NUTRI-METABOLOMICS

Effect and Exposure Markers of Apple and Pectin Intake

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PhD Thesis

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Title:
Nutri-Metabolomics. Effect and Exposure Markers of Apple and Pectin Intake.

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PREFACE

The first year of this PhD project was conducted at Department of Toxicology and Risk Assessment, The National Food Institute, Technical University of Denmark (FOOD, DTU). The two last years of the project were carried out at Quality and Technology (Q&T), Department of Food Science, Faculty of Life Science, and in collaboration with Department of Human Nutrition (IHE), Faculty of Life Science, University of Copenhagen. The project has been sponsored by a large European project called ISAFRUIT (Thematic Priority 5 – Food Quality and Safety of the 6th Framework Programme of RTD), and by SYSDIET, a Nordic Centre of Excellence in systems biology supported by the Nordic Council of Ministers, as well as NuBI, a Nutrigenomics data-integration grant from the Danish Ministry of Food, Agriculture and Fisheries. ISAFRUIT aims to reveal the biological explanation for the epidemiologically well-established health effects of fruits, and apples were selected as the study subject. SYSDIET support the work with multivariate analyses and NuBI supports the establishment of various 'omics platforms. The project has been supervised by Professor Søren Balling Engelsen and Professor Lars Ove Dragsted from University of Copenhagen.

I am grateful to my two supervisors, Søren, for your valuable knowledge, help and inspiration in regard to spectroscopy and multivariate analysis. Lars, for all kind of support during this process and for always keeping your door open for a fruitful and exciting scientific discussion.

I would like to thank my colleagues at Q&T, IHE and FOOD for a very pleasant, humorous and professional working environment. Special thanks to Flemming and Francesco for introducing me to NMR analysis and to my research group at IHE for valuable discussions and cheerful times.

I am grateful for the support from friends and family during this process and to Louise and Petrine for proof reading. Finally, thanks to Casper and Noah for your patience and for taking my mind elsewhere.

SUMMARY

Consumption of nutrients and other bioactive compounds from food interact with numerous targets, metabolic pathways and physiological functions in the organism and hereby potentially reduce or increase the risk of diseases. Analytical methods that can handle multiple responses may therefore seem particular beneficial compared to the univariate approaches most often used in nutrition research. Metabolomics is a new technique that allows measuring a large number of metabolites present in a given biological sample and the metabolic effect of e.g. a specific food intake can hereby be explored in a more global way than with traditional methods.

The aim of this project has been the establishment of a metabolomics platform utilising Mass Spectrometry (MS), Nuclear Magnetic Resonance (NMR) spectroscopy and chemometrics to investigate health potentials of apple and apple-pectin intake.

An explorative metabolomics approach was employed in Paper I to identify exposure and effect markers of 24 Fisher rats fed a diet supplemented with fresh apple or apple-pectin for 4 weeks. Urine was analyzed by liquid chromatography and mass spectrometry (LC-MS) and metabolites that responded to the apple or pectin diets were selected and classified as either exposure or effect markers based on response patterns. Quinic acid, m-coumaric acid and (-)epicatechin were identified as exposure markers and hippuric acid as one of the effect markers of apple intake. Pyrrole-2-carboxylic acid and 2-furoylglycine were identified as pectin exposure markers while 2-piperidinone was recognized as a pectin effect marker. None of these metabolites have been related to intake of pectin or other fibre products before. The metabolism and potential health aspects of these markers are discussed in this paper.

A targeted NMR-based metabolomics approach was employed in Paper II as an alternative, fast and reliable method to quantify cholesterol distribution in the different lipoprotein fractions in rats. Plasma from two rat studies (n = 68) was used in determining the lipoprotein profile by an established ultracentrifugation method and proton NMR spectra of replicate samples were obtained. From the ultracentrifugation reference data and the NMR spectra, interval partial least-square (iPLS) regression models were constructed in order to predict the amount of cholesterol in high, low and very low density lipoprotein (HDL, LDL and VLDL) as well as the total plasma cholesterol. The iPLS approach yielded fine regression models and was used to determine HDL, LDL, VLDL and total cholesterol in a study where 24 rats had been supplemented with two doses of apple-powder. A dose of 20% apple-powder significantly lowered HDL cholesterol. Thus, this method seems to be a strong and efficient way to quantify lipoprotein cholesterol in rat studies.

In Paper III the NMR-based PLS regression models developed in Paper II were used to investigate the cholesterol distribution in plasma lipoproteins in the same rat study as described in Paper I. Additionally, faecal bile acid excretion, plasma activities of selected hepatic enzymes and gene expression of antioxidant enzymes in the liver were investigated. LDL, HDL and total cholesterol as well as total and primary bile acids were significantly reduced in the apple group. Secondary bile acids showed a significant reduction after apple intake. Pectin did not exhibit any effects on cholesterol metabolism but significantly up-regulated plasma alkaline phosphatase (AlP). Both apple and apple-pectin intake revealed significant effects on genes involved in the hepatic glutathione redox cycle, indicating a higher capability to handle oxidative stress.

Overall, these investigations indicate that fresh apple may have health beneficial effects on cholesterol metabolism but from our results pectin cannot be appointed as the major decisive apple component that causes this effect. However, the investigations were conducted with rat models and it is important to stress cautious extrapolation to humans. The utilisation of the MS and NMR-based metabolomics approaches have served as competent platforms during these studies and the metabolomics technology seems very promising in further unravelling of the interplay between dietary intake and health status.

RESUMÉ

Indtag af næringsstoffer og andre bioaktive stoffer fra fødevarer påvirker adskillige metaboliske processer og fysiologiske funktioner i organismen og kan herigennem potentielt øge eller minske risikoen for at udvikle sygdom. Analytiske metoder, der kan håndtere mange responser samtidigt, er derfor særligt attraktive i forhold til de univariate metoder, som oftest anvendes i ernæringsforskning. Metabolomics er en ny teknik, hvor ideen er at måle størsteparten af de stoffer/metaboliter, der er tilstede i en given biologisk prøve. Herved kan den metaboliske effekt af f.eks. en bestemt fødevare undersøges i en større helhed, end det er muligt med traditionelle metoder.

Formålet med dette projekt har været at etablere en metabolomics platform, der anvender massespektrometri (MS), kernemagnetisk resonans (NMR) spektroskopi og kemometri for herigennem at undersøge sundhedsrelaterede egenskaber af æble og æble pektin.

En eksplorativ metabolomics tilgang blev anvendt i Artikel I for at identificere eksponerings og effekt markører fra 24 Fisher rotter der havde indtaget en kost tilsat frisk æble eller æble pektin gennem 4 uger. Urinen blev analyseret vha. væske-kromatografi og massespektrometri (LC-MS), og metabolitter, der reflekterede kosten tilsat æble eller pektin, blev udvalgt og klassificeret som enten eksponerings eller effekt markører på baggrund af deres respons mønster. Quinasyre, m-cumarsyre og (-)epicatechin blev identificeret som eksponerings markører og hippursyre som en af effekt markørerne for æbleindtag. Pyrrol-2-carboxylsyre and 2-furoylglycin blev identificeret som pektin eksponerings markører, hvorimod 2-piperidinon blev fundet som en effekt markør. Ingen af disse har tidligere været relateret til indtag af pektin eller andre fiber produkter. Metabolismen og potentielle sundhedsmæssige aspekter af disse markører diskuteres i artiklen.

En kvantitativ NMR-baseret metabolomics tilgang blev anvendt i Artikel II som en alternativ, hurtig og pålidelig metode til at kvantificere kolesterol-fordelingen i forskellige lipoprotein fraktioner i plasma fra rotter. Plasma fra to rottestudier (n = 68) blev anvendt til at bestemme lipoprotein profilen vha. en veletableret ultracentrifugerings metode og desuden blev proton NMR spektrer optaget af den samme prøve. Interval partial least-square (iPLS) regressions modeller blev opbygget ud fra ultracentrifugering reference data og fra NMR spektrene for at bestemme mængden af kolesterol i høj-, lav- og meget lav densitet lipoproteiner (HDL, LDL and VLDL) og total kolesterol i plasma. iPLS-metoden resulterede i gode regressions modeller, og blev brugt til at bestemme HDL, LDL, VLDL og total kolesterol i et forsøg, hvor 24 rotter havde fået tilsat to doser af tørret æble pulver til fodret. En dosis på 20% æble pulver

reducerede signifikant HDL kolesterol. Den anvendte metode vurderes som en kompetent og effektiv måde at kvantificere kolesterol i de forskellige lipoprotein fraktioner i rotte studier.

I Artikel III blev de NMR-baserede PLS regressions–modeller, der var udviklet i Artikel II, brugt til at undersøge kolesterol-fordelingen i plasma lipoproteiner i det samme rottestudie, som er beskrevet i Artikel I. Derudover blev galdesyre udskillelse i fæces undersøgt samt aktiviteten af udvalgte plasma enzymer og genekspression af antioxidant enzymer i leveren. LDL, HDL and total kolesterol samt total og primære galdesyrer var signifikant reduceret i æble gruppen. Sekundære galdesyrer viste en signifikant sænkning efter æble indtag. Pektin havde ingen effekter på kolesterol metabolisme, men opregulerede signifikant alkalisk fosfatase (AlP) i plasma. Både æble- og pektin-indtag viste signifikante effekter på gener involveret i leverens glutathion redox cyklus, hvilket tolkes som en forbedret evne til at håndtere oxidativt stress.

Forskningen præsenteret i denne afhandling antyder at indtag af frisk æble har fordelagtige helbredsmæssige effekter på kolesterol metabolisme, og ud fra resultaterne ser pektin ikke ud til at være den afgørende komponent i æble, der inducer denne effekt. Undersøgelserne præsenteret her er foretaget i rotter, og der må udvises forsigtighed med at overføre resultaterne direkte til mennesker. Anvendelsen af MS- og NMR-baseret metabolomics har i disse undersøgelser vist sig som kompetente analytiske platforme, og metabolomics teknologien som helhed vurderes som meget lovende i forhold til den fremtidige forståelse af samspillet mellem kost-indtag og sundhedsstatus.

LIST OF PUBLICATIONS

PAPER I

Kristensen, M., Engelsen, S.B. and Dragsted, L.O. 2010. LC-MS metabolomics top-down approach reveals new exposure and effect biomarkers of apple and apple-pectin intake. Submitted to Metabolomics.[1]

PAPER II

Kristensen, M., Savorani F., Ravn-Haren, G., Poulsen, M., Markowski, J., Larsen, F.H., Dragsted, L.O. and Engelsen, S.B. 2010. NMR and interval PLS as reliable methods for determination of cholesterol in rodent lipoprotein fractions. Metabolomics, 6:129–136.

PAPER III

Kristensen, M., Jensen, R.I., Krath, B.N, Markowsky, J., Poulsen, M. and Dragsted, L.O. 2010. Effects of apple and apple-pectin feeding on cholesterol metabolism and antioxidant response in healthy rats. Submitted to British Journal of Nutrition.

SUPPLEMENTAL MATERIAL

Gürdeniz, G., **Kristensen, M**., Skov, T., Bro R. and Dragsted, L.O. The effect of LC-MS data processing methods on the selection of plasma biomarkers in fed vs. fasted rats. 2011. Submitted to Analytical and Bioanalytical Chemistry.

[1] This paper was accepted after submission of the thesis and before press. Some changes have been made in the manuscript during the revision process and to avoid disturbance in relation to the thesis context the accepted version is enclosed as supplemental material instead of replacing the submitted manuscript.

OTHER PUBLICATIONS BY THE AUTHOR

Roldán-Marín, E., Krath, B.N., Jensen, R.I., **Kristensen, M**., Poulsen, M., Cano, M.P., Sánchez-Moreno, C. and Dragsted, L.O. 2010. An onion by-product affects plasma lipids in healthy rats. Journal of Agricultural and Food Chemistry, 58(9), 5308-5314.

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Kristensen, M., Krogholm, K.S., Frederiksen, H., Duus F., Cornett C., Bügel, S.H. and Rasmussen S.E. 2007. Improved synthesis methods of standards for quantitative determination of total isothiocyanates from broccoli in human urine. Journal of Chromtogarphy B, 852: 229–234.

Kristensen, M., Krogsholm, K.S., Frederiksen, H., Bügel, S.H. and Rasmussen, S.E. 2007. Urinary excretion of total isothiocyanates from cruciferous vegetables shows high dose-response correlation and may be a useful biomarker of ITC exposure. European Journal of Nutrition, 46: 377–382.

LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
AcCoA	Acetyl coenzyme A
BA	Bile acids
C	Cholesterol
CETP	Cholesterol ester transfer protein
CoA	Coenzyme A
COMT	Catechol-*O*-methyltransferases
CVD	Cardiovascular disease
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
FID	Free induction decay
GC	Gas chromatography
HDL	High-density lipoprotein
HMDB	Human Metabolome Data Base
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
iPLS	Interval partial least square
LC	Liquid chromatography
LDL	Low-density lipoprotein
LDL-R	Low-density lipoprotein cholesterol receptor
LPH	Lactase phloridizin hydrolase
MLR	Multiple linear regression
MS	Mass spectrometry
m/z	Mass to charge ratio
NMR	Nuclear magnetic resonance

PCA	Principal component analysis
PLS	Partial least square
PLS-DA	Partial least square discriminate analysis
QTOF	Quadropole time-of-flight
RCT	Reverse cholesterol transport
RF	Radio frequency
RMSE	Root mean square error
SCFA	Short chain fatty acid
SULT	Sulfotransferase
SGLT1	Sodium-dependent glucose transporter 1
TAG	Triacylglycerides
TOF	Time-of-flight
TSP	3-Trimethylsilylpropionic acid
UGTs	Uridine-5´-diphosphate glucuronosyltransferases
UPLC	Ultra high pressure liquid chromatography
VLDL	Very low-density lipoprotein

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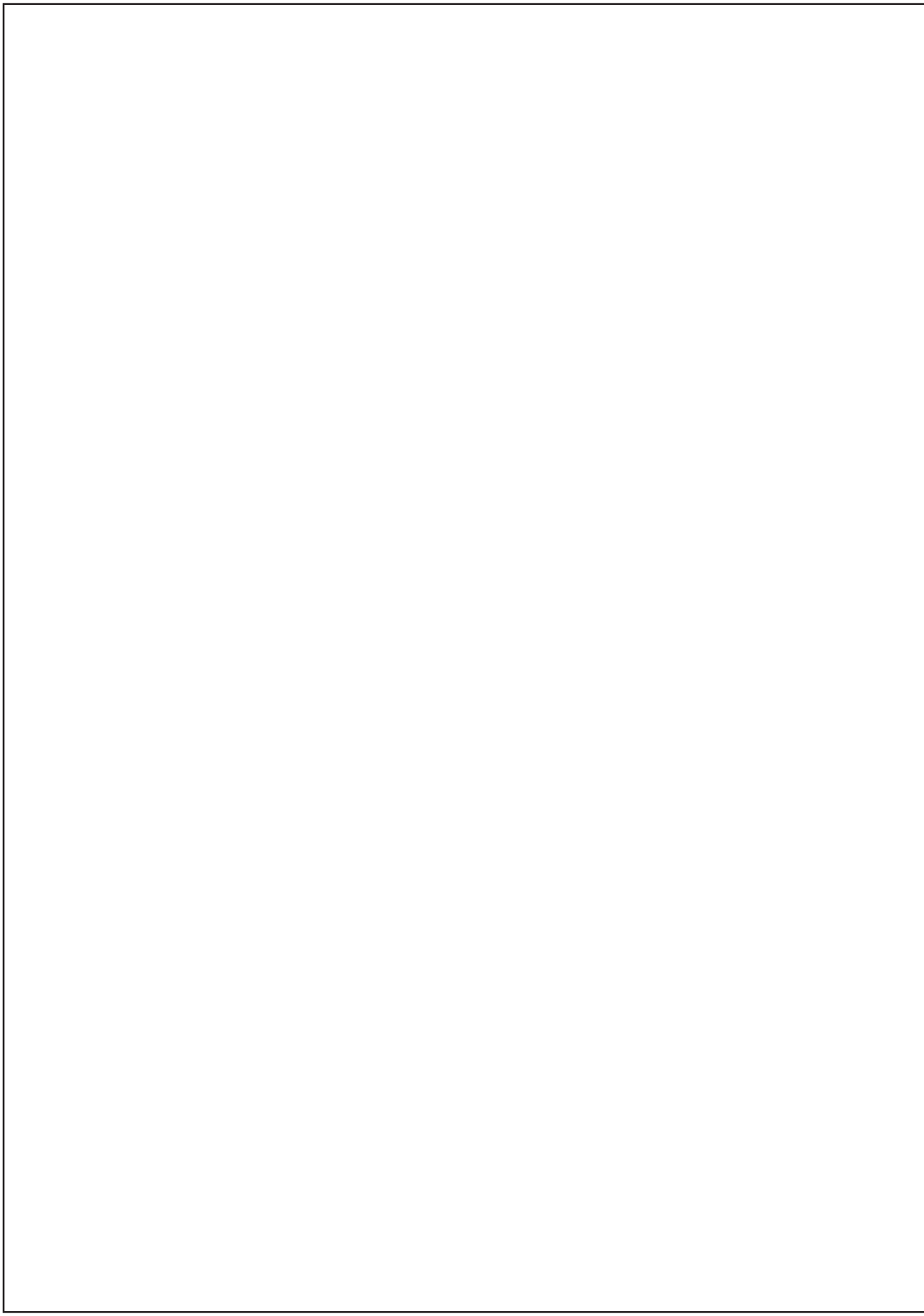


1 INTRODUCTION

1.1 BACKGROUND

The inter-play between dietary intake and disease has been investigated for many years with gradually more refined measuring methods being developed over time. Consumption of nutrients and other bioactive compounds from food will interact with numerous targets, metabolic pathways and functions in the organism and hereby potentially reduce or increase the risk of disease. Methods that can handle multiple responses may therefore be particularly beneficial compared to the classical univariate approaches most often used in nutrition research. Metabolomics is one of the latest developed approaches to access environmental influence on living systems, and this technique enables simultaneously measurement of large part of the metabolites present in a given biological sample, whereby the metabolic effect of e.g. a specific food intake can be explored in a more global way than with traditional methods (Scalbert *et al.*, 2009). This approach offers a unique possibility to measure the real end-points of physiological regulatory processes, the metabolites, either by use of nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) techniques and with subsequent exploration of the metabolic profiles with multivariate statistical analysis for biomarker identification. However, many factors may have a crucial influence on the final result and minimisation of unwanted sources of variation is very important in establishment of a reliable metabolomics platform. When this technology is properly established, the metabolomics approach may reveal new biomarkers, alterations in biochemical pathways and highlight associations between diet and disease risk. The measurement of metabolite profiles may also be applied profitably in a more targeted way to subtract quantitative information of *a priori* known effect markers, and the dual applicability of the metabolomics technology makes it a very suited and versatile tool in investigations of e.g. food intake and the corresponding physiological responses in living organisms.

In this project apple was selected as the nutritional subject, and its physiological responses were explored by means of the metabolomics technique. Apple remains one of the most consumed fruits in the Western World, and the health impact from intake of this fruit seems particularly relevant to investigate. Apple has a historical reputation of being a healthy component as illustrated by the popular expression, “an apple a day keeps the doctor away”, and several lines of scientific evidence suggest that apple and apple products posses a wide range of biological activities that may contribute to health beneficial effects against cancer, asthma, obesity, diabetes and cardiovascular diseases (CVD) (Boyer & Liu, 2004). However, the active factors and mechanisms responsible for these potential health promoting actions still remain unclear. In particular, an inverse association between apple intake and cholesterol metabolisms seems convincing (Aprikian *et al.*, 2001; Judd & Truswell, 1982; Sable-Amplis

et al., 1983a), and it has been reported that the cell wall polysaccharide, pectin, may be the fraction responsible for a lipid-lowering effect of apple consumption (Gonzalez *et al.*, 1998). Metabolomics analysis of biological samples from *in vivo* investigations of apple and pectin intake may shed new light on health aspects related to apple intake and assist in elucidation of mechanisms and bioactive components of this fruit.

1.2 AIM OF THE THESIS

The purpose of this project has been establishment of a reliable metabolomics platform utilising MS, NMR spectroscopy and chemometrics to investigate effects of apple intake. The project was divided into the following parts:

- Identify metabolomics exposure and effect markers of apple and pectin intake from a rat experiment (Paper I).
- Establish NMR-based Partial Least Square (PLS) regression models for rapid and reliable quantification of the plasma lipoprotein profile in rats (Paper II).
- Apply the NMR-based PLS regression model to the same rat study as in Paper I and to rats supplemented with apple-powder. Hereby to investigate the effect on cholesterol metabolism of dried apple (Paper II), fresh apple and pectin intake (Paper III).
- Evaluate the health effects of apple intake through the various markers.

1.3 THESIS OUTLINE

The thesis consists of an introductory part followed by three papers (Paper I, II and III). A co-authoring paper (not yet published) is enclosed as supplemental material. Papers I and III are based on the same animal experiment. In the introductory part some experimental results from the papers are presented to highlight general concepts.

Chapter1 emphasises the importance of novel tools in nutrition research, explains why apple was selected as the nutritional case and provides the general aims of the thesis.

Chapter 2 serves as an introductory text to the field of metabolomics research and provides a brief theoretical background of the different methods used in this project as well as the considerations of 'good practice' when performing nutri-metabolomics experiments.

Chapter 3 describes the chemical composition of an apple and the absorption, metabolism and potential mechanisms of action in relation to CVD of proposed bioactive apple components.

Chapter 4 provides an overview of results and discussion of Paper I, II and III and further considers aspects and reflections that did not found their way into the papers.

Chapter 5 summarises with a conclusion of the thesis and provides the perspectives for the future use of metabolomics in nutrition research.

2 METABOLOMICS

2.1 METABOLOMICS IN NUTRITION STUDIES

Metabolomics is a term used to describe the study of small molecule intermediates and products of metabolism present in biofluids, tissues and cellular extracts. The word is coined in analogy with genomics and proteomics, and while these two terms reveal possible functions of a biological system, metabolomics represents its actual state (Giovane *et al.*, 2008). The word *metabolome* was introduced for the first time by Oliver *et al*. (1998) as the set of low-molecular-mass compounds synthesised by an organism. A few years later the term *metabolomics* was introduced, as the identification and quantification of every single metabolite in a biological system (Fiehn, 2002). The two terms *metabolomics* and *metabonomics* are often used intertwined. They were initially defined separately with origins in plant science and pharmacology, respectively, but in effect mean the same, and the word metabolomics is now more widely accepted (Metabolomics Society, 2010) and will be the term used in the ensuing sections. The word *nutri-metabolomics* is used in this thesis to cover *in vivo* metabolomics studies in relation to nutrition.

The metabolome consists of a large number of small metabolites (< 1,500 Da) belonging to a variety of different compound classes, such as amino acids, peptides, organic acids, lipids, nucleotides etc. The exact number of metabolites from humans is unknown but is estimated to be around 20,000 with wide concentration ranges spreading over nine orders of magnitude (Giovane *et al.*, 2008). Several players have an impact on the metabolome in humans and animal, and the metabolome can be divided into 1) the endogenous metabolome, which includes the metabolites produced by cells or tissues in the host, 2) the xenometabolome, which includes foreign metabolites derived from e.g. drugs and dietary compounds, 3) the food metabolome with the metabolites deriving from digestion of food and 4) the microbial metabolome produced by the gut microbiota (Manach *et al.*, 2009). Altogether, this leaves a complex metabolome signature depending on genetics and on diet as well as environmental variations that the host has been exposed to.

The first published study in which the metabolomics approach was used in a nutritional experiment applied NMR technology to measure the effect of dietary soy supplementation (Solanky *et al.*, 2003), and after this several other NMR-based nutri-metabolomics studies have been conducted e.g. Holmes *et al.* (2008), Lenz *et al.* (2004), Stella *et al.* (2006). MS-based nutri-metabolomics had its beginning a little later with one of the first studies investigating polyphenol concentrations in human urine after intake of polyphenol-rich beverages (Ito *et al.*, 2005), and more studies have followed (Paper I; Fardet *et al.*, 2008a; Fardet *et al.*, 2008b; Shen *et al.*, 2008; Gürdeniz *et al.*, 2011). Typically, the different types of

metabolomics analysis can be separated into two major groups: non-targeted and targeted, depending on the aim of the research, and these approaches are briefly described below.

2.1.1 Non-targeted analysis

The non-targeted, or explorative, metabolomics approach provides a hypothesis-free global overview of abundant metabolites. During a non-targeted approach, the compounds are not initially identified, and the features of all potential compounds are considered for further analyses. This approach is often referred to as *metabolic fingerprinting*, since the intention is not to identify each observed metabolite, but instead to compare 'fingerprints' or patterns of changes in response to e.g. dietary intake or disease status (Dettmer *et al.*, 2007). However, a completely 'true' non-targeted analysis is never possible, since the chosen analytical method and experimental perturbation always affects the metabolite outcome. After selection of metabolites of interest from the non-targeted analysis a more targeted approach is required for biological interpretation. Identification and to some extent quantification of the selected metabolites is necessary in order to provide biological insight and understanding of underlying mechanism of action.

2.1.2 Targeted analysis

The targeted metabolomics approach focus on identified metabolites or pre-selected metabolic pathways. The term targeted metabolomics analysis in this thesis covers what is sometimes called *targeted analysis, targeted profiling* or *quantitative metabolomics* in the literature. The central thing for these terms is that analytical peaks (or latent peak regions) are initially identified and subsequently quantified. This kind of analysis is characterised as a hypothesis-driven approach rather than a hypothesis-generating. The term *metabolic profiling* is often used for a partly non-targeted approach where the metabolomics data are scanned for specific compounds normally collected in a reference library, but at least some of the metabolites may not be known in advance. However, the metabolic profiling approach is not used in this project and will not be considered further.

2.2 THE METABOLOMICS PIPELINE

To obtain fruitful and reliable results from metabolomics studies, numerous factors have to be carefully considered. These aspects are summarised in Figure 1, which illustrates the workflow of a metabolomics study. The following sections will consider the issues that need

special concern when working through the metabolomics pipeline, and examples from Paper I-III will be given when appropriate.

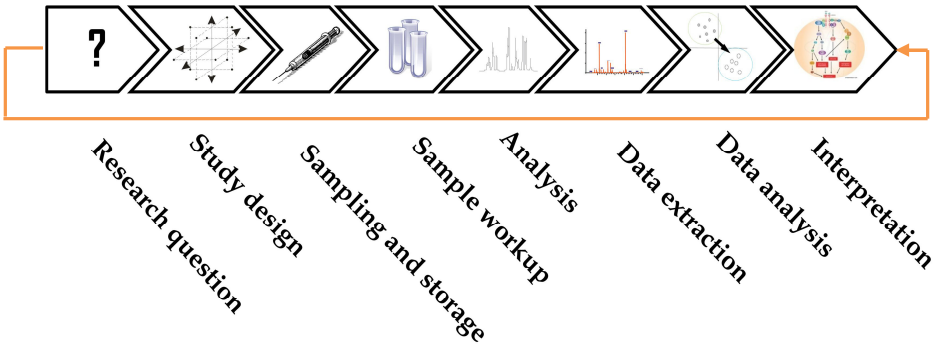


Figure 1. Illustration of the metabolomics pipeline, the workflow of a metabolomics study.

2.3 STUDY DESIGN AND SAMPLING STRATEGIES

2.3.1 Study design

Selection of an adequate study design is a recurring issue in all experimental studies; however, the high dimensionality of 'omics data means that it needs special attention in these types of studies. A general problem in metabolomics studies is the relatively low number of samples compared to the number of variables, and this rectangular shape of the data can be problematic in data analysis and hereby in extracting the correct biological information. Therefore, the highest possible number of samples should be on aim when designing a study.
In nutri-metabolomics studies it seems particularly important to control the dietary intake due to the large diversity of compounds present in different food items. Additionally, because of the often high inter-individual variation (especially in human studies), a full cross-over design should be preferred to parallel studies (Scalbert *et al.*, 2009). The most commonly used biological samples for nutritional metabolomics studies are the easy accessible samples; urine, saliva and blood/plasma/serum (Giovane *et al.*, 2008), and sampling time is an important issue to consider in the study design in regard to the research question asked, especially for urine due to high diurnal variation (Maher *et al.*, 2007).

2.3.2 Sample collection

Metabolomic experiments are most often designed to capture a snapshot of the metabolome, and the objective of sampling is to inhibit or stop metabolic flux to allow the snapshot to be representative of the metabolome before sampling. Therefore, great care must be taken to preserve the original information and variance in the biological sample, and any degradation of metabolites after sample collection should be avoided to the greatest possible extent, to ensure appropriate quantification and reproducibility among samples.

The work included in this thesis explores urine and plasma samples analysed by ultra high pressure liquid chromatography quadropole time-of-flight MS (UPLC-QTOF-MS) and ^{1}H NMR spectroscopy, and the best possible preservation procedures for these sample has been worked out as a compromise between the two analytical techniques. A proton-free preservative is preferred to limit interference with ^{1}H NMR spectra, and addition of NaN_3 was selected as the urinary preservative (Paper I) as recommended from investigations by Lauridsen *et al.* (2007). This was confirmed by Saude & Sykes (2007), who showed that NaN_3 reduced the changes in metabolite concentration when urine was kept at room temperature. Addition of NaN_3 to the sample collection devise (as described in Paper I) is only possible prior to collection in animal studies and not in human studies, due to safety issues of this highly toxic chemical. Urine will most often be contaminated with microorganisms, and the added preservative, but also cooled conditions, will minimise the microbial conversion of metabolites and in this way keep them representative for the biological situation they originally derived from. Keeping the urine below 5˚C is recommended (Maher *et al.*, 2007) and a urinary cooling method was developed for collection of 24 hour rat urine as described in Paper I.

When considering blood sample collection, the microbial aspect is less important, but instead enzymatic metabolite degradation may be pronounced. Blood samples should therefore be handled as cold as possible to preserve the metabolic snapshot in the most optimal way. For plasma samples, the anticoagulant to be used must be considered carefully, hereby avoiding possible unwanted peaks in the mass or NMR spectrum and additionally reducing oxidation of plasma to the highest possible extent. EDTA, heparin and citrate are the normal anticoagulants to choose from. Heparin is the preferred plasma anticoagulant to be used in NMR-based metabolomics experiments due to low introduction of interfering peaks (Beckonert *et al.*, 2007) whereas no general recommendations is present for LC-MS based metabolomics. However, for targeted lipidomics by LC-MS the used of EDTA is recommended to minimise loss of lysophospholipids (Seppanen-Laakso & Oresic, 2009). Both urine and plasma samples should be handled quickly and stored preferably at -80˚C,

where they can be kept for at least 9 months without significant changes in the metabolic profile (Beckonert *et al.*, 2007).

2.3.3 Sample preparation

Sample workup before analysis depends on the type of sample being analysed, the analytical method and whether specific metabolites or all metabolites are of interest. For non-targeted LC-MS analysis of urine, no specific sample preparation (besides centrifugation) has to be employed, but dilution with water and/or filtration is a possibility to protect the LC-MS system. On the contrary, plasma contains a lot of proteins which will need removal before LC-MS metabolomics studies. Different plasma and serum deproteinisation methods in combination with LC-MS profiling were investigated by Want *et al.* (2006) and Bruce *et al.* (2008) who found that protein precipitation with respectively, 100% methanol and 80% methanol resulted in the highest number of metabolites and reproducibility. During establishment of the LC-MS metabolomics platform in our laboratory a high-throughput plasma deproteinisation method was further developed from the results of Bruce *et al.* (2008) and Want *et al.* (2006), and the procedure is presented in Gürdeniz *et al.* (2011) (see supplemental material). Regarding NMR-based metabolomics, no particular pre-treatment is necessary for plasma samples besides dilution with a deuterated lock solvent. Addition of the reference compound 3-trimethylsilylpropionic acid (TSP), which is normally used in ^{1}H NMR experiments, is not recommended in plasma or other samples with high protein content due to protein binding and hereof much reduced signals (Beckonert *et al.*, 2007). The natural occurrence of α-glucose was used as reference compound in Paper II as suggested by Pearce *et al.* (2008). In ^{1}H NMR analysis of urine samples, special concerns has to be focused on minimising the chemical shift due to difference in pH between the samples, and a buffer should be applied to the sample. Typically, a phosphate buffer in D_2O and with TSP as a reference compound is used. Generally, samples should be kept cold while queued for analysis, and it is recommended to run one or two aliquots of a representative biofluid sample across the whole run as quality control measure (Beckonert *et al.*, 2007).

2.4 ANALYTICAL PLATFORMS

The metabolome is dynamic, changing from second to second. Although the metabolome can be defined readily, it is not currently possible to analyse the entire range of metabolites by a single analytical method and multiple analytical platforms are needed to increase the coverage of the metabolome. LC-MS, GC-MS and ^{1}H NMR spectroscopy are the most suited and commonly most used platforms for metabolomics studies (Oresic, 2009). LC-QTOF-MS

utilising electro spray ionisation (ESI) and NMR spectroscopy were the platforms selected in this research project. The basic principles of these two techniques are initially described, and their individual application for metabolomics are discussed at the end of this section.

2.4.1 UPLC-QTOF-MS

UPLC-QTOF-MS is a hyphenated technique, initially taking advantage of chromatography whereby it is possible to separate constituents of complex mixtures into single components (chromatographic peaks) and subsequently introduction of the fractionated eluate into a mass spectrometer for measurement of mass in relation to charge (m/z) of molecules and atoms.

2.4.1.1 Ultra high Pressure Liquid Chromatography

Liquid chromatography in general is a very efficient separation technique, where molecules are separated by using small differences in their distribution in two-phase systems, consisting of a mobile and a stationary phase. Aqueous solutions of acetonitrile and methanol are the most common mobile phases, and molecules dissolved in the mobile phase are separated as the mobile phase passes through the stationary phase, depending on their distribution coefficient in the two phases. By reversed phase chromatography, which is the method applied in Paper I, separation mechanism depends on the hydrophobic interaction between the molecules in the mobile phase and the immobilised hydrophobic ligand in the stationary phase. Experimental conditions are designed initially to favour adsorption of the molecules from the mobile phase to the stationary phase and subsequently, the mobile phase composition is modified to favour desorption of the molecules from the stationary phase back into the mobile phase (Plumb *et al.*, 2004; Poole, 2003).

One of the primary drivers for the growth of the chromatographic technique has been the evolution of packing materials used to improve separation between peaks. Compared to the more classical high pressure liquid chromatography, the recently developed UPLC technology takes additional advantage of chromatographic principles in running separations by using columns packed with smaller particles and/or higher flow rates for increased speed, resulting in improved resolution and sensitivity (Plumb *et al.*, 2004).

2.4.1.2 Quadropole Time-of-Flight Mass Spectrometry

The main features of a mass spectrometer consist of: an ion source, where the analytes are ionised and transferred to the high vacuum of the mass spectrometer; a mass analyser where ions are separated according to mass to charge ratio; a detector to measure the ion current

(amount of ions) or the ion number (by counting) as a function of time (Villas-Boas *et al.*, 2007). A QTOF mass spectrometer is illustrated in Figure 2.

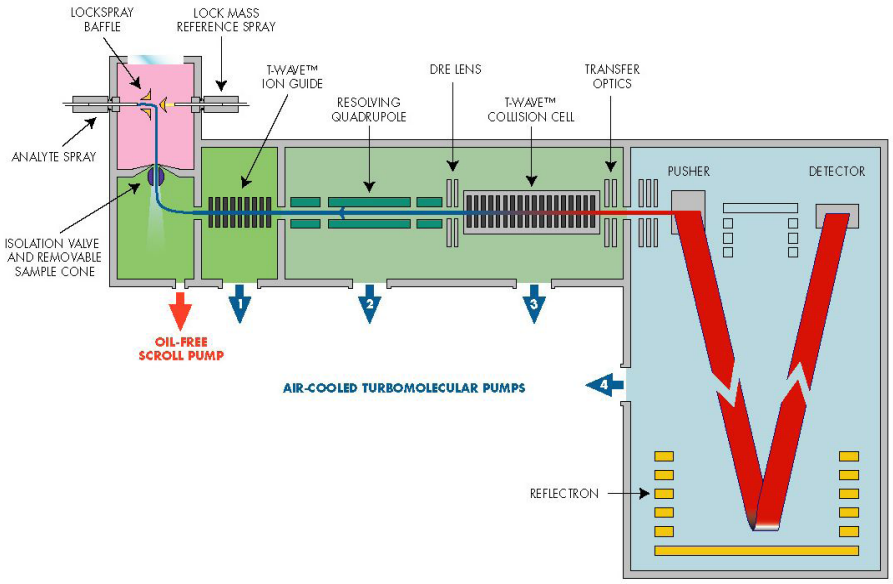


Figure 2. Schematic illustration of a Waters Q-TOF Premier mass spectrometer with a single V reflectron flight path. From Waters (2005) with permission.

When the sample elutes from the chromatographic system, the sample is injected into the ion source where the molecules are converted to a charged or ionised form. Various different ion source techniques are used in metabolomics with the electro spray ionisation (ESI) being the most commonly used when coupled to liquid chromatography (Dettmer *et al.*, 2007). ESI involves the passage of a solution through a needle held at high voltage relative to a counter electrode. The fine mist of droplets that emerge from the needle tip possess a net positive or negative charge determined by the polarity of the needle and are attracted to the entrance of a mass analyser (Villas-Boas *et al.*, 2007).

From the ion source the ions are guided into the mass analyser. The QTOF technology provides both a quadropole and time-of-flight mass analysers with an intermediate collision cell for possible fragmentation. A quadrupole mass analyser consists of four metal rods arranged in parallel where those opposite to one another are electrically connected by a radio frequency (RF) voltage supply. This creates an alternating electrical field between the rods. The charged molecules enter the quadrupole axially after they have been accelerated to a

required linear energy. Once inside the quadrupole they start spinning within an imaginary cylinder created by the RF voltages. The diameter of the imaginary cylinder depends on the mass-to-charge ratio (m/z) of the ion and the RF voltage. Only ions within a certain m/z range will survive all the way through the quadrupole (Villas-Boas *et al.*, 2007). In the study reported in Paper I the quadropole was operated as an ion filter, allowing the ions from 50-1000 m/z to pass through the quadropole for accurate measurement by the TOF. The TOF is a high resolution MS instrument and functions by applying high voltage pulses to orthogonally accelerate ions into a high vacuum flight tube and a reflectron to reflect them back towards a detector. The mass-to-charge ratio is related to time-of-flight with smaller m/z's reaching the detector first (Waters, 2005), and mass spectra can be created with a mass resolution up to 10,000.

2.4.1.3 Application of UPLC-QTOF-MS in metabolomics experiments

The excellent sensitivity and high selectivity of a UPLC-QTOF-MS platform makes this instrument a great candidate for explorative metabolomics experiments of non-volatile compounds in a solution. The high resolution allows detection of metabolites of the same nominal mass but different monoisotopic mass, and, combined with a 5 ppm mass accuracy, the molecular formula can tentatively be determined of many metabolite peaks. The UPLC chromatographic separation minimises the overlap of peaks, which again improves mass accuracy, and additionally this method facilitates high-throughput analysis (e.g. 6 min/sample for the study in Paper I). The chromatographic separation provides very efficient knowledge of the polarity of an unknown molecule, and the elution time is an important characteristic in structure elucidation of unknown and/or isomeric compounds.

A major issue and disadvantage encountered in ESI is what is known as *matrix effects* or *ion suppression*, and e.g. when analysing complex mixtures like urine and plasma, one analyte may be much more efficiently ionised than others (stealing more charge that expected from the concentration) resulting in suppression of other compounds. This will result in some types of compounds being quantitatively over estimated and others under estimated (Villas-Boas *et al.*, 2007). Therefore, the best quantitative results may be observed by use of isotopically labelled reference metabolites for each metabolite in a targeted analysis, but this is not a usable approach for non-targeted profiling (Scalbert *et al.*, 2009). Compared to triple quadropole MS, ion-trap-MS and NMR, the TOF-MS has a limited dynamic range and is therefore not suited for highly quantitative purposes.
Analysis of the sample in both positive and negative ionisation mode will result in numerous overlapping analytes detected in the two modes but also a significant amount of unique compounds and it is highly recommended to do ionisation in both modes in non-targeted

analysis to obtain a broad coverage of the metabolome (Dettmer *et al.*, 2007). Additionally, a minimum of two analytical replicates should be obtained when running ESI LC-MS metabolomics experiments, since the matrix effect may cause some slip in the detection of analytes.

2.4.2 ^{1}H NMR spectroscopy

2.4.2.1 Nuclear magnetic resonance spectroscopy

High-resolution NMR spectroscopy is capable of providing detailed information on solution-state molecular structures based on atom nuclear interactions and properties. The theory of NMR was initially proposed by Pauli in 1924 who suggested that certain atomic nuclei should have the properties of spin and magnetic moment and that exposure to a magnetic field would consequently lead to the splitting of their energy levels (Pauli, 1924). However, it was first in 1946 that the NMR phenomena was experimentally discovered independently by Block & Packard (1946) and Purcell *et al.* (1946) and they were later awarded the Nobel price in physics 1952.

Subatomic particles (electrons, protons and neutrons) can be considered as spinning on their axes. In atoms such as ^{12}C and ^{16}O, where the number of neutrons and protons are both even, these spins are paired against each other, such that the nucleus of the atom has no overall spin and cannot be detected by NMR. However, in some atoms, such as ^{1}H and ^{13}C, where the number of neutrons and/or the number of protons is odd, then the nucleus has a half-integer spin (i.e. 1/2, 3/2, 5/2), and the nucleus does possess an overall spin measurable by NMR (Lambert & Mazzola, 2004; Stryer, 1995).

NMR spectroscopy functions by the application of strong magnetic fields and RF pulses to the nuclei of atoms. All nuclei are electrically charged, and any that have a spin generate a small magnetic field. When an external magnetic field is applied, an energy transfer is possible from the low-level to a high-energy level of the nuclei. The energy transfer takes place at a frequency that corresponds to the RF, and when the spin returns to its low-level state, energy is emitted at the same frequency. The signal that matches this energy transfer is measured in several different ways and processed in order to give an NMR spectrum for the nucleus concerned. The precise resonant frequency of the energy transition is dependent on the effective magnetic field at the nucleus, and this field is affected of shielding by electrons orbiting the nucleus. Consequently, nuclei in different chemical environments absorb energy at slightly different resonance frequencies, and this effect is referred to as the chemical shift. This also means that sample conditions, such as pH and ion strength, will affect the observed

spectrum. The chemical shift for ^{1}H NMR is determined as the difference in fractional units, δ (ppm), between the resonance frequency of the observed proton and that of a reference compound (e.g. α-glucose set at 5.23 ppm in Paper II). The measured chemical shift of most protons is typically in the range of 0-10 ppm. A particular proton usually gives rise to more than one NMR signal because of the influence of non-equivalent neighbouring protons, an effect called *spin-spin coupling*. The signal intensity depends on the number of identical nuclei, and thus inherently quantitative (Dunn & Ellis, 2005; Lambert & Mazzola, 2004; Stryer, 1995). An example of a ^{1}H NMR spectrum of rat plasma from 0-6 ppm is shown in Figure 3. A recurring issue in NMR measurement of biofluids is the extreme high signal of water which reduces metabolic information in the spectrum. To eliminate this, a water-suppression pulse sequence can be applied as illustrated in Paper II.

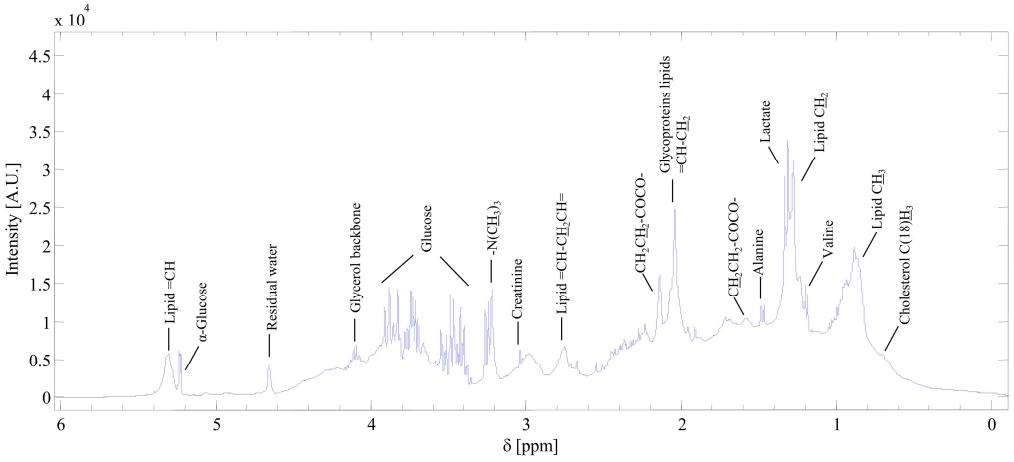


Figure 3. An average ^{1}H NMR spectrum of rat plasma at 311 K including assignment of the most prominent peaks.

2.4.2.2 Application of NMR in metabolomics experiments

NMR spectroscopy is a nondestructive and noninvasive technique with a high reproducibility and ability to simultaneously quantify multiple classes of metabolites. ^{1}H NMR spectroscopy exhibits high non-selectivity, meaning e.g. that this technique excels in identifying all proton-containing compounds in a sample. Because of the high natural abundance of ^{1}H (~99.985%), it high gyromagnetic ratio and its prevalence in metabolites, this nucleus is the most used for metabolomics experiments in NMR measurements (Beckonert *et al.*, 2007; Moco *et al.*, 2007). Generally, metabolomics studies of biofluids have shown high reproducibility when

using NMR and in most cases only one analytical replicate is sufficient per time point (Beckonert *et al.*, 2007).

The major disadvantage of NMR spectroscopy, as compared to MS, is the low sensitivity and resolution of this technique. From this point NMR application is not a first-choice for explorative metabolomics analysis to identify new biomarkers. Thus, the development of instruments with higher magnetic field strength and cryogenically cooled probes has pushed the limits of detection (Keun, 2006), improving their use in non-targeted metabolomics. In this case the later spectral comparison demands that the spectrum acquisition and control of conditions should be very precise. Small changes in pH, temperature and presence of impurities or degradation of sample material should be minimised since these factors may lead to detection of false metabolic changes and hereby incorrect selection of potential biomarkers (Moco *et al.*, 2007).

The nature of NMR as a quantitative technique due to the number of nuclear spins is directly related to the intensity of the signal, makes a targeted metabolomics approach an evident option. Biofluid NMR analysis is also often done with *a priori* knowledge of what the data will reveal about a specific target. It would be expected that the response pattern of several analytes is reflective of a physiological change in e.g. disease status or dietary habits, and the comprehensive nature of an NMR metabolome data set may enable a global evaluation of the systemic response. This can be useful in itself but the pattern may also be searchable for specific analyte information that, solely or in combination, can provide new mechanistic relevance (Robertson, 2005).

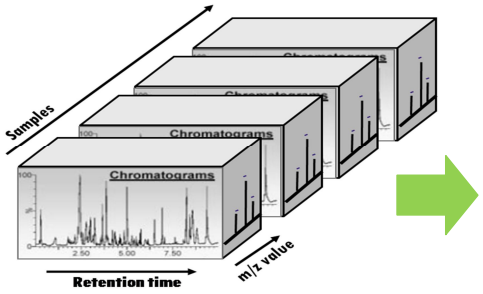
2.5 DATA EXTRACTION AND PREPROCESSING

The complexity and richness which are some of the key qualities of metabolomics data also makes data extraction and analysis very complicated. Since the metabolome changes from a dietary intervention may be rather discreet (e.g. compared to a medical intervention) data extraction errors will have a dramatic impact on the outcome of a study and therefore needs great attention.

2.5.1 Data extraction of LC-QTOF-MS data

Metabolomics raw data from MS systems are normally collected in centroid spectra or at least transformed to this format from continuous spectra as the first thing to reduce spectra

complexity before peak extraction. The software that obtains LC-MS data usually stores data as non-uniform sample data files, each consisting of a two-dimensional (2D) intensity matrix represented by scan number or retention time in the first dimension and m/z values in the second dimension. These data can be transformed to uniform length and combined as a 3-dimentional (3D) array (mass x scan x sample) for later multi-way data analysis, such as parallel factor analysis (Smilde *et al.*, 2004). However, the statistical analysis of this 3D high-resolution array demands extreme computational power and is therefore difficult to handle. Consequently, this kind of data is usually processed as a collapsed two-way data matrix where specific retention times with corresponding mass (a feature or marker) serve as the first dimension and samples as the second dimension (see Figure 4).



Rt. time	Mass	Sample #1	Sample #2	Sample #3	Sample #4	…
1,7686	**458,0276**	0,1893	0	1,3816	1,1824	
2,2397	**433,1481**	2,8412	3,1691	9,7891	8,306	
1,5782	**415,1156**	0,2038	0	0,1509	0	
1,5117	**401,1095**	0,5289	0,5715	0,3131	0,4461	
⋮	⋮					

Figure 4. Three-dimensional structure of LC-MS raw data (left) and the two-dimensional structure (right) of the collapsed dataset after Markerlynx^{TM} data extraction.

Various different software products are available to assist in data extraction, and an overview and description of commercial and freely available software up until 2007 is described in Katajamaa & Oresic, (2007). Data can also be extracted by used of in-house built software/algorithms, where Matlab (MatWorks) is a suitable and flexible environment, although it demands highly experienced user knowledge.

The central aspects of data extraction include matching the peaks extracted from the different samples and aligning all masses and scans across the entire data set. A reasonable threshold level should be applied to reduce noise and to be able to identify significant markers among the peaks. Values lower than the threshold are then considered as zero, and to reduce disturbance of these zero values in the subsequent data analysis, variables with a low number of non-zero values in all groups should be removed as suggested by Bijlsma *et al.* (2006) and in Paper I.

The commercial software, Markerlynx^{TM} (Waters), was used in Paper I and it was found that different preprocessing parameters resulted in extraction of several non-identical features. As a compromise two different data sets, preprocessed with different parameters, were extracted and subsequently combined. Peters *et al.* investigated the impact of parameter selection in different software packages (Markerlynx^{TM}, MZmine and MetAlign) by used of spiked and non-spiked control samples to evaluated the number of retrieved spiked compounds together with the number of false positive (Peters *et al.*, 2009). They recommended introduction of such samples in a metabolomics sample run for optimal parameter selection. Gürdeniz *et al.* (2011) found that data extracted by two different preprocessing approaches (MarkerLynx^{TM} and in-house built extraction by Matlab) caused large differences in the rank of selected markers, but the majority of them were found by the two quite different preprocessing methods (Gürdeniz *et al.*, 2011). This work also concluded that to achieve successful biomarker detection it is important to inspect the quality of the raw data (shift in mass and retention time) and preprocess according to its specific structure.

2.5.2 Data extraction of NMR data

NMR signals are collected as a function of time. The decaying signal that follows a pulse is called the free induction decay (FID). The chemical shift can be derived from the FID by utilising a Fourier transformation, whereby the time domain is converted into the frequency domain (Lambert & Mazzola, 2004). However, prior to Fourier transformation data is typically zerofilled and apodised to a certain line broadening. Hereafter, NMR spectra needs to be corrected for deviations from a flat horisontal baseline and phase errors. The employed NMR software can usually do this automatically but especially the phase errors may be more appropriately corrected by hand.

Different factors (e.g. sample pH, temperature and minor instrumental drifts) may cause chemical shift variations, and the overall variation between samples needs to be compensated by a shift of the entire spectra by use of an internal reference compound. If this shifting is not sufficient a co-shifting algorithm can be applied (as in Paper II), whereby spectral alignment are performed in spectral intervals, hereby preserving the shape of the peaks. Additionally, before data analysis the residual water signal should be removed.

2.5.3 Normalisation, centering and scaling of metabolomics data

Data normalisation (scaling between samples) and scaling between variables is typically applied to remove unwanted systematic bias in ion or signal intensity measurements while

retaining the interesting biological information. The sources of obscuring variation may arise from inhomogeneity of samples, minor differences in sample preparation, instrumental pertubation and also data extraction steps may introduce an additional error (Sysi-Aho *et al.*, 2007).

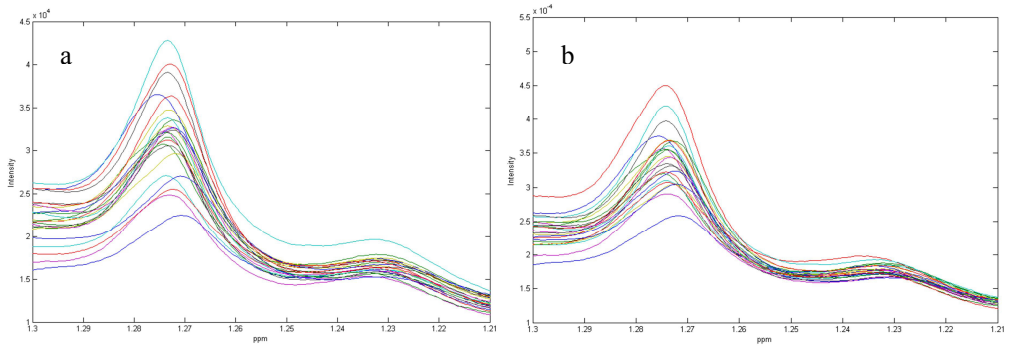


Figure 5. Chemical-shift referred (a) and co-shifted and normalised (b) ^{1}H NMR spectra of 24 rat plasma samples (data from Paper III). The peak (~1.27 ppm) refers to the CH_2 groups of different lipids in lipoprotein particles.

Each sample is usually normalised to unit sample intensity sum (as in Figure 5) or to unit sample vector length (Euclidean norm), since normalisation to a single or few selected variables will not be appropriate representatives for the chemically diverse metabolites profile present in these types of samples (Katajamaa & Oresic, 2007). However, these statistical normalisation approaches may not always be the most optimal procedure, since metabolite concentration increase in one group is not automatically balanced by a decrease of another group. A novel and very promising normalisation approach has been suggested by Sysi-Aho *et al.* (2007) utilising optimal assignment of multiple internal and/or external standards across multiple sample runs to help determine how the standards are correlated, which variation is specific to a particular standard, and which patterns of variation are shared between the measured metabolites and the standards. From this a mathematical model was developed to detect the systematic variation of metabolites as a function of variation of standard compounds. This advanced normalisation method was evaluated on LC-QTOF-MS metabolomics data, but the same strategy was considered applicable to other analytical platforms used in metabolomics as well.

Before multivariate data analysis the data matrix is normally mean centered in order to focus on the difference between the samples rather than the direction of the overall variance. Centering converts all the concentrations to fluctuations around zero instead of around the mean of the metabolite concentration and hereby adjusts for offset variation between the high

and low abundant metabolites (van den Berg *et al.*, 2006). The addition of a scaling method should also be considered before data analysis in order to adjust for the fold difference between the detected metabolites. The most commonly used scaling methods for metabolomics data are autoscaling and pareto scaling. The first method employs the standard deviation as the scaling factor, whereas the square root of the standard deviation is the scaling factor for pareto scaling (van den Berg *et al.*, 2006). It should always be considered that each type of data pretreatment emphasises different aspects of the experimental data, and each approach has both advantages and disadvantages.

2.6 DATA ANALYSIS

Metabolomics data obtained from spectroscopy and spectrometry typically contains thousands of variables from each sample. Variables attained from NMR spectroscopy are normally highly correlated, whereas in mass spectrometry data, the individual variables are not directly correlated but hyphenated to a chromatographic dimension that sorts the variables by polarity, allowing some relation to the neighbouring variable.

The multidimensionality of this type of data is difficult to comprehend and visualise, and invoke for analytical techniques which can extract the relevant information. Chemometric methods are here an obvious choice due to their ability to decompose complex multivariate data into simpler and potentially interpretable structures (Wold, 1987). Depending on the aim of the analysis, unsupervised or supervised methods may be applied and assist in e.g. obtaining an overview of data, in variable selection, in group classification or to relate the data set to a reference value for construction of prediction models. The following section aims at introducing the data analytical approaches applied in this project.

2.6.1 Principal component analysis

Principal component analysis (PCA) was first introduced in statistics by Pearson in 1901 (Pearson, 1901) with a geometric interpretation of 'lines and planes of closest fit to systems of point in space', and Hotelling (1933) further developed PCA to its present stage. PCA can be generally described as a method that reveals the internal structure of a data set in a way which best explains the variance in the data. Mathematically a PCA model can be written as:

$$X = T \cdot P' + E$$

where X is the data matrix representing samples and variables decomposed into a score matrix (T) and a transposed loading matrix (P'). The E matrix contains the residuals, the part of the data not 'explained' by the principal component model. In this way, the score and loading matrix contains the systematic variation with respect to samples and variables, leaving the unsystematic variation in the residual (Wold, 1987). PCA offers a reduced dimensional model that summarises the major variation in the data into few axes, and in this way, systematic variation is captured in a model that can be used to quickly visualise which samples in the data set are similar or dissimilar to each other. From this, possible spectral loadings causing any treatment-related separation may be identified. In Paper I, PCA was used as an initial explorative method to investigate to which extent the different treatments (apple and pectin) could be discriminated by the urinary metabolite profile. PCA was also used in a non-metabolomics context in Paper III to obtain an overview of the variance structure of classical health related biomarkers and physiological data. Additionally, in both studies PCA was used to investigate for 'outliers', by inspection for highly deviating samples with respect to residual and hotelling values, but none such were detected.

2.6.2 Partial least square regression

The most commonly used chemometric method for quantification is partial least square (PLS) regression. This method is a very robust and powerful algorithm that can analyse data with numerous strongly correlated X-variables (e.g. spectra) and also simultaneously model one or several Y-variables (e.g. a response variable/biomarker) (Wold *et al.*, 2001). This enables establishment of a linear model that can predict Y from the measured spectra in X. Like PCA, PLS regression generates a linear model of the data, but where PCA models the major variation in the data itself, PLS derives a model that describes the correlation between the X variables and a feature (Y variable) of interest (Keun, 2006).

In Paper II, PLS regression was successfully applied in modelling of NMR spectra and cholesterol content in lipoprotein fractions from rat plasma samples. Cholesterol concentration in the main plasma lipoprotein fractions could hereby be predicted in unknown samples after NMR measurement (illustrated in Paper II and III). The development of these prediction models took advantage of the interval partial least square (iPLS) regression developed by Nørgaard *et al.* (2000), which is an extension of PLS regression. The iPLS regression model splits the NMR spectrum into a number of intervals, and PLS models are calculated towards the response variable for each interval. The predictive performance of the PLS model for each interval is compared with the predictive performance of the full spectrum model. The advantage of this approach is that the limited intervals contains less interference

from irrelevant parts of the spectrum and provides more precise and easier interpretable models with a comprehensive overview of which spectral regions are best correlated with the response variables.

PLS is often used as a classification tool in metabolomics studies by applying the discriminant analysis approach (PLS-DA). For this analysis, a class vector is constructed of one variable of each class with a value of 1 if the sample belongs to a particular class and 0 if not. By regression against this class vector, latent variables can be derived that separate the classes from each other. This method has been applied to the data in Paper I (see Figure 6) but was not the final selected approach in the submitted manuscript. It seems worth mentioning that PLS-DA can suffer severely from overfitting, since the number of samples used in metabolomics applications is usually much smaller than the number of variables, and this can easily lead to chance classifications. Consequently, the PLS-DA algorithm can separate two groups comprised completely of random data, and focus on the validation process is particularly important in this analysis (Westerhuis *et al.*, 2008).

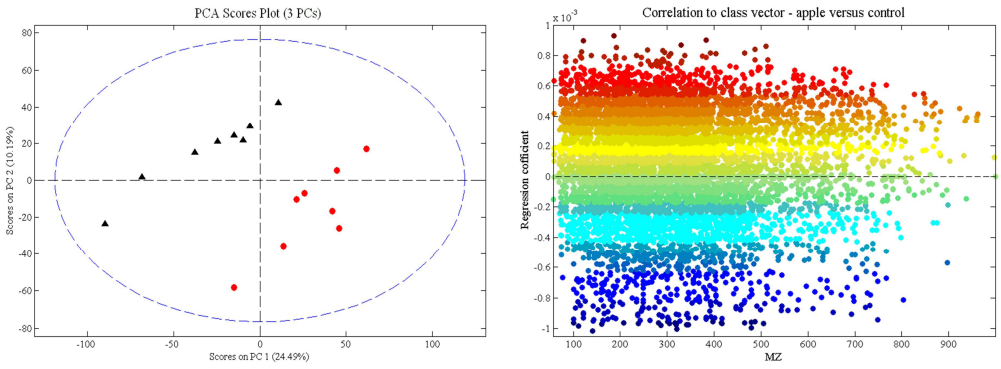


Figure 6. PCA score plot illustrating control (●) and apple (▲) rat urine sample and PLS-DA loading plot with 4010 metabolites detected by UPLC-QTOF-MS in negative ionization mode. The colour intensity in the PLS-DA loading plot illustrate the variables correlation to a class vector. Data is autoscaled and validated by use of random segmented cross validation. Data from Paper I.

2.6.2.1 Validation

Validation of chemometric models is a very central issue to ensure construction of reliable models and estimates of e.g. prediction error and to determine the optimal number of components. In this way, overfitting, meaning that the model classifies the training data well but future samples are classified poorly, may be avoided. Cross validation may be applied

when the number of samples is limited, and all samples have to be used in the calibration model. By this approach the X data matrix is divided into a number of segments containing one or more samples (full or segmented cross validation, respectively). One by one the segments are left out, and the model is calibrated with the remaining sample and used to predict the samples in the omitted segment (Wold *et al.*, 2001). Random segmented cross validation was used in Paper II for development of the PLS calibration models and to determine the optimal number of components to be used.

As a stronger validation method, test set validation can be used when a study contains enough samples to be divided into a calibration set and a validation test set. Here, the calibration set is used to build the model, and the test set is subsequently applied to estimate the prediction error. Test set validation was used in Paper II, where the PLS calibration models was build from 40 samples, and an independent test set consisting of 20 samples was applied to the model to test the model performance in future predictions. The often used estimate of prediction error is the root mean square error (RMSE), which mimics the traditional standard deviation and is described in detail in Paper II.

2.6.3 Variable selection

In explorative metabolomics approaches the aim is typically to identify and select relevant variables from the chemometric methods described previously in this section. Especially supervised methods are used, where *a priori* knowledge is used to select variables that are considerably different between two different samples groups and may be new biomarker candidates when identified. The PLS-DA is one approach to select potential biomarkers, and this was initially used for biomarker selection in Paper I. Additionally, application of the multiple linear regression (MLR) model, forward stepwise selection (described in Paper I) was attempted on this data. Both methods resulted in the selection of a very high number of promising metabolites, but the identification process of these hundreds of metabolites seemed to be an unstructured and highly time-consuming task. Instead, a more biological and top-down selection procedure was initiated, where variables were selected on the basis of their response behaviour and homogeneity between two classes (control and apple or control and pectin). The variables selected for identification were divided into *exposure* markers and *effect* markers, where exposure markers should have only zero values in the control group and positive responses in all animals in the comparing group. Effect markers were defined as markers that had a baseline response in all animals in one group and a significantly up- or down-regulated response in all animals in the comparing group. One misclassification was allowed in each group in order to tolerate small measurement errors of the MS instrumentation. Figure 7 illustrates the response pattern of what is classified as an exposure and effect marker.

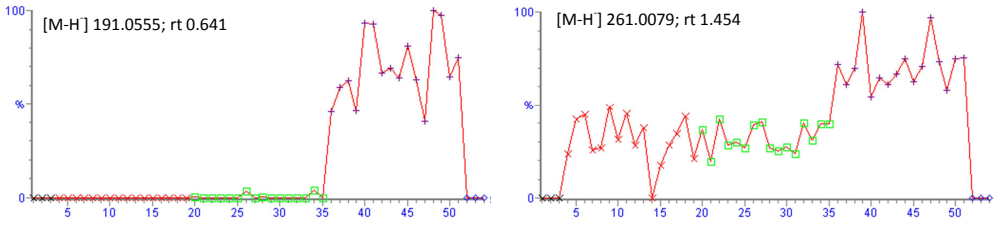


Figure 7: Example of exposure (left) and effect (right) marker selected from urinary UPLC-QTOF-MS analysis. Control (×), pectin (□) and apple (+) samples.

This kind of variable selection may have particular suitability in studies with numerous very clear markers, as it can be found in animal studies, due to isogenetic animal strains and controllable dietary habits. In human intervention studies more pronounced inter-individual variation and diverging habits will most likely entail a more blurred response profile, and in this case this 'exposure and effect marker' approach may not be the optimal selection.

Forward stepwise selection was also applied to identify a potential dose-response relationship between any of the urinary variables and the pectin intake (zero in the control group, 1 in the apple group and 16.5 in the pectin group) in Paper I. This method selects variables in a stepwise manner based on their capability to improve an MLR model established between the chromatographic features and the pectin dose (described in more detail in Paper I). Due to the relatively high independence of variables in an LC-MS data set, the forward stepwise selection method may be well suited for this type of data. However, this method is also prone to overfit due to the low number of samples as compared to the number of variables, and careful validation is an important issue in this case.

2.7 METABOLITE IDENTIFICATION

Metabolite identification is an essential part of most metabolomics studies, but since this task is difficult and a time consuming step at the end of the metabolomics pipeline, it is sometimes ignored or left unfinished (Scalbert *et al.*, 2009). Without compound identification no new or confirming metabolic information is gained, and the goal of a metabolomics study is not achieved.

The effort required for identification of metabolites depends on the scope of the study, be it targeted or explorative. If the search is for known metabolites the identification involves

comparing the experimental data with that of pure standards. If the metabolites can be predicted, then the metabolic identification involves finding representative standards and searching for the predicted metabolites. If nothing is known about the metabolites in the experimental data, as in explorative studies, the metabolite identification is much more complicated. This last approach is most widespread in MS metabolomics due to the high sensitivity and hereby the possibility to discover new and low-abundant metabolites. The explorative metabolite identification was investigated in Paper I and resulted in identification of metabolites linked to apple or pectin intake in rats. The identification strategy in this study took advantage of the accurate mass measurement and fragmentation pattern obtained from the QTOF instrument. The m/z value of the selected exposure and effect markers was searched in the Human Metabolome Data Base (HMDB). The database (version 2.5) contains over 7900 metabolite entries including both water-soluble and lipid soluble metabolites (Human Metabolome Database, 2010). If one or several metabolite hits matched the accurate mass of a searched metabolite, this was taken further in the identification process. The particular isotopic pattern in the mass spectra was inspected by use of the Markerlynx^{TM} elemental composition software, where particular the natural ^{13}C abundance is taken into account. Then fragment ions in the raw data were considered by applying a mass fragment tool (MassFragment^{TM}, Waters) and finally an authentic standard of the proposed compounds was analysed by the UPLC-MS system to verify retention time and the fragmentation and/or adduct-forming pattern.
However, several of the metabolites were left as tentatively identified, since the pure standard of these are not commercially available.

NMR metabolite identification in this project has been limited to peak assignment from comparison of chemical shift with previous work (Paper II, Figure 2). However, the NMR technique can elucidate chemical structures and provide highly specific evidence for the identification of an unknown molecule, if they are at a high enough quantity (Moco *et al.*, 2007). For most organic compounds in biological samples, the acquisition of one-dimensional ^{1}H NMR spectrum is not sufficient for full structure elucidation, and more advanced NMR measurements like homonuclear ^{1}H-2D spectra or heteronuclear 2D spectra are very helpful for identification of unknown compounds (Dunn & Ellis, 2005). However, this task was beyond the scope of this project.

2.8 BIOLOGICAL INTERPRETATION

When a metabolite has been identified, a relevant biological interpretation should be drawn related to the research question. Information about numerous biochemical pathways and metabolites interacting in these pathways is available in e.g. the KEGG pathway database (http://www.genome.jp/kegg/pathway) or the Nutritional Metabolomics Database (http://www.nugowiki.org). Additionally in the HMDB database most metabolites are described briefly in a 'MetaboCard' designed to contain chemical, clinical and biochemistry data. Besides these approaches the literature has to be thoroughly searched to put the identified metabolite into an appropriate biological context. When/if the identified metabolite is placed into a metabolic pathway this may lead to identification of additional unknown metabolites belonging to the same pathway, and the data set can be searched again now in a more targeted approach.

Biological interpretation of urinary metabolites from apple and pectin intervention in rats is discussed in Paper I, just as the more classical biomarkers in relation to apple-powder, fresh apple and pectin intake are discussed in Paper II and III. The biological interpretation of all these markers is jointly discussed in the 'Results and Discussion' section.

3 POTENTIAL DISEASE PREVENTION FROM APPLE INTAKE

Apple was selected as the nutritional case to be investigated during the establishment of our metabolomics platform. To enable biological interpretation of both well-established CVD risk markers and newly discovered metabolomics markers, some background knowledge of apple is needed. The following section describes the composition of apple, absorption and metabolism of presumed bioactive apple components as well as their physiological effects and potential mechanisms of action in relation to prevention of CVD.

3.1 COMPOSITION OF AN APPLE

Apples are primarily composed of water and carbohydrate with fructose accounting for the main part of the sugars and sucrose and glucose as minor parts. The macronutrient composition of an apple (the 'Shampion' cultivar) can be seen in Table 2 in Paper III. Apples contain several micronutrients as well, with the most predominant being pro-vitamin A (β-carotene), vitamin C and E, folic acid, magnesium and potassium (National Food Institute, 2010). Apples contain normally >2 g fibre/100 g and different phenolic compounds (see Table 2, Paper III), and in particular these two components are linked with potential health effects of apple intake (Gonzalez *et al.*, 1998; Nagasako-Akazome *et al.*, 2005), for which reason their composition is further detailed in the following.

3.1.1 Fibres in apples

The fibre part in apples can be divided into a soluble and an insoluble fraction. The insoluble fibres account for the major part and is made of cellulose, which consists of repeating monomers of glucose attached end to end. The cellulose framework is interpenetrated by a cross-linked matrix consisting of lignin, hemicelluloses, pectin and structural glycoproteins (Thakur *et al.*, 1997). Cellulose, lignin and to some extent hemicelluloses contribute to the insoluble fibre fraction of apple (Rani & Kawatra, 1994). Pectin is a highly hydrophilic polysaccharide that account for the majority of the soluble fibre fraction in apples. It has a complex structure and apple-pectin exhibits a high degree of esterification and has a very high content of branched side chains (Thakur *et al.*, 1997) (see Figure 8).

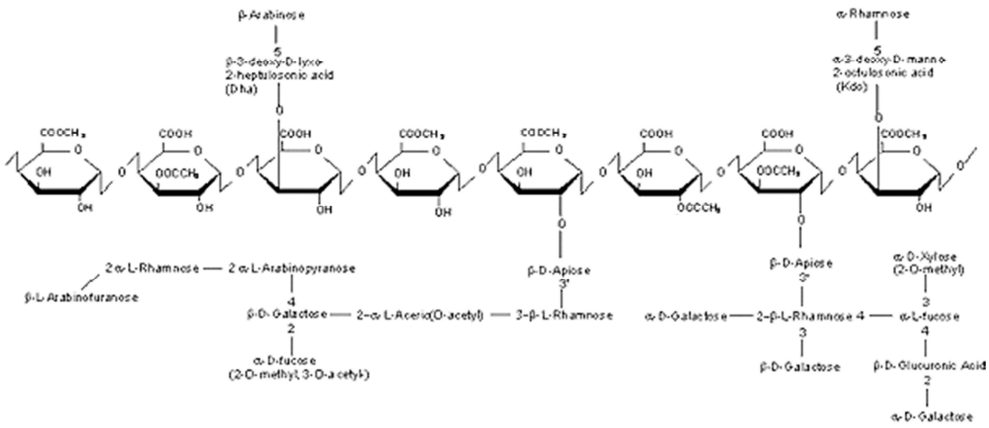


Figure 8. Example of a branched pectin structure with a poly-α-(1-4)-D-galacturonic acid backbone with partial methylation, acetylation and four different types of branching. From Sigma Aldrich (2010) with permission.

3.1.2 Phytochemicals in apples

Apples contain various phytochemicals, which are secondary plant metabolites and not considered as nutrients in mammals. The majority of these phytochemicals are phenolic compounds and only to a very limited extent phytosterols (Normen *et al.*, 1999). As illustrated in Table 2, Paper III, the most predominant phenols are: procyanidins, which consist mainly of condensed (-)-epicatechin units and/or (+)-catechin; the flavanol epicatechin; the flavonol quercetin as glycoside and the phenolic acid chlorogenic acid. Epicatechin, quercetin and chlorogenic acid are shown in Figure 9-11. An intake of 2-3 apples a day may provide an intake of 100-150 mg/day of total phenolic compounds (calculated from Table 2, Paper III).

The majority of polyphenols are present as glycosides and/or esters with exception of the catechins and proanthocyanidins. The concentration of these compounds may depend on many factors, such as cultivar of the apple, growth conditions, harvest time and storage of the apple (van der Sluis *et al.*, 2001). The phenolic compounds are found in much higher concentrations in the peel than in the flesh. Quercetin conjugates are exclusively present in the peel, whereas chlorogenic acid tends to be higher in the flesh than in the peel (Escarpa & Gonzalez, 1998).

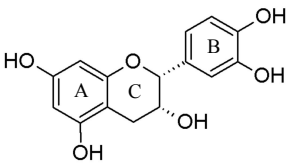


Figure 9. (-)Epicatechin

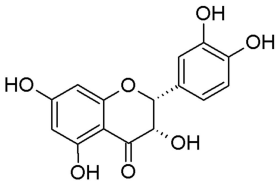


Figure 10. Quercetin

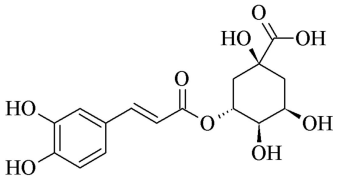


Figure 11. Chlorogenic acid

3.2 ABSORPTION, METABOLISM AND MECHANISM OF ACTION OF APPLE COMPONENTS

3.2.1 Fibre

3.2.1.1 Absorption and metabolism

The water-insoluble fibre fraction in apples (cellulose, lignin and some hemicelluloses) is resistant to hydrolysis by the human digestive enzymes. Cellulose and hemicelluloses may to a limited extent be fermented by the microbiota in the colon, whereas lignin passes undegraded. Pectin, as the main soluble fibre fraction in apples, has high gelling properties and forms a viscous solution in the small intestine. Pectin is, like the insoluble fibres, resistant to hydrolysis by the human digestive enzymes but it is rapidly and completely fermented by the microbiota in the proximal part of colon. The end products of this fermentation are short chain fatty acids (SCFA) together with CO_2, CH_4 and H_2 (Spiller, 2001). The SCFAs are organic fatty acids with up to 6 carbon atoms with acetate, butyrate and propionate being produced at the highest rate (Wong *et al.*, 2006). Pectin seems to induce particularly high production of acetate (Schweizer & Edwards, 1992). SCFAs are very efficiently absorbed in caecum and colon by direct diffusion or cellular uptake involving Na^+ and K^+. Acetate is rapidly transported to the liver and to a lesser extent to the muscle cells, where it functions as fuel. Propionate functions as a primary substrate for hepatic gluconeogenesis, and butyrate serves as the preferred fuel of the colonic epithelial cells (Wong *et al.*, 2006).

3.2.1.2 Mechanism of action inducing physiological effects of apple fibre

One of the most investigated physiological properties of soluble fibres is its ability to lower blood cholesterol. Several human and animal studies have been conducted with pectin supplementation from various sources, and most investigations find a significant reduction in cholesterol (Judd & Truswell, 1982; Keys *et al.*, 1961; Stasse-Wolthuis *et al.*, 1980; Sable-

Amplis *et al.*, 1983b; Kay & Truswell, 1977) others find no effect (Aprikian *et al.*, 2003; Sable-Amplis *et al.*, 1983b; Schwab *et al.*, 2006; Trautwein *et al.*, 1998). There are typically two suggested mechanism whereby pectin may exhibit cholesterol-lowering effect. One suggested mechanism involves interference with lipid and/or bile acid metabolism. The gel-forming properties of pectin may bind bile acids plus cholesterol and prevent the (re)absorption in the small intestine (Kay & Truswell, 1977), leading to increased excretion of bile acids via faeces. As a consequence, hepatic conversion of cholesterol into bile acids will increase, hepatic pools of free cholesterol will decrease and endogenous cholesterol synthesis will increase. This is thought to increase activity of 7-α-hydroxylase and HMG-CoA reductase to compensate for the loss of bile acids and cholesterol from the liver stores. Furthermore, hepatic LDL cholesterol receptors become upregulated to restore the hepatic cholesterol pool, and this will lead to decreased serum LDL cholesterol concentrations (Theuwissen & Mensink, 2008). This is a proposed mechanism for water-soluble fibres in general, and Figure 12 gives an overview of this proposed regulation.

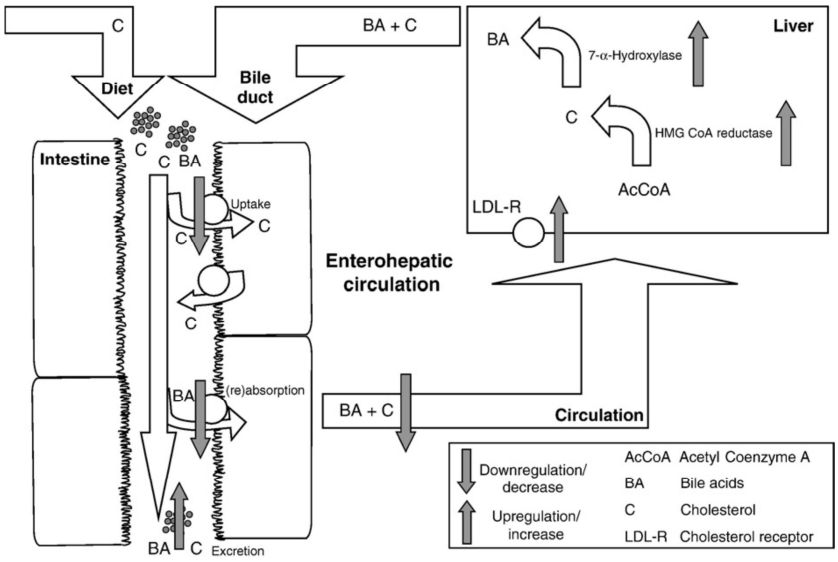


Figure 12. A proposed cholesterol-lowering mechanism of water-soluble fibres like pectin. The soluble fibers form a gel in the intestinal lumen, whereby (re)absorption of cholesterol and bile acids may be decreased. This leads to an increased faecal output of these two components. As a result hepatic conversion of cholesterol into bile acids increases, hepatic pools of free cholesterol decrease and endogenous cholesterol synthesis increases. In addition, hepatic LDL cholesterol receptors are up-regulated to re-establish hepatic free cholesterol stores. These processes will ultimately lead to decreased serum LDL cholesterol concentrations (from Theuwissen & Mensink, 2008).

The other suggested mechanism whereby pectin may exhibit a cholesterol-lowering effect links to effects of the SCFA produced by fermentation of microbiota in the colon. Propionate has been reported to inhibit cholesterol synthesis in the liver (Rodwell *et al.*, 1976; Venter *et al.*, 1990), and this is the main argument in the SCFA-cholesterol lowering theory. However, consensus is not established in this area. Propionate is at the same time found as a substrate for hepatic gluconeogenesis and in this way it seems to have two opposite and competing effects on the gluconeogenesis (Wong *et al.*, 2006). Acetate is hypothesised as a primary substrate for cholesterol synthesis. Wolever *et al.* (1989) studied the effect of rectal infusion of SCFA on lipid metabolism, and subjects given infusion of two doses of a mixture of acetate and propionate (90:30 nmol and 180:60 nmol) showed a dose-dependent increase in serum total cholesterol and triglyceride level. Another study by the same research group showed that acetate infused alone (180 nmol) produced a significant rise in total and LDL cholesterol (Wolever *et al.*, 1991). The authors concluded that these findings revealed indirect evidence that SCFA is utilised in lipid synthesis and that the exact effect of SCFA may depend on the ratio of propionate and acetate. The infusion method used in these studies can be debated, since the dosage rate may not simulate that of the SCFA produced by the colonic microbiota, and in general the effects of SCFA in cholesterol metabolism may still be regarded as unclear.

No cholesterol-lowering effects have been seen by cellulose and hemi-cellulose directly, but since these fibres are fermented to some extent, the resultant SCFAs may also have a certain impact with respect to this proposed mechanism. However, the insoluble fibre fraction is primarily regarded as being responsible for an increased stool bulk and helping to regulate bowel movements.

3.2.2 Phenolics and polyphenols

3.2.2.1 Absorption and metabolism

Various factors have an impact on the bioavailability of polyphenols. As stated earlier, the majority of polyphenols are present as glycosides in the apple, and this influences absorption in the gut. The bioavailability and metabolism of the most predominant apple phenolics are detailed here.

Procyanidins: Procyanidins are found as the B2 dimer (epicatechin-(4β-8)-epicatechin) in apple (INRA, 2010). The high molecular weight of procyanidins seems to hinder the intestinal absorption (Donovan *et al.*, 2002), and they pass unaltered into the colon where they can be catabolised by the gut microbiota. Appeldoorn *et al.* (2009) conducted an *in vitro*

fermentation of different purified procyandins (also the B2 dimer) with human microbiota and found 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone as the main metabolites. Bioavailability and metabolism have not yet been investigated with pure procyanidins in humans, but in accordance with Appeldoorn *et al.*, we tentitatively identified dihydroxyphenyl-γ-valerolactone as a urinary marker from apple supplemented rats (Paper I). 5-(3,4-dihydroxyphenyl)-γ-valerolactone was also identified by Li *et al.* (2000) as a major human urinary metabolite after intake of (-)-epicatechin, and some extent of microbial breakdown of procyanidins to monomer (-)epicatechin and further to 5-(3,4-dihydroxyphenyl)-γ-valerolactone seems plausible.

Catechins: Especially (-)epicatechin, but also its isomer (+)catechin, is prevalent in apples to some extent. These catechins are believed to have a relatively high bioavailability and can be absorbed directly in the small intestine. The absorbtion and metabolism of catechin was investigated by Donovan *et al.* (2001) via an *in situ* model of small intestinal perfusion in living rats, and absorption and metabolism of epicatechin are believed to proceed in the same way. These authors suggested that catechin enters the enterocytes by passive diffusion, and here they are primarily glucuronidated and/or to a lesser extent methylated. From the enterocytes the conjugated catechins are transported to the liver, where further re-/de-glucuronidation/methylation or sulphation can occur. The glucuronated and methylated forms, are the circulating forms whereas the glucuronated+sulphated forms primarily are thought to be eliminated by bile (Figure 13). The formation of sulphate, glucuronide and/or methylated metabolites occur through the respective action of sulfotransferase (SULT), uridine-5´-diphosphate glucuronosyltransferases (UGTs) and catechol-*O*-methyltransferases (COMT) (Crozier *et al.*, 2009).

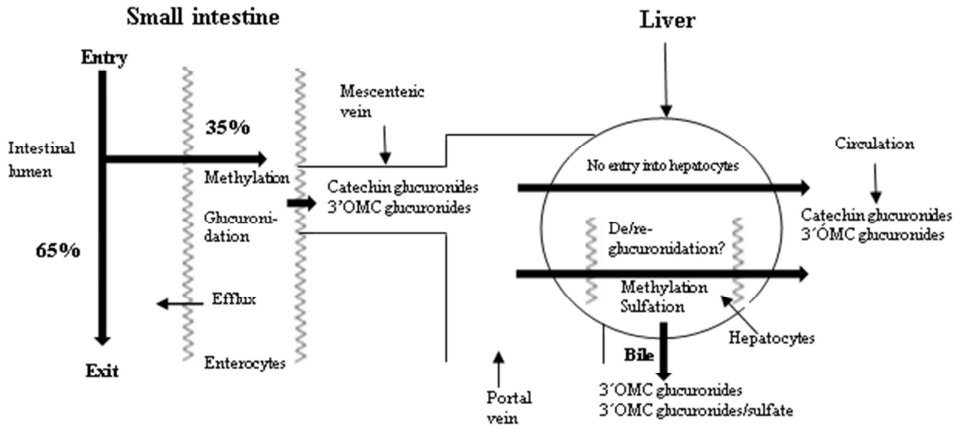


Figure 13. A schematic representation of the possible mechanisms of absorption and metabolism of catechin in rats. Abb.: 3´OMC, 3´-*O*-methylcatechin (From Donovan *et al.* (2001)).

Natsume *et al.* (2003) has elucidated the chemical structure of (-)-epicatechin metabolites in human and rat urine after oral administration of this compound, and the major circulating metabolites in humans were found as: epicatechin-3´-*O*-glucuronide, 4´-*O*-methylepicatechin-3´-*O*-glucuronide, 4´-*O*-methylepicatechin-5- or 7-*O*-glucuronide, and in rats: epicatechin-3´-*O*-glucuronide, epicatechin-7-*O*-glucuronide and 3´-*O*-methyl-epicatechin-7-*O*-glucuronide. The aglycones epicatechin was also found in both humans and rats. The authors stated that the difference in catechin metabolism between humans and rats was that the glucuronidation of epicatechin occurs at the 7´ position of the A ring for rats and at the 3´position of the B ring in humans.
(-)Epicatechin was identified as a urinary exposure marker from apple intake in Paper I, and the epicatechin glucuronide, methylated epicatechin and catechin glucuronide were also recognised, although only tentitatively identified, because lack of authentical standards hindered confirmation of retention time for these compounds.

Quercetin**:** Quercetin is primarily found as quercetin-3-glycoside, rutinoside, rhamnosides, xylosides and galactosides in apple (Boyer & Liu, 2004; INRA, 2010). Some glycosides are able to be absorbed in the small intestine, and there are two possible routes by which glucoside conjugate can be hydrolysed and the resultant aglycones can be formed in the epithelial cells. One possibility is that the glycoside is hydrolised by lactase phloridizin hydrolase (LPH) in the brush-border of the small intestine epithelial cells and hereafter enters the cell as aglycone by passive diffusion. Alternatively, the intact glycoside conjugate may be transported into the epithelial cells by the sodium-dependent glucose transporter SGLT1, where after cytosolic β-glucosidase can mediate hydrolysis (Crozier *et al.*, 2009). Quercetin-3-glycoside is thought to utilise the LPH, whereas the quercetin rutinoside, rhamnosides, xylosides and galactosides are not easily hydrolysed, and most likely pass unchanged through the small intestine and may be degraded/hydrolysed by the microbiota in colon (Boyer & Liu, 2004). Like the catechins, quercetin is subjected to glucuronidation, sulphation and/or methylation before passage into the blood stream and further phase II metabolism occurring in the liver by hepatocytes, which contain b-glucuronidase activity (Mullen *et al.*, 2006). Some of the quercetin conjugates may be recycled back into the intestine via the bile, but most will be excreted in urine (Crozier *et al.*, 2009).

Chlorogenic acid**:** Only very small amounts of chlorogenic acid are believed to be absorbed intact in the intestine, and the majority appears to be metabolised by the gut microbiota in the colon (Gonthier *et al.*, 2003). Gonthier *et al.* (2003) found that rats supplemented with chlorogenic acid primary increased excretion of hippuric acid and m-coumaric acid. Quinic acid is also a known microbial metabolite of chlorogenic acid, and this metabolite was found

as an apple exposure marker in Paper I. However, quinic acid is also naturally present in apples and the origin of this phenol may not only derive from chlorogenic acid.

3.2.2.2 Mechanism of action inducing physiological effects of apple polyphenols

The interest in health effects of dietary polyphenols have primarily been driven from epidemiological studies that indicate an inverse relationship between intake of polyphenol rich foods and different diseases such as CVD, diabetes and cancers. Based on this, numerous *in vitro* studies have been performed with the aglycone form of polyphenols showing promising disease preventive effects of these compounds. However, most circulating polyphenols are glucuronidated, methylated and/or sulphated but there are only limited studies elaborating on the biological properties of the conjugated derivatives (Crozier *et al.*, 2009). The lack of commercially available compounds complicates improvements in this area. Studies where the biologically relevant substances are used to investigate mode of action in relation to health effects are considered in the following.

Procyanidins: Since the procyanidins are not absorbed, they do not exhibit a direct systemic response but during their passage in the intestine they may exert some effects through interactions with other components, such as lipids. However, a potential health effect of these structures may most likely be attributable not to direct actions of procyanidins themselves but to actions of some of their microbial metabolites that can be more readily absorbed. Only very limited investigations have been conducted with the presumed procyanidin metabolite, 5-(3,4-dihydroxyphenyl)-γ-valerolactone. Li *et al.* (2000) suggested antioxidative activities of this compound based on the chemical structure (Figure 14), and a study by Unno *et al.* (2003) found that 5-(3,4-dihydroxyphenyl)-γ-valerolactone had stronger antioxidant potential than vitamin C *in vitro*, but no studies have been performed elaborating on the biological effects of this compound *in vivo*.

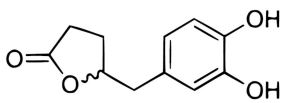


Figure 14. 5-(3,4-dihydroxyphenyl)-γ-valerolactone from Li *et al.* (2000).

Catechins: A study by Spencer *et al.* (2001) compared the epicatechin aglycone with 3´-*O*-methylepicatechin and epicatechin glucuronides (epicatechin-7- and epicatechin-5-*O*-β-D-glucuronides) regarding the ability to protect against oxidative stress of primary cultures of

neurones. They found that the epicatechin glucuronides were not able to protect against oxidative stress as it was the case for the native form of epicatechin and 3´-*O*-methylepicatechin. Cren-Olive *et al.* (2003) studied the ability of 3´-*O*-methylcatechin and 4´-*O*-methylcatechin to protect LDL from *in vitro* oxidation and found these metabolites less efficient in protection than catechin. The authors concluded that their results did not support a direct physiological relevance of catechins as antioxidants in lipid processes. No animal or human studies have been conducted with an isolated epicatechin or catechin supplement to elaborate on health effects of these compounds.

Quercetin: Kawai *et al.* (2008) developed a monoclonal antibody targeting the quercetin-3-*O*-glucoronide in humans and demonstrated the target sites of the metabolite that specifically accumulates in macrophage-derived foam cells in atherosclerotic lesions in human arteries. At this location the quercetin conjugate was found to be converted to the aglucone quercetin that subsequently reduced the lesion size. Manach *et al.* (2004) have reported on, the existence of intermolecular bonds between serum albumin and quercetin conjugates in the blood stream, and this mechanism slows the elimination of quercetin from the body, whereby a prolonged systemic effect is possible. The reported half-life of quercetin ranges from 11 to 28 hours, whereas it appears shorter for catechins (3-8 hours) (Manach *et al.*, 2005).

Generally, the evidence of potential disease preventive mechanism of apple polyphenols is very limited, and more *in vitro* and in particular *in vivo* investigation, are needed to reveal health effects of these substances.

4 RESULTS AND DISCUSSION

This section initiates with methodological considerations of the research that is the basis for Paper I, II and III in this project. The reader is referred to the individual papers for a detailed survey of the results, and the aim of this section is to link together the results of Paper I-III and to consider aspects and reflections that did not found their way into the papers.

4.1 METHODOLOGICAL CONSIDERATION

4.1.1 Study design

This project deals with four different animal experiments, whereof two were used purely to build NMR-based PLS calibration models (Paper II), and these models were utilised to predict cholesterol in different lipoprotein fractions in two other rat studies; one with different doses of apple-powder (Paper II) and one where fresh apple and apple-pectin were used as supplements (Paper I and III).

The animal study used for Paper I and III has some limitations in its study design, especially with regard to its use in the explorative metabolomics approach (Paper I). The study had a parallel design, but the isogenic nature of the animals, as compared to humans, may justify the choice of design to some extent. However, our attempt to detect pectin dose response markers would have benefitted from a cross-over designed study whereby the exact individual response for each dose level could have been measured for each rat. Additionally, inclusion of more animals (n=24) in the studies would have aided in the data analysis and interpretation of this study.

4.1.2 Rat studies and extrapolation to humans

The use of animal studies has its peculiarities and limitations as compared to human studies. The rats selected for these studies had a relatively standardised genotype, and their habits are more controllable compared to humans. This will induce a lower level of variation and make potential biological effects or metabolome biomarkers clearer. However, the genetic differences between rats and humans may result in some effects and physiological responses that are completely ignored, because the rat is insufficiently sensitive to the specific treatment. Rats have a higher metabolism rate than humans, and there will be some deviation in metabolism, e.g. they seem to methylate dietary phenols far more extensively than humans (Crozier *et al.*, 2009). Regarding cholesterol metabolism, the rats are deficiency in cholesterol

ester transfer protein (CETP) (Ha & Barter, 1982), and the major part of cholesterol is carried in the HDL particles in contrast to humans where LDL particles carry most cholesterol. These factors clearly state that there are many cautions to be taken when extrapolating data from animal to humans. However, investigation of organs, such as the liver and intestine is not possible in humans to the same extent as in rats, and the experimental conditions can be much more controlled. These aspects make animal models suited to study mechanisms of action but confirming experiments in humans is always recommended.

4.1.3 Considerations with regard to selected markers

In Paper I the markers were selected depending on their MS intensity response pattern as either effect or exposure markers. The effect markers should ideally be an expression of changes in the endogenous metabolome or the microbial metabolome of the host induced by e.g. apple intake. Identification of the specific metabolite can provide insight into metabolic pathways that are affected by apple intake, and this may generate new knowledge of the site of action for a potential health effect of apples. This will give rise to several new questions and highlight new places to search for mechanistic answers. However, the effect marker response may also derive from the food metabolome and illustrate imbalances in the standard feed between the groups.

Exposure markers are ideally an expression of changes in the xenometabolome, which correspond to compounds not used in the energy metabolism. Identification of these metabolites can imform about compounds that the organism has been exposed to and how these have been metabolised (at least the last step) before they are excreted. From previously conducted studies it is possible to elaborate on potential health effects from exposure of the specific compound, but the key quality of this type of markers is that, they may later be used as biomarkers of the specific food item. Thus, this will involve quantitative analysis and the specific marker should be validated for uniqueness in its food group and subsequently in dose-response investigations. The rat study in Paper I uncovered numerous exposure markers, and it would not be expected to find a similar result in a human intervention study. The collection of these markers may be used to unravel the presumed more blurred response behaviour of markers in human studies investigating apple or even fruit-related interventions. Despite the metabolic differences between rats and humans, it is thought possible to identify some of the apple and pectin related markers in humans as well, and potentially these could be combined selectively by multivariate modeling to search for associations between response patterns and dietary intake. An apple, pectin or fruit exposure or intake biomarker, considering several metabolites at the same time, could hereby be developed.

Several more classical health related biomarkers were measured in the rat study used for non-targeted metabolomics and they were reported in Paper III. A PCA was constructed with these markers to give an overview of the rats response variation in the different markers (Figure 3, Paper III). The traditional biomarkers and physiological data could be combined with e.g. the metabolome effect markers in a PCA (autoscaled data), and potential co-variance between these two types of markers could be identified as markers with nearby location in the multivariate space. This could highlight metabolomics markers of particular interest and obvious candidates for identification. After identification, a causal biological connection between the traditional marker and the metabolome marker may at best be verified.

4.2 EVALUATION OF EFFECTS OF APPLE AND PECTIN INTAKE

4.2.1 Effect of apple and pectin on cholesterol metabolism markers

4.2.1.1 Apple and cholesterol metabolism

The main findings in relation to cholesterol distribution in the different lipoprotein fractions in Paper II and III showed that rat feeding with a moderate amount of fresh apple during 4 weeks reduced total, HDL and LDL cholesterol compared to the control group, whereas a 10% and 20% apple-powder dose only showed significant reduction of HDL cholesterol for the high dose. The amount of apple used in these studies can be estimated to correspond to a human intake of 3-4 apples/day for the fresh apple, and the 10% apple-powder dose corresponds to the same amount. Considerable evidence has shown a clear association between decreased total and LDL cholesterol and reduced risk of CVD (Briel *et al.*, 2009), and based on this, intake of fresh apples seems to be favourable to improve cardiovascular health. The explanation of why the same effect is not found in apple-powder supplemented rats may most likely relate to the formulation of the apple supplement or the standard feed, since these rats were the same age, same strain and kept at the same conditions. The standard feed differed slightly between the studies, since fructose and sucrose were balanced in the apple-powder study and not in the fresh apple study. The finding of non-significantly elevated VLDL cholesterol and triacylglyceride TAG in the fresh apple group compared to the control could be caused by the high fructose content in apples. When fructose is consumed it will enter the hepatic glycolytic pathway, and in contrast to glucose metabolism, fructose can serve as an unregulated source of both glycerol-3-phosphate and acetyl-CoA, facilitating enhanced VLDL and triglyceride production in the liver (Havel, 2005). However, TAG was not measured in the apple-powder study, and VLDL appeared to rise in the 20% apple-powder group in the same way as seen in the fresh apple study, indicating that the fructose balancing

difference does not give a straightforward clarification on the divergences between the two studies. The formulation of the apple supplement, dried and grounded apple-powder versus fresh apple, is most likely the cause of the different results between the studies.

Just as high total and LDL cholesterol have been declared as independent risk markers of CVD in humans, so has a low HDL cholesterol (Grundy *et al.*, 2004). The significant decrease of HDL cholesterol observed in both Paper II and III seems surprising, since we expected a rise in HDL, when LDL and total cholesterol were decreased. This may be brushed aside as a coincidence or due to the rats CETP deficiency and the different distribution of cholesterol between HDL and LDL as compared to humans. However, from the literature (human and rat studies) it seems striking that a lowering in total and LDL cholesterol is not always accompanied with an increase in HDL cholesterol (Ohashi *et al.*, 2005; Aprikian *et al.*, 2001; Briel *et al.*, 2009), and a more varied view on cholesterol metabolism may be needed. A study by Ohashi *et al.* (2005) has shown that very low HDL cholesterol levels can be present in rodents, where atherosclerosis is markedly reduced. This result was explained by hepatic over-expression of the scavenger receptor (SR-BI) in the reverse cholesterol transport (RCT) pathway. The RCT pathway delivers free cholesterol from macrophages or other cells to the liver or intestine. Major constituents of the RCT pathway include acceptors, such as HDL and apolipoprotein A-I, and enzymes, such as lecithin cholesterol acyltransferase and CETP, which regulate cholesterol transport. Introduction of exogenous active compounds, e.g. from apple in rats as well as in humans, may induce or decrease activity and production of enzyme, transporters and receptors acting in the RCT pathway. Lewis & Rader (2005) stated that the flux of cholesterol through the RCT pathway may be a more important determinant of cardiovascular disease risk than steady-state HDL cholesterol concentrations. The HDL cholesterol-lowering effect we observed from the apple treatment may be due to increased activity of players in the RCT pathway, which thereby might cause a higher throughput and lower net cholesterol concentrations in HDL particles. Additionally, in this investigation (Paper II and III), we only measured the main lipoprotein fractions, and since what is classified as a fraction (e.g. HDL) spans over several sub-fractions with dynamic change in size and density, this has to be taken into account as well. There is substantial evidence that different HDL sub-fractions have differing functional properties (Ansell, 2007; Briel *et al.*, 2009), and their varying effects most likely affect their relation to cardiovascular protection. Therefore, indiscriminate evaluation of the main HDL fraction as ‘good’ or ‘bad’ does not seem reliable and future studies should evaluate the risk related to HDL by considering subfractions as well. The targeted metabolomics approach applying NMR spectroscopy and PLS modelling could here serve as an elegant and time-saving alternative to the troublesome separation of subfractions by ultracentrifugation.

4.2.1.2 Pectin and cholesterol metabolism

An apple-pectin supplement was introduced in the study in Paper III, and this facilitates interpretation of pectin as a cholesterol decreasing component of apple as proposed by the two cholesterol-lowering mechanisms stated in chapter 3.2.1.2. Pectin was not found to exhibit a lowering effect on total and LDL cholesterol and did not significantly increase the total faecal bile acids excretion. This finding was supported in the study by Aprikian *et al.* (2003), who also found no effect of apple-pectin on total plasma cholesterol. However, these authors found that hepatic cholesterol significantly decreased and faecal neutral sterol excretion significantly increased by the pectin treatment. From this result it seems likely that apple-pectin may enhance neutral sterol excretion (cholesterol and different metabolites hereof) to a higher degree than bile acid excretion. This is confirmed by an earlier study (Gonzalez *et al.*, 1998), where apple-pectin was shown to increase cholesterol in faeces. Investigation of hepatic cholesterol and neutral sterol excretion was unfortunately not examined in the Paper III investigation, and these aspects would have strengthened our understanding of pectins influence on cholesterol metabolism in this study.

A high dose of apple-pectin was used in our investigation and since pectin seems to particularly induce production of acetate (Schweizer & Edwards, 1992), this may to some extend explain why LDL and total cholesterol are not decreased to the same extent in the pectin group as in the apple group. As stated in chapter 3.2.1.2, acetate may be a substrate for cholesterol synthesis and hereby cause a higher total and LDL cholesterol in the pectin group.

4.2.1.3 Pectin as an isolated apple component

From our investigations pectin does not seem to be the main cause of a plasma cholesterol-lowering effect of apple, but it may still be one of the active players in inducing this effect. Performing nutritional experiments with purified components may not always be comparable to how these components are and act when located in their original matrix. Apple-pectin is typically extracted from dried apple pomace, and the native pectin is made soluble through heated acid extraction. After this, the pectin is precipitated with alcohol from the aqueous phase and dried (Obi-Pectin AG, 2010; Thakur *et al.*, 1997). These procedures will undoubtably introduce some deviating characteristics of pectin as compared to their structure in the fruit, and pectin may potentially lose its 3 dimensional structure during processing. Furthermore, one of the pectin markers identified in the metabolomics analysis in Paper I (2-furoylglycine) indicates that the cleaning procedure may give rise to furan derivatives. In Paper III we reported a significant increase in plasma alkaline phosphatase in the pectin

group, indicating that some adverse health effects may be caused by the high pectin dose, and the furan derivative may be speculated to partially cause this effect.
Compounds occurring naturally in the apple may even trail pectin through the purification procedure, and a urinary metabolite from the study in Paper I (pyrrole-2-carboxcylic acid) indicates a high intake of hydroxyproline in the pectin group. Apple fruit tissue has a high content of readily soluble glycoproteins, rich in hydroxyproline, and Knee (1973) found this amino acid still present in the pectin fraction after the purification process. Therefore, when introducing isolated components in interventions, the knowledge of purity and comparability of the component in the source material is a crucial factor in interpretation of results and involved mechanisms. Specific for pectin, it remains questionable if it is at all possible to isolate and use this component to obtain results that are comparable to the component embedded in the whole food matrix.

4.2.2 Metabolomics exposure and effect markers of apple and pectin intake

4.2.2.1 Apple exposure and effect markers

Epicatechin, one of the main polyphenols in apples was, not surprisingly, detected as a urinary exposure marker of this fruit (Paper I). The epicatechin glucuronide, methylated epicatechin and catechin glucuronide were also tentitatively identified, and illustrates the phase II metabolism of the parent compound. Only limited research has been conducted to clarify potential health effects of these compounds (Crozier *et al.*, 2009; Manach *et al.*, 2004), and it seems likely that the mammalian phase II enzymatic protection mechanism neutralises most health beneficial effects of catechins by glucuronidation. The compound dihydroxyphenyl-γ-valerolactone was also tentitatively identified and is possibly a microbial metabolite originating from procyanidin and epicatechin. This metabolite and the epicatechin aglycone may more likely be contributors to the health effects of apple that we observed in Paper III, especially in regard to the increased hepatic gene expression related to glutathione synthesis as well as glutathione utilisation that potentially demonstrate a higher ability to handle oxidative stress in the apple fed rats.

Since apple contains a relatively high level of quercetin glycosides some metabolites with this origin was expected, but none were found. Quercetin conjugates are exclusively present in the peel, and since all rats did not have the same preference for the eating the apple peel (as stated in Paper III), this may have caused an uneven quercetin exposure among the animals. The procedure for marker selection based on a consistent response among all rats in a group and was not able to select such markers.

Several markers were identified that very likely have their origin from chlorogenic acid (quinic acid, m-coumaric acid, hippuric acid and potentially 3-hydroxyhippuric acid). Hippuric acid and 3-hydroxyhippuric acid were present as effect markers (high response in the apple group and low response in the control group), and this may indicate a higher efficacy of specific metabolic pathways of the gut microbiota and glycine conjugation system in liver and kidney. An effect on the composition and efficacy of the gut microbiota in the present study is therefore indicated and is in accordance with previously published findings from this study, where apple intake was shown to affect caecal microbial composition by applying a PCA to data from denaturing gradient gel electrophoresis profiles of 34 different bacteria strains (Licht *et al.*, 2010).

4.2.2.2 Pectin exposure and effect markers

Pectin is thought to be completely fermented by the intestinal microbiota and the resulting SCFA primarily used as fuel in different compartments of the organism. Consequently, it was not expected to discover any pectin exposure markers in our metabolomics analysis, except potential residues of SCFA and metabolites hereof. However, 39 pectin exposure markers were detected, whereof two were identified (pyrrole-2-carboxylic and 2-furoylglycine). These two markers emphasise the influence of the pectin purification method and how this may affect what we at first think is completely comparable with the unisolated component in the original material. This highlights the value of the non-targeted and hypothesis-free metabolomics approach; it promotes new and unexpected findings that may be helpful in interpretation of results from a specific intervention.

4.2.2.3 Catecholamine metabolism

Several apple and pectin effect markers were tentatively identified, and four of these seemed to be catecholamine metabolites that may describe changes in the hormonal metabolism after the apple and pectin diet. In the apple group 3-methoxy-4-hydroxyphenylethyleneglycol sulphate was increased compared to the control group. This compound is the major metabolite of norepinephrine (Goldstein *et al.*, 2003), and to support our identification a fragment ion with a mass of 165.0557 m/z was found, and it seems likely to be the unconjugated parent ion (loss of the sulphate group, SO_3^-). Homovanillic acid sulphate, as another catecholamine metabolite that originates from L-dopa, was also tentatively identified as an effect marker that increased in the apple group. Metanephrine, which is a catechol O-methyltransferase derivative of epinephrine (adrenalin), was found to decrease in the apple group compared to the control group. Additionally, in the pectin group we found an upregulation of hydroxyphenylacetylglycine and methoxytyrosine, which are both metabolites that originate

from L-dopa (Goldstein *et al.*, 2003). Several different enzymes are active in catecholamine metabolism, and it seems likely that these are affected in different ways by circulating bioactive components from apple and pectin intake. It may cautiously be hypothesised that the physiological response to apple-pectin intake (from fresh apple and purified) especially affects gene(s) or enzyme(s) related to conversion of L-dopa and norepinephrine, and other apple components affect gene or enzyme systems related to liberation of metanephrine and epinephrine. Both norepinephrine and epinephrine have been subject to substantial research attempting to establish a link between various health disorders and the catecholaminerigic system, but their mechanisms of action are still highly debated (Kasparov & Teschemacher, 2008). Special focus has been on the function of the cardiovascular system, and drugs that interact with norepinephrine and epinephrine receptors are widely used in cardiovascular medicine (Kasparov & Teschemacher, 2008).

4.2.2.4 Cholesterol metabolism

The tentatively identified apple effect marker, 3-methylglutaconic acid, seems very interesting with regard to the total and LDL cholesterol lowering effect observed for apple in Paper III. 3-methylglutaconic acid is an intermediate metabolite in the mevalonate shunt, the isoprenoid biosynthetic pathway that appears to participate in the regulation of cholesterol synthesis (Marinier *et al.*, 1987). Mevalonate is the direct product of the rate-limiting step in cholesterol synthesis, which is catalysed by HMG CoA reductase and 3-methylglutaconic acid is produced in a co-pathway to the mevalonate-cholesterol pathway (Pappu *et al.*, 2002). The down-regulation of 3-methylglutaconic acid in the apple group is in concordance with the high, although non-significant, decrease in the *hmgcr* gene expression described for these rats in Paper III, and it is expected that also mevalonate is downregulated with reduced cholesterol synthesis as a consequence. If these findings hide the truth, there are some divergences with our discovery of increased total bile acid excretion in the apple group (Paper III), since this is thought to induce increased cholesterol synthesis. We did not find 3-methylglutaconic acid or other directly related cholesterol markers being up- or down-regulated in the pectin group, and it appears to be other bioactive components or factors than pectin that are responsible for a potential reduction in cholesterol synthesis. In general, the reduction of plasma cholesterol by apple seems more complex than that illustrated by generally known mechanisms, and it seems evident that more/other players in cholesterol metabolism should be investigated more comprehensively.

4.2.3 Why does an apple a day keep the doctor away?

From the studies included in this project fresh apple seems to be a health promoting food item with the ability to reduce total and LDL cholesterol, increase total and primary bile acids, decrease secondary bile acids, and a higher capability to handle oxidative stress is indicated due to effects on gene expression responses related to glutathione formation and utilisation. Additionally, urinary excretion of phenolics, polyphenols and potential metabolites involved in catecholamine and cholesterol metabolism point tentatively towards health promoting abilities of fresh apple, but since no firm evidence excists considering these markers, this is only mentioned cautiously as possible health effects. It is important to state that these results were obtained from rat studies and that extrapolation to humans should be done carefully considering the aspects stated in section 4.1.2.

To answer the question of 'why does an apple a day to keep the doctor away?' the soluble fibre fraction of apple, namely pectin, was inspected. Our investigations could not ascribe this component major health promoting effects, with regard to cholesterol metabolism, but it is worth mentioning that a much higher pectin dose was used as compared to the pectin dose naturally occuring in the apple. Consistent with our results Aprikian *et al.* (2003) found no effect of apple-pectin on total plasma cholesterol in rats, but a combined treatment with apple polyphenols, and apple-pectin showed a significant plasma cholesterol lowering effect. The plasma cholesterol-lowering effect we observed with fresh apple, and not with pectin alone (Paper III), may be caused by a combined effect of the polyphenols and pectin present in whole apple. However, we did not find total or LDL cholesterol lowering potential of dried apple-powder, which contains both pectin and polyphenols, and the formulation of the bioactive components in the whole fruit matrix may instead be suggested as responsible for the health beneficial effects. It seems evident that the bioavailability of both the polyphenol fraction and the fibre fraction from apple are highly dependent on the composition and the competences of the host intestinal microbiota, and the apple matrix as a whole may be speculated to have a particularly beneficial prebiotic effect.

5 CONCLUSION

During the research in this project MS and NMR-based metabolomics were employed as new approaches to evaluate the health effects of apple and pectin intake. The results from the three papers included in this thesis can be summarised as follows:

- The application of a non-targeted MS-based metabolomics approach (Paper I) demonstrated that intake of apple and apple-pectin had a high impact on the urinary metabolome. Numerous clear exposure and effect markers of apple and apple-pectin intake were found and several new apple-related urinary metabolites were identified. Most of the excreted metabolites were products of diverse metabolic processes including phase II glucuronidation, glycine-conjugation and/or microbial metabolism.

- A targeted NMR-based metabolomics approach facilitated construction of chemometric models (Paper II) capable of fast and reliable prediction of cholesterol in different lipoprotein fractions of stored rat plasma. Application of these models demonstrated a significant HDL cholesterol lowering effect of dried apple-powder in rats.

- Based on the chemometric prediction models in Paper II, cholesterol in the different plasma lipoprotein fractions was predicted from rats fed with fresh apple or apple-pectin (Paper III). Apple intake induced a significant decrease in plasma LDL, HDL and total cholesterol whereas pectin intake did not induce any significant changes in this aspect. Non-metabolomics measurements illustrated that apple increased excretion of bile acids and revealed significant effects on genes involved in the hepatic glutathione redox cycle indicating a higher capability to handle oxidative stress. Pectin only affected expression related to glutathione utilisation.

In general, both the non-targeted and targeted MS and NMR-based metabolomics approach have served as powerful platforms during these studies, and the metabolomics technology seems very promising in further deconvolution of the interplay between dietary intake and health status. The investigation revealed overall that particularly fresh apple appears to have health beneficial effects on cholesterol metabolism, but from our results pectin cannot be appointed as the major apple component that caused this effect. The formulation of the bioactive components in the apple fruit matrix and the interaction with the intestinal microbiota seems of key importance for the potential health effects of apple. However, since these investigations were conducted with rat models, it is important to stress careful extrapolation to man, and confirming experiments are warranted in humans.

6 PERSPECTIVES

Metabolomics is a discipline dedicated to the study of metabolites, their dynamics, composition, interactions and responses to interventions or to changes in the environment. Numerous factors along the metabolomics pipeline have to be considered in the establishment of this technique to achieve successful and reliable results. Once firmly established, there seems to be various ways to use this technique, especially the use of every analytical run in a multipurpose approach seems promising. In this way the maximum yield can be gained from expensive intervention studies and possible new correlating variables or patterns among multiple samples and measurements can be revealed. For instance, plasma NMR spectra could provide a metabolite profile of around 100 known compounds, and at the same time the lipoprotein profile (even the subclasses of the lipoproteins) could be examined. Several other well-established disease risk markers may possibly be predicted at the same time from these same spectra. These results could again be compared with data from MS-based metabolomics, from which a much higher number of metabolites could be identified. However, the identification procedure of metabolites of interest is continuously a difficult and time-consuming task, but fortunately databases are constantly growing in reported metabolites, making the task somewhat easier for the researcher. When a metabolite is appropriately identified it would be of high benefit to quantify this by a targeted analysis. In fact, the maximum gain of metabolomics may be obtained when qualitative and quantitative analysis is combined. The knowledge of metabolite identity and their quantitative perturbations will provide information that can be very useful in interpretation of affected biochemical pathways.

A final way to combine and correlate data should be mentioned. An ultimate challenge includes integration of the different ‘omics technologies: proteomics, genomics and metabolomics, to obtain a more complete picture of health status and in this way to unravel links between disease prevention and dietary intake.

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PAPER I

LC-MS metabolomics top-down approach reveals new exposure and effect biomarkers of apple and apple-pectin intake

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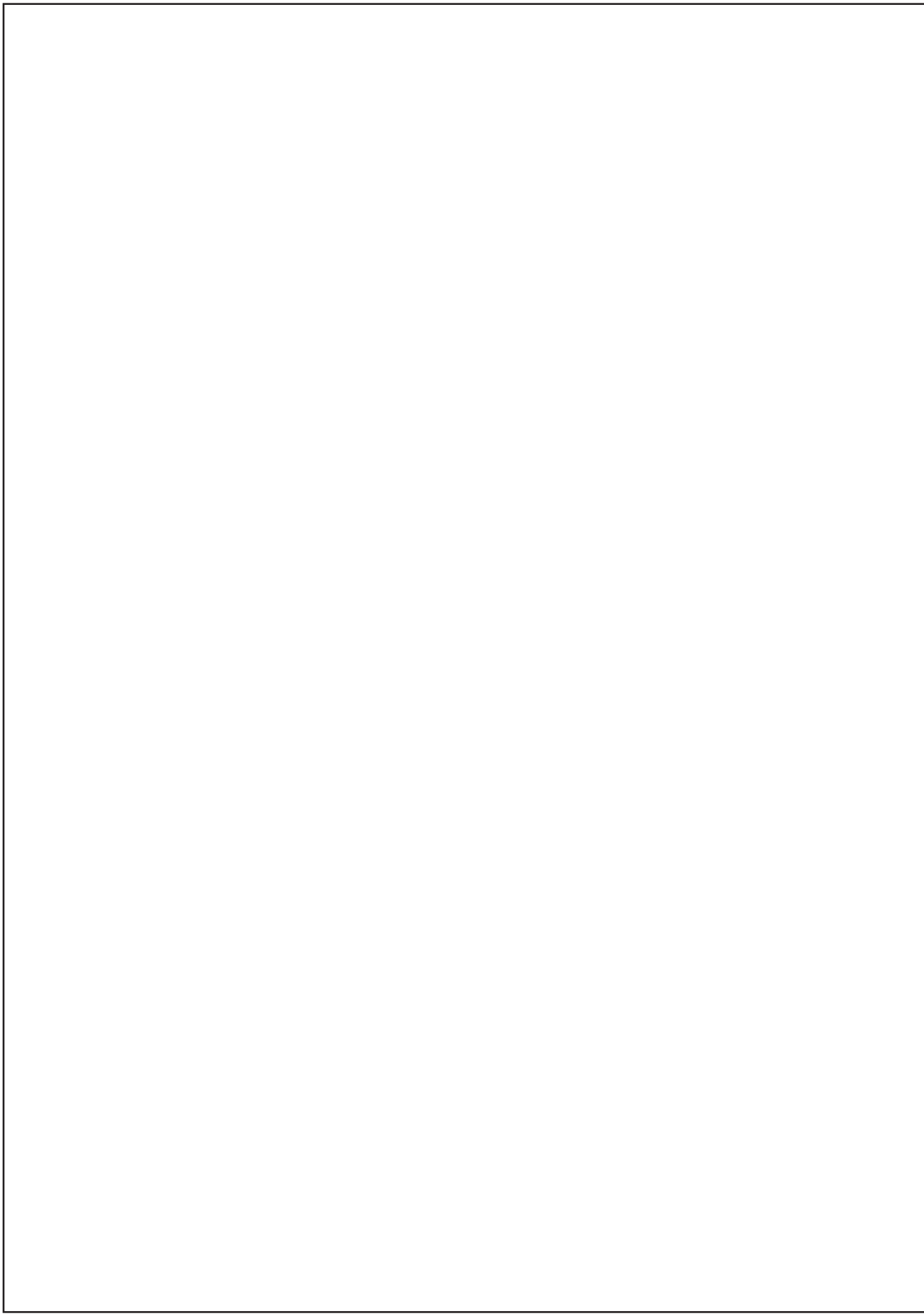

LC-MS metabolomics top-down approach reveals new exposure and effect biomarkers of apple and apple-pectin intake

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ABBREVIATED TITLE: LC-MS metabolomics markers of apple and pectin intake

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Abstract

In order to investigate exposure end effect markers of fruit and fruit fibre intake we investigated how fresh apple or apple-pectin affects the urinary metabolome of rats. Twenty-four Fisher 344 male rats were randomized into 3 groups and fed a standard diet with different supplementations added in two of the groups (7% apple-pectin or 10 g raw apple). After 24 days of feeding, 24 hour urine was collected and analyzed by UPLC-QTOF-MS in positive and negative ionization mode. Metabolites that responded to the apple or pectin diets were selected and classified as either exposure or effect markers based on their response patterns. An initial principal component analysis (PCA) of all detected metabolites showed a clear separation between the groups and during marker identification several new apple and/or pectin markers were found. Quinic acid, m-coumaric acid and (-)epicatechin were identified as exposure markers and hippuric acid as one of the effect markers of apple intake. Pyrrole-2-carboxylic acid and 2-furoylglycine were identified as pectin exposure markers while 2-piperidinone was recognized as a pectin effect marker. None of them has earlier been related to intake of pectin or other fibre products. We discuss these new potential exposure and effect markers and their interpretation.

Keywords: Metabolomics · LC-MS · apple · pectin · exposure and effect biomarkers

1 Introduction

It is well known that fruit consumption has preventive effects on degenerative diseases and especially cardiovascular disease (Bazzano et al., 2002; Liu et al., 2000), however the causal factors and their mechanisms of action are not well known. Apples represent one of the major fruits consumed throughout the western countries and the disease preventive factors of this fruit seem particularly relevant to investigate. Consumption of nutrients and other bioactive compounds from fruit will most likely interact with several physiological functions and metabolic pathways in the organism and hereby reduce the risk of disease. Methods that can handle multiple responses therefore seem particularly beneficial compared to the classical univariate approaches most often used in nutrition research. Metabolomics aim for measurement of all metabolites present in a given biological sample and by use of this technique the metabolic effect of e.g. apple intake can hereby be explored in a top-down manner compared to more targeted analytical methods. The open-minded approach of metabolomics has great potential to generate new hypotheses and thereby to improve our mechanistic understanding of why 'an apple a day keeps the doctor away'. Compared to a human study, the rats in this investigation are expected to exhibit a much lower level of background variation due to their isogenic nature and controllable habits and consequently a larger number of exposure- and effect related features in the recorded metabolome profiles. This may ease interpretation of effects in future human intervention studies where exposure to apple, pectin or fruit intake in general may be partially hidden in the large inter-individual variation. The combination of several features recorded in rat studies as related to apple exposure or effect would therefore help to identify more robust biomarkers in humans. Besides being helpful in our mechanistic understanding of dietary effects of apple intake, such objective biomarkers will be useful for the estimation of apple or fruit intake in samples from epidemiological studies, where the current methods based on questionnaires are prone to bias. Improved markers of intake should be useful to identify possible associations between dietary apple intake and disease prevention at the population level.

In the research presented here we want to focus on the cell wall polysaccharide, pectin, as a potentially disease preventive component in apples and in many other fruits. Pectins are presumed to prevent the reabsorbtion of bile acids in the intestine and to enhance steroid excretion so that more cholesterol is diverted into the bile acid pool (Ahrens et al., 1986).

However, pectins from different plant origins have a large structural diversity and thus possibly varied health effects, which presumably is the main reason why previous animal studies reporting on pectin feeding and plasma cholesterol have been inconsistent (Aprikian et al., 2003; Aprikian et al., 2001; Aprikian et al., 2002; Trautwein et al., 1998; Yamada et al., 2003). Thus, there is a need to sort out mechanisms and active components in order to understand the physiological effect of apple intake and of its associated pectin component. In this study we investigate the urinary metabolome following feeding of fresh apple and apple-pectin to rats in a nutritionally balanced feeding trial.

2 Materials and Methods

2.1 Materials

All apples used were from a single batch of the variety Shampion, grown in Skierniwice, Poland. Apple-pectin was a commercial unrefined product kindly provided by Obi-Pectin AG (Basel, Switzerland).

2.2 Animal study and sample collection

Twenty-four Fisher 344 male rats were randomized into 3 groups and all rats were fed a standard diet with different supplementations added in two of the groups. One group had 7% apple-pectin added to the diet, one group 10 g of raw apple and one group had no supplementation added to the diet (control). The diet was balanced so that all animals received the same amount of macro- and micronutrients (details to be published elsewhere). After 24 days of feeding, urine was collected in a collection vessel preconditioned with 1ml 1mM NaN_3 to avoid microbial growth. The collection vessel was surrounded by an insulated container filled with dry-ice to ensure that the urine kept a temperature below 5°C during a 24 hour collection period. The dry ice was replenished every eight hours during the collection period. Each urine sample was diluted with a fixed volume of 3 mL water used to wash the collection device in the metabolism cage and then weighed and immediately frozen at -80°C.

2.3 LC-QTOF-MS analysis

Before analysis the samples were thawed, filtered through a 40 μm Millipore filter (Millipore, Billerica, Massachusetts) and distributed randomly into a 96-well auto-injector tray. The tray was centrifuged to precipitate debris and 10 μL of each sample were injected into an UPLC (Waters, Milford, Massachusetts) with a 1.7μm C18 BEH column (Waters) operated with a 6.0 min gradient from 0.1% formic acid to 0.1% formic acid in 20% acetone: 80% acetonitrile. The eluate was analyzed in duplicate by Waters Premier QTOF-MS in both negative and positive modes. Ionization of molecules was achieved by applying a voltage of 2.8 or 3.2 kV to the tip of the capillary in negative or positive mode, respectively. This represents relatively soft ionization conditions but optimised so that fragmentation can occur and be helpful in our later structural interpretation. Data were collected in centroid mode using leucine-enkephalin as a lock-spray to calibrate mass accuracy every 10s. A blank (0.1%

formic acid) and a metabolomics standard containing 40 different physiological compounds were analyzed three times during the sample run. This standard was used to check mass error (<20 ppm) and retention time shift (<0.05min) during the run and when running authentic standards for verification.

2.4 Data preprocessing of LC-MS data

The raw data were extracted and aligned in retention time- and mass-direction by MarkerLynx^{TM} (Waters) by using two different processing conditions as detailed below, to discover as many important peaks as possible. Markerlynx^{TM} works by customized predefinition of several parameters and applies a peak picking algorithm to select potential markers. In the following the detected metabolites are termed 'features' when collected after the peak picking and alignment procedure and 'markers' after selection by data analysis. Two sets of parameters for data processing were used: A retention time window of 0.05 (0.1) s, a mass window of 0.05 (0.02) Da, a noise elimination level of 3 (6) standard deviations above background and an intensity threshold of 20 (30) cps. The first method resulted in 5350 features in the negative mode and 7668 features in the positive mode and the second method (parameters in brackets) resulted in 5574 and 8783 features in the negative and positive modes, respectively. The two datasets were exported to Excel (Microsoft) and after removing overlapping features the combined matrix consisted of 7380 and 12775 features in negative and positive mode, respectively. Duplicate sample analyses were combined as described by Bijlsma et al. (2006) meaning that if both measurement values were zero the combined value was zero and if both values were nonzero, the combined value was equal to the average of the two measurement values. If one replicate has a nonzero value and the other replicate is zero, the combined value is set to the nonzero measurement. The rationale for this procedure is that the combined value is most likely closer to the nonzero value since the measured zero value is expected to be due to a slip in the peak picking or because the analyte was not measured in the MS (e.g. momentary ion suppression). Moreover, due to the threshold level applied in the data processing step with the MarkerLynx^{TM} software some 'false' zero values will be present in the dataset. Consequently, before performing explorative analysis the data were divided into subsets (control/apple and control/pectin) and if a feature had more than 20% zero values within one of the groups in both subset it was excluded from the dataset (adapted from Bijlsma et al. (2006)) leaving 4010 and 7353 features in the negative and positive modes,

respectively. Finally, before data analysis the data was normalized to unit vector length (Euclidean norm) to reduce variations caused by instrumental variation, concentration difference in the urine samples etc. When using this type of normalization the 'closure' effect should always be considered, concerning that the true depletion of some peaks between samples will automatically be reflected in increased intensities for other peaks (and *vice versa*). However, since non-targeted metabolomics data are unlikely to be dominated by few high or low intensity variables, the risk of closure is considered minimal (Backstrom et al., 2007) and this normalization approach has earlier been applied successfully to other explorative metabolomics investigations (Nielsen et al., 2010; Scholz et al., 2004).

2.5 Data analysis of LC-MS data

A principal component analysis (PCA) was performed in Matlab (Matlab version R2009a, Matworks) for the whole dataset. Features were then divided into *exposure* markers and *effect* markers for apple and pectin intake, respectively. The selection criterion for exposure markers was that they should have only zero values in the control group and positive responses in all animals in the apple and/or pectin group (see Figure 1 marker #2 for an example). One misclasification was allowed in each group in order to tolerate small measurement errors of the MS instrumentation. In contrast, effect markers were defined as markers that have a baseline response in all animals in one group and a significantly up- or down-regulated response in all animals in the comparing group (one misclasification is allowed in each group). Simple binomial calculations give a P=0.00124 for a chance finding indicating that we can expect less than 5 false positives among the negative mode features and less than 10 in the positive mode. Less than one of these would be found among features without misclassifications. Figure 1 marker #3 illustates an effect marker.

Since pectin is present in the fresh apple it was investigated if any features display an apparent dose-response relationship to apple-pectin, given that a factor of 16.5 was the mean difference in apple-pectin consumed by the pectin group as compared with the apple group. The dose-response relationship was investigated using forward stepwise selection (Nørgaard et al., 2000). This method selects variables in a stepwise manner based on their capability to improve a multiple linear regression model (MLR) established between the chromatographic features and the pectin dose. By this method all markers are first tested individually in univariate linear regression models with the pectin dose as the dependent variable. All these

models are cross validated, and the variable with the lowest RMSECV is chosen. Then, all two-variable MLR models are investigated on the basis of the chosen variable in combination with all the remaining variables, one by one. All these models are cross validated, and the variable that (in combination with the first chosen variable) gives the lowest RMSECV is chosen. This procedure was continued as long as RMSECV decreased each time a new variable was introduced. A Partial Least Square (PLS) regression (Martens & Næs, 1993) model with the selected metabolites and the pectin dose as the dependent variable was then built to validate the dose response relationship.

2.6 Marker identification

After selection of exposure and effect markers the primary focus was to identify as many markers as possible. The nature of a QTOF-MS instrument allows very accurate mass measurement but despite the high data quality the chemical identification part of this kind of untargeted metabolomics experiments remains a highly laborious task involving interpretation of isotope patterns and fragmentation patterns, database or literature search and finally experimental verification of the selected markers by co-elution experiments. The lack of commercial availably authentic standards for some markers hinders their identification and these markers are left as tentatively identified. For some markers there are no known compounds that fit the characteristics observed by MS and these markers will need more advanced identification experiments which are beyond the scope of the present paper. Therefore, only a part of the markers listed in this publication will be chemically identified at the present time. As the first step in the identification work, retention time and response behaviour was compared between the markers to detect potential interrelated fragment-ions. Hereafter, the measured mass of a particular marker was searched in a database and the results compared to the isotopic fit in the mass spectra by use of the Markerlynx^{TM} elemental composition software. Then fragment ions were taken into consideration by looking directly in the raw data and by applying a mass fragment tool (MassFragment^{TM}, Waters) and finally a pure standard of the proposed compounds were analyzed by the UPLC-MS system to verify retention time and the fragmentation and/or adduct-forming pattern.

3 Results and Discussion

3.1 Exposure and effect marker identification

The initial PCA model of the urinary rat metabolome data (negative mode) is shown in Figure 1. The first 2 components of this model accounted for approximately 40% of the variation in the data. Urine samples from the different groups were separated by PCA with complete separation between the apple group and control/pectin groups (Figure 1).

Inset Figure 1

The total number of exposure and effect markers is shown in Figure 2 and their m/z values are listed in supplemental material, Table 1S and 2S. As seen from Figure 2 we observed 119 apple and 39 pectin exposure markers combined from positive and negative mode, with no overlapping markers between the groups. For the metabolites selected as effect markers 52 are found up-regulated in the apple group compared to the control group and 33 are found to be down-regulated. Likewise for the pectin group 42 markers are found as up-regulated pectin effect markers and 19 as down-regulated effect markers. When searching for overlapping apple and pectin effect markers we found 3 and 1 for the up- and down-regulated markers, respectively. Altogether, this is a high number of unambiguous exposure or effect markers and it is not expected to be possible to receive a similar result in a human intervention study. Therefore, the collection of these markers may be used to unravel the presumed more blurred response behavior of markers in human studies investigating apple or even fruit-related interventions. If it is possible to identify some of the markers found in this study, these could be combined selectively by multivariate modeling to search for response patterns.

Inset Figure 2

The location of the identified exposure and effect markers in the multivariate space is illustrated as different colors on the loading plot in Figure 1. Inspection of the PCA loading plot reveals that it is not only the features selected as exposure and effect markers, which are responsible for the grouping in the score plot. Other features may show an even stronger effect on the multivariate discrimination between the sample groups. This is because PCA reflects the overall variation in the data across the 24 rats and thus is insensitive to ’imperfections‘ of single features. The PCA shows no clear separation between the exposure

and effect markers in the loading plot, not even for the 'perfect' markers with no misclassifications, but it can be observed that e.g. up-regulated apple effect markers (Apple Effect Markers) and down-regulated apple effect markers (Ctrl-Apple Effect Markers) are mirrored across the center of the model and likewise for the apple exposure and pectin effect/exposure markers (Figure 1, colored marker groups). The single feature that contributes most strongly to distinguish the apple samples from the other samples is highlighted as #1 in the loading plot of Figure 1, and its analytical pattern across the 24 samples is shown in the upper right insert. Despite its clear response behavior this feature was not selected as an exposure marker by our present conditions due to a inconsistent response in the control group. Several other features with similar characteristics appear in the dataset, but will not be the target for this present investigation.

Our definition of an exposure marker in this present work represents potential biomarkers of apple or fruit/plant-based diet intake in general as well as of pectin intake. However, quantification of the markers and proper validation studies are needed before these markers can be used as true exposure biomarkers in e.g. observational studies. The term effect marker covers metabolites that are believed to reflect changes in the endogenous or gut microbial metabolome of the host and in this way may enlighten how apple and pectin intake physiologically affects the organism. In the variable selection procedure we distinguish between exposure and effect markers based on zero values or a constant baseline level. Though, before identification of markers it cannot be ruled out that exposure markers are not truly effect markers and *vice versa*. This investigation applied a low threshold level in the data extraction step in order to eliminate too much noise in the data. Without the threshold the dataset will be too large and unmanageable. If a true effect marker has a response that is lower than the threshold level this will appear as zeros in the data set and it will mistakenly be classified as an exposure marker. Likewise some effect markers may be regarded as wrongly classified if they are markers of dietary factors existing at different levels in both the control and the apple or pectin groups, as observed in the case of hippuric acid. However, this marker may also be seen as an effect of diet on the metabolic capacity of the microbiota and of the endogenous glycine-conjugation systems. Nevertheless, for the majority of the markers identified this does not appear to be a problem and the quality grading of markers was effective to ease the systematization and interpretation of unknown compounds, see the section below on identified markers.

3.2 Features exhibiting pectin dose-response relation

Since the fresh apple dose contains pectin in the relationship 1:16.5 compared to the pure pectin dose this accommodates the selection of features displaying a dose response relationship. By the use of forward selection the positive and negative mode features were investigated for a response relationship with the pectin dose. Approximate dose-response relationships were observed for 3 of the markers in the positive mode and the result of the PLS model built on these 3 selected markers is shown in Figure 3. The correlation coefficient (r^2=0.95) is very high although the samples show considerable deviations from the expected means within each group. The markers were forward selected and the first selected marker has the highest correlation to the pectin dose vector and is also selected as a pectin effect marker (134.0967 m/z). This marker appears to be a fragment ion of another pectin effect marker with mass 239.1386 m/z and also a fragment of 197.1286 m/z shows the same response pattern between the samples at this specific retention time (2.21 min). Unfortunately, it has not been possible to identify this compound but the isotopic pattern indicates that the chemical structures of these compounds are most likely: $C_{12}H_{19}N_2O_3$ (239.1386 m/z), $C_{10}H_{17}N_2O_2$ (197.1286 m/z) and $C_9H_{12}N$ (134.0967 m/z). Our present study was not designed as a dose response study and even though the pectin had the same origin the animals were fed with either isolated pectin or pectin in fresh apple and this may not leave the same physiological response. This probably explains why we do not find numerous markers with pectin dose response characteristics. Another possibility is that pectin is degraded more slowly when it is fed as a component of the apple food matrix and that the concentration of pectin degradation products in different segments of the gut would therefore only partially be reflected by the total pectin dose in the food.

Inset Figure 3

3.3 Identification and interpretation of markers

In the following the markers identified to date (listed in Table 1) will be discussed with regard to their origin, metabolism and whenever possible, their health related perspectives. Only the markers verified with an authentic standard will be discussed in details whereas the tentatively identified markers (markers in italic font in Table 1) will only be discussed to a very limited extent.

Inset Table 1

Quinic acid and *m*-coumaric acid were found as exposure markers for apple intake, in good consistency with the presence of these compounds or their precursors in fresh apple. To the best of our knowledge, quinic acid has not earlier been reported as a urinary metabolite after apple intake but the compound is present in apples and many other different plants, where it is a key intermediate in the biosynthesis of aromatic compounds (Humle, 1956). However, the excreted quinic acid may also in part originate from gut microbial degradation of chlorogenic acid (Gonthier et al., 2003), which has been quantified by HPLC-analysis of fresh apple material from the same batch of Shampion apples used in the present study at a level of approximately 70 mg/kg (data to be published elsewhere). *m*-Coumaric acid derives from gut microbial dehydroxylation of caffeic acid, another chlorogenic acid metabolite. In previous targeted investigations (Mennen et al., 2006) a correlation between apple consumption and urinary excretion of *m*-coumaric acid was reported. A study by Gonthier et al. (2003) showed that rats supplemented with either chlorogenic acid, caffeic acid or quinic acid had an increased excretion of several different metabolites (inclusive hippuric acid) after intake of chlorogenic and caffeic acid whereas the only urinary metabolite derived from quinic acid was hippuric acid. The authors (Gonthier et al., 2003) conclude that this clearly indicates that the quinic acid moiety in chlorogenic acid is the major precursor of hippuric acid. Hippuric acid is formed by aromatization of quinic acid into benzoic acid by the gut microbiota and subsequent conjugation with glycine in the liver and kidney. Figure 4 illustrates these described formations of quinic acid, m-coumaric acid and hippuric acid from chlorogenic acid. Hippuric acid is formed by many other microbial metabolic routes and is also present as an identified effect marker in our data with higher response in the apple group compared to the control group. The tentative identification of 3-hydroxyhippuric acid as an apple effect marker could indicate that it is another metabolite derived from chlorogenic and caffeic acids, although it may also have an origin from microbial degradation of e.g. dietary catechin and epicatechin (de Pascual-Teresa et al., 2010), which are also identified in this study. The presence of hippuric acid and 3-hydroxyhippuric acid as effect markers may also indicate a higher efficacy of specific metabolic pathways of the gut microbiota and the host glycine conjugation system. An effect on the composition of the gut microbiota in the present study is therefore indicated and in accordance with previously published findings (Licht et al., 2010).

Until recently quinic acid and hippuric acid were believed to have no biological effects but investigations by Pero et al. (2009) have revealed that quinic acid via microbial conversion nutritionally supports the synthesis of tryptophan and nicotinamide in the intestine, and that this in turn leads to DNA repair enhancement and NF-kB inhibition via increased nicotinamide and tryptophan production in humans. This may be an important health aspect to consider when evaluating health effect of apple and other plant products containing quinic acid. It remains to be established whether hippuric acid is a surrogate marker for the activation of these pathways.

Inset Figure 4

We also found an apple exposure marker at the retention time of (-)epicatechin (=1.78 min) with a m/z value of 139.0397 corresponding to the retro-Diels-Alder fragmentation patteren that usually occurs when performing MS analysis of flavan-3-ols (Shaw & Griffiths, 1980). The detected marker derives from the A-ring of epicatechin and this parent molecule was not itself detected as a feature but its mass peak was visible when inspecting mass spectra from the apple group. Epicatechin and its isomer catechin are well-known components in apple and unlike most other flavonoids, catechins are not on a glycosylated form in the source material (Escarpa & Gonzalez, 1998). Only a minor part of ingested catechins are thought to be circulating and excreted as the unconjugated form since these compounds are primarily glucuronidated in the enterocytes after absorbtion and even often further deglucuronidated and methylated and/or re- glucuronidated or sulphated in the liver before excretion (Donovan et al., 2001). Accordingly, we found the epicatechin glucuronide and catechin glucuronide among our exposure markers although the retention times (and positions of glucoronide groups) of these markers are not verified due to lack of commercial standards. However, as expected, their elution time is prior to the elution time of their unconjugated forms (catechin =1.68 min and epicatechin = 1.78 min) and their fragmetation pattern is comparable to what is observed in a targeted MS/MS experiments with these compounds (Schroeter et al. 2006). Methyl epicatechin was also tentatively identified with a longer retention time than epicatechin and a retro-Diels-Alder fragmentation patteren confirming its identity. The metabolite, dihydroxyphenyl-γ-valerolactone, was also tentatively identified and this compound has earlier been identified as a major human urinary metabolite after intake of (-)-

epicatechin and this lactone metabolite appears to be produced by intestinal microorganisms (Li et al., 2000). Several in vitro studies have been performed with the aglycone form of catechins to elaborate on the health aspects of epicatechin and catechin consumption. However, since most of the circulating polyphenols are conjugated after absorbtion or metabolised by the gut microbiota, there is a lack of studies to elaborate on the biological properties of the conjugated or metabolised derivatives. It is reasonable to assume that the mammalian phase II enzymatic mechanism would reduce most health effects of catechins by increasing water solubility and excretion, although minor amounts of the native aglycone and of methylated catechins will always be present in circulation.

The metabolites we have identified as pectin markers are compounds that have not earlier been linked with pectin or other fibre products.

Pyrrole-2-carboxylic acid was identified as an exposure marker of pectin intake by a convincing fit of isotope and fragmentation patterns and by co-elution of an authentic standard. The compound has previously been found in rat and human urine after administration of the D-isomers of hydroxyproline and its biotransformation to pyrrole-2-carboxylic acid is thought to be catalyzed by D-amino acid oxidase in the kidney (Heacock & Adams, 1974; Heacock & Adams, 1975). Apple fruit tissue contains readily soluble glycoproteins, rich in hydroxyproline (Knee, 1973) which trail pectin in the industrial extraction and purification process of apple pectin (Kravtchenko et al., 1992). The rats in the pectin group will therefore have a high exposure to hydroxyproline with subsequent metabolite production of pyrrole-2-carboxcylic acid. It would be expected to see low levels of pyrrole-2-carboxcylic acid also in the apple and control group, since hydroxyproline is present in fresh apple and also is a nonessential amino acid in mammals. Low intensity mass peaks can be detected in these groups from raw data but the signals are not strong enough to reach the threshold level for data processing into metabolome features. To the best of our knowledge no previous studies with food-related interventions have reported on the existence of this metabolite in urine or any other biofluids. Since pyrrole-2-carboxcylic acid is either formed in the colon by microbes or in the kidneys, the main metabolite circulating after hydoxyproline intake may either be hydroyproline itself or pyrrole-2-carboxcylic acid. Only two studies have reported on the health related effect of pyrrole-2-carboxylic acid. One related pyrrole-2-carboxylic acid in urine with lung cancer in miners from the uranium

industry (Svojtkova et al., 1982). Another study reported that pyrrole-2-carboxcylic acid at a dose of 200 mg/kg in rats and rabbits caused a suppression of platelet aggregation (Komiyama et al., 1986). Future investigations on the effects of pectin or other plant cell wall constituents should take the potentially high hydroxyproline-intake and its metabolism into consideration as potentially bioactive effectors.

2-Furoylglycine was also identified as a pectin exposure marker by the use of an authentic standard and we could identify a glycine fragment (74.0242 m/z) at the same retention time. 2-Furoylglycine is an acyl glycine and an earlier uncontrolled study showed presence of this metabolite in urine of 20 normal adults (Pettersen & Jellum, 1972). To detect if the precursors of this compound was of exogenous dietary origin these authors also provided a male adult with a simple synthetic diet (tripalmin, triolein, sucrose and water) for 3 days. No 2-furoylglycine was detected in the urine after 2 days but the compound reappeared when an ordinary diet was reintroduced. From these findings it was suggested that furan derivatives or their precursors were introduced into food by cooking when reduced sugars are heated in the presence of free amino groups. Purified apple pectin carries a lot of neutral sugar molecules and some proteins (Kravtchenko et al., 1992) and the same Maillard reaction may happen either during the industrial pectin extraction procedure where the apple pomace is boiled in hot acid or during the hot drying process giving rise to furan derivatives such as furfural (2-furanaldehyde). An alternative explanation is that furfural which is naturally present in apple and apple products have affinity for pectin and may be concentrated with the pectin fraction during industrial processing. Further investigations are needed to identify the exact source of the 2-furane-precursor in pectin and to ascertain whether it is specific to pectin intake. 2-Furoylglycine was found to be the primary urinary metabolite in rats after oral administration of furfural and furfuryl alcohol. The latter appears to be oxidised to furfural which is further oxidised to furoic acid (Nomeir et al., 1992). Furoic acid is conjugated with glycine to form 2-furoylglycine by the enzyme acyl-CoA:glycine N-acyltransferase, which is located in the mitochondria of liver and kidney tissue (Knights et al., 2007). In this study we identified other acyl glycines that have been conjugated in the same way; hippuric acid, 3-hydroxyhippuric acid and hydroxyphenylacetylglycine. The last two have not been verified by authentic standards but glycine fragments were observed in the raw data at their specific retention times indicating the expected fragmentation. In general, the amino acid conjugation serves to

inactivate reactive acyl-CoA thioesters of carboxylic acids of endogenous and exogenous origin (Knights et al., 2007) and these acyl glycines will probably exhibit relatively low physiological activity. However, their precursors and intermediate metabolites may be more likely to affect health status prior to their enzymatic conversion.

2-Piperidinone was identified as an upregulated effect marker of pectin intake in this study. To the best of our knowledge no previous studies have reported on the existence of this metabolite in urine or blood. The compound has been identified in a forensic study in the decomposition fluids from pig carcasses (Swann et al., 2010). 2-Piperidinone was also found in the anal sac secretions of different animals and it was discussed that this compound could be formed by the elimination of water from the precursor 5-aminovaleric acid by microbial fermentation processes (Albone et al., 1976; Burger et al., 2001). However, from the studies conducted to date it is not possible to decide if 2-piperidinone or its precursors are of dietary origin or is exclusively an endogenous or microbial metabolite. We have previously shown that pectin in this study caused a marked change in the caecum microbiota (Licht et al., 2010). We are currently investigating the relationships between these changes and the metabolomic patterns in fecal water and urine.

Among the effect markers that we have tentatively identified there are several catecholamine metabolites (3-methoxy-4-hydroxyphenylethyleneglycol sulphate, homovanillic acid sulphate, metanephrine, hydroxyphenylacetylglycine and methoxytyrosine) indicating changes in the hormonal metabolism or in metabolite transport after the apple and pectin diets. The identities of these markers seem likely with respect to accurate mass, elemental composition and fragment patterns but no coelution experiments with pure standards have been performed, again due to lack of commercial standards. Firm conclusions will therefore have to await the confirmation of an effect of apple intake on the excretion of these hormonal effector compounds.

4 Conclusion

By applying an untargeted MS-based metabolomics approach it has been demonstrated that intake of apple and apple pectin has a high impact on the urinary metabolome. Numerous clear exposure and effect markers of apple and apple-pectin intake have been found and several new apple-related urinary metabolites have been identified in this study. Most of these excreted metabolites are products of diverse metabolic processes including phase II glucuronidation, glycine-conjugation and/or microbial metabolism and a combination of several of the markers recorded in this rat study could ease identification of more robust biomarkers in human studies. Additionally, the markers identified in this study should shed new light on health interpretations of fruit intake in previous as well as future conducted studies. The explorative top-down metabolomics approach employing a division into effect and exposure markers seems promising as a powerful tool in formation of new ideas and hypothesis to deconvolute the interplay between dietary intake and health status.

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LEGEND TO FIGURES

Fig. 1 PCA score (top) and loading plot (lowest) of PC1/PC2 with all features measures in negative mode (n = 4010). Data are mean centred and two times pareto scaled. Diagram 1-3 illustrates the corresponding response pattern of a selected feature and markers from the loading plot.

Fig. 2 Venn diagrams showing summarized number of selected exposure (left) and effect markers (right) obtained from positive and negative ionization mode.

Fig. 3 PLS model (1 PLSC, mean centred) based on 3 metabolites selected by forward selection of features from positive mode. Black triangles represent the control group, filled red circles the apple group and white diamonds the pectin group.

Fig. 4 Formation pathway of *m*-coumaric acid, quinic acid and hippuric acid. Structures drawn in ACD/ChemSketch ver. 12.0 (www.acdlabs.com).

Table 1 Summary of identified metabolites

Metabolite	Molecular formula	m/z – in pos or neg mode (+/-)	Rt time (min)	↑↓[a]
Apple exposure markers				
Quinic acid	C7H12O6	191.0555 (-)	0.641	↑
m-Coumaric acid	C9H8O3	163.0393 (-)	2.227	↑
(-)-Epicatechin	C15H14O6	139.0397[b](+)	1.779	↑
Epicatechin glucuronide	C21H22O12	465.1045 (-)	1.671	↑
Methyl epicatechin	C16H16O6	305.1008 (+)	2.031	↑
Dihydroxyphenyl-γ-valerolactone	C11H12O4	209.0799 (+)	1.735	↑
Catechin glucuronide	C21H22O12	465.1043 (-)	1.578	↑
Apple effect markers				
Hippuric acid	C8H9NO	134.0606[c](-)	1.893	↑
3-Hydroxyhippuric acid	C9H9NO4	196.0633 (+)	1.579	↑
3-Methoxy-4-hydroxyphenyl ethyleneglycol sulfate	C9H10O6S	245.0108[d](-)	1.761	↑
Homovanillic acid sulfate	C9H10O7S	261.0079 (-)	1.454	↑
Metanephrine	C10H15NO3	198.1132 (+)	2.493	↓
3-Methylglutaconic acid	C6H8O4	143.0351 (-)	1.389	↓
Pectin exposure markers				
Pyrrole-2-carboxylic acid	C5H5NO2	110.0244 (-)	1.606	↑
2-Furoylglycine	C7H7NO4	168.0314 (-)	1.523	↑
Pectin effect markers				
2-Piperidinone	C5H9NO	100.0758 (+)	1.373	↑
Hydroxyphenylacetyl glycine	C10H11NO4	210.0762 (+)	1.516	↑
3-Methoxytyrosine	C10H13NO4	210.0769 (-)	2.391	↑

[a]Up- or down-regulated response of marker.[b]Retro-Diels-Alder MS-fragment, [b]2-Phenylacetamide;well-known dautherion of hippuric acid and [c]Water loss. Names in italics refer to compound identifications that are highly probable due to isotope- and fragmentation pattern, however not verified by an authentic standard.

FIGURE 1

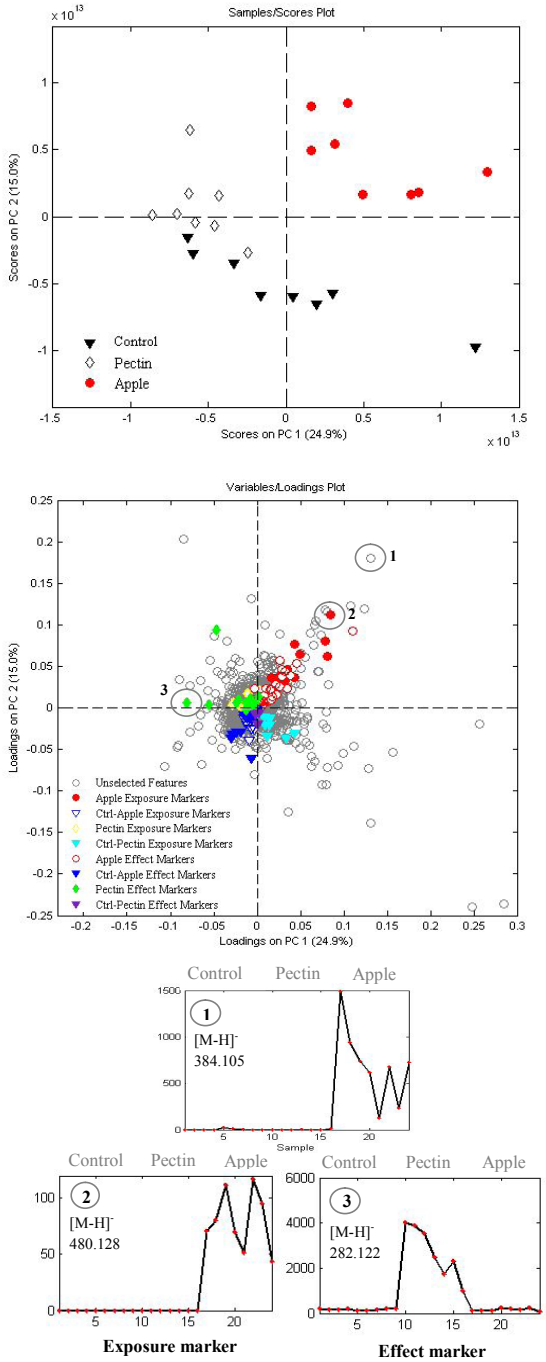


Figure 1. PCA score (top) and loading plot (lowest) of PC1/PC2 with all features measures in negative mode (n = 4010). Data are mean centred and two times pareto scaled. Diagram 1-3 illustrates the corresponding response pattern of a selected feature and markers from the loading plot.

FIGURE 2

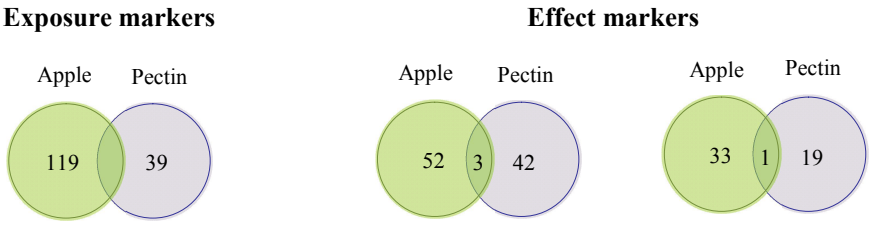

Exposure markers
Apple
Pectin
119
39
Effect markers
Apple
Pectin
52
3
42
Apple
Pectin
33
1
19

FIGURE 3

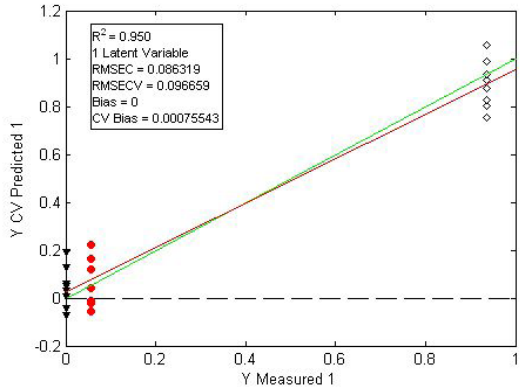
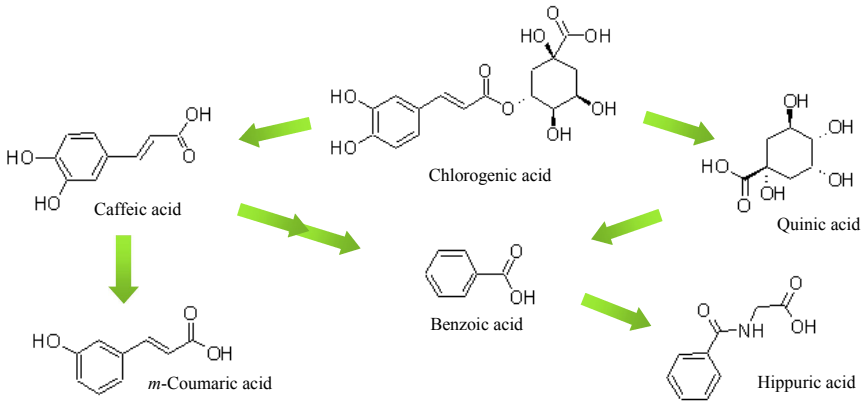

R2 = 0.950
1 Latent Variable
RMSEC = 0.086319
RMSECV = 0.096659
Bias = 0
CV Bias = 0.00075543
Y CV Predicted 1
Y Measured 1
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.2
0.4
0.6
0.8
1

FIGURE 4


Caffeic acid
Chlorogenic acid
Quinic acid
Benzoic acid
m-Coumaric acid
Hippuric acid

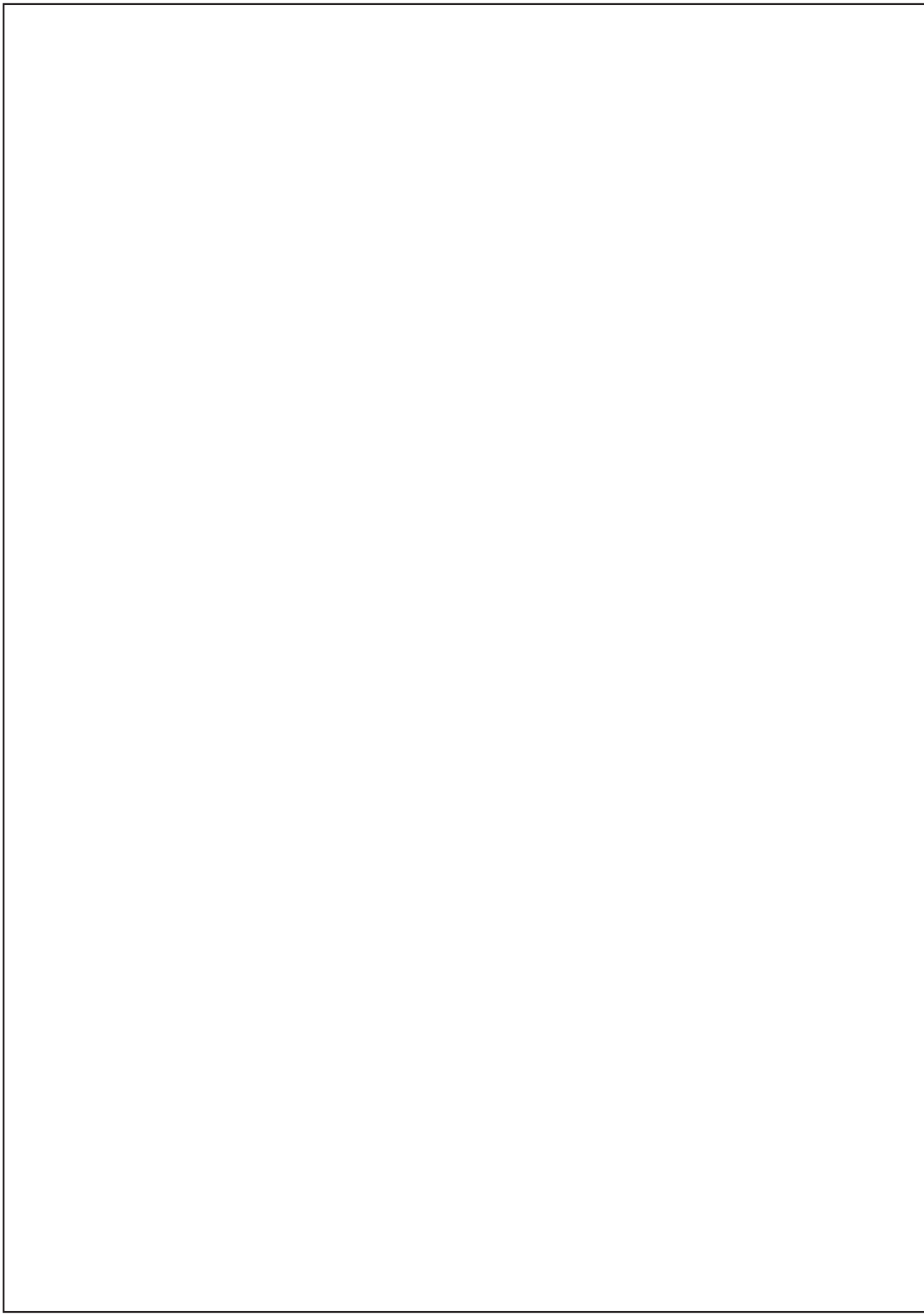
SUPPLEMENTAL MATERIAL

Table 1S Observed *exposure* markers for apple and pectin intake. Markers are selected if only zero values appear in one group and responses are measured in all animals in the comparing group (bold font means perfect classification and normal font means one misclasification is allowed in each group). Markers are listed in the order of perfect markers first and subsequently markers are ranked according to the highest mean response values. All m/z values represent measured mass (negative and positive ionization modes, respectively).

	Apple markers		**Pectin markers**	
Rank No.	Negative ionization mode $[M-H]^-$	Positive ionization mode $[M+H]^+$	Negative ionization mode $[M-H]^-$	Positive ionization mode $[M+H]^+$
1	**289.0387**	**149.06**	**303.0923**	**197.1286**
2	**191.0555**	**305.1018**	**268.085**	**295.1296**
3	**465.1043**	**455.106**	**343.116**	**157.1223**
4	**367.1054**	**109.0655**	**269.0197**	**139.1118**
5	**479.1205**	**210.0398**	110.0244	305.1109
6	**725.1395**	**272.1138**	168.0314	129.1391
7	**748.2204**	**407.0946**	159.0318	72.0805
8	**283.1534**	**95.0501**	295.1405	95.0698
9	**407.194**	**305.1008**	264.9698	283.1296
10	**526.3048**	**105.0382**	265.1153	268.0791
11	**353.1828**	**83.085**	240.0916	853.5921
12	**505.1206**	**351.1075**	263.0973	445.0923
13	**341.124**	**439.1216**	524.1376	345.1291
14	**239.1664**	**621.2308**	185.0509	329.1541
15	**465.1045**	**459.1606**	585.2152	201.1252
16	**245.0944**	**369.1494**	224.0916	100.023
17	**140.975**	**514.2309**	453.285	556.8564
18	**577.0857**	170.0437	616.1503	97.1004
19	**143.0711**	209.0799		359.2565
20	**543.141**	223.0964		445.2658
21	**767.2019**	305.1038		953.1418
22	**543.1411**	177.061		
23	**237.1537**	191.0705		
24	273.0424	445.1443		
25	100.0393	170.029		
26	369.1185	346.15		
27	692.1282	96.0465		
28	513.0159	139.0397		
29	163.0393	416.1553		
30	287.0256	385.1122		
31	491.0351	139.04		
32	317.0378	367.0996		
33	165.0556	105.0375		
34	557.0969	95.0493		
35	487.1462	421.1105		
36	281.1384	367.101		
37	499.0771	336.1111		
38	479.12	350.8857		
39	653.1094	481.1354		
40	301.0386	100.0247		
41	543.9475	373.2733		
42	267.0012	358.0762		
43	463.0867	437.0856		
44	191.0682	239.0596		
45	248.5909	337.1614		
46	345.1464	528.3171		
47	441.1968	660.8224		
48	248.7767	291.2371		
49	295.0272			
50	447.0658			
52	295.0291			
54	209.0704			
55	403.196			
56	382.011			
57	475.059			
58	411.0803			
59	803.2265			
60	417.1736			
61	105.0381			
62	303.0571			
63	477.991			
64	395.1915			
65	361.15			
66	579.0778			
67	331.9478			
68	185.0134			
69	723.1206			
70	449.2082			
71	276.6633			

Table 2S Observed *effect* markers of apple and pectin intake. Effect markers are here defined as markers that have a baseline response in all animals in the control group and this response is up- or down-regulated in all animals in the comparing group (bold font means perfect classification and normal font means one misclasification is allowed in each group). Markers are listed in the order of perfect markers first and subsequently markers are ranked according to the highest difference in mean response between the compared groups. All m/z values represent measured mass (negative and positive ionization modes, respectively).

	Negative ionization mode $[M-H]^-$				**Positive ionization mode $[M+H]^+$**			
	Apple markers (m/z)		**Pectin markers (m/z)**		**Apple markers (m/z)**		**Pectin markers (m/z)**	
Rank No.	Upregulated by apple intake	Downregulated by apple intake	Upregulated by pectin intake	Downregulated by pectin intake	Upregulated by apple intake	Downregulated by apple intake	Upregulated by pectin intake	Downregulated by pectin intake
1	**245.0108**	**208.0631**	**212.000**	**269.1055**	**142.0543**	**105.0698**	**82.014**	**282.2774**
2	**305.0435**	**74.024**	**281.114**	**383.1253**	**209.0761**	**229.1219**	**239.1386**	**247.1335**
3	**134.0606**	**307.1179**	**80.9643**	**307.1179**	**112.8979**	**229.1255**	**134.0967**	**265.2519**
4	**369.1158**	**216.0872**	**288.0185**	**309.1156**	**223.1036**	**83.0488**	**114.0913**	**254.2472**
5	**319.0571**	**390.1311**	**252.0379**		**216.0648**	**122.0423**	**261.1311**	**228.2317**
6	**100.0247**	**357.1003**	**282.0461**		**165.0549**	**226.1437**	**112.8979**	**247.1344**
7	**142.0508**	**114.0923**	**321.155**		**123.0457**	**233.1178**	**261.1326**	**167.1079**
8	**397.1047**	**143.0351**	**470.0596**		**121.0456**	**427.1876**	**100.0758**	**237.2211**
9	**121.0399**	**162.0224**	**308.0786**		**196.0482**	**198.1132**	**210.0762**	**330.2602**
10	**107.0496**	**222.1127**	**691.9436**		**196.0633**	**188.1278**	**441.3339**	**219.2104**
11	**186.0308**	**215.1286**	176.9862		**68.9982**	**113.0953**	**333.1492**	242.2493
12	**261.0079**	**224.127**	210.0769		**85.0291**	251.1292	**400.7675**	265.0661
13	**119.0497**	**145.0143**	391.2486		175.0734	612.2042	**555.2775**	82.0653
14	**121.0288**	**329.1623**	393.2659		272.1088	839.4365	**445.3128**	265.0659
15	**171.1022**	307.0971	232.8866		235.1155		**635.4167**	184.1014
16	**410.0157**	291.0972	413.0417		199.1092		**558.6806**	
17	**589.08**	510.7663	530.0668		191.0568		**542.6993**	
18	**331.049**	329.1006	465.1657		195.1009		171.1199	
19	**173.0577**	292.0861	343.0596		269.0989		195.1009	
20	**366.7686**				422.1653		613.3418	
21	**458.0696**				390.1471		422.1653	
22	383.0951						390.093	
23	415.1156						723.4687	
24	184.0982							
25	209.0312							
26	175.0613							
27	118.9312							
28	191.0467							
29	343.1352							
30	403.0368							
31	165.0193							



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ORIGINAL ARTICLE

PAPER II

NMR and interval PLS as reliable methods for determination of cholesterol in rodent lipoprotein fractions

NMR and interval PLS as reliable methods for determination of cholesterol in rodent lipoprotein fractions

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Abstract Risk of cardiovascular disease is related to cholesterol distribution in different lipoprotein fractions. Lipoproteins in rodent model studies can only reliably be measured by time- and plasma-consuming fractionation. An alternative method to measure cholesterol distribution in the lipoprotein fractions in rat plasma is presented in this paper. Plasma from two rat studies ($n = 68$) was used in determining the lipoprotein profile by an established ultracentrifugation method and proton nuclear magnetic resonance (NMR) spectra of replicate samples was obtained. From the ultracentrifugation reference data and the NMR spectra, an interval partial least-square (iPLS) regression model to predict the amount of cholesterol in the different lipoprotein fractions was developed. The relative errors of the prediction models were between 12 and 33% and had correlation coefficients (r) between 0.96 and 0.84. The models were tested with an independent test set giving prediction errors between 19 and 46% and r between 0.96 and 0.76. Prediction of High, Low and Very Low Density Lipoprotein (HDL, LDL and VLDL) and total cholesterol was conducted in a study where rats had been supplemented with two doses of air-dried apple-powder. No significant difference in LDL, VLDL and total cholesterol was observed between the groups. The high apple-powder (20%) group had significantly lower HDL cholesterol (11%, $P = 0.0452$) than the control group. It is concluded that the iPLS approach yielded excellent regression models and thus univocal established chemometric analysis of NMR spectra of rat plasma as a strong and efficient way to quantify lipoprotein fractions in rat studies.

Keywords Lipoproteins · NMR · iPLS · Apple · Targeted metabolomics

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1 Introduction

It is well known that risk of cardiovascular disease is related to distribution of cholesterol in different lipoprotein fractions (Castelli 1996). Diet is strongly influencing cholesterol distribution and rodent studies are often used to clarify and explore underlying mechanisms of dietary health effects including effects on cholesterol distribution. The reference method for determining this distribution is based on lipoprotein fractionation followed by detection of cholesterol in the different fractions. Kits are available for human samples to determine triglycerides as well as total, low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol directly on isolated plasma or serum (Roche Diagnostic, Germany). Very low density lipoprotein (VLDL) and LDL cholesterol may be determined by the Friedewald formula (Friedewald et al. 1972). Since commercial lipoprotein kits for rodents are not available, and the Friedewald formula does not apply for them,

researchers need to find other ways to explore effects on the lipoprotein profile in rodents. A common practice is to use human lipoprotein kits instead. However, the lipoprotein profile of rats differs significantly from those of humans, since HDL is the major carrier of cholesterol in rats, whereas LDL carries most of the cholesterol in humans (Davis and Vance 1996). Thus, the use of human lipoprotein kits in rat studies may most likely cause invalid results. The limited volume of blood available is another important issue, when setting up rodent studies to explore, e.g. the lipoprotein profile. The standard methods to determine cholesterol content in the different lipoprotein fractions are ultracentrifugation or gel electrophoresis (Baumstark et al. 1990; Contois et al. 1999) which are very time and labour consuming techniques, and require considerable amounts of plasma. Furthermore, only fresh plasma may be used for lipoprotein fractionation and a methodology to determine lipoprotein cholesterol distribution in stored frozen plasma is desirable. Therefore, an alternative method to measure the lipoprotein profile in rodents is needed. Previous studies in humans (Dyrby et al. 2005; Petersen et al. 2005; Bathen et al. 2000) have taken advantage of the proton nuclear magnetic resonance (NMR) spectra of plasma since these contains latent information about the concentration of lipoproteins and the use of partial least-square (PLS) regression models may allow specific extraction of this information. In the present study we employ this approach on rat plasma samples, which is to our best knowledge still unexplored. We report here its application to samples from rats fed different doses of dried apple powder.

2 Materials and methods

2.1 Materials

The apple pomace and powder used in the present study were produced from the apple variety Shampion, grown in Skierniwice, Poland. The apple pomace used in Study B was the remaining from production of clear juice. The pomace was freeze-dried and ground. The apple powder for Study C was produced by air-drying and grinding of the apples. Macronutrient, total phenolics and pectin composition of the apple products is shown in Table 1.

2.2 Study design

Three different rat studies were exploited in this investigation and they are summarized in the following. Study A and B were used to build calibration models, whereas the cholesterol distribution in the different lipoproteins were

Table 1 Composition of the experimental diets

Ingredients (g/kg feed)	Study A[e] and Study B control	Study B 2.1% pomace	Study B 6.3% pomace	Study C control	Study C 10% apple-powder	Study C 20% apple-powder
Na-caseinate	200	200	200	200	200	200
Sucrose	100	93	78	100	75	50
Fructose	–	–	–	54	27	–
Cornstarch	456	446	426	402	370	338
Soybean oil + AEDK	50	50	50	50	50	50
Soybean oil	20	20	20	20	20	20
Corn oil	80	80	80	80	78	76
Cellulose	50	46	37	50	36	22
Mineral mixture[a]	32	32	32	32	32	32
Vitamin mixture[b]	12	12	12	12	12	12
Apple pomace[c]	–	21	65	–	–	–
Apple-powder[d]	–	–	–	–	100	200

[a] Containing in mg/kg diet: 2500 Ca; 1600 P; 3600 K; 300 S; 2500 Na; 1500 Cl; 600 Mg; 34 Fe; 30 Zn; 10 Mn; 0.20 I; 0.15 Mo; 0.15 Se; 2.5 Si; 1.0 Cr; 1.0 F; 0.5 Ni; 0.5 B; 0.1 B; 0.1 V; 0.07 Co

[b] Containing in mg/kg diet: 5000 (IU) vitamin A; 1000 (IU) vitamin D_3; 50 (IU) vitamin E; 5 thiamin; 6 riboflavin; 8 pyridoxol; 2 folic acid; 0.3 D-biotin; 0.03 vitamin B-12; 20 pantothenate; 2600 cholinhydrogentartrat; 400 inositol; 40 nicotinic acid; 1 phylloquinine; 40 *p*-aminobenzoic acid; 1000 methionine; 2000 L-cystine

[c] Dry matter (g/100 g), macronutrient (%), total phenolics (mg/kg) and pectin (g/kg) composition of apple pomace: 92.5 dry matter; 6.6 protein; 3.8 fat; 1.6 ash; 21.1 carbohydrates; total dietary fiber 59.5; total phenolics 3659.5; 32 total pectins; 7.3 water soluble pectins

[d] Dry matter (g/100 g), macronutrient (%), total phenolics (mg/kg) and pectin (g/kg) composition of apple pomace: 99.0 dry matter; 1.8 protein; 0.6 fat; 1.1 ash; 81.2 carbohydrates; total dietary fiber 13.2; total phenolics 2407.0; 97 total pectins; 20 water soluble pectins

[e] 10.1 g and 20.3 g cholesterol/kg is mixed in the diet for group 2 and 3, respectively, in Study A

Table 2 Performance of the different iPLS models; HDL, LDL, VLDL, IDL and total cholesterol

Calibration models							Test set models		
Lipoprotein fraction	r	RMSECV (mmol/L)	PLSC[a]	Reference interval	Reference mean	Relative RMSECV (%)[b]	r	RMSEP (mmol/L)	Relative RMSEP (%)[c]
HDL	0.90	0.068	6	0.35–1.08	0.574	11.76	0.95	0.098	19.20
LDL	0.84	0.037	4	0.03–0.29	0.156	23.49	0.76	0.039	22.39
VLDL	0.96	0.083	7	0.07–1.28	0.248	33.37	0.92	0.083	46.42
IDL	0.46	0.037	2	0.02–0.20	0.094	39.55	0.48	0.036	33.42
Total	0.89	0.200	4	0.60–2.44	1.151	17.42	0.81	0.233	22.97

[a] Number of partial least square components

[b] Relative RMSECV calculated as RMSECV/relative mean value of reference

[c] Relative RMSEP calculated as RMSEP/relative mean value of reference

predicted in Study C by the calculated statistical models (Table 2). All rats were Fisher 344 rats from Charles River (Sulzfeld, Germany) and the experiments were carried out under the supervision of the Danish National Agency for Protection of Experimental Animals.

Study A: Eighteen male rats (nine 4 weeks old rats and nine 11 weeks old rats) were randomized into 6 groups. The rats were fed diets that had the same macro- and micronutrients composition for 4 weeks (Table 1) but differed in cholesterol content (0, 1 and 2%) to induce a high variation in the lipoprotein profiles. *Study B*: Fifty male rats (4 weeks old) were randomized into 5 groups (average weight comparable between the groups), one control group and four groups supplemented for 4 weeks with 2.1 or 6.3% apple pomace, with and without seeds respectively. The diet was balanced and depending on whether the rats received 0, 2.1 or 6.3% apple pomace, they were fed a standardized diets with slightly different composition (Table 1) to ensure that all animals received the same amount of macro- and micronutrients. Details of this study will be reported elsewhere since in the present context the plasma samples are just used as reference material to build robust calibration models. *Study C*: The objective of this study was to investigate if apple-powder has a dose–response effect on cholesterol distribution in the different lipoproteins. Twenty-four male rats (4 weeks old) were randomized into 3 groups by weight and supplemented with 0, 10 or 20% apple-powder for 4 weeks. The rats were fed slightly different purified diets to balance the nutrient compositions of the different supplementations to the control group (Table 1).

2.3 Collection and handling of plasma samples

Blood was collected just after sacrifice by decapitation after CO_2/O_2 anesthesia directly from the vena jugularis into a heparin coated funnel and subsequently into 4 ml vials containing heparin as an anticoagulant. The blood was centrifuged at $3000 \times g$, 4°C for 10 min. For Study A and B 200 µl plasma was stored at −80°C until NMR analysis and 1 ml plasma was used for serial ultracentrifugation to separate HDL, LDL, VLDL and intermediate density lipoprotein (IDL) as previously described by Baumstark et al. (1990). However, to evaluate the common use of human cholesterol test kit for rat plasma, a small volume of plasma was taken from each sample in Study A to measure total cholesterol (test kit # 14899232, Roche Diagnostic GmbH, Mannheim, Germany), cholesterol content in LDL (test kit # 03038661, Roche) and HDL (test kit # 04713109). The plasma fraction from Study C was portioned into cryo tubes and stored at −80°C until NMR analysis.

2.4 NMR data acquisition and preprocessing

Plasma samples were thawed on ice and 100 µl plasma was transferred to a 5 mm NMR tube and 450 µl D_2O was added. NMR spectra were acquired on a Bruker Avance 400 spectrometer (9.4 T) (Bruker Biospin Gmbh, Rheinstetten, Germany) operating at 400.13 MHz for ^1H. The probe was a broad band inverse detection probe head equipped with z-gradients designed to 5 mm NMR tubes. All experiments were performed at 311 K, which corresponds to the body temperature of rats. Tuning, matching and shimming were performed prior to data acquisition for each sample. Data were accumulated by utilizing a pulse sequence using pre-saturation of the water resonance during the recycle period followed by a composite 90° pulse (Bax 1985). The data was collected using a relaxation delay of 5 s and 128 scans and for each sample free induction decay of 32 K complex data points were accumulated using a spectral width of 8278.15 Hz corresponding to an acquisition time of 1.97 s. This resulted in a total experimental time of 15 min for each sample. Prior to Fourier transformation the data set was zerofilled to 64 K points and apodized by 0.3 Hz Lorentzian line broadening

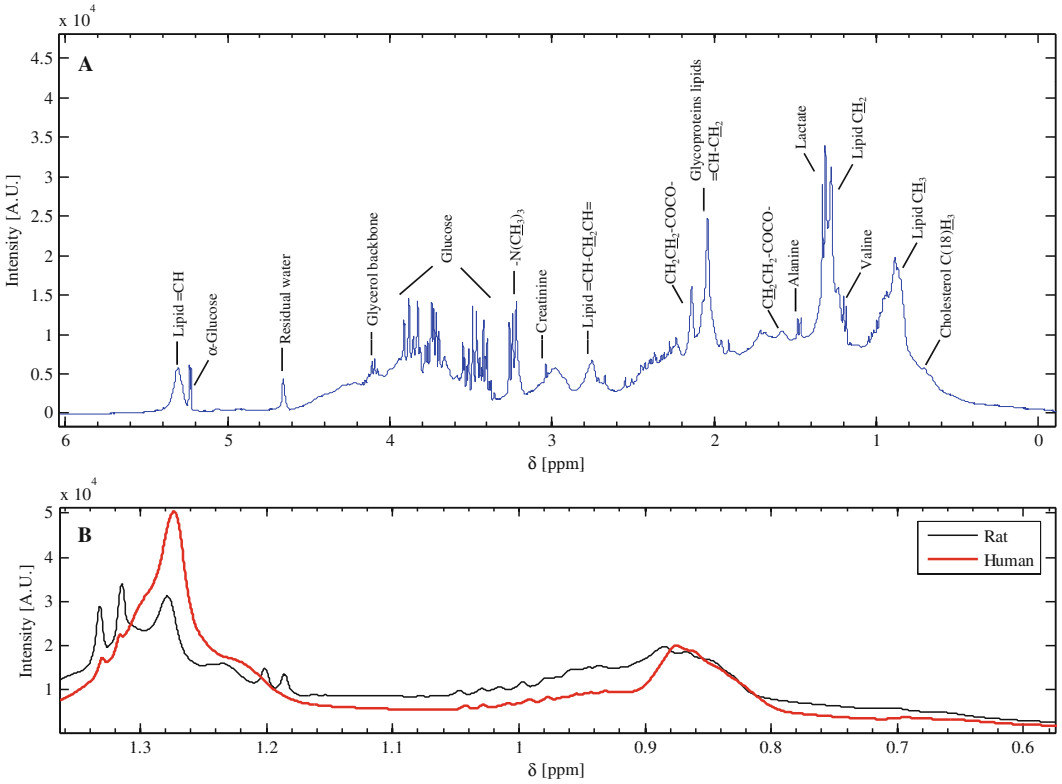

Fig. 1 An average ^{1}H NMR spectrum of rat plasma at 311 K including assignment of the most prominent peaks (**a**). The average rat and an average human NMR profile in the selected area (0.6–1.35 ppm) for iPLS modelling (**b**)

and thereafter baseline- and phase corrected manually. All spectra referenced according to α-D-glucose at 5.23 ppm. The spectral area chosen for multivariate data analysis was 0–6 ppm with exclusion of the 4.5–4.8 ppm region dominated by the residual water region (Fig. 1). After preliminary data analysis the region 0.6–1.4 ppm was selected for subsequent analysis. To correct for spectral misalignment the entire dataset was Co-shifted (Correlation optimized shifting) using an in-house developed algorithm (www.models.life.ku.dk). In this procedure the sample spectra are aligned towards a reference spectrum by simple 'left–right' shifting of the signal vector until the best correlation is found with respect to a predetermined window. Furthermore, after alignment data were normalized to unit area.

2.5 Data analysis

To build robust calibration models 40 samples were selected from Studies A and B, to ensure maximal variation of reference data, and 20 samples were used for an independent test set. Eight samples were not included in the models due to odd reference value or poor NMR spectrum quality.

From the ultracentrifugation based reference data and the NMR spectra from Studies A and B, interval partial least-square (iPLS) regression models (Nørgaard et al. 2000) were developed for predictions of the amount of cholesterol in the different lipoprotein fractions. One iPLS model was developed for each lipoprotein fraction. The iPLS regression model splits the NMR spectrum into a number of intervals and then PLS models are calculated towards the response variable for each interval. The predictive performance of the PLS model for each interval can then be compared with the predictive performance of the full spectrum model. This gives complete overview of which regions are best correlated with the response variables in the regression equation (Fig. 2). To evaluate the performance of the prediction models the Root Mean Square Error (RMSE) in combination with the correlation

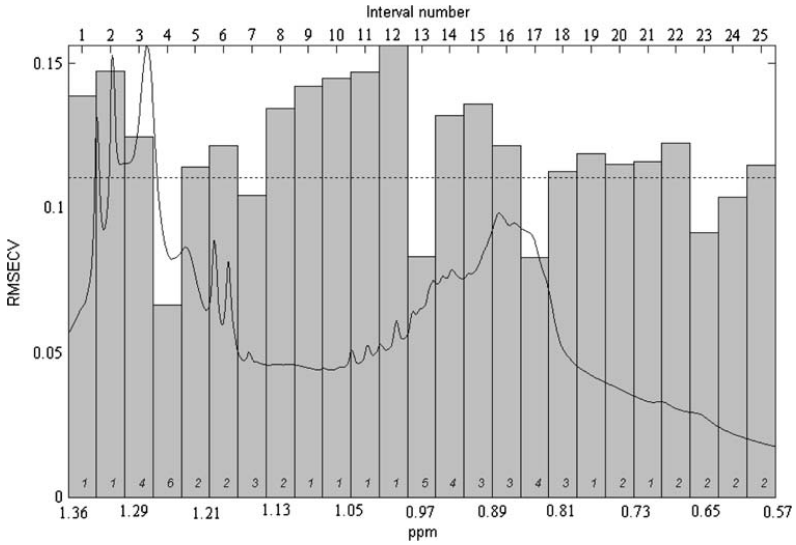

Fig. 2 iPLS plot with interval number versus RMSECV, superimposed on the average spectrum for HDL cholesterol prediction. Data is divided into 25 intervals and PLS models are calculated for each interval. Digits in the columns indicate number of components used in each local PLS model. The *dotted line* marks the RMSECV (8 components) of the global model

coefficient (r) are used as a measure. RMSE is defined as follows:

$$\mathrm{RMSE} = \frac{\sqrt{\sum \left(y_{\mathrm{pred}} - y_{\mathrm{ref}}\right)^2}}{N}$$

where y_{pred} is the predicted value, y_{ref} is the laboratory measured value, and N is the number of samples. RMSECV is the Root Mean Square Error of Cross Validation and RMSEP is the Root Mean Square Error of Prediction calculated on the independent test set. If the model is good the RMSECV and RMSEP should be quite similar. Future predictions are expected to be within ± RMSEP. To validate our predictions, repeated random cross validation with 6 segments and 100 repetitions was used. All chemometric models were computed in MATLAB (Math Works Inc., Massachusetts, US) and predictions were calculated in LatentiX version 2.0 (Latent5, Copenhagen, Denmark).

The results from cholesterol prediction of Study C were tested for normal distribution by Shapiro-Wilks test, and the homogeneity of variance among groups was evaluated by judgement of standardised residual plots. Normally distributed data were further analysed by analysis of variance (GLM) followed by a least significant difference test, whereas data that were not normally distributed were compared using Wilcoxons nonparametric tests (HDL data). A probability, $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SAS Enterprise Guide 3.0.2.414 software (SAS Institute Inc., Cary, NC).

3 Results

Measurement of cholesterol content in HDL and LDL by the human cholesterol kit and ultracentrifugation showed a large disagreement between the two methods (Fig. 3a, b) whereas measurement of total cholesterol had much higher agreement ($r = 0.86$) between the two techniques (Fig. 3c).

An example of the 400 MHz ^{1}H NMR spectra of the rat plasma is displayed in Fig. 1. Broad resonances from protein and lipoprotein contribute strongly to the spectra. Assignment of the spectra is done according to previous investigations (Ala-Korpela 2007; Tang et al. 2004) with the most important resonances for this research, being the cholesterol backbone –C(18)H_3 at 0.70 ppm and the broad signals around 0.9 and 1.3 ppm which refers respectively to the –CH_3 and –CH_2 groups of triglycerides, cholesterol compounds and phospholipids.

Dividing the spectra into intervals enabled us to find the spectral regions best predicting the reference data. Figure 2 shows an iPLS plot for HDL cholesterol prediction with interval numbers versus root mean square error of cross-validation (RMSECV) superimposed on the average spectrum. Interval 4 (1.24–1.27 ppm) has the lowest prediction error (RMSECV) compared to the rest of the intervals, but also interval 17 exhibit an improved prediction model. Interval 4 and 17 includes resonances from –CH_2 groups and –CH_3 groups, respectively, of triglycerides, cholesterol compounds and phospholipids. However, since interval 4 perform slightly better than interval 17 (higher correlation

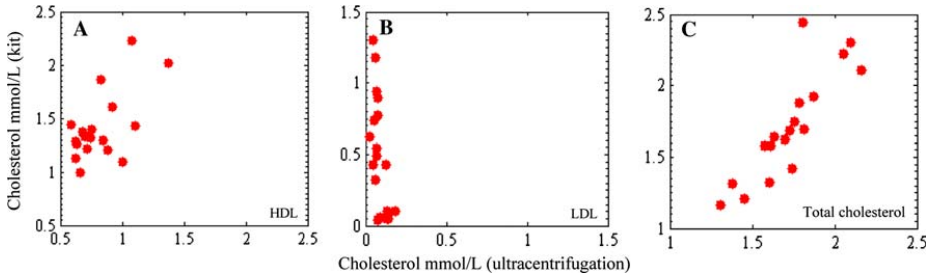

Fig. 3 Comparison of cholesterol amount (mmol/L) measured by ultracentrifugation (*x*-axis) and human cholesterol kit (*y*-axis) in HDL (**a**; $r = 0.62$), LDL (**b**; $r = -0.61$) and as total cholesterol (**c**; $r = 0.86$)

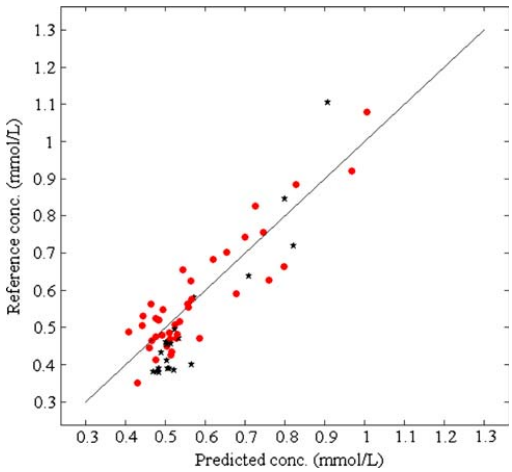

Fig. 4 Reference cholesterol content in the HDL lipoprotein fraction versus cross validated (repeated random) predicted concentration using 6 components. Filled circles are samples from the calibration set ($r = 0.90$) and black stars are the samples from the independent test set ($r = 0.95$)

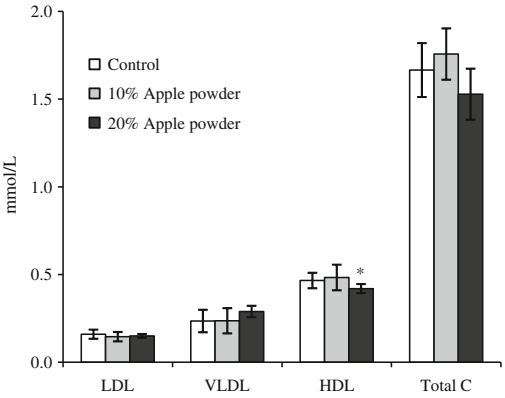

Fig. 5 Shows the cholesterol content (mmol/L) in LDL, VLDL, HDL fraction and total cholesterol between the groups determined by the PLS models in Table 2. *Statistically significant different ($P < 0.05$) from the control group

coefficient and lower prediction error) the subsequent data analysis is performed using interval 4 with 6 PLS components. The results of this PLS model is illustrated in Fig. 4, where the predicted versus reference values (mmol/L) are plotted and additionally, the result from the prediction of an independent test set is also shown. The correlation coefficient of the model was high ($r = 0.90$) and with a low relative prediction error (relative RMSECV 11.76%). For the independent test set correlation between the predicted and reference values was even higher ($r = 0.95$) than that for the calibration model but the prediction error was also higher (relative RMSECV 19.20%). Table 2 summarizes the performance of all the iPLS models (HDL, LDL, VLDL, IDL and total cholesterol). The RMSECV of the calibration models were between 12 and 40% and had correlations coefficients (r) between 0.46 and 0.96. Testing the calibration models with an independent test set provided prediction errors (RMSEP) between 19 and 46% and correlation coefficients (r) between 0.48 and 0.96.

The calibration models were then used to predict plasma total cholesterol and the cholesterol content in HDL, LDL and VLDL lipoproteins in Study C. In the subsequent study the IDL model was abandoned due to the combination of low correlation between predicted and reference values and a high prediction error (RMSECV).

The prediction results of Study C are illustrated in Fig. 5 and shows that no significant difference in LDL, VLDL and total cholesterol was observed between the groups. The high dose apple-powder group had significantly lower HDL cholesterol content (11%, $P = 0.0452$) than the control group.

4 Discussion

The present investigation have established ^{1}H NMR based iPLS models to estimate total cholesterol concentration in plasma and the distribution of cholesterol within different lipoprotein particles (VLDL, LDL and HDL) in the rat plasma. The NMR based approach has shown a high level of agreement with an established ultracentrifugation method and at the same time the expenditure of plasma volume is reduced by a factor of 10 (100 µl vs. 1 ml). This significant reduction is of general interest due to the limited amount of plasma available from rodent studies. It is common practice to use human cholesterol test kits for estimation of cholesterol fractions in rodent studies, but as shown in the present work, this approach does not seem valid for quantification of cholesterol content in HDL and LDL in rat plasma. However, the kits seem applicable to quantify total cholesterol in rat studies.

The consistency between concentrations of total cholesterol, VLDL and HDL cholesterol measured by the ultracentrifugation and predicted by iPLS was high ($r = 0.89$, 0.96 and 0.90, respectively). A little lower correlation was observed for LDL ($r = 0.84$) and this may be due to lower amounts of this fraction in rat plasma presumably causing a slightly more inaccurate measurement by the reference method and NMR than what is seen for total cholesterol and for the HDL and the VLDL fractions. Prediction of the independent test set in each model gave quite similar correlation coefficients, except for LDL and total cholesterol where the correlation decreased by 8%. This could probably be avoided by using a larger test set than the one available for the present study. The relative prediction error (RMSECV) of the VLDL measurement is moderately higher than for the other accepted models (IDL not taken into account here) and this trend was also observed in a previous study on human blood by Petersen et al. (2005). However, the cause of this phenomenon is not clear but might be due to problems with an accurate measurement of this fraction by the ultracentrifugation method. Overall, the results from this study are in agreement with previously published studies where chemometric methods for the quantification of human lipoproteins from NMR spectra were used (Dyrby et al. 2005; Petersen et al. 2005). However, in this study models using the full NMR spectrum could not be obtained wherefore selection of minor parts (intervals) of the spectrum was required in order to obtain reasonable prediction models of the cholesterol distribution in rat plasma. It is important to observe that the two intervals with the lowest RMSECV represent the $–CH_2$ groups and $–CH_3$ groups of the same lipids in a particular lipoprotein fraction as are both displaced in chemical shift due to the slower diffusion as elegantly demonstrated by Dyrby et al. (2005) using diffusion edited NMR and PARAFAC modeling.

When using the calibration models to estimate cholesterol distribution in Study C we observed that the apple-powder treatments did not have any significant effects on LDL, VLDL and total cholesterol but a significantly decreased HDL cholesterol in the 20% apple-powder group. No dose–response effect was observed on the lipoprotein profile between the 10 and 20% apple-powder dose. This indicates that the apple-powder has to be consumed in quite large amounts before any effect is reflected in the lipoprotein profile.

A previous study by Aprikian et al. (2002) where lean Zucker rats were fed 20% lyophilized apples for 3 weeks showed no significant effect of the apple diet on the lipoprotein profile of lean rats. In our study the rats were on the apple-powder diet for one week more than those in the referred study (Aprikian et al. 2002). Also, different apple varieties, processing (air-dried versus freeze-dried) and different rat strains were used. These variations may have caused the significant effect on HDL cholesterol of dried apple in our study compared to no effect on the lean rats in the study of Aprikian et al. (2002).

The diet used in this study was regulated in sucrose/fructose and total fiber content to obtain a similar sugar- and total fiber composition in the control and apple diets. The remaining potential bioactive ingredients in apples, e.g. polyphenols and soluble fiber content (pectin) was not regulated and may be the responsible components for the significantly lowered HDL cholesterol in the high apple-powder group. A rat study by Aprikian et al. (2003) suggested that apple pectin and polyphenols act in synergy since no total plasma cholesterol lowering was observed when clean apple pectin was introduced but a significantly lowering effect was found when introducing apple polyphenol and pectin jointly. This has conformity to our findings where total cholesterol is lowered, although non-significantly, in the high apple-powder group compared to the control group. Unfortunately, HDL cholesterol was not measured separately in the study by Aprikian et al. (2003).

When performing human intervention studies with potential cholesterol lowering compounds a decrease in LDL and total cholesterol and additionally an increase or no change in HDL cholesterol would be expected. Compared to humans, rats are naturally deficient in cholesterol ester transfer protein activity (Ha and Barter 1982), which is an important factor in the reverse cholesterol transport pathway (RCT). In RCT cholesterol is delivered from macrophages or other cells to the liver or to excretion in the intestine. Rats may have developed some compensatory mechanisms for this deficiency and this should be taken into account when evaluating physiological effects of dietary interventions in rats. Introduction of exogenous active compounds, e.g. from apple may induce or decrease activity and production of enzymes, transporters and

receptors acting in the RCT pathway. Hereby the flux of cholesterol through the RCT pathway may be up regulated, resulting in a higher throughput and lower net cholesterol concentrations in HDL particles. However, the actual mechanisms causing the characteristics of the lipoprotein profile in this study needs further investigation to elucidate the implicated pathways.

Summarizing, this study shows that the iPLS approach is in good accordance with spectral interpretation and that chemometric analysis of NMR spectra of rat plasma seems to be a feasible and facile way to quantify lipoprotein fractions in small amounts of stored, frozen plasma from rodent studies. Additionally, this work also illustrates a significant HDL cholesterol lowering effect of dried apple-powder in rats.

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PAPER III

Effects of apple and apple-pectin feeding on cholesterol metabolism and antioxidant response in healthy rats

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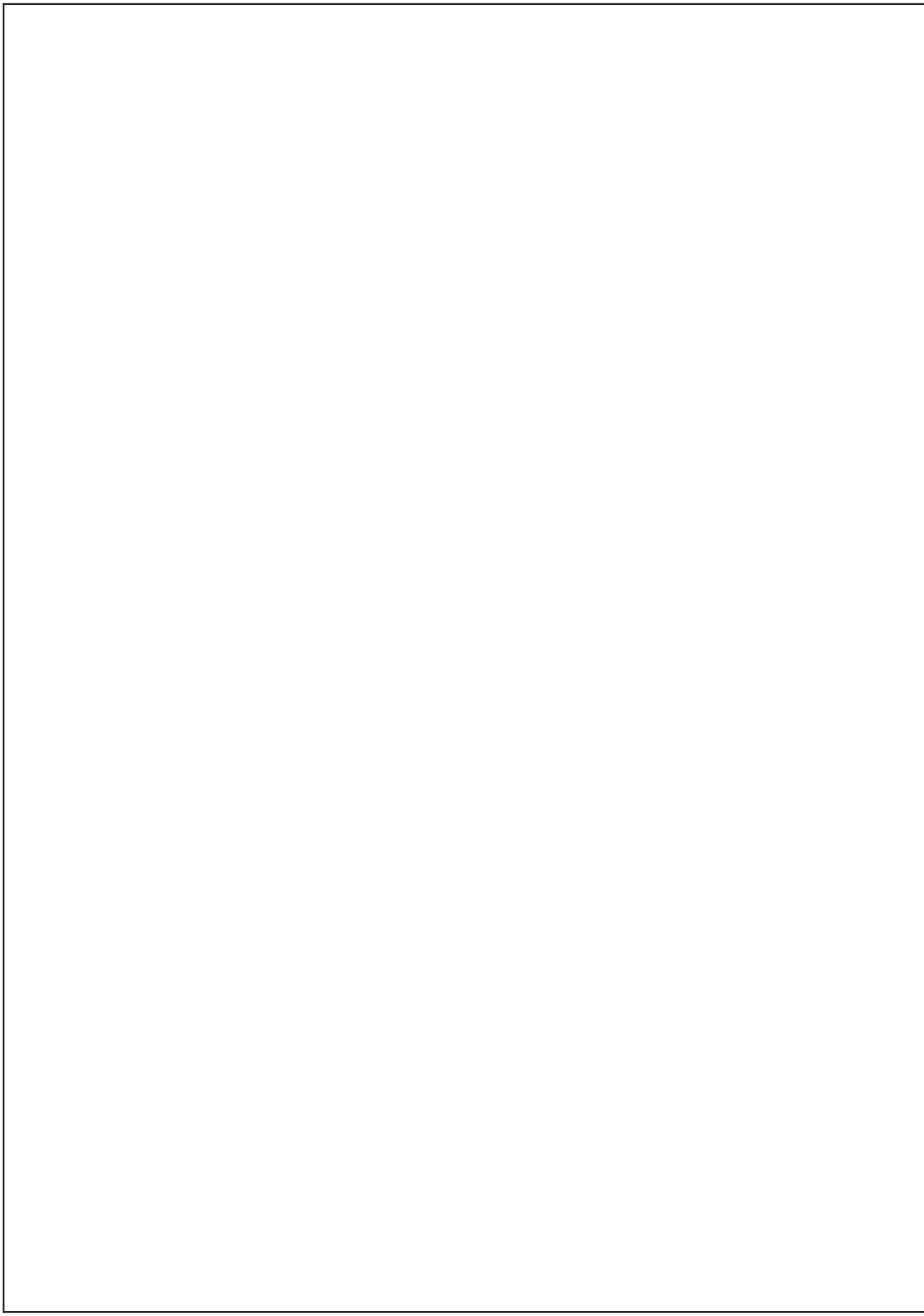

Effects of apple and pectin feeding on cholesterol metabolism and antioxidant response in healthy rats

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Key words: Apple, Pectin, Cholesterol metabolism, Antioxidant enzymes, Gene expression

Abbreviations: ALAT, alanine aminotransferase; AlP, alkaline phosphatase; *Cat*, catalase; GGT, γ-glutamyl transpeptidase; *Gr,* gene expression of glutathione reductase; *Gxp1*, gene expression of glutation peroxidase; Hb, haemoglobin; HDL, high-density lipoprotein; *Hmgcr*, gene expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low-density lipoprotein; *Nqo1*, NAD(P)H:quinine oxidoreductase; TAG, triacylglycerides; TC, total cholesterol; VLDL, very low-density lipoprotein

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Abstract

Apple intake has a general reputation of being disease preventive but potential mechanisms and active components inducing a possible health effect is unclear. In this study we wanted to investigate how feeding with fresh apple or with crude apple-pectin to rats affected cholesterol metabolism, bile acid excretion, and hepatic gene expression. Twenty-four Fisher 344 male rats were randomized into 3 groups and fed a purified diet with different supplementations added in two of the groups (7% apple-pectin or 10 g raw apple) for 4 weeks.

Total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol was significantly reduced in the apple group compared to the control group. Total and primary 24-h bile acid excretion in feces was significantly increased in the apple group whereas fecal concentrations of secondary bile acids showed a significant reduction following apple feeding. Pectin did not exhibit any effects on cholesterol metabolism but a significant up-regulation of plasma alkaline phosphatase (AlP) was observed. Hepatic gene expression of glutation peroxidase (*Gxp1*) and glutathione reductase (*Gr*) was significantly up-regulated in the apple and pectin fed rats. Expression of γ-glutamate cystein ligase catalytic subunit (*Gclc*) in the liver was only up-regulated in the apple group. In conclusion, fresh apple increased excretion of bile acids without concomitant up-regulation of cholesterol biosynthesis, leading to an overall decrease in plasma cholesterol. Pectin feeding had no such effects. Apple feeding also increased hepatic gene expression related to glutathione synthesis as well as glutathione utilization. Pectin only affected expression related to glutathione utilization.

Introduction

The evidence of health beneficial effects of fruit and vegetables are continually growing and especially an inverse association with cardiovascular diseases (CVD) is becoming well-established[1,2]. Based on these findings the current dietary advice in Europe and America is to consume five or more portions of fruit and vegetables each day and fruits in particular appear as food products with wide popularity since it is often eaten fresh as an easy and convenient snack. Apple remains one of the most consumed fruits in the western world and the health impact from intake of this fruit seems very relevant to investigate. Apple has a historical reputation of being a healthy component in the diet and scientific evidence has been able to support this assertion in some regards[3,4] although the active factors and mechanisms responsible for these health promoting actions still remain uncertain. Previous human intervention studies[5-7] have linked apple intake with a lower risk of developing CVD although the quality of these studies do not meet todays' design and quality demands. Some of the early risk markers of CVD are related to plasma lipids: high levels of plasma triacylglycerides (TAG), total cholesterol (TC), low-density and very low-density lipoprotein (LDL and VLDL) along with low levels of high-density lipoprotein (HDL) cholesterol and the ability of apple to reduce some of these main CVD risk factors need further confirmation. Most of the earlier conducted animal studies investigating the effect of apple on these risk markers have used freeze-dried apple, apple pomace or purified constituents and to the best of our knowledge no previous animal studies have been investigating effects of freshly cut apple.

The cholesterol-lowering properties of apples have partially been ascribed to the fibre moiety of the fruit where particular pectin is believed to play a major role[8]. Apple-pectin can be extracted as a by-product from apple juice production and this fibre component is frequently used in various food products due to its high viscosity. Recently highly methoxylated apple-pectin with ultra low viscosity has been developed[9] to be applied as a functional food ingredient that improves mouthfeel and helps to increase fibre contents in e.g. beverages. Daily human intake of pectins from various sources may therefore, in some cases, exceed many-folds the natural intake from moderate consumption of apples or other fruits. Pectins in general are presumed to prevent the reabsorbtion of bile acids in the intestine and to enhance steroid excretion, diverting more

cholesterol into the bile acid pool[10]. However, pectins from different plant origins have a large structural diversity, thus possibly having varied health effects, and previous animal studies reporting on pectin feeding and plasma cholesterol have been inconsistent[11-13].

Apples also contain a variety of phenolic compounds (catechins, anthocyanidins, dihydrochalcones, etc.) which exhibit antioxidative properties *in vitro*[14] but whether these effects have biological relevance *in vivo* is not well examined. Additionally, some researchers claim that dietary fibres, including pectin, may exhibit antioxidant properties[15,16] or that the polyphenols reduce cholesterol[17]. Since the liver has a central role for regulation of plasma lipids and antioxidant systems this organ seems particularly relevant to investigate in this context. Therefore, in the present study we aimed to investigate markers related to cholesterol synthesis, status and loss, as well as hepatic gene expression responses related to glutathione formation and utilization, which are both health related aspects possibly affected by intake of whole raw apple or apple-pectin.

Materials and methods

Chemicals

All chemical reagents used were analytical grade from Fluka (Steinheim, Germany), Merck (Darmstadt, Germany) and Sigma-Aldrich (Brøndby, Denmark). Ethanol (96%) was purchased from De Danske Spritfabrikker, Aalborg, Denmark. Water is MilliQ (Millipore, Bedford, MA) with >18 Ω resistivity. The bile acids: dehydrocholic acid, 13C glycocholic acid, ursodeoxycholic acid, chenodeoxycholic acid, litocholic acid were purchased from Sigma-Aldrich (Brøndby, Denmark). Tauroursodeoxycholic acid, glycoursodeoxycholic acid, taurocholic acid, glycocholic acid, taurochenodeoxycholic acid, cholic acid, taurodeoxycholic acid, glycodeoxycholic acid, deoxycholic acid were purchased from Merck (Darmstadt, Germany). Alpha-muricholic acid and beta-muricholic acid were obtained from Steraloids (Newport, Rhode Island, USA).

Apple and pectin analysis

Apples used in the present study were of the variety 'Shampion' and were delivered from an orchard near Skierniewice, Poland. The chemical composition of this apple variety is shown in Table 1. The apple-pectin used was a commercial, unrefined pectin, kindly provided by Obi-Pectin AG (Basel, Switzerland).

Rat study design and sample collection

Twenty four healthy male Fisher 344 rats, which is an inbreed strain with very limited genetically variance, were obtained from Charles River (Sulzfeld, Germany). The rats were fed either a control diet; a modified control diet added 7% of crude apple-pectin, or a modified control diet together with 10g of fresh apple per animal/d during four weeks. Every diet was based on a purified rodent diet produced at the National Food Institute, Technical University of Denmark and was nutritionally balanced as described in Table 2. Animal experiments were carried out under the supervision of the Danish National Agency for the Protection of Experimental Animals. All animal study procedures have been approved by the Institutional Committee for Animal Experimentation and the National Food Institute has been approved for this type of experiment with rodents by the Danish Ministry of Justice. Faeces samples were collected after 24 d of

feeding while the rats were housed singularly in metabolic steel cages with a device to separate urine from faeces. Total faeces from a 24-h collection were stored frozen at -80 ºC until analysis. After 4 weeks on the experimental diets the animals were fasted overnight. The next day the rats were anesthetized in CO_2/O_2 and sacrificed by decapitation. Immediately after the decapitation, blood was collected into two different vials, and the liver, colon and caecum removed. The control group of this experiment was shared with another experiment on onion and onion fibre that has been published previously[18].

Processing of blood samples

One mL of blood was collected into a PAXgene blood RNA tube for purification of RNA from the white blood cells (WBC) (BD Denmark A/S, Brøndby, Denmark). The rest of the blood was collected in vacutainer™ tubes containing heparin as an anticoagulant. After 10 min of incubation on ice the samples were centrifuged at 1500*g* for 10 min at 4 °C. Plasma was removed for later analysis of enzymes, triacylglycerides and lipoproteins. The erythrocyte fraction was haemolysed by adding an equal volume of ice-cold water. All collected fractions were immediately frozen at –80 °C.

Biochemical analysis of plasma TAG, TC and markers of hepatic function

Alkaline phosphatase (AlP), alanine aminotransferase (ALAT), gamma glutamyl transferase (GGT), TC and TAG concentrations were measured in rat plasma samples using an automated Roche/Hitachi 912 analyzer at 37 ºC in accordance with the instructions of the manufacturers (Roche Diagnostic GmbH Mannheim, Germany).

^{1}H NMR analysis and chemometric models for quantification of cholesterol contents in plasma lipoproteins

Cholesterol content in HDL, LDL and VLDL were analyzed in rat plasma samples. For ^{1}H NMR analysis, plasma samples were thawed on ice and 100 µL plasma was transferred to a 5 mm NMR tube and 450 µL D_2O was added. NMR spectra were acquired on a Bruker Avance 400 MHz spectrometer (9.4 T) (Bruker Biospin Gmbh, Rheinstetten, Germany) at 311K, which corresponds to the body temperature of rats. Cholesterol content in HDL, LDL and VLDL lipoproteins were

then predicted by previously developed chemometric models based on NMR data and interval Partial Least Square models from 60 Fisher 344 rats[19].

Haemoglobin analysis

On the day of analysis the 50% haemolysates were thawed slowly on ice and diluted 2.5x in water and sonicated 10s on ice. The samples were further diluted to 40x in 100 mM KH_2PO_4 buffer pH 7.4 containing 1 mM DTT and 1 mM EDTA. Haemoglobin (Hb) were determined spectrophotometrically on an Automated Roche/Hitachi 912 Analyzer (Roche Diagnostic A/S, Hvidovre, Denmark) at 37 ºC using Drapkins Reagent (Randox HG980) as recommended by the manufactor (Randox Laboratories Ltd., Crumlin, UK.

Sampling of liver, RNA isolation and quantitative real-time PCR

The rat liver was removed, weighted and grinded in liquid N_2 to a fine powder which was stored at -80 ºC. On the day of analysis total RNA was isolated from 30 mg liver powder using Qiagen RNeasy Mini kit according to the protocol described by the manufacturer (Qiagen, Hilden, Germany). Reverse transcriptase reactions were performed using Random Hexamer and SuperScript™ II Reverse Transcriptase kit according to the manufacturer's instructions (Invitrogen).

Relative mRNA expression was quantified by Real-time PCR on an ABI 7900HT FAST System as described previously[20]. TaqMan® Gene Expression Assays used were the following: Eukaryotic 18S rRNA Endogenous Control (catalog number 4352930E); rat catalase (*Cat*) (catalog number Rn00680386_m1), rat γ-glutamate cystein ligase catalytic subunit (*Gclc*) (catalog number Rn00689048_m1); rat glutathione peroxidase (*Gpx1*) (catalog number Rn00577994_g1), rat glutathione reductase (*Gr*) (catalog number Rn01482160_m1); and rat NAD(P)H:quinine oxidoreductase (*Nqo1*) (catalog number Rn00566528_m1) and rat 3-hydroxy-3-methylglutaryl coenzyme A reductase (*Hmgcr*) (catalog number Rn_00695772_g1).

Bile acids analysis by LC/MS and transit time measurement

The concentration of bile acids in faeces samples was measured by a novel LC/MS/MS method (Jensen *et al.*, in prep.). Briefly, total faces were weighed, homogenized with 14 volumes (w/v) of

water to slurry, and 0.3 g samples were aliquoted. 0.3 mg of this homogenate was added 13C glycocholic acid as internal standard and extracted three times with acetonitrile. The eluate was diluted with 0.1% formic acid and concentrated on an Oasis HLB 3cc column (Waters, Milford, MA). The acetonitrile eluate was evaporated to dryness and redissolved in 20% acetonitrile, 24% methanol, 0.1% formic acid (80% mobile phase A). Samples and standards were analysed on an Acquity UPLC with a TQ detector (Waters, operated in MRM mode with a gradient from phase A to B (100% acetonitrile) over 5 min. Between run CV% for the internal standard (n=48) was 13.5%. The individual compounds were quantified using QuanLynx version 4.1 (Waters) based on internal standards and external calibrants. Based on the analytical results for the individual primary and secondary bile acids these were summed for each rat.

One week before sacrifice the transit time was measured as described in Roldán-Marin *et al.*[21]

Statistical analysis

The data were analyzed for normal distribution using the Shapiro-Wilcks W-test and for homogeneity of variance Levenes test ($P>0.05$) was used. Some data had to be log transformed in order to meet these criteria. The normally distributed and variance homogenous data were analysed by ANOVA. If significant differences were found between groups further comparisons were done using least square means, whereas data that were not normally distributed were compared using Wilcoxons nonparametric tests. We used the SAS statistical package v. 9 (SAS Institute, Cary, NC, USA) and consider a *P*-value below 0.05 significant.

Principal component analysis (PCA) was conducted on all of the effect variables (except gene expression markers since these were only performed for five animals per group) including earlier published effect marker on the gut environment[22] by use of Matlab (MatWorks). Variables were autoscaled before analysis. Residual and Hotelling plots were used to search for possible outliers.

Results

Animal weight and feed intake

Animals were increasing their weight stably during the trial and there were no significant differences between groups. The liver weight was significant lower in the apple group (P=0.0123) as compared to the control group (Table 3). The relative liver weights did not differ significantly between the groups. The animals in the apple group had a significantly lower intake of feed than the other groups, due to their intake of apples given beside the diet (Table 4). The rats in the apple group consumed 9.3 ± 1.4g (mean ± SD, range 8.9 ± 1.7 to 9.8 ± 0.6) per day of the apple pieces offered with no differences between the study weeks. A night video recorded for one of the cages revealed that both rats in the cage were eating apple and apparently sharing the pieces offered but that the apple skin was the last to be consumed. The skin was also the most common leftover in the cages. The pectin group did not differ from the control group with respect to feed intake. The pectin intake was 6.5g/week in the pectin group and pectin intake from apples could be calculated to reach 0.4g/week in the apple group.

Lipids

The cholesterol distribution in the different lipoprotein fractions are shown in Figure 1. Cholesterol content in the LDL fraction was significantly lower (P = 0.0005) in the apple group as compared to the control group and the apple group had also significantly lower HDL cholesterol and TC (P = 0.0002 and P = 0.0018, respectively) than the control group. Apple pectin was without effects on the plasma lipids, except for a slight, but statistically non-significant decrease in TAG and VLDL cholesterol.

Bile acid in feces and transit time

Bile acid excretion was significantly affected by the feeding with whole apple, increasing total bile acid (P = 0.0196) as well as primary bile acid (P = 0.0171) in feces during 24 h (Figure 2). The secondary bile acid excretion was non-significantly decreased in the total 24-h feces samples in the apple group whereas the fecal concentration (umol/g) of secondary bile acids was significantly reduced (P<0.05) compared to the control group (data not shown). Pectin showed no

significant changes when compared to the control group although a trend towards increased excretion of primary and total bile acids was observed. Transit time was not affected significantly by any of the treatments (data not shown).

Markers of liver function and gene expression

Plasma activities of the liver enzyme AlP was significantly up-regulated in the pectin group compared to the control group ($P = 0.0009$) where as ALAT and GGT were not significantly up- or down-regulated in any of the groups (Table 5). Hb concentration, expressed as g/l of erythrocytes, was not affected by the apple or pectin treatment.

The hepatic gene expression of antioxidant enzymes showed a significant up-regulation of *Gclc* ($P = 0.009$), *Gxp1* ($P = 0.028$) and *Gr* ($P = 0.028$) activity in the apple fed rats (Table 5). The activity of *Gpx1* and *Gr* were also significantly up-regulated in the pectin group ($P = 0.028$ and 0.047, respectively). Hepatic gene expression of *Hmgcr*, *Nqo1* and *Cat* were not significantly affected by any of the treatments.

Multivariate analysis

To obtain an overview of all measured biomarkers a PCA was conducted. A PCA summarizes the major variation in the data into a few axes and in this way systematic variation can be captured and used to visualize which samples in a data set are similar or dissimilar to each other, and which variables (biomarkers) are having high impact of potential clustering of samples. Figure 3 shows a combined scores and loading PCA plot (biplot) of all samples and effect markers, respectively. The 3 first principal components explain approximately 54% of the variation in the data and illustrate a clear separation between the pectin and the apple group by principal component 1 (PC1) and PC3. Rat #3 seems to be an outlier, deviating from the rest of the control group with a much higher PC2 score value, however it did not exhibit outlier behavior from residual and Hotelling plots (data not shown). The control and the pectin group is overlapping and especially the plasma lipids markers (TC, LDL, HDL, VLDL, TAG) and the liver weight seems responsible for the separation between the apple fed rats and those in the control or pectin groups.

Discussion

In the current study we report on different biological responses caused by fresh apple and apple-pectin in healthy rats.

We found that a moderate level of apple intake during 4 weeks reduced total, HDL and LDL cholesterol compared to the control group. Substantial evidence has shown a clear association between decreased total and LDL cholesterol and reduced risk of CVD and from this point of view, intake of whole apples seems to be favorable to improve cardiovascular health. To the best of our knowledge no earlier rat studies have explored cholesterol effects after intake of fresh apple. Previous rat studies conducted by Aprikian *et al.*[23] showed a significant lowering of total plasma cholesterol in healthy rats after intake of freeze-dried (lyophilized) apples. Another study by the same authors found no significant effect of a 20% lyophilized apple diet on the lipoprotein profile of lean rats but for obese hypercholesterolemic rats, fed the same diet, total and LDL cholesterol was significantly lowered after apple intake[24]. In this last study HDL cholesterol was reduced by 28% in the obese rats fed apples, but whether the decrease was significant is not stated in the paper. Salgado *et al.*[25] observed an increase in the amount of HDL cholesterol and decrease in total and LDL cholesterol in Wistar rats after intake of 5, 15 and 25% apple-powder in a cholesterol-containing diet during 30 d. However, the measurement method of HDL and LDL cholesterol in this study is of doubtful quality due to use of commercial cholesterol kits and the Friedewald formula in rat studies, as earlier pointed out[19]. In humans we would expect an increase in HDL cholesterol when total and LDL cholesterol is lowered by an exogenous agent, but in our rat study HDL was significantly decreased. In contrast to humans, rats are naturally deficient in cholesterol ester transfer protein activity[26] and may have developed some compensatory mechanisms for this deficiency. These aspects have to be taken into account when evaluating physiological effects of dietary interventions in rats. HDL plays a major role in the reverse cholesterol transport pathway (RCT) which delivers free cholesterol from macrophages or other cells to the liver or intestine. Lewis and Rader [27] stated, that the flux of cholesterol through the RCT pathway, and hereby the activity of enzymes, transporters and receptors, may be a more important determinant of cardiovascular disease risk than steady-state HDL cholesterol concentrations. The HDL cholesterol lowering effect we observed from the apple treatment may

be due to increased activity of players in the RCT pathway which thereby cause a higher throughput and lower net cholesterol concentrations in HDL particles. However, these mechanisms have to be investigated further to draw firm conclusions on implicates players and pathways.

Pectin intake was not found to affect cholesterol distribution in any significant manner in the present study. Previous animal studies reporting on apple-pectin feeding and plasma cholesterol have been inconsistent; Trautwein *et al.*[28] did not observe any effect of introducing apple-pectin to cholesterol-fed hamsters whereas Samble-Amplis *et al.*[29] found a significant reduction in plasma TC in hamsters when apple-pectin was added to a cholesterol diet at a very high dose level. In consistency with our results Aprikian *et al.*[30] found no effect of apple-pectin on total plasma cholesterol in Wistar rats. In the same study, treatments with apple polyphenols showed no cholesterol effect by polyphenols alone but a significant plasma cholesterol lowering effect was observed when introducing polyphenol and pectin jointly. This highlights that apple-pectin and polyphenols may act in synergy to exhibit a stronger cholesterol lowering potential, directing us to a possible explanation of our results. The plasma cholesterol lowering effect we observe by apple, and not by pectin alone, may be caused by a combined effect of the polyphenol and pectin present in whole apple.

Pectins in fruit are frequently thought to prevent the reabsorbtion of bile acids in the intestine and to enhance steroid excretion, diverting more cholesterol into the bile acid pool[31]. However we did not find a significant increase of total fecal bile acids excretion in the pectin supplemented group and this finding was supported in the study by Aprikian *et al.*[32] which likewise found no effect of apple-pectin on total plasma cholesterol.

The apple treatment was found to increase total and primary bile acid excretion corroborating our finding of a significant decrease in total plasma cholesterol. Increased bile acid excretion may to some extent be a consequence of increased bile acid production and this might explain the decrease in plasma cholesterol by apples. Free cholesterol is the preferred substrate for 7α-hydroxylase, which is the rate-limiting enzyme in bile acid synthesis, and a higher bile acid production in the liver will remove more cholesterol from plasma. Sable-Amplis *et al.*[33,34] measured the activity of 7α -hydroxylase in hamsters and found an enhanced activity of this

enzyme after intake of fresh apple. Increased bile acid excretion is often compensated by an increased cholesterol synthesis. In our study we examined hepatic gene expression of *Hmgcr* which is the gene coding for 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme for cholesterol synthesis. This gene was non-significantly down-regulated in the apple group, indicating that a compensatory up-regulation was clearly not observed.

In support of our findings, Sembries *et al.*[35] and Aprikian *et al.*[36] observed a significant increase in total faecal bile acid excretion in rats after feeding with an apple extraction juice and lyophilized apple, respectively. The former study differentiated between primary and secondary bile acids and found significantly decreased faecal excretion of secondary bile acids. Secondary bile acids are formed after enzymatic deconjugation and dehydroxylation of primary bile acids in colon by anaerobic bacteria. Secondary bile acids are toxic to several cell systems at physiological concentrations and have shown tumour-promoting capacities in animal experiments[37]. The significantly lower concentration of secondary bile acids that we observed in the apple group may be secondary to the change in the excretion of primary bile acids, or reflect a change in intestinal milieu or gut microbiota. Analysis of the effects of apple feeding on the caecal environment and the denaturing gradient gel electrophoresis profile of the microbiota from this present study has earlier been published[22] and results from that study are included in the PCA presented here (Figure 3). Butyrate concentration was significantly increased, pH in caecum content was significantly decreased in both the pectin and apple group and the composition of the microbiota was altered. Some of these factors may be involved in the lower excretion of secondary bile acids in the apple group. A lower pH value in faeces probably reflects a higher concentration of short chain fatty acids and a decreased pH has earlier shown to reduce the activity of 7α-dehydroxylase, one of the enzymes responsible for converting primary to secondary bile acids[38]. The concentration of excreted secondary bile acids does not decrease in the pectin group and it seems reasonable to speculate that pectin *per se* does not strongly affect bile acid excretion and to consider other fractions in the apple as more likely to be responsible for the effect. Polyphenols reaching the caecum and colon can be extensively metabolised by the microbiota to various phenolic acids. This may change the chemical or biological environment affecting bile acid metabolism. Another hypothesis is that a greater proportion of primary bile

acids is excreted by binding to unabsorbed polyphenols during passage through the small intestine[39], hereby leaving fewer free bile acids for conversion into secondary bile acids.

The effect of apple intake on glutathione-related gene expression has not been reported previously. We observed here that apple and pectin can affect genes involved in the hepatic glutathione (GSH) redox cycle. The genes encoding GSH peroxidase and reductase were significantly up-regulated in both the apple and pectin groups indicating a higher capability to handle oxidative stress in these rats. GSH is synthesised enzymatically with γ-glutamate cystein ligase catalytic subunit (Gclc) as the rate-limiting enzyme[40]. We found expression of *Gclc* to be significantly up-regulated in the apple group in concordance with the up-regulation of *Gpx1* and *Gr*. *Gclc* expression was non-significantly higher in the pectin fed rats as compared to controls. This points towards other factors than pectin as responsible for the elevation of *Gclc* expression by apple. *In vivo* studies by others have shown that various extracted polyphenols fractions are capable of modulating GSH synthesis[41], indicating that the apple polyphenol fraction may be a key player in the observed effect.

Additionally, AlP activity in plasma was significantly elevated in the pectin group. AlP is a widely used marker for liver disorders, but its activity is also increased in atherosclerosis and peripheral vascular disease, and AlP can serve as an inflammatory marker[42]. The increased AlP activity by pectin might therefore be interpreted as an adverse health effect. No change in AlP activity was observed in the apple group and it is considered that either the high pectin dose or some changed physico-chemical properties of the purified pectin may be responsible. Urinary metabolites have earlier been investigated from this study (data to be published elsewhere) and here 2-furoylglycine was identified as an exposure marker of apple-pectin. This metabolite is a conjugate of a furan derivative which is thought to be up-concentrated during pectin cleaning or formed as a Maillard product under industrial pectin extraction. This compound might cause toxic effects in hepatic or endothelial cells, and hereby contribute to the elevated AlP activity, but further studies are needed to investigate this. Based on the findings in this study, consumption of high doses of isolated apple-pectin does not seem to positively affect markers of cardiovascular health.

The summarising PCA plot (Figure 3) gives an overview of the results described in this study. It is noticeable that the apple and pectin groups appear as relatively inhomogeneous groups indicating a high response variation in the different markers. The rats lived two in a cage and possible one rat was more dominant and eating more feed that the other. This could especially be problematic in the apple group due to the way fresh apple was given as a supplement in this study. From the surveillance the rats seemed to share the accessible apple pieces relatively equally but some variation in apple intake between the rats is unavoidable and some animals may have more preference for the peel or flesh than others. However, by inspection of data from the cohabitating rats there is no indication that the major variation lies within the cages (Figure 3). The relative caecum weight seems to have high impact on the pectin samples and this is in good consistency with the fact that a high-fibre diet will evoke increased gut movement and subsequently to larger caecum muscle weight. Additionally, other gut environmental variables, such as caecum β-glucuronidase, caecum β-glucosidase, butyrate, propionate and secondary bile acids, appear to cluster in the pectin area giving good sense since pectin is exclusively used as fuel for the microbiota and affected the microbial composition as well as its physiologically active byproducts.

Conclusion

The administration of a moderate dose of fresh apple showed significant *in vivo* effects on cholesterol metabolism and especially the reduction of LDL and total cholesterol as well as increased faecal excretion of bile acids supports observational evidence that fresh apple as part of fruit in general is an important dietary factor decreasing the risk of cardiovascular diseases. Pectin was not found as the responsible cholesterol lowering component of apple and the relatively high dose of apple-pectin introduced in this study does not strongly support pectin as a functional food ingredient. However, both apple and apple-pectin intake revealed significant effects on genes involved in the hepatic GSH redox cycle indicating a higher capability to handle oxidative stress. Longer-term and more detailed analyses of these effects in model organisms and future human intervention studies are needed to fully interpret and corroborate the effects observed in this study and to ascertain that they also relate to human health.

Acknowlegdement

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The author's responsibilities were as follows: L.O.D. and M.P. designed this study; J.M. provided the apples; M.P. was responsible for the animal study protocol and diets; M.K. conducted the NMR-based lipoprotein cholesterol measurement and predictions, R.I.J. was in charge for the bile acid analysis; B.N.K. was responsible for the gene expression and antioxidant enzyme analysis, L.O.D. provided support for the SAS statistical analysis and M.K. conducted the multivariate analysis; M.K. wrote the first draft and L.O.D. supervised the following drafts. All authors approved the final draft and all authors declared that they had no conflict of interest.

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TABLE 1 Composition of animal feed

Ingredients	Control group (g)	Pectin group (g)	Apple
Na-caseinate	200	200	232
Sucrose	100	100	60
Cornstarch	456	386	465
Soybean oil+AEDK	50	50	60
Soybean oil	20	20	20
Corn oil	80	80	92
Cellulose	50	50	22
Mineral mixture[a]	32	32	37
Vitamin mixture[b]	12	12	12
Apple pectin	-	70	-
Fresh apple[c]	-	-	10g/d

a: Containing in mg/kg diet: 2500 Ca; 1600 P; 3600 K; 300 S; 2500 Na; 1500 Cl; 600 Mg; 34 Fe; 30 Zn; 10 Mn; 0.20 I; 0.15 Mo; 0.15 Se; 2.5 Si; 1.0 Cr; 1.0 F; 0.5 Ni; 0.5 B; 0.1 B; 0.1 V; 0.07 Co. **b**: Containing in mg/kg diet: 5000 (IU) vitamin A; 1000 (IU) vitamin D_3; 50 (IU) vitamin E; 5 thiamin; 6 riboflavin; 8 pyridoxol; 2 folic acid; 0.3 D-biotin; 0.03 vitamin B-12; 20 pantothenate; 2600 cholinhydrogentartrat; 400 inositol; 40 nicotinic acid; 1 phylloquinine; 40 p-aminobenzoic acid; 1000 methionine; 2000 L-cystine. **c**:see apple composition in table 2.

Table 2 Composition of apples used for the study

Soluble solids (%)	13.5
Energy value (kcal/100g)	52.16
Dry weight (g/kg)	163
Protein (%w/w)	0.2771
Fat (%w/w)	0.0978
Ash (%w/w)	0.163
Methylated carbohydrates (%w/w)	12.551
Total dietary fibre (%w/w)	2.0701
Soluble dietary fibre (%w/w)	0.6683
Insoluble dietary fibre (%w/w)	1.4018
Sum of sugars (g/kg)	107
Saccharose (g/kg)	28.8
Glucose (g/kg)	19.7
Fructose (g/kg)	59
Sorbitol (g/kg)	3.7
Titratable acidity (g/kg)	3.3
Malic acid (g/kg)	3.6
Citric acid (mg/kg)	69
Total pectins (g/kg)	5.8
Water-soluble pectins (g/kg)	1.4
Catechin (mg/kg)	9.9
Epicatechin (mg/kg)	108.1
Procyanidins (mg/kg)	151.6
Total dihydrochalchone glycosides (g/kg)	26.8
Phloridzin (mg/kg)	14.7
Chlorogenic acid (mg/kg)	68.1
p-Coumaryl quinic acid (mg/kg)	7.1
Quercetin glycosides (mg/kg)	88.6
Quercetin aglycon (mg/kg)	<1
Sum of phenolics (mg/kg)	460.2

Table 3. Animal weights and liver weight by week of the study

	Week 1	Week 2	Week 3	Week 4	Week 5	Liver Weight
Control	130 ± 10.1[a)]	169 ± 12.4	203 ± 14.5	225 ± 13.6	235 ± 13.7	8.3±0.3
Pectin 7%	129 ± 7.8	165 ± 8.9	198 ± 8.9	215 ± 3.4	231 ± 5.3	7.9±1.0
Apple 10g/d	128 ± 8.8	155 ± 10	187 ± 11.1	213 ± 12.2	218 ± 13.3	7.0±0.9* [b)]

a) Numbers are mean animal weights in g ± standard deviations.
b) Differences to control group, * P<0.05

Table 4. Animal feed intake by week of the study

	Week 1	Week 2	Week 3	Week 4	Total
Control	97. ± 5.3 [a)]	98. ± 7.0	90. ± 8.2	84. ± 6.1	370 ± 9.8
Pectin 7%	88. ± 4.2* [b)]	96. ± 5.2	86. ± 11.9	102 ± 9.1**	373 ± 12.3
Apple 10g/d	69. ± 5.2**	84. ± 5.5**	81. ± 11.1	68. ± 8.4**	303 ± 22.4**

a) Numbers are mean animal feed intake in g ± standard deviations.
b) Differences to control group, * P<0.05, **P<0.01

Table 5. Effects of apple-pectin and apple on plasma activities of hepatic enzymes (AlP, ALAT and GGT), on haemoglobin concentration (Hb) and on hepatic gene expression of antioxidant enzymes (*Hmgcr, Gclc, Nqo1, Gpx1, Gr and Cat*)

Marker	Control	Apple-Pectin	Apple
AlP (UI/L)	753.6 ± 71.6	895.9 ± 78.0*	756.14 ± 57.0
ALAT (UI/L)	91.14 ± 8.21	93.375 ± 10.8	99.375 ± 13.0
GGT (UI/L	2.4 ± 0.59	3.16 ± 2.30	2.47 ± 0.43
Hb (g/L)	20.91 ± 4.83	23.98 ± 5.77	20.69 ± 5.05
***Hmgcr*[a]**	0.94 ± 0.69	0.86 ± 0.47	0.62 ± 0.17
***Gclc*[a]**	0.76 ± 0.42	1.56 ± 0.66	2.18 ± 0.51*
***Nqo1*[a]**	0.79 ± 0.25	2.40 ± 2.54	0.85 ± 0.67
***GPx1*[a]**	0.80 ± 0.30	1.61 ± 0.60*	1.35 ± 0.26*
***Gr*[a]**	0.73 ± 0.27	1.22 ± 0.33*	1.20 ± 0.19*
***Cat*[a]**	0.95 ± 0.36	1.14 ± 0.58	1.19 ± 0.40

Results are expressed as mean ± SD, n=8. *P<0.05 as compared with the control group. [a]Gene expression (n=5) is given relative to the endogenous reference 18S rRNA and a calibrant. AlP, alkaline phosphatase; ALAT, alanine aminotransferase; GGT, γ-glutamyl transferase; Hb, Haemoglobin concentration; *Hmgcr*, 3-hydroxy-3-methylglutaryl coenzyme A reductase; *Gclc*, γ-glutamate cystein ligase catalytic subunit; *Nqo1*, NAD(P)H:quinine oxidoreductase *Gr*, Glutathione reductase; *Gpx1*, Glutathione peroxidase; *Cat*, catalase.

FIGURE 1

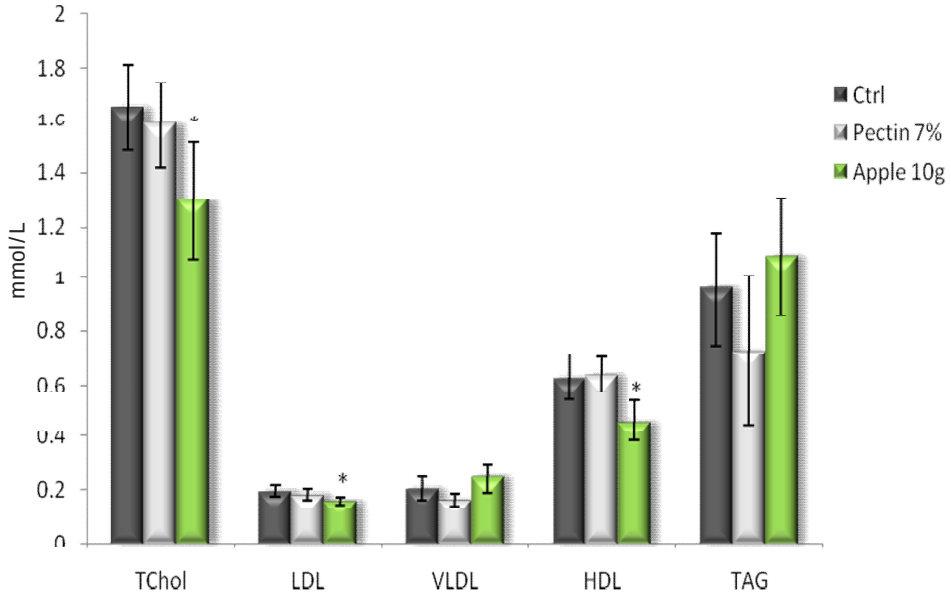

Figure 1. Rats were fed with 7% pectin in the feed, 10g fresh raw apple, or a control feed balanced to ascertain similar nutrient intakes in all groups. Data shown are means of total cholesterol, cholesterol contents in different lipoprotein fractions (HDL, LDL and VLDL) and triglyceride in plasma. Error bars showing standard deviations. *Significant differences between apple and control group ($P<0.05$).

FIGURE 2

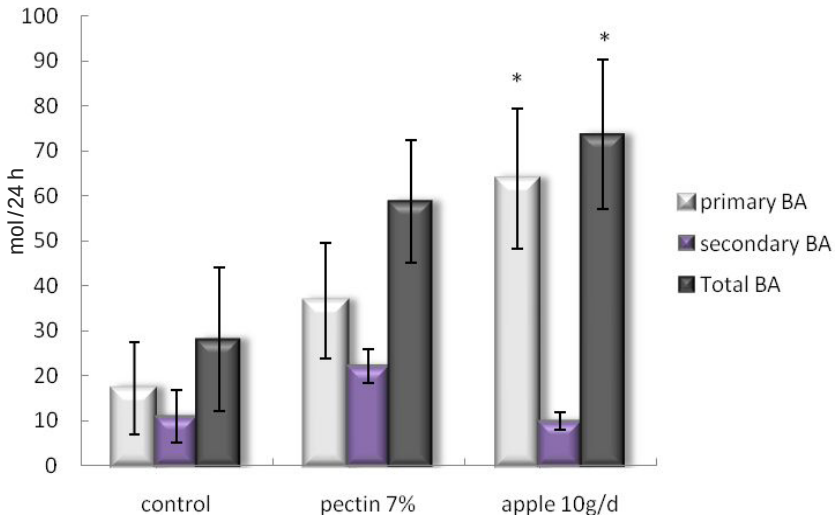

Figure 2. Rats were fed with 7% pectin in the feed, 10g fresh, raw apple, or a control feed balanced to ascertain similar nutrient intakes in all groups. Data shown are means of total 24 h fecal excretion of primary, secondary and total bile acid with error bars showing standard errors. *Significant differences between apple and control group ($P<0.05$).

FIGURE 3

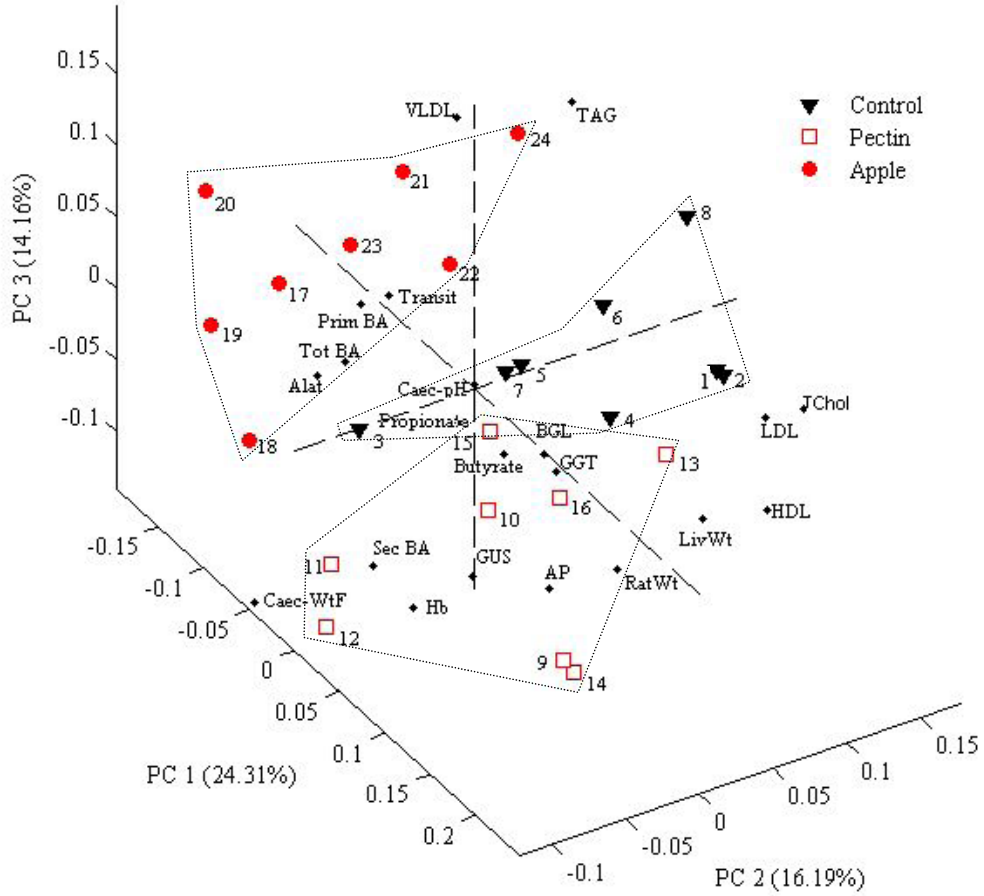

Figure 3. Principal component analysis (PCA) of rat samples after feeding with apple and apple-pectin. Scores and loadings are combined (biplot). The numbers next to the sample marker illustrate animal number and they have been living in the cage with a rat with the consecutive number (1 and 2; 3 and 4;...). Abbreviations: ALAT, plasma alanine aminotransferase; AlP, plasma alkaline phosphatase; BGL, caecum β-glucuronidase; GUS, caecum β-glucosidase activity; Caec-WtE, caecum weight (empty); GGT, plasma γ-glutamyl transpeptidase; Hb, erythrocyte total haemoglobin; HDL, plasma HDL cholesterol; LDL, plasma LDL cholesterol; VLDL, plasma VLDL cholesterol; Liver-Wt, total liver wet weight; RatWt, total rat weight at sacrifice; Tot BA, total bile acid excretion; Prim BA, total primary bile acid excretion; Sec BA, total secondary bile acid excretion; TAG, plasma triacylglycerides; Transit, gut transit time.

SUPPLEMENTAL MATERIAL

The effect of LC-MS data processing methods on the selection of plasma biomarkers in fed vs. fasted rats

Gürdeniz, G., Kristensen, M., Skov, T., Bro R. and Dragsted, L.O.

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LC-MS metabolomics top-down approach reveals new exposure and effect biomarkers of apple and apple-pectin intake

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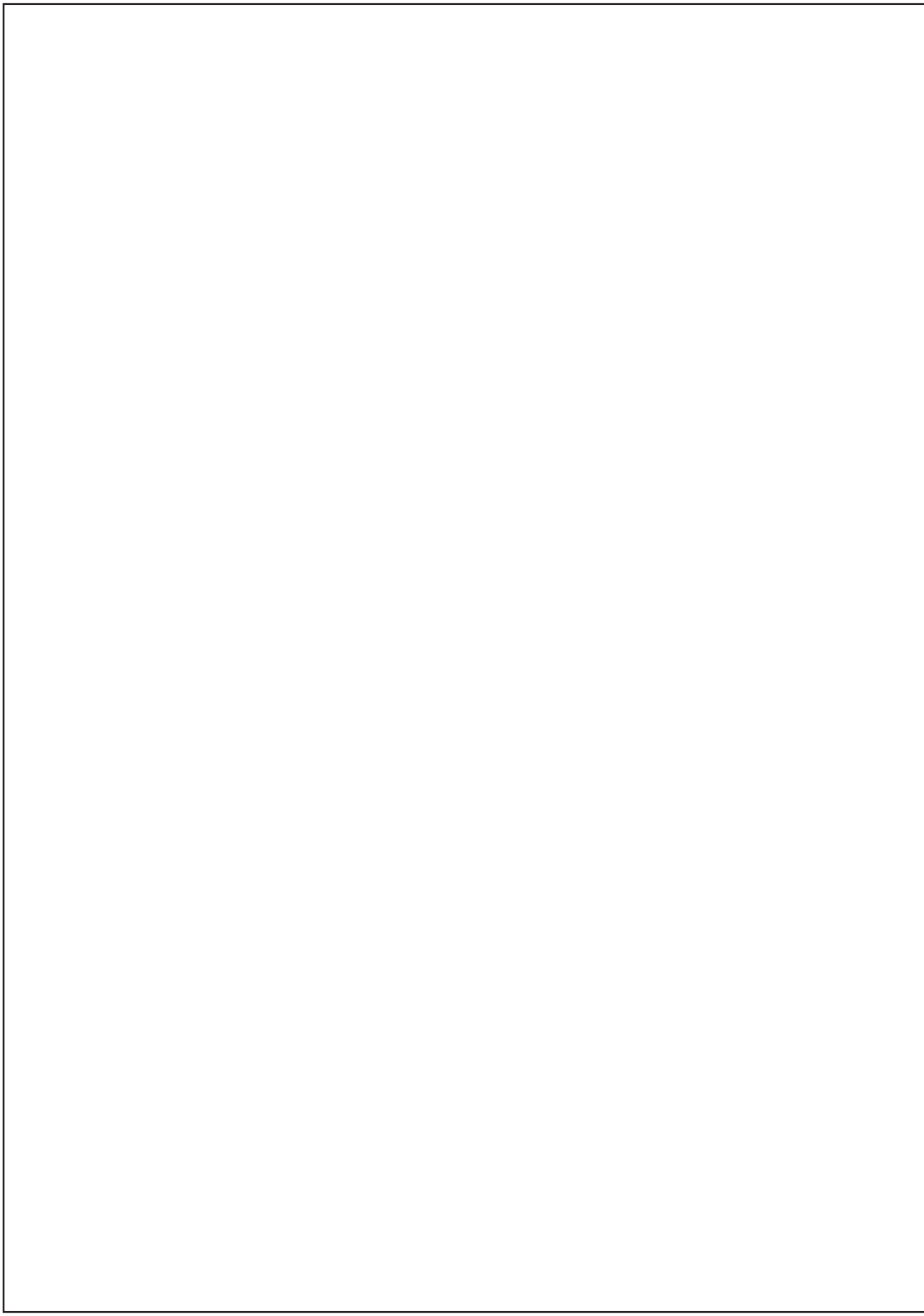

The effect of LC-MS data processing methods on the selection of plasma biomarkers in fed vs. fasted rats

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Abstract

The metabolic composition of plasma is affected by time passed since the last meal and by individual variation in metabolite clearance rates. Rat plasma in the fed and fasted states was analyzed with liquid chromatography quadrupole-time-of-flight mass spectrometry (LC-QTOF) for an untargeted investigation of metabolite patterns. This data set was used to investigate the effect of data processing using four different methods; MarkerLynx, MZmine, XCMS and a customized processing method that performs binning of m/z channels followed by summation through retention time. Direct comparison of the markers selected by partial least square analysis (PLS-DA) from each processed data, resulted in relatively few overlapping markers. Further identification revealed that differences were due to markers representing adducts or daughter ions of the same metabolite. Moreover, many markers identified by only one or two of the methods were members of the same chemical subclasses as those identified by the others, e.g. lyso-phosphatidylcholines (LPC) and lyso-phosphatidylethanolamines (LPE), which were found more abundant in the fed state. Carnitine and acetylcarnitine were also identified by all methods as biomarkers. The results illustrate that as long as proper parameter settings is attained, the data processing method has limited effect on the extracted biological information.

Keywords *Plasma ·Fasting vs. fed states· LC-QTOF · Data processing methods*

Introduction

In dietary interventions and frequently also in observational studies, blood samples are collected in order to relate nutritional conditions with metabolic markers. The blood is obtained from individuals either in the fasted or postprandial state, depending on the hypothesis being tested. The fasting state, typically following an overnight fast, is considered to be more reproducible and can be defined as a baseline level for metabolic studies. However, imbalances in diet-dependent metabolism may not be detectable in the fasted state [1]. On the other hand, determination of the metabolic response in the extended postprandial state, which is the normal metabolic situation of human beings throughout the day, is more challenging as the individual variability is high [2]. The meal frequency is a function of the metabolic rate and an overnight fasting period in rats having an eight times higher rate of energy metabolism than humans may therefore represent a more extreme condition than overnight fasting in humans. A rat model may therefore be convenient to study the major differences between the fasting and the fed states, the latter defined as the state of rats following a normal *ad libitum* meal pattern. A rat model also offers full control of the food intake in the study subjects.

In this study, an untargeted metabolomics based approach to study the metabolic differences between rat plasma at fasted and fed states was performed. Metabolomics is defined as the process of monitoring and evaluating changes in metabolites during biochemical processes and has become an emerging tool to understand responses of cells and living organisms with respect to their gene expression or alterations in their lifestyles and diets [3]. Application of metabolomics in nutritional studies offers the opportunity to have a broader understanding of biochemical variation with respect to a specific diet intake.

By nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), a wide range data of metabolites and other compounds can be detected in various human biofluids. These approaches can be either untargeted through total data capture or highly targeted, such as measuring a large number of defined lipids. MS based instruments, with higher sensitivity compared to NMR [4;5], are an increasingly important tool in metabolomics studies. Liquid chromatography (LC) coupled with time-of-flight (TOF) MS offers high resolution, reasonable sensitivity and improved data acquisition for complex sample mixture analyses. The system has served as a powerful tool in many other studies focusing on untargeted metabolic profiling of biofluids [6-8].

LC-MS analysis produces large amount of data with complex chemical information. An important task is to arrange data in a way so that relevant information can be extracted. The complexity of LC-MS data brings out the concept of data handling which can be roughly summarized in two basic steps [9]. The first step is *data processing,* which includes the methods to go from raw instrumental data to clean data. The next step is *data analysis,* including conventional data scaling and normalization methods followed by univariate and multivariate methods applied to screen the patterns in the processed data.

Recently many software tools (commercially or freely available) have emerged to process LC-MS data. All of these tools aim to provide user-friendly platforms and high speed automated data processing. The basic principles of these software packages have recently been summarized [9]. To be able to obtain high efficiency in data processing, the software tool employed should be understood and its parameter settings are required to match the structure of the specific data set.

Existence of various data processing tools brings out concerns on whether all these tools perform in a similar way. There are some studies attempting to define quality parameters for comparison of peak detection [10;12] or alignment [13] algorithms of different data processing tools, however, comparison of algorithms was outside of the scope of this study. The question to be addressed in this study is whether there is agreement between the biological information as represented by the biomarkers extracted by processing the same data set with different data processing methods using similar settings. Therefore we chose to compare the potential biomarkers extracted from an LC-QTOF generated dataset of plasma analyzed in the fed and fasted state using four different software packages; (1) MarkerLynx (MassLynx (Waters, Milfold, MA)), (2) MZmine [14], (3) XCMS[15] and (4) a customized method that is implemented in MATLAB (The Mathworks, Inc., MA, US). MarkerLynx is a commercial software whereas XCMS and MZmine are freely available software tools. MATLAB is also commercially available but represents here any programming tool offering simple, customized processing. For the customized method we applied a simpler algorithm, which is comprised of m/z binning and retention time collapsing. The applicability of this method for LC-MS data has been illustrated in other studies[16-17] but an extensive comparison with three other softwares have not been published previously.

In this study UPLC-QTOF profiles of rat's plasma in the fasted and fed states were analyzed for two different purposes: (1) to identify the effect of data processing on the final

biomarker selection, and (2) to interpret the biology behind the biomarkers identified for the two states based on each data processing.

Materials and Methods

Animal Study and Sample Collection

Eighty male Fisher 344 rats (4 weeks old) were obtained from Charles River (Sulzfeld, Germany). The animals had a one week run-in period to adapt to the standardized diet. The rats were subsequently randomised into five groups of 16 rats, each with equal total body weights and then fed five different diets which were all nutritionally balanced to give exactly the same amounts of all important macro- and micronutrients [18]. After 16 weeks all rats were sacrificed by decapitation after CO_2/O_2 anesthesia. Before sacrifice 56 of the animals had fasted for 12 hours and 24 of the animals were given access to food up until termination. Blood samples were collected immediately after sacrifice directly from the *vena jugularis* into a heparin coated funnel drained into 4 mL vials containing heparin as an anticoagulant. The blood was centrifuged at 3000 g, 4^{o}C for 10 min. The plasma fraction was aliquoted into 2 mL cryotubes and stored at -80^{o}C until further processing. The animal experiment was carried out under the supervision of the Danish National Agency for Protection of Experimental Animals.

Plasma pre-processing and LC-QTOF Analysis

Removal of plasma proteins was performed before LC-MS analysis of the plasma metabolites. The plasma samples were thawed on ice and 40 µL of each sample was added into a 96-well Sirocco™ plasma protein filtering plate (#186002448, Waters) containing 180 µL of 90% methanol 0.1% formic acid solution, and the plates were vortexed for 5 minutes to extract metabolites from the plasma protein precipitate. A 96-well plate for the ultra-performance liquid chromatograms UPLC autosampler (Waters, cat # 186002481) was placed underneath the protein filtering plate and vacuum was applied to the plates (using a manifold) whereby the rubber wells in the Sirocco™ plates opened and the crash solvent including metabolites dripped into the 96-well UPLC plate. When the filtering plates were dry, 180 uL of a 20% acetone 80% acetonitrile 0.1 % formic acid solution was added to each well to further extract metabolites from the precipitated protein and vacuum was connected until dryness. The

solvent was evaporated from the UPLC plates by using a cooled vacuum centrifuge and the dry samples were redissolved in 200 µL milliQ acidic water before analysis. A blank sample (0.1% formic acid) and a standard sample containing 40 different physiological compounds (metabolomics standard) was also added to spare wells to evaluate possible contamination and/or loss of metabolites in the filtering procedure.

10 µL of each sample were injected into the UPLC equipped with a 1.7µm C18 BEH column (Waters) operated with a 6.0 min gradient from 0.1% formic acid to 0.1% formic acid in 20% acetone: 80% acetonitrile. The eluate was analyzed in duplicates by TOF-MS (QTOF Premium, Waters). The instrument voltage was 2.8 or 3.2 kV to the tip of the capillary and analysis was performed in negative or positive mode, respectively. In the negative mode desolvation gas temperature was 400°C, cone voltage 40 V, and Ar collision gas energy 6.1 V; in the positive mode we used the same settings except for collision energy of 10 V). A blank (0.1% formic acid) and the metabolomics standard were analyzed after every 50 samples during the run.

Authentic Standards

L-carnitine, linoleic acid and gamma-linolenic acid were purchased from Sigma Aldrich (Copenhagen, Denmark). 1-acyl LPC(18:1), 1-acyl LPE(18:1), PC(16:0/18:1) and PE(16:0/18:1) were obtained from Avanti Lipids (Alabaster, AL, USA). For the synthesis of acetyl L-carnitine, carnitine acetyltransferase from pigeon and acetyl coenzyme A were purchased from Sigma Aldrich. Acetylation of L-carnitine was performed as described by Bergmeyer et al. [19]. The 2-acyl lyso-forms were synthesized with phospholipase A1 from Thermomyces lanuginosus (Sigma Aldrich). Phospholipase A1 hydrolyzes the acyl group attached to the 1-position of PC(16:0/18:1) and PE(16:0/18:1) so that acyl-2 LPC(18:1) and LPE(18:1) were produced. The description of the method was given by Pete et al.[20]. Briefly, the phospholipids and fatty acids were initially diluted in iso-propanol to 100 mg/L and further dilution was performed in water to 2 mg/L whereas other standard compounds were diluted in water to 10 mg/L. For the chemical verification of identified metabolites, one plasma sample from a rat in the fasted and another from the fed state were spiked with LPC(18:1) and LPE(18:1) individually before analysis by the procedure outlined above.

Raw Data

The MassLynx^{TM} (Version 4.1, Waters, Milford, MA, USA) software collected centroided mass spectra in real time using leucine-enkephalin as a lock-spray standard injected every 10s to calibrate mass accuracy. Each of the 80 samples was analyzed in duplicates. For negative mode both measurements were included in the data analysis. However, for the positive mode only 24 samples with duplicates and 52 samples without duplicates were included based on an initial outlier detection considering the instrumental error that occurred during analysis. In this case the outliers had very low intensity due to injection errors.

The software stores data as non-uniform sample data files, each consisting of a two-dimensional intensity matrix represented by scan number or retention time (0-6 min) in the first dimension and m/z values (non-uniform) in the second dimension. The raw data was converted to an intermediate netCDF format with the DataBridge^{TM} utility provided with the MassLynx software.

Software Tools for Data Processing

Raw data was transferred to MarkerLynx^{TM} (Version 4.1, Waters, Milford, MA, USA) directly from MassLynx whereas netCDF files were imported to MZmine [14] and XCMS [15]. The data processing parameters were given in electronic supplementary material (Table 1S) for the three software tools. Final outcome from the software tools was feature sets where each feature is denoted with its exact m/z and retention time. Feature sets were transferred to the MATLAB interface for further data analysis.

Custom Methods for Data Processing

An alternative data processing was performed with MATLAB (Version 7, The Mathworks, Inc., MA, US). To transfer netCDF files to MATLAB, the iCDF function [16;21] was employed. The steps of the custom data processing are shown in Fig. 1. As the first step, binning was performed on the m/z dimension as described by Nielsen et al. [16].

Alignment and offset correction was applied only to positive mode data as the instrumental response was observed to be significantly lower during the duplicate runs in the positive mode. To correct for instrumental response differences, prior alignment was performed using ICOshift [22]. The lower response of duplicates was corrected by calculating the difference matrices between each duplicate set, averaging and adding the average difference to the matrix with the lower response. Here it is assumed that the first injection of a sample holds the correct instrumental response whereas its duplicate with lower response is the one being corrected. The effect of this procedure is shown in electronic supplementary material (Fig. 1S).

A threshold level was applied for the elimination of small peaks/intensities lower than the analytical detection level. The values lower than a certain threshold level was considered as zero. The strategy to define the threshold was as follows: (1) The first median value of the whole data set (excluding zeros) was calculated (2) That median was evaluated as a threshold (by the ability of principle component analysis (PCA) score plots to fully separate the fasted vs. the fed state, data not shown) (3) The next median was calculated by using only those data from the whole data set that were higher than the previous median, and again the corresponding PCA scores plot (not shown) was evaluated. (4) This procedure was iterated until a good separation was achieved by PCA. The threshold level of the 4th median with the value of 16.17 in the negative mode and 24.85 in the positive mode were selected as adequate.

This strategy for calculation of thresholds by medians provided a good estimation for setting the threshold parameters in other software tools (MarkerLynx, MZmine and XCMS).

To enable the application of subsequent two-dimensional data analysis methods, the intensity values of each sample matrix were summed (or collapsed) throughout the retention time index. The resulting data matrix (two-dimensional) is described by samples vs. m/z bins (Fig. 1) and is also referred to as feature sets throughout this paper.

Data Analysis

The feature sets processed by MZmine, XCMS and customized methods were normalized to Unit length (in MATLAB) whereas MarkerLynx processed data were normalized to a total sum of 10,000 (the default setting in MarkerLynx).

Afterwards, mean centering combined with Unit scaling as well as Pareto scaling was performed on the feature sets (Fig. 2). Unit scaling employs the standard deviation as the scaling factor whereas the square root of the standard deviation is the scaling factor in Pareto scaling.

The PLS_Toolbox (version 5.3, Eigenvector Research, Inc., MA, US) was used to implement the data analysis. PCA [23] was applied individually on feature sets obtained from each data processing method for general visualization of discrimination of samples from rats in the fasted vs. fed states.

PLS-DA is based on the development of a PLS model [24] to predict class membership of a data set X with a y vector including only 0 and 1 (1 indicates that one sample belongs to a given class). The PLS-DA model was cross validated by 500 times iteratively excluding random subsets from the data matrix. The number of latent variables (LV) was determined to minimize the classification errors using cross validation (CV).

Variable (Feature) Selection

A rough and effective variable reduction procedure was performed specifically during MarkerLynx and custom data processing by only keeping a feature if it had a nonzero measurement in at least 80% of the intensity values recorded within one of the two subsets; otherwise the feature was removed (80% rule) [25]. This rule is not directly applicable for softwares with gap filling procedures.

Further variable selection was performed with PLS-DA. The features or m/z bins with larger regression coefficients were considered as more discriminative between fasted and fed states, these are regarded as the potential biomarkers. Due to the fact that the selected variables will be later considered as potential biomarkers it is important to be confident with them, so we applied an advanced method for cross validation. Instead of applying only a single cross-validated PLS-DA model on all samples we performed repeated submodel testing every time PLS-DA was performed. This implies taking out samples randomly (here 10% were taken out at a time), constructing a PLS-DA model on the remaining 90% samples and repeating this 1000 times. By doing this the influence of each feature is comprehensively tested. For each model the features are given a rank in the order of their regression coefficients and the final rank of each feature for all the 1000 submodels were summarized with one number using the product of the 1000 ranks per feature. This gives a much more robust ranking, hereby suggesting a solution to the global model problem in the paper on cross model validation methods by Westerhuis et al. [26].

Selection of potential biomarkers was performed in several steps as illustrated in Fig. 2. Each processed feature set was scaled individually with Unit as well as Pareto scaling to create two subsets. PLS-DA was applied on each subset as described above after each kind of scaling and the 200 (arbitrary number) features and m/z bins with the largest absolute regression coefficient products were picked out from each (Fig. 2, step 1). Then, these 2 x 200 features were combined for each feature set omitting duplicates (Fig. 2, step 2), and PCA was used to ascertain that the resulting sets gave good separation between samples from fed and fasted states. The three sets were unit scaled and a final variable selection was performed with PLS-DA (Fig. 2, step 3), again using cross model validation as described above. The 25 top rank features from each feature set, i.e. those with highest absolute regression coefficient products, were selected as they may potentially represent biomarkers. However, since these features might be daughter ions, adducts, summed ions, etc. we choose here to simply call them markers whereas after identification the compounds represented by these markers in the top rank feature sets will be termed biomarkers.

Marker Identification

The initial identification of markers was performed according to their exact mass compared with those that were registered in the Human Metabolome Database [27]. Possible fragment

ions were investigated by an automated tool to look for such ions in any recorded spectrum using a mol-file format of a candidate compound (MassFragment^{TM}, Waters). Further confirmation of candidate biomarkers was obtained by verification of the retention time and fragmentation pattern of an authentic standard (see authentic standards section above). The authentic standards were in some cases selected as one representative of biomarkers belonging to the same chemical compound class, i.e only one LPC out of a series was confirmed by a standard. Additionally, acyl-1 and acyl-2 LPC(18:1) and LPE(18:1) were spiked into two plasma samples collected in the fed and the fasted states, respectively, at a concentration of 0.5 mg/L for a more reliable confirmation.

Results and Discussion

An Overview of Data Processing Methods

All the softwares employed here were able to produce a feature set that were showing some separation of samples from the fasted and fed states as shown in PCA scores plot (Fig. 3). MarkerLynx and MZmine are both user friendly tools for users who do not want to go into R, MATLAB, or similar programming tools. Processing the data with MarkerLynx requires just a few user-defined settings, however the software does not provide any possibility for checking the success of any data processing step. In comparison, MZmine provides a powerful visualization side that can be considered as quite useful for tuning the settings. Visualization of peak detection results is also included in the XCMS package in R. An important missing part of MarkerLynx is that it does not contain any gap filling algorithm resulting in many zero values in the final extracted feature set. Zeros may obscure the later data analysis step and may result in incorrect definition of ‘effect markers’ as ‘exposure markers’, because ‘true’ zeros as well as smaller and larger peaks missed by the algorithm are given the same zero value [28].

The number of features obtained from each processing method is given in electronic supplementary material (Table 1S). We aimed to extract a similar number of features from the data processed by each software for either mode (positive or negative). This is a difficult task since each software tool has its own specific algorithm that requires optimization with user defined settings so they cannot be operated in exactly the same manner. For instance the

algorithms of MZmine and XCMS include gap-filling, so they have few zero entries and it makes no point to implement an 80% rule based on zeros. The effect of the 80% rule on the reduction of the number of features by the other methods is shown for comparison in electronic supplementary material (Table 2S). For positive and negative modes, approximately 50% of features (MarkerLynx) and 70% of m/z bins (custom processing) were eliminated with the 80% rule. In positive mode we seemed to succeed in getting similar numbers of features with the different software tools were obtained, however, the numbers varied somewhat in the negative mode.

An Overview of Data Analysis Methods

The scaling method (Unit- or Pareto-scaling) had an effect on the PCA scores plots for the feature sets created by the four software tools but neither of the scaling methods was superior in all cases. Here superior implies the better separation of fasted and fed groups by PCA scores plot. For instance, Pareto scaling performed better for MarkerLynx processed data whereas Unit scaling gave a better description of fasted-fed discrimination for XCMS processed data (electronic supplementary material, Fig. 2S). In addition, by comparing the selected 200 features using each of the two scaling methods before PLS-DA from any software and mode (Fig. 2, step 1), only 40-60% were found to be in common (electronic supplementary material, Fig. 3S). Consequently, the scaling methods are strongly affecting the feature selection. Since the intention in this study was to compare different processing algorithms in an objective manner it is an important point to satisfy the commonality in the whole subsequent data analysis pipeline. Hence, instead of selecting the better scaling method for each feature set we decided to combine the results from both scaling methods as illustrated in Fig. 2, step1. PLS-DA was then applied to each of the sets to select the 200 top rank features before combining them. The CV classification errors from the output PLS-DA models did not exceed 4% (with maximum of 6 LV) for any of the subsets produced by Unit scaling or Pareto scaling. The combined sets for each software and mode had 277-332 resulting features after including the unique ones.

In this study, we aimed to detect possible differences between two states for any feature regardless of its concentration, so in the third step we treated the combined data only with Unit scaling prior to the final variable selection step (Fig. 2, step 3). The main advantage of Unit scaling is that it is able to remove the influence of the signal intensity on the final rank of

the markers [29]. The crucial point is to keep in mind that the choice of scaling method depends on the biological question to be answered, the properties of the data set, and the data analysis method. Different strategies were used for the data analysis steps 1 and 3 because the data structures and the questions posed at these steps were different as explained above.

After the last scaling each feature set was evaluated with PLS-DA and the 25 features having the highest rank were extracted as markers potentially representing biomarkers discriminating the fasted and fed states.

Comparison of Data Processing Methods

We compared first the custom method with the three dedicated software methods. The algorithm of the custom processing method differs from the others by not having any peak detection and alignment steps. It can therefore be considered as a more primitive method, however, many markers were found to be in common with the other three methods. The point was to evaluate whether a very different data processing method would succeed in detecting similar markers. When custom processing was compared with each of the other methods we observed quite a large overlap which was generally larger for the positive than for the negative mode (Fig. 4a). In the positive mode the overlap with XCMS seemed slightly lower compared to other two methods.

A further comparison of the 25 markers for positive and negative mode data from each of the three dedicated software tools is illustrated in two Venn diagrams (Fig. 4b). In general these three methods seem to have only 3-6 markers in common among the top 25 markers detected in the negative and positive mode. This is the same level of overlap seen for each of these methods with the custom method (Fig. 4a). There is a trend towards a larger difference between XCMS and each of the other methods in the pairwise comparisons. So in consequence all of the data processing methods seem to miss out potentially important markers observed to be ranked among the top-25 markers by the other methods. In fact, only 3-4 markers would be observed to be in common if four research groups would investigate the same biological phenomenon using different softwares for data processing, provided they had recorded similar LC-MS data. On top of this comes the difference caused by parameter settings and other factors in the metabolomics experiment.

The 25 markers from each method and their various ranks are compared in Tables 1 & 2 for the negative and positive modes, respectively. The higher rank values for the selected markers

are also added in these tables for reference (Table 1 & 2, column 3). Obviously, the markers selected here have very different ranks with the four data processing methods. The consequence is that there is no basis for putting too much emphasis on the rank in PLS-DA methods. Consider that in many metabolomics studies, PLS-DA regression coefficients or VIP cut-offs have commonly been employed for marker selection. In order to make a rational selection of the cut-off and reduce the overoptimistic variable selection by PLS-DA additional statistical methods would be needed [30].

Another perspective in the comparison of different data processing methods is illustrated in electronic supplementary material (Fig. 4S). In this Figure, each row represents the rank of one marker from Table 1 (column 3) for all four different data processing methods. The first impression from electronic supplementary material (Fig. 4Sa) may be that the number of black regions (undetected peaks) might seem alarmingly high for some of the methods. It is important here to state that the custom data processing leads to a number of false positives, which were not detected as peaks by any of the other methods, thereby explaining part of the black regions. The false positives are probably a consequence of summation across retention times, causing baseline noise to increase and cover the significant differences in peak intensities. MarkerLynx and MZmine have similar patterns for negative mode data. XCMS seemed to have higher numbers of undetected peaks. One crucial remark is that the detection of peaks depends very much on the data processing settings of each software algorithm. Although we attempted to attain the largest possible similarity in the processing parameters of MarkerLynx, MZmine and XCMS, we were aware that it is not possible to obtain exactly the same results, since each method is based on different algorithms. To illustrate this point, we processed MZmine with less conservative settings and constructed the heat map again leading to a new pattern much more similar to XCMS (figure not shown). So in reality it may be possible to obtain more or less the same patterns with all three softwares, depending on their individual parameter settings. This points to the user defined settings as the major effector rather than the peak-detection algorithm, and also underlines that it is not possible to conclude that any of the three software algorithm performs less well than the others since their performance depends on the proper optimization of experimental settings. Moreover, combining feature sets from several settings during data processing with any software is likely to improve marker detection in untargeted metabolomics. In this study the patterns of fasted and fed state were very clear in the feature set whereas in many other metabolomics studies

this may not be the case. Improper settings of data processing parameters may therefore obscure the extraction of the relevant information. Proper settings are based on careful inspection of raw data as well as insight into the function of the software parameters. The heat map for positive mode data is shown in electronic supplementary material (Fig. 4Sb) and shows a pattern very similar to that for negative mode.

Trend views for some of the markers with high ranks are presented as electronic supplementary material (Fig. 5S). These raw data plots show that for most of these markers the differences between intensities in the fed and fasted states are visible to the naked eye.

Biomarker patterns

Three patterns are immediately visible for markers of the fed state in Tables 1 & 2. The first of these is the presence of sets of isomers having very similar masses but slightly different retention times, indicating that some specific groups of isomers are typical markers. The slight mass difference may be attributed to the mass accuracy of the instrument. Examples are clusters at 512.29, 478.29 and 590.35 in the negative mode and at 468.32, 520.34, and 522.36 in the positive mode. In many cases the earlier eluting isomeric form was not detected in the XCMS processed dataset, possibly because they are much smaller peaks. Considering the parameters set while processing the data with XCMS (electronic supplementary material, Table 1S), additional filtering or a too high *bw* parameter (for setting the RT shift) might be the cause of not detecting those peaks. Furthermore, these patterns are always spotted with the custom data processing as they were included into the same m/z bin thereby intensifying their relative importance. As can be seen from Tables 1 & 2, the possible isomers were therefore given the same rank for the custom data processing.

Another pattern in the marker sets is the presence of peaks with mass differences corresponding to 2 or 4 hydrogen atoms but with different retention times. These pairs are observed in both modes (e.g. 476/478, 520/522 or 586/590 in negative mode, and 506/508 or 520/522/524 in the positive, Table 1 & 2). These clusters and patterns are all observed for compounds with retention times in the same (unpolar) range pointing towards series of lipids with varying levels of saturation (-2 for each double bond) and similar patterns can also be observed for changes in chain lengths (+ 26 for adding –CH=CH-) as the underlying biomarkers.

Pattern recognition therefore identified lipids as potential discriminative markers between plasma samples collected in the fasted and fed states. This confirms an expected finding and further identification of some of the lipids as well as some of the more polar peaks were therefore perused.

Marker identification

Most of the masses belonging to the lipid-related patterns and clusters in the positive mode fit well with the masses expected for positively charged LPCs and LPEs of varying chain lengths and degrees of saturation. Lysophosphatidylcholine (LPC) is a plasma lipid that has been recognized as an important cell signaling molecule and it is produced by the action of phospholipases A1 and A2, by endothelial lipase or by lecithin-cholesterol acyltransferase (LCA) which transfers one of the fatty acids from phosphatidylcholine to cholesterol. LCA has a well-known function in catalyzing the transfer of fatty acids to free cholesterol in plasma for the formation of cholesteryl esters [31]. In the rat LPCs with more saturated acids are formed mainly in the plasma whereas unsaturated LPC is formed from PCs in the liver. We observe here a mixture of both saturated and unsaturated LPCs indicating that the source may be dual. The cytolytic and pro-inflammatory effects of LPCs are well known so their level is closely regulated. However, in blood plasma the LPCs form complexes with albumin and lipoproteins, especially LDL and are therefore not as likely to cause direct cell injury [32]. Another action of LPCs seems to be related to increased insulin resistance [33]. A slow clearance of postprandial lipids is known to be a risk factor for diabetes but the LPCs might be a lipid fraction contributing more strongly to this action. It is interesting in this context to note that Kim et al. identified LPCs as the major discriminative compounds of plasma species separating fasting plasma from obese/overweight and lean men [7]. They reported higher levels of saturated LPCs and lower level of unsaturated LPCs in the plasma of obese or overweight men. We found a similar profile here in lean rats as reported for the lean humans in the study of Kim et al. with a higher level of unsaturated LPCs. The unsaturated LPCs have been found also to pass the blood-brain barrier and to be important vehicles for delivering unsaturated lipids to the brain [34]. We speculate that the high level of unsaturated LPCs in the postprandial state of healthy individuals might be a part of the satiety signaling system which is malfunctioning in obesity. The LPCs appear usually in two isomeric forms, as 1-acyl or 2-acyl LPCs. The true separation of isomeric groups of LPC(18:1) in a fed state plasma

sample is illustrated in electronic supplementary material (Fig. 6S). These isomers were unstable and spontaneously positionally isomerized as also recognized in 1-acyl authentic standards of LPC and LPE(18:1) where 9% of the authentic standard was detected as another peak belonging to the 2-acyl form. For the confirmation of the 2-acyl LPC form, standards of PC and PE(16:0/18:1) were hydrolyzed by phospholipase A1. In addition to the 2-acyl LPC and LPE(18:1) the 7% of the acyl group spontaneously migrated to the 1-acyl position electronic supplementary material (Fig. 6S). Croset et al. studied the significance of positional acyl isomers of unsaturated LPCs in blood [35]. They concluded that 50% of PUFA was located at the 2-acyl position where they are available for tissue uptake, and that they can be re-acylated at the 1-acyl position to form membrane phospholipids. The lipid species observed here as important for the fed and fasting states reflect also that we would only be able to extract the more polar lipids and lipids with m/z below 1000 daltons. We can therefore not conclude here that the LPCs, LPEs and free fatty acids are the major discriminative lipid species and lipidomics studies have previously reported less polar lipid classes which may have m/z above 1000 daltons, such as PCs, sphingomyelins and triacylglycerols [36;37]. With our current method, we are able to identify PCs but they were not discriminative in this study.

A group of carnitine based compounds were also detected as markers in the positive mode data. The main function of carnitine is to assist the transport and metabolism of fatty acids in mitochondria, where they are oxidized as a major source of energy [38]. In plasma samples from the fasting state levels of L-carnitine was found to be lower whereas acetyl-L-carnitine is higher. During fasting an elevated concentration of acetyl coenzyme A favors the production of acetyl-L-carnitine and the ketone body, 3-hydroxybutanic acid, and these were identified as characteristic markers for the fasting state [39].

One of the amino acids, isoleucine, was found to be strongly discriminating between the fed and fasted states. Isoleucine belongs to the group of branched-chain amino acids which have been implicated in altered protein catabolism, insulin resistance and obesity [40;41]. However, only isoleucine was associated with the fed state here whereas at least leucine did not display similar differences (data not shown). It seems therefore that isoleucine may be a marker of recent food ingestion and decrease with fasting.

Many adduct or daughter ions were also included in our markers as given in Table 1 & 2. In the negative mode, LPCs form ion pairs with formate ions from the UPLC-solvent rendering

them neutral and by loss of a hydrogen ion the same compounds as observed in the positive mode may be observed in the negative mode with a net increase in mass by 44 Daltons (formate – H^+) and with the same RT. Instead LPEs and free fatty acids were detected only with loss of a hydrogen atom (Table 2). In the positive mode data, LPCs were observed not only in proton bound form but also as sodium and potassium adducts or even dimers (Table 2). The loss of CH_3 from the choline end of parent LPCs forms the characteristic LPC fragments in negative mode data (Table 1). In positive mode the LPC fragments were observed with the removal of one molecule of water from the parent compound (Table 2). One conclusion from this study is that in LC-MS based metabolomics studies a single molecular species is represented together with its parent ion and its inter-convertible isomers, their adduct forms, and the fragment ions. In many cases different adducts or fragment ions of the same metabolite may emerge with a higher or lower rank than the parent ion, and this is an important cause of differences in the ranking orders between methods. To illustrate the higher commonality at the metabolite level, we established a new rank for each metabolite (each metabolite attained the lowest rank value from among its representative adducts, fragments or isomers). The unidentified features were considered as representing the same metabolite as long as they are within the range of an 0.02min retention time window. The metabolite ranks of different methods are represented in Fig. 5. Fig. 5 illustrates that there are a higher number of undetected metabolites for MarkerLynx which may be a result of the parameter selection or the peak detection algorithm. For both negative and positive mode data, the rank patterns were much more similar between different methods at the metabolite level than at the feature level (electronic supplementary material, Fig. 4S). Metabolites with a high rank with any one method also tend to attain higher rank values (yellowish color on Fig. 5) for the other methods. Thus, it seems correct to conclude that different data processing methods employed in this study provide 36% to 64% common markers (Fig. 4a), but the commonality is actually much higher at the metabolite level as different features (adducts or fragment ions), selected from the different methods actually represent the same metabolite.

The observation that all these related ions come up with high ranks and that their high ranks are shared between the positive and negative modes as in this study, would strengthen not only the confidence in the identification step but also in our variable selection method.

Conclusions

We aimed here to explore the effect of four data processing methods on the pattern of final biomarkers. The selection of proper experimental parameters based on the specifics of the dataset is the key for obtaining a high quality data analysis, regardless of applied data processing method. The better parameter setting is a matter of experience and wrong settings may obscure the extraction of relevant information. Combination of several such settings with any of the data processing methods may increase the coverage of relevant markers.

Here we defined the markers as the 25 features having the highest regression coefficients by each data processing method. Although the comparison of the selected markers from different data processing methods revealed many different features, further chemical identification revealed that they were often just adducts or daughter ions representing the same biomarker compound. Another point is that many of the biomarkers identified were biologically closely related so that any of the softwares and procedures applied here could identify biomarkers explaining a major part of the biological processes causing the differences between the fasting and the fed states.

The major lipid classes, LPCs, LPEs and free fatty acids, emerged as discriminative between fed and fasted states in rats. The high level in the postprandial state of LPCs, generally known to be pro-inflammatory, is interesting and their importance for low-grade inflammation should be further explored. L-carnitine and acyl carnitines were also found as important markers and the shift from free to acylated carnitine during fasting might be useful for the development of a marker to follow the switch from lipid storage to lipid degradation from feeding to fasting by using the ratio between the two.

Electronic supplementary material

The online version of this article contains supplementary material, which is available to authorized users.

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Figure Captions

Fig. 1 Custom data processing scheme

Fig. 2 The scheme summarizing the steps for the selection of markers in data analysis. Steps 1 to 3 were applied on each feature set extracted using any processing method (^{2}variable selection)

Fig. 3 PCA scores plots of negative mode data processed with MarkerLynx (A), MZmine (B), XCMS (C) and customized methods (D)

Fig. 4 (a) Bar plot illustrating the number of markers in the intersection of custom and other three data processing software tools (A: MarkerLynx, B: MZmine, C:XCMS). (b) Venn diagrams illustrating the number of common and method specific markers extracted from three software tools (left: positive mode; right: negative mode)

Fig. 5 Heatmap illustrating the comparison of metabolite ranks between four different methods for (a) negative and (b) positive mode data. Each row represents the lowest value rank of a metabolite for four different methods (Table 1,3rrd column). The markers were sorted in ascending order based on the rank obtained with method A (A: MarkerLynx; B: MZmine, C: XCMS and D: Custom data processing). (yellow to red: rank 1-25; claret red: rank higher than 25; black: not detected; green: detected)

Tables

Table 1. Retention times and measured masses of the markers obtained from MarkerLynx, MZmine, XCMS and custom data processing of negative mode data that contributed most to the separation of samples in fasted and fed states.

RT (min)	Measured m/z	Rank	Group	Suggested Compound	Suggested Adduct	Monoisotopic mass
0.64017	105.02	$nd^A110^B12^Cd^D$	fed	U1		
1.3652	103.039	$30^A15^B75^C49^D$	fasted	3-hydroxybutanoic acid	$[M-H]^-$	104.0473
1.3662	59.01	$40^A46^B6^C9^D$	fasted	3-hydroxybutanoic acid fragment ($-C_2H_4O$)		
1.37	418.125	$nd^And^Bnd^C25^D$	fasted	U2		
1.37	576.3235	$nd^And^Bnd^C19^D$	fasted	U2		
1.3638	259.9988	$54^A49^B24^C3^D$	fasted	U2		
1.3748	130.09	$39^A52^B22^C115^D$	fed	Isoleucine	$[M-H]^-$	131.0946
1.8788	178.0495	$23^A13^B18^C69^D$	fasted	Hippuric acid*	$[M-H]^-$	179.0582
2.4654	623.8663	$31^A25^Bnd^C10^D$	fed	U3		
2.4665	365.0683	$11^A33^Bnd^C48^D$	fed	U3		
2.4715	343.08	$5^A8^B3^C2^D$	fed	U4		
2.4715	623.36	$18^A38^B9^Cd^D$	fed	U4		
2.68	198.1021	$nd^And^Bnd^C14^D$	fed	U5		
3.5713	580.33	$nd^A88^B21^C129^D$	fed	U6		
4.0573	512.2995	$8^A12^Bnd^C29^D$	fed	LPC(14:0)	$[M+FA-H]^-$	467.3012
4.0577	452.2779	$21^And^Bnd^Cd^D$	fed	LPC(14:0) fragment ($-CH_3$)		
4.099	562.3133	$nd^A11^Bnd^Cd^D$	fed	U7		
4.1104	586.3145	$nd^A7^Bnd^C1^D$	fed	LPC(20:5)	$[M+FA-H]^-$	541.3168
4.1232	309.2035	$2^A20^B2^C303^D$	fed	LPC(20:5) fragment ($-C_7H_{22}NO_5P$)		
4.1578	979.6007	$24^A41^Bnd^C8^D$	fed	LPC(14:0)	$[2M+FA-H]^-$	467.3012
4.158	512.2978	$9^A9^B5^C29^D$	fed	LPC(14:0)	$[M+FA-H]^-$	
4.1581	452.2772	$13^A14^Bd^Cd^D$	fed	LPC(14:0) fragment ($-CH_3$)		
4.1622	514.31	$nd^A44^B10^C291^D$	fed	U8		
4.177	474.2612	$nd^A16^Bnd^Cd^D$	fed	LPC(18:3) fragment ($-C_3H_7$)		
4.1789	502.2917	$20^A22^Bnd^C13^D$	fed	LPC(18:3) fragment ($-CH_3$)		
4.1811	562.3148	$27^A1^B92^Cd^D$	fed	LPC(18:3)	$[M+FA-H]^-$	517.3168
4.1826	526.2948	$10^A10^B4^Cd^D$	fed	LPC(20:5) fragment ($-CH_3$)		
4.1847	586.32	$3^A6^B1^C1^D$	fed	LPC(20:5)	$[M+FA-H]^-$	541.3168
4.1948	538.3145	$25^A40^Bnd^C11^D$	fed	LPC(16:1)	$[M+FA-H]^-$	493.3168
4.2285	562.3143	$47^A21^B11^Cd^D$	fed	LPC(18:3)	$[M+FA-H]^-$	517.3168
4.2288	502.29	$d^A27^B17^C13^D$	fed	LPC(18:3) fragment ($-CH_3$)		
4.23	634.2	$nd^And^Bnd^C24^D$	fed	U9		

4.2467	455.21	$nd^{A}91^{B}27^{C}17^{D}$	fed	U10		
4.28	450.26	$nd^{A}nd^{B}nd^{C}22^{D}$	fed	LPC(16:1) fragment ($-C_3H_7$)		
4.2863	538.31	$35^{A}36^{B}14^{C}11^{D}$	fed	LPC(16:1)	$[M+FA-H]^-$	493.3168
4.2868	606.3	$38^{A}56^{B}13^{C}12^{D}$	fed	U11		
4.2878	478.29	$22^{A}58^{B}19^{C}d^{D}$	fed	LPC(16:1) fragment ($-CH_3$)		
4.3265	311.22	$14^{A}26^{B}7^{C}d^{D}$	fed	U12		
4.3474	476.2776	$16^{A}5^{B}nd^{C}4^{D}$	fed	LPC(18:2) fragment ($-C_3H_7$)		
4.3517	504.3093	$6^{A}3^{B}$ $nd^{C}32^{D}$	fed	LPC(18:2) fragment ($-CH_3$)		
4.3525	564.3296	$4^{A}2^{B}nd^{C}16^{D}$	fed	LPC(18:2)	$[M+FA-H]^-$	519.3325
4.3538	995.6027	$nd^{A}nd^{B}nd^{C}7^{D}$	none	Unknown		
4.4171	588.3309	$200^{A}17^{B}183^{C}258^{D}$	fed	U13		
4.4341	476.2775	$17^{A}24^{B}8^{C}4^{D}$	fed	LPC(18:2) fragment ($-C_3H_7$)	$[M-H]^-$	477.2856
4.4422	168.3515	$15^{A}64^{B}nd^{C}d^{D}$	fed	U14		
4.443	168.6234	$12^{A}167^{B}nd^{C}20^{D}$	fed	U14		
4.4435	564.3302	$137^{A}18^{B}50^{C}16^{D}$	fed	LPC(18:2)	$[M+FA-H]^-$	519.3325
4.45	335.3454	$nd^{A}nd^{B}nd^{C}21^{D}$	none	Unknown		
4.45	335.45	$nd^{A}nd^{B}nd^{C}6^{D}$	none	Unknown		
4.45	335.7682	$nd^{A}nd^{B}nd^{C}5^{D}$	none	Unknown		
4.5061	552.3273	$19^{A}47^{B}15^{C}188^{D}$	fed	U15		
4.5908	590.35	$37^{A}35^{B}16^{C}d^{D}$	fed	LPC(20:3)	$[M+FA-H]^-$	545.3481
4.5912	658.33	$43^{A}57^{B}25^{C}18^{D}$	fed	U16		
4.644	478.294	$1^{A}4^{B}nd^{C}d^{D}$	fed	LPE(18:1)*	$[M-H]^-$	479.3012
4.6916	590.3466	$26^{A}23^{B}nd^{C}d^{D}$	fed	LPC(20:3)	$[M+FA-H]^-$	545.3481
4.7	595.3	$nd^{A}nd^{B}nd^{C}23^{D}$	none	Unknown		
4.7306	478.2934	$7^{A}19^{B}d^{C}d^{D}$	fed	LPE(18:1)*	$[M-H]^-$	479.3012
4.7322	999.65	$36^{A}nd^{B}23^{C}d^{D}$	fed	LPE(18:1)*	$[2M+FA-H]^-$	
5.1378	277.2154	$32^{A}36^{B}25^{C}42^{D}$	fasted	Gamma-Linolenic acid*	$[M-H]^-$	278.2246
5.2203	338.2677	$nd^{A}nd^{B}nd^{C}15^{D}$	fasted	U17		
5.377	279.2322	$28^{A}31^{B}20^{C}82^{D}$	fasted	Linoleic acid*	$[M-H]^-$	280.2402

^{A},MarkerLynx; ^{B},MZmine; ^{C},XCMS; ^{D},Custom data processing; 'U', Unidentified compound; *, identity confirmed with authentic standards; 'd', detected but not given a rank because the feature was omitted in step 1 of the data processing procedure (Fig. 1); 'nd', not detected by the software peak-finding algorithm.

Table 2. Retention times and measured masses of the markers obtained from obtained from MarkerLynx, MZmine, XCMS and custom data processing of positive mode data that contributed most to the separation of samples in fasted and fed states.

RT (min)	Measured m/z	Rank	Group	Suggested Compound	Suggested Adduct	Mono-isotopic mass
0.51917	112.1138	$nd^A62^B21^C226^D$	fasted	U1		
0.6134	162.1128	$40^A41^B37^C12^D$	fed	L-Carnitine*	$[M+H]^+$	161.1052
0.66298	116.0719	$44^A16^B20^C9^D$	fed	U2		
0.66313	70.0658	$nd^A21^B40^C17^D$	fed	U2		
0.822	143.1201	$55^A42^B24^C135^D$	fasted	U3		
0.8881	204.125	$7^A31^B7^C16^D$	fasted	L-Acetylcarnitine*	$[M+H]^+$	203.1158
0.8881	144.1036	$28^And^B16^C318^D$	fasted	L-Acetylcarnitine* fragment ($-C_2H_4O_2$)		
0.9003	60.0816	$13^And^Bnd^Cd^D$	fasted	L-Acetylcarnitine* fragment ($-C_6H_{12}NO_3$)		
0.9012	85.028	$17^A64^B10^C175^D$	fasted	L-Acetylcarnitine* fragment ($-C_5H_{13}NO_2$)		
0.9013	145.051	$4^A19^B3^C110^D$	fasted	L-Acetylcarnitine* fragment ($-C_6H_9N$)		
1.1552	248.1521	$d^A56^B15^C25^D$	fasted	U4		
1.6373	231.1215	$nd^Ad^B1^Cd^D$	fasted	U5		
1.8878	105.0348	$100^A80^B6^C244^D$	fasted	U6		
2.63	570.3439	$20^And^B5^Cd^D$	fed	U7		
3.2285	552.3341	$91^A32^B12^Cd^D$	fed	U8		
3.2795	107.0871	$d^A74^B19^Cd^D$	fasted	U9		
3.2797	121.1028	$114^And^B25^Cd^D$	fasted	U9		
3.2798	163.15	$57^A63^B23^Cd^D$	fasted	U9		
3.2798	205.1613	$73^A85^B18^Cd^D$	fasted	U9		
3.409	550.3199	$nd^And^B13^Cd^D$	fed	U10		
3.4286	536.3383	$1^And^Bnd^C14^D$	fed	U10		
3.5823	518.3278	$nd^And^B17^C15^D$	fed	U11		
3.7402	534.321	$nd^A24^B9^Cd^D$	fed	U12		
4.0664	468.3109	$5^A33^Bnd^C6^D$	fed	LPC(14:0)	$[M+H]^+$	467.3012
4.1187	542.328	$nd^A3^Bnd^C3^D$	fed	LPC(20:5)	$[M+H]^+$	541.3168
4.1213	564.3085	$nd^A11^Bnd^C2^D$	fed	LPC(20:5)	$[M+Na]^+$	541.3168
4.1588	468.3101	$2^A13^B2^C6^D$	fed	LPC(14:0)	$[M+H]^+$	467.3012
4.1626	957.6048	$11^And^Bnd^Cd^D$	fed	LPC(14:0)	$[2M+Na]^+$	467.3012
4.1642	985.641	$9^And^Bnd^C9^D$	fed	LPC(14:0)		
4.1643	506.2667	$14^A23^B31^Cd^D$	fed	LPC(14:0)	$[M+K]^+$	467.3012
4.1645	490.2931	$8^A6^B8^C7^D$	fed	LPC(14:0)	$[M+Na]^+$	467.3012
4.1649	935.6182	$10^A17^Bnd^C24^D$	fed	LPC(14:0)	$[2M+H]^+$	467.3012
4.1911	564.3099	$12^A7^B22^C2^D$	fed	LPC(20:5)	$[M+Na]^+$	541.3168
4.193	524.3201	$19^And^Bnd^C129^D$	fed	LPC(20:5) fragment ($-H_2O$)		

4.1946	476.2937	$6^{A}nd^{B}nd^{C}71^{D}$	fed	LPC(20:5) fragment ($-CH_6O_3$)		
4.197	542.3258	$15^{A}8^{B}11^{C}3^{D}$	fed	LPC(20:5)	$[M+H]^+$	541.3168
4.2287	540.3107	$49^{A}27^{B}27^{C}4^{D}$	fed	LPC(18:3)	$[M+Na]^+$	517.3168
4.2291	518.3258	$23^{A}2^{B}42^{C}15^{D}$	fed	LPC(18:3)	$[M+H]^+$	517.3168
4.292	987.6565	$nd^{A}nd^{B}nd^{C}20^{D}$	fed	LPC(16:1)	$[2M+H]^+$	493.3168
4.299	266.6	$nd^{A}nd^{B}nd^{C}13^{D}$	none	Unknown		
4.3015	532.2859	$21^{A}18^{B}93^{C}d^{D}$	fed	LPC(16:1)	$[M+K]^+$	493.3168
4.3022	516.3092	$16^{A}15^{B}57^{C}8^{D}$	fed	LPC(16:1)	$[M+Na]^+$	493.3168
4.3022	494.327	$24^{A}35^{B}47^{C}11^{D}$	fed	LPC(16:1)	$[M+H]^+$	493.3168
4.3516	566.3244	$nd^{A}12^{B}nd^{C}169^{D}$	fasted	U13		
4.3583	503.3221	$nd^{A}22^{B}4^{C}105^{D}$	fed	LPC(18:2) fragment (-OH)		
4.3587	520.3422	$37^{A}1^{B}nd^{C}1^{D}$	fed	LPC(18:2)	$[M+H]^+$	519.3325
4.3588	502.3252	$nd^{A}9^{B}nd^{C}23^{D}$	fed	LPC(18:2) fragment ($-H_2O$)		
4.3672	337.2576	$22^{A}20^{B}14^{C}86^{D}$	fed	LPC(18:2) fragment ($-C_5H_{14}NO_4P$)		
4.4408	997.6404	$32^{A}44^{B}38^{C}5^{D}$	fed	LPC(P-16:0)	$[2M+K]^+$	479.3376
4.442	519.5459	$nd^{A}nd^{B}nd^{C}21^{D}$	none	Unknown		
4.4453	520.3401	$3^{A}5^{B}67^{C}1^{D}$	fed	LPC(18:2)	$[M+H]^+$	519.3325
4.5116	508.3451	$35^{A}14^{B}58^{C}d^{D}$	fed	LPE(20:1)	$[M+H]^+$	507.3325
4.5972	546.3587	$18^{A}25^{B}nd^{C}132^{D}$	fed	LPC(20:3)	$[M+H]^+$	545.3481
4.6527	339.2921	$nd^{A}4^{B}36^{C}239^{D}$	fed	LPC(18:1)* fragment ($-C_5H_{14}NO_4P$)		
4.654	975.6608	$66^{A}nd^{B}nd^{C}10^{D}$	fed	U14		
4.654	522.359	$321^{A}nd^{B}nd^{C}18^{D}$	fed	LPC(18:1)*	$[M+H]^+$	521.3481
4.684	519.045	$nd^{A}nd^{B}nd^{C}22^{D}$	none	Unknown		
4.7278	522.3558	$25^{A}48^{B}134^{C}18^{D}$	fed	LPC(18:1)*	$[M+H]^+$	521.3481

^{A},MarkerLynx; ^{B},MZmine; ^{C},XCMS; ^{D},Custom data processing; 'U', Unidentified compound; *, identity confirmed with authentic standards; 'd', detected but not given a rank because the feature was omitted in step 1 of the data processing procedure (Fig. 1); 'nd', not detected by the software peak-finding algorithm.

FIGURE 1

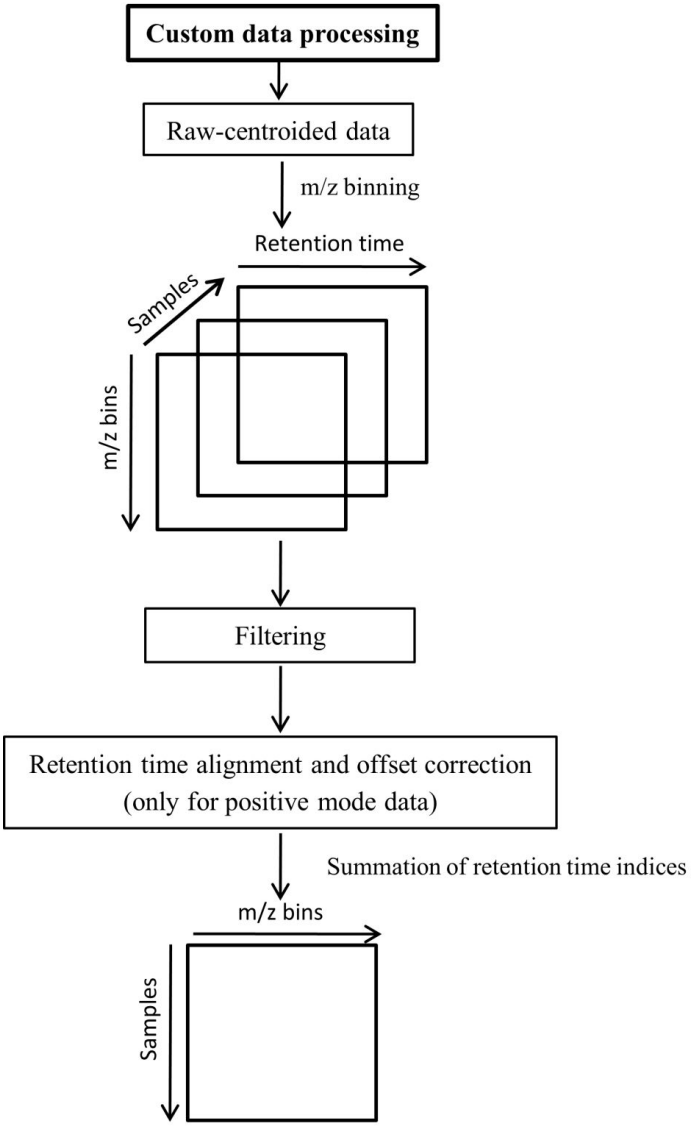

Custom data processing
Raw-centroided data
m/z binning
Retention time
Samples
m/z bins
Filtering
Retention time alignment and offset correction
(only for positive mode data)
Summation of retention time indices
m/z bins
Samples

FIGURE 2

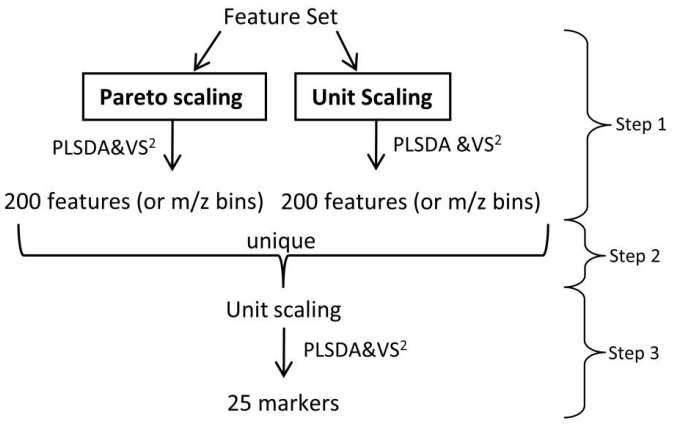

Feature Set
Pareto scaling
Unit Scaling
PLSDA&VS2
PLSDA &VS2
200 features (or m/z bins)
200 features (or m/z bins)
unique
Unit scaling
PLSDA&VS2
25 markers
Step 1
Step 2
Step 3

FIGURE 3

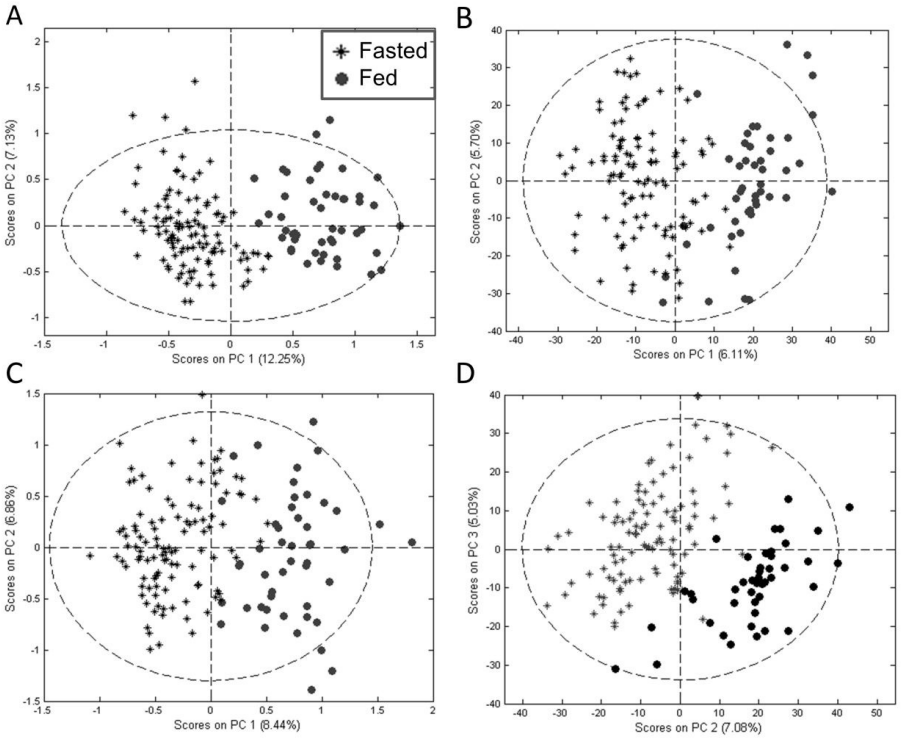
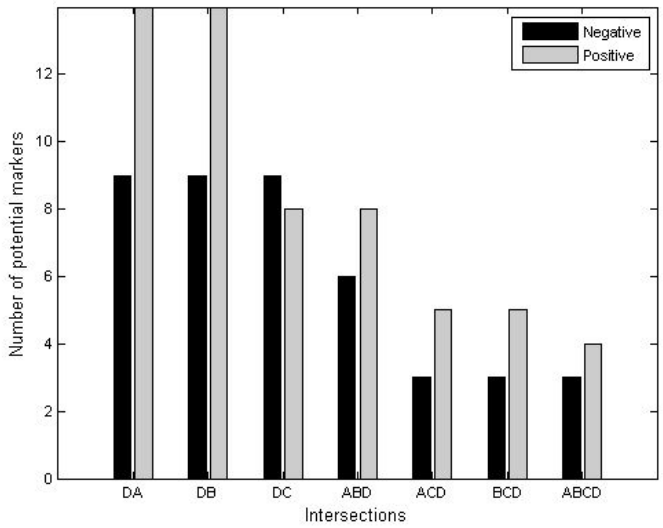

A
Fasted
Fed
Scores on PC 2 (7.13%)
Scores on PC 1 (12.25%)
B
Scores on PC 2 (5.70%)
Scores on PC 1 (6.11%)
C
Scores on PC 2 (6.86%)
Scores on PC 1 (8.44%)
D
Scores on PC 3 (5.03%)
Scores on PC 2 (7.08%)

FIGURE 4

a


Negative
Positive
Number of potential markers
0
2
4
6
8
10
12
DA
DB
DC
ABD
ACD
BCD
ABCD
Intersections

b

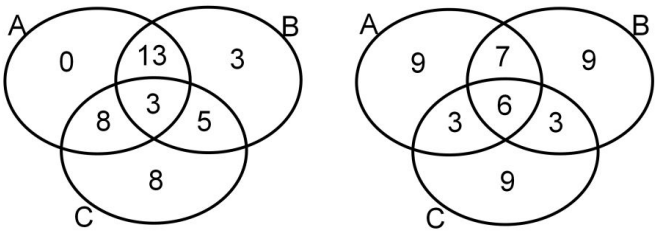
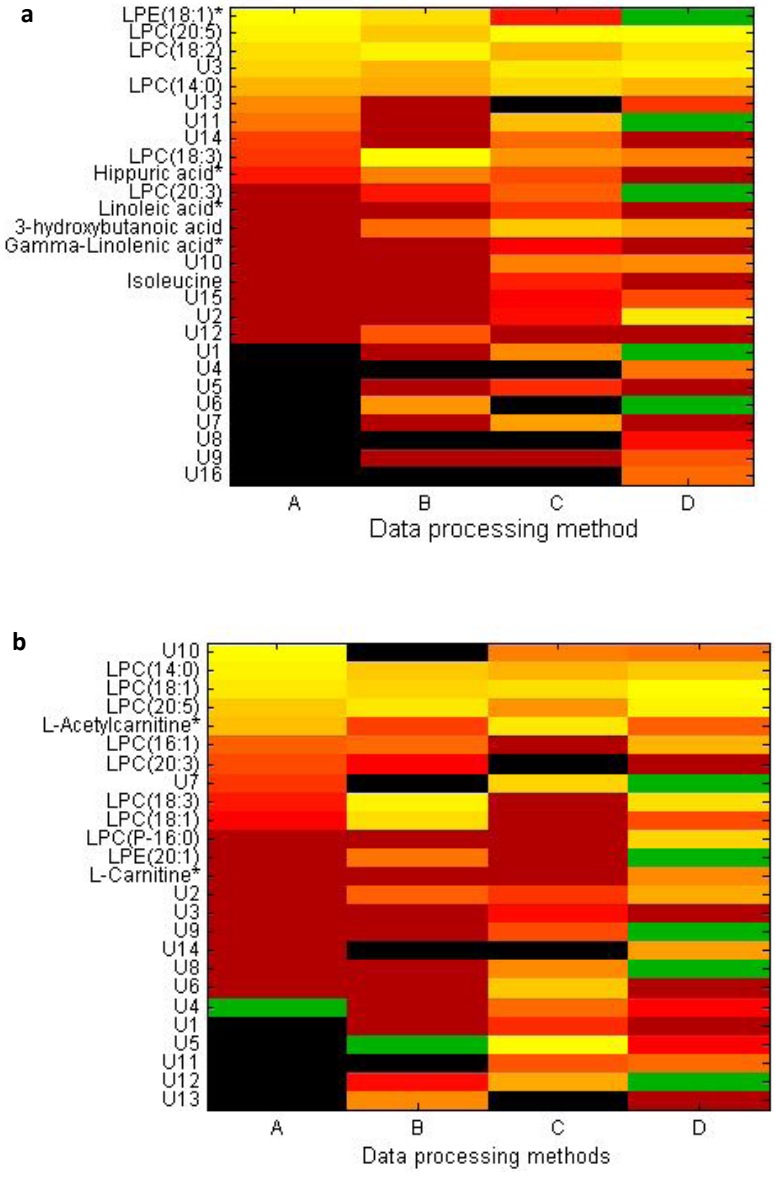

A
B
C
0
13
3
3
8
5
8
A
B
C
9
7
9
6
3
3
9

FIGURE 5


a
LPE(18:1)*
LPC(20:5)
LPC(18:2)
U3
LPC(14:0)
U13
U11
U14
LPC(18:3)
Hippuric acid*
LPC(20:3)
Linoleic acid*
3-hydroxybutanoic acid
Gamma-Linolenic acid*
U10
Isoleucine
U15
U2
U12
U1
U4
U5
U6
U7
U8
U9
U16
A
B
C
D
Data processing method
b
U10
LPC(14:0)
LPC(18:1)
LPC(20:5)
L-Acetylcarnitine*
LPC(16:1)
LPC(20:3)
U7
LPC(18:3)
LPC(18:1)
LPC(P-16:0)
LPE(20:1)
L-Carnitine*
U2
U3
U9
U14
U8
U6
U4
U1
U5
U11
U12
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Data processing methods

Metabolomics
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ORIGINAL ARTICLE

LC–MS metabolomics top-down approach reveals new exposure and effect biomarkers of apple and apple-pectin intake

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Abstract In order to investigate exposure end effect markers of fruit and fruit fibre intake we investigated how fresh apple or apple-pectin affects the urinary metabolome of rats. Twenty-four Fisher 344 male rats were randomized into three groups and fed a standard diet with different supplementations added in two of the groups (7% apple-pectin or 10 g raw apple). After 24 days of feeding, 24 h urine was collected and analyzed by UPLC-QTOF-MS in positive and negative ionization mode. Metabolites that responded to the apple or pectin diets were selected and classified as either potential exposure or effect markers based on the magnitude and pattern of their response. An initial principal component analysis (PCA) of all detected metabolites showed a clear separation between the groups and during marker identification several new apple and/or pectin markers were found. Quinic acid, *m*-coumaric acid and (-)epicatechin were identified as exposure markers of apple intake whereas hippuric acid behaved as an effect marker. Pyrrole-2-carboxylic acid and 2-furoylglycine behaved as pectin exposure markers while 2-piperidinone was recognized as a potential pectin effect marker. None of them has earlier been related to intake of pectin or other fibre products. We discuss these new potential exposure and effect markers and their interpretation.

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1 Introduction

It is well known that fruit consumption has preventive effects on degenerative diseases and especially on cardiovascular disease (Bazzano et al. 2002; Liu et al. 2000), however the causal factors and their mechanisms of action are not well known. Apples represent one of the major fruits consumed throughout the western countries and the disease preventive factors of this fruit seem particularly relevant to investigate. Consumption of nutrients and other bioactive compounds from fruit will most likely interact with several physiological functions and metabolic pathways in the organism and hereby reduce the risk of disease. Methods that can handle multiple responses therefore seem particularly beneficial compared to the classical univariate approaches most often used in nutrition research. Metabolomics aim for measurement of all metabolites present in a given biological sample and by use of this technique the metabolic effect of e.g., apple intake can hereby be explored in a top-down manner compared to more targeted analytical methods. The open-minded approach of metabolomics has great potential to generate new hypotheses and thereby to improve our mechanistic understanding of why 'an apple a day keeps the doctor away'. Compared to a human study, the rats in this investigation are expected to exhibit a much lower level of background variation due to their isogenic nature and controllable habits and consequently a larger number of exposure- and effect related features in the recorded metabolome profiles. This may ease interpretation of effects in future human intervention studies where exposure to apple, pectin or fruit intake in general may be partially hidden in the large

inter-individual variation. The combination of several features recorded in rat studies as related to apple exposure or effect would therefore help to identify more robust biomarkers in humans. Besides being helpful in our mechanistic understanding of dietary effects of apple intake, such objective biomarkers would be useful for the estimation of apple or fruit intake in samples from epidemiological studies, where the current methods based on questionnaires are prone to bias. Improved markers of intake should be useful to identify possible associations between dietary apple intake and disease prevention at the population level.

In the research presented here we want to focus on the cell wall polysaccharide, pectin, as a potentially disease preventive component in apples and in many other fruits. Pectins are presumed to prevent the reabsorbtion of bile acids in the intestine and to enhance steroid excretion so that more cholesterol is diverted into the bile acid pool (Ahrens et al. 1986). However, pectins from different plant origins have a large structural diversity and thus possibly varied health effects, which presumably is the main reason why previous animal studies reporting on pectin feeding and plasma cholesterol have been inconsistent (Aprikian et al. 2001, 2002, 2003; Trautwein et al. 1998; Yamada et al. 2003). Thus, there is a need to sort out mechanisms and active components in order to understand the physiological effect of apple intake and of its associated pectin component. In this study we investigate the urinary metabolome following feeding of fresh apple and apple-pectin to rats in a nutritionally balanced feeding trial.

2 Materials and Methods

2.1 Materials

All apples used were from a single batch of the variety Shampion, grown in Skierniwice, Poland. Apple-pectin was a commercial unrefined product kindly provided by Obi-Pectin AG (Basel, Switzerland).

2.2 Animal study and sample collection

Twenty-four Fisher 344 male rats were randomized into three groups and all rats were fed a standard diet with different supplementations added in two of the groups. One group had 7% apple-pectin added to the diet, one group 10 g of raw apple and one group had no supplementation added to the diet (control). The diet was balanced so that all animals received the same amount of macro- and micronutrients (details to be published elsewhere). After 24 days of feeding, urine was collected in a collection vessel preconditioned with 1 ml 1 mM NaN_3 to avoid microbial growth. The collection vessel was surrounded by an insulated container filled with dry-ice to ensure that the urine kept a temperature below 5°C during a 24 h collection period. The dry ice was replenished every 8 h during the collection period. Each urine sample was diluted with a fixed volume of 3 ml water used to wash the collection device in the metabolism cage and then weighed and immediately frozen at −80°C.

2.3 LC-QTOF-MS analysis

Before analysis the samples were thawed, filtered through a 40 µm Millipore filter (Millipore, Billerica, Massachusetts) and distributed randomly into a 96-well auto-injector tray. The tray was centrifuged to precipitate debris and 10 µl of each sample were injected into an UPLC (Waters, Milford, Massachusetts) with a 1.7 µm C18 BEH column (Waters) operated with a 6.0 min gradient from 0.1% formic acid to 0.1% formic acid in 20% acetone: 80% acetonitrile. The eluate was analyzed in duplicate by Waters Premier QTOF-MS in both negative and positive modes. Ionization of molecules was achieved by applying a voltage of 2.8 or 3.2 kV to the tip of the capillary in negative or positive mode, respectively. This represents relatively soft ionization conditions but optimised so that fragmentation can occur and be helpful in our later structural interpretation. Data were collected in centroid mode using leucine-enkephalin as a lock-spray to calibrate mass accuracy every 10 s. A blank (0.1% formic acid) and a metabolomics standard containing 40 different physiological compounds were analyzed three times during the sample run. This standard was used to check mass error (<20 ppm) and retention time shift (<0.05 min) during the run and when running authentic standards for verification.

2.4 Data preprocessing of LC–MS data

The raw data were extracted and aligned in retention time- and mass-direction by MarkerLynx™ (Waters) by using two different processing conditions as detailed below, to discover as many important peaks as possible. MarkerLynx™ works by customized predefinition of several parameters and applies a peak picking algorithm to select potential markers. In the following the detected ions are termed 'features' when collected after the peak picking and alignment procedure and 'markers' after selection by data analysis. Two sets of parameters for data processing were used: A retention time window of 0.05 (0.1) s, a mass window of 0.05 (0.02) Da, a noise elimination level of 3 (6) standard deviations above background and an intensity threshold of 20 (30) cps. The first method resulted in 5,350 features in the negative mode and 7,668 features in the positive mode and the second method (parameters in

brackets) resulted in 5,574 and 8,783 features in the negative and positive modes, respectively. The two datasets were exported to Excel (Microsoft) and after removing overlapping features the combined matrix consisted of 7,380 and 12,775 features in negative and positive mode, respectively. Duplicate sample analyses were combined as described by Bijlsma et al. (2006) meaning that if both measurement values were zero the combined value was zero and if both values were nonzero, the combined value was equal to the average of the two measurement values. If one replicate has a nonzero value and the other replicate is zero, the combined value is set to the nonzero measurement. The rationale for this procedure is that the combined value is most likely closer to the nonzero value since the measured zero value is expected to be due to a slip in the peak picking or because the analyte was not measured in the MS (e.g., momentary ion suppression). Moreover, due to the threshold level applied in the data processing step with the MarkerLynx™ software some 'false' zero values will be present in the dataset. Consequently, before performing explorative analysis the data were divided into subsets (control/apple and control/pectin) and if a feature had more than 20% zero values within one of the groups in both subset it was excluded from the dataset (adapted from Bijlsma et al. (2006)) leaving 4,010 and 7,353 features in the negative and positive modes, respectively. Finally, before data analysis the data was normalized to unit vector length (Euclidean norm) to reduce variations caused by instrumental variation, concentration difference in the urine samples etc. When using this type of normalization the 'closure' effect should always be considered, concerning that the true depletion of some peaks between samples will automatically be reflected in increased intensities for other peaks (and vice versa). However, since non-targeted metabolomics data are unlikely to be dominated by few high or low intensity variables, the risk of closure is considered minimal (Backstrom et al. 2007) and this normalization approach has earlier been applied successfully to other explorative metabolomics investigations (Nielsen et al. 2010; Scholz et al. 2004).

2.5 LC–MS data analysis and pattern recognition for potential exposure and effect markers

A principal component analysis (PCA) was performed in Matlab (Matlab version R2009a, Matworks) for the whole dataset. Features were then divided into potential *exposure* markers and *effect* markers for apple and pectin intake, respectively. The selection criterion for potential exposure markers was that they should have only zero values in the control group and positive responses in all animals in the apple and/or pectin group (see Fig. 1 marker #2 for an example). One misclassification was allowed in each group in order to tolerate small measurement errors of the MS instrumentation. In contrast, potential effect markers were defined as markers that have a baseline response in all animals in one group and a significantly up- or down-regulated response in all animals in the comparing group (one misclassification is allowed in each group). Simple binomial calculations give a $P = 0.00124$ for a chance finding indicating that we can expect less than five false positives among the negative mode features and less than 10 in the positive mode. Less than one of these would be found among features without misclassifications. Figure 1 marker #3 illustrates the pattern of a potential effect marker.

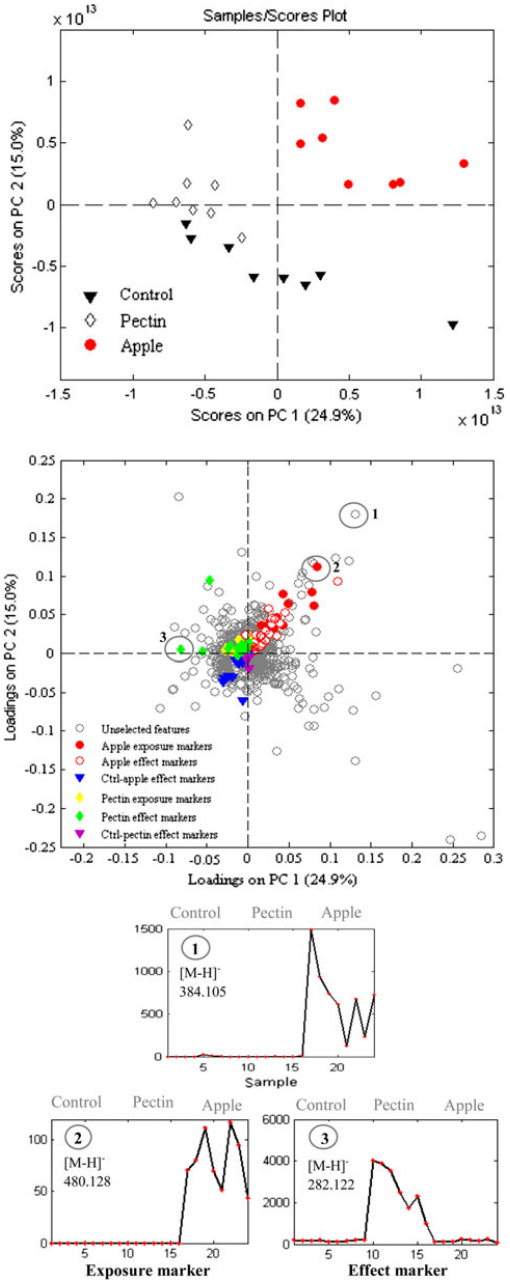
2.6 Marker identification

After selection of potential exposure and effect markers the primary focus was to chemically identify as many of these markers as possible. The nature of a QTOF-MS instrument allows very accurate mass measurement but despite the high data quality the chemical identification part of this kind of untargeted metabolomics experiments remains a highly laborious task involving interpretation of isotope patterns and fragmentation patterns, database or literature search and finally experimental verification of the selected markers by co-elution experiments. The lack of commercially available authentic standards for some markers hinders their identification and these markers are left as tentatively identified. For some markers there are no known compounds that fit the characteristics observed by MS and these markers will need more advanced identification experiments which are beyond the scope of the present paper. Therefore, only a part of the markers listed in this publication will be chemically identified at the present time. As the first step in the identification work, retention time and response behaviour was compared between the markers to detect potentially interrelated fragment-ions. Hereafter, the measured mass of a particular marker was searched in a database and the results compared to the isotopic fit in the mass spectra by use of the Markerlynx™ elemental composition software. Then fragment ions were taken into consideration by looking directly in the raw data and by applying a mass fragment tool (MassFragment™, Waters) and finally a pure standard of the proposed compounds were analyzed by the UPLC-MS system to verify retention time and the fragmentation and/or adduct-forming pattern.

3 Results and discussion

3.1 Potential exposure and effect marker identification

The initial PCA model of the urinary rat metabolome data (negative mode) is shown in Fig. 1. The first two components of this model accounted for approximately 40% of



◀ **Fig. 1** PCA score (*top*) and loading plot (*lowest*) of PC1/PC2 with all features measures in negative mode ($n = 4010$). Data are mean centred and two times pareto scaled. *Diagram 1–3* illustrates the corresponding response pattern of a selected feature or markers from the loading plot. Unselected feature refers to a feature that is not selected as an exposure or effect marker by the presented data analysis method

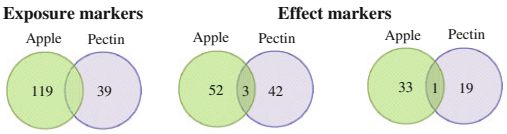


Fig. 2 *Venn diagrams* showing summarized number of selected exposure and effect markers (*left*; exposure markers, *center*; up-regulated effect markers and *right*; down-regulated effect markers) obtained from positive and negative ionization mode

the variation in the data. Urine samples from the different groups were separated by PCA with complete separation between the apple group and control/pectin groups (Fig. 1).

The total number of exposure and effect markers is shown in Fig. 2 and their *m/z* values are listed in supplemental material, Table 1S and 2S. As seen from Fig. 2 we observed 119 potential apple and 39 potential pectin exposure markers combined from positive and negative mode, with no overlapping markers between the groups. For the metabolites selected as potential effect markers 52 are found up-regulated in the apple group compared to the control group and 33 are found to be down-regulated. Likewise for the pectin group 42 markers are found as up-regulated pectin effect markers and 19 as down-regulated effect markers. When searching for overlapping apple and pectin effect markers we found 3 and 1 for the up- and down-regulated markers, respectively. Altogether, this is a high number of unambiguous exposure or effect markers and it is not expected to be possible to obtain a similar result in a human intervention study due to the less controllable eating habits and less isogenetic nature of human subjects as compared to animal models. Therefore, the collection of these markers may be used to unravel the presumed more blurred response behavior of markers in human studies investigating apple or even fruit-related interventions. If it is possible to identify some of the markers found in this study, these could be combined selectively by multivariate modeling to search for interesting response patterns.

The location of the identified exposure and effect markers in the multivariate space is illustrated as different colors on the loading plot in Fig. 1. Inspection of the PCA loading plot reveals that it is not only the features selected as exposure and effect markers, which are responsible for the grouping in the score plot. Features that were not selected by our method may show an even stronger effect on the multivariate discrimination between the sample groups. This is because PCA reflects the overall variation in the data across the 24 rats and thus is not sensitive to

inhomogeneous responses between the animals. The PCA shows no clear separation between the potential exposure and effect markers in the loading plot, but it can be observed that e.g., up-regulated apple effect markers (Apple effect markers) and down-regulated apple effect markers (Ctrl-apple effect markers) are separated from each other in the loadings plot (Fig. 1, colored marker groups). The single feature that contributes most strongly to distinguish the apple samples from the other samples is highlighted as #1 in the loading plot of Fig. 1, and its analytical pattern across the 24 samples is shown in the upper right insert. Despite its clear response behavior this feature was not selected as a potential exposure marker by our present criteria due to several non-zero values in the control group. Several other features with similar characteristics appear in the dataset, but will not be the target for this present investigation.

3.2 Identification and interpretation of markers

In the following the markers identified to date (listed in Table 1) will be discussed with regard to their origin and metabolism. Only the markers verified with an authentic standard will be discussed in details whereas the tentatively identified markers (markers in italic font in Table 1) will only be discussed to a very limited extent.

Quinic acid and *m*-coumaric acid were found as exposure markers for apple intake, in good consistency with the presence of these compounds or their precursors in fresh apple. The excreted quinic acid may originate directly from quinic acid in the apple, where it is a key intermediate in the biosynthesis of aromatic compounds (Humle 1956). It may also in part originate from gut microbial degradation of chlorogenic acid (Gonthier et al. 2003). This compound has been quantified by HPLC-analysis of fresh apple material from the same batch of Shampion apples used in the present study at a level of approximately 70 mg/kg (data to be published elsewhere). *m*-Coumaric acid derives from gut microbial dehydroxylation of caffeic acid, another chlorogenic acid metabolite. In previous targeted investigations (Mennen et al. 2006) a correlation between apple consumption and urinary excretion of *m*-coumaric acid was reported. A rat metabolism study by Gonthier et al. (2003) indicated that the quinic acid moiety in chlorogenic acid is the major precursor of hippuric acid. Hippuric acid is formed by aromatization of quinic acid into benzoic acid by the gut microbiota and subsequent conjugation with glycine in the liver and kidney. Figure 3 illustrates these described formations of quinic acid, *m*-coumaric acid and hippuric acid from chlorogenic acid. Hippuric acid is formed by many other microbial metabolic routes and is also present as an identified effect marker in our data with higher response in the apple group compared to the control group. The tentative identification of 3-hydroxyhippuric acid as an apple effect marker could indicate that this is another metabolite derived from chlorogenic and caffeic acids, although it may also have an origin from microbial degradation of e.g., dietary catechin and epicatechin (de Pascual-Teresa et al. 2010). The presence of hippuric acid and 3-hydroxyhippuric acid as effect markers may also indicate a higher efficacy of specific metabolic pathways of the gut microbiota and of the host glycine conjugation system. An effect on the composition of the gut microbiota in the present study is therefore indicated and in accordance with previously published findings (Licht et al. 2010).

We also found an apple exposure marker at the retention time of (-)epicatechin (=1.78 min) with a m/z value of 139.0397 corresponding to the retro-Diels-Alder fragmentation pattern that usually occurs when performing MS analysis of flavan-3-ols (Shaw and Griffiths 1980). The detected marker derives from the A-ring of epicatechin and this parent molecule was not itself detected as a feature but its mass peak was visible when inspecting mass spectra from the apple group. Epicatechin and its isomer catechin are well-known components in apple and unlike most other flavonoids, catechins are not in a glycosylated form in the source material (Escarpa and Gonzalez 1998). Only a minor part of ingested catechins are thought to be present in the circulation and excreted as the unconjugated form since these compounds are primarily glucuronidated in the enterocytes after absorption and even often further deglucuronidated and methylated and/or re- glucuronidated or sulphated in the liver before excretion (Donovan et al. 2001). Accordingly, we found the epicatechin glucuronide and catechin glucuronide among our exposure markers although the retention times (and positions of glucoronide groups) of these markers are not verified due to lack of commercial standards. However, as expected, their elution time is prior to the elution time of their unconjugated forms (catechin = 1.68 min and epicatechin = 1.78 min) and their fragmentation pattern is comparable to what is observed in a targeted MS/MS experiments with these compounds (Schroeter et al. 2006). Methyl epicatechin was also tentatively identified with a longer retention time than epicatechin and a retro-Diels-Alder fragmentation pattern confirming its identity. The metabolite, dihydroxyphenyl-γ-valerolactone, was also tentatively identified and this compound has earlier been identified as a major human urinary metabolite after intake of (-)-epicatechin and this lactone metabolite appears to be produced by intestinal microorganisms (Li et al. 2000).

The metabolites we have identified as pectin markers are compounds that have not earlier been linked with pectin or other fibre products.

Pyrrole-2-carboxylic acid was identified as an exposure marker of pectin intake by a convincing fit of isotope and

Table 1 Summary of identified metabolites

Metabolite	Molecular formula	Theoretical monoisotopic mass	Measured mass (±)[a]	Fragments (atom loss)	*RT* time (min)	Difference in mean response between control and apple/pectin group (↑↓)[b], relative intensity
Apple exposure markers						
Quinic acid	$C_7H_{12}O_6$	192.0634	191.0555 (−)	–	0.641	251 (↑)
m-Coumaric acid	$C_9H_8O_3$	164.0473	163.0393 (−)	147.0446 (–O) 119.0497 ($–CO_2$)	2.227	209 (↑)
(-)-Epicatechin	$C_{15}H_{14}O_6$	290.0790	291.0876 (+)	139.0397[c] ($–C_8H_8O_3$)	1.779	57 (↑)
Epicatechin glucuronide	$C_{21}H_{22}O_{12}$	466.1111	465.1045 (−)	137.0238 ($–C_{14}H_{16}O_9$) 175.0243 ($–C_{15}H_{14}O_6$) 297.0613 ($–C_8H_8O_4$)	1.671	33 (↑)
Methyl epicatechin	$C_{16}H_{16}O_6$	304.0947	305.1008 (+)	139.0395 ($–C_9H_{10}O_3$)	2.031	47 (↑)
Dihydroxyphenyl-γ-valerolactone	$C_{11}H_{12}O_4$	208.0735	209.0799 (+)	123.0446 ($–C_4H_6O_2$)	1.735	313 (↑)
Catechin glucuronide	$C_{21}H_{22}O_{12}$	466.1111	465.1043 (−)	137.0239 ($–C_{14}H_{16}O_9$) 175.0242 ($–C_{15}H_{14}O_6$) 297.0610 ($–C_8H_8O_4$)	1.578	202 (↑)
Apple effect markers						
Hippuric acid	C_8H_9NO	179.0582	178.0498 (−)	134.0606[d] ($–CO_2$) 77.0395 ($–C_3H_3NO_3$)	1.893	1941 (↑)
3-Hydroxyhippuric acid	$C_9H_9NO_4$	195.0532	196.0633 (+)	136.0408 ($–C_2H_4O_2$)	1.579	22 (↑)
3-Methoxy-4-hydroxyphenyl ethyleneglycol sulfate	$C_9H_{10}O_6S$	264.0304	245.0108[e] (−)	79.9569 ($–C_9H_{11}O_4$) 163.0392 ($–H_4O_4S$)	1.761	25469 (↑)
Homovanillic acid sulfate	$C_9H_{10}O_7S$	262.0147	261.0079 (−)	79.9572 ($–C_9H_8O_4$) 181.0510 ($–O_3S$)	1.454	158 (↑)
Metanephrine	$C_{10}H_{15}NO_3$	197.1052	198.1132 (+)	165.0527 ($–CH_7N$) 137.0615 ($–C_2H_7NO$)	2.493	31 (↓)
3-Methylglutaconic acid	$C_6H_8O_4$	144.0422	143.0351 (−)	111.0091 ($–CH_4O$) 99.0446 ($–CO_2$)	1.389	52 (↓)
Pectin exposure markers						
Pyrrole-2-carboxylic acid	$C_5H_5NO_2$	111.0320	110.0244 (−)	66.0349 ($–CO_2$)	1.606	91 (↑)
2-Furoylglycine	$C_7H_7NO_4$	169.0375	168.0314 (−)	74.0242 ($–C_5H_2O_2$)	1.523	57 (↑)
Pectin effect markers						
2-Piperidinone	C_5H_9NO	99.0684	100.0758 (+)	84.0800 (–O)	1.373	24 (↑)
Hydroxyphenylacetyl glycine	$C_{10}H_{11}NO_4$	209.0688	210.0762 (+)	76.0385 ($–C_8H_6O_2$) 109.0654 ($–C_3H_3NO_3$) 137.0591 ($–C_2H_3NO_2$)	1.516	45 (↑)
3-Methoxytyrosine	$C_{10}H_{13}NO_4$	211.0845	210.0769 (−)	137.0600 ($C_2H_3NO_2$)	2.391	66 (↑)

Names in italics refer to compound identifications that are highly probable due to isotope- and fragmentation pattern, however not verified by an authentic standard

[a] Positive (+) or negative (-) ionisation mode

[b] Up- or down-regulated response of marker

[c] Retro-Diels-Alder MS-fragment which is found as the marker ion

[d] 2-Phenylacetamide, a well-known dautherion of hippuric acid and the one found as marker

[e] Water loss

fragmentation patterns and by co-elution of an authentic standard. Figure 4 shows an extracted ion chromatogram and mass spectrum with pyrrole-2-carboxylic acid. The compound has previously been found in rat and human urine after administration of the D-isomers of hydroxyproline and its biotransformation to pyrrole-2-carboxylic

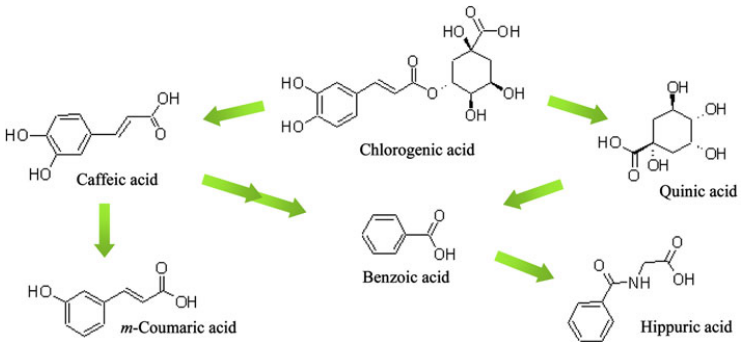


Fig. 3 Formation pathway of *m*-coumaric acid, quinic acid and hippuric acid. Structures drawn in ACD/ChemSketch ver. 12.0 (www.acdlabs.com)

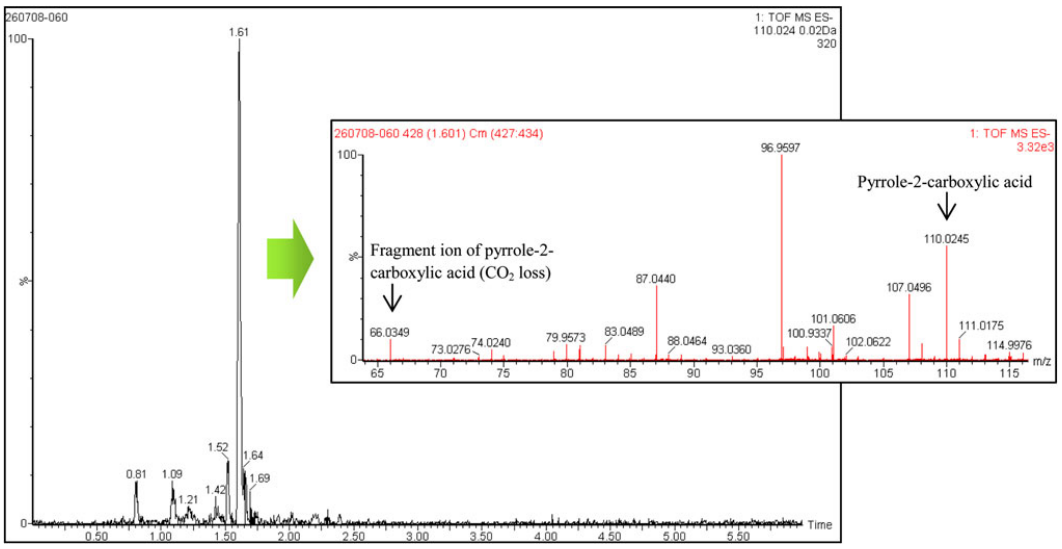


Fig. 4 Extracted ion chromatogram of pyrrole-2-carboxylic acid and mass spectrum of the peak with retention time at 1.61 min

acid is thought to be catalyzed by D-amino acid oxidase in the kidney (Heacock and Adams 1974, 1975). Apple fruit tissue contains readily soluble glycoproteins, rich in hydroxyproline (Knee 1973) which trail pectin in the industrial extraction and purification process of apple pectin (Kravtchenko et al. 1992). The rats in the pectin group will therefore have a high exposure to hydroxyproline with subsequent metabolite production of pyrrole-2-carboxcylic acid. To the best of our knowledge no previous studies with food-related interventions have reported on the existence of this metabolite in urine or any other biofluids. Since pyrrole-2-carboxcylic acid is either formed in the colon by microbes or in the kidneys, the main metabolite circulating after hydoxyproline intake may either be hydroyproline itself or pyrrole-2-carboxcylic acid.

2-Furoylglycine was also identified as a pectin exposure marker by the use of an authentic standard and we could identify a glycine fragment (74.0242 *m*/*z*) at the same retention time. 2-Furoylglycine is an acyl glycine and an earlier uncontrolled study showed presence of this metabolite in urine of 20 normal adults (Pettersen and Jellum 1972). To detect if the precursors of this compound was of exogenous dietary origin these authors also provided a male adult with a simple synthetic diet (tripalmin, triolein, sucrose and water) for 3 days. No 2-furoylglycine was detected in the urine after 2 days but the compound reappeared when an ordinary diet was reintroduced. From these findings it was suggested that furan derivatives or their precursors were introduced into food by cooking when reduced sugars are heated in the presence of free amino

groups. Purified apple pectin carries a lot of neutral sugar molecules and some proteins (Kravtchenko et al. 1992) and the same Maillard reaction may happen either during the industrial pectin extraction procedure where the apple pomace is boiled in hot acid or during the hot drying process giving rise to furan derivatives such as furfural (2-furanaldehyde). An alternative explanation is that furfural which is naturally present in apple and apple products have affinity for pectin and may be concentrated with the pectin fraction during industrial processing. Further investigations are needed to identify the exact source of the 2-furane-precursor in pectin and to ascertain whether it is specific to pectin intake. 2-Furoylglycine has been found previously to be the primary urinary metabolite in rats after oral administration of furfural and furfuryl alcohol. The latter appears to be oxidised to furfural which is further oxidised to furoic acid (Nomeir et al. 1992). Furoic acid is conjugated with glycine to form 2-furoylglycine by the enzyme acyl-CoA:glycine *N*-acyltransferase, which is located in the mitochondria of liver and kidney tissue (Knights et al. 2007). In this study we identified other acyl glycines that have been conjugated in the same way; hippuric acid, 3-hydroxyhippuric acid and hydroxyphenylacetylglycine. The last two have not been verified by authentic standards but glycine fragments were observed in the raw data at their specific retention times indicating the expected fragmentation.

2-Piperidinone was identified as an up-regulated effect marker of pectin intake in this study. To the best of our knowledge no previous studies have reported on the existence of this metabolite in urine or blood. The compound has been identified in a forensic study in the decomposition fluids from pig carcasses (Swann et al. 2010). 2-Piperidinone was also found in the anal sac secretions of different animals and it was discussed that this compound could be formed by the elimination of water from the precursor 5-aminovaleric acid by microbial fermentation processes (Albone et al. 1976; Burger et al. 2001). However, from the studies conducted to date it is not possible to decide if 2-piperidinone or its precursors are of dietary origin or is exclusively an endogenous or microbial metabolite. We have previously shown that pectin in this study caused a marked change in the caecum microbiota (Licht et al. 2010). We are currently investigating the relationships between these changes and the metabolomic patterns in fecal water and urine.

Among the effect markers that we have tentatively identified there are several catecholamine metabolites (3-methoxy-4-hydroxyphenylethyleneglycol sulphate, homovanillic acid sulphate, metanephrine, hydroxyphenylacetylglycine and methoxytyrosine) indicating changes in the hormonal metabolism or in metabolite transport after the apple and pectin diets. The identities of these markers seem likely with respect to accurate mass, elemental composition and fragment patterns but no coelution experiments with pure standards have been performed, again due to lack of commercial standards. Firm conclusions will therefore have to await the confirmation of an effect of apple intake on the excretion of these hormonal effector compounds.

3.3 Importance of markers for other studies

In the variable selection procedure we distinguish between potential exposure and effect markers based on zero values or a constant baseline level. However, without unambiguous identification of the markers it cannot be ruled out that potential exposure markers are in reality effect markers and vice versa. This investigation applied a threshold level in the data extraction step in order to eliminate too much noise in the data. Without the threshold the dataset will be too large and unmanageable. If a true effect marker has a response that is lower than the threshold level this will appear as zeros in the data set and it will mistakenly be classified by our selection procedure as an exposure marker. The identification of a number of potential exposure and effect markers gives us an opportunity to evaluate whether the pattern-based classification leads to biologically plausible results and points to markers, which might serve as true exposure and effect markers. While the apple exposure markers are likely metabolites of apple components and therefore most likely markers of apple or fruit/vegetables in general, they would also be affected by the metabolic capacity of the exposed subject and might therefore show a more variable response in other species and particularly in humans. They might therefore also have features that make them markers of effects such as metabolic capacity or genetic polymorphisms. The pectin exposure markers could potentially be markers of food contaminant exposures or they may be seen as effect markers, i.e., products of specific microorganisms, which increase with pectin consumption. For all exposure markers proper validation studies with human volunteers would therefore be needed before these markers can be used in e.g., observational studies. In analogy some effect markers may be wrongly classified if they are markers of dietary components existing at different levels in both the control and the apple or pectin groups, as observed in the case of hippuric acid. However, this marker may also be seen as an effect of diet on the metabolic capacity of the microbiota and of the endogenous glycine-conjugation systems, so classification as an effect marker is not altogether misleading. So the classification into exposure and effect markers would depend on the biological system investigated. Nevertheless, for the qualitative grading of markers, our classification was effective and has eased the systematization and interpretation of unknown compounds.

4 Conclusion

By applying an untargeted MS-based metabolomics approach it has been demonstrated that intake of apple and apple pectin has a high impact on the urinary metabolome. Numerous potential exposure and effect markers of apple and apple-pectin intake have been found and several new apple-related urinary metabolites have been identified in this study. Most of these excreted metabolites are products of diverse metabolic pathways including phase II glucuronidation, glycine-conjugation and/or microbial metabolism and a combination of several of the markers recorded in this rat study could ease identification of more robust exposure biomarkers for human studies. Additionally, the markers identified in this study should shed new light on health interpretations of fruit intake in previous as well as future conducted studies. The explorative top-down metabolomics approach employing a division into potential effect and exposure markers was effective for the rat study and seems promising as a tool in formation of new ideas, however, the classification of markers has to be validated in subsequent human studies.

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