Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

**Faculty of Natural Resources and Agricultural Sciences**
**Department of Food Science**

# Quantification of Short Chain Fatty Acids in Serum and Plasma

Kvantifiering av kortkedjiga fettsyror i serum och plasma

*Jonna Skoglund*

Independent Project in Food Science• Bachelor Project • 15 hec • Ground G2E
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## Abstract

Type 2 diabetes (T2D) has become a major problem around the world. Current research has linked a high dietary fibre (DF)- intake with a number of health benefits and possible prevention of T2D. One proposed mechanism is via short chain fatty acids (SCFA), which are produced from fermentation of DF in the colon by the microflora. A high DF intake will cause higher concentration of SCFA in the blood. In order to investigate the role of T2D it is of importance to have an easy, robust fast and accurate method for determine the concentration of SCFA in a small volume serum and plasma from fasted individuals. In the protocol used the sample preparation relies on the ability for proteins to form a precipitate with phosphoric acid and extraction of supernatant in organic solvent. The concentration is measured by gas chromatography-mass spectrometry (GC-MS). The aim of the present study was brief to further develop and validate an existing rapid GC-MS based method to determine the concentration of SCFA in serum from human subjects, including an extension of an existing protocol for SCFA analysis in 400 µl to the reduced volume of 200 µl was evaluated, the agreement of method, using plasma or serum for determination of SCFA in a small population if individuals (n=30) was evaluated. The measured SCFAs were acetate, butyrate, iso-butyrate, propionate, valerate, iso-valerate and caproate. The method developed could analyse 200 µl serum and plasma with acceptable precision. The accuracy was tested with spiking acetate at 106.6, 53.3 and 26.7 µM and the other SCFA at 10.7, 5.3 and 2.7 µM. The recovery were between 80.1-107.7 %. The fasting concentration of acetate was 86.0-313.3 µM, 19.4-28.5 µM for propionate, 2.6-4.7 µM for iso-butyrate, 9.2-29.1 µM for butyrate, 11.2-44.4 µM for iso-valerate, 0.2-0.4 µM for valerate and 1.4-9.7 µM for caproate, of the 30 subjects. It was a good agreement between serum and plasma concentration for butyrate. For propionate, valerate, acetate, iso-butyrate, iso-valerate and caproate there were a significant difference.



*Keywords:* Short Chain Fatty Acids, Type 2 Diabetes, GC-MS, Dietary Fibre, Serum, Plasma

## Sammanfattning

Typ 2 diabetes har blivit ett omfattande problem världen runt. Rådande forskning har sett samband mellan en diet rik på kostfiber och minskad ohälsa samt en möjlig förebyggande effekt mot att utveckla diabetes typ 2. En föreslagen mekanism är via kortkedjiga fettsyror. Ett högt intag av kostfiber resulterar i en högre koncentration av kortkedjiga fettsyror i blodet. För att undersöka typ 2 diabetes roll är det viktigt att ha en enkel, robust och noggrann metod vid mätning av koncentrationen kortkedjiga fettsyror i en liten volym i serum och plasma. Den använda metoden är baserad på proteiners förmåga att bilda en fällning med fosforsyra och sedan extraktion med en organisk syra. Koncentrationerna av fettysorna uppmätts med gaskromatografi- masspektrometri (GC-MS). Syftet med denna studie var kortfattat att vidareutveckla och validera en befintlig GC-MS baserad metod för att noggrant bestämma koncentrationen av kortkedjiga fettsyror i humant serum, inklusive att utveckla metoden för 400 µl och till reducerad volymen (200 µl) men även att utvärdera sambandet mellan koncentration av kortkedjiga fettsyror i serum och plasma i en liten population (n=30). De kortkedjiga fettsyror som analyserades var acetat, butyrat, iso-butyrat, propionat, valerat, iso-valerat och kaproat. Den utvecklade metoden kunde analysera 200 µl serum och plasma med god precision. Noggrannheten mättes genom att addera lösning med 106,6, 53,3 och 26,7 µM acetat och de andra fettsyrorna 10,7, 5,3 och 2,7 µM, utbytet låg mellan 80,1-107.7%. Koncentrationerna var vid fasta för de 30 individerna mellan 86,0-313,3 µM för acetat, 19.4-28.5µM för propionat, 2.6-4.7µM för iso-butyrat, 9.2-29.1µM för butyrat, 11.2-44.4µM för iso-valerat, 0.2-0.4µM för valerat och 1.4-9.7µM for kaproate. Ett tydligt samband mellan butyrat koncentrationen serum och plasma uppvisades. För resterande kortkedjiga fettsyrorna var där en signifikant skillnad.

*Nyckelord:* Kortkedjiga fettsyror, typ 2 diabetes, GC-MS, Kostfiber, Serum, Plasma

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# Abbreviations

| | |
|---|---|
| ACE | Acetate |
| BUT | Butyrate |
| CARP | Caproate |
| CV | Coefficient of variation |
| DF | Dietary Fibre |
| FFAR2 | Free fatty acid receptor 2 |
| FFAR3 | Free fatty acid receptor 3 |
| GC-MS | Gas Chromatography- Mass Spectrometry |
| ISO-BUT | Iso-Butyrate |
| ISO-VAL | Iso-Valerate |
| OGTT | Oral Glucose Tolerance Test |
| PRO | Propionate |
| SCFA | Short Chain Fatty Acids |
| SD | Standard deviation |
| T2D | Type 2 Diabetes |
| VAL | Valerate |

# 1 Introduction

## 1.1 Health and Dietary Fibre

Lifestyle diseases such as metabolic syndrome (Kaur, 2014), obesity and type 2 diabetes (T2D) are currently increasing worldwide (Wilde *et al*. 2004). A more sedentary lifestyle together with a higher energy intake is one of the reasons, but diet is also considered to be an important modifiable risk. Through their association with health benefits, an increase in dietary fibre (DF)-intake has been discussed as one way to prevent and treat lifestyle diseases (Mudgil & Barak 2013). Treating mice with $\beta$-glucan have for example showed an improved glucose tolerance, decrease in insulin resistance and hyperinsulinemia, which all are risk factors of the metabolic syndrome (Choi *et al.* 2010). Similar results have been displayed through dietary interventions in humans with muffin high in β-glucan and resistant starch (Behall *et al.* 2006). β-Glucan is one of few food constituents that are excepted to carry a health claim because of its maintenance ability of blood lipid levels (EG 432/2012). Moreover, giving T2D patients psyllium, consisting of high amounts of the DF mucilage (Sangeethapriya & Siddhuraju 2014), was shown to result in decreased glucose absorption and reduction of total and cholesterol LDL (Sierra *et al.* 2002). A high fibre diet, particularly insoluble fibre, also gives an increase of faecal bulk and decrease in transit time, which promotes a normal laxation (Dahl *et al.* 2005).

The DF definition has widely been discussed since it is not only based on chemical structure. The concept also includes a physical aspect, where the main physical function includes bulking, viscosity, fermentability and non-digestibility in the small intestine (Mudgil & Barak 2013). Codex Alimentarius have tried to evaluate the DF definition and have proposed a definition in 2009; it requires a certain structure, non-digestibility and physiological health benefits, among others, for a food constituent to be defined as DF (Codex 2009).

DF exists in largest amounts in cereals, legumes, fruit and vegetables (Bartoli *et al.* 2007). They are the indigestible part of carbohydrates and include cellulose, hemicelluloses, pectin's, hydrocolloids, $\beta$-glucans, gum, mucilages, lignin, α-

galactosides, resistant starch and non-digestible oligosaccharides such as inulin (Mudgil & Barak 2013).

DF can be divided into fermentable and non-fermentable where pectin, gums, mucilages (Bartoli *et al.* 2007) and *β*-glucan are fermentable (Schroeder *et al*. 2013). The solubility of DF differs depending on structure. Oats, fruits, vegetables and pulses are rich in more soluble DFs (Mudgil & Barak 2013) such as β-glucans, some hemicelluloses, pectin, gums and mucilage's (Jiménez-Escrig & Sánchez-Muniz 2000) while less- or non-soluble DF often exists in the skin of fruit and vegetables (Mudgil & Barak 2013), examples are celluloses, lignin, arabinoxylan, some hemicellulose and resistant starch (Jiménez-Escrig & Sánchez-Muniz 2000).

## 1.2 Dietary Fibre Degradation and SCFA

Since adequate enzymes are lacking from the human endogenous metabolism, DF are resistant to human endogenous digestive enzymes, and consequently, cannot be absorbed in the small intestine. They can, however, influence absorption and digestion of nutrients indirectly, by affecting the accessibility, gastric emptying, digestion and nutrient absorption kinetics and thereby affect the levels of blood glucose, insulin, blood lipids, cholesterol and microflora in the gastrointestinal tract through other mechanisms (Den Besten *et al*. 2013).

Fermentable DFs are fermented by gut microbial enzymes primarily in the upper colon. The fermentability relies on structure bonds within the fibre molecules and fibre solubility, where soluble DFs are more easily fermented (Mudgil & Barak, 2013). A wide range of metabolites are produced from this microbial fermentation, such as carbon dioxide and methane, but also a high concentration of short chain fatty acids (SCFA) (Canfora *et al*. 2015). Although digestible carbohydrates and DF are the main substrates for fermentation, indigestible proteins, resistant starch and other substrates can also be microbially fermented in the colon (Soldavini & Kaunitz 2013). SCFA are defined as volatile 1-6 carbon fatty acids that have a straight or branched conformation (Rios-Covián *et al.* 2016), where straight SCFAs are mainly derived from carbohydrates and branched SCFAs from proteins (Soldavini & Kaunitz 2013).

Approximately 90% of the SCFA formed are absorbed in the colon and transported through the hepatic vein to the liver (den Besten *et al*. 2013; Soldavini & Kaunitz 2013) and the residual amount is secreted through the faeces (Canfora *et al.* 2015). Acetate, butyrate and propionate are the three most common SCFA (Figure 1) and together constitute 90-95% of the SCFAs in the colon. Other SCFAs, iso-butyrate, valerate, iso-valerate and caproat, are present in considerable lower concentrations (Topping & Clifton 2001).

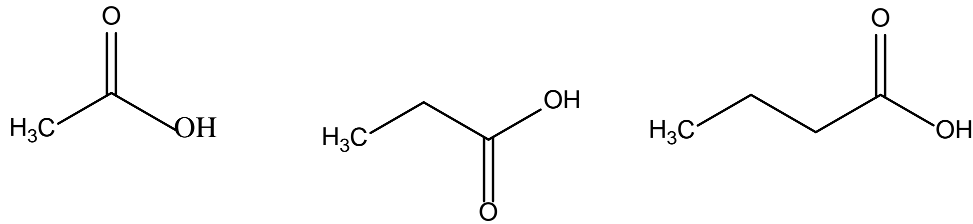



Figure 1. The three most abundant SCFA. Acetate, Propionate and Butyrate.

Together all SCFA constitute of 5-10% of the human energy requirement (Soldavini & Kaunitz, 2013) and general effect in the colon include the facilitation of water and electrolyte absorption (Rios-Covián *et al.* 2016). The most abundant SCFA in the gastrointestinal tract is acetate. It reaches circulation and can be used as energy by the host or acts as substrate for hepatic lipogenesis and cholesterol biosynthesis (Brüssow & Parkinson 2014). Acetate can protect the host by improving epithelial cells and intestinal defence (Fukuda *et al.* 2011). Propionate acts as substrate in the hepatic gluconeogenesis and is involved in the cholesterol production (Brüssow & Parkinson 2014). Most of the butyrate is metabolized by the enterocytes in the colon and is used locally as energy source. This stimulates cell renewal, which improves the mucin production and the mucus barrier. Only small amounts reach the blood circulation and contribute as energy to the host (Pryde *et al.* 2002). Butyrate-producing bacteria are also believed to affect the blood glucose regulation and lipid metabolism (Puddu *et al*. 2014).

A high intake of DF causes higher concentration of SCFA in the gastrointestinal tract and in the blood (De Filippo *et al.* 2010). The rate of production of the individual SCFA is dependent on substrate and thus related by the diet, whereas the distribution of SCFAs produced, i.e. the SCFA profile, is determined by microbiota composition, molecular weight of the substrate and the amount of undigested matter that reaches the colon (den Besten *et al.* 2013; De Filippo *et al.* 2010). Oat bran containing a great amount of $\beta$-glucan and kidney beans with a lot of resistant starch and $\alpha$-galactosides yield the highest amount of SCFA (McBurney *et al.* 1988).

The turnover rate of SCFA in blood is rapid: 29, 4 and 0.3 mmol/kg respectively for acetate, propionate and butyrate (Pouteau *et al.* 2007), effectively resulting in low but variable concentrations of SCFA in the blood (Table 1).

*Table 1. Physiological concentrations of the three most common SCFA in fasting blood plasma, blood serum and blood; expressed in mean ± standard deviation (SD) (µmol/l)*

| | **Acetate** | **Propionate** | **Butyrate** | |
|---|---|---|---|---|
| Portal blood | 258±98 | 88±68 | 29±19 | Cummings *et al*. 1987 |
| Portal plasma | 128.0±70.8 | 34.4±23.3 | 17.6±18.4 | Peters *et al.* 1992 |
| Venous blood serum | 245±9.4 | 13±0.5 | 2.1±0.1 | Jakobsdottir *et al*. 2013 |
| Venous blood serum* | 174 | 13 | 14 | Zhao *et al.* 2007 |

*No mean available

The activity and composition of the gut microbiota is dependent, among others, on the diet and this affects the production and ratio of the SCFA (Rios-Covián *et al.* 2016). Disruption in the microbiota composition and activity has been shown to be one of the important factors behind the increase in metabolic disorders (Leser & Mølbak 2009). Further studies have shown difference in microbiota between adults with T2D and non-diabetic adults (Larsen *et al.* 2010) and a recent study on mice has shown that the diet can affect the composition of the microbiota more than host genotype (Carmody 2015).

Microbiota perturbation has been associating with T2D in a Chinese study, where subject with T2D displayed a lower level of butyrate producing bacteria (Qin *et al.* 2012) and similarly in a European setting (Karlsson *et al.* 2013). These changes have further been associated with obesity, insulin resistance and T2D (Musso *et al.* 2010) and have generated an interest in altering the microbiota composition by dietary means as a possible route to health benefits.

## 1.3 SCFA Metabolic Effects and Type 2 Diabetes

Type 2 diabetes (T2D) is the most common type of diabetes and is also increasing on a global level (WHO 1999). It is preceded by a successive decrease in insulin sensitivity; which leads to metabolic dysregulation and finally T2D (Smushkin & Vella 2010). Insulin is the most important hormone for glucose regulation and decreased or disrupted insulin functionality will affect carbohydrate, fat and protein metabolism. In a healthy individual the insulin producing β-cells can adapt to changes in insulin level, but in the diseased state the β-cells are damaged and can no longer adapt adequately to elevated blood glucose, thus leading to hyperglycaemia (Stumvoll *et al.* 2005). For diagnosis of T2D glucose level in the blood while fasting or 2 hours after an oral glucose tolerance test (OGTT) with an intake of 75 g glucose is measured. The limit for T2D is when at fasting state the glucose is ≥7.0 mmol $1^{-1}$ in plasma samples and ≥11.1 mmol $1^{-1}$ after OGTT (WHO 1999).

The involvements of SCFA in numerous metabolic processes have generated a hypothesis of a possible mechanistic link to the metabolic syndrome and subsequent T2D. The complete mechanism and action is not fully understood but the free fatty acid receptors 2 (FFAR2, or GP43) and 3 (FFAR3, or GP41) are sug-

gested since SCFA are known to trigger and promote these receptors (Puddu *et al.* 2014). FFAR2 has higher affinity to propionate than other SCFA whereas FFAR3 is equally activated by acetate, propionate and butyrate. Both FFAR2 and FFAR3 are expressed in adipose tissue, where the appetite supressing hormone leptin is present. Research has shown an increase in leptin secretion in tissues when the concentration of SCFA is high (Stoddart *et al.* 2008). These results correspond with the reduced obesity and insulin resistance reported in mice when treating with butyrate diet supplement (Gao *et al*. 2009).

## 1.4 Method to Measure SCFA in Serum

To gain an increased understanding of the kinetics and partitioning of SCFA in the different biocompartments it is important to be able to accurately measure circulating levels of SCFA. Moreover, circulating concentrations of SCFA will reflect a high or low consumption of DF in the diet (Zhao *et al.* 2005). Since *in vivo* measurements of SCFAs are a cumbersome task, various easier options have been developed. A number of methods have been used when measuring SCFA in faeces and blood e.g. enzymatic methods and a number of chromatography systems where gas chromatography (GC) is the most common (Zhao *et al.* 2005).

To be applicable to different study designs and sampling schemes, it is relevant to be able to measure SCFA in serum as well as plasma. The relation between plasma and serum concentrations is of importance when comparing studies made on different substances; to compare populations. An alternative reason to use serum instead of plasma is the possible easier injection of samples because of its inferior protein content. A reduction in sample volume is necessary to allow smaller samples to be analysed.

## 1.5 Aim of the Thesis

The aim of the thesis was to further develop and validate a rapid Gas Chromatography-Mass Spectrometry (GC-MS) based method to accurately measure SCFA concentrations in serum from human subjects. Specific objectives included: i) to extend an existing protocol for SCFA analysis in 400 µl plasma to a smaller sample volume (200 µl) of serum; ii) to evaluate the agreement between methods using plasma or serum for determination of SCFA concentration in a small population of individuals (n=30).

# 2 Materials and Method

## 2.1 Samples

The method was performed on fasting blood serum from three healthy volunteers. Samples were stored in -80°C and thawed in room temperature prior to analysis. EDTA-plasma and serum were obtained from 30 subjects' participants in a pilot study to compare the SCFA concentration agreement in serum and EDTA-plasma. These samples were stored at -20°C prior to analysis.

## 2.2 Chemicals

*Reagents:* Acrylic acid (Sigma no. 127230), meta-phosphoric acid (Merck no. 1.00546), propyl formate (Sigma-Aldrich no. W294306-1KG-K), sodium carbonate and sodium hydroxide (Merck no. 1.06469.1000).
*SCFA standards:* Caproic acid (Fluka no. 21530), iso-valeric acid (Fluka no. 59850), sodium acetate (Merck no. 6268), sodium butyrate (Ardrick no. 30.341.0), sodium iso-buryrate (Fluka no. 58360), sodium propionate (Sigma no. P 1880) and valeric acid (Fluka no. 94530).

## 2.3 Preparation of Serum and Plasma Samples

In brief, 100 µl of internal standard solution (150 µM acrylic acid, 1500 µM m-phosphoric acid) was added to 200 µl serum or plasma. Samples were vortexed (5 min; VWR VX-2500 Multi-Tube Vortexer) followed by centrifugation (30 min, 20817 g; Eppendorf centrifuge 5417C, rotor: FA-45-30-11) and left for 30 min (4°C) to solidify the precipitate. In total, 100 µl of the clear supernatant was transferred into a new tube and 100 µl washed propyl formate was added. Samples were vortexed (5 min) followed by centrifugation (10 min, 20817 g) before transferring 50 µl of the organic layer into GC vials for analysis. Blank samples were prepared similarly with the exception that no serum or plasma was added.

## 2.4 GC-MS analysis

SCFA quantified employing a recently developed GC-MS method (Wu personal communication). In brief, 2 µl sample was injected (splitless) into a straight glass liner, held at 200 °C. Helium (2 ml/min) was used as carrier gas in a ZB-FFAP column (30 m, 0.25 mm ID, no. 7HG-G009-11; Phenomenex, USA). The initial oven temperature of 55 °C was maintained for 4 min, then ramped to 130 °C at 50 °C/min and held for 3.7 minutes and finally raised to 250 °C at 30 °C/min and held for 2 minutes. Electron ionization (70 eV and 250 °C) mode was used. All samples, standards and blanks were analysed randomly at the GC-MS system. Finnigan Trace GC Ultra Gas Chromatograph with a Finnigan Trace DSQ II mass detector (Thermo Fisher Scientific, Waltham, MA, USA) were used. Integrations were performed automatically with Xcalibur 2.0.7(Thermo Fisher Scientific, Waltham, MA, USA).

The current method was developed and evaluated using 400 µl plasma in each sample. In this laboratory the volume were reduced to 200 µl; accuracy (by recovery study) and precision (by coefficient of variation; CV) were estimated. A value below 15% is considered acceptable for most analytical methods (FDA 2011). In total eight quality plasma control samples with known concentration were tested, four with 200 µl and four with 400 µl (one sample could not be included due to waste of sample).

Validation of the method was made for serum by calculating accuracy (by recovery) and precision (by CV). The accuracy was estimated by adding standard solution with known concentration at three levels; low, medium and high according to Table 2.

*Table 2. Concentrations of spiking solution for validation of GC-MS method for quantification of SCFA in serum*

| Spiking solution | Concentration acetate (µM) | Concentration SCFA C3-C6 (µM) |
|---|---|---|
| Low | 26.7 | 2.7 |
| Medium | 53.3 | 5.3 |
| High | 106.6 | 10.7 |

A comparison between SCFA concentrations in serum and plasma was made by quantifying the concentration from the same individual (n=30). Bland-Altman plots present the result; the technique displays the difference of SCFA concentration between the two substances (Giavarina 2015). A t-test was also made to determine the difference between serum and plasma concentration.

# 3 Result and Discussion

## 3.1 Comparison Between 200 µl and 400 µl Plasma

The result showed good recovery when using 200 µl-sample. The CV was between 2-25% for all analytes and all values except for propionate were below 15% of variation (Table 3). The deviant number is due to one sample with much lower concentration than the others. The variation when using 400 µl was between 0.6-6% i.e. all values are below 15% of variation. When comparing the two different volumes the result were similar with regard to precision.

The volume used is extremely crucial when quantifying. The small amount of 200 µl makes it more difficult to perform the analysis, both analytically and the human factor has an impact on the result. The slightly higher variation of the 200 µl-sample might be due to this. Alternative reason in difference of variation between the volumes might be the loss of one of the 400 µl-samples. Despite this a triplicate should be enough for observing a credible trend.

*Table 3. Recovery of SCFA in serum. Within batch variation when using, 200µl and 400µl and recovery for 200µl.*

| Sample Name | ACE | PRO | ISO-BUT | BUT | ISO-VAL | VAL | CARP |
|---|---|---|---|---|---|---|---|
| CV QC200 (%) | 4.0 | 25.3 | 12.7 | 2.10 | 5.5 | 3.6 | 4.4 |
| CV QC400 (%) | 2.1 | 5.2 | 5.7 | 0.6 | 0.9 | 0.8 | 5.9 |
| Recovery 200 (%) | 100.4 | 77.8 | 98.5 | 94.7 | 88.5 | 106.1 | 98.3 |

## 3.2 Recovery Test

The first test of recovery showed an uneven recovery between SCFA and in most cases the recovery rate was found too low (Table 4). During the test propyl formate was added before the spiking solutions. This might have contributed to an increase in evaporation of SCFA due to their volatility. Additional reason was maybe too long time between sample preparation and injection of sample into GC.

*Table 4. First recovery test with samples spiked at three different levels: high, medium and low*

| Sample | ACE | PRO | ISO-BUT | BUT | ISO-VAL | VAL | CARP |
|---|---|---|---|---|---|---|---|
| $Recovery_{Low}$ (%) | 13.9 | 87.0 | 104.8 | 85.6 | 73.4 | 70.9 | 71.7 |
| $Recovery_{Medium}$ (%) | 8.0 | 78.9 | 81.3 | 97.9 | 71.3 | 68.9 | 78.9 |
| $Recovery_{High}$ (%) | -44.7 | 73.1 | 77.0 | 125.0 | 68.0 | 68.6 | 85.0 |

During the second recovery test the spiking was added before propyl formate. This gives a protective surface, which captures and keeps the volatile SCFA in the tube. The time between sample preparation and injection of sample in to GC were reduced. As a result, the second test showed an even and high recovery between 80-112% (Table 5). The CV was small between spiking levels and quartets (Table 6).

*Table 5. Second recovery test after spiking samples with high, medium and low amounts of SCFA*

| Sample | ACE | PRO | ISO-BUT | BUT | ISO-VAL | VAL | CARP |
|---|---|---|---|---|---|---|---|
| $Recovery_{Low}$ (%) | 80.1 | 93.7 | 104.5 | 94.3 | 94.2 | 92.9 | 82.3 |
| $Recovery_{Medium}$ (%) | 92.0 | 111.8 | 101.1 | 97.4 | 96.4 | 93.6 | 80.8 |
| $Recovery_{High}$ (%) | 95.0 | 107.7 | 99.5 | 95.7 | 96.5 | 93.7 | 81.3 |

*Table 6. The coefficient of variance from the second recovery test*

| Sample | ACE | PRO | ISO-BUT | BUT | ISO-VAL | VAL | CARP |
|---|---|---|---|---|---|---|---|
| $CV_{Low}$ (%) | 4.1 | 1.9 | 0.5 | 0.1 | 0.6 | 1.0 | 2.4 |
| $CV_{Medium}$(%) | 4.5 | 1.2 | 1.3 | 1.4 | 1.8 | 1.9 | 2.0 |
| $CV_{high}$ (%) | 3.0 | 1.6 | 1.0 | 1.1 | 0.3 | 2.2 | 2.4 |

## 3.3 Plasma vs Serum

The concentration of acetate, propionate and butyrate in serum are within earlier measured concentration (Table 1). Iso-butyrate is also within range comparing with earlier measurements (Jakobsdottir *et al.* 2013; Zhao *et al.* 2007). The concentration of iso-valerate is in the lower segment of concentrations measured. The concentration of valerate is considerable lower in this study than previous (Jakobsdottir *et al.* 2013; Zhao *et al.* 2007). Studies measuring caproate have not been found.

*Table 7. The concentration range and mean concentration for each SCFA in serum*

| | ACE | PRO | ISO-BUT | BUT | ISO-VAL | VAL | CARP |
|---|---|---|---|---|---|---|---|
| Range (µM) | 86.0-313.3 | 19.4-28.5 | 2.6-4.7 | 9.2-29.1 | 11.2-44.4 | 0.2-0.4 | 1.4-9.7 |
| Mean (µM) | 181.2±46.4 | 23.6±8.4 | 14.8±0.4 | 3.2±4.2 | 23.1±6.5 | 0.4±0.1 | 3.5±1.6 |

Results from the comparison of analysis of SCFA in plasma and serum is presented in Bland-Altman plots (Figure 2-8). The samples display a wide range of each SCFA concentration because of individual DF intake. A good agreement was found between plasma and serum for butyrate (Figure 6). For propionate (Figure 8) and valerate (Figure 4) the concentrations did not differ that much between individuals, while we have a systematically low concentration of iso-butyrate (Figure 5) and iso-valerate (Figure 3), and higher concentration of acetate (Figure 2) and caproate (Figure 7).

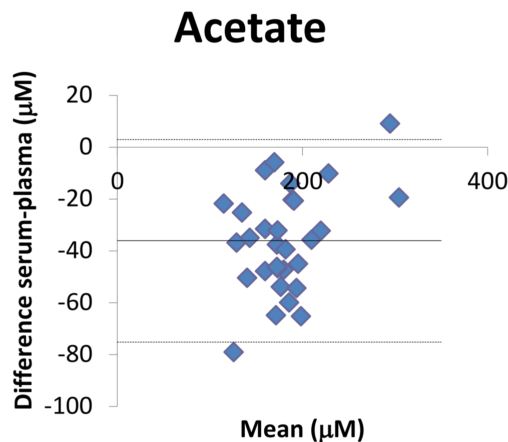
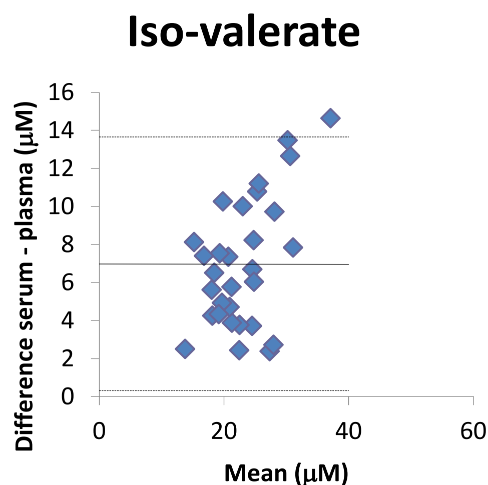



Figure 2. Bland-Altman plot of the difference between concentration of acetate determined by serum concentration minus plasma concentration on the Y-axis and average of the two substances on the X-axis. The dotted lines show the higher and lower limit of agreement.



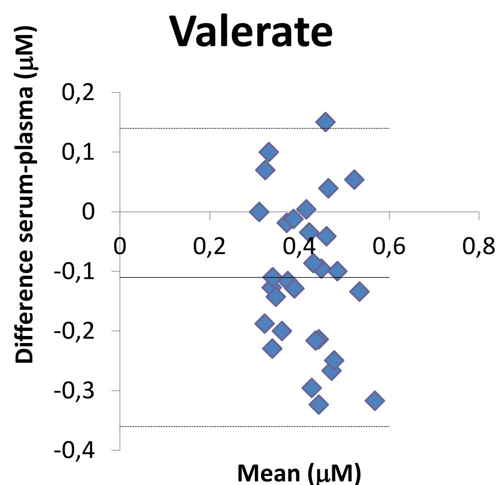
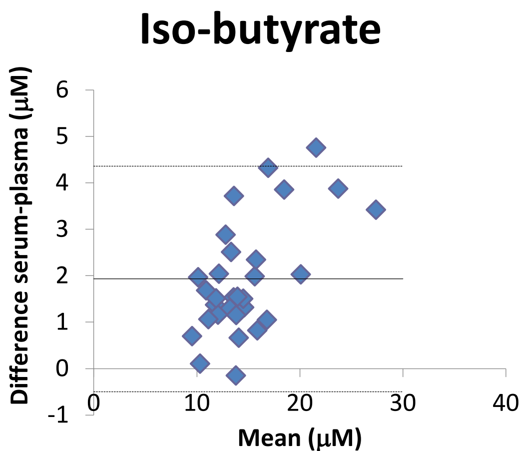




Figure 3. Bland-Altman plot of the difference between concentration of iso-valerate determined by serum concentration minus plasma concentration on the Y-axis and average of the two substances on the X-axis. The dotted lines show the higher and lower limit of agree-

Figure 4. Bland-Altman plot of the difference between concentration of valerate determined by serum concentration minus plasma concentration on the Y-axis and average of the two substances on the X-axis. The dotted lines show the higher and lower limit of agreement.



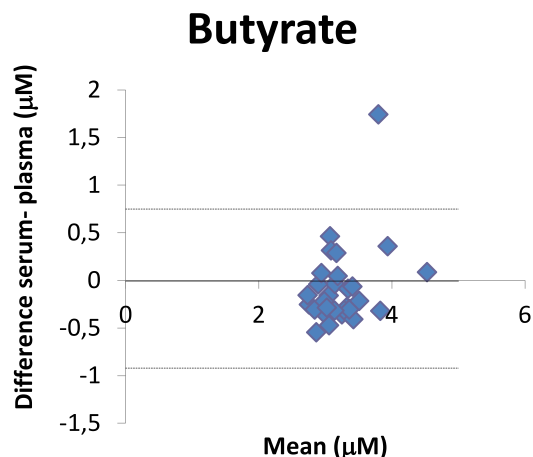




Figure 5. Bland-Altman plot of the difference between concentration of iso-butyrate determined by serum concentration minus plasma concentration on the Y-axis and average of the two substances on the X-axis. The dotted lines show the higher and lower limit of agreement.

Figure 6. Bland-Altman plot of the difference between concentration of butyrate determined by serum concentration minus plasma concentration on the Y-axis and average of the two substances on the X-axis. The dotted lines show the higher and lower limit of agreement.

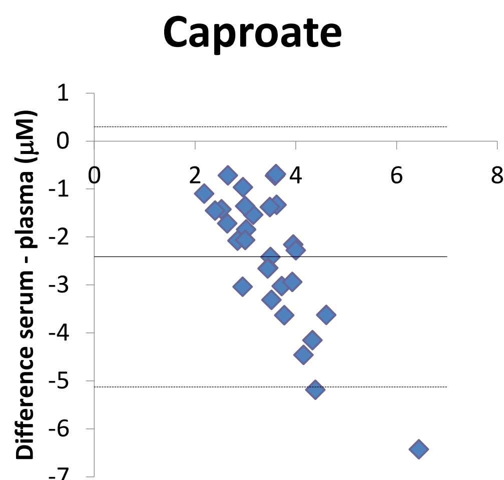



Figure 7. Bland-Altman plot of the difference between concentration of caproic acid determined by serum concentration minus plasma concentration on the Y-axis and average of the two substances on the X-axis. The dotted lines show the higher and lower limit of agreement.

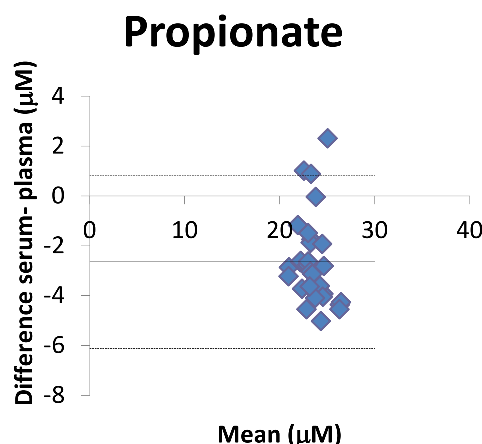



Figure 8. Bland-Altman plot of the difference between concentration of propionate determined by serum concentration minus plasma concentration on the Y-axis and average of the two substances on the X-axis. The dotted lines show the higher and lower limit of agreement.

The t-test confirms the result and shows a significant difference between concentrations in serum and plasma for all SCFA except for butyrate (Table 8).

*Table 8. Result for the t-test between serum and plasma, a value <0.05 is considered as a significant difference*

| | ACE | PRO | ISO-BUT | BUT | ISO-VAL | VAL | CARP |
|---|---|---|---|---|---|---|---|
| P-value | 7.7E-11 | 5.4E-09 | 2.1E-09 | 0.30 | 4.4E-12 | 6.7E-05 | 1,9E-10 |

The samples used in this analysis were from a previous research project. The samples have earlier been thawed and then frozen again at least one time. Due to this, unwanted interactions between SCFA and protein may have occurred and probably some evaporation of SCFA. The changes might be due to the different chain lengths and branching of the SCFAs.  This might also be the reason for the somewhat lower concentrations of valerate and iso-valerate. Possible changes might also be due to the different deprotenization methods used when converting blood to serum or plasma.

## 3.4 Further Studies

During calculations of the concentration the blank value is subtracted, in this study the blank was constructed by the same means as samples, but with the exception

that no serum or plasma was added. A more accurate way of construction blanks would be by adding ex water to gain the same volume of each sample. To avoid unpredictable interactions it would be optimal to use fresh blood serum or plasma to minimize the possible risk for inadequate result and to be able to compare concentrations from different studies. To gain a sufficient result of the comparison more studies are needed.

# 4 Conclusion

The developed method was able to analyse SCFA concentrations in human serum with good precision and accuracy. The reduced amount to 200 µl was not limiting. Further studies are though needed before a conclusion regarding agreement between SCFA concentrations in serum and plasma can be made.

# 5 References

Behall, K.M., Scholfield, D.J., Hallfrisch, J.G. & Liljeberg-Elmståhl, H.G. (2006) Consumption of both resistant starch and beta-glucan improves postprandial plasma glucose and insulin women. *Diabetes Care* 29(5), 976-981.

Den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.J. & Bakker, BM. (2013) The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research* 54(9), 2325-2340.

Bartoli, R., Josep Mañé, Cabré, E., Lorenzo-Zúñiga, V., Planas, R., Ausina, V. & Gassull, M.A. (2007) Effect of the administration of fermentable and non-fermentable dietary fibre on intestinal bacterial translocation in ascetic cirrotic rats. *Clinical nutrition* 26(3), 383-387.

Brüssow, H. & Parkinson, S.J. (2014) You are what you eat. *Nature Biotechnology* 32(3), 243-245.

Canfora, E.E., Jocken, J.W. & Blaak, E.E. (2015) Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature Reviews Endocrinology* 11(10), 577-91.

Carmody, R.N., Gerber, G.K., Luevano, J.M., Gatti, D.M., Somes, L., Svenson, K.L. & Turnbaugh, P.J. (2015) Diet dominates host genotype in shaping the murine gut microbiota. *Cell Host and Microbe* 17(1), 72-84.

Choi, J.S., Kim, H., Jung, M.H., Hong, S. & Song, J. (2010) Consumption of barley beta-glucan ameliorates fatty liver and insulin resistance in mice fed a high-fat diet. *Molecular Nutrition & Food Research* 54(7), 1004-1013.

Codex Alimentarius Commission (2009) Report of the 30th session of the Codex committee on nutrition and foods for special dietary uses ALINORM 09/32/26 November 2008, Appendix II.

Commission regulation of establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and children´s development and health (EU) 2012 Brussels. The European Parliament.

Cummings, H.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), 1221-1227.

Dahl, W.J., Lockert, E.A., Cammer, A.L. & Whiting, S.J. (2005) Effects of flax fiber on laxation and glycemic response in healthy volunteers. *Journal of Medicinal Food* 8(4), 508-5011.

De Filippo, C., Cavalieri D., Di Paola M., Ramazzotti, M., Poulett, J.B Massart, S., Collini, S., Pieraccini, G. & Lionetti, P. (2010) Impact of diet shaping gut microbiota revealed by comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Science of the United States of America* 107(33), 14691-14696.

Food and Drug Administration, FDA (2001) Guidance for Industry: Bioanalytical Method Validation Rockville, MD: US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J.M., Topping, D.L., Suzuki, T., Taylor., T.D., Itoh, K., Kikuchi, J., Morita, H., Hattori, M. & Ohno, H. (2011) Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 469(7331), 543-547.

Gao, Z., Yin, J., Zhang, J., Ward, R.E., Martin, R.J., Lefevre, M., Cefalu W.T. & Ye, J. (2009) Butyrate improves insulin sensitivity and increases energy expenditure in mice. *American Diabetes Association* 58(7), 1509-1517.

Giavarina, D. (2015) Understanding Bland Altman analysis. *Biochemia Medica* 25(2), 141-151.

Hong, Y.H., Nishimura, Y., Hishikawa, D., Tsuzuku, H., Miyahara, H., Gotoh, C., Choi, K.C., Feng, D.D., Chen, C., Lee, H.G., Katoh, K., Roh, S.G. & Sasaki, S. (2005) Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. *Endocrinology* 146(12), 5092-5099.

Jakobsdottir, G., Holst-Bjerregaard, J., Skovbjerg, H. & Nyman, M. (2013) Fasting serum concentrations of short-chain fatty acids in subjects with microscop-ic colits and celiac disease: no difference compared with controls, but between genders. *Scandinavian Journal of Gastroenterology* 48(6), 696-701.

Jiménez-Escrig, A. & Sánchez-Muniz, F.J. (2000) Dietary fibre from edible seaweeds: Chemical structure, physicochemical properties and effects on cholesterol metabolism. *Nutrition Research* 20(4), 585-598.

Karlsson, F.H., Tremaroli, V., Nookaew, I., Bergström, G., Behre, C.J., Fagerberg, B., Nielsen, J. & Bäckhed, F. (2013) Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* 498(7452), 99-103.

Kaur, J. (2014) A comprehensive review on metabolic syndrome. *Cardiology Research and Practice* 2014.

Larsen, N., Vogensen, F.K., Frans, W.J., van den Berg, F.W.J., Nielsen, D.S., Andreasen, A.S., Pedersen, B.K., Abu Al-Soud, Q., Sørensen, S.J., Hansen, L.H. & Jakobsen, M. (2010) Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLOS ONE* 5(2).

Leser, T.D. & Mølbak, L. (2009) Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environmental microbiology* 11(9), 2194-2206.

McBurney, M., Thompson, L.U., Cuff, D.J. & Jenkins, D.J.A. (1988) Comparison of ileal effluents, dietary fibres, and whole foods in predicting the physiological importance of colonic fermentation. *American Journal of Gastroenterology* 83(5), 536-540.

Musso, G., Gambino, R. & Cassadet, M. (2010) Obesity, diabetes and gut microbiota. *Diabetes Care* 33(10), 2277-2284.

Mudgil, D. & Barak, S. (2013) Composition, properties and health benefits of indigestible carbohydrate polymers as dietary fibre: A review. *International Journal of Biological Macromolecules* 61, 1-6.

Peters, S.G., Pomare, E.W. & Fisher, C.A. (1992) Portal and peripheral blood short chain fatty acid concentration after caecal lactulose instillation at surgery. *Gut* 33(9), 1249-1252.

Pouteau, E., Rochat, F., Jann, A., Meirim, I., Sanchez-Garcia, J., Ornstein, K., German, B. & Ballévre, O. (2007) Chicory increases acetate turnover but not propionate and butyrate peripheral turnover in rats. *British Journal of Nutrition* 99(2), 287-296.

Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S. & Flint, H.J. (2002) The microbiology of butyrate formation in the human colon. *FEMS Microbiology Letters* 217(2), 133-139.

Puddu, A., Sanguineti, R., Montecucco, F. & Viviani, G.L. (2014) Evidence for the gut microbiota short-chain fatty acids as key pathophysiological molecules

improving diabetes. *Mediators Inflammation* 2014, 1-9.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., Peng, Y., Zhang, D., Jie, Z., Wu, W., Qin, Y., Xue, W., Li, J., Han, L., Lu, D., Wu, P., Dai, Y., Sun, X., Li, Z., Tang, A., Zhong, S., *et al.* (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes *Nature* 490(7418), 55-60.

Rios-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de Los Reyes-Gavila´n, C.G. & Salazar, N. (2016) Intestinal short chain fatty acids and their link with diet and human health *Frontiers in Microbiology* 7(185).

Rodríguez, J.M., Murphy, K., Stanton, C., Ross, R.P., Kober, O.I., Juge, N., Avershina, E., Rudi, K., Narbad, A., Jenmalm, M.C., Marchesi, J.R. & Collado, M.C. (2015) The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health and Disease* 26.

Sangeethapriya, M. & Siddhurju, P. (2014) Health related functional characteristics and antioxidant potential of mucilage (dietary fiber) from *Zizyphus mauritiana*. *Food Science and Human Wellness* 3(2), 79-88.

Schroeder, N., Marquart, L.F. & Gallaher, D.D. (2013) The role of viscosity and fermentability of dietary fibres satiety- and adiposity-related hormones in rats. *Nutrients* 5(6), 2093-2113.

Sierra, M., García, J.J., Fernández, N., Diez, M., Calle, A.P., Álvarez, J.C, Carriedo, D., Castro, L.J., de la Torre, M., González, M., González, M.A., Morán, V., Prieto, C. & Sahagún, A.M. (2002) Therapeutic effect of psyllium in type 2 diabetic patient. *European Journal of Clinical Nutrition* 56(9), 830-842.

Smushkin, G. & Vella, A. (2010) What is diabetes type 2. *Medicine* 38(11), 597-601.

Soldavini, J. & Kaunitz, J.D. (2013) Pathobiology and potential therapeutic value of intestinal short-chain fatty acids in gut inflammation and obesity. *Digestive Diseases and Sciences* 58(10), 2756-2766.

Stoddart, L.A., Smith, N.J. & Milligan, G. (2008) Free fatty acid receptors FFA1, -2 and -3: Pharmacology and pathophysiological functions. *Pharmacological Reviews* 60(4), 405-417.

Stumvoll, M., Goldstein, B.J. & van Haeften, T.W. (2005) Type 2 diabetes: principles of pathogenesis and therapy. *The Lancet* 365(9467), 1333-1346.

Topping, D.L. & Clifton, P.M. (2001) Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. *Physiological Reviews* 81(3), 1031-1064.

Vrieze, A., Out, C., Fuentes, S., Jonker, L., Reuling, I., Kootte, R.S., van Nood, E., Holleman, F., Knaapen, M., Romijn, J.A., Soeters, M.R., Blaak, E.E., Dallinga-Thie, G.M., Reijnders, D., Ackermans, M.T., Serlie, M.J., Knop, F.K., Holst, J.J., van der Ley, C., Kem, I.P., Zoetendal, E.G., de Vos, W.M., Hoekstra, J.B., Stroes, E.S., Groen, A.K. & Nieuwdorp, M. (2014) Impact of oral vancomycin on gut microbiota, bile acid metabolism and insulin sensitivity. *Journal of Hepatology* 60(4), 824-831.

WHO (1999) *Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications.* Geneva: Department of Noncommunicable Disease Surveillance.

Wild, S., Roglic, G., Green, A., Sicree, R. & King, H. (2004) Global prevalence of diabetes. *Diabetes Care* 27(5), 1047-1053.

Zhao, G., Liu, J-F., Nyman, M. & Jönsson, J.A. (2007) Determination of short-chain fatty acids in serum by hollow fiber supported by liquid membrane extraction coupled with gas chromatography. *Journal of Chromatography* 846(1-2), 202-208.

Zhao, G., Nyman, M. & Jönsson, J.A. (2005) Rapid determination of short-chain fatty acids in colonic content and faeces of human and rats by acidified water-extraction and direct-injection gas chromatography. *Biomedical Chromatography* 20(8), 674-682.