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KAISA RANINEN

EXHALED BREATH ANALYSES IN MONITORING DIETARY EFFECTS OF RYE

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ABSTRACT

Exhaled breath is a potential non-invasive sampling matrix to give new information on the metabolic effects of diets. Foods contain numerous components which after digestion and metabolism by gut microflora are absorbed to the circulation, and can be excreted to exhaled breath. Various technologies have been developed for analysing volatile organic compounds (VOCs). However, standardized methodologies for exhaled breath VOC analysis are rare, and only a few pilot studies have been conducted to monitor the effects of diets on exhaled breath VOCs.

Wholegrain cereals, rich in dietary fibre and related bioactive compounds, are associated with decreased risk of chronic diseases, obesity, type 2 diabetes, cardiovascular disease, and certain cancers. Rye is a remarkable source of grain fibre in Finnish diet, mostly consumed as sourdough fermented wholegrain rye bread, and studied especially for its beneficial effects on glucose metabolism. However, the underlying physiological mechanisms behind the health effects are complex and unclear.

This doctoral thesis explored the potential of exhaled breath VOC analysis in studying the health effects of rye and grain fibre in dietary interventions. Three different VOC analysis technologies were applied to exhaled breath analysis and tested in three dietary intervention studies monitoring the effect of rye and grain fibre on exhaled breath VOCs. In Study I, targeted exhaled breath VOCs were monitored with gas chromatography – mass spectrometry (GC-MS), in Study II, changes in the exhaled breath VOC profiles were monitored with aspiration ion mobility spectrometry (AIMS), and in Study III, a non-targeted analysis was

performed with comprehensive two-dimensional GC-MS (GCxGC-MS) to explore exhaled breath compounds relating to wholegrain diets.

The tested methodologies showed potential for different research targets. The targeted GC-MS analysis was found to be suitable to detect changes in specific exhaled breath VOCs, and it could be developed further to analyse gut fermentation related exhaled breath VOCs in standardized protocols. The AIMS technology detected changes in exhaled breath VOC profiles, and it could be developed further for identifying VOC profiles for specific metabolic states relating to diets. The GCxGC-MS has improved separation for compounds and was found to be sensitive enabling exploring of new compounds from the exhaled breath.

Specific exhaled breath biomarker compounds for rye were not detected in our study. However, there were some coherent changes in the exhaled breath VOC profiles and in the fermentation related VOCs, which would be interesting to study further. Based on our findings, it seems that the exhaled breath VOCs have potential to give new information on the metabolic effects of foods and their associations with health effects.

Medical Subject Headings: Breath Tests; Diet; Dietary Fiber; Digestion; Exhalation; Gas Chromatography-Mass Spectrometry; Gastrointestinal Microbiome; Ion Mobility Spectrometry; Secale; Whole Grains; Volatile Organic Compounds.

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TIIVISTELMÄ

Hengitysilman yhdisteet voivat antaa uutta tietoa ruokavalioiden vaikutuksista. Ruoka sisältää lukuisia molekyylejä, jotka ruoansulatuksen ja suolistomikrobiston käsittelyn jälkeen imeytyvät verenkiertoon ja voivat erittyä hengitysilmaan. Haihtuvien orgaanisten yhdisteiden eli VOC-yhdisteiden analysoimiseksi on kehitetty erilaisia analyysimenetelmiä. Standardoidut hengitysilman mittausmenetelmät ovat kuitenkin harvinaisia, ja ruokavalion vaikutusta hengitysilman VOC-yhdisteisiin on tarkasteltu toistaiseksi vain muutamissa pilottitutkimuksissa.

Täysjyväviljat sisältävät ravintokuitua ja kuitukompleksiin kiinnittyneitä bioaktiivisia yhdisteitä, joiden runsaan saannin on arveltu vähentävän kroonisten sairauksien, kuten tyypin 2 diabeteksen, sydän- ja verisuonitautien ja tiettyjen syöpien riskiä. Ruisleipä on merkittävä viljakuidun lähde suomalaisessa ruokavaliossa, jolla on havaittu suotuisia vaikutuksia erityisesti glukoosiaineenvaihduntaan. Rukiin ja viljakuidun terveysvaikutusten mekanismeja ei kuitenkaan vielä tunneta kaikilta osin.

Tässä väitöskirjatutkimuksessa tarkasteltiin mahdollisuuksia hyödyntää hengitysilman VOC-yhdisteitä rukiin ruokavaliovaikutusten tutkimiseksi. Kolmea erilaista VOC-yhdisteiden mittaustekniikkaa sovellettiin hengitysilmanäytteille ja testattiin kolmessa ruokavaliotutkimuksessa. Ensimmäisessä tutkimuksessa tarkasteltiin muutoksia valikoiduissa hengitysilman yhdisteissä kohdennetulla kaasukromatografia – massaspektrometri (GC-MS) -analyysillä, toisessa tutkimuksessa tarkasteltiin muutoksia uloshengitysilman VOC-profiileissa aspiraatioioniliikkuvuusspektrometrialla (AIMS), ja kolmannessa tutkimuksessa

pyrittiin tunnistamaan täysjyväviljaruokavalioihin liittyviä yhdisteitä hengitysilmasta laajalla kaksivaiheisella GC-MS:llä (GCxGC-MS).

Kehitettyjen menetelmien havaittiin soveltuvan erilaisiin tutkimustarkoituksiin. Kohdennettu GC-MS-analyysi soveltuu valikoitujen VOC-yhdisteiden muutosten tarkasteluun standardoidussa tutkimusasetelmassa. Menetelmää ja tutkimusasetelmaa voitaisiin kehittää edelleen analysoimaan erityisesti suoliston fermentaatioon liittyviä hengitysilman yhdisteitä. AIMS-teknologialla havaittiin muutoksia hengitysilman VOC-profiileissa, ja sitä voitaisiin kehittää edelleen erilaisten ruokavalioihin liityvien aineenvaihdunnallisten tapahtumien tai tilojen tunnistamiseen. GCxGC-MS-menetelmä puolestaan soveltuu uusien yhdisteiden etsimiseen hengitysilmasta herkkyytensä ja erottelukykynsä vuoksi.

Spesifisiä hengitysilman biomarkkeriyhdisteitä rukiille tai viljakuidulle ei tutkimuksessamme löytynyt. Havaitsimme kuitenkin ruokavalioon liittyviä yhtenäisiä muutoksia sekä hengitysilman VOC-profiileissa että joissakin suolistofermentaatioon liittyvissä yhdisteissä, jotka olisivat mielenkiintoinen jatkotutkimuskohde. Tutkimustemme perusteella näyttää siltä, että hengitysilman VOC-yhdisteet voivat tarjota uutta tietoa elintarvikkeiden terveysvaikutusten takana olevista aineenvaihdunnan tapahtumista.

Yleinen suomalainen ontologia: aineenvaihdunta; haihtuvat orgaaniset yhdisteet; hengitys; kaasukromatografia; massaspektrometria; ravintokuitu; ruis; ruoansulatus; ruokavaliot; suolistomikrobisto; terveysvaikutukset.

“Välillä menee hermot,
mutta ei saa luovuttaa.”

-Tanhupallo-

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I Raninen KJ, Lappi JE, Mukkala ML, Tuomainen T-P, Mykkänen HM, Poutanen KS and Raatikainen OJ. Fiber content of diet affects exhaled breath volatiles in fasting and postprandial state in a pilot crossover study. Nutrition Research 36: 612-619, 2016.

II Raninen K, Lappi J, Kolehmainen M, Kolehmainen M, Mykkänen H, Poutanen K and Raatikainen O. Diet-derived changes of sourdough fermented rye bread in exhaled breath aspiration ion mobility spectrometry profiles. International Journal of Food Sciences and Nutrition 68(8):987-996, 2017.

III Raninen K, Nenonen R, Järvelä-Reijonen E, Poutanen K, Mykkänen H and Raatikainen O. Comprehensive two-dimensional gas chromatography–mass spectrometry analysis of exhaled breath compounds after whole grain diets. Molecules 26(9):2667, 2021.

CONTENTS

ABBREVIATIONS

AIMS	Aspiration ion mobility spectrometry
API	Atmospheric pressure ionization
AR	Alkylresorcinols
AX	Arabinoxylans
BCFA	Branched-chain fatty acid
BMI	Body mass index
CAR	Carboxen
C-IBS	Constipation predominant irritable bowel syndrome
DF	Dietary fibre
DMS	Differential mobility spectrometry
DTIMS	Drift-time ion mobility spectrometry
EBC	Exhaled breath condensate
ECD	Electron capture detector
EE	Energy expenditure
E-NOSE	Electronic nose
ESI	Electrospray ionization
FAIMS	Field asymmetric waveform ion mobility spectrometry
FFA	Free fatty acid
FID	Flame ionization detector
FPD	Flame photometric detector
GC	Gas chromatography
GCxGC	Comphehensive two-dimensional gas chromatography
GLP-1	Glucagon-like peptide-1
HDL	High-density lipoprotein
IC	Indirect calorimetry
IMR	Ion molecule reaction
IMS	Ion mobility spectrometry
IRMS	Isotope ratio mass spectrometry
IS	Internal standard
LDL	Low-density lipoprotein
MCC	Multicapillary column
MDGC	Multidimensional gas chromatography

MESI	Membrane extraction with a sorbent interface
MIMS	Membrane inlet mass spectrometry
MRP	Maillard reaction product
MS	Mass spectrometry
NDIRS	Non-dispersive isotope selective infrared spectrometry
NO	Nitrogen oxide
NSP	Non-starch polysaccharides
NTD	Needle trap devices
PDMS	Polydimethylsiloxane
PID	Photo ionization detector
PP	Postprandial
PPM	Parts per million
PPT	Parts per trillion
PTFE	Polytetrafluoroethylene
PTR	Proton transfer reaction
RQ	Respiratory quotient
RS	Resistant starch
RT	Retention time
SCFA	Short chain fatty acid
SESI	Secondary electrospray ionization
SIBO	Small intestinal bacterial overgrowth
SIFT	Selected ion flow tube
SIM	Selected ion monitoring
SPME	Solid phase micro extraction
TD	Thermal desorption
TOF	Time of flight
TWIMS	Traveling-wave ion mobility spectrometry
VOC	Volatile organic compound
WE	Water-extractable
WG	Wholegrain
WGR	High-fibre diet with wholegrain rye bread
WGW	High-fibre diet with wholegrain wheat bread
WU	water-unextractable
WW	Low-fibre diet with white wheat bread
WW+BRB	High-fibre diet with white wheat bread enriched with bioprocessed rye bran

1 INTRODUCTION

Exhaled breath is a potential non-invasive matrix to monitor metabolic changes induced by dietary modifications. Foods contain numerous molecules which after digestion and metabolism by gut microflora are absorbed to the circulation, and if having a suitable vapor pressure and solubility can be excreted to exhaled breath. Various technologies have been developed for analysing volatile organic compounds (VOCs) from exhaled breath. However, standardized methodologies for exhaled breath VOC analysis are rare, and only a few pilot studies have been conducted to monitor the effects of diets on exhaled breath VOCs.

Wholegrain (WG) cereals, rich in dietary fibre (DF) and related bioactive compounds, are a good source of high-quality carbohydrates, being consistently associated with decreased risk of chronic diseases, obesity, type 2 diabetes, cardiovascular disease, and certain cancers. Rye is a remarkable source of grain fibre in the Finnish diet, an average Finn eating rye 15 kg per year [1], mostly in form of sourdough fermented WG rye bread. Although high intake of rye has been associated with beneficial health effects, the underlying physiological mechanisms are complex and unclear [2]. People have varying metabolic responses to diets because of individual physiology and gut microflora. Therefore, there has been a growing interest in metabolomics approaches offering a window on metabolic mechanisms behind the various dietary responses. Exhaled breath VOCs are potential biomarkers to complement the knowledge of the metabolic effects of rye and grain fibre and their association with health effects.

This doctoral thesis investigated analysis methodologies for studying exhaled breath VOCs relating to diet. Three different VOC analysis technologies were applied to exhaled breath analysis. The methodologies were tested in three dietary intervention studies monitoring effects of rye and grain fibre on exhaled breath VOCs.

2 REVIEW OF THE LITERATURE

2.1 EXHALED BREATH ANALYSIS

2.1.1 Composition of exhaled breath

Breath is a mixture of excreted gases from circulation and inhaled gases from the environment [3]. The exhaled breath air is mostly consisted of nitrogen (about 74%), oxygen (about 15 %), carbon dioxide (about 5 %), water (about 6 %) and inert gases (about 1 %) [4-6]. Furthermore, there is a small fraction (typically less than 0.005 %) of various volatile metabolites occurring in concentrations in ppm (parts per million) to ppt (parts per trillion) i.e., µmol/l-pmol/l range [6], including inorganic molecules e.g. nitrogen oxide, ammonia and carbon monoxide, and VOCs e.g. small hydrocarbons, alcohols, aldehydes, acids, esters, ketones, furan and ether compounds, nitrogen and sulphur containing compounds, and benzyl and phenyl hydrocarbons [7]. There are about 3500 VOCs detected in exhaled breath [8-15], and of those about 900 compounds are considered to be related to healthy human body [7,16]. Inter-individual variation in the composition of breath is wide [14,17]. The typical number of compounds in a single breath sample is about 200 and it is assumed that only a dozen of compounds is present in every breath sample [14].

Monitoring exhaled breath VOCs is usually based on the assumption that concentrations of volatile metabolites in exhaled breath correlate with their concentrations in blood [18]. VOCs are carried from pulmonary circulation to alveoli in accordance with the gas partial pressures [19]. To be excreted to breath, a compound must be volatile, i.e., having low boiling point and high vapour pressure [20], and have a suitable solubility to go through the blood-air barrier in lung alveoli [21]. Many VOCs tend to condensate from gas phase into liquid phase when they are exhaled to lower temperature. The exhaled breath condensate (EBC) is also studied as a potential diagnostic matrix [22].

Exhaled breath VOCs can have exogenous or endogenous origin [3,23]. They can be derived from the environmental exposure via inhaling, food and beverages, medicines, and cosmetics, or be produced in metabolism by somatic cells or by body microbiota including microbes throughout the oral and nasal cavities and in the gastrointestinal tract [24]. Because VOCs are molecules with simple structure, they can be by-products of many different metabolic processes and usually have

multiple sources. Distribution and levels of some endogenous breath VOCs have been studied including acetone [25-28], acetaldehyde [29-31], ammonia [32-34], methanol [30,35,36] ethanol [29,37,38], propanol [39,40], isoprene [27,41-44], hydrogen cyanide [45,46], alkanes [47,48] and aldehydes [49].

2.1.2 **Methodology in exhaled breath analysis**

There is not an analytical method capable to measure all the breath volatiles, i.e., the analysis method always defines which compounds can be analysed. The choice of the analysis method depends primarily on the aim, whether it is identification of compounds, quantification of the specified VOC or detecting changes in VOC profiles. There are multiple analysis methodologies applied to monitor exhaled breath VOCs, but not many standardized methodologies for their sampling or analysis [50-52]. Breath analyses are classified as direct reading (on-line/real time methods), where sampling and analysis are done with a united device, and indirect reading, where samples are stored and analysed usually later in the laboratory [53]. Various methodologies used in exhaled breath VOC analysis are presented in Figure 2.1.

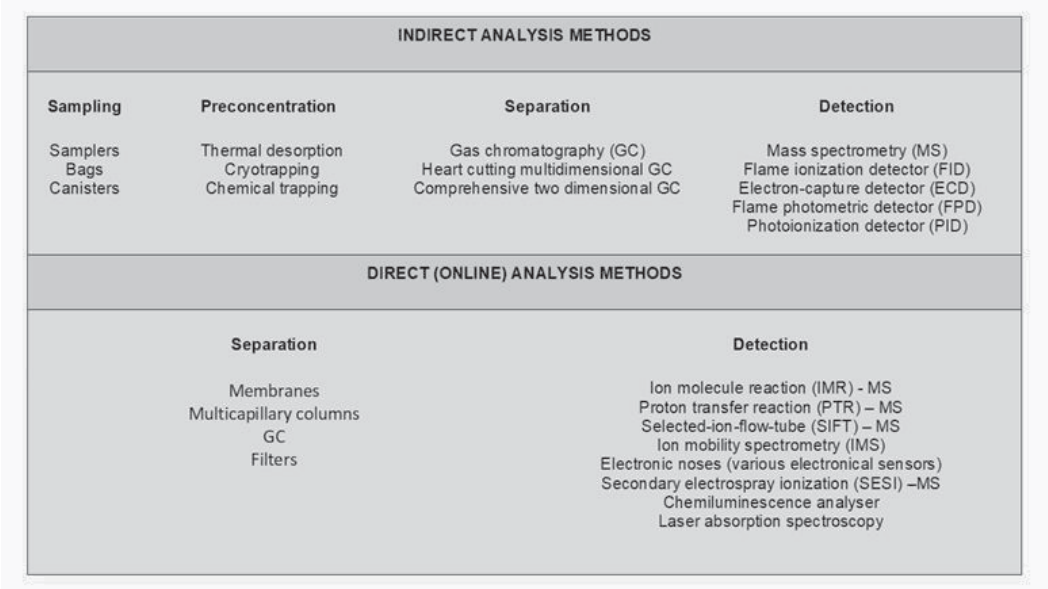

Figure 2.1. Available methodologies in analysing exhaled breath volatile organic compounds.

Sampling

Sampling is challenging in breath analysis. Exhaled breath sample must be taken with a standardized sampling protocol concidering also the physiological state of subject (e.g, digestion state, tension state) and the quality of surrounding background air (e.g. humidity, temperature, air quality). Blowing technique, heart rate and pulmonary ventilation have effect on the excretion of exhaled breath compounds [54-57].

There are various methods for breath sampling. Breath samples can be collected from upper airways, from alveoli or from total breath [58]. Upper airway collection is used for sampling nitrogen oxide (NO) released in the airways, whereas alveolar air contains molecules from circulation and therefore can indicate metabolic status or exposure. The volume of expired breath with normal breathing, the tidal volume, is about 500 ml [19], which is consisted of 150 ml death space air from the upper airways and 350 ml alveolar air.

Exhaled breath contains compounds of endogenous and exogenous origin [23]. Therefore, Phillips et al [14] determined the concept of alveolar gradient to describe which compounds are endogenous. The alveolar gradient is abundance of compound in breath minus abundance of compound in background air. Positive alveolar gradient signifies endogenous compounds, which indicates that rate of synthesis in body is greater than clearance, and negative alveolar gradient signifies the opposite. However, subtracting the background compounds from exhaled breath compounds is questionable because most of the breath VOCs originate both from endogenous and environmental sources and exposure can happen during a long period.

There are two main approaches used to minimize the effect of compounds inhaled from the background air: 1) breathing pure air before sampling to washout the airways and 2) subtracting measured background air computationally from exhaled air [58]. The first needs devices to prepare pure air and adequate sampling system to keep the room air totally away from sample until it's analysed, which is challenging to put into practise. The latter requires sampling of background air simultaneously with exhaled breath. In both cases, it should be noticed that VOCs have different clearance rates and therefore it is almost impossible to washout or substract all the previously exposed VOCs from exhaled breath. Also, the sampling conditions must be considered when comparing the levels in the background air and exhaled breath since temperature and humidity can affect the sampling and preconcentration steps of compounds [59,60]. The temperature of breath air is about 33 ˚C and the relative humidity variates between 42-91 % [61]. Because the ambient

air is usually colder and drier, water of breath air tends to condensate and dilutes some hydrophilic compounds to EBC, which can remain onto surfaces of sampling devices and decrease the signal for these compounds. High humidity can also attenuate absorption of VOCs to the sorption materials [62].

Indirect breath sampling methods exploit different samplers, glass tubes, bags, canisters, adsorbent tubes, and cryogenic traps [53]. The material of sampling devices, valves, fittings, and tubes must be inert not releasing VOCs that can contaminate the samples [58]. Stainless steel, brass, glass, and Teflon are widely used materials, whereas rubber and some plastics are not recommendable.

Preconcentration

Because the most exhaled VOCs are excreted in low concentrations, preconcentration techniques are needed to improve the sensitivity of analysis [63]. There are three different kinds of approaches for preconcentration: cryogenic trapping where the VOCs are frozen and released rapidly, chemical trapping where the sample is transported through a reagent solution which catch the specific compounds and adsorptive trapping where the molecules are attached and released on the surface of different kind of adsorbents by changing temperature[64].

Adsorptive trapping includes thermal desorption (TD) tubes and solid phase micro extraction (SPME) needles [65], which are widely used, and newer needle trap devices (NTD) [66]. Adsorptive trapping is used to extract and concentrate VOCs from a complex matrix prior to GC analysis. In TD, stainless steel or glass tubes are packed with one or multiple beds of solid adsorbents. Sample air is passed through the tube, where the adsorbents trap the compounds until they are released in the analyses. Organic polymers, activated charcoals, graphitized carbons and carbon molecular sieves have been used as adsorbent material [5]. SPME device is a simple fibre which is coated with an extracting phase (polymer or sorbent) [67]. According to phase used, SPME extracts different kinds of molecules (volatile and non-volatile) from liquid and gas phase. After extraction, the compounds are desorpted from the fibre by increasing the temperature, typically by injecting the fibre to the hot GC injector. NTD is based on the same idea than SPME, but adsorptive material is packed inside the needle and gas sample is actively drawn into the absorbent with a gas-tight syringe or pump [68]. It should be considered in adsorptive trapping techniques that the sorption kinetics is dependent on sampling conditions, e.g., temperature, humidity and competitive sorption with other compounds [59,62]. Therefore, the changes in sampling conditions can affect to the adsorption and challenge the quantitative analysis.

Different membranes are used in the direct sampling devices to select compounds to be analysed [69]. Membrane extraction with a sorbent interface (MESI) simulates the membrane of the alveoli, which passes the compounds from circulation to the expired air, having a silicone membrane [70]. MESI has a non-polar membrane being suitable for the analysis of non-polar compounds. The high humidity of breath is not interfering with the analysis, because water does not go through the non-polar membrane. MIMS (membrane inlet mass spectrometry) is a membrane connected to mass spectrometry [71]. Multicapillary columns (MCC) are used in direct sampling devices to separate the compounds and delaying the humidity [72].

Technologies in breath analysis

Traditionally breath analysis has utilized GC [73], which is a technology for separating volatile compounds. In GC, a sample is introduced into a column covered with a liquid or solid stationary phase and transported in a gas flow through the column [74]. VOCs interact with a stationary phase, proceed along the column at different rate and are released in specific temperature based on their chemical properties. Therefore, the choice of GC column determinates which compounds can be analysed [75]. Multidimensional GC-devices (MDGC) have more than one GC column and thus can separate wider range of different types of molecules [76,77]. There are two types of MDGCs: heart cutting MDGC and comprehensive GCxGC. In heart-cutting system, there is a valve modulator between GC columns and selected discrete portions from the first chromatograph separation are directed to the second column for further separation [78]. In comprehensive GCxGC, all the compounds eluating from the first column are stopped and released with a cryogenic modulator at a few second intervals to the second column resulting in a two-dimensional chromatogram [79]. MDGCs have been used especially for the non-targeted profiling, also for monitoring breath VOCs [8,80-85].

Different detectors are used with GC, such as flame ionization detector (FID), flame photometric detector (FPD), photo ionization detector (PID), and electron-capture detector (ECD), but only mass spectrometry (MS) can identify detected compounds [86]. Therefore GC-MS has become a standard technology in identifying VOCs in any sample matrixes. The identification is usually based on mass spectral libraries but must be assured with pure standard compounds, or at least with retention indices, because mass spectra can be very similar with multiple compounds [87]. There are different types of MS detectors [88], in which the quadrupole MS is perhaps the most used, but in recent years TOF (time of flight) -

MS has become more popular especially in identifying VOCs in breathomics studies [89]. GC-MS has been widely used in breath analysis being sensitive and selective, and superior in identifying compounds, but not very convenient for clinical studies requiring expensive and robust laboratory devices and specialized lab personnel for their use. GC-MS analysis is mainly indirect, and therefore samples must be stored and transported to the laboratory for the analysis, which is challenging with the gaseous samples. Therefore, this technology is mainly for research purposes, for example in exploring biomarker VOCs to be studied further.

Various online monitoring technologies have been developed for breath analysis [90]. These are convenient in rapid analysis detecting changes in targeted VOCs. The most used online techniques are based on mass spectrometric methods with chemical ionization and ion-molecule reactions (IMR) or ion mobility spectrometry (IMS).

SIFT-MS (selected ion flow tube – mass spectrometry) is a technique developed by Smith and Španěl [91] for rapid real time breath analysis. In SIFT-MS device, a microwave plasma ion source creates positive ions (H_3O+, NO+, or O_2+). These precursor ions are transported through a quadrupole mass filter via a fast-flowing inert gas to a flow tube, where they react with sample VOCs. The IMR result in characteristic product ions, which are detected and counted by a quadrupole mass spectrometer.

PTR-MS (proton transfer reaction – mass spectrometry), developed by Lindinger et al [92], consists of an ion source that is directly connected to a drift tube and a quadrupole or TOF type MS detector. In contrast to SIFT-MS no mass filter is interconnected. In PTR-MS, water vapour is ionized in the ion source creating H_3O^+-primary ions in a short drift tube. A vacuum pump is continuously drawing air in the reaction drift tube transporting primary ions and sample VOCs, which are interacting and performing proton transfer reaction. Finally, the protonated molecules are detected at the end of the drift tube. PTR-MS can detect only the molecules which have higher proton affinity than water (> E = 166.5 kcal/mol or 7.16 eV) and the total concentration of VOCs must not exceed 10 ppm. PTR-MS detects molecular masses of protonated compounds but cannot separate compounds having a same mass, such as 1-propanol and 2-propanol [39].

Ion mobility spectrometry (IMS) is based on mobility of ionized compounds in electric field in gas matrix [93]. Molecular mass, structure and electric charge of the molecule have effect on ion mobility. There are four types of IMS technology: drift-time ion mobility spectrometry (DTIMS), aspiration ion mobility spectrometry

(AIMS), differential-mobility spectrometry (DMS, also called field-asymmetric waveform ion mobility spectrometry, FAIMS) and traveling-wave ion mobility spectrometry (TWIMS). IMS devices have been combined with mass spectrometry to identify compounds, but due to the occurrence of ion-molecule reactions and relatively poor resolution it is rather used to monitor changes in gaseous samples or processes [94]. IMS technologies have been utilized in electronic noses (e-nose) [95] being sensitive, giving rapid responses, and not requiring large instruments. However, IMS is sensitive to humidity, which must be taken into account especially when sampling exhaled breath. MCCs has been successfully used in combination with IMS to improve resolution of compounds and removing the water in humid samples [96].

Different types of e-noses have been tested for breath analysis [97]. The idea of an e-nose is to simulate the human olfactory system and detect odours (VOC profiles) instead of individual VOCs. This is achieved by using a bunch of selective electronic sensors, such as optical sensors, piezoelectric sensors, metal oxide semiconductors and conducting film polymers. E-noses are typically low cost, miniaturized, ease-to-use equipment. However, they require training for recognizing the odours, for example for cancer prediction training with a group of patients and a healthy control group.

Secondary electrospray ionization – mass spectrometry (SESI-MS) combines atmospheric pressure ionization – mass spectrometry (API-MS) and electrospray ionization (ESI) technique [98,99]. SESI-MS can detect compounds with wide mass range (5-40 000 m/z) being also very sensitive (low ppt-levels) and having a good resolution. However, the ionization process is sensitive to water. This technology has been tested for determining the etiology of lung infections [100,101] and composition of amino acids [102] and fatty acids [103] in breath.

There are also optical analysis methods for analysing breath VOCs. Colorimetric sensor arrays, developed especially for the diagnosis of lung cancer are based on chemical colour-changing reaction between sample compounds and chemically sensitive compounds on disposable cartridge [104,105]. These are utilized in optoelectronic noses [106,107]. Chemiluminescence analyser, utilized in measuring exhaled NO, is based on the chemical reaction between NO and oxygen, where light is emitted [108,109]. Also, various technologies based on laser absorption spectroscopy have been utilized in developing diagnostic monitoring technologies based on breath VOCs [110].

2.2 BREATH ANALYSIS IN NUTRITIONAL STUDIES

2.2.1 Breath tests in nutrition research

There are essentially two types of breath tests utilized in nutritional and gastroenterological studies: hydrogen and 13C-isotope breath tests [111]. Moreover, indirect calorimetries utilize breath air to determine the energy expenditure [112]. Recently, there have been interest to monitor also breath methane to understand the role of methanogens to gastrointestinal functions. Development of breath analysis methods has increased the interest to utilize also exhaled breath VOCs in monitoring gastrointestinal disorders [113-115] and blood glucose levels relating to diabetes [116]. However, there are so far only few studies which have monitored the effects of diets on exhaled breath VOCs [117-119].

Hydrogen as biomarkers of bacterial fermentation

Hydrogen is released in the fermentation of dietary carbohydrates by intestinal bacteria [24]. A typical protocol in hydrogen breath test is to inject a carbohydrate substrate or a meal and to measure breath hydrogen level in standardized sampling points. When the substrate has reached the colon, it is degraded and fermented by bacteria and can be monitored in breath hydrogen.

Hydrogen breath tests are used to monitor fermentation of carbohydrates commonly for diagnosing carbohydrate maldigestions (intolerances) or bacterial overgrowth, or for determining orocoecal transit time i.e., the time that food item transit from ingestion to the ileocecal valve for being fermented by the cecal microflora [120]. Breath hydrogen levels can be utilized for assessing fermentation rate of foods for example in monitoring individual postprandial gastrointestinal function and symptoms [121]. Hydrogen breath tests are also used for detecting small intestinal bacterial overgrowth (SIBO). Sugars are normally absorbed in the small intestine and are not fermented because bacteria usually colonize only the colon. However, in the SIBO the number of bacteria is increased also in the small intestine causing fermentation of sugars and increased production of hydrogen [122].

Hydrogen breath tests are probably the most common breath tests used, although they have been criticized for their limited clinical value. The interpretation of the results is challenging because of inter- and intra-individual variation in digestion, gut fermentation, and their relations to clinical symptoms[123].

For example, people having methanogens in gut microflora can have false negative result in the hydrogen breath test [124]. Therefore, some studies have grouped people as hydrogen producers and non-producers based on hydrogen breath test when studying effects of DF [125,126].

Methane is associated to intestinal motility

Methane is produced by methanogens, which are one of the three branches of the phylogenetic evolutionary descent in the Kingdom Archaea, having properties of both prokaryotes and eukaryotes [122]. Three phylotypes have been detected in humans: *Methaninobrevibacter smithii* and *Methanospaera stadmagnae* in the intestine [127] and *Methannobrevibacter oralis* in the oral cavity [128]. In methanogenesis, carbon dioxide is reduced to methane using hydrogen or other electron donor produced in anaerobic bacterial fermentation [129]. Methane producing status varies between individuals. Studies indicate that 35 – 65 % of healthy adults are methane producers [130,131]. Methane producing status has been associated to intestinal motility as delayed transit time [132,133], and to constipation [134], diverticulosis [135,136], and constipation-predominant irritable bowel syndrome (C-IBS) [137].

Isotope breath tests in studying metabolic pathways

Isotope breath tests are based on the use of isotope forms of carbon or other atoms, not common in nature [111]. They can be used to label a specifically designed substrate which is a part of the metabolic pathway of interest. The released isotope is monitored in breath in standardized sampling points by high resolution isotope ratio mass spectrometers (IRMS) or non-dispersive isotope selective infrared spectrometers (NDIRS), which are capable to separate isotopes. ^{13}C (carbon-13) is a natural, stable, and the most used isotope in breath tests but also the radioactive ^{14}C has been used in some protocols. ^{13}C-breath tests are utilized in studying transport, digestion, absorption, oxidation processes, enzymatic activities or orocecal transit time. However, the most common isotope breath test is propably the urease breath test for diagnosing *Helicobacter pylori* infection [138]. The idea of this test is to inject isotope-labelled urea and to monitor from breath if ^{13}C is released or not, since *Helicobacter pylori* have urease enzyme which can degrade urea and release ^{13}C.

Indirect calorimetry measures energy expenditure

Calorimetries are used to assess individual energy needs. Direct calorimetries measure the produced heat, whereas indirect calorimetries (ICs) calculate the energy expenditure (EE) utilizing the consumed oxygen and produced carbon dioxide [139]. IC is a gold standard method to assess the metabolic rate with high reproducibility and accuracy. The respiratory gases can be collected in several ways and analysed with electronic gas analyser. The EE can be calculated with Weir equation and respiratory quotient, RQ, as the ratio between volumes of carbon dioxide and oxygen. The RQ reflects the energy substrate used. In recent years, handheld IC devices have also been introduced [112].

2.2.2 **Biomarker compounds related to metabolism**

Breath odour has been utilized for diagnosis purposes for a long time, for example sweet fruity odour (acetone) of breath is related to uncontrolled diabetes and musty, fishy reek (nitrogen-containing compounds) to advanced liver disease [16]. Along the development of analytical technology, there have been attemps to find specific biomarker compounds for diseases. Although some exhaled breath VOCs are related to metabolic processes or specific physiological status (Table 2.1), none of them are recognized as an unambiguous biomarker compound for diagnostic purposes. Therefore, research has focused nowaways to monitor changes in VOC profiles instead of exploring individual biomarker compounds [140,141]. The breath-based metabolomics aimed at identifying VOC patterns for diagnosis of medical and physiological conditions is called breathomics [89,142].

Table 2.1. Endogenous exhaled breath compounds related to metabolism or physiological states.

Compound	Metabolic origin	Physiological state	References
Acetaldehyde	oxidation of ethanol	fatty liver	31,143
Acetone	lipolysis or lipid peroxidation: decarboxylation of acetoacetate	fasting, ketosis, ketoacidosis (diabetes), fat loss	144-147
Ammonia	breakdown of proteins (deamination)	liver function, renal function	33,148,149
Ethane, pentane, other alkanes	lipid peroxidation	oxidative stress, inflammation, lung cancer	113,150–152
Ethanol	bacterial fermentation	fatty liver, metabolic syndrome, acute postprandial response	6,153-156
Isoprene	mevalonic pathway of cholesterol synthesis	hypoglycemia, liver fibrosis, hypoxia	43,157-160
Methanol	digestion, colonic microflora	consumption of ethanol/aspartame/fruits	35,161,162
Nitric oxide (NO)	oxidation of L-arginine by nitric oxide synthase	inflammation	163,164
2-propanol	converted from acetone by hepatic alcohol dehydrogenase	fasting, ketosis, ketoacidosis (diabetes), fat loss	165-167
Short chain fatty acids (SCFAs)	bacterial fermentation	acute postprandial response, liver function	80,98,168,169
Sulphur-containing compounds	metabolism of methionine	liver function	170-173

2.2.3 **Effects of fasting and feeding on breath compounds**

The feeding state has an effect on breath VOCs [118] and is therefore an important confounding factor in breath analysis. Eating a meal starts digestion and anabolic processes in the body, whereas in fasting state catabolic pathways occur. In addition metabolic prosesses by gastrointestinal microflora are part of food digestion, for example eating a meal triggers fermentation of sugars by oral microflora yielding VOCs such as short chain fatty acids (SCFAs) and ethanol [80], and when the undigested food i.e. DF enters the gut the colon microbes produce similar compounds in the colon fermentation. Some of the VOCs produced in the colon are absorbed to the circulation and may end up to the exhaled air as such or after being metabolized to other compunds [125,168,169]. The complex interactions between diet, metanolism, body microflora and expired breath metabolites is described in the article by Ajibola et al [118].

Fasting affects especially breath acetone concentration [174] but also other VOCs [146,175]. There are a few studies (Table 2.2) which have monitored the acute effect of meal or glucose ingestion to exhaled breath VOCs. These are done either to evaluate the effects of eating on exhaled breath VOCs as a confounding factor or in attempt to monitor glucose level from exhaled breath especially relating to diabetes.

Recently, Neyrick et al [125] studied the effect of single meal containing insoluble fibre, chitin-glucan, on exhaled breath VOCs. They studied the effects of meals containing either the chitin-glucan or maltodextrin on exhaled breath VOCs in 30 minutes, and 2 and 4 hours after the meal and 1, 3, 5 and 7 hours after the second meal. Chitin-glucan increased breath methanol and decreased breath ethane levels after the first meal and increased breath butyric acid, pentane, triethylamine, 2,3-butanedione and 3-hydroxybutanone levels in 3 hours after the second meal. The study indicate that DF affect the exhaled breath volatiles mainly 5-10 hours after ingestion. The same has been demonstrated with breath hydrogen levels in the second meal studies with WGs[176].

Table 2.2. Effect of meal or glucose ingestion on exhaled breath VOCs.

Ingestion	Subjects*	Effect	Reference
High-protein meal	6	Acetone ↓ Ammonia ↓ immediately, ↑ after 0.5-5 hours (above fasting levels) Methanol → Ethanol ↑ immediately, → after 1–2 hours (at fasting levels) Isoprene →	[175]
High-protein meal	30/24	Ammonia ↓ immediately, ↑ 0.5–5 hours (above fasting levels) Ethanol ↑ after 30 min, → after 1–2 hours (at fasting levels) Acetone ↑ after 30 min→ after 1–5 hours	[153]
Protein drinks (6 g and 60 g protein)	17	Trimethylamine ↑ steadily increased for 5 hours Indole ↑ steadily increased for 3-5 hours Methanethiol ↑ 6-fold immediately, ↓ 30 min, → for 5 hours Dimethylsulfide → for 5 hours (except after high protein intake immediately ↑) Acetone ↑ after 2 hours → for 5 hours (with high protein intake) 2-propanol ↑ (with high protein intake) 2-pentanone ↓ 1,5-3 hours, ↑ (back to fasting levels) Phenol ↑ steadily increased for 5 hours	[177]
High-fat meal	20	Nitric oxide ↑	[178]
High-fat meal	18 diabetic children	Methyl nitrate ↑ after 45–120 min	[179]
Standardized breakfast containing maltodextrine or chitin-glucan	15	Maltodextrine: Acetic acid, propionic acid, butyric acid, caproic acid, valeric acid, isovaleric acid, methanol, pentane, triethyl amine, 2-3-butanedione, 3-hydroxybutanone ↓ and ethane ↑ compared to fasting levels until 4 hours Chitin-glucan: otherwise the same than with maltodextrine, but methanol ↑ and ethane ↓	[125]
75 g glucose (oral)	10	Acetone ↓ Ethanol ↑ immediately to 30 min, → after 1–2 hours (at fasting levels)	[180]

(Table continues on next page)

Table 2.2. (continued)

Ingestion	Subjects*	Effect	Reference
75 g glucose (oral)	16, normal, diabetic, and impaired glucose tolerance subjects	Acetone ↓ Acetoin ↑ Diacetyl ↑ in most, ↓ in some	181
75 g glucose (oral)	5	Acetone ↓ Ammonia ↓ after 30 min, ↑ after 1–2 hours (staying below fasting levels) Methanol ↑ after 30-60 min, ↓ after 1,5–2 hours (staying above fasting levels) Ethanol ↑ after 30 min, ↓ after 1–2 hours (below fasting levels) Propanol (mainly 2-propanol) ↑ after 30 min, ↓ after 1–2 hours (below fasting levels) Isoprene →	182
15 g glucose (oral)	3	Propionate and Butyrate ↑ after 15 min (in 2/3 of subjects)	98
75 g glucose (oral)	10	Acetone ↓ Volatile sulfide compounds ↓	183
75 g glucose (oral)	3 males	Ethanol ↑ immediately to 15 min and again after 20 min, ↓ after 25 min below fasting levels until 55 min 1-propanol ↑ immediately, ↓ after 10 min below fasting levels until 55 min Acetic acid ↑immediately, ↓ after 10 min below fasting levels until 55 min Propionic acid ↑ immediately, ↓ after 10 min below fasting levels until 40 min Butanoic acid ↑ immediately, → after 10 min (at fasting levels) 2,3-butanedione and acetoin ↑ immediately, → after 20 min (at fasting levels)	80
25 g glucose (intravenous)	10	Acetone ↑ immediately, ↓after 60–120 min (below fasting levels) Ethanol ↑ immediately, → after 30-60 min (at fasting levels), (↑ momentary at 90 min sampling point) Methyl nitrate ↓ (↑momentarily at 30 min sampling point) Xylene ↑ immediately, → after 15-120 min (at fasting levels) Ethyl benzene↑ immediately, → after 15–120 min (at fasting levels)	184

↑ = concentration increased, ↓ = concentration decreased, → = concentration remained stable, *healthy adults unless otherwise stated

2.2.4 **Effect of diet on breath compounds**

Experiments made with animals have demonstrated the impact of diet on exhaled breath composition [185,186]. Thus far, there have been only few studies monitoring the effects of diet on the exhaled breath VOCs in human subjects. Ketogenic and restricted energy diets increase breath acetone [144,187,188] and isopropanol [165] levels. When there are not carbohydrates available to maintain adequate blood glucose levels, fatty acids are used as a primary energy source and ketone bodies (aceto-acetate, β-hydroxybutyrate and acetone) are generated in the liver [147]. Acetone is again reduced to isopropanol by hepatic alcohol dehydrogenase enzyme [189]. There are also commercial devices available for breath acetone measurement, which utilize various acetone sensor technologies e.g. metal oxide semiconductors [187,190].

Two studies have demonstrated changes in the exhaled breath VOC profiles in gluten-free diet compared to normal diet. Baranska et al [191] observed statistically significant changes in exhaled breath VOC profiles (12 compounds) between gluten-free and normal diet in healthy people, although they also demonstrated a large heterogeneity in responses to the dietary change. Aprea et al [192] compared exhaled breath profiles of people having a celiac disease following a gluten-free diet and healthy people with normal diet. They found no statistically significant changes in exhaled breath VOC profiles between diets but could separate the groups using the PCA-DA model. These studies indicate that there are consistent changes in VOCs due to gluten-free diet despite large inter-individual variation.

Diets which are supposed to reduce oxidative stress and lipid peroxidation have been shown to attenuate breath alkane levels. Habib et al [193] demonstrated in rats that dietary restriction without malnutrition decreased the level of exhaled ethane. Miller et al [194] observed a reduction of breath ethane in people having a diet rich in fruits and vegetables, and a further decrease when using also low-fat dairy products. Lipid peroxidation is associated to exhaled breath pentane levels, and dietary supplementation of vitamin E or β-carotene in diet has been shown to decrease these levels [195,196]. Also, in the study of Baranska et al [191] breath octane was decreased in gluten-free diet, and in the study of Biagini et al [197] breath pentane, hexane and hexanal in vegan diet. The researchers are discussing if these could be related to decreased oxidative stress due to the diets.

The consumption of fruits has an effect also on breath methanol levels [36,162,198], as have consumption of artificial sweetener aspartame [161] and alcoholic beverages [36,199]. Biaglini et al [197] observed increased breath methanol levels in vegan diet, which they discussed to be due to increased consumption of fruits and vegetables. Methanol is released in the colon in the degradation of pectin, DF component high

in fruits [200], both the natural and the pure form of pectin increasing the methanol in breath [162]. However, methanol is presented in all breath samples, the main source considered to be degradation of unabsorbed complex carbohydrates by colonic microbes [35].

2.3 METABOLIC EFFECTS OF RYE

2.3.1 Chemical characteristics of rye

Rye (*Secale cereale* L.) is mainly consumed as WG rye containing all the anatomical parts of the grain: bran, endosperm, and germ. Rye grain and WG rye flour consist of starch (55-70 %), DF (15-25 %), protein (8-16 %), fat (2-3 %), minerals (ash content ca. 2%) and water [201-203]. The composition varies between different rye varieties and growing conditions [204,205].

Rye fibre

Both WG and refined rye flours contain a significant amount of DF compared to other common cereals (Table 2.3.). About 50 % of the rye DF is arabinoxylans (AX), hemicelluloses with a structure of copolymer of pentose sugars, xylose, and arabinose [206]. AX are a reservoir of various phenolic acids, which are covalently linked to the polymers. Most of the cereal AX are water insoluble (water-unextractable AX, WU-AX), but some are soluble (water-extractable AX, WE-AX) differing its physicochemical and functional properties, such as viscosity and fermentability [207]. Rye bran contains more than double the amount of WE-AX compared with wheat bran [207], and there is higher proportion of WE-AX in the rye endosperm compared to bran [2].

Rye contains the highest amount of fructan (3,6-6,4 %) of the common cereals [203,208]. Fructan is a highly fermentable polymer of fructose. However, high temperature and low pH in baking process, as well as microbial activity, break the glycosidic bonds of fructose polymers and therefore decrease the amount of fructose polysaccharides [209,210] and increase the amount of oligosaccharides (<10 monosaccharide units) in rye bread. Oligosaccharides are highly fermentable and thus can cause discomfortness for some people because of rapid gas formation [211].

Rye also contains glucose polymers β-glucan (2-3 %) and cellulose (1-3 %), and lignin (1-2 %) [212,213]. β-glucan is a soluble DF demonstrated to have cholesterol-lowering potential but being less-extractable in rye compared to oat affecting to its viscosity properties [214]. Cellulose and lignin are water-insoluble DF. Lignin is a three-dimensional highly heterogeneous polymer of phenylpropanes filling the spaces in the cell walls between the celluloses and hemicelluloses [215].

Table 2.3. DF composition of WG rye, wheat, oat, maize and rice and refined flours of rye and wheat (g/100g DM) [216]

	WG					Refined flour	
	Rye	Wheat	Oat	Maize	Rice	Rye	Wheat
DF	**20,5**	14,2	9,8	11,6	2,5	10,1	4,3
Total NSP	**18,5**	12,4	7,6	9,5	1,4	9,6	4,0
AX	**9,6**	7,1	2,1	4,7	0,4	4,3	2,1
Cellulose	1,3	1,9	0,6	**2,0**	0,3	0,5	0,2
β-Glucan	2,0	0,6	**3,8**	0,1	0,1	0,8	0,2
Fructan	**3,1**	0,9	0,1	0,5	<0,1	2,3	0,6
Others[1]	**2,5**	1,9	1,0	2,2	0,9	1,7	0,9
RS	0,3	0,3	0,2	**1,0**	0,3	0,3	0,3
Lignin	1,7	1,5	**2,0**	1,1	0,8	0,2	0

DF = dietary fibre, WG = wholegrain, DM = dry matter, NSP = non-starch polysaccharides, AX = arabinoxylans, RS = resistant starch, [1]non-cellulosic residues galactose, mannose, uronic acids and glucose not accounted for as β-glucan or cellulose

Rye phytochemicals

Rye DF is rich in a variety of phytochemicals i.e. non-nutrient bioactive compounds that are produced in plants; lignans, alkylresorcinols (AR), phenolic acids, benzoxazinoids, phytosterols, tocols, flavonoids, and carotenoids [216-220]. Most of these are in the DF complex in the outer bran layers, in the pericarp seed coat and aleurone layers.

Lignans are water soluble diphenolic compounds with various structures usually found as a part of the lignin polymers [221]. In WG rye, there are 1-2 mg/100 g lignans, which is more than in other common cereals [222-224]. However, the lignan content varies a lot in rye and other cereals between different cultivars, and e.g., because of environmental conditions, grain size and extraction method used in analysis [223,225]. Typical lignans in rye bran are syringaresinol, pinoresinol, lariciresinol, 7-hydroxymatairesinol and mediorecinol, respectively, but there are also several other lignans, such as matairesinol and secoisolariciresinol [223]. Lignans can be directly absorbed from the small intestine [226], but they can also be metabolized to enterolignans e.g., enterolactone and enterodiol by the gut microflora [227]. Lignans are phytoestrogens studied especially for their cancer-preventive properties [221].

AR consist of long aliphatic chains of different lengths and resorcinol-type phenolic rings [228,229] occurring in numerous plants and microorganisms [229]. Rye is rich in AR with the length of the alkyl chain C15–C27 and variable degree of saturation (saturated, mono- and diunsaturated) odd-numbered homoloques C17, C19, and C21 being the dominant [230,231]. AR have been utilized as biomarkers for estimating intake of WG wheat and rye in diet [232]. They are revealed to have wide range of antimicrobial, anticancer, antilipidemic, and antioxidant activities [233].

Phenolic acids in rye are phenylpropanoids e.g., ferulic acic, sinapic acid and p-coumaric acid, respectively, and in less amount's vanillic acid, syringic acid and p-hydroxybenzoic acid [234]. Phenolic acids can be released from DF complex by microbiota esterases in the large intestine, when they can be absorbed to the circulation or be metabolized by the microbiota to propionic, acetic, and benzoic acid derivatives [235]. Phenolic acids may exhibit antioxidant, anticancer, anti-inflammatory, and antimicrobial activities [236].

Benzoxazinoids are allelopathy chemicals containing benzene and oxazine structures [237]. Within eatable plants, they are characterized thus far only in WG rye and wheat, especially in rye bran and wheat germ [238,239]. Benzoxazinoids can be

converted into phenylacetamides that are found in WG rye bread and in urine and plasma samples after consumption of rye bread [240-242].

Phytosterols are steroid compounds having a phenandrene structure and studied because of their serum cholesterol-lowering properties [243]. They occur in rye as free sterols and steryl conjugates, i.e., steryl esters with fatty acids or phenolic acids and steryl glycosides. Rye is a good source of phytosterols compared to other cereals [204,205], the main phytosterols including sitosterol, campesterol, stigmasterol, sitostanol, and campestanol.

Tocols, i.e., tocopherols and tocotrienols are lipid-soluble compounds consisting of a chromanol ring and 16-carbon side chain and having E vitamin activity [243]. Tocol levels in rye are comparable to other cereals, α-tocopherol and α-tocotrienol being the major tocols.

Processing affects chemical composition and physicochemical properties of rye products [244-247]. Rye is usually consumed as bread, although nowadays there is increasing number of various rye products because of consumers´ interest for its nutritional and health benefiting properties [201]. Rye grains are usually milled to flours. Various types of flour can be obtained by milling process possessing different extraction rates, wholemeal (100%), brown (85–98%) and white/refined/endosperm (72–80%) [248]. The extraction rate of flour decreases according to the outer layers of grain removed, therefore decreasing the DF content and related phytochemicals.

In the Eastern-Europe and Russia, rye bread is commonly made using sourdough fermentation [249], where lactic acid bacteria produce several metabolites, e.g., organic acids, exopolysaccharides, and enzymes. This affects texture, aroma and self life of bread, protein and starch fractions and bioavailability of some nutrients. Several process parameters including temperature, time of fermentation and composition of starter cultures determine the properties of sourdough rye bread [250,251]. In sourdough, increased acidity affects enzyme activities and degratation of starch and protein [252]. Starch is partially gelatinized and crystallized [253] and amylose forms a layer surrounding the starch granules [254]. This increases the amount of resistant starch (RS) in rye bread, i.e., starch that reaches the large intestine and thus can be fermented by gut microflora[255]. Sourdough fermentation also increases water solubility of arabinoxylan and free phenolic acids from DF complex increasing their bioavailability [235].

In baking process, the Maillard reaction happens in the bread crust because of heating, formating Maillard reaction products (MRPs) [248]. In the Maillard reaction,

amino groups of amino acids, peptides or protein react with reducing sugars, formating various amino acid derivates in different stages of reaction, e.g., Amadori compounds, various fluorescence compounds and coloured melanoidins [256].

2.3.2 **Health effects of rye**

Intake of WG foods has been associated to beneficial health effects and prevention of chronic diseases, such as type 2 diabetes, coronary heart disease, and cancer, and also contribute to gastrointestinal health and body weight management [257,258]. However, it is not usually possible to conclude whether the causality seen in epidemiological studies is actually due to intake of WG, the bran or cereal DF [259]. WGs are heterogeneous group consisting of various cereals with varying DF content (Table 2.3) therefore having versatile physiological effects [260].

DFs are non-digestible components in diet possessing beneficial physiological functions [261] mediated from the gastrointestinal tract. The functional properties of DF depend on their physicochemical properties e.g., particle size, solubility, water-holding capacity, viscosity, bulking ability, swelling, cation exchange properties, and fermentability [262,263]. DF affect the physiology of the gastrointestinal tract in several complex ways [264,265]. DF affect to the digestion of food and the bioavailability of nutrients and other compounds, and they interact with the gut microbiota affecting to their growth (prebiotic effect) and metabolism. The production of SCFAs in the fermentation of gut microbes is considered to be one of the key mechanisms related to health effects of DF [266-268]. Cereal DF contains, unlike isolated or synthetizised fibres, associated phytochemicals in the DF complex [261], which may be responsible some of the physiological effects related to rye [236,269].

Among the WGs, rye is superior both in DF content and associated phenolic compounds [2,220]. Rye fibre contains both insoluble and soluble DF fractions [270], the distribution varying in rye products principally based on extraction rate of used flour [271], and due to prosessing [272]. Moreover, sourdough fermentation may contribute to some of the beneficial effects related to rye bread [273].

Dietary intervention studies are conducted to investigate risk markers of chronic diseases and to reveal physiological and metabolic mechanisms behind the health effects of diets, because appearance of the disease may take several years or decades and there are multiple confounding factors related. Most intervention studies with rye compare the effects of diets with WG rye and refined

grain foods. In case of studying effects in glucose metabolism, these usually include postprandial measurements after a standardized meal.

Effects on bowel function and gut microflora

DF is an essential nutrient, and the recommended intake for adults typically is 25-35 g/d [274] . The recommended levels are usually considered to be adequate for normal laxation. Rye fibre possesses many of the physiological effects related to bowel function. It decreases intestinal transit time, increases stool bulk, and is fermentable by colonic microflora [275,276] . Rye fibre has an official approaved health claim in the EU: "Rye fibre contributes to normal bowel function".

Especially insoluble DF increases fecal bulk [277], which is preferable to bowel health. Larger fecal bulk dilutes harmful compounds such as carcinogens and mutagens and decreases the intestinal transit time reducing the contact between the harmful compounds and epithelial cells. In long run this can result in a lower risk of colon cancer [278]. In addition, some fibres may alter the metabolism of colon carcinogens, resulting in detoxified products and thus reducing colon carcinogenesis [278].

Fermentable DF is nourishment for the colonic bacteria yielding increased bacterial mass in the colon and production of SCFAs [277]. Soluble DF is typically fermented readily by the colonic bacteria, whereas insoluble DF can take a longer time to be extracted and fermented [264,279]. Colonic fermentation decreases colonic pH [280], which promotes the growth of beneficial bacteria [281], improves absorption of some minerals [282,283] and inhibits conversion of primary bile acids to carsinogenic secondary bile acids [284]. Rye fibre has resulted in modest but beneficial changes of gut microbiota in different studies [285-288]. Changes in microflora may be related also to the function of gut barrier, and low-grade inflammation [289,290].

The SCFAs, acetate, propionate, and butyrate, are physiologically important end products of colonic fermentation, and the main energy source of the gut epithelial cells [291]. Butyrate seems to have a key role mediating the effects of the gut microbiota on the immune system and may be a protective factor in colon carcinogenesis and developing of inflammatory bowel disease [292]. Consumption of rye increases fecal butyrate levels [275,276,285]. Moreover, high intake of DF from rye [293,294] or barley [295-297] has been shown to increase postprandial levels of plasma SCFAs, especially butyrate in the following meal, which may have positive affects to glucose and lipid metabolism [298,299]. In turn, gut-derived propionate is used for the

hepatic synthesis of odd-chain fatty acids [300] e.g., pentadecanoic acid and heptadecanoic acid, which are associated with a lower incidence of ischemic heart disease [301] and type 2 diabetes [302-304].

Effects on glucose metabolism

WGs are associated with beneficial effect on glucose metabolism and prevention of diabetes, however the mechanism being complex and unclear [305,306]. Hyperinsulinemia impairs β-cell function and increases insulin resistance, ultimately leading to the onset of diabetes [201]. Rye products have been demonstrated to lower the insulin demand, induce attenuated and prolonged blood glucose response, and reduce subclinical inflammation [176].

Ingestion of rye bread reduces postprandial insulin response regardless of the glucose response, i.e., less insulin is required for the regulation of postprandial glucose excursion. The phenomenon is called "the rye factor" [2], although its physiology is not fully understood. The effect is seen with WG, wholemeal or endosperm rye bread [298,307,308] in meal tests, but also after the meal not containing any rye if rye is consumed in previous meal [176,294] or diet [293]. The demonstrated second meal and dietary effects indicate that the effect is mediated from gut, although the single-meal tests have shown that there are also short-term effects. The rye fibre and the related bioactive compounds [309], the amino acids of rye [307,310], and the processing and structure of rye bread [254,311] are suggested to be behind the rye factor.

The compact structure of rye bread may slow the digestion and reduce uptake of glucose by the intestine, leading to a lower rate of glucose appearance in the blood [312]. This is seen especially in the early phase of postprandial glucose response when compared to white wheat bread [313]. Also, the structures of the starch granules and the bread matrix differ between rye and wheat breads yielding to reduced rate of starch hydrolysis and more resistant starch in rye bread [254]. Fermentable DF, including resistant starch, can increase the levels of circulating SCFAs [293,294], which might have association to peripheral insulin sensitivity, whole body lipolysis and glucagon-like peptide-1 (GLP-1) concentration [314]. Fermentation of grain DF is slower compared to isolated DF [261] and the dense structure of rye bread may further slow it down because of slower digestion and bigger particles entering the colon [312], enabling the fermentation to happen not only in the proximal but also in the distal colon [315].

Also, the polyphenols have a role in beneficial effects of rye to carbohydrate metabolism [269]. Growing evidence indicates that various dietary polyphenols may influence carbohydrate metabolism, the possible mechanisms including inhibition of carbohydrate digestion and glucose absorption, stimulation of insulin secretion, modulation of glucose release from the liver, activation of insulin receptors and glucose uptake, and modulation of intracellular signalling pathways and gene expression [269,316]. Effects of polyphenols are mostly transmitted from the gut. Gut microbiota releases polyphenols from DF complex increasing their bioavailability, and converts them also to other compounds, such as plant lignans to mammalian lignans [220]. Food processing affects colonic degradation of DF and bioavailabilty of rye phytochemicals, which may explain modest effects of some rye products in meal tests compared to sourdough fermented rye bread [235,245].

2.3.3 **Metabolites related to rye intake**

Several metabolites have been associated with the consumption of rye (Table 2.4). These are studied especially with the intention of finding exposure biomarkers or biomarkers of effects for WGs and rye [317]. Exposure biomarkers are needed to assess the intake of specified foods for example to confirm the compliance of diet in dietary intervention studies, whereas biomarkers of effects can reveal individual metabolic responses of diets or dietary components. Biomarker compounds are typically detected from plasma and urine, but also from serum, adipose tissue, erythrocytes, or feces. However, so far there are no dietary biomarker compounds identified from exhaled breath.

There are no biomarkers of total WG intake, but a few compounds have been linked to the intake of certain WGs [232,317,318]. Especially AR and their metabolites have been associated with the intake of WGs. Odd-numbered AR are the most utilized biomarker compounds for WG wheat and rye [319], whereas even-numbered AR for quinoa [320], and avenanthramides and avenacosides for oats [321,322]. The sum of AR homologs with 17–25 carbon alkyl-chains, i.e., total AR, reflects total whole-grain wheat and rye intake, whereas the AR homolog profile can be used as a biomarker of the intake of whole-grain rye to whole-grain wheat intake [232]. Especially 5-heptadecylresorcinol (AR-C17) is associated to intake of rye [323]. However, the half-life of AR in plasma is rather short (4–5 h) and therefore they reflect only a short-term intake [324,325]. Also, different metabolites of AR, and their conjugates are studied as biomarker candidates for WG wheat and rye intake. Benzoxazinoid derived phenylacetamides have been associated especially to

intake of sourdough fermented rye bread [242], because processing of grains increases the bioavailability of these coumpounds [238,239].

Besides the exposure biomarkers, there are also some biomarkers of effects related to rye (Table 2.4). These could give information about the relationships between the consumption of dietary components and health outcomes. For example, there are large interpersonal variation in the levels of mammalian lignan enterolactone associated with reduced risk of several cancers as well as cardiovascular disease and total and cause-specific mortality regardless the intake of lignans [326]. It is stated that this may be one explanation behind the inconsistency of the reported health effects associated with high lignan intake.

Table 2.4. Metabolites associated to intake of rye

Metabolites	Sample type	References
Total AR	Plasma Erythrocyte membranes Adipose tissue	327-334
AR homologues	Plasma Urine Erythrocyte membranes Lipoproteins	324,325,329,332,333
AR metabolites		
DHBA	Urine Plasma	332,335-341
DHPPA	Urine Plasma	332,335,337-342
DHCA	Urine	343-345
DHPPTA	Urine	343-345
DHBA-glycine	Urine	343-345
DHCA-amide	Urine	344
DHCA-sulfate	Urine	241
DHPPA-sulfate	Urine	241,346
3,5-dihydroxyphenylethanol sulfate	Urine	241
3,5-Dihydroxyhydrocinamic acid sulfate	Urine	241
Benzoxazinoids		
BOA	Plasma (Pigs) Urine (Pigs) Feces (Pigs, Rats)	347,348
MBOA	Plasma (Pigs)	347
AAPO	Urine (Pigs, Rats) Feces (Pigs, Rats)	347,348

(Table continues on the next page)

Table 2.4. (continued)

Metabolites	Sample type	References
HBOA-glc	Plasma (Pigs, Rats) Urine (Pigs, Rats) Feces (Pigs, Rats)	347,348
HBOA-glc-hex	Plasma (Pigs) Urine (Pigs, Rats) Feces (Pigs, Rats)	347,348
HBOA-glucuronide	Urine Urine (Rats) Plasma (Rats)	242,348
HMBOA	Urine (Pigs)	347
HMBOA-glc	Plasma (Pigs) Urine (Pigs, Rats)	347,348
DIBOA	Plasma (Pigs) Urine (Pigs, Rats) Feces (Pigs, Rats)	347,348
DIBOA-glc	Plasma (Pigs, Rats) Urine (Pigs, Rats) Feces (Pigs, Rats)	347,348
DIBOA-glc-hex	Plasma (Pigs) Urine (Pigs, Rats) Feces (Pigs, Rats)	347,348
DIBOA-sulfate	Urine	241
DIBOA-glucuronide	Plasma (Rats) Urea (Rats)	348
DIMBOA-glc	Plasma (Pigs) Urine (Rats)	347,348
Benzoxazinoid derived phenylacetamides		
HPAA-sulfate	Urine	240,242,346
HHPAA	Urine	346
HHPAA-sulfate	Urine	240,242,346
HHPAA-glucuronide	Urine	242
Phenylacetylglutamine derivative	Urine	242
Creatinine	Urine	242
N-feruloylglycine-sulfate	Urine	242
Ferulic acid-4-sulfate	Urine	241
2-aminophenol sulphate	Urine	241
Indolylacryloylglycine	Urine	241
Enterolactone	Plasma Serum Urine	276,349

(Table continues on the next page)

Table 2.4. (continued)

Metabolites	Sample type	References
Enterolactone glucuronide	Urine	[241]
Caffeic acid sulfate	Urine	[346]
Heptanedioic acid	Urine	[346]

AR = alkylresorcinols, DHBA = 3,5-dihydroxy-benzoic acid, DHPPA = 3-(3,5-dihydroxyphenyl)-1-propanoic acid, DHCA =3,5-dihydroxycinnamic acid, DHPPTA = 5-(3,5-dihydroxyphenyl) pentanoic acid, BOA = benzoxazolin-2-one, MBOA = 6-methoxy-benzoxazolin-2-one, AAPO = 2-acetylaminophenoxazin-3-one, HBOA = 2-hydroxy-1,4-benzoxazin-3-one, HBOA-glc = 2-β-D-glucopyranosyloxy-1,4-benz-oxazin-3-one, HBOA-glc-hex =2-β-[Hexosepyranosyl1(1→4)-D-β-glucopyranosyloxy]-1,4-benz-oxazin-3-one, HMBOA = 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one, HMBOA-glc =2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one, DIBOA = 2,4-dihydroxy-1,4-benzoxazin-3-one, DIBOA-glc = 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one, DIBOA-glc-hex = 2-β-[Hexosepyranosyl1(1→4)-D- β-gluco-pyranosyloxy]-4-hydroxy-1,4-benzoxazin-3-one, DIMBOA-glc = 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one; HPAA = N-(2-hydroxyphenyl) acetamide, HHPAA = 2-hydroxy-*N*-(2-hydroxyphenyl) acetamide

3 AIMS OF THE STUDY

The main objective was to explore the potential of exhaled breath analysis in studying the health effects of rye and grain fibre in dietary interventions. The changes in exhaled breath VOCs after diets rich in WG rye were investigated using various analytical technologies.

The specific aims were as follows:

1. To examine changes in exhaled breath VOCs with targeted GC-MS analysis in relation to dietary intake of grain fibre (Study I).
2. To examine changes in exhaled breath aspiration IMS profiles in relation to dietary intake of grain fibre and sourdough fermented rye bread (Study II).
3. To explore exhaled breath VOCs by non-targeted GCxGC-MS analysis in relation to intake of WG and rye (Study III).

4 SUBJECTS AND METHODS

4.1 DIETARY INTERVENTIONS

4.1.1 **Study protocols and subjects**

Exhaled breath analyses were performed in three dietary intervention studies during 2009-2015 in the Institute of Clinical Nutrition and Public Health in the University of Eastern Finland: the RAVIDI study in the spring 2009, the FIBREFECTS study in the spring 2012 and the RYEBREATH study in the spring 2015 (Table 4.1, Figure 4.1,). The study protocols were approved by the Research Ethics Committee, Hospital District of Northern Savo. Each individual provided a written informed consent before participation in the study.

Table 4.1. Dietary intervention studies

	Study I	Study II	Study III
	RAVIDI	FIBREFECTS	RYEBREATH
Participants	7 men (age 24-45)	4 men, 3 women (age 38-61)	2 men, 7 women (age 21-59)
Diets	WW vs WGR	WW vs WGR vs WW+BRB	WW vs WGR vs WGW
Analysis method	targeted GC-MS analysis of 15 exhaled breath VOCs	exhaled breath AIMS profiles	non-targeted GCxGC-MS analysis of exhaled breath VOCs

WW = low-fibre diet with white wheat bread, WGR = high-fibre diet with wholegrain rye bread, WW+BRB = high-fibre diet with white wheat bread enriched with bioprocessed rye bran, WGW = high-fibre diet with wholegrain wheat bread, GC-MS = gas chromatography – mass spectrometry, VOC = volatile organic compound, AIMS = aspiration ion mobility spectrometry, GCxGC-MS = comprehensive two-dimensional gas chromatography – mass spectrometry

Study I

In Study I (the Ravidi study), a randomized crossover study with two diets differing in the DF content was carried out (Figure 4.1). The diet periods were in randomized order and lasted one week, and there was a 2-week washout period between the diets, during which the subjects followed their habitual diet. The exhaled breath was analysed at the end of the diet periods in the fasting state and in the postprandially after a standardized test meal.

Seven healthy (no diagnosed chronic diseases and no evidence of disturbed glucose metabolism) non-smoking Finnish men were recruited through advertisements in the campus area of the University of Eastern Finland. They were 23 to 46 years old (average 32) and had BMI (body mass index) 21.7 to 32.5 kg/m^2 (average 26.0).

The participants were advised to maintain their body weight and lifestyle habits throughout the study except the adviced modifications in the diet. The compliance was monitored with four-day dietary records, weighing, and with use of activity computers (Polar Electro, Kempele, Finland) which recorded physical activity during the diet periods.

Study II

In Study II (the Fibrefects study), a randomized crossover study was carried out with two high-fibre diets differing in types of bread (Figure 4.1). After a 4-week low-fibre period, 4-week high-fibre periods with either sourdough fermented WG rye bread or wheat bread enriched with bioprocessed rye bran were followed in randomized order. The exhaled breath was analysed in the end of the diet periods in the fasting state and postprandially after a standardized test meal.

Eight participants were randomly selected to give exhaled breath samples from a group of 21 participants recruited to the FIBREFECTS study [293]. These participants were healthy Finnish men and women reported having gastrointestinal symptoms (flatulence, bloating, discomfort, constipation, or diarrhea) after ingestion of rye bread. One participant did not complete the study protocol and therefore the final analysis was done with 4 men and 3 women, aged 38 to 61 years (average 54.3) and BMI from 19.2 to 29.3 kg/m^2 (average 25.5).

The participants were advised to maintain their body weight and habitual lifestyle throughout the study except the adviced modifications in the diet. The compliance was monitored with recorded amount of eaten test breads and other

grain products in a daily questionnaire, and with four-day dietary records and weighing in each diet period.

Study III

In Study III (the RyeBreath study), a randomized crossover study with two high-fibre diets differing in DF source was carried out (Figure 4.1). One-week high-fibre periods with WG rye or wheat bread were run in randomized order and one-week low-fibre diet periods preceeded each high-fibre diet. The exhaled breath was analysed in the end of the diet periods in the fasting state.

Nine healthy non-smoking Finnish participants were recruited to the RyeBreath study through advertisements in the campus area of the University of Eastern Finland. They were 21 to 59 years old (average 31) and had BMI 18.7 to 29.0 kg/m^2 (average 23.0), regular eating and exercise habits, and no diagnosed diseases. The participants were advised to maintain their body weight and lifestyle habits throughout the study except the adviced modifications in the diets.

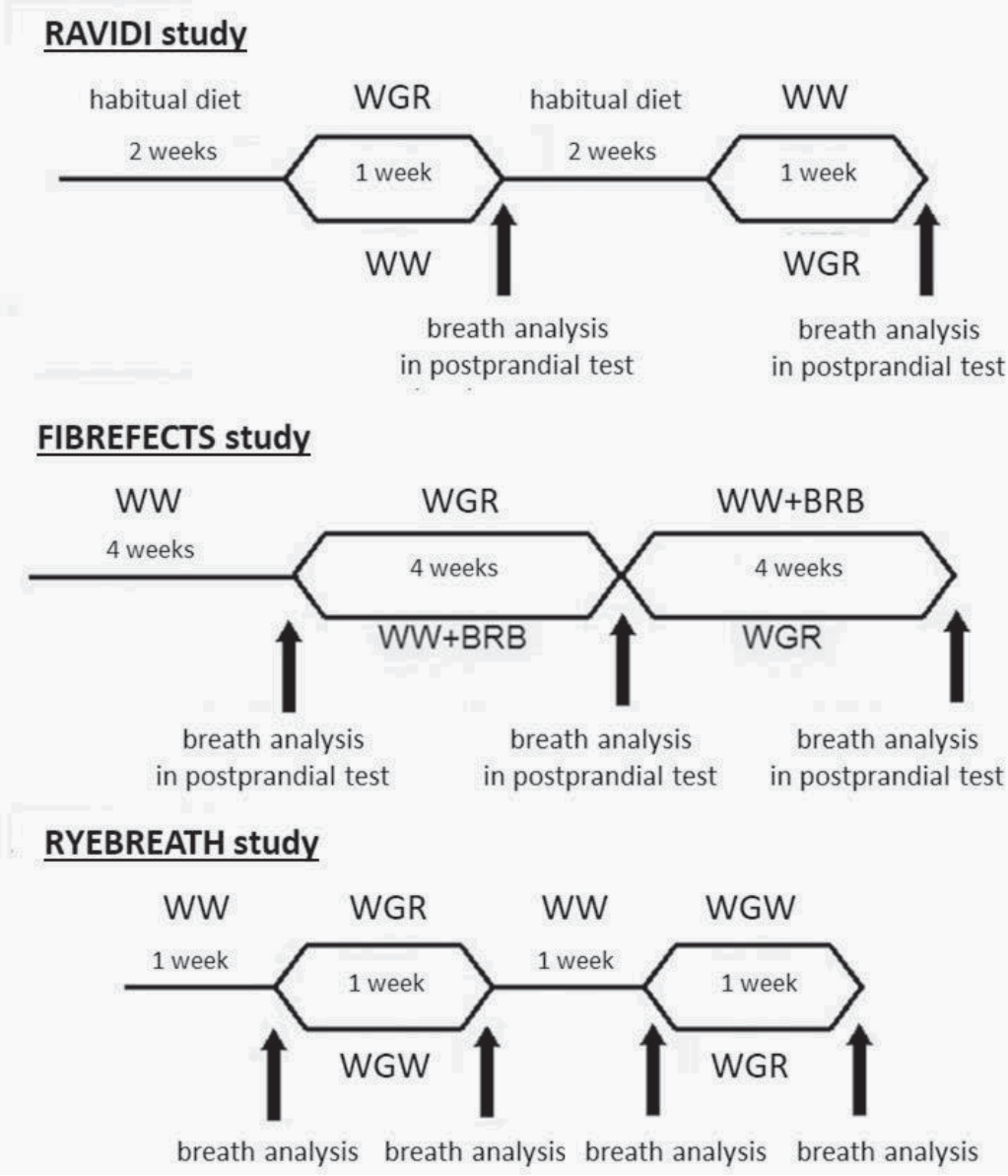

Figure 4.1. Study protocols.

4.1.2 **Diets**

A dietician advised the participants on the practical management of the diets in all three studies. The compliance with the diets was checked by four-day food records filled in during the diet periods. The food records always included one weekend day. The food records were analysed for nutrient intakes using Diet32 software (Aivo Finland Oy, Turku, Finland).

Study I

The participants were asked to maintain their habitual diet otherwise, except the intake of DF was instructed. The recommended amount of DF was adjusted according to the energy intake of each participant which was calculated using the formula of Mifflin-St Jeor [350]. During the low DF diet (WW) the participants were advised to consume white wheat bread and other low DF cereal products, such as white rice and pasta, aiming to DF intake of 7.5 g per 1000 kcal, whereas during the high DF diet (WGR) the subjects were instructed to consume WG rye bread and other high-fibre cereal products such as brown rice, WG pasta, and muesli, aiming to DF intake of 15 g per 1000 kcal.

The participants were advised to limit the intake of fruits and vegetables to 3-4 servings per day, depending on their estimated energy intake. They were also advised to limit berry intake to 1 dl per week, and not to eat oat or barley porridge, or oatpasta during the diet periods. In addition, they received instructions not to use foods that affect strongly on bowel function, such as legumes, plums, dried fruits, seeds, brans, and licorice.

The intakes of energy, protein, fat, and carbohydrates during the test diet periods were maintained at the same level as in the participants' habitual diet. Intake of DF was significantly different between the WW and WGR periods (16.9 g/d and 43.7 g/d, respectively, Wilcoxon test $p = 0.018$).

Study II

The participants were advised to follow three diets differing in type of bread. During the WW period the participants were advised to consume daily 6-10 slices (20-25 g/slice) of white wheat bread (commercial breads) with 100% white wheat flour (Vaasan Oy, Kuopio, Finland). During the high DF periods, the participants were asked to consume daily 6-10 slices (25-30 g/slice) of sourdough WG rye bread (in WGR) or wheat bread which was enriched with bioprocessed (fermented) rye

bran (in WW+BRB). The breads for the high DF diets were baked at VTT Technical Research Centre of Finland, (Espoo, Finland) as described in [293]. The number of bread slices was adjusted according to the individual energy requirement of the participants.

Food items producing gastrointestinal symptoms were avoided during the intervention, and vegetables, fruits, and berries without or with only a low content of readily fermentable carbohydrates were favored. One to two small portions of other grain products than the test breads were allowed daily. Occasional intake of non-grain fibre supplements such as dried and soaked plums, linseeds, or sugar beet fibre was allowed during the WW period to avoid constipation.

The intake of DF was lower during the WW period compared to the WGR and WW+BRB periods (24 vs 34 and 32 g/d, respectively, Friedman test $p = 0.004$), and similar between the high-fibre periods (Wilcoxon´s test $p = 0.176$). The intake of energy and proportions of energy from fat, protein and carbohydrates were stable during the intervention, except a minor decrease in the supply of carbohydrates during the WW+BRB period (mean intakes in g/d: WW 237, WGR 223, WW+BRB 206, Friedman test $p = 0.018$). There was no difference in the carbohydrate content between the WW and WGR diets (Wilcoxon´s test $p = 0.237$).

Study III

The participants were advised to follow three diets during the intervention differing in amount and source of DF. In low DF diet periods (WW1 and WW2) participants were adviced to consume 5-7 slices of white wheat breads, to avoid WG products and not to consume any rye. In high rye DF diet (WGR) participants were adviced to consume 5-7 slices of WG rye breads and in high wheat DF diet (WGW) 7-8 slices of WG wheat breads and to favour WG products, but not to consume any rye in the WGW period. The number of bread slices was adjusted according to the individual energy requirement of the participants. Participants received two different commercial breads in each period and recorded number of eaten breads.

The intakes of energy, protein, fat, and carbohydrates during the test diet periods were maintained at the same level during the intervention. Intake of DF was significantly different between the low DF and high DF periods (WW1 23.6 and WW2 24.6 g/d vs WGR 36.2 and WGW 43.4 g/d, Friedman test $p < 0.001$).

4.2 EXHALED BREATH MEASUREMENTS

4.2.1 **Sampling**

End-tidal air samples were taken with Bio-VOC® samplers (Markes International Ltd, UK), which are designed for capturing alveolar breath concentrated with VOCs excreted from circulation. Participants were trained to give an adequate sample. Before the sampling participants were sitting still without talking for a few minutes. Then they were told to give a constant blow, as long as it is possible to blow, to give an alveolar breath sample. The participants fasted overnight (approx. 12 hours) before the measurements, and they were advised to avoid exhausting exercise, sauna bathing and heavy meals during 24 hours and to abstain from alcohol consumption during 48 hours before the sampling days. To minimize bacterial fermentation in the mouth, they were asked to brush their teeth with toothpaste in the morning and again without toothpaste in the appointment before the first breath sampling.

Samples were handled depending on analysis method (Figure 4.2). In SPME-GC-MS–analysis the breath samplers were closed with adapters with septa, the VOCs were preconcentrated with SPME and introduced to the GC-MS by manual SPME injection (Study I). When using aspiration IMS technology, the the breath sampler was introduced to the inlet of device immediately after sampling through a multicapillary column (Study II). In GCxGC-MS (comprehensive two-dimensional gas chromatography –mass spectrometry) -analysis exhaled breath samples were injected to TD (thermal desorption) liners using the Bio-VOC sampler as a gas syringe and preconcentrated samples were introduced to GC-MS with automated injection in TD liners (Study III).

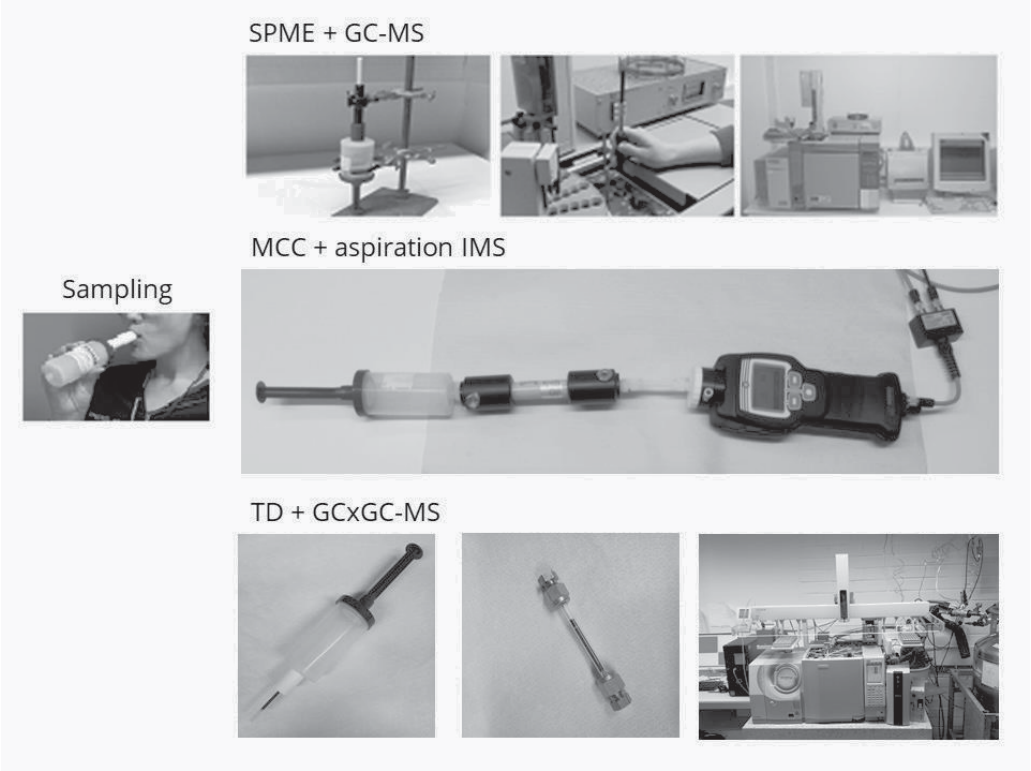

Figure 4.2. Exhaled breath sampling, preconcentration and analysis methods used in the studies. In all studies, alveolar breath samples were taken with Bio-VOC sampler. In Study I, breath VOCs were preconcentrated with solid phase microextraction (SPME) and analysed with gas chromatography -mass spectrometry (GC-MS). In Study II, exhaled breath was analysed with aspiration ion mobility spectrometry (IMS) having multicapillary column (MCC) to delay the humidity. In Study III, breath VOCs were preconcentrated with thermal desorption sorbent tubes (TD) and analysed with comprehensive GC-MS (GCxGC-MS).

SPME extraction (Study I)

The Bio-VOC sampler was closed immediately after the sampling with adapter containing a septum (self-made by sculpting from PTFE (polytetrafluoroethylene) rods, Thermogreen LB-2 Septum, Sigma-Aldrich, St. Louis, USA), and the internal standard (IS, 0.11 µg/µl acetone-d6, Euriso-top, Saint-Aubin, France) in Milli-Q ultrapure water (Millipore, Bedford, USA) was injected into the sampler through the septum. The exhaled VOCs and IS were extracted by using a 75-µm carboxen-polydimethylsiloxane (CAR-PDMS) SPME fibre which was inserted into the Bio-VOC sampler through the septum and kept there for 30 minutes at room temperature.

Because sampling was done outside the analysis laboratory, extra attention was paid to storage of the samples. SPME portable field sampler (Supelco, Bellefonte, Pensylvania, USA) was used, which can store the SPME needle into the septum, thereby minimizing desorption of the extracted compounds and absorption of the background compounds during the transportation. The septum for the portable field sampler was changed after cleaning the SPME fibre (300°C/10 min in the injection port of GC) and after the extractions. The SPME samplers were transported in fixed polytetrafluoroethylene tubes (Vink Finland, Kerava, Finland) which were plugged with Viton stoppers (VWR International, Radnor, Pennsylvania, USA). All samples were analysed within 2-5 hours of sampling.

MCC with aspiration IMS (Study II)

A multicapillary column (MCC, BEKO Technologies GmbH, Neuss, Germany) was used because the IMCell in the ChemPro100i is sensitive for water. By using the MCC, the AIMS response of exhaled breath VOCs was achieved before the response of the humidity. The MCC was installed to the inlet of ChemPro100i having the flow rate 1.3 l/min. Active carbon filter (Pall Corporation, Port Washington, NY, USA) was used in the inlet of MCC before and between the measurements to maintain the background air constant. Immediately after the breath sampling, the Bio-VOC sampler was placed to the inlet of MCC for 7 seconds.

TD extraction (Study III)

An alveolar exhaled breath sample was injected to thermal desorption sorbent tube (Fritted Liner packed with Tenax GR, mesh 80-100, GL Sciences, Eindhoven, The Netherlands) using the Bio-VOC sampler as a gas syringe and adapter (self-made by sculpting from PTFE rod) to connect the sampler and the liner tightly. After sampling the TD liner was closed with a storage cap (Brass Liner Blanking Cap, GL Sciences, Eindhoven, The Netherlands) and analysed within 2-5 hours.

4.2.2 **Targeted GC-MS analysis (Study I)**

Several VOCs were identified from exhaled breath samples in our preliminary studies (not published), and 15 compounds were selected for further examination in the dietary intervention. Alcohols, SCFAs, branched-chain fatty acids (BCFAs),

acetoin, diacetyl, and phenol were selected because these are related to gut fermentation [351,352]. Acetone was selected because it is one of the most studied compounds in exhaled breath and related to glucose metabolism [353]. The VOCs were identified based on their specific RTs and specific ions formed in mass spectrometry analysis using the Wiley275 mass spectrum library (Hewlett Packard Ltd, Wilmington, USA). The recognition was ascertained with commercial analytical grade standard solutions from Supelco (Bellefonte, USA); except 1-propanol from Merck (Darmstadt, Germany), ethanol from Altia (Helsinki, Finland), and solvent methanol from Avantor Performance Materials (J.T Baker, Center Valley, USA).

Analyses were performed with a GC-MS (GC 5890 series II Plus + MS detector 5972 series, Hewlett Packard Ltd, Wilmington, USA) with Nukol capillary column (0.25 µm thick, 0.25 mm internal diameter, 30 m long, Supelco, Bellefonte, USA) using Helium 4.6 (AGA, Espoo, Finland) as a carrier gas with flow rate 1 ml/min. The injection was done in 300°C in splitless mode (split vent closed for a minute). The GC oven was programmed to be 45°C for 3 minutes, then raised 15°C/min to 200°C and kept there for 5 minutes. The detector temperature was 280°C.

Detection was done in the following selected ion monitoring (SIM) windows: retention time (RT) window in minutes 2.1-3.5 and ion 58 for acetone, and 64 for acetone-d6 (IS); RT 3.5-4.6 and ion 45 for 2-propanol, and ion 46 for ethanol; RT 4.6-5.0 and ion 86 for diacetyl; RT 5.0-6.5 and ion 59 for 1-propanol; RT 6.5-8.5 and ion 56 for 1-butanol; RT 8.5-9.5 and ion 88 for acetoin RT 9.5-14.5 and ion 60 for acetic acid, butyric acid, valeric acid, isovaleric acid, and hexanoic acid, and ion 74 for propionic acid and 2-methylbutyric acid; and RT 14.5-18.33 and ion 94 for phenol. Areas of the GC peaks were calculated from the selected ion of each compound with the MSD Productivity ChemStation software (Hewlett Packard Ltd, Wilmington, USA). The signals were proportioned to the peak area of IS to control the variability in sampling and analysis.

4.2.3 **Aspiration IMS analysis (Study II)**

Aspiration IMS analysis were done using the ChemPro®100i online analyser (Environics OY, Mikkeli, Finland). The ChemPro100i is a type of e-nose based on AIMS technology [354]. The principle of the technology is following: sample air is taken inside the device with an internal pump with a constant flow, the compounds are ionized by an Am-241 source and detected by IMCell. Ionized compounds form clusters and move along with the air flow orthogonally and are detected in the IMCell containing 8 AIMS channels for positively charged clusters

and 8 for negatively charged clusters, which form the AIMS spectrum. The output of the AIMS spectrum is electric current (pA) collected on each channel separately. ChemPro100i gives also the AIMS sum signal, which is calculated as the sum of the absolute values of all ion channels (absolute change of all AIMS channels in pA). There are also extra sensors in the ChemPro100i; semiconductor cells, metal oxide sensors and a field effect transistor sensor, but those were not used in our study.

The signal was levelled as zero before the sampling and relative signals (changes from the filtered background air in pA) from the 14 AIMS channels were used in data analysis (no signal in channels 8 and 16). The AIMS profile (= individual values from 14 AIMS channels in pA) was taken from the time point displaying the maximum value of the AIMS sum signal (the sum of the absolute values of 14 AIMS channels).

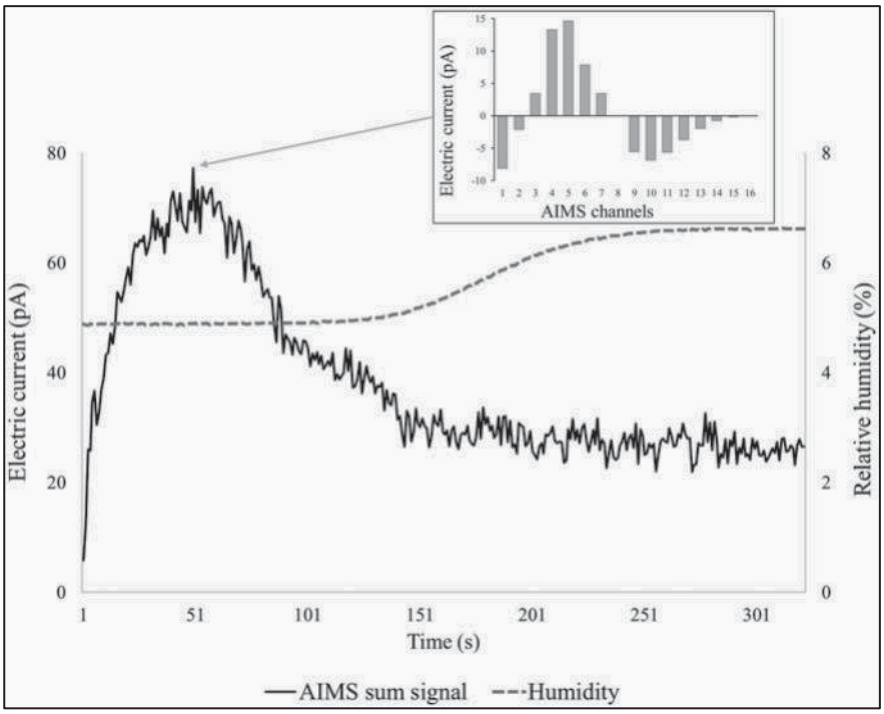

Figure 4.3. Exhaled breath AIMS signal measured with ChemPro100i gas detector. Representative signals of AIMS sum signal and delayed humidity because of the multicapillary column. The AIMS profile was taken from the the maximum point of the sum signal.

4.2.4 **Non-targeted GCxGC-MS analysis (Study III)**

TD-GCxGC-MS -analysis method was developed for non-targeted analysis of exhaled breath VOCs. Analysis was performed with a GCxGC-MS device consisting of GCMS-QP2010 Ultra, AOC-5000 Plus injection system (Shimadzu Scientific Instruments, Columbia, USA), Optic-4 multi mode inlet (GL Science, Eindhoven, The Netherlands) and ZX-1 thermal modulator (Zoex Corporation, Houston, USA). The injection was done with automated AOC-5000 Plus, which transported the TD liners to the inlet of Optic injector. The temperature of the inlet was at the beginning 35 °C for 2 minutes and then rose to the 200 °C at the rate 18 °C/min. The split was 5 and the injection was done in high-pressure mode allowing the pressure of the inlet decrease temporaly during the injection. The sample was preconcentrated to the cryotrap at -100 °C for 7 minutes and released rapidly at 200°C (temperature rise 60 °C/s) to the GC.

VOCs were separated on two serial capillary columns; polar Nukol (0.25 µm thick phase, 0.25 mm internal diameter, 30 m long, Supelco, Bellefonte, USA, same than in Study I) and non-polar Zebron ZB-35HT Inferno (0.8 µm/ 0.18 mm/ 1 m, Phenomenex Torrance, California, United States), separated by a cryogenic Zoex modulator. The modulation was done with 8 seconds modulation time and 10-30 % filling of the 5 L dewar of the liquid nitrogen, i.e., a small part of the second column is freezed for 8 seconds gathering the compounds coming from the first column, and a hot pulse releases the compounds into the second column in 8 seconds intervals. A carrier gas was Helium 4.6 (AGA, Espoo, Finland) with column pressure 150 kPa, column flow 2.14 ml/min and linear velocity 45.5. The GC oven was programmed to be 35°C for 10 minutes, then raised 3°C/min to 200°C. The duration of the GC program was 70 min. The detection was done with MS SCAN 35-300 m/z, event time 0.02 s and scan speed 20 000 unit/sec. Temperature of the ion source was 200°C and MS interface 220°C.

The data was analysed using ChromSquare 2.2 data analysis software. All the visible blobs in two-dimensional chromatograph were manually selected for identification. The tentative identification was performed by comparing their mass spectra with data from NIST 11 Mass Spectral library (The National Institute of Standards and Technology, Maryland, USA), Wiley Registry 10th Edition (John Wiley & Sons, Hoboken, USA) and Flovour & Frangrance Natural & Synthetic Compounds GCMS library FFNSC 2 (Shimadzu Corp., Kyoto, Japan). The identification was checked for each blob by the researcher, but it was not confirmed with analytical standards or retention indices.

4.3 REFERENCE MEASUREMENTS

Study I

Fasting and postprandial blood samples were taken in the end of the diet periods parallel to exhaled breath samples. The blood samples were analysed for plasma glucose, and serum total cholesterol, HDL (high-density lipoprotein) and LDL (low-density lipoprotein) cholesterols, and triglycerides with KoneLab 20XT Clinical Chemistry Analyser (Konelab, Thermo Fisher Scientific, Vantaa, Finland) using the enzymatic photometric (glucose hexokinase) method for glucose, and commercial kits (Thermo Electron Corporation, Vantaa, Finland) for lipids. Serum insulin was analysed with a chemiluminescent immunoassay (Advia Centaur Immunoassay System, Siemens Medical Solution Diagnostics, Tarrytown, USA). Weight of the subjects was measured in study visits and the physical activity was monitored during the diet periods with activity computers (Polar Electro, Kempele, Finland).[195]

Study II

Exhaled breath hydrogen, plasma glucose and insulin, and serum free fatty acids (FFAs) were analysed in fasting and postprandial states parallel to exhaled breath samples. Plasma SCFAs, acetate, propionate, butyrate and isobutyrate were analysed in the fasting state and at 30 min postprandially, and serum triglycerides, total, HDL, and LDL cholesterols in the fasting state. Exhaled breath hydrogen was analysed with Gastro+ Gastrolyzer (Bedfont Scientific, Kent, UK). Plasma glucose, serum FFAs, cholesterols, and triglycerides were analysed with KoneLab 20XTi Clinical Chemistry Analyser (Konelab, Thermo Fisher Scientific, Vantaa, Finland) using the enzymatic photo-metric (glucose hexokinase) method for glucose, enzymatic colorimetric method for FFAs, and commercial kits (Thermo Electron Corporation, Vantaa, Finland) for cholesterols and triglycerides. Insulin was analysed with a chemiluminescent immunoassay (Advia Centaur Immuno-assay System, Siemens Medical Solution Diagnostics, Tarrytown, USA). Plasma SCFAs, acetate, propionate, butyrate and isobutyrate were measured with a GC method [355] with slight modifications using 2-ethyl butyrate (FLUKA no. 03190; Sigma Aldrich, St. Louis, USA) as an internal standard.

Study III

No reference measurements were made in Study III.

4.4 STATISTICAL ANALYSIS

Statistical analyses were performed using the SPSS software (SPSS Inc, Chicago, USA) or Matlab environment (R2011a, The Math Works Inc., Natick, USA). The statistical significance of the differences of individual parameters were analysed with non-parametric tests for related samples; Wilcoxon signed-rank test for two variables [356] and Friedman test for more than two variables [357,358]. Spearmans´s rho was used to analyse correlations between variables. Differences and correlations were considered significant at two-sided $p<0.05$, except in Study I $p<0.10$. All the observed trends (p-values < 0.10) were mentioned, and no correction methods were used to compensate for multiple comparisons problems, since the number of participants were small, and the research were explorative (hypothesis-generating rather than hypothesis-testing).

The exhaled breath aspiration IMS data were monitored with multivariate methods. The data (signals of 14 AIMS channels) was pre-processed with varience scaling and by the principal component reduction [359] in the Matlab environment. The variance scaling (zero mean, unit variance) was performed in two ways; by scaling the AIMS channels separately, when changes are detected in the intensity of each channel between samples (AIMS signal intensity) and by scaling each breath sample separately, when changes are detected in the ratios of AIMS channels between samples (AIMS profiles). Two to four principal components were selected for further processing based on Kaiser Criterion (i.e eigenvalue > 1). The statistical significance of the differences between diets were tested with dependent Hotelling´s T-test [360] using the principal components as inputs.

5 RESULTS

5.1 TARGETED GC-MS ANALYSIS (STUDY I)

5.1.1 **Effect of WG rye diet on exhaled breath VOCs**

The diet-induced changes in most of the targeted exhaled breath VOCs were variable between individuals. However, the signals of 2-methylbutyric acid in the fasting state and 1-propanol at 120 minutes after the test meal were lower after the WGR compared to the WW diet (Wilcoxon test, p = 0.091 for both). Also, exhaled phenol was higher after the WGR compared to the WW diet when all the exhaled breath samples were compared between the diets (Wilcoxon test, p = 0.010).

Exhaled breath 2-methylbutyric acid was lower after the WGR diet compared to WW diet in 6 of 7 participants in the fasting state (Figure 5.1). However, the postprandial levels were variable between indiduals, and there were no differences between the diets when all the exhaled breath samples were compared (Friedman´s test, p = 0.509).

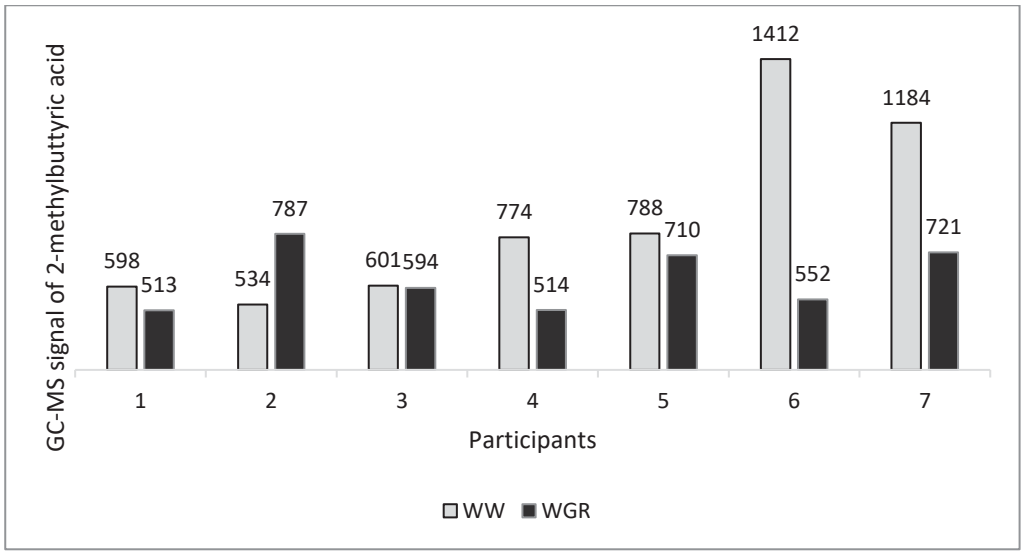

Figure 5.1 GC-MS signal intensities of exhaled breath 2-methylbutyric acid after the low-fibre (WW, dietary fibre 16.9 g/d) and high-fibre (WGR, dietary fibre 43.7 g/d) diets in seven participants in the fasting state (Study I)

Exhaled breath 1-propanol levels were lower after the WGR diet compared to WW diet when all the exhaled breath samples were compared (Friedman test, p = 0.076, Figure 5.2), but the difference reached the statistical significance only at 120 min after the test meal (Figure 5.3).

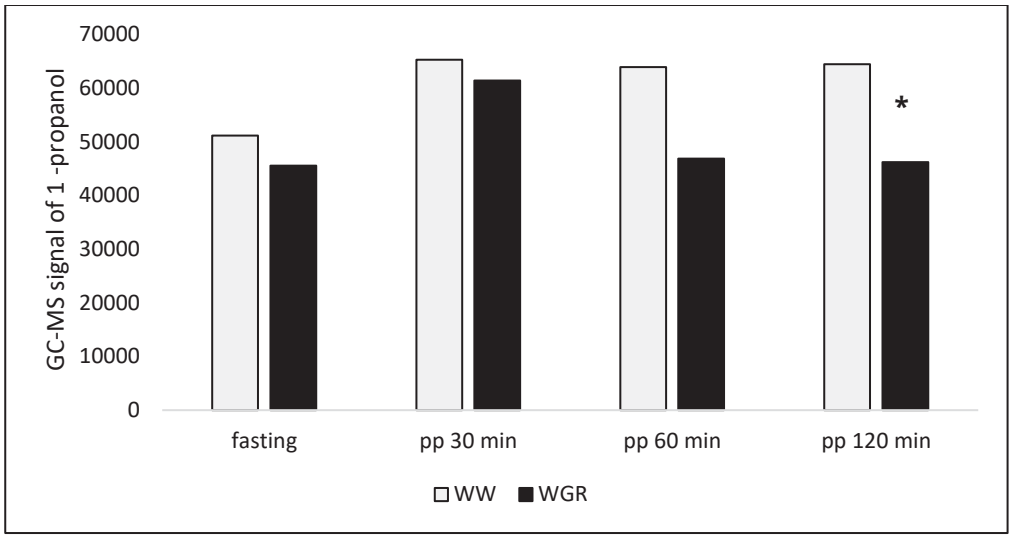

Figure 5.2 Average GC-MS signal intensities of exhaled breath 1-propanol after the low-fibre (WW, dietary fibre 16.9 g/d) and high-fibre (WGR, dietary fibre 43.7 g/d) diets in seven participants in the fasting state and postprandially after the test meal (pp), *p < 0.10 (Study I)

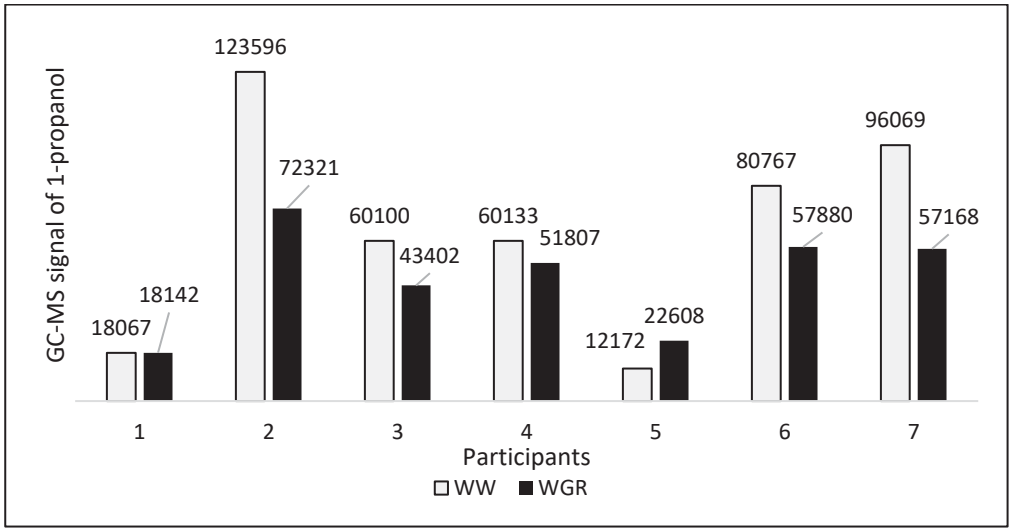

Figure 5.3 Average GC-MS signal intensities of exhaled breath 1-propanol after the low-fibre (WW, dietary fibre 16.9 g/d) and high-fibre (WGR, dietary fibre 43.7 g/d) diets in seven participants at 120 minutes after the test meal (Study I)

Exhaled breath phenol was higher after the WGR diet compared to the WW diet when all the exhaled breath samples were compared between the diets (Wilcoxon test, p = 0.010). Although the difference did not reach statistical significance at any separate sampling point, the phenol was higher in 6 of 7 participants at 60 minutes after the meal and 5 of 7 participants at 120 minutes after the test meal.

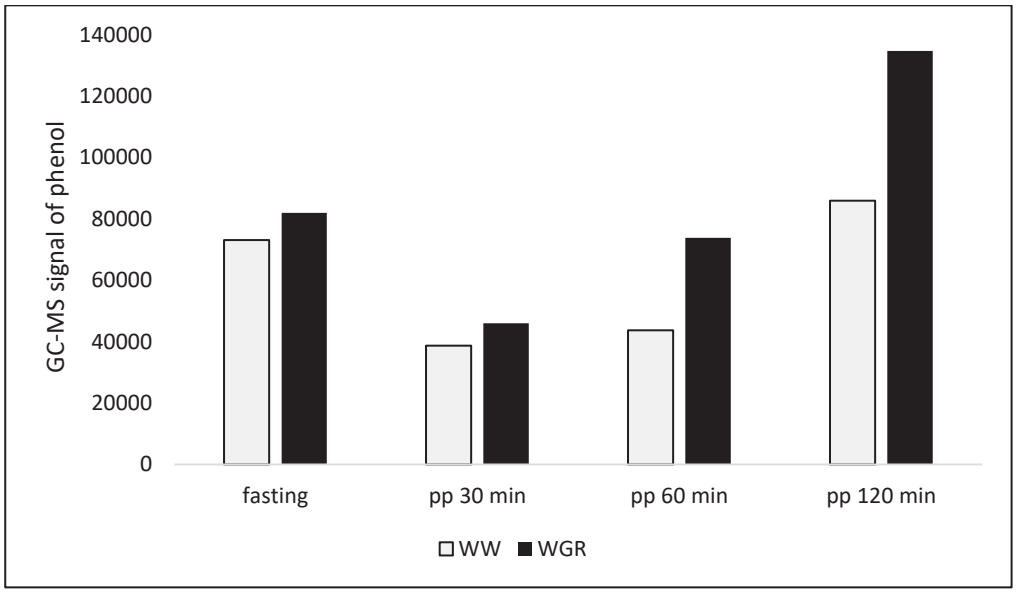

Figure 5.4 Average GC-MS signals of exhaled breath phenol after the low-fibre (WW, dietary fibre 16.9 g/d) and high-fibre (WGR, dietary fibre 43.7 g/d) diets in seven participants in the fasting state and in the postprandially after the meal (pp) (Study I)

5.1.2 **Effect of the test meal on targeted breath VOCs**

The postprandial changes were similar in most subjects in exhaled breath acetone, ethanol, 1-propanol, 1-butanol, acetoin, diacetyl, phenol, propionic acid, and butyric acid, whereas changes in acetic acid, 2-methylbutyric acid, isovaleric acid, valeric acid, hexanoic acid, and 2-propanol were inconsistent between individuals.

Acetone decreased postprandially after the test meal being statistically significantly different between the fasting state and at 30, 60, and 120 minutes (Wilcoxon test, p-values 0.091, 0.063, and 0.018, respectively) after the WGR diet but after the WW diet only between time points 0 and 120 minutes (p=0.063).

The postprandial change in exhaled ethanol reflected that of plasma glucose, increasing significantly after both diets at 30 minutes after the test meal (0 vs 30 min, p = 0.018). Similarly, 1-propanol increased after both diets at 30 minutes after

the test meal (WGR p = 0.028 and WW p = 0.091), but unlike ethanol, it did not return to the fasting level at 60 minutes after the test meal after the WW diet (Figure 5.2). On the contrary, 1-butanol decreased postprandially below the fasting levels after both diets (statistically significant after the WGR diet at 0 vs 60 min, p = 0.063, and after the WW diet at 0 vs 30 min, p = 0.018, and 0 vs 120 min, p = 0.043).

Acetoin increased postprandially after both diet periods (statistically significant at 0 vs 30 min, p = 0.046 and 0.018 after the WGR and WW diets, respectively), whereas diacetyl decreased (statistically significant at 0 vs 120 min, p = 0.091 and 0.018 after the WGR and WW diets, respectively).

Phenol decreased significantly after the WW diet at 30 and 60 minutes after the test meal (p = 0.043 and 0.028, respectively), and increased at 120 minutes after the test meal (60 vs 120 min, p = 0.018 after both diets). Also, exhaled propionic acid and butyric acid increased at 30 minutes in most subjects after the test meal (WGR 6/7 and WW 5/7 subjects) similarly to ethanol and acetoin, although the increase did not reach statistical significance (p = 0.128).

5.2 EXHALED BREATH ASPIRATION IMS PROFILES (STUDY II)

5.2.1 **Effect of WG rye diet on breath profiles**

The exhaled breath AIMS signal intensities (variance scaled each AIMS channel separately) in the fasting state decreased after the WGR diet being statistically significantly different compared to the WW and WW+BRB diet periods (Hotelling´s t-test for principal components of AIMS channels´ signals, p = 0.023 and 0.026, respectively) (Figure 5.5). Signal intensities in the fasting state decreased in all AIMS channels except in channel 3, producing statistically significantly lower AIMS sum signal after the WGR diet compared to the WW diet (Wilcoxon test, p= 0.043) in 6 of 7 participants (Figure 5.6). Also, the AIMS signals intensities at 30 minutes after the test meal tended to be different between the WGR and WW diet periods (Hotelling´s t-test for principal components of AIMS channels, p=0.078).

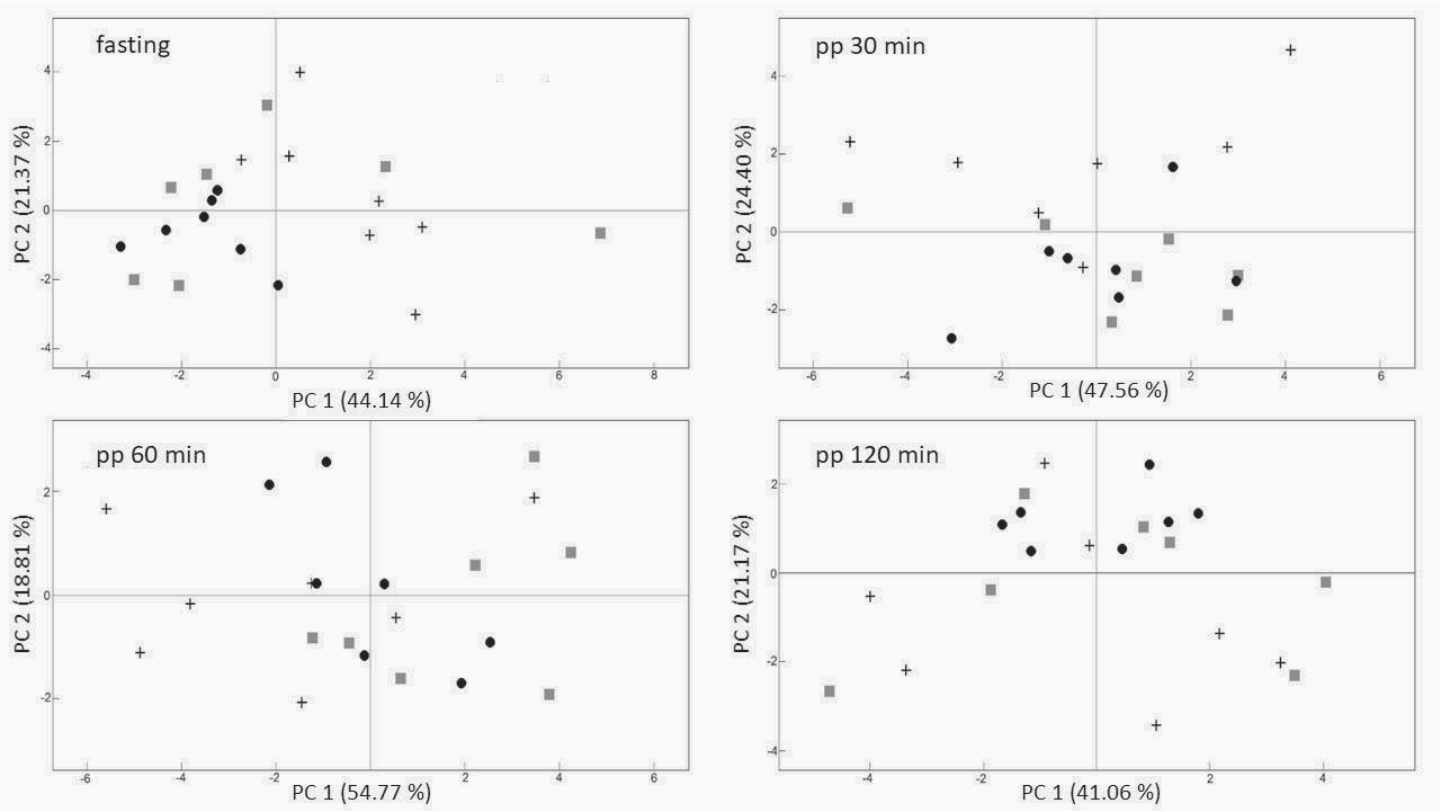

Figure 5.5 PCA Score Plots for AIMS signal intensities of exhaled breath samples of seven participants in fasting state, and postprandially after the test meal (pp) after three 4-week diet periods; WW = control diet with white wheat bread (cross), WGR = high DF diet with sourdough wholegrain rye bread (black dot), WW+BRB = high DF diet with white wheat bread enriched with bioprocessed rye bran (grey box). The score plots (not published before) are made with free version of LatentiX software (LatentiX Aps, Denmark). (Study II)

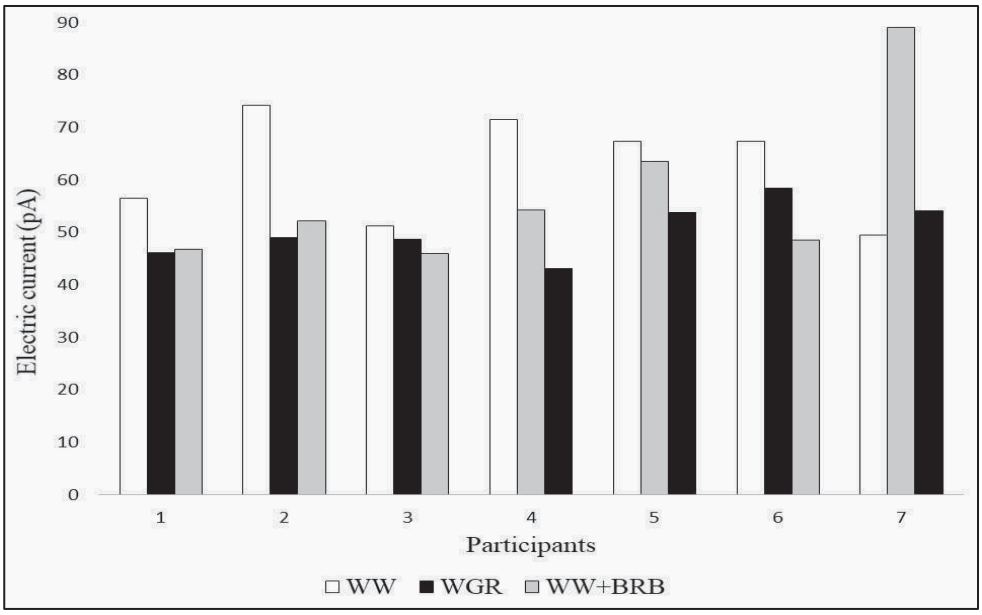

Figure 5.6 Average AIMS sum signals of exhaled breath fasting samples of seven participants after three 4-week diet periods. WW = control diet with white wheat bread; WGR = high DF diet with sourdough wholegrain rye bread; WW+BRB = high DF diet with white wheat bread enriched with bioprocessed rye bran. The order of the columns is according to the order of diets in each participant. (Study II)

Also, the exhaled AIMS profiles (variance scaled each sample separately) tended to be different between the WGR and WW diets in the fasting state (Hotelling´s t-test for principal components of AIMS channels, $p = 0.065$) and 30 minutes after the test meal ($p = 0.074$), and between the WW+BRB and WW diets at 30 minutes after the test meal ($p = 0.098$).

5.3 NON-TARGETED GCXGC-MS ANALYSIS (STUDY III)

About 260 VOCs were detected from exhaled breath samples from nine participants with the selected analysis protocol. In these, about 40 were common VOCs, presenting in more than half of the samples. Only 13 of these were found in all exhaled breath samples, i.e. carbon dioxide, isoprene, acetone, ethanol, 1-butanol, 2-propanol, benzene, benzaldehyde, methyl vinyl ketone, 2-butanone, phenol, hexanoic acid, and acetonitrile. Only 86 VOCs were tentatively identified by

their MS spectra, while about 150 VOCs remained unidentified, as their MS spectra were not found in the MS libraries.

There were no specific VOCs relating to WGR diet, i.e., there was not a compound which was present in all the exhaled breath samples after the WGR diet, but not after the WW or WGW diets. Diphenyl ethanedione and 5-dodecyl-dihydro-(3H)-furanone were detected only after the WGR diet, however, only in 2 of 7 exhaled breath samples.

There were some derivatives of benzoic acid and phenolic compounds detected in exhaled breath samples only after the WG diets. Phthalic acid or phthalic anhydride (similarity index 93 for both) was found in 57% of the breath samples after the WGR diet, and in 11 % of the breath samples after the WGW diet, but in none of the breath samples after the WW diet. Benzoic acid was detected in 29 % of the breath samples during the WGR diet and in 11% during the WGW diet, but in none of the breath samples after the WW diet. Also, some furanones (γ-lactones) were detected in the exhaled breath only after the WG diets: 5-dodecyldihydro-(3H)-furanone (in two participants after the WGR diet), dihydro-4-hydroxy-2(3H)-furanone (in one participant after the WGR and WGW diets) and dihydro-5-tetradecyl-2(3H)-furanone (in one participant after the WGR and WGW diets).

5.4 REFERENCE MEASUREMENTS

Study I

The activity level and the energy expenditure, monitored by the Polar activity computers, remained stable throuhout the study. Mild changes were observed in bowel function and body weight after the diet periods: the frequency of defecation increased (WW 1.3 per day, WGR 2.1 per day, Wilcoxon test $p = 0.028$) and body weight decreased slightly during the WGR diet (WW 85.2 kg, WGR 84.3 kg, Wilcoxon test $p = 0.091$). There were no significant differences between the diets in the blood parametres, except decrease in postprandial insulin response at 60 minutes after the WGR diet (Wilcoxon´s test, $p = 0.043$).

Study II

The fasting level of exhaled breath hydrogen tended to decrease during the WGR diet (WW 7.4 ppm, WGR 2.3 ppm and WW+BRB 6.1 ppm, Friedman test $p = 0.060$, between the WGR and WW with Wilcoxon test, $p < 0.05$), although this was not

seen in the large population in the FIBREFECTS study [293] (result not published). The fasting blood parameters remained stable during the study, except a minor decrease in plasma glucose after the high-fibre periods (WW 5.2 mmol/l, WGR 4.7 mmol/l and WW+BRB 4.8 mmol/l, Friedman test p = 0.015), and slight increase in plasma triglycerides (WW 1.0 mmol/l, WGR 1.3 mmol/l and WW+BRB 1.1 mmol/l, Friedman test, p = 0.060), plasma SCFA (WW 78 µmol/l, WGR 100 µmol/l and WW+BRB 98 µmol/l, Friedman test p = 0.066) and plasma acetate (WW 74 µmol/l, WGR 96 µmol/l and WW+BRB 95 µmol/l, Friedman test p = 0.066). No correlations were found between the exhaled breath AIMS signals and breath hydrogen or plasma glucose. However, there was a borderline significant associations between fasting AIMS sum signal and plasma SCFA (Spearman´s correlations 0.379, p = 0.090) and plasma acetate (Spearman´s correlations 0.395, p = 0.077).

6 DISCUSSION

The main objective of this thesis was to explore the potential of exhaled breath analysis in studying health effects of rye and grain fibre in dietary interventions. This was implemented by investigating the changes in exhaled breath VOCs after diets rich in WG rye using various VOC analysis technologies.

6.1 RESULTS

6.1.1 Effect of WG rye diet on exhaled breath VOCs

In Study I, the changes in 15 targeted exhaled breath VOCs were monitored between the WW and WGR diets. Consistent changes were seen in three exhaled breath VOCs: 2-methylbutyric acid, 1-propanol and phenol.

2-Methylbutyric acid is one of the BCFAs formed during fermentation of amino acid isoleucine [361]. The subjects consumed sourdough fermented WG rye bread during the WGR period, having therefore more DF than in the WW period. It is likely that high DF content in the WGR diet accelerated fermentation and production of SCFAs in the gut [362] leading to decreased colon pH and attenuated production of BCFAs. It is demonstrated previously that increased production of BCFAs are associated with adaptation of microbes to elevated pH conditions [363]. Gut fermentation was not monitored in Study I, but in Study II, fasting plasma SCFAs and acetate tended to increase and isobutyrate decrease after the high-fibre diets, althought the differences were not statistically significant in the small study group. It is noteworthy, that decreased levels of exhaled breath 2-methylbutyric acid after the WGR diet were seen only in the fasting state. It is possible that meal is a confounding factor to monitor fermentation related VOCs since some fermentation can occur also in the mouth. This could be seen in the rise of exhaled breath ethanol, propionic acid, and butyric acid in the postprandial test. Also the sampling time can be crucial when observing fermentation related compounds. It is demonstrared that DF affects the exhaled breath volatiles mainly 5-10 hours after the ingestion [125,176]. Therefore the effect of DF may be not observed if monitored at the time when fermentation is not occuring actively. This can explain also the attenuated breath hydrogen levels after the WGR in the fasting state in Study II. To our knowledge, 2-methylbutyric acid has not been detected from exhaled breath previously [7], and it should be studied further to

understand its role in metabolism and to confirm its relation to gut fermentation. Production of SCFAs is suggested to play a key role in the health effects of DF and WGs, and 2-methylbutyrate is therefore an interesting compound to study further.

The attenuated level of exhaled 1-propanol after the WGR diet as compared with the WW diet was an unexpected new finding. Although 1-propanol is a common compound in human breath, its role in human metabolism is vague. Elevated levels of exhaled 1-propanol have been associated with lung cancer [364] and halitosis [365], but the underlying mechanisms are unknown. Recently, Biagini et al [197] discussed that increased level of breath 1-propanol could be related to bacterial fermentation of amino acids threonine and isoleucine [366,367]. This suggests that in addition to 2-methylbutyric acid, exhaled breath 1-propanol could also indicate increased intestinal fermentation of proteins when less DF is available. However, further studies are needed to confirm these findings and the relations of these breath VOCs to gut fermentation.

We also observed that exhaled breath phenol tended to be higher in the WGR diet compared to WW diet especially at later postprandial states. This is an interesting finding, which should be studied further, although the result didn´t reach statistical significance at separate sampling points with seven participants. The WGR diet contained WG rye rich in various phenolic compounds, such as phenolic acids, AR and lignans [220]. It has been shown that consumption of rye increases levels of various phenolic compounds in body, such as AR in plasma and ferulic acid in urine [232]. It is possible that these compounds are further metabolized and partly excreted via exhaled breath as phenol. Phenol was detected in all the exhaled breath samples in Studies I and III, indicating that it is a common compound in exhaled breath and likely to have multiple sources. Phenol is known to be produced also in fermentation of amino acid tyrosine in the human large intestine, especially at neutral to alkaline pH in the distal colon [368]. Concequently, it is not likely that phenol is increased in the WGR diet due to amino acid fermentation.

In Study II, the exhaled breath AIMS signal intensities were different in diets differing in DF content in the fasting state. Especially the WGR diet could be distinquished from the WW diet with exhaled breath profiles, both with AIMS signal intensities and AIMS profiles. The same observation was demonstrated also in our previous study [369], indicating that high intake of sourdough fermented rye bread causes a metabolic change, which can be monitored from exhaled breath. There was more variance in the exhaled breath AIMS profiles after the WW and WW+BRB diets compared to WGR. This can be seen in the PCA score plot figures

(Figure 5.5) where the exhaled breath AIMS profiles in the WGR are close to each other. This suggests that there might be specific exhaled breath VOCs related to WGR diet. On the other hand, the decreased AIMS signals in the WGR diet indicate that there was decreased concentration of AIMS sensitive VOCs in the exhaled breath [354,370]. However, the limitation of the AIMS technology is that it does not identify the detected compounds, only changes in the VOC profiles.

In Study III, specific VOCs relating to WG rye diet were not detected. Our study strengthened the impression that there are wide differences in the composition of exhaled breath between individuals, as concluded in other studies [7,14]. There were also a huge number of unidentified VOCs, whose MS spectra could not be found in the MS libraries. This indicates that there might still exist numerous unidentified molecules in the exhaled breath because GC-MS is a standard technology for identifying VOCs, and identification is mainly based on the MS libraries. One reason for that can be that most GC-MS analyses are made by using non-polar columns, which separate non-polar VOCs. Also, there are not many non-targeted approaches which have monitored exhaled breath with GCxGC systems [8,80-85,371].

We did not find any specific VOC relating to WGR diet. However, a few benzoic acid and phenolic derivatives, as well as furanones, were detected from exhaled breath samples only after the WG diets. It is possible that these VOCs are degradation products of phenolic compounds such as phenolic acids, AR and lignans from the DF complex in the bran. Phenolic compounds can be metabolized to various compounds by colonic fermentation and metabolism [372], for example, benzoic acid can be formed from rye phenolics [362]. However, it is metabolized further to hippurate within a few hours from ingesting by liver and kidneys. Therefore, it cannot be a feasible biomarker compounds for intake of rye or WG. However, the fermentation related VOCs are interesting to study further to reveal possible mechanisms behind the health effects of rye and DF. In these studies, orocecal transit time and timing of eating WG should be controlled to monitor these VOCs from exhaled breath.

6.1.2 **Effect of a single meal on exhaled breath VOCs**

Ingesting a meal can be a confounding factor to breath tests since some of the consumed food can remain in the oral cavity and lead to released VOCs [373,374]. A standardized meal test is commonly used in nutritional studies to monitor postprandial glucose metabolism, and health effects of rye bread are expected to associate to attenuated postprandial insulin levels [298,307,308]. Therefore in Studies I and II we performed exhaled breath analyses also postprandially after a

stardardized meal. In tudy I, some fermentation related exhaled breath VOCs (ethanol, 1-propanol, acetoin, propionic and butyric acid) increased 30 minutes after the meal in most participants. Postprandial increases in these compounds have been observed also in other studies [80,98,153,175,180-182]. The increased levels of these VOCs are speculated to be mainly due to the residues of the ingested food and their bacterial fermentation in the mouth. This can be one cause although in our study the participants were brushing their teeth and spurting with water before and after the meal. However, elevated level of exhaled breath ethanol has been detected also after intravenously administered glucose without an oral injection [184]. Moreover, we observed a difference in levels of 1-propanol between the WGR and WW diets in later postprandial phase, although the test meal was the same after both diet periods. This suggest that postprandial increase in these VOCs can be associated also to increased metabolic activity in the gut or in the liver. It is also noteworthy that we did not detect similar postprandial increase in all the compounds known to be released in bacterial fermentation, such as acetic acid.

There were consistent changes also in breath acetone, 1-butanol, diacetyl and phenol. Acetone levels decreased after the meal. This was expected since the major source of exhaled acetone is decarboxylation of acetoacetate which arises from lipolysis and ingesting a meal likely reduces the lipolysis. The same has been observed also in other studies [175,180-183]. However, in some meal studies with high intake of proteins, the acetone level has rised postprandially since acetone is produced also in the breakdown of glycogenic amino acids [153,177].

Exhaled breath 1-butanol and diacetyl decreased postprandially. However, it is unclear what caused the postprandial reduction of these compounds. 1-butanol can be generated by gut microflora, e.g, by lactic acid bacteria [375]. The elevated levels of these compounds in exhaled breath are associated in a few studies to inflammation [376,377] and lung cancer [378], where 1-butanol is discussed to be an oxidative product of butane and having an anti-inflammatory effect. Exhaled breath 1-butanol levels seem to decrease also shortly after various intravenous injections in rat studies [376,377], although these studies were not actually monitoring this. Diacetyl is produced during fermentation of lactic acid bacteria [379] and yeasts [380], and it is related also to the chronic polymicrobial infections in lungs in persons suffering from cystic fibrosis [381]. Exhaled diacetyl was reported to increase after glucose ingestion in most participants but decreased in some participants [181]. Diacetyl is an aroma compound associated to a buttery flavour especially in dairy products [382] and detected to be one of the key aromas also in white wheat bread

[383]. However, increased postprandial diacetyl levels were not observed in our standardized meal test containing white wheat bread.

Phenol was slightly decreased postprandially (statistically significant only after the WW diet), but at 120 minutes after the meal an increase was observed. Also, Pugliese et al [177] reported increased exhaled breath phenol levels after the intake of protein drinks. They postulated that this was due to the breakdown of amino acids such as tyrosine by intestinal bacteria.

In Study II, the different sampling points (fasting and postprandial sampling points 30, 60 and 120 minutes after the meal) could not be separated with the PCA from the exhaled breath AIMS profiles. However, there were some consistent patterns observed when AIMS profiles were compared during the same diet, for example the AIMS profiles between the postprandial sampling points 30 and 60 minutes after the meal could be separated with PCA from each other in WW, WGR and WW+BRB diet, but not from AIMS profiles in fasting state or postprandially 120 minutes after the meal (the results not published). The difference between the pp30 and pp60 can reflect the observed postprandial chances in VOCs in Study I, but this should be confirmed in parallel measurements. However, AIMS technology seems not to be applicable to monitor postprandial changes in exhaled breath due to increased variation in breath VOCs after the meal.

6.2 METHODOLOGICAL CONSIDERATIONS

6.2.1 Dietary interventions

All the dietary interventions in this thesis work had a randomized crossover design, i.e., the participants followed all the study diets, and at least two of the diets per study were in randomized order. However, the study designs varied. In Study I, there were one-week WW and WGR diets in randomized crossover design having two-week washout periods before both diet periods. In Study II, there were four-week WGR and WW+BRB diets in randomized crossover design, and four-week WW period in the beginning of the study. In Study III, there were one-week WGR and WGW periods in randomized crossover design and one-week WW periods before the WG diets. The randomized crossover design is preferable when monitoring dietary changes. When changes are compared between the diets within the same participant under a controlled protocol, it is possible to monitor

individual responses and identify consistent changes despite individual dietary responses and interindividual variation in VOCs.

Nevertheless, all the interventions included the WW diet containing white wheat bread and the WGR diet containing sourdough fermented rye breath, and there were significant differences in the DF intake between the diets (17 vs. 44, 24 vs. 34, and 24/25 vs. 36 g/day, in Studies I, II and III, respectively). The diets in the interventions were successful since there were no differences in other dietary factors than in the DF intake. However, in Studies II and III, the DF levels in the WW diets were at the minimum levels of dietary recommendations [274] because the participants consumed plenty of fruits and vegetables thus receiving DF, and probably also phenolic compounds from other sources than cereal DF. The bread intake was controlled in Studies II and III, but not in Study I, although also in Study I, the participants were advised to choose rye products during the high-fibre diet. The amount of eaten WG rye, or rye DF was not controlled, although it could have been monitored for example by analyzing the plasma AR. It is therefore possible, that the sources of DF varied between individuals and increased the variation also in exhaled breath VOCs.

The dietary interventions and the reference measurements in Studies I and II were planned to monitor the metabolic effects of WGR diets, the focus being on glucose metabolism. Therefore, the postprandial tests were included. The exhaled breath measurements, being non-invasive, were easy to add to the dietary intervention protocols. However, it seems that exhaled breath analyses are not optimal in postprandial meal tests, since ingesting a meal changes the levels of some breath VOCs temporarily, especially those associated with gut fermentation. Whether this is due to oral fermentation or increased metabolic activity in the body, it increases the interpersonal variation. Therefore, we decided to take only fasting samples in Study III. However, based on the results of our studies, it could be more interesting to monitor exhaled breath at the time when ingested fibre enters the colon and is fermented [125,176], i.e., postprandially 5-10 hours after the meal. Breath hydrogen measurements could be utilized to monitor the fermentation status. Also, the fecal microbiota and its methane-producing status could be analysed to reveal their role in individual responses to rye DF. However, it should be always considered in study interventions that the protocol is not too laborious for participants. Otherwise, it can weaken the research compliance.

The number of subjects in our studies was very small, ranging from seven to nine. This is typical in studies analyzing exhaled breath VOCs (e.g., in Table 2.2), since there are multiple confounding factors to be controlled and analysis should

be done mainly in the laboratories. It is also common in exploratory studies, where the focus is to find new phenomena and associations to study further. When the number of subjects is small, the interindividual variation has a large effect, and it is difficult to attain statistically significant results. Therefore, not many conclutions can be drawn from the results of these pilot studies. Instead, they can indicate which changes in exhaled breath are potential targets to further studies.

6.2.2 **Exhaled breath measurements**

The analysis methologies suitable for different kinds of research targets were used, developed and piloted during the PhD study. In Study I, a method was built for monitoring targeted exhaled breath VOCs, in Study II, a type of e-nose was applied to exhaled breath analysis to monitor changes in exhaled breath AIMS profiles, and in Study III, a non-targeted analysis was performed to explore exhaled breath VOCs.

Sampling

In all the piloted analysis methods, sampling was done with commercial breath sampler designed to capture the alveolar breath. This was chosen, because we wanted to monitor endogenous VOCs, and alveolar breath is rich of compounds coming from the circulation. The Bio-VOC sampler was easy to use, and could be applied with various preconcentration methods, in Study I with SPME, and in Study III with sorbent liners, whereas in Study II, it was tested with the ChemPro100i handheld chemical detector. To the SPME application, teflon adapters with septa were made to close the breath samplers immidiately after sampling and to connect SPME devices compactly to the breath samplers during the preconcentration. Teflon adapters were sculptured also for the TD sorbent liners to make TD liners to fit better to the outlet of the breath samplers. The disadvantage of the Bio-VOC sampler is, that the sample must be transferred quickly further to the sorbent material or to the online analysis, otherwise it will be lost. The Bio-VOC sampler has been evaluated by Kwak et al [384].

The sampling and the handling of the sample are crucial steps in breath analysis. However, they are difficult to control. The breath sampling was controlled in all our studies only by watching. The blowing technique was shown by the researcher to the participants. However, the participants could not practise the blowing because it could have affected the VOC concentrations. Therefore, it is

possible that there were minor individual differences in breath sampling. However, it was obvious that the participants gave an alveolar breath sample every time.

Deuterated acetone was used as an IS in Studies I and III. In Study I, the IS was injected into the Bio-VOC sampler before the SPME. In that study, the coefficient of variation for IS was 18,3%, which covers the variation in SPME and GC-MS analysis, however, not in breath sampling. In Study III, the IS was injected into the TD sorbent tube with the Bio-VOC sampler before the exhaled breath sampling and monitored only qualitatively, i.e., it was checked by the researcher, that the IS was included in all the samples, and the signal seemed to be at adequate level and coming at expected RT. However, the signal was not quantified, and the variation could not be calculated. On the other hand, this was not crucial in Study III, since the exhaled breath VOCs were monitored only qualitatively, i.e., if they were present in the sample or not.

Preconcentration is needed in indirect VOC analysis. The preconcentration is momentous since it defines which VOCs are gaptured from the sample and can be analysed. It is important that the circumstances in the preconcentration process are stable. Therefore, extra attention was paid to make preconcentration conditions as constant as possible. This was time consuming and required strict protocols in the preconcentration, but also in conditioning and transporting steps. In Study I, the preconcentration was done with the SPME utilizing the portable field samplers, and in Study III with sorptive TD liners utilizing storage gaps for the liners. Attention was paid also to materials not releasing VOCs and room air to be as odorless as possible. We made a lot of pretests (not reported) to find optimal conditions and protocols to the preconcentration and made compromised choices that were confirming the preconcentration for many interesting compounds rather than optimized preconcentration for a single compound. For example, the chosen SPME fibre, CAR-PDMS, was found to be suitable to capture many interesting compounds, and the preconcentration time was tested to be long enough for all the targeted VOCs. However, we didn´t make comprehensive validations for the preconcentration methods because this would have required mimiching the conditions in exhaled breath sampling and testing also the possible competitive adsorptions for the VOCs. Therefore, the preconcentration methods tested in these studies are rather premethods which could be developed further for more specified research targets. Because of the complicated sorption kinetics of the preconcentration methods [59,62], we think that these are not fitting very well for exhaled breath sampling, concerning the hight humidity of the breath and large variation in exhaled breath compounds. However, these are needed if the VOC

sample is not possible to analyse online, and in some cases also to increase the sensitivity of the analysis.

GC-MS analyses

GC-MS technology is a standard technology to identify VOCs, and since it was an obvious choise to start with. In Study I, a kind of traditional GC-MS having one column and quadrupole type MS was applied to monitor changes in targeted VOCs. Various GC columns were pretested (not reported), and finally the Nukol column was chosen, because it was developed especially for separating free fatty acids (e.g., SCFAs) and other polar compounds. A set of compounds were selected for the targeted analysis because they were known to be related to gut fermentation or glucose metabolism, and they were also possible to detect with the developed method. The sensitivity for the detection was increased by using SIM mode. Otherwise, the signals would have been too small with some of the compounds. However, using the SIM mode, it is not possible to detect other than the targeted VOCs. The changes in VOCs were detected in signal intensities, since we noticed that the quantification of exhaled breath compounds is a challenging task. To use the SPME-GC-MS method for quantitative breath analysis, it should be calibrated with each compound of interest in conditions comparable to the breath air (same temperature and humidity), examining also the possible competitive sorption by VOCs during SPME preconcentration. However, the quantification of the VOCs was not necessary for detecting changes in the levels of compounds.

In Study III, GCxGC-MS was applied to monitor which VOCs are present in the exhaled breath samples after the diets. The MS detection was done with the SCAN mode instead of SIM, and the GC separation and the data analysis were completely different compared to the GC-MS method used in Study I. This technology is designed for qualitative monitoring for complex sampling matrixes; however, it has not been tested for breath analysis before. In GC separation, we used the same first column than in Study I, and the stationary phase in the second column was chosen to be chemically different to enable additional chromatographic separation. However, various options for the column set were not tested. It is noteworthy, that selection of absorption material in the preconcentration and choice of the GC columns determine which VOCs can be detected. The GCxGC technology had improved separation for the VOCs, e.g., three compounds were detected with very similar MS spectrum with isoprene but with slightly different RTs. It is noteworthy that these compounds can be erroneously identified as isoprene, and therefore interfere the quantification of

isoprene if they are not separated in the GC analysis. Also, the sensitivity was improved due to cryogenic modulation, and thus the number of compounds was increased compared to tradional GC. With the protocol used in Study I, the number of breath VOCs analysed in the SCAN mode was about 40 (not reported), whereas with GCxGC it was about 260. However, this technology is not optimal for quantitative detection. In complex matrices, it is typical that there are compounds in various concentrations. Since there are VOCs, which signals are overloading and cannot be quantified or even idenfied, and if the sensitivity is decreased or sample is diluted, the VOCs with lower concentration are missed. The quantification for the VOCs could be done with separate GC methods optimal for the targeted VOCs. The two-dimensional data was analysed with the Chromsquare software, which is suitable for visual monitoring of the data, and for library identification of the selected blobs, but not very handy for further data analysis with two-dimensional data. This is another reason that even the levels of the VOCs weren´t monitored in Study III. Nonetheless, I think that it would be more relevant to monitor changes in their levels rather than searching for specific biomarker compounds, since exhaled breath VOCs might have multiple sources. However, non-targeted volatomic analysis can be used to select the relevant target compounds to study further.

GC-MS technologies are mainly for research purposes, for example in exploring biomarker VOCs to be studied further. GC-MS methods are sensitive and selective, and therefore superior in identifying compounds, but not very convenient for clinical studies requiring expensive and robust laboratory devices and specialized lab personnel for their use. Perhaps, for these reasons, the GC-MS analyses have not become as routine analytics for breath VOCs but remain as a popular research tool.

AIMS analysis

We had tested the aspiration IMS technology before for food quality assessment and monitoring of microbial fermentation and growth [385-386]. In the Ravidi study, we tested it for the first time to detect changes in exhaled breath profiles [369], and found it to be promising to detect changes in exhaled breath after dietary changes. In Study II, the methodology was applied with minor changes for the Fibrefects intervention [235].

Only the IMCell and the humidity sensor were utilized for the measurements, although there are also some other sensors involved in ChemPro100i. IMCell is known to be sensitive for water. Therefore, the MCC was used to delay the water, which is always high in exhaled breath samples. This was working well since the

AIMS signal from the breath VOCs came before the humidity signal giving a signal peak or slope to the AIMS signals. We ended up taking the AIMS signals from the maximum point of the AIMS sum signals and compared these in the PCA analysis. The signal was zero levelled to the filtered background air every time before the analysis, which mean that the signal levels were related to the room air and therefore changes in the room air (e.g, humidity level or occuring VOCs) can affect the signals. However, attention was paid to keep them as constant as possible, and a carbon filter was used in the inlet of the device before the sampling because of that. We found the AIMS having potential to detect changes in the exhaled breath, although we used it in very simple way. It is noteworthy, that there are also many alternative ways to monitor the AIMS signal. The signals could be taken from each AIMS channels separately, and from the whole signal slopes instead of the selected time point. Also, the information from the various sensors could be involved. These would require sophistecated data analysis tools to interpret the signals. Nonetheless, we think that there is potential in the technology to develop it further to the exhaled breath analysis.

7 CONCLUSIONS

The results obtained in this PhD thesis showed that high-fibre diet containing WG rye affects the composition of exhaled breath, and consequently exhaled breath analysis can be used in studying the health effects of rye and grain fibre. Based on these findings, it seems the exhaled breath VOCs have potential to give new information on the metabolic effects of foods and the associated health effects.

Consistent changes were seen after the WG rye diet in exhaled breath 1-propanol, 2-methylbutyric acid and phenol, and in the exhaled breath AIMS profiles. Based on the non-targeted GCxGC-MS analysis, it seems that benzoic acid derivatives, phenolic compounds and furanones can be related to WG diets. The fermentation-associated compounds should be studied further. Especially, compounds related to the fermentation of amino acids could be indicative of low- DF diets.

Timing is critical in breath sampling when monitoring effects of dietary components. In our study, it was possible to separate the exhaled breath AIMS profiles between the different diets only in the fasting state, since ingesting a meal increased variation especially in the fermentation-associated exhaled breath VOCs. The effects of WGs to exhaled breath VOCs would be interesting to monitor at the time when ingested DF is potentially fermented in the gut, i.e., 5-10 hours after the meal.

Based on our findings, it seems that diet induced changes should be monitored rather in the VOC profiles than in individual compounds. Exhaled breath VOCs typically have multiple sources and people display large variation in exhaled breath VOCs. Furthermore, it seems that there are plenty of unidentified exhaled breath VOCs. GC-MS devices are superior to identify the VOCs. However, online analysis methods, like e-noses, are more promising in developing rapid monitoring tools for example for identifying metabolic states relating to diets. However, applicable data-analysis tools are essential to identify the patterns in the VOC profiles, which could be related to diet-associated metabolic factors.

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ORIGINAL PUBLICATIONS (I – III)

I

Fiber content of diet affects exhaled breath volatiles in fasting and postprandial state in a pilot crossover study

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Fiber content of diet affects exhaled breath volatiles in fasting and postprandial state in a pilot crossover study

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ABSTRACT

Our pilot study examined the potential of exhaled breath analysis in studying the metabolic effects of dietary fiber (DF). We hypothesized that a high-fiber diet (HFD) containing whole grain rye changes volatile organic compound (VOC) levels in exhaled breath and that consuming a single meal affects these levels. Seven healthy men followed a week-long low-fiber diet (17 g/d) and HFD (44 g/d) in a randomized crossover design. A test meal containing 50 g of the available carbohydrates from wheat bread was served as breakfast after each week. Alveolar exhaled breath samples were analyzed at fasting state and 30, 60, and 120 minutes after this meal parallel to plasma glucose, insulin, and serum lipids. We used solid-phase microextraction and gas chromatography–mass spectrometry for detecting changes in 15 VOCs. These VOCs were acetone, ethanol, 1-propanol, 2-propanol, 1-butanol, acetic acid, propionic acid, butyric acid, valeric acid, isovaleric acid, 2-methylbutyric acid, hexanoic acid, acetoin, diacetyl, and phenol. Exhaled breath 2-methylbutyric acid in the fasting state and 1-propanol at 120 minutes decreased ($P = .091$ for both) after an HFD. Ingestion of the test meal increased ethanol, 1-propanol, acetoin, propionic acid, and butyric acid levels while reducing acetone, 1-butanol, diacetyl, and phenol levels. Both DF diet content and having a single meal affected breathVOCs. Exploring exhaled breath further could help to develop tools for monitoring the metabolic effects of DF.

1. Introduction

Epidemiological studies demonstrate that intakes of dietary fiber (DF) and whole grain foods is inversely related to many chronic diseases including type 2 diabetes [1]. Rye bread, a major source of whole grain and DF in the Northern Europe, has beneficial effects on postprandial insulin responses [2,3] and glucose metabolism after prolonged use [4]. The

Abbreviations: BCFA, branched-chain fatty acid; DF, dietary fiber; GC-MS, gas chromatography–mass spectrometry; HFD, high-fiber diet; IS, internal standard; LFD, low-fiber diet; RT, retention time; SCFA, short-chain fatty acid; SPME, solid-phase microextraction; VOC, volatile organic compound.

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mechanisms behind these associations are unclear but may involve gut-related events [5,6]. Production of short-chain fatty acids (SCFAs) and release of phenolic compounds by gut microbes are maybe involved in the improvement of glucose metabolism [7,8]. However, it is challenging to study these mechanisms because of the large variability in human gut microbiota and anaerobic conditions in the gut. New noninvasive tools are warrended to study the gut-related effects of DF.

Effects of nutrients are usually investigated using blood and urine analysis, but exhaled breath is also a potential matrix of increasing interest because of noninvasive sampling and instant responses. There are almost 900 volatile organic compounds (VOCs) in the exhaled breath of healthy humans [9]; thus, it is possible to find new biomarker compounds in exhaled breath relating to a specific metabolic event or quality of diet. Nevertheless, there are essentially only 2 types of breath tests used in the nutritional and gastroenterology studies: ^{13}C-isotope and hydrogen [10], although also methane [11,12] and some VOCs [13] have been considered essential in monitoring gastrointestinal disorders. Thus far, there have been only a few study monitoring the effects of diet on the composition of exhaled breath [14–18]. Many possibilities of measuring microbial VOCs exist for health applications [19,20], such as analyzing VOCs in fecal samples from gastrointestinal patients [21,22]. Exhaled breath could be used to study the gut-related metabolic effects of DF because the bacterial fermentation of DF produces numerous volatile metabolites [6].

Our aim was to examine the potential of exhaled breath analysis to study the metabolic effects of DF. We hypothesized that a diet high in DF containing whole grain rye increases microbial fermentation in the gut, which in turn increases the levels of these VOCs in the circulation and in exhaled breath. Furthermore, we postulated that consuming a single meal changes the levels of these compounds in exhaled breath postprandially by activating the digestion and absorption processes in the gut. To test these hypotheses, a pilot dietary intervention with crossover design with 2 diets different in DF content was conducted; postprandial tests in the end of the diet periods were carried out, and the levels of selected VOCs were monitored from exhaled breath.

2. Methods and materials

2.1. Protocol

A randomized crossover study with 2 different diets was performed. The test diets were a low-fiber diet (LFD) and a high-fiber diet (HFD) both lasting for a week. The subjects followed their habitual diet during a 2-week washout period between the diet periods. They were advised to maintain their body weight and lifestyle habits throughout the study. The compliance was monitored by weighing and using 4-day food records and activity computers (Polar Electro, Finland), which recorded physical activity during the diet periods. Blood and exhaled breath samples were taken in postprandial tests at the end of the diet periods. The study protocol was approved by the Research Ethics Committee, Hospital District of Northern Savo. Each individual provided a written informed consent before participation in the study.

2.2. Study participants

Seven men were recruited with campus advertising in the University of Eastern Finland. The subjects were healthy (no diagnosis of chronic diseases and no evidence of disturbed glucose metabolism), nonsmokers, and 25 to 46 years old (average age 32) and had an average body mass index 26.0 kg/m^2 (21.7-32.5 kg/m^2) and regular eating and exercise habits.

2.3. Diets

The recommended energy level for each subject was calculated using the formula of Mifflin-St Jeor [23], and the amount of DF was adjusted to 7.5 g/1000 kcal (4180 kJ) during the LFD and 15 g/1000 kcal (4180 kJ) during the HFD period. During the LFD, low-fiber cereal products such as white wheat bread, white rice, and pasta were recommended, and during the HFD, high-fiber cereal products such as wholegrain rye bread, wholegrain pasta, and muesli were recommended. The subjects were advised to limit their fruit and vegetable intake to 3-4 servings per day, depending on their estimated energy level, and the intake of berries to 1 dL/wk and to avoid oat and barley porridge, and oat pasta during the diet periods. In addition, they received instructions to avoid foods that affect bowel function, such as legumes, plums, dried fruits, seeds, brans, and licorice. Otherwise, the subjects were supposed to maintain their habitual diet. Compliance with the diets was monitored by 4-day food records maintained by the subjects during the diet periods. Diet32 software (Aivo Finland Oy, Turku, Finland) was used to calculate nutrient intake.

2.4. Postprandial test

Postprandial tests were performed at the end of the diet periods in the morning after an overnight fast of about 12 hours. The subjects were advised to avoid exhausting physical exercise, sauna bathing and heavy meals the previous 24 hours and to abstain from alcohol ingestion 48 hours before the tests. To standardize the bacterial fermentation in the mouth, the subjects were asked to brush their teeth with a toothpaste in the morning of the test day and again without a toothpaste before the first breath sample and after the test meal.

The subjects consumed a test meal containing 50 g of the available carbohydrates (106 g of sliced white wheat bread) and 3 dL energy-free juice (Funlight, Felix Abba Oy Ab, Turku, Finland). The bread was stored frozen and thawed at room temperature before the test. The subjects were advised to consume the test meal within 10 minutes and to use equal amount of time during both test meals.

Blood and breath samples were collected before the meal (fasting) and at 30, 60, and 120 minutes after starting the meal. Venous blood samples were analyzed in the Research Institute of Public Health (University of Eastern Finland, Kuopio, Finland) for glucose and insulin from plasma, and total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides from serum.

2.5. VOC analysis of exhaled breath samples

Alveolar breath samples (150 mL) were taken using the Bio-VOC samplers (Markes International Ltd, Rhondda Cynon Taff, UK). The subjects were trained to blow deeply and slowly through the sampler after normal breathing. The Bio-VOC samplers were closed immediately with Teflon adapters (self-made) with septa (Thermogreen LB-2, Sigma-Aldrich, St. Louis, Missouri, USA). The internal standard (IS, 0.11 μg/μL acetone-d_6, Euriso-top, Saint-Aubin, France) in Milli-Q ultra-pure water (Millipore, Bedford, Massachusetts, USA) was injected into the samplers through the septum.

The exhaled VOCs and IS were extracted by using a 75-μm carboxen-polydimethylsiloxane fiber which was inserted into the Bio-VOC sampler through the septum and kept there for 30 minutes at room temperature. Because sampling was done outside the analysis laboratory, extra attention was paid to storage of the samples. We used a solid-phase microextraction (SPME) portable field sampler (Supelco, Bellefonte, Pensylvania, USA), which stored the SPME needle into the septum, thereby minimizing the desorption of the extracted compounds and absorption of the background compounds during the transportation. The septum was changed after cleaning the fiber (300°C/10 min in the injection port of gas cromatography) and after the extraction. The SPME samplers were transported in fixed polytetrafluoroethylene tubes (Vink Finland, Kerava, Finland) which were plugged with Viton (VWR International, Radnor, Pennsylvania, USA). All samples were analyzed within 2-5 hours of sampling.

Several VOCs were identified from exhaled breath samples in our preliminary studies (not published), and 15 compounds were selected for further examination in the dietary intervention. Alcohols, SCFAs, branched-chain fatty acids (BCFAs), acetoin, diacetyl, and phenol were selected because these are related to gut fermentation [24,25]. Acetone was selected because it is one of the most studied compounds in exhaled breath and related to glucose metabolism [26]. Analysis was performed on a gas chromatography–mass spectrometry (GC-MS) device (GC 5890 series II Plus + MS detector 5972 series, Hewlett Packard Ltd, Wilmington, Delaware, USA) with Nukol capillary column (0.25 μm thick, 0.25 mm internal diameter, 30 m long, Supelco, Bellefonte, Pensylvania, USA) using Helium 4.6 (AGA, Espoo, Finland) as a carrier gas with flow rate 1 mL/min. The injector temperature was 300°C, and split vent was closed for a minute (splitless operation). The GC oven temperature was 45°C for 3 minutes, then raised 15°C/min to 200°C, and kept there for 5 minutes. The detector temperature was 280°C. Detection was done in the following selected ion monitoring windows: retention time (RT) window in minutes 2.1-3.5 and ion 58 for acetone, and 64 for acetone-d_6 (IS); RT 3.5-4.6 and ion 45 for 2-propanol, and ion 46 for ethanol; RT 4.6-5.0 and ion 86 for diacetyl; RT 5.0-6.5 and ion 59 for 1-propanol; RT 6.5-8.5 and ion 56 for 1-butanol; RT 8.5-9.5 and ion 88 for acetoin RT 9.5-14.5 and ion 60 for acetic acid, butyric acid, valeric acid, isovaleric acid, and hexanoic acid, and ion 74 for propionic acid and 2-methylbutyric acid; and RT 14.5-18.33 and ion 94 for phenol (Fig. 1).

The VOCs were identified based on their specific RTs and specific ions formed in mass spectrometry analysis using the Wiley275 mass spectrum library (Hewlett Packard Ltd, Wilmington, Delaware, USA). The recognition was ascertained with commercial analytical grade standard solutions from Supelco (Bellefonte, Pensylvania, USA); however, 1-propanol was from Merck (Darmstadt, Germany), ethanol was from Altia (Helsinki, Finland), and solvent methanol was from Avantor Performance

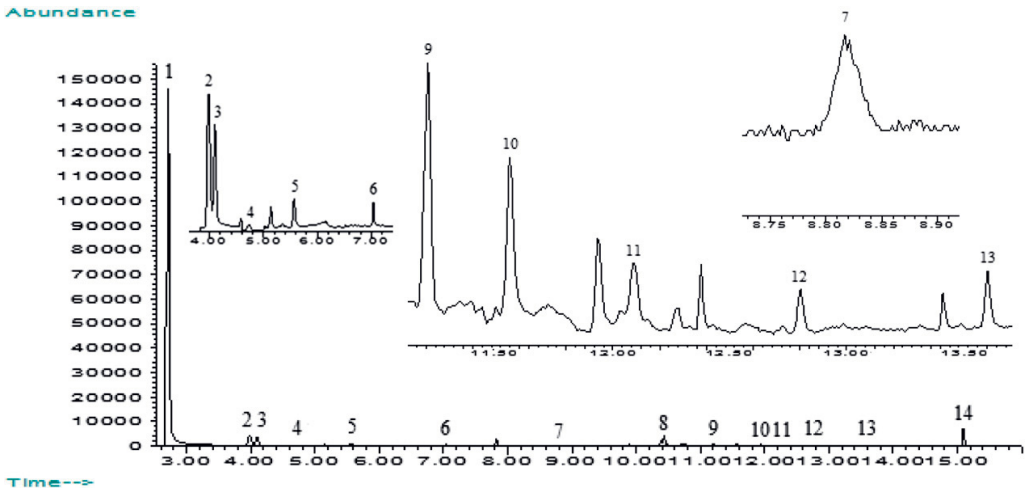

Fig. 1 – GC-MS single ion monitoring cromatogram and 3 zoomed chromatograms of a presentative exhaled breath sample (350 mL) spiked with internal standard (0.11 μg/μL acetone-d_6) and extracted onto SPME fiber (75 μm carboxen-polydimethylsiloxane). Chromatogram peaks of compounds with characteristic ion for each compound (in brackets): (1) acetone (58) and acetone-d_6 (64), (2) 2-propanol (45), (3) ethanol (46), (4) diacetyl (86), (5) 1-propanol (59), (6) 1-butanol (56), (7) acetoin (88), (8) acetic acid (60), (9) propionic acid (74), (10) butyric acid (60), (11) 2-methyl butyric acid (74) and isovaleric acid (60), (12) valeric acid (60), (13) hexanoic acid (60), and (14) phenol (94).

Materials (J.T Baker, Center Valley, Pensylvania, USA). Areas of the GC peaks were calculated from one particular ion of each compound with the MSD Productivity ChemStation software (Hewlett Packard Ltd, Wilmington, Delaware, USA). The signals were proportioned to the peak area of IS to control the variability in sampling and analysis.

2.6. *Statistical analyses*

Nonparametric tests were used to analyze the significance of changes in different time points or between diets (Wilcoxon test with 2 dependent variables and Friedman test with more than 2 dependent variables). Instead of the conventional hypothesis testing limit $P < .05$, differences were considered significant at 2-sided $P < .10$ to reveal interesting parameters for further research despite the small study group. Statistical analysis was performed using the SPSS 19.0 software (SPSS Inc, Chicago, Illinois, USA).

3. Results

3.1. *Nutritional value of diets, energy expenditure, weight, and bowel function*

The intakes of energy, protein, fat and carbohydrates during the test diet periods were maintained at the same level as in the subjects' habitual diet (Table 1). As expected, intakes of total, soluble, and insoluble DF were significantly different between the HFD and LFD periods.

There were no changes between the test diet periods in the activity level and energy expenditure measured by activity computers (Table 1). Mild changes were observed in bowel function and body weight after the diet periods: the frequency of defecation increased and body weight decreased slightly during the HFD.

3.2. *Effects of diets on blood biochemistry*

There were no significant differences between the diets in the fasting values of plasma glucose and insulin, serum total cholesterol, low-density lipoprotein, high-density lipoprotein cholesterol, or serum triglycerides. Postprandial responses in plasma glucose after the single meal were not different between the diet periods, whereas there were reduction in insulin response at 60 minutes in the HFD ($P = .043$).

3.3. *Effects of diets on exhaled breath VOCs*

Signals of 2-methylbutyric acid in the fasting state were lower in the HFD compared with the LFD (Table 2). In the postprandial state, 1-propanol was lower in the HFD (Fig. 2, $P = .042$, Friedman test for all postprandial samples) but reached the statistical significance only at 120 minutes after the meal (means ± SD in the HFD 46,190 ± 19,670 and in the LFD 64,410 ± 40,180; $P = .091$). The diet-induced changes in the other exhaled breath VOCs were variable.

3.4. *Effects of meal on exhaled breath VOCs*

The postprandial changes were similar in most subjects in acetone, ethanol, 1-propanol, 1-butanol, acetoin, diacetyl, phenol, propionic acid, and butyric acid (Fig. 2), whereas changes in acetic acid, 2-methylbutyric acid, isovaleric acid, valeric acid, hexanoic acid, and 2-propanol were inconsistent between individuals. Acetone decreased in the postprandial state, being significantly different in the HFD between fasting state and 30, 60, and 120 minutes after the meal (*P* values .091, .063, and .018, respectively) but in the LFD only between 0 vs 120 minutes ($P = .063$). The postprandial change in exhaled ethanol reflected that of plasma glucose, increasing signifi-

Table 1 – Dietary content, physical activity, weight, and bowel function in 2 × 1–week diet intervention with LFD and HFD in 7 healthy men [a]

	LFD	HFD	*P* value [b]
Nutrients			
Energy (MJ/d)	12.3 ± 2.1	12.0 ± 1.9	.612
Fat (E%)	28 ± 3.3	30 ± 4.9	.309
Carbohydrates (E%)	52 ± 5.6	50 ± 4.4	.672
Protein (E%)	18 ± 3.5	19 ± 3.2	.499
Alcohol (E%)	2.1 ± 4.2	0.8 ± 1.2	.581
Dietary fiber (g/d)	16.9 ± 5.1	43.7 ± 6.3	**.018**
Insoluble fiber (g/d)	12.4 ± 3.7	35.0 ± 5.0	**.018**
Soluble fiber (g/d)	4.5 ± 1.3	8.5 ± 1.4	**.018**
Physical activity [c]			
Physical activity level	1.6 ± 0.2	1.6 ± 0.1	.446
Energy expenditure (MJ/d)	12.8 ± 0.5	12.7 ± 1.0	.398
Weight (kg)	85.2 ± 18.0	84.3 ± 17.4	**.091**
Frequency of defecation (1/d)	1.3 ± 0.4	2.1 ± 0.7	**.028**

[a] Values are means ± SD.
[b] Significance of difference analyzed with Wilcoxon test; differences were considered significant (in bold) at 2-sided $P < .10$.
[c] Approximated with activity computers worn during diet intervention.

Table 2 – Signal levels [a] of exhaled breath compounds in 2 × 1–week diet intervention with LFD and HFD diets in 7 healthy men in fasting state

	LFD	HFD	*P* value [b]
Acetone	3,718,350 ± 2,516,560	4,565,430 ± 3,759,880	.612
Ethanol	63,960 ± 48,090	53,910 ± 19,910	.735
1-Propanol	51,120 ± 37,140	45,550 ± 25,130	.866
2-Propanol	492,240 ± 504,940	368,550 ± 291,390	.866
1-Butanol	7650 ± 2090	7210 ± 2560	.735
Acetic acid	43,360 ± 22,890	47,000 ± 32,550	1.000
Propionic acid	5470 ± 4620	5560 ± 4990	.499
Butyric acid	1440 ± 660	1370 ± 1130	.612
Valeric acid	810 ± 370	790 ± 700	.735
Isovaleric acid	360 ± 140	330 ± 230	.600
2-Methylbutyric acid	840 ± 330	630 ± 110	**.091**
Hexanoic acid	1050 ± 540	1020 ± 740	.612
Acetoin	460 ± 210	420 ± 330	.866
Diacetyl	10,980 ± 5140	10,190 ± 6910	1.000
Phenol	73,090 ± 48,470	81,620 ± 67,680	.866

[a] Values are means ± SD of the GC-MS signals normalized with the signal of internal standard.
[b] Significance of difference analyzed with Wilcoxon test; differences were considered significant (in bold) at 2-sided $P < .10$

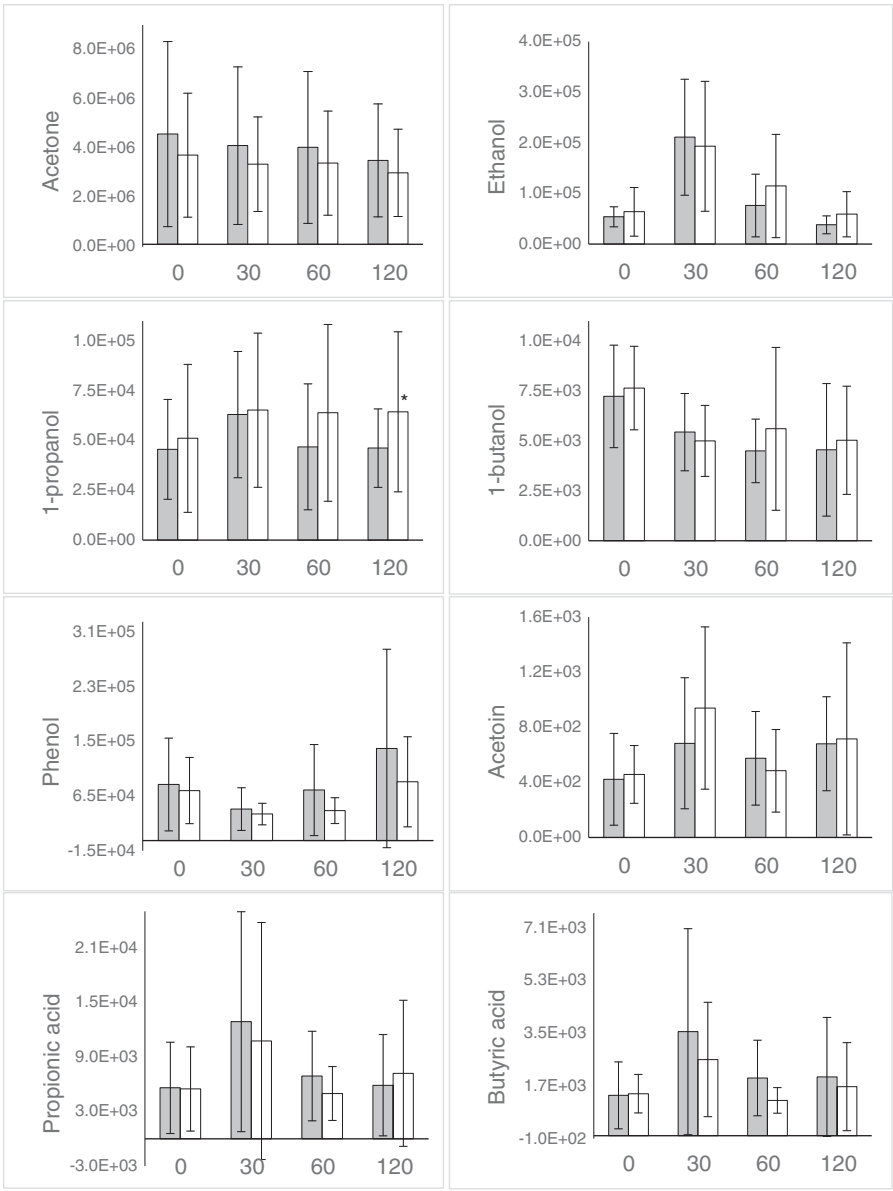

Fig. 2 – Postprandial changes in the signals of exhaled breath VOCs after a week of HFD (gray columns) and LFD (white columns) in 7 healthy men in a crossover design. Exhaled VOCs were analyzed in fasting state (0) and postprandially 30, 60, and 120 minutes after the test meal. Means are presented as columns and ± SD as error bars. *The significance of difference, $P < .10$ between diets analyzed with Wilcoxon test.

cantly in both diets at 30 minutes after the test meal (0 vs 30 minutes, $P = .018$). Similarly 1-propanol increased in both diets at 30 minutes after the standard meal (in the HFD $P = .028$ and and in the LFD $P = .091$), but unlike ethanol, it did not return to the fasting level at 60 minutes after the meal in the LFD. On the contrary, 1-butanol decreased postprandially below the fasting levels after both diets (statistically significant in the HFD 0 vs 60 minutes, $P = .063$, and in the LFD 0 vs 30, $P = .018$, and 0 vs 120 minutes, $P = .043$). Acetoin increased postprandially during both diet periods (statistically significant in 0 vs 30 minutes, $P = .046$ in the HFD and $P = .018$ in the LFD), whereas diacetyl decreased (statistically significant in 0

vs 120 minutes, $P = .091$ in the HFD and $P = .018$ in the LFD). Phenol showed a statistically significant decrease in the LFD at 30 and 60 minutes after the test meal ($P = .043$ and .028, respectively) and an increase at 120 minutes after the meal in both diets (60 vs 120, $P = .018$ in both). Also, exhaled propionic acid and butyric acid increased at 30 minutes in most subjects after the meal (in the HFD in 6/7 and in the LFD in 5/7) similarly to ethanol or acetoin, although the increase was not statistically significant ($P = .128$ in all).

4. Discussion

The present findings suggest that exhaled breath is a potential matrix to study the metabolic effects of DF. A diet high in DF from rye had effect on studied exhaled breath volatiles, as we hypothesized, although the changes in many compounds were individual. Also, consuming a single meal had an effect on the levels of the compounds, indicating that the state of digestion must be taken into account in the study protocol in studies on metabolic effects of nutrients.

High-fiber diet reduced the fasting level of exhaled 2-methylbutyric acid and the postprandial response of 1-propanol. 2-Methylbutyric acid is one of the BCFAs formed during fermentation of amino acids [27]. However, in the present study, both diets provided the same amount of protein. It is possible that the gut microflora prefers polysaccharides when these are available in the gut, thereby decreasing the fermentation of protein. In our intervention, the subjects were consuming sourdough fermented whole grain rye bread during the HFD, having therefore more arabinoxylooligosaccharides and other DF than in the LFD. This leads to accelerated fermentation and production of SCFAs in the gut and increased colon acidity [28]. Higher pH after the LFD could be the reason for the increased 2-methylbutyric acid because increased production of BCFAs is associated with adaptation of microbes to elevated pH conditions [29]. Van Craeyveld et al [30] demonstrated that a high degree of polymerization of arabinoxylooligosaccharides is associated with decreased concentration of BCFAs in rats feces. Increased DF content in the gut can also decrease absorption of BCFAs into the circulation during an HFD. To best of our knowledge, 2-methylbutyric acid has yet been undetected from exhaled breath previously [9], and it should be studied further to understand its role in metabolism. The attenuated postprandial response of exhaled 1-propanol after the HFD as compared with the LFD was an unexpected new finding, and no explanation was revealed in the present study. Although 1-propanol is a common compound in human breath, its role in human metabolism is vague. Elevated levels of exhaled 1-propanol have been associated with lung cancer [31] and halitosis [32], but the underlying mechanisms are unknown. It is possible that exhalation of 1-propanol is related to metabolic events associated with an LFD, for example, lower fermentative activity in the gut. More studies are needed to confirm the present finding and to increase understanding of the mechanisms involved. Overall, these results indicate that ingestion of a single meal releases 1-propanol into the breath and a low-DF intake maintains the exhaled 1-propanol at a higher level than a high-DF intake.

The postprandial test with a standardized meal is a common procedure to study glucose metabolism in dietary interventions. It can give extra information because some of the dietary changes are related to the postprandial metabolism and thus are not evident in the fasting state. The exhaled breath measurements, being noninvasive, were easy to add to this protocol. Postprandial changes of some exhaled breath VOCs, including acetone, ammonia, isoprene, methanol, ethanol, propanol (mainly isopropanol), methyl nitrate, xylene and ethyl benzene, propionic acid, and butanoic acid, have been monitored previously after a meal [33,34] or after ingesting a single dose of glucose [35–38] with healthy or diabetic subjects [34,39,40], mainly in an attempt to measure plasma glucose noninvasively [41–43]. However, to our knowledge, this is the first study to monitor the postprandial changes in 1-propanol, 1-butanol, BCFA, acetoin, diacetyl, and phenol in the exhaled breath. Changes observed in VOCs in earlier studies have been somewhat contradictory, and we suggest that this conflict arises from individual metabolic responses of the body and its microbiota. Nevertheless, it seems that ethanol, xylene, and methyl nitrate as well as SCFAs tend to increase after a meal or ingested glucose in most subjests, whereas acetone and ammonia decrease and isoprene stays stable. Our results support these findings. The residues of the ingested food and bacterial fermentation in the mouth or throat [36,38] can partly explain increased postprandial levels of exhaled breath VOCs. These do not, however, explain completely the rises of these coumpounds because elevated levels of exhaled breath ethanol have been detected also after intravenously administered glucose without an oral injection [35]. Our results on postprandial excretion of 1-propanol support this. The test meal was the same after both diet periods; thus, it is unlikely that the postprandial decrease in 1-propanol in the HFD was due to reduced fermentation in the mouth. We suggest that increased metabolic activity in the alimentary tract after ingestion of a meal could explain, at least partly, the boluses of ethanol, 1-propanol, acetoin, propionic acid, and butyric acid. It is also noteworthy that we found no similar postprandial increase in all the compounds known to be released in bacterial fermentation such as acetic acid. Overall, it is evident that ingestion of a meal affects exhaled breath compounds.Therefore, it is important to standardize a sampling protocol by taking the timing of the last meal into account. The present study shows that the standardized postprandial test can give a new perspective to exhaled breath measurements revealing effects of diet on postprandial metabolism.

The GC-MS–based analysis method was chosen because of reliable identification of compounds and the possibility to carefully control the repeatability of the analysis. Sampling exhaled breath is challenging [44]. The commercial Bio-VOC sampler was chosen for being easy to use and sampling the end-tidal air. The Bio-VOC is intended to be used with thermal desorption tubes, but like Poli et al [45], we found it suitable also for SPME preconcentration. SPME is an extraction technology developed by Grote and Pawliszyn [46] and used widely also for preconcentration of exhaled breath VOCs [47–49]. Deuterated acetone was used as an internal standard for normalizing the signal intensities of VOCs and thus reducing the variation in the analysis. The coefficient of variation was 18.3% for IS, which can result from differences in the SPME

extraction (temperature, humidity, condition of fiber), storage and transporting of samples, variation in the GC-MS conditions, or competitive sorption among VOCs. The SPME involves complicated sorption kinetics [50], making quantification of compounds challenging. Therefore, we ended up using this technique only for compound identification and for monitoring the changes of VOCs to evaluate the dynamics of the compounds induced by diet. To use the SPME-GC-MS method for quantitative breath analysis, SPME should be calibrated with each compound of interest in conditions comparable to the breath air (same temperature and humidity), examining also the possible competitive sorption by VOCs during SPME preconcentration. In our experience, the breath sampling and analysis protocol used in our study is suitable for studies in which only a few compounds are detected and when the analysis can be carried out immediately after sampling. It is an option for preliminary screening of new biomarker compounds from exhaled breath. Despite the small number of study subjects, statistically significant changes were observed in postprandial test. We also found some trends in the changes of exhaled breath VOCs related to diets, indicating that these VOCs are potentially related to the nutritional metabolic changes; more studies are needed to confirm these findings. The randomized crossover design used in our dietary trial is suitable for breath studies. When changes are compared between the diets within the same subjects under a controlled protocol, it is possible to monitor individual responses and identify consistent changes despite individual VOC levels. Because of large individual differences in VOC levels and in their changes after a diet and a single meal, we recommend inclusion of breath hydrogen and methane measurements and analyses of fecal microbiota in further studies. It would be interesting to study whether microbiota or its methane-producing status affects the responses to high DF.

In conclusion, our pilot data show that VOC levels are affected by HFD and ingestion of a single meal. Thus, we recommend that exhaled breath is further studied in developing tools for monitoring the metabolic effects of DF and rye.

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II

Diet-derived changes of sourdough fermented rye bread in exhaled breath aspiration ion mobility spectrometry profiles

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Diet-derived changes by sourdough fermented rye bread in exhaled breath aspiration ion mobility spectrometry profiles in individuals with mild gastrointestinal symptoms

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ABSTRACT

The potential of utilizing exhaled breath VOC (volatile organic compound) profiles in studying diet-derived metabolic changes was examined. After a 4-week initial diet period with white wheat bread (WW), seven participants received in randomized order high-fibre diets containing sourdough whole grain rye bread (WGR) or white wheat bread enriched with bioprocessed rye bran (WW+BRB), both for 4 weeks. Alveolar exhaled breath samples were analysed with ChemPro®100i analyser (Environics OY, Mikkeli, Finland) at the end of each diet period in fasting state and after a standardized meal. The AIMS signal intensities in fasting state were different after the WGR diet as compared to other diets. The result suggests that WGR has metabolic effects not completely explained by the rye fibre content of the diet. This study encourages to utilize the exhaled breath VOC profile analysis as an early screening tool in studying physiological functionality of foods.

Keywords: Exhaled breath; VOC profile, Aspiration ion mobility spectrometry; rye; diet

1. Introduction

Changes in metabolism after dietary modifications are usually studied using targeted analyses of blood and urine samples. However, exhaled breath is an interesting matrix because of its non-invasive sampling and rapid responses from metabolism (Amann et al. 2014). Breath research has traditionally been focused on finding specific biomarker compounds for diseases (Kurada et al. 2015; Pereira et al. 2015; Schmidt & Podmore 2015; van der Schee et al. 2015), but this is challenging because of large inter-individual variation in breath volatile organic compounds (VOCs) (Amal et al. 2013; Phillips et al. 1999) and technological limitations in sampling and analysing breath volatiles (Lourenço & Turner 2014; Mathew et al. 2015). There are more than 3400 detected compounds in exhaled breath (Rattray et al. 2014), of which about 900 are common in healthy people (de Lacy Costello et al. 2014). However, only a few pilot studies have thus far been conducted using exhaled breath in monitoring dietary effects, notably those of Smith and Španěl et al. (Smith et al. 1999; Smith et al. 2011; Španěl et al. 2011; Španěl & Smith, 2011), Blake and Galassetti et al. (Galassetti et al. 2005; Lee et al. 2009; Minh et al. 2012; Minh et al. 2011) and van Shooten et al. (Baranska et al. 2013). Current technologies (Mathew et al., 2015; Sun et al. 2016) detect typically only a small portion of these compounds in one analysis and metabolomic analysis of breath VOCs requires both expertise and expensive laboratory equipment (Rattray et al., 2014; Smolinska et al., 2014). Because of these challenges, analysing of VOC profiles (Baranska et al. 2013; Kistler et al. 2014) and using electronic noses (Bikov et al. 2015) detecting the changes have attracted more interest. These are profitable as an early screening tool to reveal new potential research targets.

The aspiration ion mobility technology (AIMS) detects changes in VOC profiles (Kanu at al. 2008). The technology was originally developed for detection of xenobiotic chemicals, but it has been utilized also for food quality assessments (Gursoy et al. 2009; Raatikainen et al. 2005), monitoring of microbial fermentation and growth (Hakalehto et al., 2009; Kolehmainen et al. 2003), microbial VOCs in building materials (Räsänen et al. 2010), and recently for human samples detecting prostate cancer from urine and cell samples (Roine et al. 2012; Roine et al. 2014). In our earlier pilot study (Raninen et al. 2015) AIMS technology was shown to have potential to detect changes in exhaled breath VOCs after specified diets.

Rye bread has been studied for its beneficial health effects (Belobrajdic & Bird 2013; Hallmans et al. 2003), but underlying mechanisms are unclear. Part of the beneficial effects are likely related to dietary fibre and gut function, but sourdough fermented rye bread has also shown beneficial effects on insulin sensitivity and inflammation unrelated to other cereals (Juntunen et al. 2003; Kallio et al. 2008; Lankinen et al. 2010). These effects are considered to be linked to rye bran containing wide variety of bioactive phytochemicals, such as alkylresorcinols, benzoxazinoids, lignans, phenolic acids, phytosterols, and tocols (Andersson et al. 2014; Bondia-Pons et al. 2009). Determining gut-related mechanisms in nutritional studies presents a complex challenge because of the large variability in human gut microbiota and anaerobic conditions in the gut. Thus new non-invasive tools are warranted to study the gut-related metabolic effects of rye bread and other cereals.

We have examined the potential of utilizing exhaled breath analysis in studying the metabolic effects of diets and detected changes in exhaled breath VOCs and exhaled breath VOC profiles (Raninen et al. 2015; Raninen et al. 2016) between a high-fibre rye bread diet period and a low-fibre wheat bread diet period. In the present study, we continued the approach by exploring changes in exhaled breath VOC profiles by AIMS after the diets containing different breads; two high-fibre diets containing either sourdough wholegrain rye bread (WGR) or white wheat bread enriched with bioprocessed rye bran (WW+BRB) and after a control diet containing white wheat bread (WW). We wanted to explore if sourdough fermented whole grain rye bread would give divergent exhaled breath VOC profile compared to wheat bread having the same amount of dietary fibre from the rye bran, hypothesizing that sourdough whole grain rye bread may have metabolic effects not related to dietary fibre.

2. Methods

2.1. Protocol

A 4-week run-in period with white wheat bread (WW) preceded two 4-week high-fibre test periods (WGR and WW+BRB) in randomized cross-over manner (Figure 1). At the end of the diet periods a postprandial test was performed. Exhaled breath samples were taken parallel with blood samples in fasting state and postprandially, and differences in exhaled breath VOC profiles after the diets were evaluated. The study was approved by the Ethics Committee of the Hospital District of Northern Savo (https://www.psshp.fi/web/en/research/research-ethics-committee; 84/2010, accepted 19 November 2010). All the participants provided written informed consent prior participating in the study.

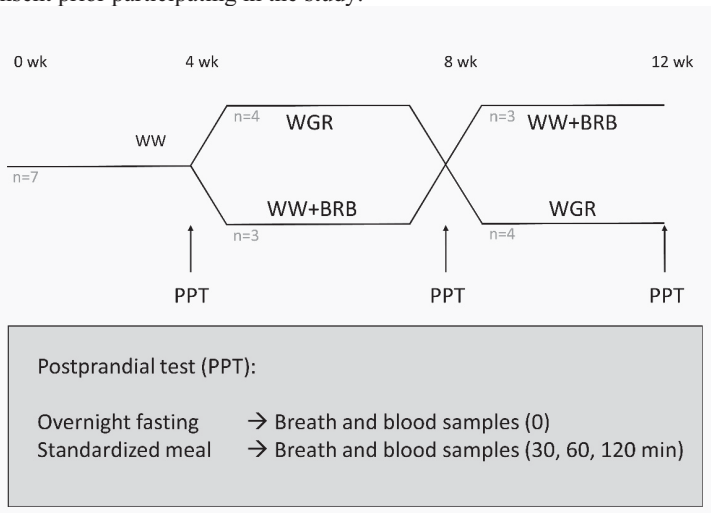

Figure 1. Study design. WW = control diet with white wheat bread; WGR = high-fibre diet with sourdough wholegrain rye bread; WW+BRB = high-fibre diet with white wheat bread enriched with bioprocessed rye bran.

2.2. Study participants

Eight persons were selected randomly from a group of 21 participants recruited to the FIBREFECTS study (Lappi et al. 2014). Participants were healthy Finnish men and women reported having gastrointestinal symptoms (flatulence, bloating, discomfort, constipation, or diarrhoea) after ingestion of rye bread. One participant did not complete the study protocol and therefore the final analysis was done with 4 men and 3 women, aged 38 to 61 years (average 54.3) and BMI from 19.2 to 29.3 kg/m2 (average 25.5 kg/m2). The participants were advised to maintain their body weight and habitual lifestyle throughout the study.

2.3. Diets

During the run-in period (WW) the participants were advised to consume daily 6-10 slices (20-25 g/slice) of white wheat bread (commercial breads) with 100% white wheat flour (Vaasan Oy, Kuopio, Finland). During the high-fibre test periods, the participants were asked to consume daily 6-10 slices (25-30 g/slice) of sourdough wholegrain rye bread (in WGR-period) or wheat bread which was enriched with bioprocessed (fermented) rye bran (in WW+BRB-period). The breads for the high-fibre diets were baked at VTT Technical Research Centre of Finland, (Espoo, Finland) as described in (Lappi et al., 2014). The number of bread slices was adjusted according to the individual energy requirement of the participants.

Food items producing gastrointestinal symptoms were avoided during the intervention. Dietary counseling was based on avoiding vegetables, fruits, and pulses containing rapidly fermentable oligo-, di- and monosaccharides and polyols, as well as foods supplemented with fructo-oligosaccharide, inulin, or galacto-oligosaccharide (Gibson et al., 2010). Instead, the subjects were asked to favor vegetables, fruits, and berries without or with only a low content of these fermentable carbohydrates. One to two small portions of other grain products than the test breads were allowed daily. Occasional intake of non-grain fibre supplements such as dried and soaked plums, linseeds, and sugar beet fibre was allowed during the run-in period to avoid constipation.

A dietician advised the participants weekly or biweekly on the practical management of the diets. The participants recorded the eaten amount of the test breads and other grain products in a daily questionnaire and filled in 4-day food records during the last week of each period. The food records were analysed for nutrient intakes using the Diet32 software (version 1.4.6.3, Aivo Finland Oy, Turku, Finland).

Based on the daily questionnaires, the subjects consumed the test breads as advised. The intake of dietary fibre was lower during the WW period than during the WGR and WW+BRB periods, and similar between the high-fibre periods (Table 1). The intake of energy and proportions of energy from fat, protein and carbohydrates were unchanged during the intervention, except a decrease in the supply of carbohydrates during the WW+BRB period (mean intakes in g/d: WW 237, WGR 223, WW+BRB 206) due to the lower starch content of the bread (Lappi et al 2014).

Table 1. Mean daily intakes[1] of energy and nutrients during the 4-weeks diet periods (n=7).

	WW	WGR	WW+BRB	p-value[2]
Energy, MJ/d	9.4 ± 2.7	8.8 ± 3.0	9.2 ± 3.3	0.867
Carbohydrates, E%	42 ± 7	42 ± 7	37 ± 9*	**0.050**
Protein, E%	22 ± 4	22 ± 3	24 ± 4	0.156
Fat, E%	32 ± 8	31 ± 8	34 ± 9	0.276
Total fibre, g/d	24 ± 8	34 ± 13*	32 ± 13*	**0.004**
Bread, g/d	138 ± 30	164 ± 59*	144 ± 65*	**0.030**
Fibre from bread, g/d	8 ± 1	19 ± 5*	17 ± 5*	**0.006**

[1] values are means ± SD; [2] Statistical significance of the difference among the diet periods analysed with Friedman's test; *Different from WW period, Wilcoxon´s test, $p < 0.05$; WW = control diet with white wheat bread; WGR = high-fibre diet with sourdough wholegrain rye bread; WW+BRB = high-fibre diet with white wheat bread enriched with bioprocessed rye bran; E%= percentage of total energy intake.

2.4. Postprandial test

At the end of each diet period a standardized meal test was done to monitor the change in the exhaled breath VOC profiles induced by diets in fasting state and postprandial states. Participants were instructed to avoid unusually large portions of food the day before the meal test and avoid consumption of alcohol for two days before the test. After an overnight fast, the participants were offered a standardized meal consisting of white wheat bread (80 g), milk-free margarine (20 g), cheese (20 g), cucumber (40 g), and juice concentrate (0.4 dl) diluted in 2.6 dl water (Lappi et al. 2014). Exhaled breath and blood samples were taken before the meal at the fasting state and at 30, 60 and 120 minutes after starting to consume the meal. To standardize the bacterial fermentation in the mouth, the participants were asked to brush their teeth with toothpaste in the morning of the test day and again without toothpaste before the first breath sample and after the test meal.

2.5. Aspiration IMS analysis of exhaled breath samples

Breath analyses were done using the ChemPro®100i online analyser (Environics OY, Mikkeli, Finland) as described in (Raninen et al. 2015). ChemPro®100i is a type of electronic nose based on AIMS technology (Utriainen et al. 2003). The principle of the technology is that sample air is taken inside the device with an internal pump with a constant flow, the compounds are ionized by an Am-241 source and detected by IMCell. Ionized compounds form clusters and move along with the air flow orthogonally and are detected in the ion mobility cell (IMCell) containing 8 AIMS channels for positively charged clusters and 8 for negatively charged clusters, which form the AIMS spectrum. The output of the AIMS spectrum is electric current (pA) collected on each channel separately. ChemPro®100i gives also the AIMS sum signal, which is calculated as the sum of the absolute pA values of all ion channels.

Alveolar breath samples (150 ml) were taken using the Bio-VOC® samplers (Markes International Ltd, Rhondda Cynon Taff, UK). The subjects were trained to give a deep slow blow through the sampler after normal breathing. Bio-VOC® samplers were placed to the inlet of ChemPro®100 right after the sampling for 7 seconds. A multicapillary column (BEKO Technologies GmbH, Neuss, Germany) was installed between the sampler and the inlet of ChemPro®100i to delay the water of exhaled breath sample. This was done because the IMCell is sensitive also for water, and by using the BEKO tube the AIMS response of exhaled breath VOCs was achieved before the response for humidity. Active carbon filter (Pall Corporation, Port Washington, NY, USA) was used in the inlet of ChemPro®100i before and between the measurements to maintain the background air constant. The flow rate was nominal 1.3 l/min. The signal was zeroed before the sampling and relative signals (changes from the filtered background air in pA) from the 14 AIMS channels were used in data analysis (no signal in channels 8 and 16). The signal values of AIMS channels (pA) were taken from the time point displaying the maximum value of the AIMS sum signal (Figure 2).

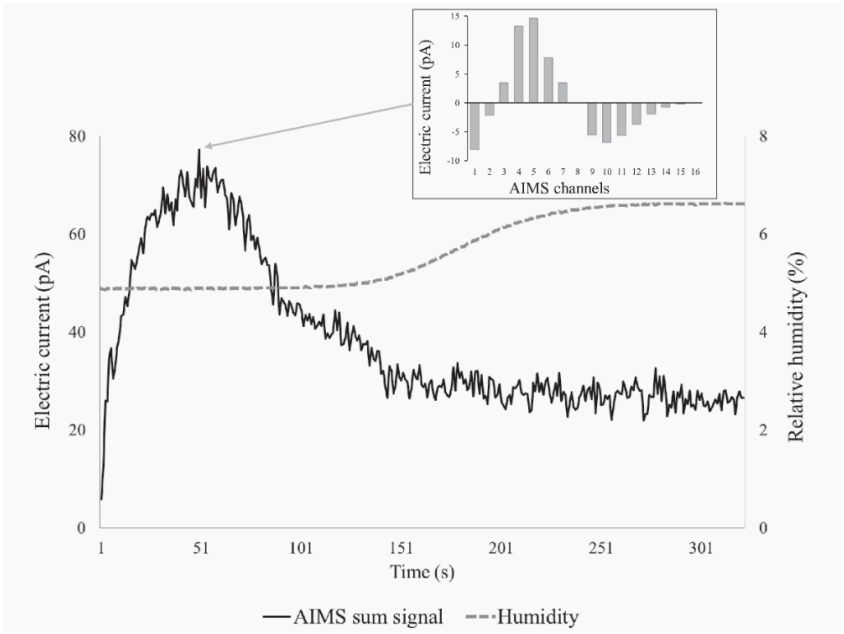

Figure 2. Exhaled breath AIMS signal. Representative signals of AIMS sum signal and humidity of the exhaled breath sample measured with ChemPro®100i and multicapillary column used. The insert figure shows the AIMS profile at the maximum point of the sum signal.

2.6. Reference measurements

Exhaled breath hydrogen, plasma glucose and insulin, and serum free fatty acids (FFAs) were analysed parallel to exhaled breath AIMS samples in fasting state (0) and postprandially 30, 60, and 120 min after the standardized meal. Plasma SCFAs (short chain fatty acids), acetate, propionate, butyrate and isobutyrate were analysed in the fasting state and at 30 min postprandially, and serum triglycerides, total cholesterol, HDL and LDL cholesterols in the fasting state. Exhaled breath hydrogen was analysed with Gastro+ Gastrolyzer (Bedfont Scientific, Kent, UK). Plasma glucose, FFAs, serum total cholesterol, HDL and LDL cholesterol, and triglycerides were analysed with KoneLab 20XTi Clinical Chemistry Analyser (Konelab, Thermo Fisher Scientific, Vantaa, Finland) using the enzymatic photometric (glucose hexokinase) method for glucose, enzymatic colorimetric method for FFAs, and commercial kits (Thermo Electron Corporation, Vantaa, Finland) for cholesterol and triglycerides. Insulin was analysed with a chemiluminescent immunoassay (Advia Centaur Immunoassay System, Siemens Medical Solution Diagnostics, Tarrytown, NY, USA). Plasma SCFAs, acetate, propionate, butyrate and isobutyrate were measured with a gas chromatography as described by Brighenti et al. (1998) with slight modifications using 2-ethyl butyrate (FLUKA no. 03190; Sigma Aldrich, St. Louis, MO, USA) as an internal standard. The intestinal microbiota does not produce 2-ethyl butyrate, and it is consequently not present in biological samples.

2.7. Statistical analyses

The exhaled breath AIMS data (consisting signals of 14 AIMS channels) were pre-processed with variance scaling (zero mean, unit variance) in two ways; by scaling the AIMS channels separately, when changes are detected in the intensity of AIMS signal between samples (AIMS signal intensity), or by scaling each breath samples separately, when changes are detected in the ratios of AIMS channels between samples (AIMS profile). The scaled AIMS data was further pre-processed by the principal component reduction (Dundeman 1989) in the Matlab environment (R2011a, The MathWorks Inc., Natick, MA, USA). Two to four principal components were selected for further processing based on Kaiser Criterion (i.e. eigenvalue > 1). The statistical significance of the differences between diets were tested with dependent Hotelling´s T-test using the principal components as inputs.

Statistical analysis of the differences and correlations in individual variables were performed using the IBM SPSS Statistics version 21. Nonparametric tests (Wilcoxon and Friedman) were used to analyse the differences between diets. Spearman´s rho was used to analyse correlations between variables. Differences and correlations were considered significant at two-sided $p < 0.05$.

3. Results

The exhaled breath AIMS signal intensities were statistically significantly different in WGR compared to WW and WW+BRB diet periods (both $p < 0.05$, Table 2) in the fasting state. Signal intensities decreased in all AIMS channels except in channel 3 (Table 3), producing statistically significantly lower AIMS sum signal after WGR compared to WW in the fasting state ($p = 0.043$) in 6 of 7 participants (Figure 3). The signals tended to be different also 30 minutes after the meal between the WGR and WW periods ($p < 0.10$).

Table 2. Statistical significances[1] (p-values) of the differences in exhaled breath AIMS signal intensities[2] and profiles[3] between the diets in fasting state (0) and at 30, 60 and 120 minutes after the standardized meal (n=7).

	0	30 min	60 min	120 min
WGR vs. WW				
AIMS signal intensity	**0.023**	0.078	0.181	0.514
AIMS profile	0.065	0.074	0.347	0.999
WW+BRB vs. WW				
AIMS signal intensity	0.731	0.114	0.239	0.920
AIMS profile	0.188	0.098	0.130	0.729
WGR vs. WW+BRB				
AIMS signal intensity	**0.026**	0.998	0.780	0.458
AIMS profile	0.520	0.707	0.568	0.687

[1] Hotelling´s t-test; [2] PCA of AIMS signals of 14 AIMS channels variance scaled each IMS channels separately; [3] PCA of AIMS signals of 14 AIMS channels variance scaled each sample separately; WW = control diet with white wheat bread; WGR = high-fibre diet with sourdough wholegrain rye bread; WW+BRB = high-fibre diet with white wheat bread enriched with bioprocessed rye bran

Table 3. Aspiration ion mobility spectrometry signals[1] of ChemPro®100i for exhaled breath samples analysed in the fasting state after the 4-week diet periods containing either white wheat bread (WW), sourdough wholegrain bread (WGR) or white wheat bread enriched with bioprocessed rye bran (WW+BRB) (n=7).

	WW	WGR	WW+BRB	p-value[2]
Sum (pA)	62.5 ± 10.0	50.5 ± 5.2*	57.2 ± 15.3	**0.050**
Channel 1 (pA)	-4.9 ±1.8	-4.5 ± 0.5	-5.1 ± 1.9	0.867
Channel 2 (pA)	-3.9 ±1.6	-3.6 ± 1.4	-3.2 ± 1.6	0.180
Channel 3 (pA)	0.5 ± 1.4	0.9 ± 1.6	1.8 ± 1.5	0.368
Channel 4 (pA)	10.1 ± 2.0	8.6 ± 1.2	8.6 ± 2.2	0.156
Channel 5 (pA)	12.4 ± 2.3	9.8 ± 1.3	11 ± 3.1	0.156
Channel 6 (pA)	6.8 ± 1.2	5.2 ± 1.1*	5.9 ± 2.3	0.066
Channel 7 (pA)	2.8 ± 0.5	1.9 ± 0.8	2.4 ± 1.3	0.180
Channel 9 (pA)	-3.9 ± 1.1	-3.5 ± 0.7	-3.7 ± 0.8	0.565
Channel 10 (pA)	-4.4 ± 1.9	-4.0 ± 0.9	-5.1 ± 0.8	0.156
Channel 11 (pA)	-4.0 ± 1.4	-3.0 ± 1.0	-4.1 ± 1.3	0.276
Channel 12 (pA)	-4.1 ± 0.8	-2.5 ± 0.6	-3.3 ± 1.3	0.156
Channel 13 (pA)	-2.6 ± 0.6	-1.6 ± 0.2	-2.2 ± 1.0	0.180
Channel 14 (pA)	-1.1 ± 0.3	-0.6 ± 0.2	-0.9 ± 0.6	0.368
Channel 15 (pA)	-0.3 ± 0.2	-0.2 ± 0.1	-0.2 ± 0.2	0.317

[1] values are means ± SD, [2] statistical significance of the difference among the diet periods analysed with Friedman's test; * different from WW period, Wilcoxon´s test, $p < 0.05$

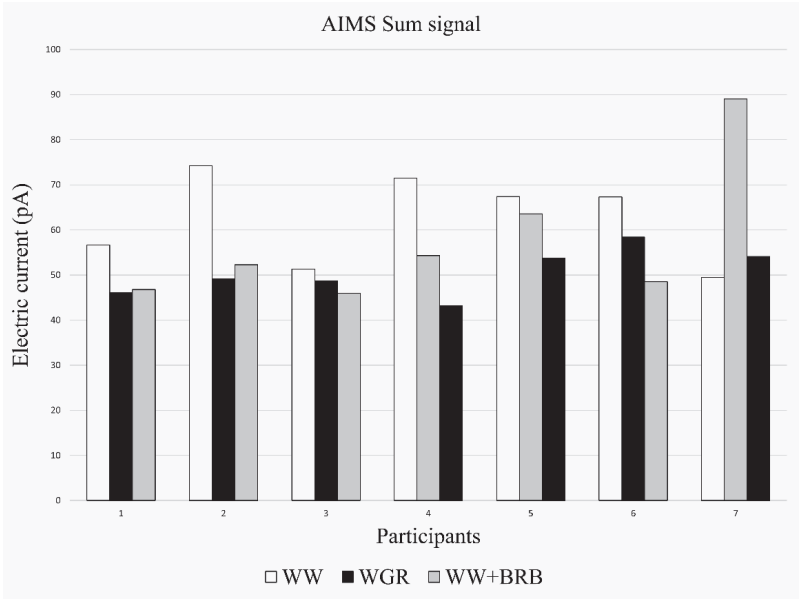

Figure 3. AIMS Sum signal in three diets. AIMS Sum signal of the exhaled breath samples of 7 participants after three diet period; WW = control diet with white wheat bread; WGR = high-fibre diet with sourdough wholegrain rye bread; WW+BRB = high-fibre diet with white wheat bread enriched with bioprocessed rye bran. The order of the columns in the figure is according to the order of diets in each individual.

The fasting level of exhaled breath hydrogen decreased slightly during the WGR period, whereas fasting plasma SCFA and acetate tended to decrease during WW ($p < 0.10$, Table 4). Other fasting blood parameters remained stable during the study, except a minor decrease in plasma glucose (Table 4). However this change was not clinically significant, and the blood glucose was maintained at normal level in all subjects throughout the study. No correlations were found between the AIMS signals and breath hydrogen or plasma glucose. There was a borderline significant associations between fasting AIMS sum signal and plasma SCFA (Spearman´s correlations 0.379, $p=0.077$) or plasma acetate (Spearman´s correlations 0.395, $p=0.090$).

The exhaled AIMS profiles tended to be different between WGR and WW in the fasting state and 30 minutes after the meal, and between WW+BRB and WW 30 minutes after the meal ($p < 0.10$, Table 2).

Table 4. Reference measurements[1] in the fasting state after the 4-week diet periods containing white wheat bread (WW), sourdough wholegrain bread (WGR) or white wheat bread enriched with bioprocessed rye bran (WW+BRB) (n=7).

	WW	WGR	WW+BRB	p-value
Breath H_2 (ppm)	7.4 ± 5.3	2.3 ± 1.4*	6.1 ± 5.0	0.060
Blood biochemistry				
p-Glucose (mmol/l)	5.2 ± 0.5	4.7 ± 0.4*	4.8 ± 0.6*	**0.015**
s-Insulin (mU/l)	7.9 ± 3.0	7.5 ± 3.6	8.4 ± 6.1	0.565
p-Triglycerides (mmol/l)	1.0 ± 0.3	1.3 ± 0.9	1.1 ± 0.3	0.066
p-Cholesterol (mmol/l)	5.3 ± 1.0	5.2 ± 0.7	5.5 ± 0.8	0.180
p-HDL-cholesterol (mmol/l)	1.5 ± 0.3	1.5 ± 0.3	1.6 ± 0.4	0.276
p-LDL-cholesterol (mmol/l)	3.2 ± 0.9	3.1 ± 0.6	3.4 ± 0.6	0.250
s-FFA (mmol/l)	0.38 ± 0.14	0.46 ± 0.08	0.55 ± 0.16	0.102
p-SCFA (µmol/l)	78 ± 20	100 ± 40	98 ± 31	0.066
p-Acetate (µmol/l)	74 ± 19	96 ± 40	95 ± 31	0.066
p-Propionate (µmol/l)	2.8 ± 0.9	3.0 ± 0.8	2.8 ± 0.5	0.254
p-Butyrate (µmol/l)	1.1 ± 0.3	1.1 ± 0.4	1.1 ± 0.3	0.961
p-Isobutyrate (µmol/l)	1.5 ± 0.5	1.3 ± 0.5	1.2 ± 0.2	0.143

[1] values are means ± SD; [2] statistical significance of the difference among the diet periods analysed with Friedman's test; * different from WW period, Wilcoxon´s test, $p < 0.05$

4. Discussion

Sourdough wholegrain rye bread in diet produced distinctive changes in the composition of exhaled breath VOCs compared to diets with white wheat bread or wheat bread enriched with bioprocessed rye bran. This supports the postulation that exhaled breath reflects the intake of different breads and is a potential matrix to study metabolic effects of diets, supporting the results of our previous studies (Raninen et al. 2015; Raninen et al. 2016). The AIMS technology of ChemPro®100i detected changes in exhaled breath induced by WGR despite no effects were observed in blood parameters.

New rapid monitoring technologies are essential to complement modern nutritional research in which metabolic responses for diets and foods at the individual rather than the population level are assessed (Hackman et al. 2014). Rapid, non-invasive and individualized feedback is an excellent way to motivate people to make adjustments in their diet and is also economical, since prevention is less expensive than treatment of diseases. We have investigated the potential of utilizing breath VOC analysis in studying the individual dietary effects of foods. Exhaled breath is an interesting matrix to study, since the sampling is non-invasive and can be done multiple times and without medical expertise. It is necessary to gain knowledge in this field to further develop the monitoring technologies. However, finding exhaled breath biomarkers for nutrition related metabolism is challenging, because many of the VOCs excreted from somatic cells or bacterial flora are also common in the environment (Phillips et al. 1994). It is almost impossible to confirm the origin of the exhaled breath compounds, and in many cases the exhaled breath VOCs have multiple sources. Perhaps for that reason there are only few recognized breath biomarkers (Boots et al. 2012).

We used the ChemPro®100i portable detector based on AIMS technology for exhaled breath analyses because it is simple to use and it detects changes in VOC profiles. Since there are hundreds of compounds in a single breath sample [9] and candidate biomarkers are not known, it is reasonable to monitor the changes in breath VOC profiles as an early screening tool. If the changes are seen in VOC profiles, it indicates that there are differences in the composition of breath VOCs, which should be studied more detailed. In our earlier study (Raninen et al. 2015), changes were detected between low fibre and high fibre diets (corresponding to WW and WGR) in the AIMS signals intensities. In the present study, we

decided to monitor the AIMS signal in more detail, detecting the changes in AIMS signal intensities and AIMS profiles. This was done by performing the variance scaling for the AIMS data in two different ways before creating PCAs. Significant changes were observed in the AIMS signal intensity, which reflects the concentration of IMCell sensitive compounds (Utriainen et al. 2003) in a sample. Thus our results indicate that there was decreased concentration of AIMS sensitive VOCs in the exhaled breath after WGR. The results of the AIMS sum signal support this: 6 of 7 participants had reduced signal after WGR. The changes in AIMS profiles between diets were, however, modest, indicating that changes in the composition of breath VOCs were minor. This is not surprising, considering that AIMS can respond to numerous VOCs and the observed change in the AIMS profile was likely due to only a small part of these compounds. The AIMS technology does not identify individual compounds, only changes in VOC profiles, which is the limitation of the technology. Identification of the responsible compounds behind these changes is the next step when exploring further the mechanisms behind the metabolic effects of rye bread.

Based on recent evidence it is possible that the health effects of whole grains and rye bread can be mediated via gut microbiota (Desai et al. 2016; Kovatcheva-Datchary et al. 2015). Although the composition of microbiota is considered to be quite stable in individuals (de Vos et al. 2012; Rajilic-Stojanovic et al. 2013), the diet may have an impact on the composition and diversity of the gut microbiota (Wu et al 2011). However, in our earlier study, we did not found differences in the composition of microbiota between diets containing WW or WGR during 12-weeks intervention with people having metabolic syndrome (Lappi et al. 2013). In the present study, we did not monitor the composition of microbiota or its metabolic activity. However, it is likely that the breads affected gut fermentation and metabolites produced by gut microbiota, as indicated by the changes in the fasting levels of breath hydrogen, and plasma SCFA and acetate. We also noticed borderline significant associations between fasting AIMS sum signal and plasma SCFA or plasma acetate. Although these results are not statistically significant in this small study population, they point towards the necessity of further studies on the subject. The role of SCFA in human physiology has been recently reviewed by Koh et al. (2016). The SCFAs can be detected also from exhaled breath (Gruber et al. 2016). However, in our previous study (Raninen et al. 2016), the diet-induced changes of breath SCFAs in low and high fibre diets (corresponding to WW and WGR) were variable among individuals.

Metabolic effects of rye bread are also considered to be linked to phytochemicals in rye bran (Andersson et al. 2014; Bondia-Pons et al. 2009). The WGR and BRB+WW diets contained the same amount of fibre from rye bran, but differences in processing of breads may have had affect the bioavailability of the compounds in rye (Koistinen et al. 2017, Liukkonen et al. 2003). Microbial and metabolic conversions of rye phytochemicals have been reviewed by Koistinen et al. (2016). It is possible that changes in breath profiles detected by AIMS are partly due to volatile metabolites of these compounds.

The participants of this study were volunteers who had reported gastrointestinal problems related to rye bread, because one aim of the main study (Lappi et al. 2014) was to monitor symptoms produced by WGR and WW+BRB diets as compared to WW diet. However, the subjects reported only few gastrointestinal symptoms during the intervention and the diets produced no differences in the frequency of defecation or in the fasting concentration of plasma SCFA in the main study. It is possible that the variation in the composition of microbiota in the present subpopulation was small, and therefore also the change was more consistent in the plasma SCFA.

Because of the small sample size of this study, it is likely that the power of the study was insufficient to detect all diet-associated metabolic changes. It is also possible that 4-week dietary periods were too short to produce long-term effects of dietary changes. The possible confounding effects, such as a wide ranges of age, BMI and the habitual lifestyle, were however minimized by the crossover study design, thus enabling each participant to serve as her/his own control. Although there might have been large variation in breath compounds between individuals as seen in other studies (Amann et al. 2014; Phillips et al. 1999) or in effects of background compounds, it was possible to find consistent changes in exhaled breath VOC profiles due to the diets when changes were monitored within individuals.

Several studies have reported reduced plasma insulin response to a single meal containing rye bread (Bondia-Pons et al. 2011; Juntunen et al. 2003; Rosen et al. 2009), suggesting that health effects of rye bread are mediated via short-term postprandial glucose metabolism. Therefore the exhaled breath analyses were performed in the postprandial test at the end of the diet periods. However, the changes in the exhaled breath VOC profiles after the diets were statistically significant only in fasting state. This result indicates that wholegrain rye bread may have also longer-term metabolic effects.

5. Conclusions

A diet containing sourdough wholegrain rye bread produces a change in exhaled breath VOC profile measured by AIMS. This adds to the growing evidence that exhaled breath can be utilized in monitoring effects of dietary modifications.

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Declaration of interest

The authors report no conflicts of interests.

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III

Comprehensive two-dimensional gas chromatography–mass spectrometry analysis of exhaled breath compounds after whole grain diets

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Comprehensive Two-Dimensional Gas Chromatography–Mass Spectrometry Analysis of Exhaled Breath Compounds after Whole Grain Diets

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Abstract: Exhaled breath is a potential noninvasive matrix to give new information about metabolic effects of diets. In this pilot study, non-targeted analysis of exhaled breath volatile organic compounds (VOCs) was made by comprehensive two-dimensional gas chromatography–mass spectrometry (GCxGC-MS) to explore compounds relating to whole grain (WG) diets. Nine healthy subjects participated in the dietary intervention with parallel crossover design, consisting of two high-fiber diets containing whole grain rye bread (WGR) or whole grain wheat bread (WGW) and 1-week control diets with refined wheat bread (WW) before both diet periods. Large interindividual differences were detected in the VOC composition. About 260 VOCs were detected from exhaled breath samples, in which 40 of the compounds were present in more than half of the samples. Various derivatives of benzoic acid and phenolic compounds, as well as some furanones existed in exhaled breath samples only after the WG diets, making them interesting compounds to study further.

Keywords: exhaled breath; whole grain; rye; comprehensive two-dimensional gas chromatography–mass spectrometry; dietary fiber

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1. Introduction

Whole grain (WG) cereals are an important source of dietary fiber (DF) and micronutrients and are therefore acknowledged as part of the healthy diet in dietary recommendations [1,2]. Epidemiological studies and their meta-analyses have consistently shown high intake of WG to lower risk of chronic diseases and mortality [3–5], and associate negatively with obesity [6,7], type 2 diabetes [8–10], cardiovascular disease [11–13], and certain cancers [14,15]. However, the underlying physiological mechanisms are complex and unclear. Phenolic compounds in the fiber matrix of bran [16] are one proposed element for the protective effects of WG. Alkylresorcinols are present in the outer layers of wheat and rye grains and are known to be absorbed by humans. They have been detected in plasma and urine, and hence have been studied as a promising biomarker for WG wheat and rye in the diet [17].

People have variable metabolic responses to diets because of individual physiology and gut microflora [18]. Therefore, there has been an increasing interest in nutrigenomics, proteomics, and metabolomics to monitor metabolism from a wider perspective. Volatomic analysis of exhaled breath, used mainly for searching noninvasive biomarkers for diseases [19–21], could be used to characterize volatile organic compounds (VOCs) relating to various diets or specific foods, such as WG cereals. This research could lead to new information on the metabolic effects of WG foods and their association with health effects.

Exhaled breath is a potential noninvasive matrix to monitor metabolic changes induced by dietary modifications [22,23]. Foods contain numerous molecules which after digestion or metabolism by gut microflora are absorbed to the circulation. If they have a suitable boiling point, vapor pressure, and solubility, they can be excreted to exhaled breath. Currently, more than 3400 compounds have been identified in exhaled breath [24,25], with an average exhaled breath sample containing about 200 detected compounds [26]. However, there is wide interindividual variation since typically only a few dozen of the compounds are detected in every exhaled breath sample.

Although diet is known to cause variation in exhaled compounds [27], so far only a few pilot studies have been conducted to monitor the effects of diets on exhaled breath VOCs. Pioneering research in developing methods for exhaled breath analysis was done by Smith and Španěl who explored the effects of a meal [28] and glucose ingestion [29] to exhaled breath VOCs, as well as the effects of ketogenic diet on breath acetone levels [30]. Galassetti, Blake and colleagues studied exhaled breath compounds relating to diabetes [31,32] and monitored the effects of high-fat meals on exhaled breath VOCs [33]. They also studied exhaled breath VOC profiles relating to blood glucose [34–36] and lipid levels [37]. van Schooten et al. applied the breath analysis to monitor gastrointestinal diseases [38–40] and demonstrated a distinctive exhaled breath VOC profile after a gluten-free diet [41].

We have earlier demonstrated changes in exhaled breath VOC profiles by aspiration ion mobility spectrometry (AIMS) in diets differing in DF content (low-fiber diet vs. high-fiber) and type of bread (white wheat bread vs. sourdough fermented whole grain rye bread vs. white wheat bread enriched with modified rye fiber) [42,43]. However, the AIMS technology, regarded as a type of electronic nose, cannot identify the compounds responsible for the changes. Gas chromatography–mass spectrometry (GC-MS) is a standard technology for identifying VOCs [24,44] and multidimensional chromatography techniques, such as comprehensive two-dimensional GC-MS (GCxGC-MS), are utilized especially for characterization of compounds in metabolomic research, due to their increased separation capability [45]. However, they are not yet utilized for monitoring exhaled breath VOCs regarding to diets.

In this study, our aim was to pilot exhaled breath analysis with GCxGC-MS to explore VOCs relating to WG diets.

2. Results

About 260 VOCs were detected in 32 exhaled breath samples from 9 persons; of these VOCs, 40 were common, being present in more than half of the breath samples (Table 1). Carbon dioxide, isoprene, acetone, ethanol, 1-butanol, 2-propanol, benzene, benzaldehyde, methyl vinyl ketone, 2-butanone, phenol, hexanoic acid, and acetonitrile were found in all samples, but large individual differences existed in the other compounds. Additionally, 86 VOCs were tentatively identified by their MS spectra (Table S1), whereas about 170 detected compounds remained unidentified.

Some derivatives of benzoic acid and phenolic compounds were detected in exhaled breath samples only after the WG diets (Table 2). Phthalic acid or phthalic anhydride (similarity index, SI 93 for both compounds) was found in 57% of the exhaled breath samples during the whole grain rye bread diet (WGR), in 11% of breath samples during the whole grain wheat bread diet (WGW), and in 6% of the background room air (BG) samples, but in none of the samples collected after the control diets containing refined wheat bread (WW). Benzoic acid was detected in 29% of breath samples during the WGR diet and in 11% of breath samples during the WGW diet, but in none of the exhaled breath samples during the WW diets and in 6% of BG samples. Furthermore, diphenyl ethanedione and benzamide were detected only after the WG diets, diphenyl ethanedione in 29% of breath samples during the WGR and benzamide in one participant during the WGR and the WGW.

Table 1. Common [1] volatile compounds in the exhaled breath samples collected from the study participants after the diet periods and their presence in the background room air samples.

	Detected in % of Samples in			
Compounds	**WGR**	**WGW**	**WW**	**BG**
Carbon dioxide	100	100	100	100
Ethanol	100	100	100	100
Hexanoic acid	100	100	100	100
Acetophenone	100	100	100	100
1-Butanol	100	100	100	97
Benzene	100	100	100	97
Benzaldehyde	100	100	100	97
Methyl vinyl ketone	100	100	100	97
2-Butanone	100	100	100	97
Acetone	100	100	100	94
Phenol	100	100	100	94
2-Propanol	100	100	100	90
Acetonitrile	100	100	100	87
Isoprene	100	100	100	42
2,3-Butanedione	100	89	100	68
Toluene	100	33	81	74
3-Pentanol/2-Propanol, 2-methyl	86	100	100	97
Butanal	86	100	100	87
Hexanal	86	100	88	87
Heptanal	86	100	81	74
Octanal	86	89	94	23
Benzaldehyde, 2/4-methyl	86	89	75	90
Pentanal	86	89	69	68
n-Hexane	86	67	88	58
Nonanal	71	78	100	94
Acetaldehyde	71	56	81	77
3,4-Dimethyl heptane	71	33	75	35
D-Limonene	71	33	69	10
1,3-Pentadiene	71	33	69	0
Benzene, 1,4-dimethyl-	71	22	56	26
Dimethyl sulfide	57	78	81	0
Decanal	57	67	56	84
Methyl cyclopentane	57	56	63	42
6-Methyl-5-hepten-2-one	57	56	88	68
1-Propanol	57	44	81	39
Octane	57	44	56	17
Ethyl acetate	57	33	75	71
Styrene	57	33	69	29
p-Cymene	57	33	69	3
Heptane	43	56	75	71

[1] present in >50% of the analyzed exhaled breath samples, WGR = whole grain rye bread diet (n = 7), WGW = whole grain wheat bread diet (n = 9), WW = refined wheat bread diet (n = 16), BG = background room air (n = 31).

Some furanones (γ-lactones) were also identified in the exhaled breath only during the WG diets: 5-dodecyldihydro-(3H)-furanone (in two participants after WGR), dihydro-4-hydroxy-2(3H)-furanone (in one participant after WGR and WGW) and dihydro-5-tetradecyl-2(3H)-furanone (in one participant after WGR and WGW).

We also detected several unidentified compounds having mass spectrum fragments 105, 77 and 51, which are typical for benzoic acid derivatives, and 107, 121, 135, and 149, typical for alkylphenols (potential degradation products of alkylresorcinols). However, none of the unidentified compounds were detected only in a particular diet period.

Table 2. Volatile organic compounds detected only in the exhaled breath samples after WG diets.

Compounds	RT	SI	Detected in % of Samples in			
			WGR	WGW	WW	BG
Phthalic acid/Phthalic anhydride	40–69	93	57	11	0	6
Benzoic acid	30–69	94	29	11	0	6
Diphenyl ethanedione	61.7	92	29	0	0	0
5-Dodecyldihydro-(3H)-furanone	63.7	92	29	0	0	0
Benzamide	65.5	94	14	11	0	0
Dihydro-4-hydroxy-2(3H)-furanone	57.5	85	14	11	0	0
Dihydro-5-tetradecyl-2(3H)-furanone	67.7	89	14	11	0	0

RT = retention time (min), SI = similarity index, WGR = whole grain rye bread diet (n = 7), WGW = whole grain wheat bread diet (n = 9), WW = white wheat bread diet (n = 16), BG = background room air (n = 31).

3. Discussion

We piloted exhaled breath analysis with GCxGC-MS to detect VOCs relating to WG diets. With this technology and the chosen method, about 260 compounds were detected from exhaled breath samples, and of these, 40 VOCs were present in more than half of the exhaled breath samples. Some benzoic acid and phenol derivatives, as well as furanone compounds, were detected more frequently after the whole grain diets.

GCxGC-MS-technology has better sensitivity and separation of compounds as compared to the traditional GC-MS, which make it suitable for non-targeted analysis of exhaled breath compounds. We have earlier analyzed exhaled breath VOCs by traditional GC-MS having the same column and same kind of sampling protocol [46], and about 40 VOCs were detected in the exhaled breath samples collected from healthy men. In the current GCxGC-MS protocol, the total number of detected compounds was approximately seven times more (about 260 compounds). The comprehensive GCxGC-MS technology is based on cryogenic modulator: effluent from the first column is trapped in the modulator for a given period (for 8 s in our method) before being released into the second column. This increases the sensitivity of the method remarkably as compared to traditional GC-MS.

GCxGC-MS technology also improved the separation of compounds as compared to traditional GC-MS. For example, we found two compounds giving almost identical mass spectra with isoprene, having only slightly different retention times. These are probably cis-1,3-pentadiene and trans-1,3-pentadiene which have been detected earlier in exhaled breath samples [47,48], or 1,4-pentadiene, which has been associated with smoking [49,50]. However, our participants were non-smokers. It is noteworthy that these compounds can be erroneously identified as isoprene, and therefore interfere the quantification of isoprene if they are not separated in the analysis. Exhaled breath isoprene has been studied extensively as a potential biomarker compound for cholesterol synthesis, though with controversial results [51,52]. It is possible that these compounds have interfered the quantification of isoprene in some studies.

Although the GCxGC-MS has advantages in sensitivity and selectivity, it also has drawbacks. Because of its sensitivity, the signal is easily overloaded when both the quantification and identification are challenged. This technology is suitable mainly for detecting compounds from challenging matrixes (having a multitude of compounds to be separated), but it is not very convenient for their quantification. The quantitative method should be optimized for each compound of interest separately, including calibration with breath mimicking conditions. Therefore, in this study we did not quantify the detected compounds. Exhaled breath VOCs might have multiple sources, and therefore it would be more relevant to monitor the changes in their levels rather than searching for specific biomarker compounds. However, nontargeted volatomic analysis can be used to select the relevant target compounds to monitor.

In total, 86 VOCs were tentatively identified from exhaled breath samples while about 150 VOCs remained unidentified, as their MS spectra were not found in the MS libraries. This indicates that there might still exist numerous unidentified molecules in the exhaled

breath because GC-MS is a standard technology for identifying volatile compounds, and identification is mainly based on the MS libraries.

There is no analytical method available to monitor all the compounds in exhaled breath. For example, breath sampling method and thermal desorption (TD) adsorbents select the compounds, and GC column determine which compounds are chromatographically separated and can be detected. In this study, we chose the polar Nukol column for the first separative column because we have found it suitable for detecting endogenous gut-related exhaled breath VOCs [46], and non-polar Zebron ZB-35HT Inferno column for the second column due to its chemically different stationary phase compared to Nukol. By choosing other columns or TD adsorbent, different compounds could have been detected. It is noteworthy that most GC-MS analyses for exhaled breath VOCs are made by using general purpose nonpolar methylpolysiloxane columns containing 5% phenyl. With our protocol, we detected some common exhaled breath compounds such as isoprene, acetone, and ethanol, but we were unable to detect, for example, ammonia and methane (too small to detect with MS SCAN 35–300 m/z), or short-chain fatty acids (not enough sensitivity with MS SCAN mode [46]), although these compounds would be interesting in the perspective of nutrition and gut health [53–55]. We detected some compounds (Table 1; 3,4-dimethyl heptane, 3-pentanol, and methyl cyclopentane), which have not been reported in exhaled breath before. However, it should be pointed out that in our study, the identification was done only by the spectral library match, and was not confirmed with standard molecules (i.e., tentative identification) or retention indices. Mass spectra can be almost identical for some compounds, for example, for structural isomers (e.g., 2-methylbutane and n-pentane) or compounds with same structure with different length of alkyl chain (e.g., undecanal and tetradecanal). Therefore, the identification of VOCs in this study must be considered with caution.

Some benzoic acid and phenolic derivatives, as well as furanones, were detected from exhaled breath samples only after whole grain diets. It is possible that these VOCs are degradation products of phenolic compounds such as phenolic acids, alkylresorcinols and lignans from the DF complex in the bran. Phenolic compounds can be metabolized to various compounds by colonic fermentation and metabolism [56]; for example, benzoic acid can be formed from rye phenolics [57]. The compounds were detected mostly in the same exhaled breath samples, which indicate the same origin for these compounds.

Benzoic acid is known to be related to various foods but considered to have relatively low levels in the alveolar exhaled breath in the fasting state, since it is metabolized by liver and kidneys to hippurate within a few hours after oral dosing [58]. However, benzoic acid is formed also from whole grains in gut fermentation, which may explain the elevated levels in the fasting state in some individuals during WG diets. The exhaled breath samples were taken in the fasting state, but the fermentation rate may have been varied based on individual orocecal transit time and timing of eating WG. Estimation of the fermentation rate by breath hydrogen measurements [59] would be relevant when studying fermentation-related exhaled breath VOCs.

It´s noteworthy that the GC parameters used were not optimal for benzoic acid and phthalic acid/anhydride. Both compounds had wide tailing chromatograph peaks. This did not interfere with the identification of compounds, but may have affected sensitivity in their detection, and partly explains why these compounds were seen only in a minority of exhaled breath samples. Other GC parameters or technology should be used for analyzing these compounds more accurately.

Furanones are known to be formed in chemical reactions during charbroiling and seed oil cooking, and in Maillard reaction between sugars and amino acids [60,61]. They can also be metabolized from grain lignans such as matairesinol or 7-hydroxymatairesinol, or from enterolactone, a mammalian lignan, which is formed in the large intestine from plant lignans [62]. All these lignans have dihydro-2(3H)-furanone in their molecule structure. Enterolactone is considered a biomarker for high lignan intake in the diet [63], but high interindividual variation has been found in its absorption and metabolism [64]. To our knowl-

edge, 5-dodecyldihydro-(3H)-furanone (CAS 730-46-1, also known as γ-palmitolactone), dihydro-4-hydroxy-2(3H)-furanone (CAS 5469-16-9, 3-hydroxy-γ-butyrolactone) or dihydro-5-tetradecyl-2(3H)-furanone (CAS 502-26-1, γ-stearolactone) have not been detected from exhaled breath before, unlike some other furanones [25,41]. Dihydro-5-tetradecyl-2(3H)-furanone has been detected from skin [25]. It would be interesting to monitor exhaled breath phenolic and furanone compounds and their levels in relation to different dietary sources, for example rye, using optimized analysis methodology for those compounds.

In this study, the randomized crossover protocol was used because the inter-individual variation of breath VOCs is known to be high [26]. In a crossover protocol, it is more likely that detected differences in breath VOCs are due to dietary changes because the other lifestyle factors are rather constant. Furthermore, most of the study participants were students or staff members of the Faculty of Health Sciences in the University of Eastern Finland and therefore likely to pay more attention to their eating than the average population in Finland. The participants consumed plenty of fruits and vegetables and received plenty of DF, and probably also phenolic compounds, also from sources other than the study breads. Therefore, the supply of DF remained higher than expected during the WW diets, being in the level of dietary recommendations. However, the total amount of consumed fruits and vegetables remained stable during the study, and there was a significant difference in the DF levels between WG and WW diets, as intended.

In conclusion, the GCxGC-MS technology, being sensitive and selective, offered some advantage for detecting exhaled breath VOCs. Benzoic acid derivatives, phenolic compounds, and furanones are potential compounds in monitoring metabolic effects of whole grains in exhaled breath. However, based on earlier reports by us [42,43] and others [22], it seems that it would be more relevant to monitor changes in the levels of multiple compounds or in VOC profiles rather than individual compounds when monitoring diet-related changes in exhaled breath VOCs.

4. Materials and Methods

4.1. Protocol

A randomized crossover manner dietary intervention was performed with 9 participants. They followed high-fiber diets containing either whole grain rye bread (WGR) or whole grain wheat bread (WGW) for 1 week in randomized order, and there were 1-week periods with refined (white) wheat bread (WW) before both test periods (Figure 1). At the end of the diet periods, exhaled breath samples in fasting state and parallel background air samples (room air samples, BG) were analyzed with GCxGC-MS technology. The RYEBREATH study was approved by the Ethics Committee of the Hospital District of Northern Savo (University of Eastern Finland, Hannu Mykkänen, 40/2015).

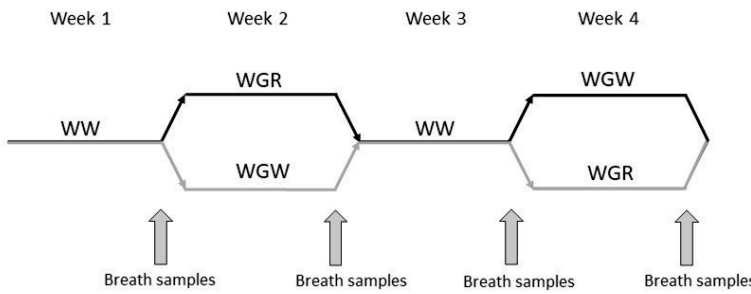

Figure 1. Study design of a randomized crossover manner dietary intervention with 9 participants. WW = control diet containing refined wheat bread; WGR = whole grain rye bread diet; WGW = whole grain wheat bread diet. Exhaled breath and parallel background room air samples were taken at the end of the diet periods.

4.2. Study Participants

The participants were recruited into the RYEBREATH study with the campus advertisements in the University of Eastern Finland. They were healthy non-smoking Finnish men (2) and women (7) aged 21 to 59 years (average 31 years) and with BMI (body mass index) between 18.7 and 29 kg/m^2 (average 23 kg/m^2). The participants were advised to maintain their body weight and habitual lifestyle throughout the study, except the devised dietary modification for cereal content. All the participants provided written informed consent prior to participating in the study.

4.3. Diets

Participants followed three diets differing in consumed grain products. They were advised to consume 5–7 slices of white wheat bread per day during WW periods, 5–7 slices of whole grain rye bread during WGR period and 7–8 slices of whole grain wheat bread during WGW period. The commercial breads used in each period were: white toasts Vaasan Iso Paahto (DF 0.9 g/slice) and Oululainen Reilu Vehnä (DF 1.2 g/slice) during the WW periods, whole grain rye breads Fazer Real Ruis (DF 4.2 g/slice) and Porokylän leipomo PikkuKartano (DF 1.6 g/slice) during the WGR period, and wholegrain wheat breads Fazer Täysjyvä Paahto (DF 1.5 g/slice) and Vaasan Täysjyvä Isopaahto (DF 2.5 g/slice) during the WGW period. The study subjects were advised to avoid whole grain products during the WW periods and not to consume any rye except during the WGR period. Food items which typically increase gut fermentation and fermentative gases in the intestines, such as beans, cabbages, and xylitol products, were avoided throughout the intervention. A master´s student in clinical nutrition advised the participants weekly on the practical management of the diets. The participants filled in 4-day food records during each diet period and recorded the eaten amount of the test breads in a daily questionnaire. The food records were analyzed for nutrient intakes using the Diet32 software (version 1.4.6.3, Aivo Finland Oy, Turku, Finland).

The intakes of energy, protein, fat, and carbohydrates during the test diet periods were maintained at the same level during the intervention (Table 3). Only intake of DF was significantly different between the WW and WG periods. The participants consumed breads on average 159 g/day during the WW periods, 208 g/day during WGR, and 200 g/day during WGW, which covered 18% of energy intake in WW1, 19% in WW2, 24% in WGR, and 23% in WGW.

Table 3. Mean daily intakes [1] of energy and nutrients during the 1-week diet periods (n = 9).

	WW1	WW2	WGR	WGW	*p*-Value [2]
Energy, MJ	9.0 ± 1.7	9.3 ± 1.6	9.0 ± 1.8	9.0 ± 1.5	0.865
Carbohydrates, E%	42 ± 2	41 ± 4	42 ± 3	41 ± 4	0.706
Protein, E%	20 ± 3	20 ± 3	19 ± 2	20 ± 3	0.254
Fat, E%	35 ± 4	36 ± 5	35 ± 4	35 ± 7	0.954
Dietary fiber, g	24 ± 8	25 ± 8	36 ± 6 *	34 ± 1 *	<0.001

[1] Values are means ± SD; [2] Statistical significance of the difference among the diet periods analyzed with Friedman's test; * Different from WW periods, Wilcoxon´s test, $p = 0.008$; WW = diet with white wheat bread; WGR = whole grain rye bread diet; WGW = whole grain wheat bread diet; E% = percentage of total energy intake.

4.4. Exhaled Breath Analysis

End-tidal exhaled breath samples were taken with Bio-VOC® samplers (Markes International Ltd., UK), which are made for capturing the last part of exhaled breath from the alveoli concentrated with VOCs excreted from the circulation (Figure 2a). Participants were trained to give an adequate sample. Before the sampling, participants brushed their teeth with toothpaste and rinsed the mouth effectively with water to stabilize the microbial fermentation in the mouth. They were sitting still without talking and breathing normally for a few minutes before sampling to standardize the ventilation. Then they gave a constant deep blow through the sampler. Exhaled breath samples were injected immediately

after sampling into TD liners (fritted glass liner packed with Tenax GR, mesh 80–100, GL Sciences, Eindhoven, The Netherlands) using the Bio-VOC® sampler as a gas syringe and adapter (self-made by sculpting from PTFE rod) to connect the sampler and the liner tightly (Figure 2b). The TD liner was closed with a storage cap (Brass Liner Blanking Cap, GL Sciences, Eindhoven, the Netherlands) and analyzed within 2–5 h (Figure 2c). The internal standard (1 µL 0.22 µg/µL acetone-d6 (Euriso-top, Saint-Aubin, France) in Milli-Q ultrapure water (Millipore, Bedford, MA, USA)) was injected to the TD liners 30–60 min before sampling by using gas tight syringe and the Bio-VOC® sampler. The background room air samples were taken before breath samples by injecting the room air with the Bio-VOC® sampler to the TD liners. The room air samples were otherwise handled in the same way as breath samples.

Analysis was performed with a GCxGC-MS device consisting of GCMS-QP2010 Ultra and AOC-5000 Plus injection system (Shimadzu Scientific Instruments, Columbia, MD, USA), Optic-4 multi-mode inlet (GL Science, Eindhoven, The Netherlands) and ZX-1 thermal modulator (Zoex Corporation, Houston, TX, USA) (Figure 2d). The injection was done with automated injection of AOC-5000 Plus to the inlet of Optic injector. The temperature of the inlet was at the beginning 35 °C for 2 min and then rose to the 200 °C at the rate of 18 °C/min. The injection was done in high-pressure mode with split 5 allowing the pressure of the inlet decrease temporarily during the injection. The injected sample was preconcentrated to the cryotrap after the injector at −100 °C for 7 min and released rapidly at 200 °C (temperature rise 60 °C/s) to the GC. VOCs were separated on two serial capillary columns; polar Nukol (0.25 µm thick phase, 0.25 mm internal diameter, 30 m long, Supelco, Bellefonte, PA, USA) and non-polar Zebron ZB-35HT Inferno (0.8 µm/0.18 mm/1 m, Phenomenex Torrance, CA, USA), separated by a cryogenic Zoex-modulator. The modulation was done with 8 s modulation time and 10–30% filling of the 5 L dewar of the liquid nitrogen. Carrier gas was Helium 4.6 (AGA, Espoo, Finland) with column pressure 150 kPa, column flow 2.14 mL/min, and linear velocity 45.5. The GC oven was programmed to be 35 °C for 10 min, then raised by 3 °C/min to 200 °C. The duration of the GC program was 70 min. The detection was done with MS SCAN 35–300 m/z, event time 0.02 s, and scan speed 20,000 unit/s. Temperature of the ion source was 200 °C and for MS interface 220 °C.

The data were analyzed using ChromSquare 2.2 data analysis software (Chromaleont, Messina, Italy). All the visible blobs in two-dimensional chromatograph were manually selected for identification. The tentative identification was performed by comparing their mass spectra with data from NIST 11 Mass Spectral library (The National Institute of Standards and Technology, Gaithersburg, MD, USA), Wiley Registry 10th Edition (John Wiley & Sons, Hoboken, NJ, USA), and Flavour & Fragrance Natural & Synthetic Compounds GCMS library FFNSC 2 (Shimadzu Corp., Kyoto, Japan). The identification was checked precisely for each blob by the researcher, but it was not confirmed with analytical standards or retention indices. Tentatively identified VOCs were reported only if they were found in more than a single exhaled breath sample. Four exhaled breath samples and five background room air samples were excluded because of technical problems in the GC-MS analysis. Siloxanes and polyethylene glycol compounds were excluded from analysis because they likely originate from column phases.

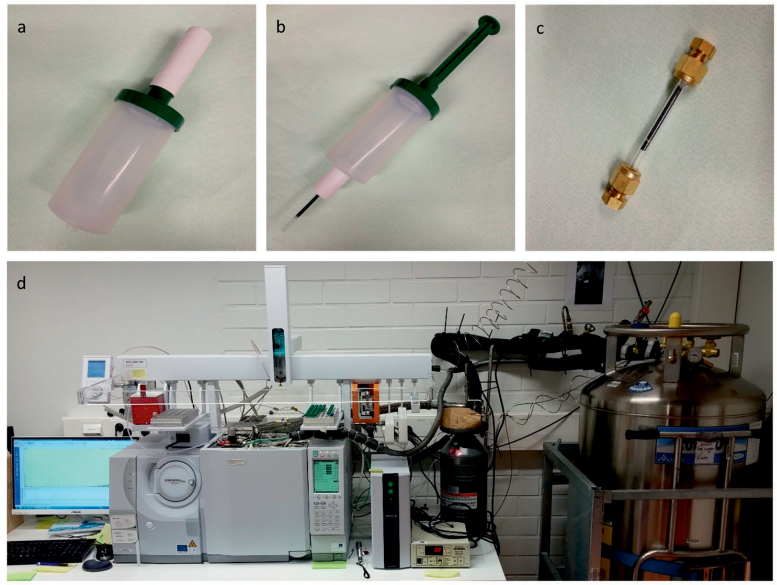

Figure 2. Exhaled breath analysis. End-tidal exhaled breath was sampled with the Bio-VOC® sampler (**a**), injected into a glass liner containing Tenax GR absorbent (**b**), removed to laboratory in a sealed liner (**c**), and analyzed with comprehensive two-dimensional gas chromatography–mass spectrometry (**d**).

5. Conclusions

Exhaled breath VOCs reflect metabolism and lifestyle, thus having large interindividual variation and a lot of so far unidentified molecules. Since diet affects exhaled breath VOCs, they could be utilized in studying the metabolic effects of diets. The GCxGC-MS technology offers some advantage in making the detection of human VOCs sensitive and selective. Exhaled breath benzoic acid derivatives, phenolic compounds, and furanones are interesting compounds to study further when exploring the metabolic effects of whole grains.

Supplementary Materials: The following are available online, Table S1: The detected VOCs (86) with tentative identification [1] from exhaled breath samples (n = 32) of nine study participants.

Author Contributions: Conceptualization, K.R., K.P., H.M. and O.R.; methodology, K.R., E.J.-R., H.M. and O.R.; validation, K.R. and E.J.-R.; formal analysis, K.R. and R.N.; investigation, K.R. and R.N.; resources, K.R., R.N., H.M., K.P. and O.R.; data curation, K.R.; writing—original draft preparation, K.R. and R.N.; writing—review and editing, K.R, E.J.-R., K.P., H.M. and O.R.; visualization, K.R.; supervision, K.P., H.M. and O.R.; project administration, K.P., H.M. and O.R.; funding acquisition, K.R., K.P., H.M. and O.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Hospital District of Northern Savo (University of Eastern Finland, Hannu Mykkänen, 40/2015, 27 January 2015).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The original data are not publicly available due to privacy of participants.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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Exhaled breath is a potential noninvasive sampling matrix to study dietary related metabolic effects.
Rye, rich in dietary fibre and related bioactive compounds, is studied especially for its beneficial effects on glucose metabolism. However, the underlying physiological mechanisms are complex and unclear.
This doctoral thesis explored the potential of exhaled breath VOC analysis
to give new information on the metabolic effects of rye and grain fibre.

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