genetic engineering has been widely used for the improvement of crops quality, but inorder to examine influence of genetic modification on the cellular composition of plant and its quality, metabolomics study should be performed (Roessner *et al*, 2009). Metabolism in plants is a complex mechanism which produces app. 200,000 to 1,000,000 metabolites. The systemic analysis of metabolome data reveals the secrets of the plant cell system which helps to evaluate genetically modified crops (Saito *et al*, 2010) and also to determine the markers of diseased and stressed plants (Hall *et al*, 2008).

Traditionally, the aim of nutritional research was to understand the relationship between health and diet but now it has broadened to prevent from disease and improve the health and performance through diet. It is known through previous studies about the advantage of consumption of certain types of food like fruits, vegetables, whole grain etc, but the real mechanism behind the preventive actions of nutrients is not well understood. Metabolomics, by the simulatneous determination of multiple metabolic endpoints can determine the functionalities of bioactive components present in the food which can further explain the preventive actions of nutrients (Schnackenberg *et al*, 2010). One of the promising approaches of metabolomics is to determine individual´s nutritional status and predict the health and disease outcomes. The metabolomic approach is extensively practised to study metabolic effects of dietary intervention as well. Furthermore, metabolomics study is widely used in food safety inorder to determine the food adulteration and contaminations. In a study, Goodacre *et al* (2003) described MS (Mass spectrometry) technique to determine contaminants in olive oil (Goodacre *et al*, 2003) Similarly, adulteration in strawberry products with cheaper apple material was determined using SPME- GC- MS by Reid *et al* (2004). (Reid *et al*, 2004). With the aid of metabolomics technique, food industry also detects the antinutritional components in food. Thus the metabolomic approach is applied in agriculture, nutritional research and food industry.

1.4.2.2 Diagnostic and drug discovery

Global metabolic profililing has been used for identification of biomarkers for clinical diagnosis of disease and also to acess the pathophysiological status of an organism (Laura K. *et al*, 2007). Biomarkers occur in biological matrices like fluids (blood, urine, cebrospinal fluid) cells or tissues and reflect the biochemical and cellular alteration occuring during disease. Thus biomarkers are widely used in diagnosis and prognosis purposes in clincial medicine (Spratlin *et al*, 2009; Vaidyanathan, 2005).

Beside diagnosis of any disease, these biomarkers also indicate the stage of the disease and help to monitor the effects of therapy. Metabolomics explain the probable mechanism of disease and reveal the distrubted biochemical pathway which also aids in drug design and development. In addition, metabolmics studies is also used to determine the response to organ transplant. Further, metabolic profiling helps to determine the toxicity of any drug early in the drug discovery process which can save

time as well as money (Laura K *et al*, 2007). Thus metabolomic approach is being applied in pharmaceutical industry. Metabolomic profiling is also utilized to predict the toxicity of any drug, the study termed as pharmacometabolomics which can further guide for the choice of therapy and direct for customized therapy (Baraldi. E *et al*, 2009).

Further, metabolomics can also enhance the knowledge in functional genomics which helps to understand changes in metabolites and alteration in metabolomic pathway from a genetic aspect as well.

1.5 Methods used in metabolic analyses:

Basically, two analytical approach have been used for the anlaysis of the metabolites in blood , urine, CSF or different tissues and organs.

- Mass spectrometry (MS)
- Nuclear Magnetic Resonance (NMR) spectrometry

Both the techniques have advantages as well as disadvantages and optimal selection of the technique depends on the aim of the study. The mass spectrometer is coupled with other chromatographic technique like GC (Gas Chromatography) LC (Liquid Chromatography) or HPLC (High Performance Liquid Chromatography) or with capillary electrophoresis in order to separate biologically complex mixtures. MS is highly selective and sensitive with detection limits in the femtomolar to attomolar range (Veenstra, 2012). It also provides chemical information of the analytes like its chemical structure, mass e.t.c (Zhentian Lei, 2011) However, the major drawbacks of this technique is lack of reproduciblity, accuracy and precision. The major drawbacks of MS are the advantages of NMR: NMR is considered as gold standard.in metabolite structural elucidation (Zhentian Lei, 2011). It is highly selective, quantitative and reproducible and versatile unlike MS. The peak area of a compound in the NMR spectrum corresponds to the concentration of specific compound; so the method is highly quantitative and accurate. Further, NMR data is unbiased and doesn't depend on the acidity or hydrophobicity of the compound (Raftery, 2006)

However, the asset of MS is the weakness of NMR; NMR is less sensitive with detection limit of micromolar but its selectivity can be increased by the use of cryoprobes that may have detection limit to nano molar concentration. (Raftery, 2006) Both the techniques have pros and cones and it is not possible to analyze all the metabolites from a single technique. Wishart et al (2008) investigated the metabolome of CSF (Cerebrospinal Fluid) by different techniques such as GS MS, NMR and LC FTMS (Liquid chromatography–Fourier transform mass spectrometry) which detected 70 diverse compounds out of 308 metabolites present in the CSF metabolome database. Each technique had their unique detection limit and was able to quantify the compounds that were unique for specific method. NMR detected 53 metabolites, GC-MS 41 and LC- FTMS 17 metabolites with 28 metabolites common in NMR and GC-MS. (Wishart D.S, 2008). This is illustrated in the fig.1-4

The combination of both the techniques can improve the identification and quantification of unknown metabolites that can enhance the scope of metabolomics (Raftery, 2006 (Vaidyanathan, 2005)

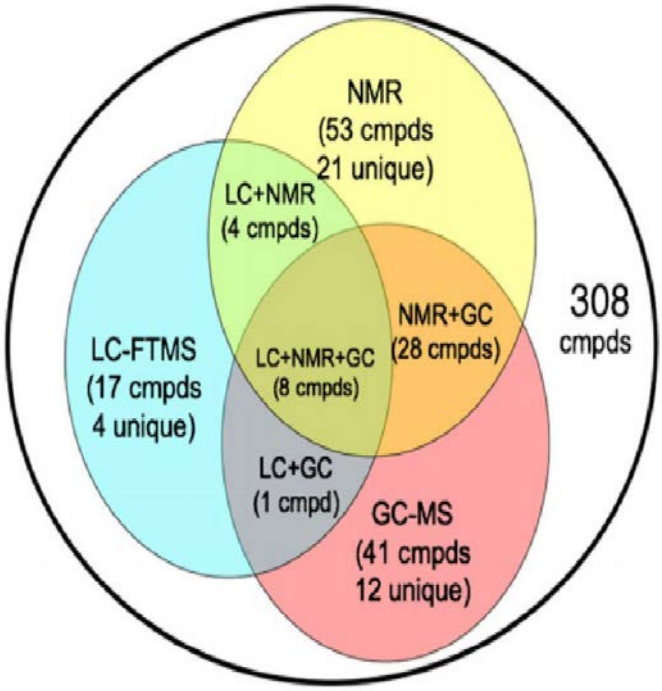

Fig 1-4 Venn diagram showing metabolites in CSF detected by various techniques

Adopted from Wishart D.S and et al, 2008

Metabolites detected in CSF by different techniques NMR, LC-FTMS, and GC-MS, with NMR having the highest detection limits of 53 compounds.

2. Objectives

The main aim of the study was to use the metabolic approach in order to identify the possible metabolites involved in the postprandial response following wheat and rye intervention. We sought to determine if there is some discrepancies in postprandial response between rye and wheat bread, which could explain the “rye factor”.

3. Methods and Methodology

a. Human intervention

This study is a continuation of the previous research (Juntunens K and *et al* ,2003) to understand the mechanism of dietary benefits of of rye compare to wheat bread.

The study subjects included 20 healthy, non diabetic post menopousal women. One woman discontinued after her first visit due to heart problems. So the subjects included in the study were 19 post menopousal women. The participants were adviced to maintain their diet and body weight and heavy exercise and large portion of foods were avoided on the day before each test. Alcohol consumption was forbidden 2 days before the test and smoking was forbidden in the morning of each test. The health profile of the subjects at the time of the study are shown in the appendix.The study protocol was approved by the Ethics Committee of Kupio University and Univesity Hospital, Finland.

b. Blood sample collection and Test meal

For the fasting samples, subjects fasted for 12 –15 hours before the test and blood samples were collected by insertion of intravenous cathetor in anticubital vein of the arm. The serum samples were collected in prechilled tubes. After collection of fasting samples, subjects received the the test meal. The test bread included endosperm rye bread and refined wheat bread. The details of nutrient composition of the test bread portion is shown in the appedix. The test bread was given randomly at the interval of 1-2 weeks. The postprandial samples were collected at 7 different time periods (15, 30, 45, 60, 90, 120, 150 and 180 min) For the present study, only three of the time periods were considered (30, 45, 60) which showed changes in the insulin concentration in previous studies.(Juntunen s K and *et al* ,2003). The study was conducted in Kuopio hospital, Finland, and the serum samples collected were send to Swedish University of Agricultural Sciences (Department of Food Science). The samples were stored at -80^{o}C until the metabolomics studies were performed.

c. Serum extraction for NMR

The serum samples collected in each intervention were used for metabolomics study by NMR. First, serum samples were extracted by ultrafiltration using nanosep centrifugal filters (Pall life science)with 3 kDa cutoff. Since glycerol is used as a preservative in the filter it should be removed by washing prior to extraction otherwise the signal of gycerol is observed in the NMR spectra which might disrupt

the analysis.The centrifugal filters were washed 10 times with 500 µl deionized water at 4000 RPM at 36^{o}C for 20 minutes. After each wash water was removed and refilled in the filter. The remaining water in the centrifugal filter was carefully removed during the last washing step by pasteur pippete but the filters was always kept moist to prevent the breakage of the filters. After washing, the filters were kept on ice and the serum samples were defrosted, vortexed and 500 µl of each samples were added in the corresponding labelled filters. Then serum samples were centrifuged at 12000 RPM at 4^{o}C until approximately 1mm visocus retentate was left in the filters which took around 5 to 6 hrs depending on the serum samples. The filtrate were then kept at -80^{o}C until the NMR analysis was carried out. Since the cut off value for the filters is 3kDa, metabolites with a lower molecular weight get separated from the macromolecules greater than 3 kDa(eg. Protein).

d. Sample preparation for NMR analysis

The extracted serum samples were defrosted, then vortexed and 310 µl was transferred to an ependorf tube (1.5 ml) and mixed with 150 µl sodium phosphate buffer (pH 7.03, 0.4M) 65 µl deionized purified water, 45 µl D_2O (deuterium water) and 30 µl internal standard *i.e* TSP (sodium-3-trimethylsilyl 2,2,3,3,-tetradeuteriopropionate 5.8mmol/L). The mixture was then votexed and transferred to NMR tubes with 3 mm diameter.

e. Metabolomics Analysis by NMR

The H^1NMR analysis of all the samples were performed using Bruker spectrometer operating at 600Mhz (Karlsruhe, Germany) equipped with cryoprobes. H^1NMR spectra of serum samples were obtained at 25^{o} C using zgesgp pulse sequence with 128 scans, acqusition time 1.82sec and a spectral widh of 17942 (Hz). The NMR spectral datas were processed by using the software Bruker Topspin 2.2 (Bruker Biospin, Germany) In order to increase the signal to noise ratio, each signal was multiplied by line broadening of 0.3 Hz and then all acquired signals of samples were converted to spectrum by using Fourier transformation. All the peaks were referenced to internal standard peak (TSP) at 0.00 ppm and spectral phase and baseline were corrected manually.

f. Statistical Analysis

The processed spectral data of all the measured samples were incorporated into a single data set using Amix 3.7.3 (Bruker Biospin GmbH Rheinstetten) which creates

the data set in the form of buckets with 0.01 ppm integral regions (buckets) between 0.5 to 8.5 ppm. Such partitioning process of the spectra is called binning or bucketing, which facilitates the spectral analysis and reduces the effect of change in chemical shift due to pH and also ensures that the same species is counted correctly across samples with such variations (S. Wishart, 2007, Craig and et al, 2006). However, such binned data is only used for the chemometric classification method and further we need to analyse the full resolution spectra for identification of the compound. (Andrew Craig, 2006) After bucketing, the spectral area which comprised of water, between 4.31 to 6.49 ppm, was deleted. For comparision of data, each spectral region was normalised to the intensity of the internal standard (TSP) by dividing the intensity of each bucket with reference standard (TSP). The normalised data were further integrated as variables into the bucket table.

Further, multivariate statistical analysis like Principle Component Analysis (PCA) and Orthogonal partial least sqares- discriminant analysis (OPLS) were performed using SIMCA-P^+ 12.0.1 software (UMETRICS, Umea, Sweden). Prior to statistical analysis, data were pretreated using mean centering and pareto-scaling. By mean centering, the average value of each variable is calculated and then subtracted from the data which improves the interpretability of the model (L. Eriksson, 2006). Further, paretoscaling was adjusted which divides the centered values by square root of standard deviation. Thus such processing of the data can detect significant differences between different groups inorder to compare the datas quantitatively (S.Wishart, 2007) The processed datas were finally analysed by PCA and OPLS-DA

PCA gives the overview of the data, and is not a classification method but a unsupervised projection method which provides a summary of the complex information and gives an idea about the sample patterns and grouping. It basicallly reveals how one samples varies from another and which variables contribute to their differences and whether the variables are correlated or not.

It represents the multivariate data table as a low dimensional plane and reveals dominating variables, trends and patterns (outliers, groups and clusters) as a graphical representation in the form of score scatter plot. The projection of the variables can also be shown in a loading scatter plot which gives information about the direction of variables in the subspace.(Trygg and *et al* 2007)

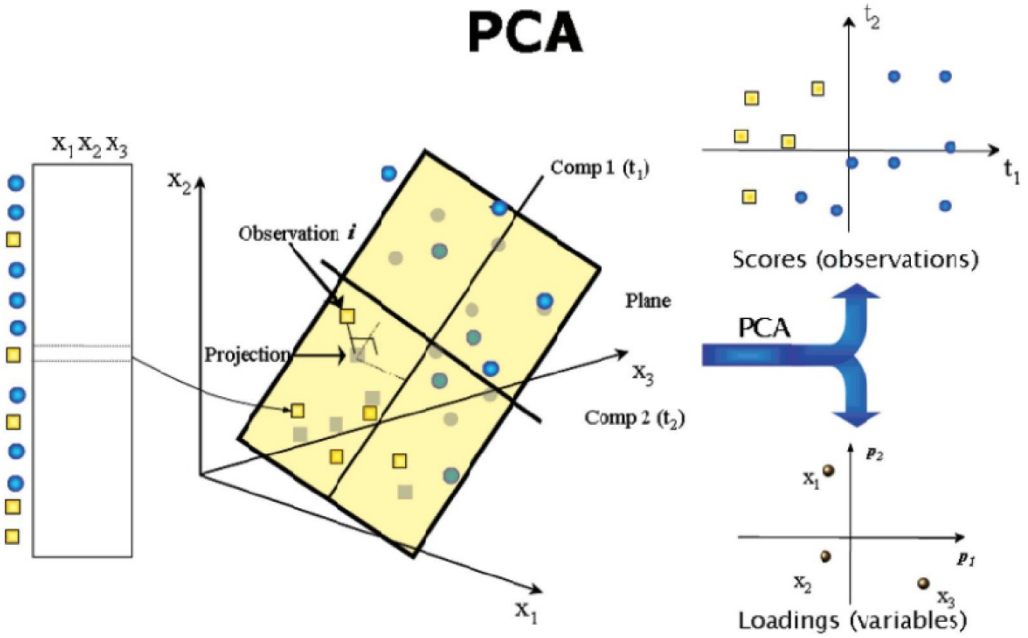

Fig 3-1 PCA model

Adopted from Trygg and et al 2007
The figure demonstarates how variables are distributed in the subspace and also shows the score scatter plot and loading plot from PCA analysis

i. Orthogonal partial Least squares-discriminant analysis (OPLS- DA)

OPLS is modification of PLS method, but produces models that are transparent and easier to interpret. It separates systematic variation in spectral data (x block) in two ways: one part which is linearly related to Y (predictive) and other which is independent or orthogonal to Y. OPLS-DA is regression analysis of PCA which also helps in the discrimination of classes (Trygg *et al*, 2007). One example of OPLS model is illustrated in Fig.3-2 where component t1p (predictive) shows the variation between the class and component t2 shows the variation within the classes (orthogonal).

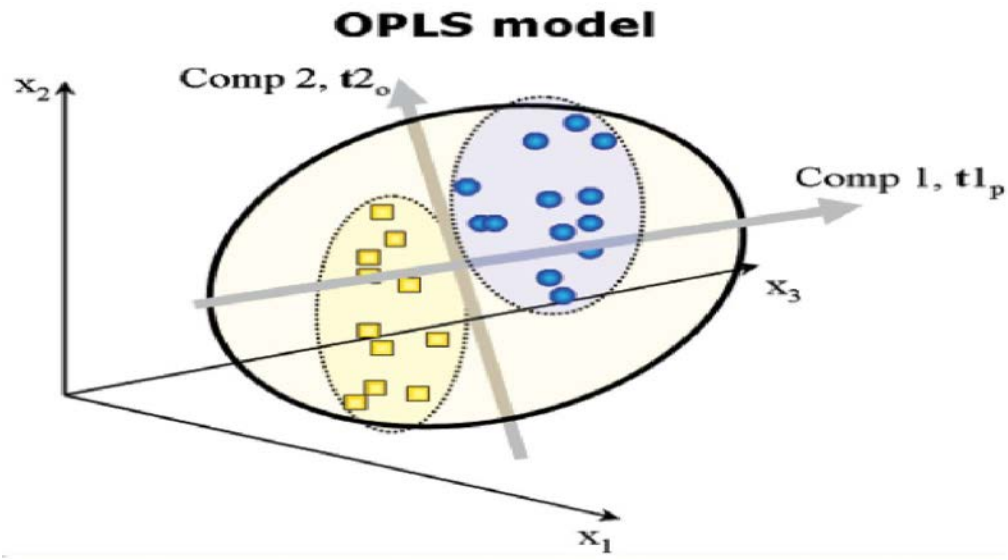

Fig 3-2 OPlS Model

(Adopted from Trygg and et al, 2007)
OPLS model that showing the variation between the classes and within the classes.

The outliers observed from the PCA scatter plot were removed before further analysis. The contribution plot and variance importance plot (VIP) were used for determining the buckets that were responsible for the separation of class. VIP depicts which variables are the most important of the model in general and VIP greater than 1 are the important variables that influence on the model thus were used for the selection of important buckets for further analysis. The VIP list also gives the confidence interval (CI) which indicates reliability of estimation. The criteria for the selection of important variable are discussed as follows:

Calculation of final VIP (VIPf)

VIPf = VIP-CI

Percentage of VIPf

% VIPf = (VIPf/ VIP)*100%

Following variables were excluded after the calculation

- Buckets with negative VIPf
- Buckets with VIP <1

The remaining buckets were considered for the further univariate analysis and also determination of the NMR signal and identification of the metabolites. The statistical significant buckets were further analyzed to determine the corresponding NMR signal by using software Bruker Topspin 1.3(Bruker Biospin GmbH, Rheinstetten,

Germany) and finally the compound was identified by using Chenomx NMR Suite software version 7.1 (Chenomx Inc, Canada)

Further, univariate statistical analysis of the identified compound having the maximum VIP was performed using Minitab version 16 (Minitab Inc, USA) to check the significance of the result and also for the pair wise comparison between different classes. The normality of the data was tested by Anderson- Darling test and log transformed when the distribution was skewed and the significance of the data was analyzed by one way ANOVA. P value <0.05 was considered statistically significant.

4. Results

a. Metabolomic analysis of plasma samples

H^1NMR spectroscopy was used to generate 1D NMR spectra of 114 samples (19 subjects); one of the H^1NMR spectra is shown in fig 4.1

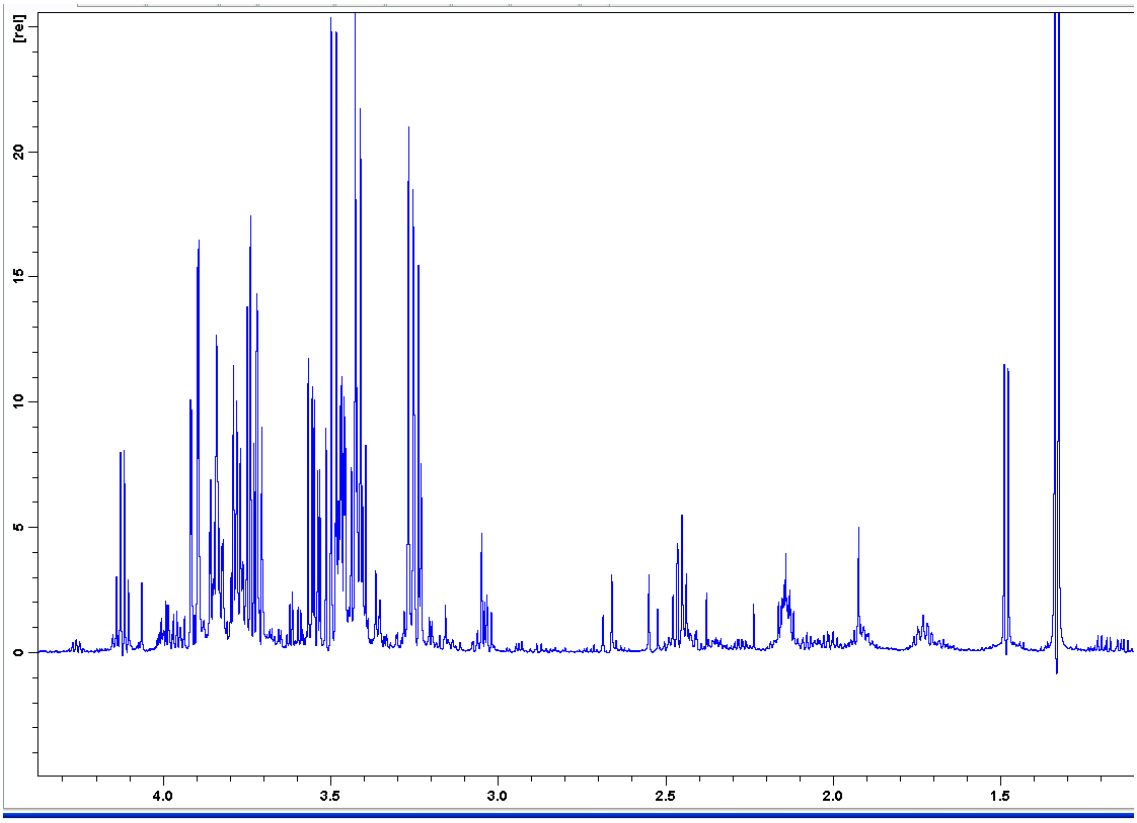

Fig 4-1 H^1NMR spectrum of serum.

The H^1NMR spectra of serum showing different metabolites at different chemical shift (ppm)

b. Statistical Analysis:

Multivariate data analysis were performed separately for each time line (30, 45 and 60 min) using PCA and OPLS. Firstly, score scatter plot of PCA model was performed to view the distribution of data as shown in fig.4.2 which depicted 92.4% of variation and clearly showed an outlier which was excluded for further analysis.

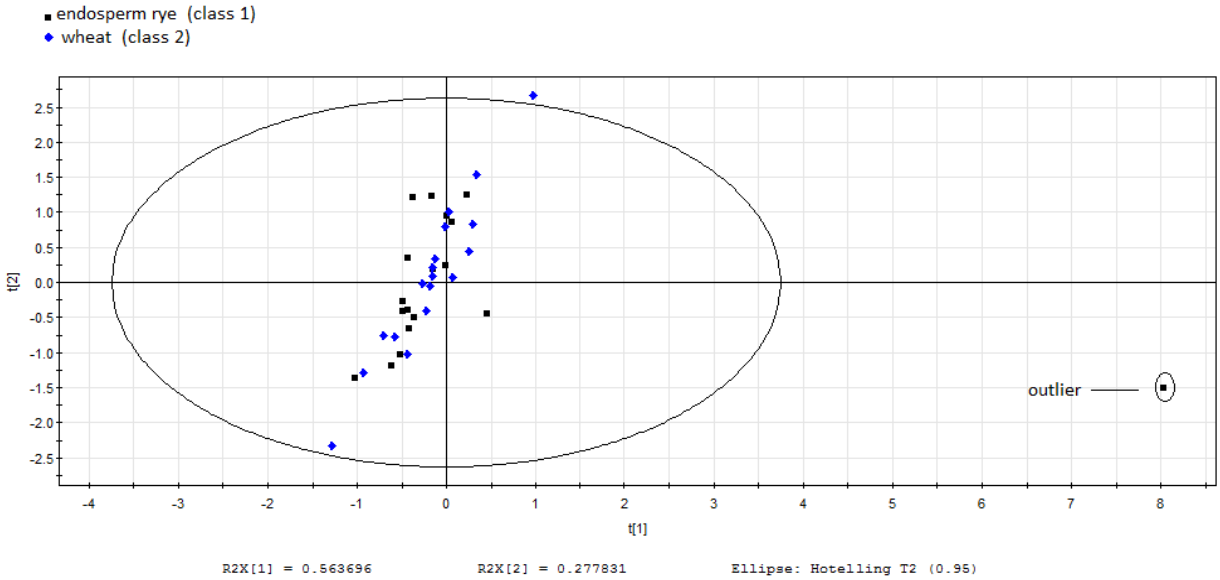

Fig 4-2 PCA score scatter plot at 30 minutes

PCA showing the distribution and patterns of the principal components and also the outlier which should be removed for further analysis

After exclusion of the outlier PCA model was refitted where the observations are well spread across a model plane which explained 82.9% total variation (Fig4-3). Here the comparison between the two classes for 30 minutes is illustrated.

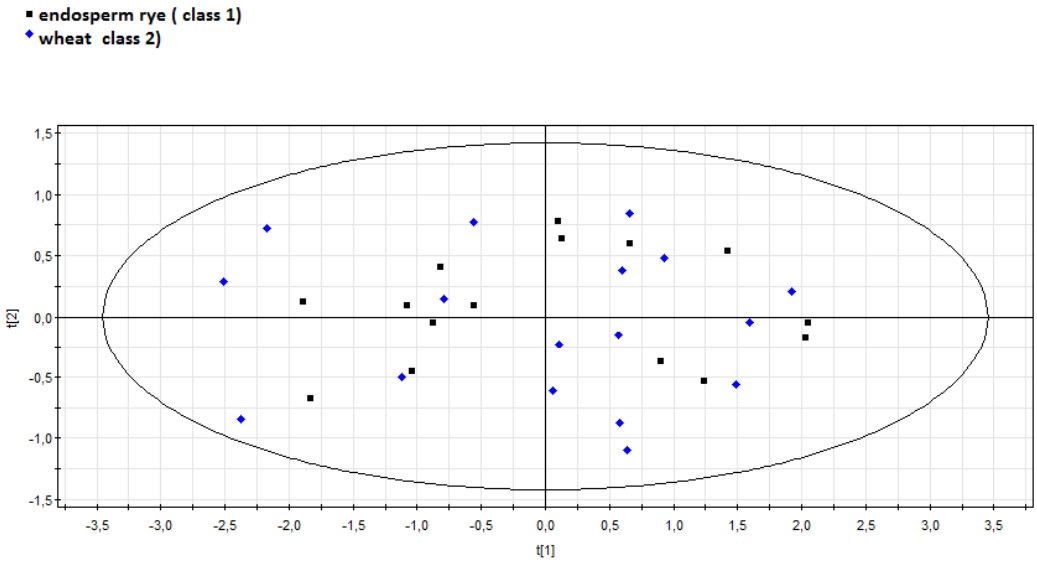

Fig 4-3 PCA score scatter plot excluding outliers at 30 minutes.

PCA plot after the outlier has been removed which shows the distribution of the components

After the PCA model an OPLS-DA model (fig. 4-4) was created where the class separation was exemplified. The OPLS model was used to create a contribution plot which is the average of spectral buckets in class 1 (endosperm rye) subtracted from the spectral buckets in class 2 (wheat) which is illustrated in fig.4-5. The positive signals represent the metabolites in the buckets that are higher in class 2 (wheat) and negative signals represents metabolites in the buckets that are higher in class 1 (endosperm rye)

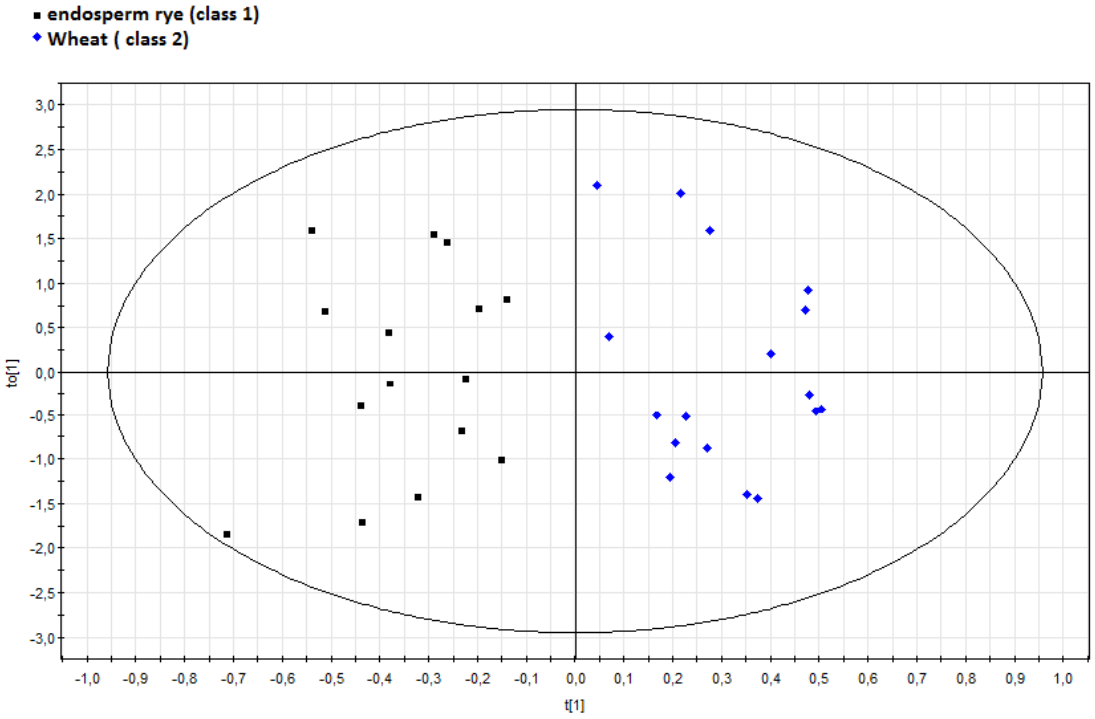

Fig 4-4 OPLS- DA score scatter plot of class 2 and class1 (30min)

OPLS-DA plot showing the distribution of the two classes (rye and wheat)

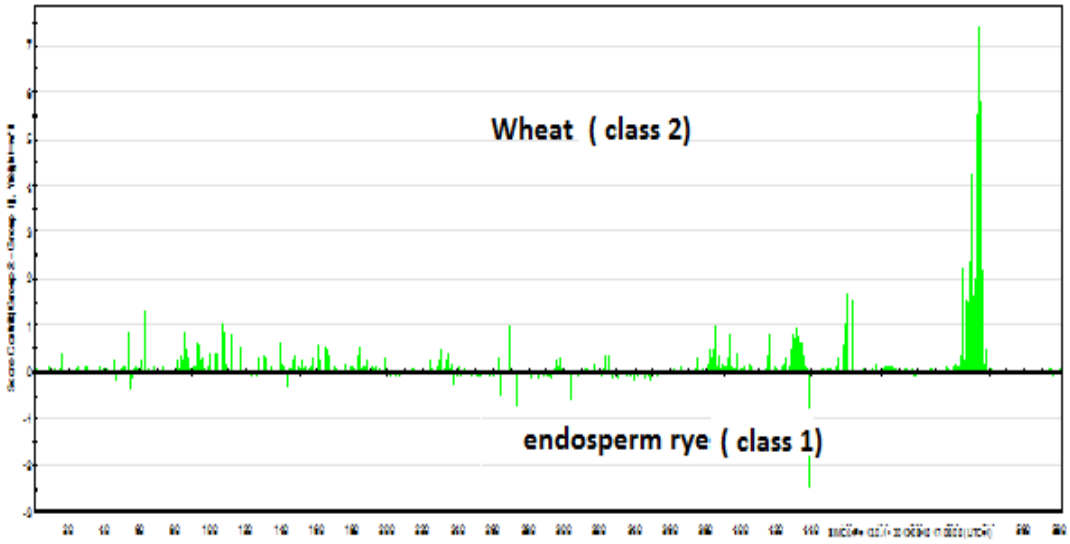

Fig 4-5Contribution plot for 30 min

The metabolites higher in wheat is observed as positive signal and higher in endosperm rye is observed as negative signal. Most of the metabolites are higher after wheat consumption

c. Identification of compound

The statistically significant NMR signals were identified by using The Chenomx NMR suite software version 7.1 (Chenomx Inc., Canada). NMR spectrum was imported into the reference library then compounds within the spectral region of the NMR signals were searched with the software which gave the probable compounds. Then identification was performed based on the shape and ratio of the signal .One of the example of identification of compound by the software is shown below Fig 4-6

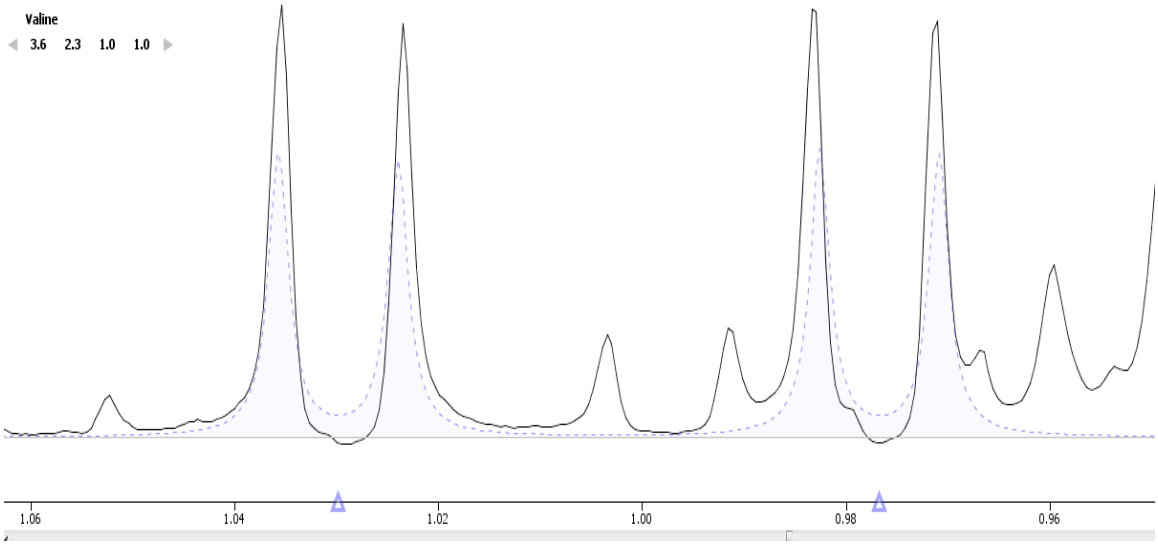

Fig 4-6 Part of NMR spectrum compared with the standard compound by Chenomx software.

The figure depicts how the chenomx software help in the identification of the compound. The dotted line represents the standard compound matched by the software i.e valine

Compounds within the spectral region (1.04ppm) were searched with the software and the dotted line in blue represent the matched signal of Valine by the software.

After identification of the compound univariate analysis (ANOVA) was conducted in class 1 and class 2 to check the significance of class discriminating metabolites. The class discriminating metabolites with its NMR signals and statistical significance are illustrated in the tables below.

Table 1 NMR based metabolomics analysis of serum from endosperm rye and wheat at 30 minutes (n=16)

Metabolite	NMR Signal(ppm)	VIP(CI)	p-Value
Dimethy Sulfon	3.157	3.537(2.294)	0.005
Isoleucine	0.942	3.48(1.776)	0.003
	1.007	5.124(2.9)	0.002
Leucine	1.73	2.647(1.634)	0.002
Phenylalanine	7.327	1.313(0.964)	0.010*
	7.381	1.216(0.844)	0.009
	7.421	1.428(1.234)	0.001*
Valine	1.039	3.824(3.037)	0.812
	1.051	4.622(3.482)	0.019
	2.5	1(0.762)	0.301
	2.5	1.143(0.962)	0.205
	3.614	2.601(2.41)	0.042
	3.621	2.289(2.273)	0.064

All discriminating metabolites were greater after intake of wheat bread compared to the rye bread
*shows the data which was log transformed before paired t test

Table 2 NMR based metabolomics analysis of serum from endosperm rye and wheat at 45 min (n=13)

Metabolite	NMR Signal(ppm)	VIP(CI)	p-Value
Dimethy Sulfon	3.157	1.965(1.644)	0.007
Glutamine	2.118	1.697(0.816)	0.008
	2.144	2.745(1.098)	0.008
	2.45	1.77(1.038)	0.024
	2.45	2.228(1.295)	0.035
Isoleucine	0.929	2.145(0.832)	0.003
	0.942	3.403(1.115)	0.000
	1.007	3.443(1.344)	0.000*
	1.97	1.79(0.829)	0.001
Leucine	0.964	6.056(1.965)	0.000
	1.67	1.533(0.572)	0.001
	1.731	2.356(0.812)	0.0001
Lysine	1.947	1.315(0.756)	0.012
Phenylalanine	7.34	1.252(0.819)	0.000
Valine	0.986	3.441(1.433)	0.002*
	1.051	3.237(1.462)	0.000*
	2.5	1.539(0.828)	0.000
	3.614	1.606(1.012)	0.002

All discriminating metabolites were greater after intake of wheat bread compared to the rye bread

*shows the data which was log transformed before paired t test.

Table 3 NMR based metabolomics analysis of serum from endosperm rye and wheat at 60 min (n= 15)

Metabolite	NMR Signal (ppm)	VIP(CI)	p-Value
Dimethy Sulfon	3.157	1.818(1.104)	0.011
Glutamine	2.133	2.851(1.239)	0.001
	2.155	2.567(1.345)	0.003
	2.45	2.179(1.017)	0.001
	2.45	2.824(1.694)	0.004
	2.45	3.047(1.759)	0.002
	6.9	1.786(0.731)	0.000
Isoleucine	0.942	3.534(1.144)	0.000
	1.019	2.693(0.738)	0.000
	0.942	1.853(1.115)	0.003
	1.97	1.7(0.803)	0.000
	1.97	1.989(0.886)	0.000
Leucine	0.964	6.704(1.507)	0.000
	1.67	1.42(0.661)	0.000
	1.705	2.756(0.646)	0.000
Phenylalanine	7.34	1.74(0.594)	0.000
	7.434	1.868(0.452)	0.000
Proline	2.012	2.777(1.357)	0.000
	2.055	1.823(1.16)	0.001
	2.077	1.83(1.154)	0.000
	2.335	1.906(0.939)	0.000
Valine	0.998	3.232(1.691)	0.000
	1.039	3.247(1.755)	0.001
	1.051	3.364(1.901)	0.001
	2.5	1.205(0.751)	0.002
	3.614	1.685(0.976)	0.003

All discriminating metabolites were greater after intake of wheat bread compared to the rye bread

There was significant rise in the AAs level after the consumption of wheat compare to rye in all the time periods (30, 45 and 60 minutes). The principal metabolites that were increased after wheat intake include branched chain AAs like leucine, isoleucine, and valine. In addition, there was also increase in the level of other AAs

like glutamine, proline and lysine. Furthermore, increase in the level of Dimethyl sulfon was also observed. The p- value was found to be significant in all these metabolites which were determined by mini tab software by using Anova test. The VIP and CI were determined by using software SIMCA which also reflected significant changes in the metabolites after intake of wheat and rye diet.

5. Discussion

In this study, rye bread consumption showed significantly lower amount of branched chain AAs (leucine, isoleucine, and valine) and certain other AAs like proline, serine phenylalanine, glutamine, lysine in comparison to wheat bread. This change in the AAs level may explain the increase in insulin response after intake of wheat bread in comparison to rye bread. In the previous study, increase in the level of insulin response was observed without change in the glucose level following wheat intervention compared to the rye, but its mechanism was unknown (Juntunens K and *et al*, 2003). Thus discrepancies in AA metabolism observed in the present study could explain the mechanism behind change in the insulin response. Certain insulin triggering AAs regulate the secretion of insulin from pancreatic β-cells *in vitro* and *in vivo*. However, AAs donot provoke insulin secretion as glucose *in vitro* but combined intake of different AAs with glucose showed synergistic effect (Newsholme P and *et al* 2006). *In vivo*, AAs from the intestinal cells and from dietary intake along with glucose induces insulin secretion, which has been observed in previous research (Loon L and *et al*, 2000). The administration of leucine and phenylalanine in presence of protein hydrolysate in diabetic patients as well as healthy people showed threefold increase in insulin secretion compared to administration of carbohydrate alone (Van LJ and *et al*., 2003). When islet of mice was incubated with mixtures of AAs at physiological concentrations, four AAs (leucine, isoleucine, alanine and arginine) were found to stimulate insulin secretion (Newsholme P and *et al*, 2006). The pancreatic β- Cells express many distinct AAs transport systems for the transport of various AAs including alanine, arginine glutamate, leucine, lysine, proline, and serine which results in the accumulation of AAs in the pancreatic β-cells. (Zhenping Liu, 2009). The long term exposure of AAs may influence gene expression in β-cell that finally alters insulin secretion. In addition to the AAs, enzymes like glutamate dehydrogenase, aspartate and amino transferase and malate aspartate shuttle also

affects the insulin secretion which also requires certain AAs for their activation. (Newsholme. P and *et al*, 2006)

The mechanism by which AAs stimulate the insulin secretion may involve both transcriptional and mitochondrial effects. There are three possible mechanisms that confer the stimulatory action of AAs (Newsholme P and *et al*, 2006; Zhenping Liu P. B., and *et al* 2009)

1) direct depolarization of membrane (cationic AAs like arginine and Lysine)
2) generation of ATP (alanine, leucine and proline)
3) depolarization of membrane by co-transport with Na+ (proline and alanine)

AAs like alanine, glutamine and glutamate activate mitochondrial metabolism which enter TCA (Tricarboxylic Acid) cycle and enhance the TCA cycle substrate. TCA cycle then produces reducing equivalent, which finally generates ATP (Adenosine Tri phosphate) after transferred to the electron transport chain (Newsholme P and *et al*, 2006; Zhenping Liu P. B., and *et al* 2009) ATP binds to the subunit of K+ channel which destabilize the channel open state and thus promotes channel closures. (Craig TJ, 2008) Thus increase in the ATP level leads to closure of K^+ channel resulting in depolarization of the membrane. Hence, voltage dependent Ca^{2+}channels open, increasing the influx of Ca^{2+} which triggers insulin exocytosis from the cell membrane. Some AAs like proline and alanine are co transported with Na+ and activates the voltage dependent Ca^2+ channels which also activates pyruvate dehydrogenase that increase the oxidation of pyruvate derived from glucose resulting in insulin secretion. Further, cationically charged AAs like arginine and lysine lead to direct depolarization of membrane at neutral pH in presence of glucose inducing insulin secretion from β cells. Other mitochondrial signals might also be generated that induces insulin secretion (Newsholme P, 2006 and *et al*; Zhenping Liu P. B., 2009 and *et al*).

Leucine can stimulate the insulin release by two of the mechanisms, one by serving as metabolic fuel and the other as an allosteric activator of glutamate dehydrogenase (GDH) (Liu Z and et al, 2008,Yang J and et al, 2010) The transamination of leucine to α- ketoisocaproate results in mitochondrial oxidation which enhances the insulin release. Leucine also activates GDH that results in oxidation of glutamate to the TCA

intermediate α ketoglutarate. A summary of model that reveals the regulation of insulin secretion in βcell by amino acid is illustrated in Fig. 5-1

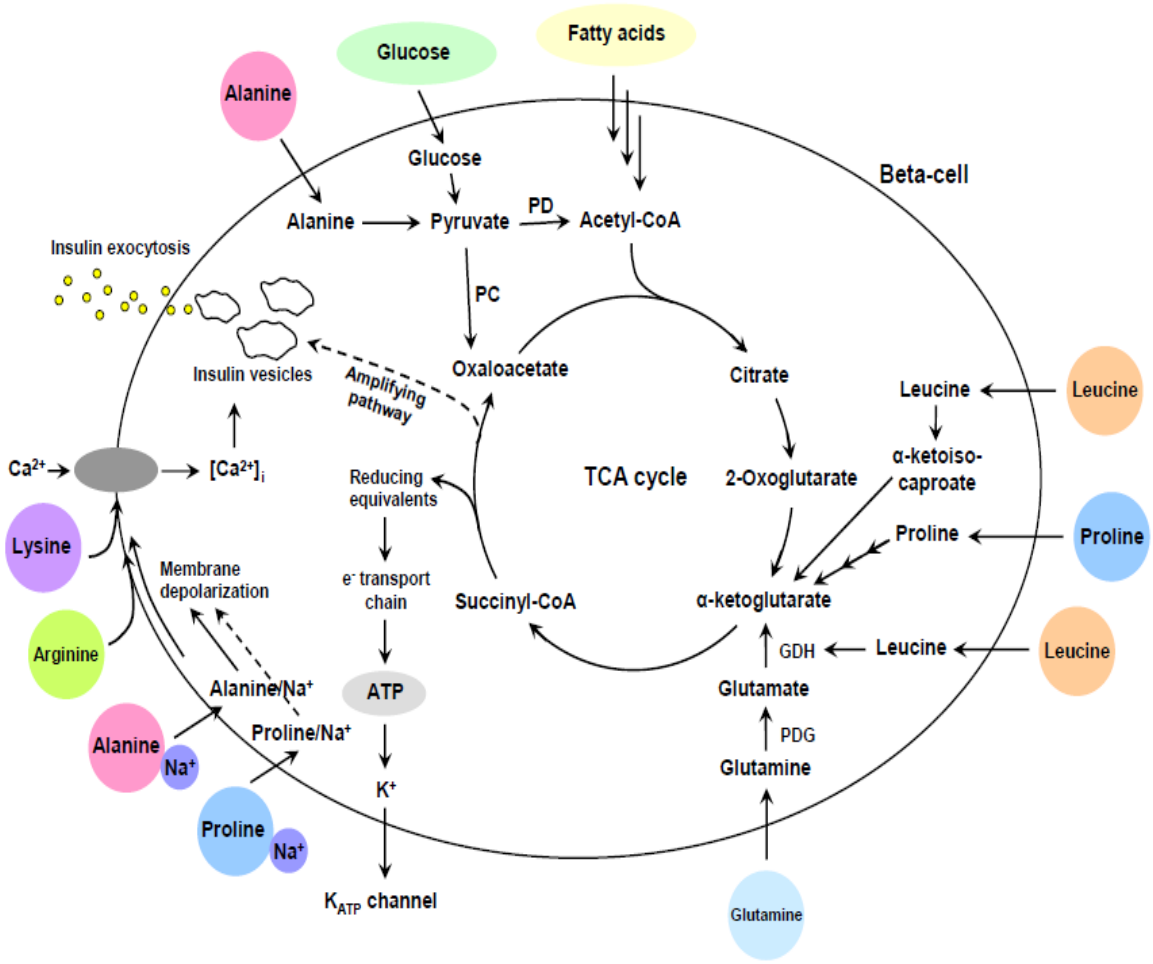

Fig 5-1 Model for insulin secretion in β cells stimulated by amino acids and glucose
Adopted from Liu Z and et al 2009
The AAs like leucine, glutamine increases the substrate for TCA cycle that enhance the ATP level which closes the K^+ channel. This leads to opening of voltage dependent Ca^+ channel , which ultimately causes insulin exocytosis from the β- cell of pancreas.

Several studies have demonstrated that Branched chain amino acid (BCAA) regulate protein synthesis by activation of mTOR (mammalian target of rapamycin) signaling pathway in pancreatic β-cells. mTOR is a serine threonine kinase which regulates cell

growth, proliferation, transcription and protein synthesis. The mTOR signaling pathway activates phosphorylation of ribososmal protein S6 which integrates

stimulation of insulin receptor and nutrient availability with protein synthesis.(Philip Newsholme, 2006) Leucine is found to be the most effective AA to activate mTOR pathway which stimulates the phosphorylation of p70 ^{S6K}and enhances the protein synthesis in pancreatic β cell. Similarly valine and isoleucine also enhance the protein synthesis by activation of p70 ^{S6K} and induce insulin secretion of pancreatic β- cell. However, leucine can stimulate gene transcription and protein synthesis in pancreatic islets by both mTOR dependent and independent pathway. (Yang J and *et al*, 2010)

Previous studies support the fact that phenylalanine can induce insulin secretion but its mechanism of action is not well understood. (Newsholme P and *et al,* 2006, Luc JC and *et al*, 2000). In addition there was also increase in the level of dimethyl sulfon after wheat consumption but the reason and its mechanism is still unknown. Thus the change in AA metabolism following the wheat and rye intervention could explain the increased insulin response after wheat consumption. The decrease in insulin response following rye intervention explains the nutritional benefits of rye compare to wheat. Many studies have revealed that hyperinsulinemia could be a risk for many metabolic abnormalities that includes insulin resistance, type II diabetes, dyslipidemia, impaired fibrinolysis and prostate cancer (Despres, JP, 1996). Thus lower levels of postprandial insulin response for prolong time can protect the pancreatic cell from overstimulation of pancreatic β-cells and improve cell function which explains the benefits of consumption of rye bread over wheat (Juntunen. S K and *et al*, 2003). In the previous study, glucose level were not different initially between wheat and rye response, but gluocse level fall below the fasting level between 2 to 3hrs of postpranadial studies. Thus decrease in the level of circulating glucose level suggests increase in hunger and stimulate the release of counterregulatory hormones. But the glucose curve in response to the rye bread remained above fasting level for longer time which may be due to the lower insulin response.Thus these findings could also explain the satiety effect of rye bread compare to wheat. However, further investigations are required to understand the detailed mechanism.

For future analysis, it would be better to compare the metabolites between rye bread of various fibres content which could give better explanation of rye factor. Furthermore, other hormones responsible for insulin secretion could also be measured to determine the other potential mechanism responsible for the insulin response and explain other benefits of consumption of rye.

6. Conclusion

The present study confers that discrepancies in amino acid metabolism could be one of the probable mechanisms that explain the decrease in insulin response following rye intervention compare to wheat. Further, it explains the advantage of consumption of rye compare to wheat.

7. Acknowledgement

I would like to express my deep gratitude to my supervisor Dr. Ali Moazzami for trusting me to be part of his project and guiding me through out my thesis period.

I would also like to give my especial thanks to my friends Maren Podszun and Ashish Lamichhane for their help and suggestion. In addition, I would like to thank all the members of Food science Department in SLU for their cordial behavior and for creating proper environment to work.

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9. Appendix

Characteristics of the subjects at the time of entry into the study

	Women
Age (y)	61 ± 4.8 (51-69)
BMI (kg/m^2)	26.0 ± 2.5 (22.5-30.2)
Systolic blood pressure (mm Hg)	125 ± 18 (96-151)
Diastolic blood pressure (mm Hg)	77 ± 9 (63-97)
Serum total cholesterol (mmol/L)	6.1 ± 0.8 (4.7-7.4)
Serum HDL cholesterol (mmol/L)	1.7 ± 0.3 (1.3-2.3)
Serum triacylglycerol (mmol/L)	1.1 ± 0.4 (0.7-2.2)
Oral-glucose-tolerance test	
Plasma glucose, 0 min (mmol/L)	5.7 ± 0.4 (5.2-6.9)
Plasma glucose, 120 min (mmol/L)	5.6 ± 1.0 (4.3-7.5)
Plasma insulin, 0 min (pmol/L)	44.4 ± 13.8 (25.3-74.5)
Plasma insulin, 120 min (pmol/L)	279.6 ± 175.8 (27.6-795.7)

- ↵1 $\bar{x}$ ± SD; minimum and maximum values in parentheses. n = 19.

Nutrient composition of the test bread portions

	Refined wheat bread	Endosperm rye bread
Portion size (g)	105.5	111.9
Available carbohydrate (g)	50	50
Total dietary fiber (g)	2.7	6.1
Insoluble dietary fiber (g)	1.5	3.1
Soluble dietary fiber (g)	1.2	3.0
Protein (g)	9.0	4.9
Fat (g)	5.2	3.4
Moisture (g)	38.2	46.7
Energy content (kJ)	1177	1056