Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences
Department of Food Science

Analysis of short chain fatty acids in faecal samples

– Development and validation of a new method

Analys av kortkedjiga fettsyror i avföringsprover

– Utveckling och validering av en ny metod

Nils Ewald

Independent Project in Food Science• Bachelor Project • 15 hec • Ground G2E
Publication / Swedish University of Agricultural Sciences, Department of Food Science, no 449
Uppsala, 2016

Analysis of short chain fatty acids in faecal samples

– Development and validation of a new method

Analys av kortkedjiga fettsyror i avföringsprover

– Utveckling och validering av en ny metod

Nils Ewald

Supervisor:	Carl Brunius, Department of Food Science, SLU
Assistant Supervisor:	Huaxing Wu, Department of Food Science, SLU Rikard Landberg, Department of Food Science, SLU
Examiner:	Lena Dimberg, Department of Food Science, SLU

Credits: 15 hec
Level: Ground G2E
Course title: Independent Project in Food Science – Bachelor Project
Course code: EX0669
Programme/education: Agricultural Programme - Food Science

Place of publication: Uppsala
Year of publication: 2016
Cover picture: Illustration by Nils Ewald, 2016
Title of series: Publication / Swedish University of Agricultural Sciences, Department of Food Science
Serie no: 449
Online publication: http://stud.epsilon.slu.se

Keywords: short chain fatty acids, SCFA, faeces, dietary fibre, quantification, gas chromatography, flame ionization detector, GC-FID.

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences
Department of Food Science

Abstract

Short chain fatty acids (SCFA) are produced by the gut microflora following the intake of complex carbohydrates and have been suggested as a contributor for some of the health benefits associated with dietary fibre (DF) intake. For further research of the mechanisms and effects of SCFA, reliable and accurate methods for analysis of SCFA in different biological samples are needed. In this project the aim was to develop and validate a rapid and ease-of use method for analysis of SCFA content in faecal samples. The contents of acetic, propionic, *iso*-butyric, *n*-butyric, *iso*-valeric, *n*-valeric and capronic acid were analysed through a method where fecal water was obtained through centrifugation and SCFA were further extracted with propyl formate. Extracts were analysed on a gas chromatograph coupled with a flame-ionization detector (GC-FID). The method was validated including estimation of accuracy and precision which were measured through calculation of recovery, deviation from linearity and by the coefficient of variance (CV), respectively. An exploratory test was also performed to analyse the distribution of SCFA in faecal matter. Moreover, the method was used to analyse SCFA concentrations in faecal samples (n=40) from two Norwegian bowel cancer projects, NORCCAP and BCSN. The results of the validation (recovery, CV and deviation from linearity) varied somewhat between the three batches, and thereby also the limits of quantification (LOQ), which were limited, but in most cases included the physiologically relevant concentrations. Through the exploratory distribution test, a significant difference in SCFA content was found between the in- and outside of the faecal sample, which emphasises the importance of homogenization of samples or consistent sampling from faecal samples. SCFA contents of faecal samples from the NORCCAP and BCSN samples were consistent with those reported by other studies, which indicate that the method produces reliable results. For a higher accuracy and precision, it is suggested that the amount of faecal material analysed is increased, followed by further validation of both lower and higher concentrations. Additionally, the effects of storage with and without buffer should also be investigated for more comparable results.

Keywords: short chain fatty acids, SCFA, faeces, dietary fibre, quantification, gas chromatography, flame ionization detector, GC-FID.

Sammanfattning

Kortkedjiga fettsyror produceras av tarmfloran efter intag av komplexa kolhydrater och har föreslagits som en bidragande förklaring till några av de hälsofördelar som förknippats med intag av kostfibrer. För vidare studier kring mekanismerna och effekterna av kortkedjiga fettsyror krävs pålitliga och precisa metoder för analys av kortkedjiga fettsyror i olika biologiska prover. I detta projekt var syftet att utveckla och validera en enkel och snabb metod för analys av kortkedjiga fettsyror i avföringsprover. Innehållet av ättik-, propan-, isobutan-, butan-, isovalerian-, valerian- och kapronsyra analyserades genom centrifugering, extrahering med propylformat och analys med gaskromatografi och flamjonisationsdetektor (GC-FID). Metoden validerades genom mätning av noggrannhet och precision som räknades ut i form av utbyte, avvikelse från standardlinje och variationskoefficient (CV). Ett ytterligare test utfördes för att undersöka hur koncentrationen av kortkedjiga fettsyror varierade i olika delar av ett avföringsprov. Dessutom användes metoden för att analysera innehållet av kortkedjiga fettsyror i prover (n=40) från två norska tarmcancer-projekt, NORCCAP och BCSN. Resultaten från valideringen (utbyte, variationskoefficient och avvikelse från standardlinjen) varierade mellan de tre omgångarna och därmed även gränserna för kvantifiering, vilka var begränsade men i de flesta fallen inkluderade de koncentrationer av kortkedjiga fettsyror som är fysiologiskt relevanta. Testet som undersökte variationen av kortkedjiga fettsyror uppvisade signifikanta skillnader mellan in- och utsidan av avföringsprovet, vilket understryker vikten av homogenisering av prover och att vara konsekvent vid provtagning av avföringsprover. De koncentrationer som uppmättes från NORCCAP- och BCSN- proverna stämde väl överens med de som rapporterats i tidigare studier, vilket indikerar att metoden ger tillförlitliga resultat. För att uppnå högre precision och noggrannhet bör mängden avföring som analyseras ökas samt en ytterligare validering på både lägre och högre koncentrationer utföras. Dessutom bör effekterna av lagring med och utan buffert undersökas för mer jämförbara resultat.

Nyckelord: kortkedjiga fettsyror, avföring, kostfibrer, kvantifiering, gaskromatografi, flamjonisationsdetektor.

Table of contents

Abbreviations

CV	Coefficient of Variance
DF	Dietary Fibre
FID	Flame Ionization Detector
GC	Gas Chromatography
IS	Internal Standard
LC-MS	Liquid Chromatography Mass Spectrometry
LLOQ	Lower Limit of Quantification
LOQ	Limit of Quantification
MS	Mass Spectrometry
SCFA	Short Chain Fatty Acids
ULOQ	Upper Limit of Quantification

ace	Acetic acid*
pro	Propionic acid*
ibut	*iso*-Butyric acid*
but	*n*-Butyric acid*
ival	*iso*-Valeric acid*
val	*n*-Valeric acid*
cap	Caproic acid*

* For the sake of simplicity, the acid name will be used for all short chain fatty acids throughout the thesis, even though they in practice may occur in their ion form during some instances.

1 Introduction

1.1 Dietary fibre and health

Cases of non-communicable diseases, chronic diseases which are not transferred from person to person, are increasing and are today the most common causes of death worldwide (WHO, 2014). Cardiovascular disease and different cancers represent 60% of the deaths caused by non-communicable diseases worldwide and these numbers also apply to the situation in Sweden (Socialstyrelsen, 2015; WHO, 2014). Reduced intake and use of alcohol and tobacco are suggested to reduce the deaths from non-communicable disease according to WHO (2014), but also a change towards a more healthy diet.

Increased intake of dietary fibre (DF) may contribute towards a healthier diet. Several studies have shown associations between increased DF intake and lower mortality from cardiovascular disease and certain types of cancer (Kim & Je, 2016). Studies have also consistently shown that the intake of DF lowers the risk for developing type-2 diabetes (Yao *et al.*, 2014). However, the underlying mechanisms and causality is uncertain and further studies on the effects of DF are needed (Kim & Je, 2016; Yao *et al.*, 2014). The production of short chain fatty acids (SCFA) by the gut microflora following DF intake and subsequent absorption and utilization of the host has been suggested as a contributor of health effects related with fibre intake (Lattimer & Haub, 2010).

1.2 Short chain fatty acids- microbial products of fibre fermentation

The gut microflora in the large intestine have an important physiological role in human physiology and health and consists of more than 10^{14} bacteria from more than 400 different species (Bourlioux *et al.*, 2003). Analysis of the 16S rRNA from a single faecal sample have shown that 95% of the species inhabiting the intestinal tracts consists of the phylogenetic groups *Bacteroides* and *Clostridium coccoides* and the subgroup *Clostridium leptum* (Suau *et al.*, 1999). Alterations of the microflora has been associated with several diseases, but it also affects several aspects of a healthy host such as organ morphogenesis, immune system matura-

tion, metabolism and behaviour (Sommer & Backhed, 2013). One of the properties of the microflora is the ability to produce hydrolytic enzymes, which allows the microbiota to digest some of the complex carbohydrates which the human enzymes has no ability to degrade and proteins that have not been absorbed earlier in the gastrointestinal tract (Macfarlane & Macfarlane, 2012). When DF are fermented by the bacterial hydrolytic enzymes, SCFA are among the main products.

SCFA are defined as straight or branched fatty acids with up to six carbons in length. However the definition varies and the upper limit may range between five and seven carbons in length (Rios-Covian *et al.*, 2016; Bergman, 1990). Among SCFA, it is primarily the contents of acetic, propionic and *n*-butyric acid (Figure 1) that are affected by the DF intake (Zhao *et al.*, 2006). These three fatty acids are also the main SCFA found in the large intestine (Cummings *et al.*, 1987). However, *iso*-butyric, *n*-valeric, *iso*-valeric and caproic acid are also present in lower concentrations in the large intestine.

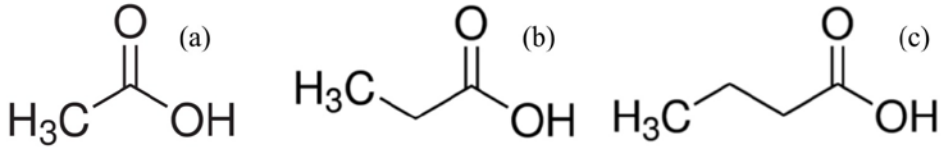

Figure 1. The SCFA found in highest concentrations in the human large intestine: a) acetic acid, b) propionic acid and c) n-butyric acid.

There is a wide range of metabolic effects of the SCFA produced in the gut and most of the effects can be linked to acetic, propionic and *n*-butyric acid (Macfarlane & Macfarlane, 2012). These three SCFA can be oxidized to provide energy and have also been shown to affect the immune system, colonic function, cholesterol metabolism, satiety and oxidative stress. Even if acetic acid is present in the highest concentrations, butyric acid seems to be of greater metabolic importance followed by propionic acid. Butyric acid has been shown to have a preventive and inhibiting effect towards colonic carcinogenesis and may also affect obesity, insulin sensitivity and ischemic stroke (Canani *et al.*, 2011).

Overall, many animal and *in vitro* studies have been made regarding the production and functions of SCFA and how they may affect human health (Canfora *et al.* (2015). However, human studies where the role of SCFA have been investigated in relation to clinical outcomes are largely lacking. Therefore, to draw further conclusion about the role of SCFA in human health more observational and intervention studies in humans are needed.

1.3 Analysis of short chain fatty acids

For further conclusions about the effects of SCFA, a better understanding of partitioning and metabolic fates of SCFA is also needed. This requires reliable analytical methods to quantitate SCFA in different biospecimen, such as blood plasma, serum and faeces. Analysis of faecal SCFA has previously been performed by chromatography coupled with either flame ionisation (FID) or mass spectrometric (MS) detection (Table 1). At earlier stages, LC-MS (Liquid Chromatography Mass Spectrometry) was used for analysis of SCFA (Chen & Lifschitz, 1989) but more recent studies are using GC (Gas Chromatography) because of the high resolution and sensitivity it provides (Zhao *et al.*, 2006). There are also similarities in the sample preparation: most methods use centrifugation at some stage to separate faecal water from solids and larger particles. However, the sample preparation is also the step where most methods differ.

Table 1. Different methods used for analysis of SCFA in faecal samples with their pros and cons.

Method	Analysis	Author	Advantages	Disadvantages
Ultrafiltration and steam distillation	LCMS and GC-FID	Chen and Lifschitz (1989)	Detects many SCFA with high recovery	Time consuming and requires much equipment
Acidification	GC-FID	Zhao *et al.* (2006)	Simple method Detects many SCFA	May contaminate the GC system easily
Solid phase micro-extraction	GC-MS	Garner *et al.* (2007)	Detects large amount of compounds Rapid method	Detects few SCFA
Ultracentrifugation and double derivatization steps	GC-MS	Gao *et al.* (2009)	Detects large amount of compounds	Detects few SCFA

A less advanced method have been used where the faecal samples were homogenized in water and acidified with hydrochloric acid followed by centrifugation (Zhao *et al.*, 2006). The supernatant was then injected through glass wool onto the GC column. A wide array of SCFA were detected, but a possible drawback is the rapid contamination from non-volatile compounds in the GC column that may occur. An earlier method was also able to detect different SCFA through a series of preparations such as stomacher homogenization and ultra-filtration followed by GC and LC-MS (Chen & Lifschitz, 1989). However, this method was very laborious, making it impractical for rapid screening of SCFA in large sample series.

More recent methods using GC-MS have been able to identify a wide variety of compounds in faecal matter. Through a simple procedure of adsorbing volatile compounds with a solid-phase micro-extraction and directly injecting into a GC-MS, it has been possible to detect SCFA and many additional compounds (Garner *et al.*, 2007). Through ultracentrifugation of faecal water followed by derivatization of the compounds by addition of ethyl chloroformate and extraction with hexane, a large amount of different compounds has also been quantified in human faecal samples (Gao *et al.*, 2009). However, an important fact was that major SCFA such as acetic and propionic acid was not analysed in any of these methods.

DF and SCFA have been inversely associated with several non-communicable diseases and other metabolic risk factors. Through the development of reliable and rapid methods for screening the contents of SCFA in large quantities of faecal samples it would be possible to further investigate these associations in interventions and larger cohort studies. There are, as mentioned, a wide variety of different methods for analysis of SCFA content in faecal matter. However, most of these suffer from shortcomings such as being too time consuming, detecting too few SCFA or potentially exposing the column too high amounts of non-volatile compounds, making the methods not qualifying as rapid screening methods.

Besides the possibility to further investigate the mechanisms and potential health benefits of SCFA, by developing more accurate and robust SCFA methods for faecal samples analysis and combining the measurements with those in other biospecimen such as blood plasma, it may be possible to further develop risk models for diseases and risk factors linked to SCFA.

1.4 Aim of the project

The aim of this project was to develop a method for analysis of SCFA in faecal samples. A method recently developed by Wu *et al*. (manuscript) for SCFA analysis in blood plasma was extended and validated for faecal samples. Faecal samples obtained from the Norwegian bowel cancer projects NORCCAP and BCSN were used within this development and validation study. To make the method applicable for larger screening quantities, a secondary aim was to address a combination of requirements: reduction or complete elimination of non-volatile compounds during the sample preparation while at the same time offering an ease-of-use and a rapid procedure for multiple sample analysis.

2 Methods

2.1 Sample collection

The Norwegian Cancer Registry is the institution under Oslo University Hospital Trust which collects information about all cancer cases in Norway. They are responsible for several national screening programs for cancer in Norway such as the Norwegian Colorectal Cancer Prevention (NORCCAP) and the Bowel Cancer Screening in Norway (BCSN) (Johnsen & Ursin, 2013). Samples collected during these two projects were analysed in this experiment.

The NORCCAP study was performed between 1999 and 2001. Norwegian women and men (n=21 000) aged 50-64 and living in Oslo and Telemark were randomly chosen from the population registry (Johnsen & Ursin, 2015). They were offered a screening examination of the large intestine to detect cancer prevalence. Half of the participants were also offered an extended screening of blood and faecal samples, which were collected for further research purposes. The faecal samples from the NORCCAP study were stored without buffer at -25 to -30°C (Knudsen, personal communication). A randomly selected subset (n=20) of the NORCCAP faecal sample set were used in the present study.

The BCSN study is currently running and was built upon the NORCCAP study. In 2012, Norwegian women and men (n=140 000) aged 50-74, living in the Norwegian counties Østfold, Akershus and Buskerud, were invited to participate in the study (Johnsen & Ursin, 2012). The study is screening for cancer in colon and rectum and aims to develop improved methods for screening colorectal cancer. Faecal samples were collected by the participants themselves with a self-administered kit where 10 mg faeces were added to 2 mL HEPES buffer. These samples were stored at -25°C (Knudsen, personal communication). For the present study, a randomly selected subset (n=20) of BCSN samples were analysed. Half of the samples were older and collected at an earlier stage compared to the rest of the samples.

Quality Control (QC) samples were collected from a 22-year old healthy male volunteer who did not participate in any of the Norwegian bowel cancer projects. The sample collection was preceded by a day of a high DF intake, consisting predominantly of whole grain products and foods rich in inulin such as onion and Jerusalem artichoke. Whole faecal samples were collected by the volunteer himself and were stored without buffer in plastic containers at -80°C.

2.2 Chemicals, reagents and standards

The HEPES buffer used contained 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; *Sigma H-3375*), 0.4% bovine serum albumin (BSA; *Medicago, 16-0026-500*), 0.095% sodium azide (*Sigma-Aldrich, CAS No. 26628-22-8*) and had pH=6.8. Acrylic acid (*Sigma 147230*) was used as internal standard (IS; 4 mM acrylic acid in 16% (w/v) metaphosphoric acid (*Merck no 1.00546*)). Propyl formate (*Sigma-Aldrich no W294306-1KG-K*) was purified by adding 50% (w/v) saturated sodium carbonate water solution before use.

In total, twelve different standard solutions were prepared and used for calibration. These standard solutions contained a mixture of seven different SCFA (Table 2) in concentrations ranging between 0.2-64 mM for acetic acid and 0.02-6.4 mM for the other six SCFA. The standard solutions were stored at +4°C.

Table 2. The compounds measured throughout the experiment and added to the standard solution.

Compound	Chemical formula	Molar mass (g/mol)	Boiling point (°C)	Manufacturer, product number
Acetic acid	$C_2H_4O_2$	60.05	118	*Merck no. 6268*
Propionic acid	$C_3H_6O_2$	74.08	141	*Sigma no. P 1880*
iso-Butyric acid	$C_4H_8O_2$	88.11	155	*Fluka no. 58360*
n-Butyric acid	$C_4H_8O_2$	88.11	164	*Aldrich? no. 30.341.0*
iso-Valeric acid	$C_5H_{10}O_2$	102.13	175	*Fluka no. 59850*
n-Valeric acid	$C_5H_{10}O_2$	102.13	186	*Fluka no. 94530*
Capronic acid	$C_6H_{12}O_2$	116.16	206	*Fluka no. 21530*

2.3 Sample preparation

Samples not stored in buffer (QC samples and NORCCAP) were diluted and homogenised in HEPES buffer before analysis. A total amount of 400 mg of faecal matter from a QC sample was weighed into 80 ml HEPES buffer. Metal beads were then added and the sample was homogenised by vortexing and shaking by hand. The obscure yellow supernatants were aliquoted (1 mL) and stored at -25°C until analysis. Similarly, 6 mg fecal matter from the NORCCAP study samples were each weighted and put into 1.2 mL HEPES buffer. Metal beads were then added to the samples, which were then vortexed, after which 1 mL yellow supernatant was further prepared for GC-MS analysis.

Internal standard mixture (100 µL) was added to 1 mL faecal extract (5 mg/mL HEPES), and vortexed for 30 seconds. Washed propyl formate (300 µL) was added and the samples were extracted by vortexing for an additional 30 seconds, followed by centrifugation for 30 minutes at 23600 g. Approximately 100 µL of the clear upper organic phase was transferred to GC vials and analysed by GC-FID. For performance monitoring, four blank samples (containing no faecal sample) and four QC samples were also prepared and analysed in each sample sequence.

2.4 Sample analysis

The samples were analysed using the method by Wu *et. al.* (manuscript), here described briefly. Propyl formate extracts (2 µL) were injected (splitless) by a CTC CombiPAL autosampler (CTC analytics, Zwingen, Switzerland) onto an Aglient 6890N GC system equipped with a flame ionization detector (GC-FID). Helium was used as carrier gas (8.0 mL/min) and separation was conducted on a ZB-FFAP column (30 m ∗ 0.53 mm ∗ 1 µm, Phenomenex, Torrance, California). A straight glass liner was used in the injector and maintained at 200 °C. The oven was maintained at 50 °C for 2 min, ramped up to 135 °C at 20 °C/min and held for 5 min, then ramped up to 240 °C at 30 °C/min and finally maintained for 5 min. The flow rates of hydrogen, air and nitrogen (makeup gas) in the FID were 30, 300 and 30 mL/min, respectively. The temperature of the FID was 200 °C. The run time for each analysis was 20 min.

Pure SCFA samples were analysed to obtain specific retention times for each SCFA, which were later used for compound identification. Standard samples were also extracted in the same way as faecal samples (1 mL pure standard was treated as 1 mL HEPES with faecal sample) and during each run, these standards were injected to monitor system performance and for calibration. Chromatograms were received from the FID, and the response (measured as area under the peaks) from samples and standard solutions was used for further calculations of concentrations.

2.5 Method validation

Validation was performed according to FDA (2001) and included testing of precision, accuracy and linearity. However, robustness testing was not performed. For precision, the coefficient of variation (CV) was estimated. Accuracy was assessed through analysis of recovery after spiking a sample with known amounts of analytes and was also evaluated by deviation from linearity of the standard curves. Validation was performed in three batches over three days.

QC samples were spiked with 100 µL standard solution at five different levels. However, in the first validation batch, only STD 8,10 and 12 were used. Samples were prepared in triplicate and at each spiking level, samples were prepared both with and without faecal QC sample (Table 3). After spiking, all samples were prepared similarly to ordinary samples with 100 µL acrylic acid in metaphosphoric acid and 300 µL propyl formate added before centrifugation and GC analysis.

Table 3. Experiment outline for the method validation. Concentrations are final and adjusted for the volume of spiking solution added. Each sample (A-L) was prepared in triplicate.

Sample	1 ml HEPES with faecal QC	1 ml pure HEPES	100 µl spiking solution	Conc. acetic acid (µM)	Conc. other SCFA (µM)
A	-	x	-	0	0
B	x	-	-	0	0
C	-	x	STD 1	20	2
D	x	-	STD 1	20	2
E	-	x	STD 3	80	8
F	x	-	STD 3	80	8
G	-	x	STD 8	400	40
H	x	-	STD 8	400	40
I	-	x	STD 10	1600	160
J	x	-	STD 10	1600	160
K	-	x	STD 12	6400	640
L	x	-	STD 12	6400	640

Results were calculated from the chromatograms obtained. All calculations were made both within the batches (intra-batch measurement) and between the batches (inter-batch measurement). The areas of samples were normalised by the measured area of the IS. The areas (*response*$_{spike}$) of samples were then subtracted with the area of the baseline (*response*$_{baseline}$) specific for each matrix (i.e. with or without QC sample). Precision, measured as CV, and the recovery were calculated using the formulas seen below. Example calculations can be seen in Appendix 7.1.

$$Cal.repsonse_{QC\ spike} = Response_{QC\ spike} - Response_{QC\ baseline}$$
$$Cal.repsonse_{HEPES\ spike} = Response_{HEPES\ spike} - Response_{HEPES\ baseline}$$

$$Precision(CV) = \frac{Standard\ deviation\ (Cal.repsonse_{QC\ spike\ 1-3})}{Mean\ value\ (Cal.repsonse_{QC\ spike\ 1-3})}$$

$$Recovery = \frac{Mean\ value\ (Cal.repsonse_{QC\ spike\ 1-3})}{Mean\ value\ (Cal.repsonse_{HEPES\ spike\ 1-3})}$$

Standard curves were constructed using the mean response of the spiked QC-samples towards the corresponding spiking concentrations. Concentration levels with a CV exceeding 20% or recovery deviating more than 20% from 100% were excluded from the standard curves. Using the standard curves, concentrations were calculated (*concentration*$_{measured}$) and compared to the concentrations added (*concentration*$_{true}$) to calculate the deviation from linearity in accordance with the formula seen below. Example calculations can be seen in Appendix 7.1.

$$Deviation\ from\ linearity = \frac{Concentration_{measured} - Concentration_{true}}{Concentration_{true}}$$

Using the measurements of accuracy and precision, the limits of quantification (LOQ) were determined. When accuracy or precision exceeded 20% (recovery counted as deviation from 100%), that concentration level was determined as the upper or lower limit of quantification (ULOQ; LLOQ) for the analyte.

2.6 Distribution of SCFA in a faecal sample

An exploratory analysis was performed to investigate the variations in SCFA concentration within a faecal sample. Samples were collected in four technical replicates from six different positions (1-6) of a faecal sample: upper, middle and lower, on the inside and outside respectively (Figure 2). The samples were analysed in the same way as the other faecal samples. The faecal sample was collected simultaneously as the QC samples, but was not the same sample used for the validation. The difference between SCFA content between the inside and outside of the faecal sample was tested using unpaired, two-tailed T-test in Microsoft® Excel for Mac 15.21 (Microsoft Corporation, Redmond, Washington).

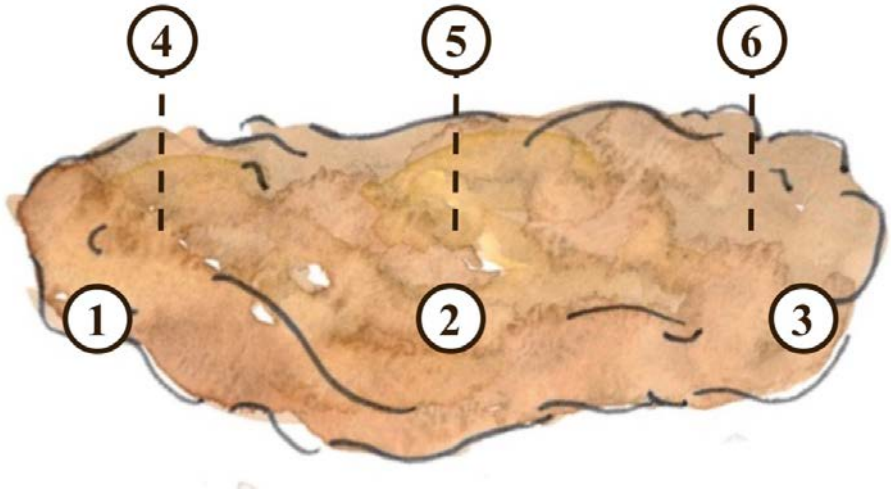

Figure 2. A schematic figure describing the positions of the samples collected from a single faecal sample. The samples were collected from a lower, middle and upper position in a peripheral position (sample 1-3) and the bulk (sample 4-6) respectively. (Illustration: Nils Ewald).

3 Results

3.1 Method validation

A high variability was observed between the results of the three validation batches, especially at the lower spiking levels (Table 4). The values of validation (recovery, CV and deviation from linearity) were in general higher at the lower spiking levels and higher during the second validation batch for all spiking levels compared to the the first and third batch. It was also observed that the inter-batch CV exceeded 20% at all spiking levels, therefore this measure was excluded when determining LOQs for the inter-batch analysis.

With the measures of accuracy and precision varying between batches, the LOQs also varied (Table 5-8). However, the ULOQ was relatively constant at 1280 µmol/g for acetate and 128 µmol/g for the other SCFA respectively and R^2-values were all close to 1. In most cases the ULOQ was determined by the lack of measurements at higher concentrations, with exception for three SCFA in the second validation batch where the recovery deviated more than 20% from 100%. The LLOQ was lower during the third validation batch compared to the second, and the LLOQ for the first batch was only determined by lack of measurements at the two lower concentrations. The varied results indicate a LLOQ of 4-80 µmol/g for acetate respectively 0.4-8 µmol/g for the other SCFA.

Table 4. Summary of the validation results on three spiking levels: low (4/0.4 µmol/g faeces), medium (80/8 µmol/g faeces) and high (1280/128µmol/g faeces). Recoveries are presented as deviation from 100%. Abbreviations: recovery (Rec), deviation from linearity (Dev) and inter-batch (Inter). Full results for all five spiking levels can be seen in Appendix 7.2.

Spiking	Batch		ace (%)	pro (%)	ibut (%)	but (%)	ival (%)	val (%)	cap (%)
Low	2	Rec	35.8	15.2	-45.4	-345.3	-82.7	-126.0	-205.2
		CV	11.6	17.0	7.1	20.5	11.9	17.4	23.4
		Dev	-176.3	23.5	357.2	1253.5	844.8	928.9	-114.1
	3	Rec	19.7	10.2	18.4	-10.5	24.8	-27.7	-53.1
		CV	26.9	11.9	0.7	18.5	7.9	10.1	17.6
		Dev	-1.2	35.2	122.7	-33.1	-7.0	-10.6	-74.1
	Inter	Rec	31.2	13.6	-10.4	-152.6	-26.5	-83.4	-142.8
		CV	47.8	46.8	27.0	70.2	53.5	56.0	68.2
		Dev	-79.4	13.3	172.4	253.7	219.9	416.5	516.3
Medium	1	Rec	4.5	2.3	-1.7	-12.1	-5.9	0.7	-11.7
		CV	2.8	0.9	2.0	3.9	2.9	3.0	3.3
		Dev	-5.5	-1.5	-0.6	5.9	1.3	2.1	0.0
	2	Rec	4.2	2.6	-6.9	-21.8	-9.7	-14.1	-18.3
		CV	1.9	3.8	1.1	4.0	1.6	1.5	2.8
		Dev	-6.9	-0.8	7.1	49.4	27.0	29.1	0.0
	3	Rec	0.4	0.1	1.6	-4.6	1.2	-3.0	-5.5
		CV	1.3	1.7	0.5	1.9	0.6	0.3	0.4
		Dev	-2.8	-1.7	-0.6	0.7	0.3	0.4	-0.3
	Inter	Rec	3.5	1.9	-2.8	-13.8	-5.6	-5.5	-12.7
		CV	30.8	27.2	25.5	26.4	25.9	25.4	28.0
		Dev	-4.2	-0.4	3.2	8.9	6.1	13.4	17.6
High	1	Rec	1.7	0.9	-1.2	-1.9	-3.5	-3.0	-5.1
		CV	0.6	0.3	0.3	0.3	0.7	0.7	0.9
		Dev	-0.1	0.0	0.0	0.0	0.0	0.0	1.8
	2	Rec	5.7	1.4	-7.1	-9.5	-16.8	-18.5	-26.9
		CV	6.3	1.2	6.7	7.5	13.5	14.4	19.9
		Dev	-0.3	0.1	0.4	0.0	0.4	0.5	-17.1
	3	Rec	1.2	14.8	1.3	0.6	1.2	0.8	1.0
		CV	0.5	0.2	0.1	0.2	0.9	1.0	2.0
		Dev	0.0	0.1	0.1	0.0	0.0	0.0	0.0
	Inter	Rec	3.2	4.7	-2.8	-4.2	-7.5	-8.1	-12.3
		CV	29.6	25.6	25.2	27.2	30.6	31.3	36.1
		Dev	-0.2	0.0	0.2	0.1	0.3	0.2	0.3

Table 5. The LOQs (µmol/g faeces) determined during the first validation batch and the limiting factors for each LOQ. R^2 is the coefficient of determination for the standard curve constructed for calculation of deviation from linearity. Only STD 8, 10 and 12 were used for spiking during this validation batch.

	ace	pro	ibut	but	ival	val	cap
LLOQ	80	8	8	8	8	8	8
Limit	-	-	-	-	-	-	-
ULOQ	1280	128	128	128	128	128	128
Limit	-	-	-	-	-	-	-
R^2	0.999936	0.999995	0.999999	1.000000	0.999996	0.999990	1.000000

Table 6. The LOQs (µmol/g faeces) determined during the second validation batch and the limiting factors for each LOQ. R^2 is the coefficient of determination for the standard curve constructed for calculation of deviation from linearity

	ace	pro	ibut	but	ival	val	cap
LLOQ	16	0.4	1.6	8	8	8	1.6
Limit	Deviation	Deviation	Deviation	Recovery	Recovery	Deviation	Recovery
ULOQ	1280	128	128	128	128	128	128
Limit	-	-	-	-	Recovery	Recovery	Recovery
R^2	0.999716	0.999991	0.999211	1.000000	0.998485	0.998240	1.000000

Table 7. The LOQs (µmol/g faeces) determined during the third validation batch and the limiting factors for each LOQ. R^2 is the coefficient of determination for the standard curve constructed for calculation of deviation from linearity. At the LLOQ for acetic acid. both recovery and precision were limiting.

	ace	pro	ibut	but	ival	val	cap
LLOQ	4	0.4	1.6	0.4	0.4	0.4	1.6
Limit	Rec, Pre	Deviation	Deviation	Precision	Recovery	Recovery	Recovery
ULOQ	1280	128	128	128	128	128	128
Limit	-	-	-	-	-	-	-
R2	0.999989	0.999988	0.999963	0.999999	1.000000	1.000000	1.000000

Table 8. The LOQs (µmol/g faeces) determined during the inter- batch validation analysis and the limiting factors for each LOQ. R^2 is the coefficient of determination for the standard curve constructed for calculation of deviation from linearity. The CV exceeded 20% for all concentration levels in the inter-batch validation, therefore the LOQs were only determined using the accuracy measurements.

	ace	pro	ibut	but	ival	val	cap
LLOQ	16	0.4	1.6	1.6	1.6	1.6	8
Limit	Deviation	-	Deviation	Recovery	Deviation	Recovery	Deviation
ULOQ	1280	128	128	128	128	128	128
Limit	-	-	-	-	-	-	-
R^2	0.999892	0.999999	0.999867	0.999836	0.999669	0.999628	0.999358

3.2 Distribution of SCFA in a faecal sample

Mean values were calculated for the concentrations measured at each position. A low standard deviation was observed and overall, the contents were higher on the inside than the the surface and some differences were seen between the lower, middle and upper positions (Table 9). The T-test showed a significant difference between the inside and surface of the faecal sample (Table 9). It was not possible to perform an ANOVA comparing the three longitudinal positions due to the low amount of samples taken and the fact that the samples from each position were technical replicates.

Table 9. Mean concentrations (µmol/g faeces) and standard deviations on six different positions of a faecal sample. The resulting P-values from the T-test comparing the contents of the in- and outside are presented below.

Position		*ace*	*pro*	*ibut*	*nbut*	*ival*	*nval*	*cap*
1	Mean	126.8	25.5	3.2	27.3	4.1	5.4	5.5
	SD	0.81	0.02	0.02	0.16	0.02	0.04	0.06
2	Mean	121.0	25.7	3.5	28.3	4.7	5.6	5.2
	SD	0.23	0.05	0.01	0.14	0.01	0.03	0.02
3	Mean	115.0	22.2	2.8	22.4	3.6	4.6	5.0
	SD	0.90	0.09	0.01	0.17	0.02	0.02	0.04
4	Mean	133.8	31.0	3.9	43.3	5.3	6.9	7.5
	SD	1.19	0.45	0.01	0.56	0.03	0.02	0.02
5	Mean	139.7	34.6	4.5	51.4	6.0	7.9	8.8
	SD	0.70	0.29	0.02	0.13	0.03	0.04	0.07
6	Mean	141.4	34.8	4.6	51.7	6.2	8.1	9.1
	SD	0.79	0.30	0.01	0.12	0.04	0.03	0.03
T-test	P-value	0.0176	0.0062	0.0132	0.0039	0.0151	0.0074	0.0153

3.3 SCFA concentrations in NORCCAP and BCSN samples

Three kinds of faecal samples were analysed; samples in the NORCCAP study stored without buffer and older respectively newer samples collected in the BCSN study stored in HEPES buffer. Boxplots were constructed (Figure 3) and a one-way ANOVA was performed using SAS® 9.4 (SAS Institute Inc., North Carolina, US) to compare the concentrations of the different sample types. One test was made using two levels: NORCCAP and BCSN samples, and one test was made with three levels: NORCCAP samples and older and newer BCSN samples. However, no significant differences were observed in any of the tests for any SCFA.

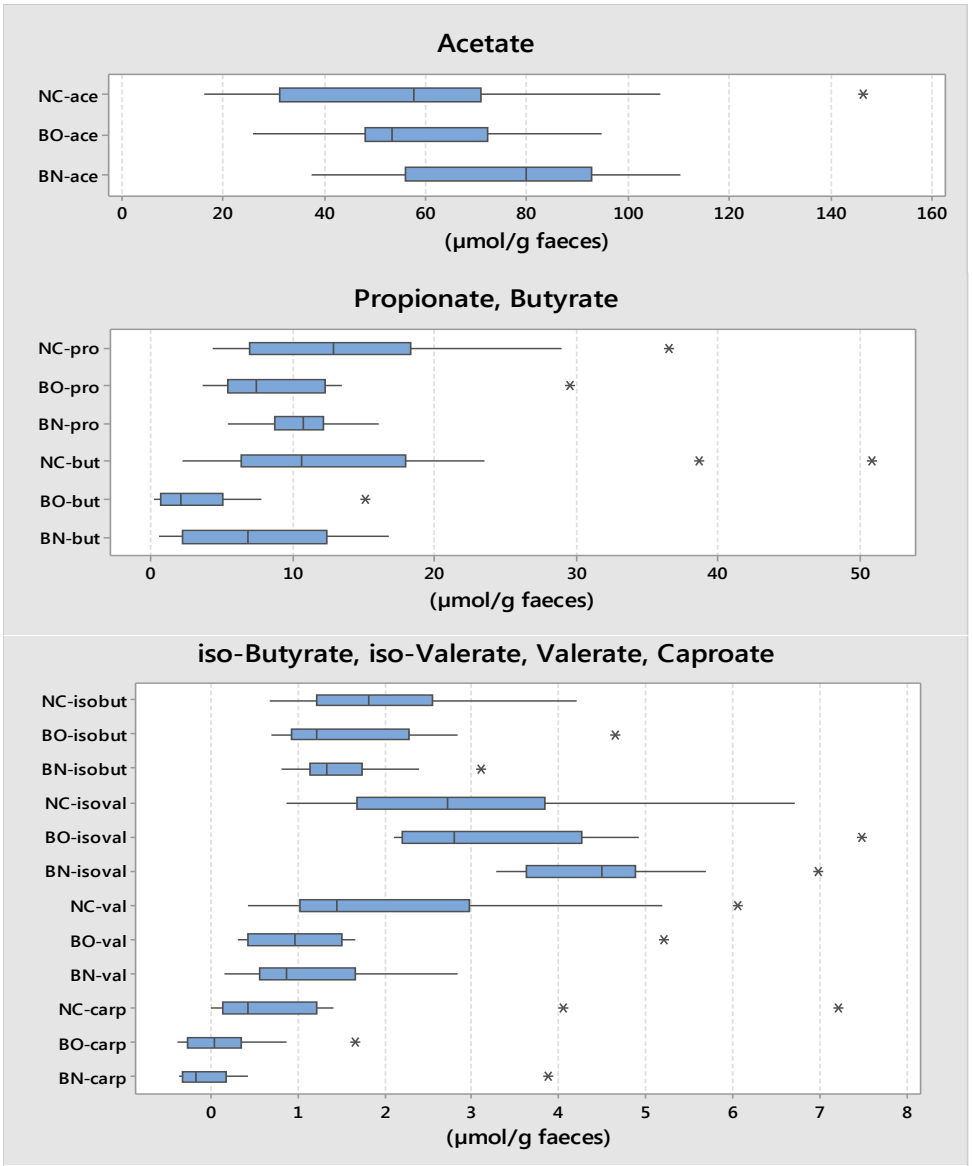

Figure 3. Boxplots describing the SCFA concentrations measured throughout the analysis of NORCCAP and BCSN samples. The stars (*) marks the outliers identified. Abbreviations used: NC (NORCCAP samples), BO (old BCSN samples) and BN (new BCSN samples).

4 Discussion

The validation showed that the method used was useful for quantification of seven SCFA in faecal samples within the range determined by the LOQs. With the sample preparation taking approximately four hours for 40 samples and the GC-FID analysis 20 minutes per sample, the method can be regarded as a rapid method suitable for screening of larger number of samples.

The ULOQ was similar between batches. In general, the accuracy and precision ranged between 0-10% at the highest concentration level with a few exceptions. This indicates that it may be possible to expand the quantification range upwards by analysing higher concentration levels. The coefficient of determination (R^2) was another factor with low variability and was in all cases close to the number 1 with only a few thousands in difference. This shows high linearity of the standard samples. However, with only five standard samples used at its highest, the R^2-value is expected to be very close to 1.

The concentration level of the LLOQ varied more between the validation batches: for acetate it ranged between 4-80 µmol/g faeces and for other SCFA it ranged between 0.4-8 µmol/g. The reason for this variation is partly explained by the first validation batch, where the two lower spiking levels were not analysed. It was also observed that the values of accuracy and precision was much higher in the second validation batch compared to other batches. This may have been caused handling errors throughout the experiment. If a more experienced person would carry out the validation it is possible that lower values of accuracy and precision would be achieved and the results between the batches would be more similar.

Even though the lowest LLOQs would be assumed (4, 0.4 and 1.6 µmol/g respectively), the sensitivity of the method is somewhat limited compared with an earlier method developed by Zhao *et al.* (2006). However, the concentrations measured in the BCSN and NORCCAP samples were within the LOQs for all SCFA with exception for *iso*-butyric acid and caproinc acid. With exception for these two SCFA, this proves the applicability of the method for physiologically relevant concentrations. It would be desirable to further expand the range of quantification for the method though, especially for *iso*-butyric and capronic acid.

Comparing with other methods, a distinguishable difference is the amount of samples used for analysis. In other methods (Zhao *et al.*, 2006; Chen & Lifschitz, 1989), 0.1 to 0.2 g of faecal sample is added per mL solvent in the initial steps of the experiment. By increasing the concentration of faecal sample analysed, it is possible that a higher sensitivity and a lower LLOQ could be achieved, which has also been suggested in earlier methods for SCFA analysis (Arellano *et al.*, 2000).

The inter-batch CV exceeded 20% for all SCFA when comparing the batches, a problem which was neither observed in the recovery nor deviation from linearity. This may indicate that the response within each batch are on the same relative levels, but between the batches the response differs, thus giving a high CV. It is possible that the column of the GC successively has been contaminated, indicating that the aim to reduce or eliminate non-volatile compounds was not fulfilled. Another possible explanation though, is the organic solvent used. A new bottle of washed propyl formate was taken into use before the third validation batch. A distinctly higher amount of upper phase as observed during this batch compared to earlier batches. A larger volume of organic phase would explain the lower response seen in the third validation batch compared to the two earlier batches, as a result of a more diluted sample. The purity of the washed propyl formate therefore seems to have a significant role for the formation of phases, which should be heavily considered for further analysis to achieve comparable results.

Before applying the method in further studies, additional validation analysis should preferably be carried out. After increasing the amount of faeces used in the method, the lower concentrations should be examined throughoutly once again. To expand the range of quantification towards higher concentrations, QC samples should also be spiked with higher SCFA concentrations.

The SCFA distribution test showed a significantly higher content of all seven SCFA on the inside compared to the outside of the faecal sample. Colonic SCFA is rapidly absorbed by the epithelial cells in the colorectal region of the intestine with only 5-10% of the produced amounts remaining in the faeces (Canfora *et al.*, 2015). Since the SCFA of the peripheral parts of the faeces naturally are more easily accessible, these are also more rapidly absorbed, which may be the reason for the observed difference between the in- and outside. The volatility of SCFA may also contribute, since the SCFA of the outside of the faeces are more prone to evaporation than those on the inside.

Even though the analysis was performed on a single faecal sample it may give important information about the characteristics of faecal matter. The analysis clearly shows that a single aliquot of faecal sample may not be representative for the whole faecal sample with respect to the SCFA content. To reduce error terms from sampling location, homogenization of the faecal sample before analysis would therefore be ideal. This may not always be possible, however. The second best opinion would then be consistent sampling from the same position of all samples, which may also be difficult, since faeces will vary in shape and consistence.

No earlier evidence has been found regarding the distribution of SCFA or metabolites in general in faecal samples. To further investigate the case, analysis of samples from a large variety of individuals is suggested. Biological replicates within each position is also suggested to make further statistical analysis possible regarding both inner and outer position, but also the longitudinal position. This way it would be possible to make more accurate measurements and obtain comparable results regarding SCFA in faeces.

The SCFA concentrations measured in the Norwegian cancer project faecal samples shows large variability, which is to be expected from a free-living population. Compared to another study (Hoverstad *et al.*, 1984) earlier performed on a Norwegian population (n=20), the SCFA concentrations lies within the same range. This further indicates that the method used in this project is valid for the concentrations which were measured. Another study (McOrist *et al.*, 2008) carried out on a small Australian population (n=8), also provides similar results regarding the concentrations. However, this study should not be seen as fully comparable, since it has been shown that the SCFA contents in faeces are significantly different between ethnical groups (Segal *et al.*, 1995).

NORCCAP samples were collected more than 15 years ago, and due to the volatility of SCFA it is likely that some SCFA have evaporated during storage. However, there is no evidence of systematically lower concentrations in these samples compared to the more recently collected BCSN samples, which makes this hypothesis less plausible. In some cases, the NORCCAP samples seem to contain even a higher amount of SCFA than the BCSN samples. During sampling it was observed that some NORCCAP samples were very dry, which indicates that water may have evaporated during the storage, giving a higher concentration of SCFA. However, the ANOVA performed showed no significant difference between the different sample types to support this theory.

When analysing the BCSN samples, it was also apparent that the volume had reduced from the original sample addition to the vials. This represents a considerable problem first because it hints at poor storage stability either of the samples or in the applied protocol. If the volume reduction was the result of solvent evaporation, the contents will also be more concentrated giving a systematic but unquantifiable bias. In some cases, the caproic acid concentrations for BCSN samples reached negative concentrations. A possible explanation is that the HEPES buffer used for storage was different from the one used for blank samples since they were produced in Norway and Sweden respectively. If this method is to be further developed, it would be essential to investigate thoroughly the effects of storage with and without HEPES buffer, and to use the same buffer in storage and analysis.

5 Conclusion

The aim of this project was to develop a rapid and ease-of-use method for analysis of SCFA contents in faecal samples, which was also achieved. The validation of the method presented showed varying results, but for most SCFA the limits of quantification included the physiologically relevant concentrations. The exploratory test performed regarding the distribution of SCFA contents in faecal matter implicates that homogenisation and the position when sampling is of importance due to the differences seen between the in- and outside of the faeces. Results obtained from the NORCCAP and BCSN faecal samples is consistent with earlier reports, indicating that the concentrations measured with the method are within the correct range. However, before further application of the method, additional validation is proposed. A larger amount of faeces should preferably be added to the method, the lower concentrations should be examined once again, and higher concentration levels should also be investigated. A further investigation is also proposed regarding the storage stability with and without HEPES buffer.

6 References

Arellano, M., Jomard, P., El Kaddouri, S., Roques, C., Nepveu, F. & Couderc, F. (2000). Routine analysis of short-chain fatty acids for anaerobic bacteria identification using capillary electrophoresis and indirect ultraviolet detection. *Journal of Chromatography B: Biomedical Sciences and Applications,* 741 (1), pp. 89-100.

Bergman, E.N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Reviews,* 70 (2), pp. 567-590.

Bourlioux, P., Koletzko, B., Guarner, F. & Braesco, V. (2003). The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium "The Intelligent Intestine," held in Paris, June 14, 2002. *The American Journal of Clinical Nutrition,* 78 (4), pp. 675-683.

Canani, R.B., Costanzo, M.D., Leone, L., Pedata, M., Meli, R. & Calignano, A. (2011). Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World Journal of Gastroenterology,* 17 (12), pp. 1519-1528.

Canfora, E.E., Jocken, J.W. & Blaak, E.E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature Reviews Endocrinolology,* 11 (10), pp. 577-591.

Chen, H.M. & Lifschitz, C.H. (1989). Preparation of fecal samples for assay of volatile fatty acids by gas-liquid chromatography and high-performance liquid chromatography. *Clinical Chemistry,* 35 (1), pp. 74-6.

Cummings, J.H., Pomare, E.W., Branch, W.J., Naylor, C.P. & Macfarlane, G.T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut,* 28 (10), pp. 1221-1227.

FDA (2001). *Guidace for Industy: Bioanalytical Method Validation*: Food and Drug Administration.

Gao, X., Pujos-Guillot, E., Martin, J.-F., Galan, P., Juste, C., Jia, W. & Sebedio, J.-L. (2009). Metabolite analysis of human fecal water by gas chromatography/mass spectrometry with ethyl chloroformate derivatization. *Analytical Biochemistry,* 393 (2), pp. 163-175.

Garner, C.E., Smith, S., de Lacy Costello, B., White, P., Spencer, R., Probert, C.S.J. & Ratcliffe, N.M. (2007). Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease. *The FASEB Journal,* 21 (8), pp. 1675-1688.

Hoverstad, T., Fausa, O., Bjorneklett, A. & Bohmer, T. (1984). Short-chain fatty acids in the normal human feces. *Scand J Gastroenterol,* 19 (3), pp. 375-81.

Johnsen, T. & Ursin, G. (2012-02-14). *Bowel Cancer Screening in Norway – a pilot study*. http://www.kreftregisteret.no/en/Cancer-prevention/Screening-for-colorectal-cancer/ [2016-04-08].

Johnsen, T. & Ursin, G. (2013-06-23). *About the organization*. http://www.kreftregisteret.no/en/General/About-the-Cancer-Registry/About-the-organization/ [2016-04-25].

Johnsen, T. & Ursin, G. (2015-10-08). *Norwegian Colorectal Cancer Prevention study no. I*. http://www.kreftregisteret.no/en/Research/Projects/NORCCAP-I/ [2016-04-08].

Kim, Y. & Je, Y. (2016). Dietary fibre intake and mortality from cardiovascular disease and all cancers: A meta-analysis of prospective cohort studies. *Archives of Cardiovascular Diseases,* 109 (1), pp. 39-54.

Lattimer, J.M. & Haub, M.D. (2010). Effects of dietary fiber and its components on metabolic health. *Nutrients,* 2 (12), pp. 1266-1289.

Macfarlane, G.T. & Macfarlane, S. (2012). Bacteria, colonic fermentation, and gastrointestinal health. *Journal of AOAC International,* 95 (1), pp. 50-60.

McOrist, A.L., Abell, G.C.J., Cooke, C. & Nyland, K. (2008). Bacterial population dynamics and faecal short-chain fatty acid (SCFA) concentrations in healthy humans. *British Journal of Nutrition,* 100 (1), pp. 138-146.

Rios-Covian, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., De Los Reyes-Gavilan, C.G. & Salazar, N. (2016). Intestinal short chain fatty acids and their link with diet and human health. *Frontiers in Microbiology,* 7.

Segal, I., Hassan, H., Walker, A.R.P., Becker, P. & Braganza, J. (1995). Fecal short chain fatty acids in South African Urban Africans and whites. *Diseases of the Colon & Rectum,* 38 (7), pp. 732-734.

Socialstyrelsen (2015). *Dödsorsaker 2014*. (Sveriges Officiella Statistik 2015-8-1). Stockholm: Socialstyrelsen (National Board of Health and Welfare).

Sommer, F. & Backhed, F. (2013). The gut microbiota [mdash] masters of host development and physiology. *Nature Reviews Microbiology*, 11 (4), pp. 227-238.

Suau, A., Bonnet, R., Sutren, M., Godon, J.-J., Gibson, G.R., Collins, M.D. & Doré, J. (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied and Environmental Microbiology*, 65 (11), pp. 4799-4807.

WHO (2014). *Global status report on noncommunicable diseases 2014*. Geneva, Switzerland: World Health Organization.

Yao, B., Fang, H., Xu, W., Yan, Y., Xu, H., Liu, Y., Mo, M., Zhang, H. & Zhao, Y. (2014). Dietary fiber intake and risk of type 2 diabetes: a dose–response analysis of prospective studies. *European Journal of Epidemiology*, 29 (2), pp. 79-88.

Zhao, G., Nyman, M. & Åke Jönsson, J. (2006). Rapid determination of short-chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct-injection gas chromatography. *Biomedical Chromatography*, 20 (8), pp. 674-682.

7 Appendix

7.1 Example calculations

Table A1. Responses for acetic acid and IS from the chromatogram of the third validation batch. A and B samples are on baseline (non-spiked), C and D are spiked with STD 1 (4 µmol/g faeces) and E and F are spiked with STD 3 (16 µmol/g faeces). A, C and E contains only HEPES, no QC, sample. B, D and F contains QC sample.

Sample	A1	A2	A3	B1	B2	B3	C1	C2	C3
Acetic acid	9.8	9	9.1	58.8	56.6	57	12.2	12.2	12.4
IS	346.4	360.9	356.4	360.2	345.4	347.7	339.7	341.1	362.3
Sample	D1	D2	D3	E1	E2	E3	F1	F2	F3
Acetic acid	58.8	59.4	59.4	21	20.9	20.8	68.3	69.2	68.3
IS	345.7	343	350.3	350.5	350.3	347.2	352.1	352.7	353.1

1. A mean value was calculated for all IS responses.

$$IS_{mean} = 358.2$$

2. All responses were normalised towards the IS of the respective sample and multiplicities with the IS_{mean}. Example for D1:

$$D_1/IS_{mean} * IS_{D1} \qquad 58.8/346.4 * 345.7 = 60.0$$

3. Mean values were calculated for the calibrated baseline samples (A and B).

$$A_{mean} = 9.4 \qquad B_{mean} = 58.7$$

4. Responses for spiked samples were subtracted with the baseline means for the specific matrix, spiked HEPES samples with A_{mean} and spiked QC samples with B_{mean}. Example for D1:

$$D_1 - B_{mean} \qquad 61.0 - 58.7 = 2.3$$

5. Mean values were calculated for each each spiking level and matrix.

$$C_{mean} = 3.2 \qquad D_{mean} = 2.6$$

6. The recovery was calculated by dividing the corrected mean response for the spiked QC sample with the spiked HEPES sample. The recovery was subtracted from 1. Example for D-samples:

$$Recovery = 1 - (D_{mean}/C_{mean})$$

$$1 - (2.6/3.2) = 0.197 = \mathbf{19.7}\%$$

7. The CV was calculated by dividing the standard deviation (SD) for the corrected response for each spiking level with the mean value for the responses. Example for D-samples:

$$CV = SD(D_{1-3})/Mean(D_{1-3})$$

$$0.7/2.6 = 0.269 = \mathbf{26.9}\%$$

8. Standard curves (figure A1) were constructed for each SCFA were the mean values (x) for each spiking level was plotted towards the corresponding concentration (y). Spiking levels with recovery or precision exceeding 20% were excluded from the standard curve. The equation and R^2-value were noted.

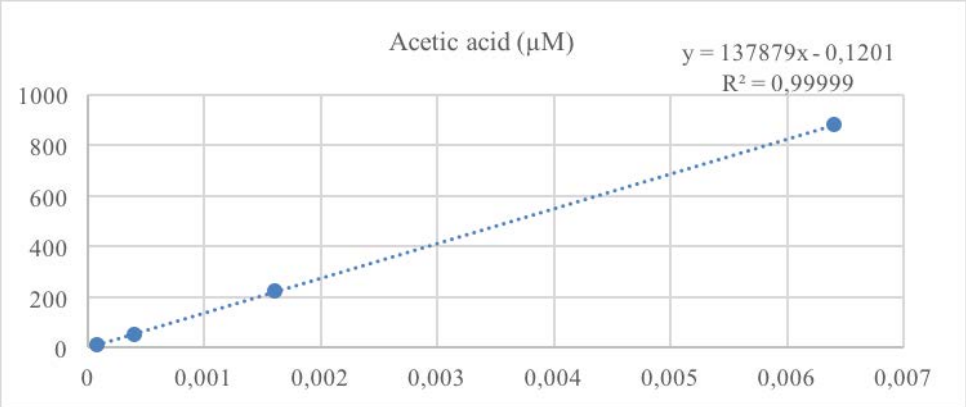

Figure A1. The standard curve for acetic acid made from the mean values of calibrated response plotted towards the corresponding concentrations.

9. The equation for the x value was calculated.

$$y = 137\,879x - 0.1201$$

$$x = (y - 0.1201)/137\,879$$

10. Using the re-calculated equation from the standard curve, a concentration was calculated for each mean response value. Example for E-samples:

$$E_{mean} = 11.1$$

$$Concentration_E = (11.1 - 0.1201)/137\,879 = 8.1 * 10^{-5}$$

11. The calculated concentration was subtracted with the true concentration and divided by the same concentration to calculate the deviation from linearity.

$$Concentration_{True\ E} = 8 * 10^5$$

$$Deviation\ from\ linearity = (Concentration_E - Concentration_{true})/Concentration_{true}$$

$$(8.1 * 10^5 - 8 * 10^5)/8 * 10^5 = 0.0141 = \mathbf{1.41}\%$$

7.2 Validation results

Table A2. Results for each spiking level from the first validation batch. The mean responses are normalised towards IS and the baseline sample. Calculated concentration are given by standard curve.

STD 8	ace	pro	ibut	but	ival	val	cap
Mean response	98.6	59.5	191.7	207.0	395.4	410.1	644.7
Calc. conc (µM)	377.9	39.4	39.8	42.4	40.5	40.9	40.0
True conc (µM)	400	40	40	40	40	40	40
Recovery (%)	4.5	2.3	-1.7	-12.1	-5.9	0.7	-11.7
CV (%)	2.8	0.9	2.0	3.9	2.9	3.0	3.3
Deviation (%)	-5.5	-1.5	-0.6	5.9	1.3	2.1	0.0
STD 10	ace	pro	ibut	but	ival	val	cap
Mean response	411.9	238.5	758.3	745.5	1519.3	1597.2	2401.5
Calc. conc (µM)	1627.6	160.8	160.3	160.0	159.3	158.9	160.0
True conc (µM)	1600	160	160	160	160	160	160
Recovery (%)	2.4	1.6	2.1	-2.5	-3.6	-2.0	-6.0
CV (%)	1.4	1.4	3.3	1.3	0.7	0.7	0.9
Deviation (%)	1.7	0.5	0.2	0.0	-0.4	-0.7	0.0
STD 12	ace	pro	ibut	but	ival	val	cap
Mean response	1607.2	945.1	3013.2	2943.0	6068.6	6436.2	9599.6
Calc. conc (µM)	6394.5	639.9	639.9	640.0	640.1	640.2	651.7
True conc (µM)	6400	640	640	640	640	640	640
Recovery (%)	1.7	0.9	-1.2	-1.9	-3.5	-3.0	-5.1
CV (%)	0.6	0.3	0.3	0.3	0.7	0.7	0.9
Deviation (%)	-0.1	0.0	0.0	0.0	0.0	0.0	1.8

Table A3. Results for each spiking level from the second validation batch. The mean responses are normalised towards IS and the baseline sample. Calculated concentration are given by standard curve

STD 1	ace	pro	ibut	but	ival	val	cap
Mean response	5.3	3.5	12.6	48.3	34.1	41.5	86.3
Calc. conc (µM)	-15.3	2.5	9.1	27.1	18.9	20.6	-0.3
True conc (µM)	20	2	2	2	2	2	2
Recovery (%)	35.8	15.2	-45.4	-345.3	-82.7	-126.0	-205.2
CV (%)	11.6	17.0	7.1	20.5	11.9	17.4	23.4
Deviation (%)	-176.3	23.5	357.2	1253.5	844.8	928.9	-114.1
STD 3	ace	pro	ibut	but	ival	val	cap
Mean response	20.8	12.8	39.4	70.4	92.8	102.7	176.4
Calc. conc (µM)	50.4	8.8	14.5	31.2	24.0	25.5	5.9
True conc (µM)	80	8	8	8	8	8	8
Recovery (%)	15.9	-3.1	-15.5	-74.8	-22.3	-39.3	-53.6
CV (%)	7.6	6.0	6.7	28.5	13.3	15.7	20.8
Deviation (%)	-37.0	9.6	80.9	290.5	199.5	218.6	-26.0
STD 8	ace	pro	ibut	but	ival	val	cap
Mean response	96.9	58.7	182.3	221.5	403.7	428.6	671.1
Calc. conc (µM)	372.4	39.7	42.8	59.8	50.8	51.6	40.0
True conc (µM)	400	40	40	40	40	40	40
Recovery (%)	4.2	2.6	-6.9	-21.8	-9.7	-14.1	-18.3
CV (%)	1.9	3.8	1.1	4.0	1.6	1.5	2.8
Deviation (%)	-6.9	-0.8	7.1	49.4	27.0	29.1	0.0
STD 10	ace	pro	ibut	but	ival	val	cap
Mean response	404.2	235.4	711.3	752.4	1512.7	1597.8	2413.4
Calc. conc (µM)	1673.5	158.7	147.9	160.0	146.5	145.5	160.0
True conc (µM)	1600	160	160	160	160	160	160
Recovery (%)	-0.3	-1.4	-4.3	-6.4	-6.0	-7.0	-8.7
CV (%)	2.3	2.2	1.1	1.5	0.9	0.9	0.7
Deviation (%)	4.6	-0.8	-7.5	0.0	-8.4	-9.1	0.0
STD 12	ace	pro	ibut	but	ival	val	cap
Mean response	1516.9	949.9	3202.4	3294.8	7261.8	7797.5	12465.4
Calc. conc (µM)	6383.8	640.3	642.8	640.0	642.7	642.9	530.8
True conc (µM)	6400	640	640	640	640	640	640
Recovery (%)	5.7	1.4	-7.1	-9.5	-16.8	-18.5	-26.9
CV (%)	6.3	1.2	6.7	7.5	13.5	14.4	19.9
Deviation (%)	-0.3	0.1	0.4	0.0	0.4	0.5	-17.1

Table A4. Results for each spiking level from the third validation batch. The mean responses are normalised towards IS and the baseline sample. Calculated concentration are given by standard curve.

STD 1	ace	pro	ibut	but	ival	val	cap
Mean response	2.6	1.8	8.6	16.3	15.4	18.0	30.1
Calc. conc (µM)	19.8	2.7	4.5	1.3	1.9	1.8	0.5
True conc (µM)	20	2	2	2	2	2	2
Recovery (%)	19.7	10.2	18.4	-10.5	24.8	-27.7	-53.1
CV (%)	26.9	11.9	0.7	18.5	7.9	10.1	17.6
Deviation (%)	-1.2	35.2	122.7	-33.1	-7.0	-10.6	-74.1
STD 3	ace	pro	ibut	but	ival	val	cap
Mean response	11.1	7.4	25.3	35.7	51.6	56.2	86.1
Calc. conc (µM)	81.1	8.9	10.0	8.0	7.9	7.9	6.7
True conc (µM)	80	8	8	8	8	8	8
Recovery (%)	8.1	0.9	10.6	-10.8	6.1	-11.3	-20.7
CV (%)	4.8	6.5	8.6	0.6	1.0	1.3	2.3
Deviation (%)	1.4	11.1	24.6	0.0	-0.9	-1.6	-15.8
STD 8	ace	pro	ibut	but	ival	val	cap
Mean response	53.5	35.1	115.5	129.9	243.5	259.3	384.5
Calc. conc (µM)	388.8	39.3	39.8	40.3	40.1	40.1	39.9
True conc (µM)	400	40	40	40	40	40	40
Recovery (%)	0.4	0.1	1.6	-4.6	1.2	-3.0	-5.5
CV (%)	1.3	1.7	0.5	1.9	0.6	0.3	0.4
Deviation (%)	-2.8	-1.7	-0.6	0.7	0.3	0.4	-0.3
STD 10	ace	pro	ibut	but	ival	val	cap
Mean response	222.2	143.7	472.7	478.2	958.2	1013.2	1466.7
Calc. conc (µM)	1612.5	158.8	157.7	159.6	159.9	160.0	160.1
True conc (µM)	1600	160	160	160	160	160	160
Recovery (%)	-1.3	-0.9	2.9	0.7	5.0	4.1	5.7
CV (%)	1.4	0.9	0.8	0.9	1.3	1.4	2.2
Deviation (%)	0.8	-0.8	-1.4	-0.2	0.0	0.0	0.1
STD 12	ace	pro	ibut	but	ival	val	cap
Mean response	882.0	581.9	1934.9	1880.2	3821.3	4033.1	5785.2
Calc. conc (µM)	6397.5	640.3	640.6	640.1	640.0	640.0	640.0
True conc (µM)	6400	640	640	640	640	640	640
Recovery (%)	1.2	14.8	1.3	0.6	1.2	0.8	1.0
CV (%)	0.5	0.2	0.1	0.2	0.9	1.0	2.0
Deviation (%)	0.0	0.1	0.1	0.0	0.0	0.0	0.0

Table A5. Results for each spiking level from inter-batch validation. The mean responses are normalised towards IS and the baseline sample. Calculated concentration are given by standard curve.

STD 1	ace	pro	ibut	but	ival	val	cap
Mean response	3.9	2.6	10.6	32.3	24.7	29.7	58.2
Calc. conc (µM)	4.1	2.3	5.4	7.1	6.4	10.3	12.3
True conc (µM)	20	2	2	2	2	2	2
Recovery (%)	31.2	13.6	-10.4	-152.6	-26.5	-83.4	-142.8
CV (%)	47.8	46.8	27.0	70.2	53.5	56.0	68.2
Deviation (%)	-79.4	13.3	172.4	253.7	219.9	416.5	516.3
STD 3	ace	pro	ibut	but	ival	val	cap
Mean response	15.9	10.1	32.4	53.1	72.2	79.5	131.2
Calc. conc (µM)	61.6	8.1	10.6	12.0	11.7	15.5	17.3
True conc (µM)	80	8	8	8	8	8	8
Recovery (%)	13.3	-1.6	-3.7	-46.4	-10.4	-27.9	-41.0
CV (%)	43.1	38.1	31.0	46.3	40.4	41.3	48.7
Deviation (%)	-23.0	0.8	32.0	50.0	46.2	93.8	116.4
STD 8	ace	pro	ibut	but	ival	val	cap
Mean response	83.0	51.1	163.2	186.1	347.5	366.0	566.8
Calc. conc (µM)	383.2	39.8	41.3	43.5	42.4	45.3	47.0
True conc (µM)	400	40	40	40	40	40	40
Recovery (%)	3.5	1.9	-2.8	-13.8	-5.6	-5.5	-12.7
CV (%)	30.8	27.2	25.5	26.4	25.9	25.4	28.0
Deviation (%)	-4.2	-0.4	3.2	8.9	6.1	13.4	17.6
STD 10	ace	pro	ibut	but	ival	val	cap
Mean response	346.1	205.9	647.4	658.7	1330.1	1402.8	2093.8
Calc. conc (µM)	1645.3	159.8	155.0	155.6	152.1	153.3	151.2
True conc (µM)	1600	160	160	160	160	160	160
Recovery (%)	0.6	-0.1	0.0	-3.1	-2.2	-2.3	-4.0
CV (%)	31.0	26.1	23.7	23.7	24.2	24.0	25.9
Deviation (%)	2.8	-0.1	-3.1	-2.8	-4.9	-4.2	-5.5
STD 12	ace	pro	ibut	but	ival	val	cap
Mean response	1335.4	825.6	2716.8	2706.0	5717.2	6089.0	9283.4
Calc. conc (µM)	6390.0	640.1	641.1	640.9	641.8	641.3	641.8
True conc (µM)	6400	640	640	640	640	640	640
Recovery (%)	3.2	4.7	-2.8	-4.2	-7.5	-8.1	-12.3
CV (%)	29.6	25.6	25.2	27.2	30.6	31.3	36.1
Deviation (%)	-0.2	0.0	0.2	0.1	0.3	0.2	0.3