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SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
Diplomski studij
Biotehnologija u medicini

Karla Soldatić

Razvoj LC-MS/MS metode u svrhu ispitivanja utjecaja ozljede leđne moždine na disbiozu i količinu kratkolančanih masnih kiselina u štakorima

Diplomski rad

Rijeka, Lipanj 2023.

Mentor: Doc. dr. sc. Christian Reynolds

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1. Izv. prof. dr. sc. Jelena Ban
2. Doc. dr. sc. Stribor Marković
3. Doc. dr. sc. Christian Reynolds

This thesis has 59 pages, 12 figures, 11 tables, and 37 references.

ABSTRACT

Spinal cord injury (SCI) has shown to induce changes in healthy gut microbiome and cause gut dysbiosis, leading to a reduction of bacteria responsible for the synthesis of short-chain fatty acids (SCFA). SCFAs have a variety of anti-inflammatory and immune properties, and it has been hypothesised that SCI leads to changes in concentration of circulating SCFAs, leading to a variety of health issues, including neural damage. Due to this, SCFAs pose as clinical markers, and the effect of SCI on SCFA concentration can be studied in rat models. SCFAs are usually quantified using gas chromatography coupled with mass spectrometry (GC-MS) because of their volatility. However, there is a growing number of quantification studies done using liquid chromatography coupled with mass spectrometry (LC-MS). To examine SCFAs as clinical markers, the derivatization and extraction methods were optimised as were parameters used for multiple reaction monitoring (MRM) using tandem mass spectrometry. The protocol included derivatisation using 3-nitrophenylhydrazine hydrochloride (3-NPH). Following derivatisation, eleven SCFA standards were injected onto a liquid chromatography tandem mass spectrometry (LC-MS/MS) system for MRM parameters optimization. Deuterated internal standards (ISTD) were used to identify and account for the matrix effect (ME) and method recovery. SCFAs from plasma samples of SCI rats and sham-control rats (n=5 per group) were extracted and analysed using the LC-MS/MS method to test whether there is a difference in SCFA concentration between sham and SCI samples. Results showed a successful chromatographic separation of all SCFA standards, however only three out of eleven SCFAs showed a statistically significant difference in concentrations between SCI and sham groups. To draw firm conclusions, the experiment should be performed on a larger sample size.

Key words: short-chain fatty acids, LC-MS/MS, derivatisation, internal standards, spinal cord injury

SAŽETAK

Istraživanja su pokazala kako ozljeda leđne moždine uzrokuje promjene u crijevnoj mikrobioti. Takve promjene uzrokuju disbiozu i smanjenje broja bakterija zaslužnih za sintezu kratkolančanih masnih kiselina. Kratkolančane masne kiseline imaju protuupalna i imunomodulatorna svojstva, te se smatra kako ozljeda leđne moždine uzrokuje promjene njihove koncentracije u cirkulaciji i uzrokuje negativne učinke na zdravlje. Zahvaljujući tome, kratkolančane masne kiseline su potencijalni klinički markeri za ispitivanje raznih bolesti, a najprikladniji model za ispitivanja su štakori. Zbog svojstva hlapljivosti, kratkolančane masne kiseline obično se kvanitificiraju koristeći plinsku kromatografiju spregnutu sa spektrometrijom masa (GC-MS), no sve veći broj ispitivanja koristi tekućinsku kromatografiju spregnutu sa spektrometrijom masa (LC-MS). Da bi se kratkolančane masne kiseline ispitivale pomoću LC-MS metode, metode derivatizacije i ekstrakcije moraju biti optimizirane. Protokol u ovom radu uključuje derivatizaciju pomoću 3-nitrofenilhidrazin hidrokorida. Nakon procesa derivatizacije, jedanaest standarada kratkolančanih masnih kiselina se injektiralo u LC-MS/MS te su optimizirani parametri za kvantifikaciju. Deuterirani interni standardi su korišteni za supresiju efekta matriksa i za određivanje oporavka metode. Uzorci plazme za kvantifikaciju kratkolančanih masnih kiselina prikupljeni su iz pet štakora s induciranom ozljedom leđne moždine (SCI), te pet štakora sa zdravom leđnom moždinom (SHAM). Rezultati su pokazali uspješno kromatografsko razdvajanje kratkolančanih masnih kiselina, no samo tri od jedanaest kratkolančanih masnih kiselina je pokazalo statistički značajnu razlika između SCI i SHAM grupe. Zbog malog broja uzoraka, ovaj se eksperiment mora ponoviti na većem broju kako bi se mogli donesti konačni zaključci.

Ključne riječi: kratkolančane masne kiseline, LC-MS/MS, derivatizacija, interni standardi, ozljeda leđne moždine

LIST OF ABBREVIATIONS

SCI	Spinal cord injury
GI	Gastrointestinal
CNS	Central nervous system
SCFA	Short-chain fatty acids
GPR	G protein-coupled receptors
FFAR	Free fatty acid receptor
MS	Mass spectrometry
GC-MS	Gas chromatography coupled with mass spectrometry
LC-MS	Liquid chromatography coupled with mass spectrometry
PRP	Platelet-rich plasma
LC-MS/MS	Liquid chromatography tandem mass spectrometry
ESI	Electrospray ionisation
CID	Collision-induced dissociation
MRM	Multiple reaction monitoring
dMRM	Dynamic multiple reaction monitoring
AABD-SH	4-acetoamido-7-mercapto-2,1,3-benzoxadiazole
TPP	Triphenylphosphine
DPDS	2,2’-dipyridyl disulphide
3-NPH	3-nitrophenylhydrazine hydrochloride
EDC	N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride
ISTD	Deuterated fatty acid internal standards
ME	Matrix effect
ESI-MS	Electrospray ionisation-mass spectrometry
MeOH	Methanol
ACN	Acetonitrile
FA	Formic acid
PCA	Principal component analysis

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1. INTRODUCTION

1.1. Gut dysbiosis in spinal cord injury

Spinal cord injury (SCI) affects more than 250 000 people in the United States alone (1). Patients suffering from SCI exhibit neurogenic intestinal dysfunction as a result of intestinal denervation (2) in addition to long-term paralysis (3). Apart from experiencing changes in bowel movement habits, the gut microbiome is significantly disturbed (2). The gut-brain axis has been a topic of interest in studying SCI for a long time, and with the development of gut microbiota sequencing technologies, the relationship between the CNS and the gut microbiome is becoming of increasingly important nature. Recently, more focus has been placed on the gut microbiome, a collection of microbes found in the gastrointestinal (GI) tract whose metabolites play a role in cardiovascular health, digestion, nutrient absorption, immune system modulation, and have anti-inflammatory properties (3,4). Furthermore, SCFAs as the gut's metabolites, play a role in neural development, such as the blood-brain barrier (BBB) formation, myelination, neurogenesis and microglia maturation, making them indispensable for normal neural functioning (2).

Gut dysbiosis, or change from its normal, healthy state to an imbalanced one, correlates with digestive dysfunction caused by SCI (4). The central nervous system (CNS) has an effect on intestinal function through multiple mechanisms (2). The CNS regulates intestinal motility, molecule transport time, permeability of the intestines, and hormone secretion (2). Changes in intestinal permeability are of particular importance because they may lead to the movement of intestinal bacteria into the bloodstream, and, in turn, cause dysbiosis (2). Kiegrl et al. have found that SCI-induced gut dysbiosis is associated with changes in quantity of immune cells in the mesenteric lymph nodes, which may alter recovery after injury (5). Dysbiosis leads to increased inflammation, hypersensitivity, and pain. Furthermore, SCFAs

have been shown to cross the BBB via monocarboxylate carriers, so any changes in the delicate gut homeostasis leads to metabolite dysregulation and possible aggravation of existing CNS injury. Approximately 11% of SCI patients are hospitalised due to GI issues (4).

The gut microbiota regulates the innate and adaptive immune systems by providing feedback and developing the immune system in neonates, but also by producing SCFAs which affect the immune system within the GI tract and outside of it by promoting the release of immune cells into the bloodstream (4).

The exact mechanism by which SCI affects the gut microbiota is still unknown, but we do know the possible pathways in which dysbiosis occurs. The gut has direct innervation to the spinal cord through multiple pathways (3). Visceral sensory afferents bring back information from the gut to the brain through neurons in the spinal cord (3). Afferent vagal fibres report on the intestinal environment' conditions, while sympathetic nerve fibres, which give autonomic input into the ENS, are found in the spinal cord's thoracic region (3). Damage to any of these pathways disturbs the homeostasis of the intrinsic ENS circuits, partly explaining why patients with SCI experience severe changes in the mobility of the gut, higher faecal retention, as well as a higher risk of infection (3).

A consequence of SCI is the change in overall diversity of the gut microbiome, particularly of SCFA-producing bacteria. Acetic and propionic acids are vital sources of energy, and when the bacteria responsible for producing them are deficient, it leads to adverse GI effects (4). Some studies showed a reduction of beneficial butyrate-producing bacteria in the gut microbiome of SCI patients, leading to an increase of pro-inflammatory cytokines and a high number of M1 macrophage phenotype. This ultimately results in neural damage (4).

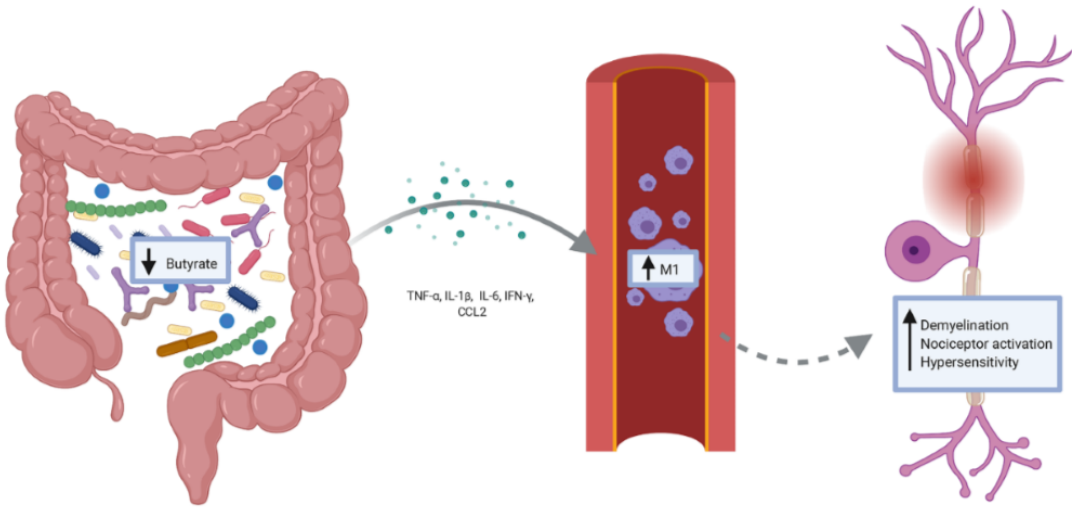


Figure 1: Following SCI, numbers of butyrate-producing bacteria are decreased, leading to an increase of inflammatory macrophages which results in demyelination, nociceptor activation and hypersensitivity (4).

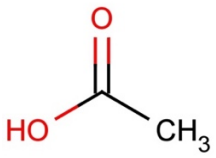
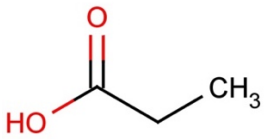
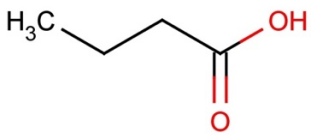
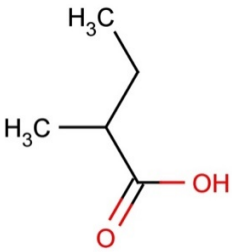
1.1. Short-chain fatty acids

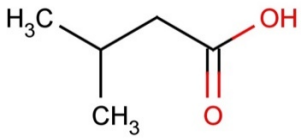
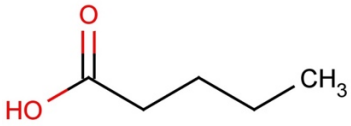
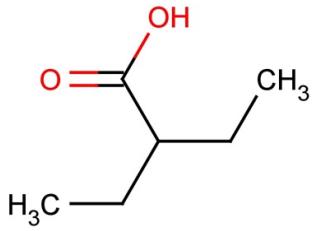
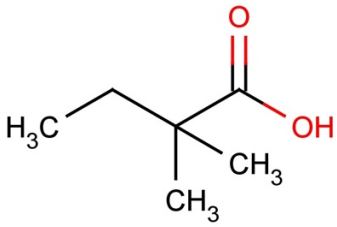
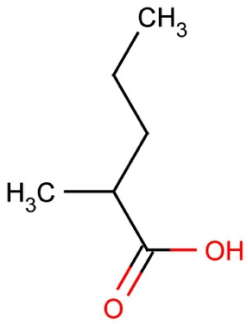
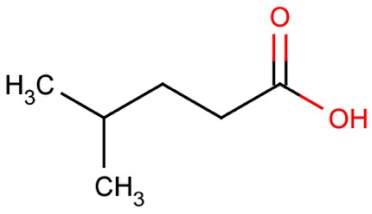
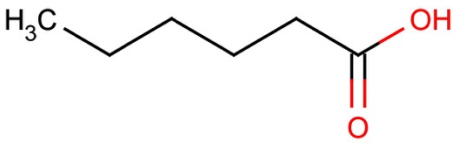
Short chain fatty acids (SCFAs) are organic monocarboxylic acids made up of up to six carbon atoms (6). They are found as straight chains or branched molecules (7). The most abundant SCFAs are acetic acid (C2), propionic acid (C3) and butyric acid (C4), as they make up 95% of total SCFAs found in the human gut (8). Acetic, propionic and butyric acids are found in a ratio of 60:20:20, respectively (9). Eleven SCFAs were used for analysis in this thesis, and their names, molecular weights, molecular structures, as well as logP and pKa values are provided in Table 1.

Gut microbiota synthesises SCFAs via anaerobic fermentation of non-digestible dietary fibre or catabolism of branched amino acids (6). SCFA production is associated with lowering of the pH of the cecum. SCFAs are absorbed in the proximal colon to give bicarbonate, which in turn raises the pH of the lumen and makes it less acidic (10). SCFAs can be metabolized by the host both locally within the gut or at distant sites within the body where they serve as a source of energy (8). Additionally, SCFAs act as

physiological regulators important for the maintenance of the intestinal epithelium (8,11). SCFAs have been observed to have a wide range of protective and therapeutic effects on the gastrointestinal environment (7). They are responsible for the maintenance of intestinal pH, the absorption of various minerals in the distal part of the colon, and promote the production of beneficial colonic microbial microflora, which in turn inhibits overgrowth of pathogenic bacteria (12). Furthermore, there is growing evidence that SCFAs have a positive effect on chronic and acute conditions that are not gut-related, but are rather anti-inflammatory responses exerted by SCFAs (7).

Table 1. Short-chain fatty acid specifications including compound name, molecular weight (g/mol), chemical structure, and logP and pKa values. Chemical structures were drawn in Marvin Sketch.

Compound name	Molecular weight g/mol	Structure	logP	pKa
Acetic acid	60,052		0,28	4,76
Propionic acid	74,08		0,33	4,88
Butyric acid	88,11		0,79	4,82
2-Methylbutyric acid	102,13		1,47	4,97

Isovaleric acid	102,13		1,16	4,80
Valeric acid	102,13		1,37	4,82
2-Ethylbutyric acid	116,16		1,68	4,69
2,2-Dimethylbutyric acid	116,16		1,84	5,03
2-Methylvaleric acid	116,16		1,80	5,05
4-Methylvaleric acid	116,16		1,65	5,09
Hexanoic acid	116,16		1,81	5,09

It has long been hypothesised that SCFAs play a role in modulating metabolism and inflammation (8,11). The identification of orphan G-protein-coupled receptors specific to SCFAs, known as GPR41 or free fatty acid receptor 3 (FFAR3), and GPR42 or free fatty acid receptor 2 (FFAR2), found in various digestive, fat and immune cells established an interest in SCFAs being important signalling molecules in disease (13). Studies have shown a possible role of SCFAs in alleviating symptoms of insulin resistance, suppressing tumorigenesis in the colon by constraining histone deacetylase, and their potential in the reduction of systemic inflammation through the synthesis of pro-inflammatory cytokines (9,14).

Faecal or serum samples are usually used as typical specimens for SCFA quantification as they are produced in the large intestine and absorbed into the bloodstream (15). However, analysis of circulating plasma SCFA levels provide an insight into the systemic and indirect effects of gut microbiota on distal immune cells, tumorigenesis in the colon and systemic inflammation (6). Therefore, SCFAs pose as potential clinical biomarkers. For this kind of assessment to be applied to clinical situations, a precise, easily reproducible and cost-effective analysis protocol is required (7). Majority of SCFA analyses done so far rely on gas chromatography coupled with mass spectrometry (GC-MS) to separate and quantify SCFAs in biological samples (15). Recently, more focus has been placed on liquid chromatography techniques coupled with mass spectrometry (LC-MS) (8,11). The most straightforward and high-yielding protocols include a derivatisation step that allows for the identification and quantification of SCFAs (7). Inflammatory diseases, including spinal cord injury (SCI) are hypothesised to affect microbiota composition and, therein, on the production of SCFAs. This thesis aims to develop and optimise an analytical method through which SCFAs can be quantified, and determine whether their concentrations are affected by SCI.

Rat or mice models of SCI are suitable for studying SCI and translating results into clinical settings, as they are a consistent and cost-effective

strategy (4,16). Rats' platelet-rich plasma (PRP) is collected and analysed as it is a valuable source of growth factors, and it indicates detrimental effects of SCI on mechanisms responsible for intrinsic repair and neuronal regeneration (17).

1.2. Liquid chromatography tandem mass spectrometry

For the detection of SCFAs, mass spectrometry (MS) is used in conjunction with a separation technique such as HPLC, as biological samples are too complex to analyse using MS alone (18).

After preparation, the samples are injected into the liquid chromatography system. High pressure forces deliver the solvent carrying the analyte to the column, where they are separated based on their polarity, ionic interactions, and size (18). The type of flow used is gradient elution because the contents of the mobile phase vary over the total run time. The flow rates should be constant and reproducible if the conditions are controlled. Afterwards, the eluent is delivered to the mass spectrometer's source, where gas phase ions are created (18). For liquid samples to be ionised, electrospray ionisation (ESI) is applied after the samples pass through the chromatography system (18,19). Flowing out of the electrode at a high voltage, the eluent containing both the analyte and the solvent, becomes charged. Gas flows heat the newly formed spray, thereby causing the evaporation of the solvent, leaving only the analyte. Ions are then derived in a gaseous phase and enter the mass spectrometer (18).

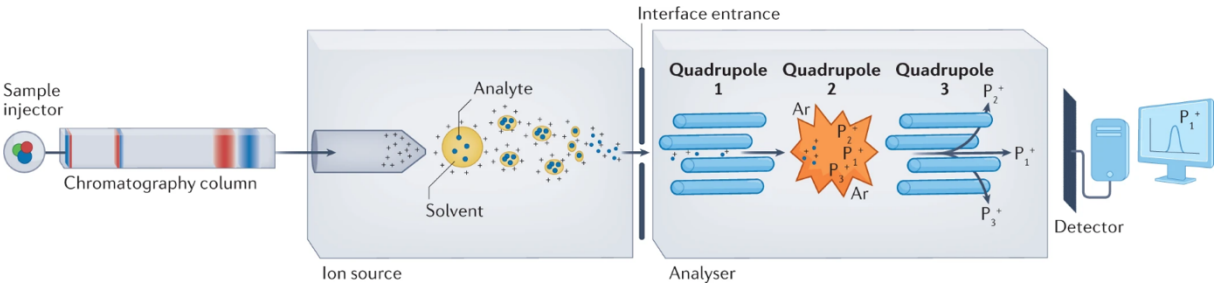


Figure 2: Electrospray ionisation in a tandem mass spectrometer (18).

MS analysis separates ions based on their mass to charge ratio (*m/z*) (18). While there are many different types of mass spectrometry-based analytical approaches, the one used in this thesis is known as the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Specifically, a triple quadrupole instrument allows for mass analysers to be used in tandem, which enables fragmentation between two mass analyses, based on the applied direct current voltage. The most important strength of the liquid chromatography tandem mass spectrometry (LC-MS/MS) is its analytic selectivity (18). Quadrupole 1 (Q1) allows only precursor ions of a specific *m/z* value to pass through the filter towards the collision cell found in quadrupole 2 (Q2). Here, collision-induced dissociation (CID), or energy used for fragmentation, breaks these specific precursor ions into smaller fragments (19). These fragmented products then enter quadrupole 3 (Q3) (18). The product ions are filtered based on a specific *m/z* value and hit the detector in the final section of the tandem mass spectrometer. The mass spectrum reflects only the specifically charged product ions (18).

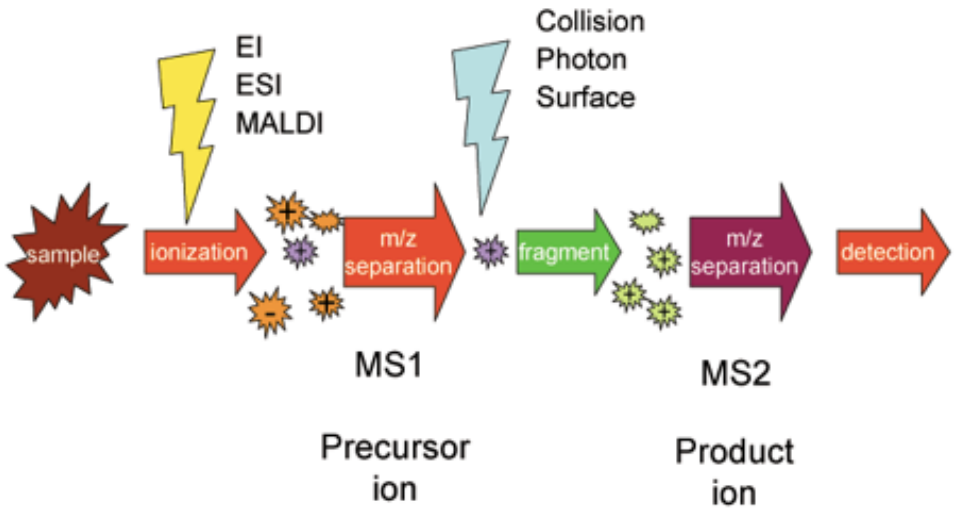
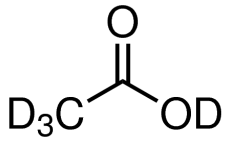
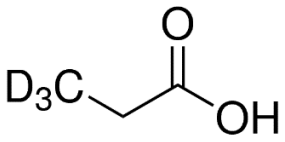
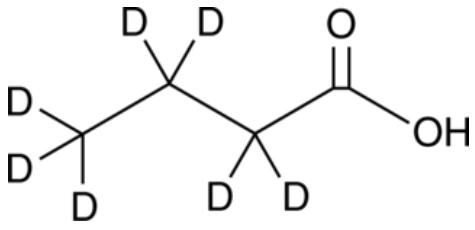


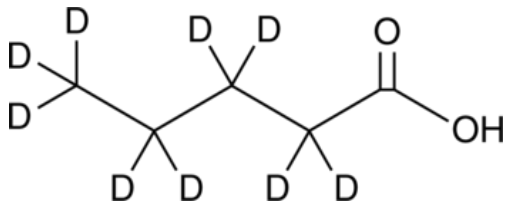
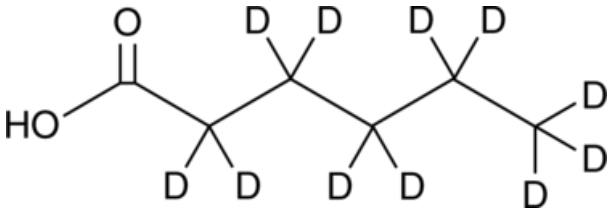
Figure 3: Schematic representation of tandem MS work principle (19).

Matrix effects (ME), known as the alterations to ionisation efficiency of target analytes in a complex mixture, are usually observed as ionisation

suppression or enhancement (20). To ensure ionisation efficiency and to normalise differences in analyte recovery during sample preparation, an internal standard is recommended for use. Isotopes labelled with deuterium (^{2}H or D) are used, and they have the same chromatographic properties as the samples, however, the two are distinguished on the mass spectrometer by the differences in their molecular weight (18). When analysing SCFAs on a mass spectrometer, using internal standards identifies and accounts for the matric effect present (21). Furthermore, the use of branched isotope-labelled SCFAs may improve quantitation accuracies for branched SCFAs (8).

Table 2. Internal standard specifications including compound name, molecular weight (g/mol) and chemical structure. Chemical structures were drawn in Marvin Sketch.

Compound name	Molecular weight g/mol	Structure
Acetic acid d_4	64,08	
Propionic acid d_3	77,10	
Butyric acid d_7	95,15	

Pentanoic acid d_9	111,19	
Hexanoic acid d_{11}	127,20	

Results of LC-MS/MS analyses are presented as a chromatogram, where data are plotted as abundance of analyte over time. Each peak on the chromatogram represents the number of ions that reach the detector (18). Usually, there is more than one chromatogram for each compound analysed. These transitions can be separate fragments if the analyte produces multiple stable product ions, but other causes also exist. Sometimes, non-measurable signals are observed and are, typically, derived from analyte's isomers, dissociation of some other, larger molecules, or a combination of a few factors (18).

Calibration of mass spectrometry analyses guarantees mass accuracy and ion resolution with a stable course to the detector (18). Calibration curves are generated using standards of known concentrations (18). For each standard, the area under the curve of each transition peak is measured and compared to the peak area of an analyte's transitions, to give a normalised response function (18). These are divided to give a ratio of the area, which is used to develop a linear least squares regression (18). This value is used to describe the area-concentration relationship of each analyte (18).

SCFAs are highly volatile, and because of their hydrophilicity, their chromatographic separation is poor, and they do not ionise efficiently. This could be combated by forming harsh conditions on the column using a strong acid, however, even then the chromatographic separation would be unsatisfactory, and the column's lifespan would be shortened (22). For the quantification of SCFAs to be performed, the fatty acids need to undergo chemical derivatisation (8).

Derivatisation is a process in which the structure of a compound, in this case SCFAs, is modified using a chemical reagent to give a new compound with improved chemical and physical properties needed for the purpose of an analysis (23). The reaction conditions are optimised in favour of the formation of the desired derivative with the highest possible yield. Derivatisation of SCFAs can be performed using a variety of compounds which act as reactive nucleophiles in the presence of a coupling agent and a base catalyst. Song et al. used reagent 4-Acetoamido-7-mercapto-2,1,3-benzoxadiazole (AABD-SH) coupled with triphenylphosphine (TPP) and 2,2'-dipyridyl disulphide (DPDS) to derivatise SCFAs in their work (24). Optimal reaction conditions were set at 4°C for 2 hours. This protocol also includes a drying process in the Speed VAC. Bihan et al. performed SCFA derivatisation using aniline solution coupled with N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), followed by keeping the reaction mixture on ice for 2 hours (25). In a study done by Sowah et al. glycine ethyl ester was used as the derivatisation agent. This method required 20 hours of incubation prior to derivatisation, and further 2 hours for derivatisation to occur (13). The majority of SCFA experimental work, which includes derivatisation prior to analysis on liquid chromatography tandem mass spectrometry (LC-MS/MS), uses 3-nitrophenylhydrazine hydrochloride (3-NPH) as the derivatisation agent of choice. Derivatisation of acylhydrazines 3-NPH is used to separate and quantify acetic, propionic, butyric, valeric and hexanoic acids and their branched forms (11). This method is straightforward and fast as it requires

the samples to be incubated for 40 minutes at 37°C. Furthermore, 3-NPH is convenient because it is compatible with LC solvents and requires relatively mild conditions (25).

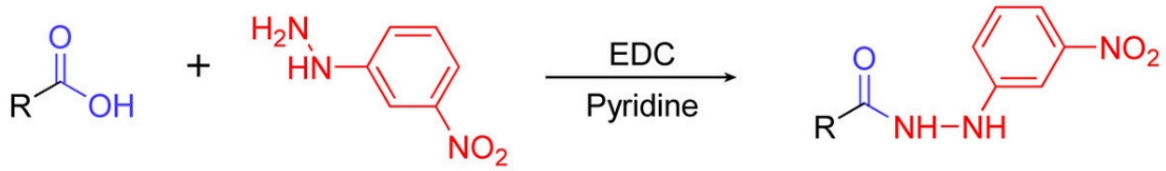


Figure 4. Derivatisation of short-chain fatty acids using 3-NPH in the presence of EDC and pyridine.

Derivatisation allows for the increase in size and hydrophobicity of SCFAs, leading to longer retention time and better separation of structural isomers. Shorter and straight SCFAs elute earlier, while longer and branched SCFAs elute later (8).

Recent advances in electrospray ionisation-mass spectrometry (ESI-MS) based lipidomics have allowed for a large number of lipid compounds from a variety of sources to be identified (26). Analysis of lipids in only the negative mode allows for the greater coverage of points on a compound's peak with increasing specificity and sensitivity, in a single scan (26). Multiple reaction monitoring (MRM) is a highly delicate technique of targeted mass spectrometry (MS) used to identify and quantify compounds based on their specific precursor lipid-to-fragment ion transitions, therefore allowing for the quantification of compounds in a complex mixture (27,28). To establish the most sensitive MRM assay, the selected MRM precursor (Q1) and fragmentor (Q3) ions are optimised by selecting the values with the most intense charge state (27). Collision-induced dissociation (CID) is the energy responsible for the fragmentation of compounds in Q2 (28). This way, a transition or an MRM Q1/Q3 ion pair is created (27). The fragmentor value affects the efficiency of the transfer of precursor ions onto the mass

spectrometer. Therefore, optimising these values ensures the strongest MRM signal (27). The main issue in using just MRM when analysing SCFAs is the fact that the fatty acids with the same number of carbon atoms have the same precursor and product ions, as well as *m/z* fragments (Table 6), and therefore cannot be distinguished one from another using MRM only. To further increase specificity and sensitivity of this method, and to quantify SCFAs, a dynamic MRM (dMRM) is used (26). dMRM provides better quality data as compared to traditional monitoring, as well as easier method modification (21). dMRM methods are based on individual compounds' retention time windows. This technique lowers the chances of multiple compounds having co-eluting peaks, which is a great concern when eluting SCFAs on any chromatographic column (27). Furthermore, dMRM is specific because it accounts for even data point spacing with adequate sampling across a peak and, therefore, gives the most accurate representation of the peak. (21). Individual MRM dwell times are adjusted to keep a constant sampling rate across all peaks, even with changing numbers of ion transitions being monitored due to variations between cycles (21). Even narrow LC peaks can be quantified using dMRM, and dMRM calibration curves show excellent sensitivity, linearity and dynamic range (18). By using this method, overall MS cycle duration can be reduced, which makes the overall method even more straightforward.

2. AIMS AND HYPOTHESES

The aims of this thesis are:

1. Evaluate the suitability of various SCFA derivatisation protocols for use in combination with the Agilent LC-MS/MS system.
2. Optimize all necessary LC/MS-MS method parameters for the separation and quantification of SCFAs, including for the distinction of fatty acids of the same molecular weight, and MRM transitions.
3. Employ the newly optimized method to quantify SCFA in plasma from a cohort of rats following chronic spinal cord injury or sham surgery.

The hypotheses of this thesis are:

1. The developed LC/MS-MS method will successfully separate short chain fatty acids that have the same molecular weight, and MRM transitions.
2. It is expected that the highest fatty acid constituent in rat plasma samples will be acetic acid.
3. SCFA concentrations will be significantly different between the sham surgery group and the group with spinal cord injury.

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

Anaesthetics carprofen, lidocaine and isoflurane used during spinal cord surgery were bought from Covetrus (Portland, ME, USA). Heparin tubes were bought from Covetrus (Portland, ME, USA). Antibiotic Baytril used to prevent urinary infections was bought from Covetrus (Portland, ME, USA).

SCFA standards: acetic acid, propionic acid, butyric acid, 2-methylbutyric acid, valeric acid, isovaleric acid, hexanoic acid, 2,2-dimethylbutyric acid, 2-ethylbutyric acid, 2-methylvaleric acid and 4-methylvaleric acid were acquired from Sigma Aldrich (St. Louis, MO, USA). Deuterated fatty acid internal standards (ISTD): acetic acid d_4, propionic acid d_9, butyric acid d_7, pentanoic acid d_9, and hexanoic acid d_{11} were acquired from CDN isotopes (Pointe-Claire, Quebec, Canada). Derivatisation reagent 4-Acetoamido-7-mercapto-2,1,3-benzoxadiazole (AABD-SH) was acquired from Tokyo Chemical Industry (Tokyo, Japan). Derivatisation catalysts triphenylphosphine (TPP) and 2,2'-dipyridyl disulphide (DPDS) were from Tokyo Chemical Industry (Tokyo, Japan). Derivatisation reagent 3-Nitrophenylhydrazine hydrochloride (3-NPH) was acquired from Sigma Aldrich (St. Louis, MO, USA). Derivatisation catalysts N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and pyridine were acquired from Sigma Aldrich (St. Louis, MO, USA).

Analytical grade acetonitrile (ACN) was acquired from VWR Chemicals (Randor, PA, USA). Analytical grade methanol and Milli-Q water were acquired from VWR Chemicals (Randor, PA, USA). Formic acid was acquired from Honeywell (Charlotte, NC, USA).

3.2. Rat plasma samples

Rat plasma samples were obtained from male Sprague-Dawley rats bought from Charles River Laboratories (Wilmington, MA, USA) and kept at the Wayne State University, Department of Emergency Medicine (Detroit, MI, USA.) The rats were unrelated, 9 weeks old and weighed 300-325 grams on the day of surgery. Spinal cord injury was induced as described by Reynolds et al.(16) Rats were anesthetised using 3-5% isoflurane. The anaesthetic depth was confirmed by the absence of paw withdrawal and corneal reflexes, and for the remainder of the surgery the isoflurane levels were reduced and maintained at 1.5-2.5%. Carprofen (5 mg/kg) was administered subcutaneously on the day of surgery and for two additional days post-operation. The rats' temperature was maintained at 37°C using a homeothermic blanket. According to Reynolds et al. a skin incision overlaying the T2-T5 vertebral segments was made, and lidocaine (0.5 mg/kg) was used to infiltrate the incision. A dorsal laminectomy was performed between T3 and T4 vertebral segments. The dura mater was excised and the spinal cord was completely transected using microscissors. Muscle and skin incisions were closed using a 3-0 suture and wound clips, respectively. After the surgery, the rats were kept in a temperature-controlled environment to recover. They received the antibiotic Baytril (10 mg/kg) for seven days to prevent urinary infections. Their bladders were expressed twice daily for two weeks. They were kept at a 12/12-hour light on, light off cycle. The temperature was kept between 18°C and 25°C, and the humidity ranged from 30% - 70%. For the sham operation, all surgical and post-operative procedures were identical to those describe above, including dorsal laminectomy, however the spinal cord was not transected.

The rats were euthanised four weeks post operation (16). Blood was collected from the heart and placed into heparin tubes to prevent blood coagulation. Blood was centrifuged for 3 minutes at 1000 x g, and the

supernatant containing the plasma was transferred into a vial and stored at -80 °C.

Rat plasma samples used for the SCFA quantification are described in Table 3. Rats with spinal cord injury are classified as SCI, while the control group with an intact spinal cord is classified as SHAM. Five plasma samples from each group were analysed.

Table 3. List of analysed rat plasma samples (n=5 per group).

Rat plasma samples	
Spinal cord injury	**Healthy spinal cord (control)**
SCI 1	SHAM 1
SCI 2	SHAM 2
SCI 3	SHAM 3
SCI 4	SHAM 4
SCI 5	SHAM 5

3.3. Extraction of short-chain fatty acids

Rat plasma (50 μL) was deprotonated using 100 μL of ice-cold isopropanol. 10 μL of 5 μM ISTD mix (acetic acid d4, propionic acid d9, butyric acid d7, pentanoic acid d9, and hexanoic acid d11) was added and plasma sample was then thoroughly mixed by vortex. After centrifugation at 14000 RPM for 15 min, 100 μL of the supernatant was removed into a new vial for derivatisation step.

3.4. Short-chain fatty acid derivatisation

Two modified methods were used for the optimisation of SCFA derivatisation. The first attempted derivatisation method was done according to Song et al. 1 mM standard solution was prepared in water (8).

Milli-Q water (380 μL) and 1 mM internal standard (100 μL) were added to a vial. For the derivatisation step, 10 μL of 20 mM AABD-SH, 20 mM TPP and 20 mM DPDS in dichloromethane were added to the mix. Standard samples were left to derivatise at room temperature for 5 minutes. The mix was dried in the Speed VAC for 2 hours and was reconstituted using MeOH (100 μL) (8). The standards were injected directly onto LC-MS/MS.

The second derivatisation method was performed according to Dei Cas et al. 100 μL of 1mM standard solution prepared in ACN was added to a new glass vial (11). 50 mM 3-NPH, 50 mM EDC and 7% pyridine were prepared in water: methanol (3:7, v/v), and stored for no more than three days at -20 degrees Celsius. 50 μL of each was added to the vial for derivatisation. Standard samples were derivatised in an incubator at 37 °C for 40 minutes. The derivatization reaction was quenched by adding 250 μL of 0,5% formic acid in water. Afterwards, the samples were directly injected onto LC-MS/MS, without the need for any drying process (11).The two optimisation methods are compared in Table 4. Plasma samples were derivatized using the same method as for standard samples.

Table 4. Two SCFA derivatization method protocols (8,11).

	Method 1	**Method 2**
Sample preparation	1 mM standard solution in Milli-Q water	1 mM standard solution in ACN
Derivatisation	100 μL standard (or plasma) solution	100 μL standard (or plasma) solution
	10 μL 20 mM AABD-SH 10 μL 20 mM TPP 10 μL 20 mM DPDS } In dichloromethane	50 μL 50 mM 3-NPH 50 μL 50 mM EDC 50 μL 7% pyridine } In ACN
	5 minutes at room temperature 2 hours drying in Speed VAC	40 minutes at 37°C
Reconstitution	100 μL MeOH	250 μL 0,5% formic acid in water

3.5. LC-MS/MS method development parameters

The analytical system used to separate and quantify each of the short-chain fatty acids, includes an HPLC Agilent 1260 system consisting of two tanks providing the mobile phase, a degasser, a binary pump, an automatic injector, a column, and a column heater connected to the Agilent 6460 triple quadrupole mass spectrometer using electrospray ionisation (ESI) (Agilent Technologies, Santa Clara, CA, USA). Chromatographic analyses were performed using the Pursuit 5 C18 150 x 2.0 mm column (Agilent Technologies, Santa Clara, CA, USA). Mobile phase consisted of (A) 0,1% (v/v) formic acid in Milli-Q water, and (B) 0,1% (v/v) formic acid in acetonitrile. Flow rate was set at 0,6 mL/min, while the column temperature

was set at 30 degrees Celsius. Injection volume was 10 μL for the analysis of each sample. Elution gradient is shown in Table 5.

Table 5. LC-MS/MS analysis elution gradient parameters. Table contains the ratio (%) of mobile phase A (milli-Q water + 0,1% formic acid) and mobile phase B (acetonitrile + 0,1% formic acid).

Time / min	A/%	B/%	Flow rate (mL/min)
0,00	90	10	
2,00	70	30	
20,00	50	50	
21,00	1	99	0,600
22,00	1	99	
23,00	90	10	
28,00	90	10	

For the mass spectrometry analysis, the following parameters were set: negative ESI mode, ion source temperature at 300 degrees Celsius, capillary voltage at 3500 V, and nozzle voltage at 500 V. Inert nitrogen under 45 psi pressure and gas flow of 5 L/min was used in the collision cell. The temperature of the drying gas was set at 250 degrees Celsius, while the flow rate was 11 L/min. Collision energies ranged from 6V to 28V, depending on the analysed SCFA. Each sample was injected and analysed in triplicates. Table 6 contains the LC-MS/MS optimisation parameters for quantitative analyses. The parameters include the ESI mode, the *m/z* precursor, the fragmentor, *m/z* fragments, collision energy (CE), retention time (R_T), delta retention time (ΔR_T), the slope (a) and y intercept (b), linearity. Underlined transitions were used for quantification.

Table 6. MRM parameters for MS analysis. Shown are: scan mode, *m/z* precursor, fragmentor (frag), *m/z* fragment values, and collision energies (CE). Underlined fragment values are used for quantification.

SCFA standard	Mode	*m/z* precursor ion	Fragm /V	*m/z* fragments	CE/V	Rt/min	ΔRt/min	Y= ax + b		R^2	Linearity (µg/mL)
								(a)	(b)		
Acetic acid	-	193,9	100	175,2	10	3,8	+/-1	34.8378	82.6221	0.995	0.01-30
				<u>151,6</u>	10						
				136,7	14						
Acetic acid-d_4	-	196,9	100	177,4	10	3,9	+/-1	25.7355	0.662	0.997	0.001-32
				152,6	10						
				<u>136,7</u>	14						
Propionic acid	-	207,9	100	189,5	6	4,6	+/-0,5	1110.3841	237.6034	0.991	0.001-0.4
				164,7	6						
				<u>136,7</u>	14						
Propionic acid-d_3	-	210,9	100	<u>167,6</u>	6	4,7	+/-0,5	241.954	1.9372	0.996	0.001-0.8
				151,6	8						
				136,7	14						
Butyric acid	-	221,9	100	178,6	8	5,5	+/-0,5	1656.9068	685.1185	0.993	0.001-0.4
				151,7	6						
				<u>136,6</u>	14						

Butyric acid-d_7	-	229	100	185,7	8	5,5	+/-0,5	934.5579	20.5729	0.995	0.001-0.5
				152,6	10						
				136,7	16						
2-Methyl-butyric acid	-	236	110	151,7	14	6,6	+/-0,25	4573.7786	2091.456	0.994	0.001-1
				136,8	10						
				106,7	24						
Valeric acid	-	236	110	192,5	6	7,2	+/-0,4	2301.1981	2143.6507	0.991	0.001-1
				151,7	10						
				136,7	16						
Isovaleric acid	-	235,9	120	192,7	8	6,85	+/-0,2	1852.5578	88.7201	0.992	0.005-1
				151,7	12						
				136,8	16						
Pentanoic acid-d_9	-	245	110	201,9	8	7,1	+/-0,6	1643.3694	213.0585	0.9914	0.001-1.1
				152,7	12						
				136,8	16						
Hexanoic acid	-	249,9	110	206,8	8	9,8	+/-0,4	2355.5685	3243.1569	0.993	0.01-1
				151,7	12						
				136,8	16						
2,2-Dimethyl-butyric acid	-	249,9	110	136,8	18	8,45	+/-0,3	3823.3542	37.6597	0.999	0.001-1
				106,7	28						

2-Ethyl-butyric acid	-	250	110	151,8 136,7 106,7	16 16 26	8,1	+/-0,3	7348.734	481.3647	0.994	0.001-1
2-Methyl-valeric acid	-	250	110	151,7 136,8 106,8	12 16 26	8,8	+/-0,25	5203.6172	735.8194	0.991	0.001-1
4- Methyl-valeric acid	-	249,9	110	231,6 151,8 136,8	12 12 18	9,3	+/-0,5	2330.0342	63.0179	0.992	0.001-1
Hexanoic acid-d_{11}	-	261	110	152,6 136,8	14 18	9,6	+/-0,6	1848.1191	24.9394	0.997	0.001-1

3.5.1. Dynamic MRM

To increase the total number of transitions that can be quantified during an MRM analysis, a time limit is added (26). Dynamic MRM allows the systems to acquire transitions only during the retention period of each eluting analyte. For this analysis, the acquisition time for each transition was set at as low as retention time +/- 0,2 for isovaleric acid, and as high as retention time +/-1 for acetic acid (Table 6). Dynamic MRM measurements segment the total number of transitions in accordance with their elution time from the column. Reducing long dwell-times reduces the signal-to-noise ratio and increases selectivity (26). Adding a time constraint in dynamic MRM enables the distinction between compounds that cannot be distinguished using the precursor ion, fragmentor and product ion values.

3.5.2. Calibration curve and linearity

A calibration curve, as a bioanalytical method, is a linear relationship between concentration and response (29). It is used to predict unknown concentrations of an analyte in a complex mixture. Linearity is the ability of an analytical process to provide test results, which are directly proportional to the concentration of the measurand, also known as the quantity to be measured, in a sample. If an analytical method is linear, the test results are directly, or according to a well-defined mathematical transformation, proportional to the concentration of the analyte in the sample within a certain range. Linearity is usually expressed as the confidence limit around the slope of the regression line.

For obtaining a calibration curve, a stock mix of SCFA standards and ISTDs in ACN was prepared. Through serial dilutions, eleven different concentrations of the mix were prepared: 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 μM. Linearity was determined by constructing calibration curves, in which the concentrations ranged: 0.01-30 μg/mL for

acetic acid; 0.001-32 µg/mL for acetic acid d_4; 0.001-0.4 µg/mL for propionic and butyric acid; 0.001-0.8 µg/mL for propionic acid d_3; 0.001-0.5 µg/mL for butyric acid d_7; 0.001-1 µg/mL for 2-methylbutyric, valeric, 2,2-dimethylbutyric, 2-ethylbutyric, 2-methylvaleric, 4-methylvaleric acids and hexanoic acid d_{11}; 0.005-1 µg/mL for isovaleric acid; 0.01-1 µg/mL for hexanoic acid; and 0.001-1.1 µg/mL for pentanoic acid d_9.
Each of the dilutions was derivatised as previously described and the dilution samples were injected onto LC-MS/MS in a triplicate.
Calibration curves were calculated using standard linear regression analysis. Linearity was calculated using the regression coefficient R^2.

3.5.3. Internal standards

The matrix effect occurs when compounds that are coeluted with the main analyte interfere with the ionisation process in the detector, thereby inducing ionisation suppression or enhancement (20). According to Tan et al., this causes detrimental effects on the reproducibility, accuracy and sensitivity of the results of an LC-MS/MS analysis (30). One of the ways in which the matrix effect can be eliminated is the addition of internal standards to the analysed sample (30).

For the recovery value calculation, the concentration of SCFA in each sample was calculated using analysed concentration of ISTD versus spiked ISTD concentration. Each of the plasma samples was spiked with 10 µL of 5 µM ISTD mix and derivatization was performed as previously described. The percentage of recovery was determined using the formulae by Tan et al below (30):

$$\%\ recovery = \left(\frac{Concentration\ of\ analyte\ recorded,\ C1}{Concentration\ of\ analyte\ added, C0)}\right) x\ 100$$

Where C_0 is the concentration of added ISTD (5 μM) and C_1 is the concentration of ISTD analysed in the plasma sample after spiking it with a known concentration and performing extraction protocol.

3.6. Statistical data analysis

Statistical data analysis was performed using Mass Hunter Qualitative analysis, version B.07.00 (Agilent Technologies, Santa Clara, CA, USA) and Microsoft Excel version 16.16.27 (Microsoft Corporation, WA, USA). Unpaired, two-tailed Student's T-test, performed in GraphPad, version 8.4.2 (Boston, MA, USA), was used to determine whether the differences in SCFA concentrations between the rats with spinal cord injury (SCI) and healthy rats (SHAM) are statistically significant. Principle component analysis (PCA) was performed in R studio, version v.4.1.2 using following packages: readxl, FactoMineR, factoextra and corrplot.

4. RESULTS

4.1. Short-chain fatty acid derivatisation

During the experimental part of this work, the SCFA derivatisation protocol was optimised, and a dynamic MRM method was developed and optimised. Furthermore, we optimised the MS-MRM method in which SCFAs from rat plasma samples were quantified using their corresponding ISTD, and, finally, the most abundant SCFA content in SCI rat plasma samples were quantified.

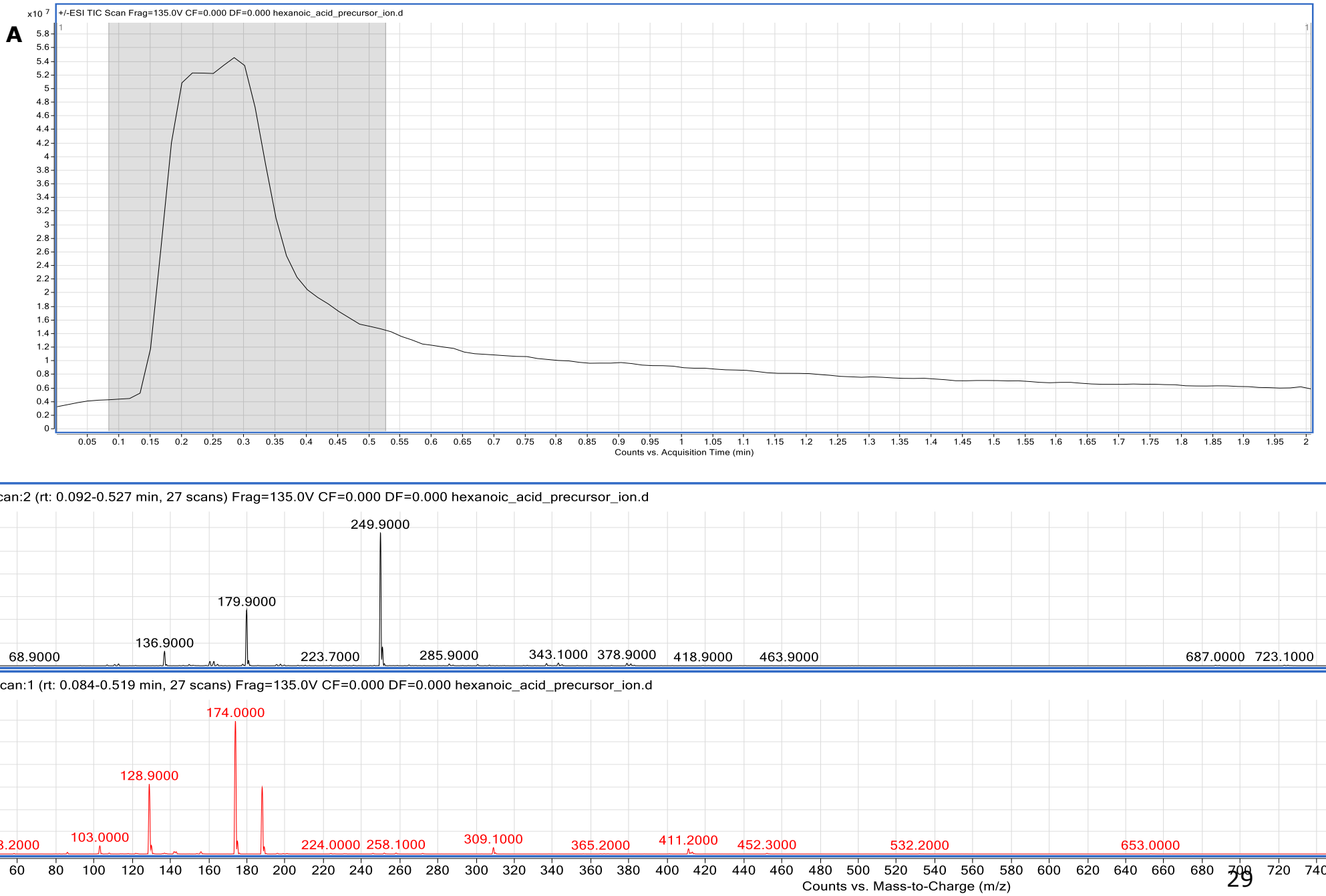
Derivatisation method 1, applied from Eun Song et al., has shown inadequate results in terms of obtaining MRM parameters following SCFA derivatisation. As they are highly volatile, it is presumed that the vast majority of SCFA content was lost during the drying process in the Speed VAC. Due to this loss, it was not possible to optimise the MRM parameters in this step. Using AABD-SH as a derivatisation agent failed to produce any ions that would have to be used for protocols following derivatisation.

Due to this, a modified version of method 2 using 3-NPH was chosen as the method for SCFA derivatisation.

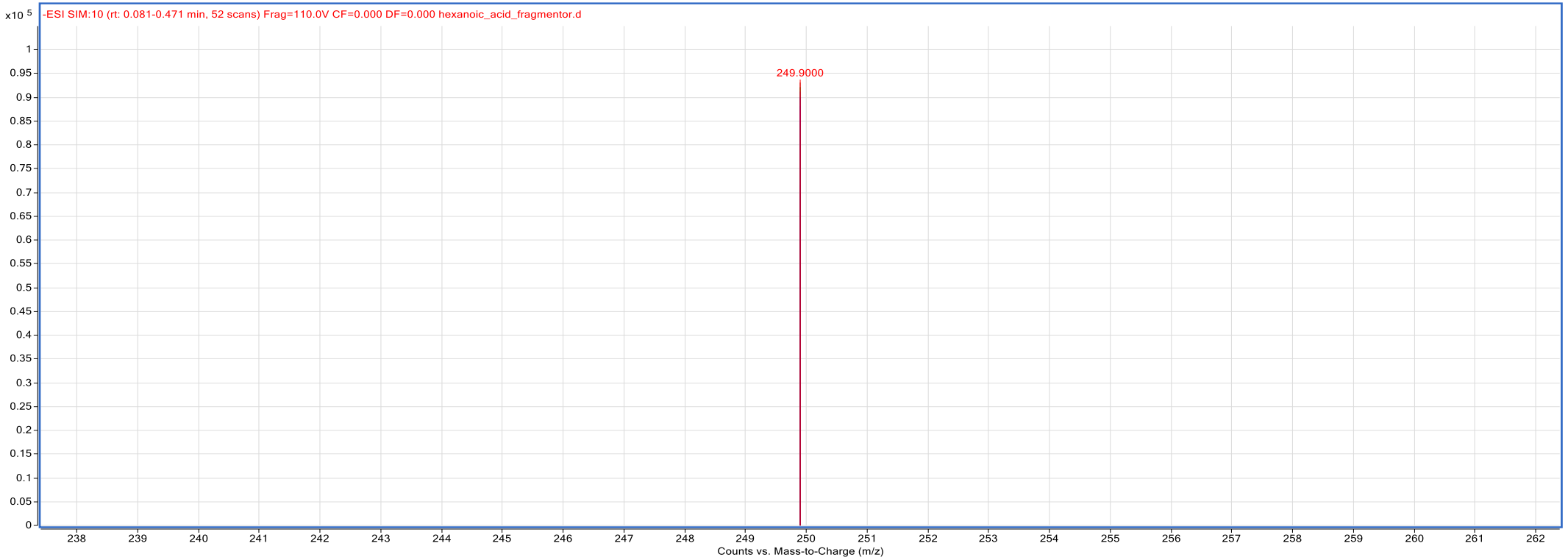
4.1.2. Optimisation of MRM parameters

Following the injection of derivatised standards onto LC-MS/MS, charged precursor ions are produced by electrospray ionization (ESI). The optimal voltage for a given analyte is determined by looking at the highest ion peak on the spectrum, which occurred at 110 V for hexanoic acid (Figure 4b). Only the precursor ions with a specific mass-to-charge (*m/z*) ratio are passed through, are used to identify the compound and ensure the emitted signal is coming from the target compound. The hexanoic acid precursor ion was determined by examining the negative mode and is represented by the highest peak on the spectrum (249.9) (Figure 4a). By applying the

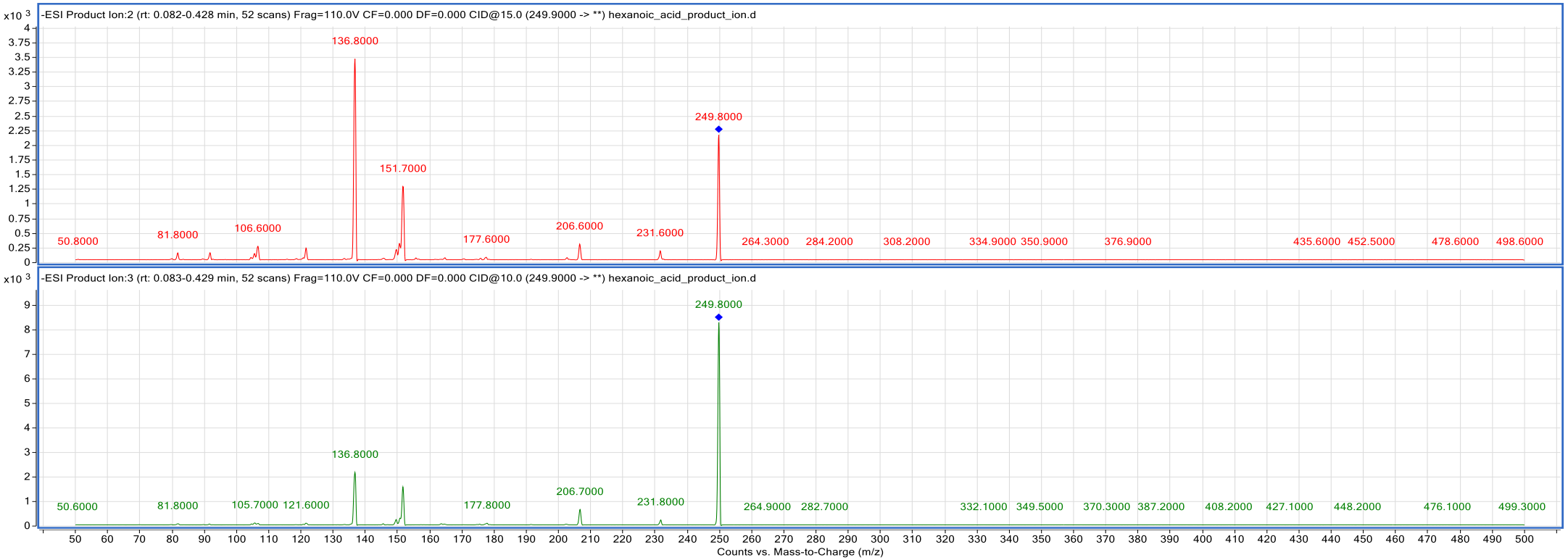
corresponding collision-induced dissociation (CID), also known as the energy needed for the breakdown of precursor ions into its product ions, an MS/MS spectrum specific to the analysed compounds' products is produced. Three hexanoic acid product ions generated for detection by the MS detector were 136.8, 151.7, and 249.8 (Figure 4c). CID value is determined by finding the highest energy peak on the spectrum, which was 8 V for hexanoic acid (Figure 4d).


A
x10 7
+/-ESI TIC Scan Frag=135.0V CF=0.000 DF=0.000 hexanoic_acid_precursor_ion.d
Counts vs. Acquisition Time (min)
x10 5
-ESI Scan:2 (rt: 0.092-0.527 min, 27 scans) Frag=135.0V CF=0.000 DF=0.000 hexanoic_acid_precursor_ion.d
249.9000
179.9000
136.9000
68.9000
223.7000
285.9000
343.1000
378.9000
418.9000
463.9000
687.0000
723.1000
x10 6
+ESI Scan:1 (rt: 0.084-0.519 min, 27 scans) Frag=135.0V CF=0.000 DF=0.000 hexanoic_acid_precursor_ion.d
174.0000
128.9000
58.2000
103.0000
224.0000
258.1000
309.1000
365.2000
411.2000
452.3000
532.2000
653.0000
Counts vs. Mass-to-Charge (m/z)

B


-ESI SIM:10 (rt: 0.081-0.471 min, 52 scans) Frag=110.0V CF=0.000 DF=0.000 hexanoic_acid_fragmentor.d
x10 5
1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
249.9000
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
Counts vs. Mass-to-Charge (m/z)

c


-ESI Product Ion:2 (rt: 0.082-0.428 min, 52 scans) Frag=110.0V CF=0.000 DF=0.000 CID@15.0 (249.9000 -> **) hexanoic_acid_product_ion.d
x10 3
136.8000
249.8000
151.7000
50.8000
81.8000
106.6000
177.6000
206.6000
231.6000
264.3000
284.2000
308.2000
334.9000
350.9000
376.9000
435.6000
452.5000
478.6000
498.6000
-ESI Product Ion:3 (rt: 0.083-0.429 min, 52 scans) Frag=110.0V CF=0.000 DF=0.000 CID@10.0 (249.9000 -> **) hexanoic_acid_product_ion.d
x10 3
249.8000
136.8000
50.6000
81.8000
105.7000
121.6000
177.8000
206.7000
231.8000
264.9000
282.7000
332.1000
349.5000
370.3000
387.2000
408.2000
427.1000
448.2000
476.1000
499.3000
Counts vs. Mass-to-Charge (m/z)

D

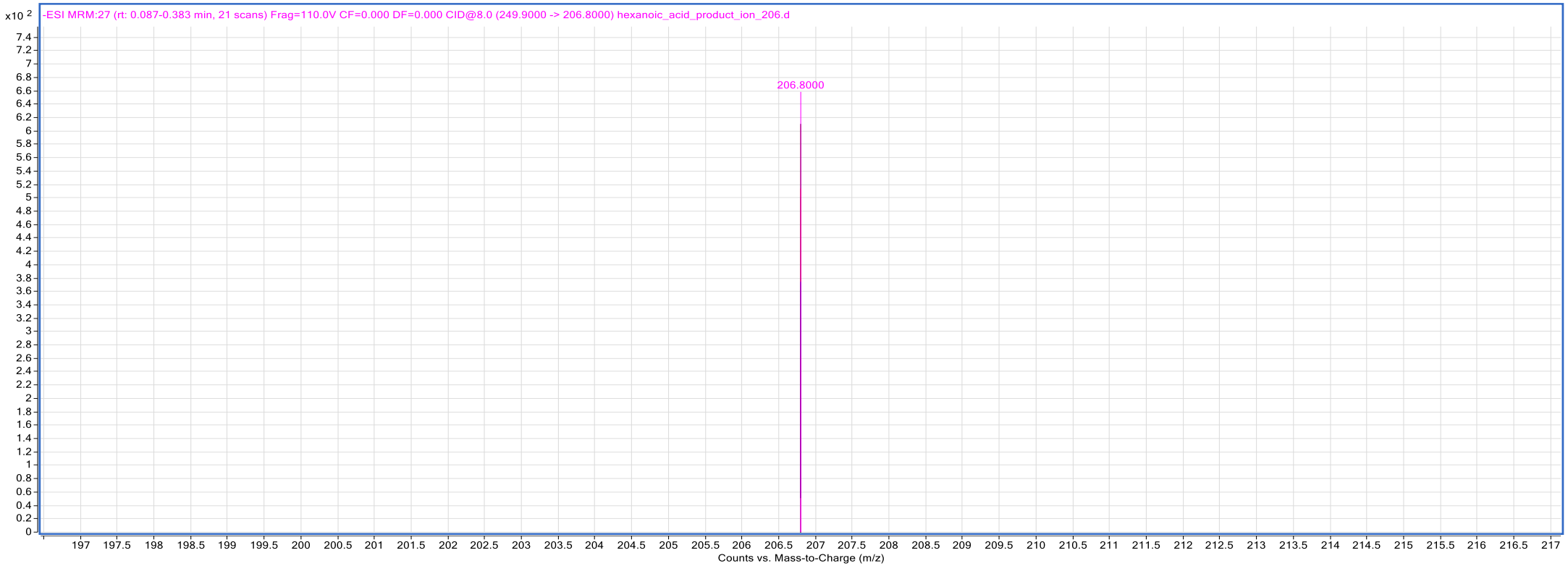
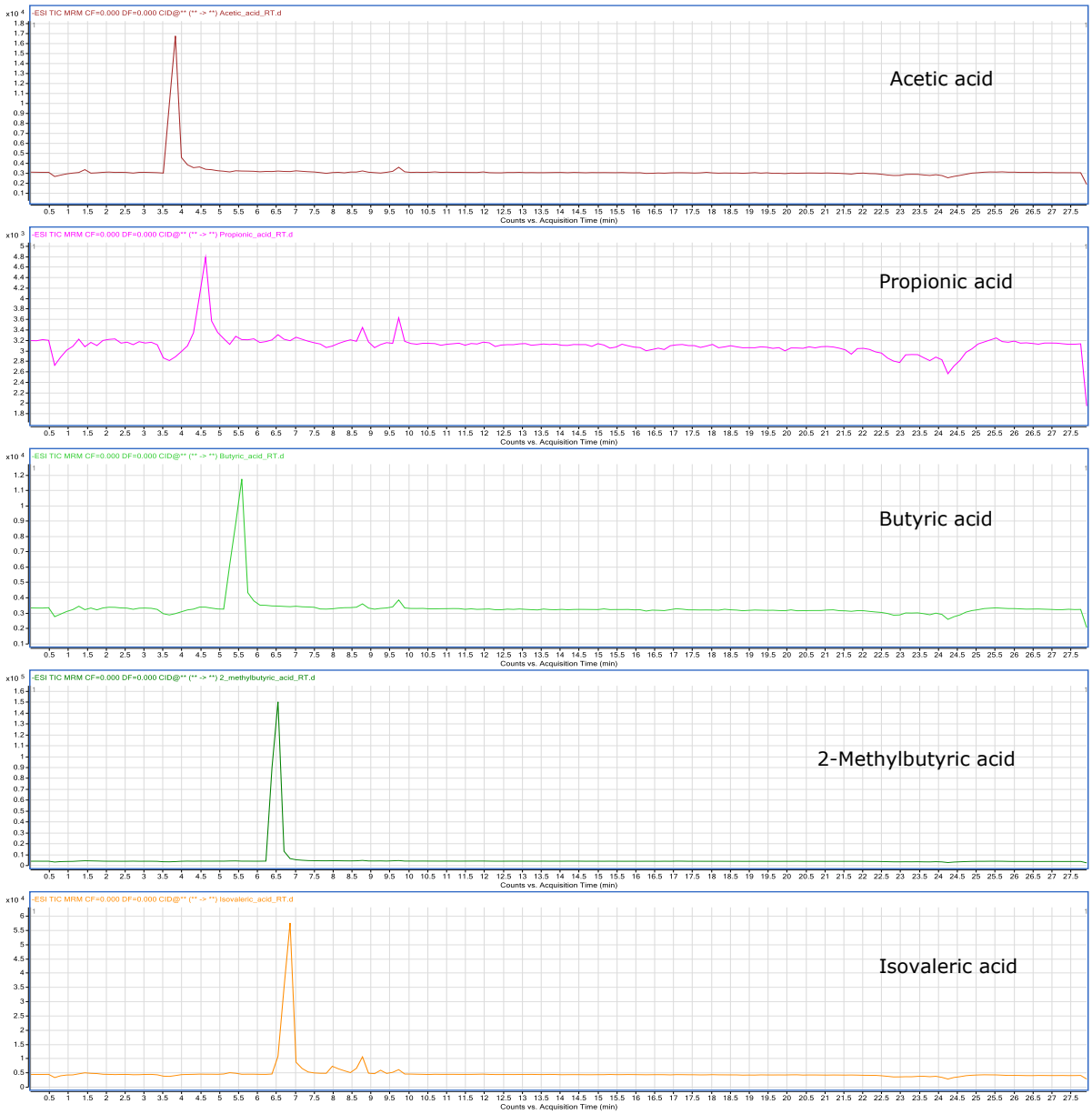


Figure 5. MS spectra for hexanoic acid standard optimisation. A) Precursor ion spectrum. B) Fragmentor value spectrum. C) Product ion spectrum. D) CID spectrum.

4.2. HLPC analysis of SCFA standards and dynamic MRM method development

4.2.1. SCFA retention time analysis

After optimising MRM parameters, each of the SCFA standards was analysed on the HPLC part of the instrument. The generated spectra (Fig.5) are used to determine retention times (R_T) of each analyte, which are necessary in developing a dynamic MRM scan.



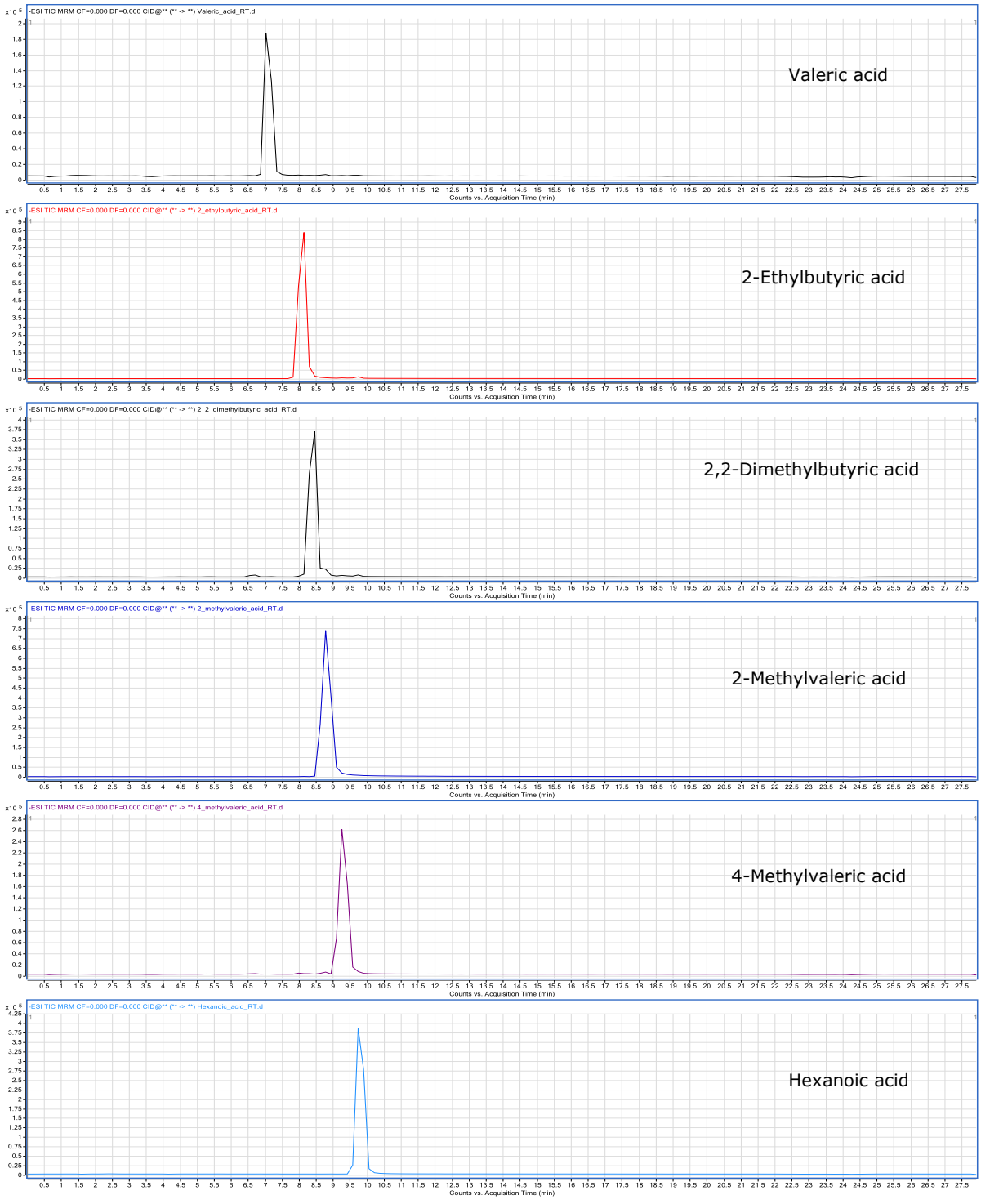


Figure 6. MS spectrum of retention times for each of the SCFA standards. The spectra are shown in the order of fastest eluting/lowest retention time (R_T) (acetic acid: 3,5-4,5 min) to slowest eluting standard/highest retention time (R_T) (hexanoic acid: 9,5-10,5 min).

4.2.2. ISTD retention time analysis

Each of the internal standards (ISTD) are used for quantification of SCFAs that have the same number of carbon atoms as the ISTD in question. The grouping is done in Table 7.

Table 7. Five ISTDs and their corresponding SCFAs in relation to the shared number of carbon atoms.

Number of carbon atoms	Internal standard (ISTD)	Short-chain fatty acid (SCFA)
2C	Acetic acid d_4	Acetic acid
3C	Propionic acid d_3	Propionic acid
4C	Butyric acid d_7	Butyric acid
5C	Pentanoic acid d_9	Valeric acid Isovaleric acid 2-Methylbutyric acid
6C	Hexanoic acid d_{11}	2-Ethylbutyric acid 2-Methylbutyric acid 4-Methylvaleric acid 2,2-Dimethylbutyric acid Hexanoic acid

An MS spectrum with retention times was also made for the five deuterated standards and is shown in Figure 7.

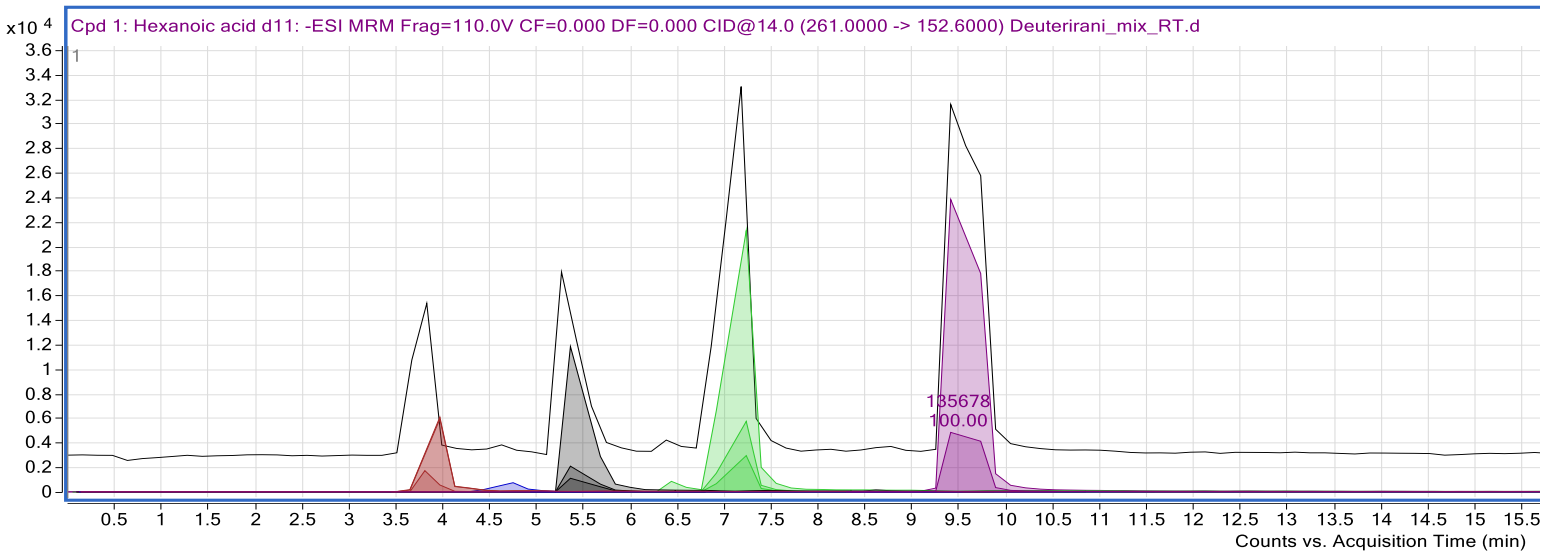


Figure 7. MS spectrum showing acetic acid d_4 (red), propionic acid d_3 (blue), butyric acid d_7 (grey), pentanoic acid d_9 (green), hexanoic acid d_{11} (purple), in order by their retention time (R_T).

4.2.3. Dynamic MRM method development

SCFAs that share the same number of carbon atoms are very similar, if not identical in molecular weight, and so they cannot be separated using MRM alone. Therefore, after eluting them on the HPLC column, and specifying their retention times in Mass Hunter Qualitative analysis programme, the spectrum in Figure 7 is developed. There are clearly separated peaks for each of the SCFA standards and ISTDs visible on the spectrum. Also, the SCFAs are clearly grouped by the shared number of carbon atoms on the MS spectrum. Acetic acid (2C), propionic acid (3C) and butyric (4C) are eluted on their own, while 2-methylbutyric acid, isovaleric acid, valeric acid (4C); 2-ethylbutyric acid, 2,2-dimethylbutyric acid, 2-methylvaleric acid (5C); and 4-methylvaleric acid, hexanoic acid (6C) are all grouped. Furthermore, logP values of each compound, for the majority of compounds on the spectrum, correlate with their elution time (Table 1).

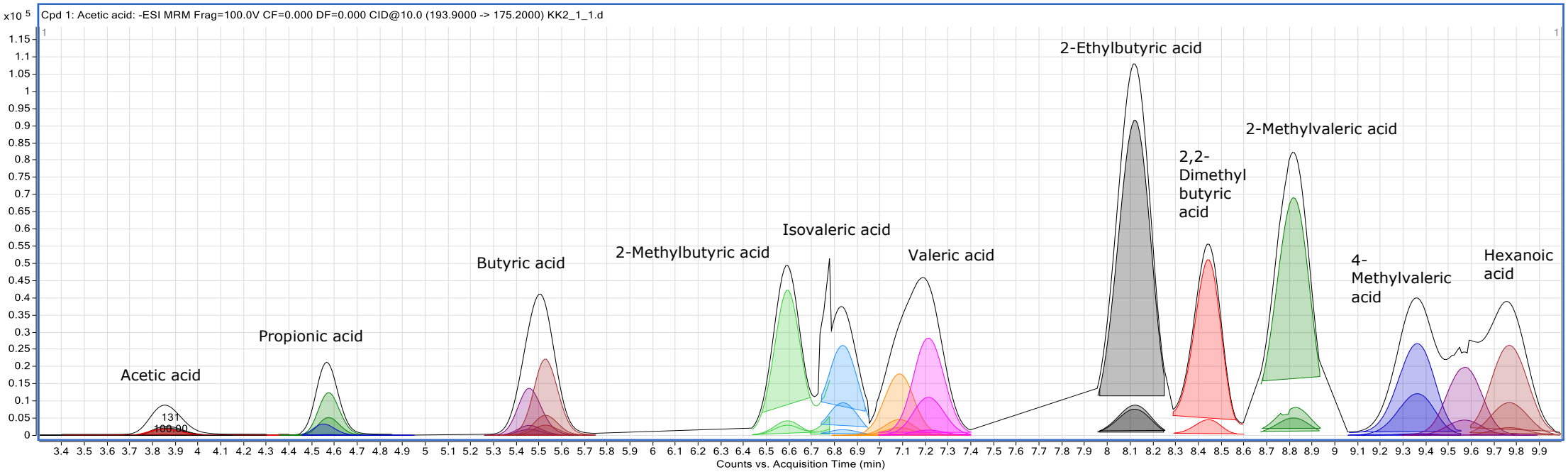


Figure 8. Dynamic MRM-MS spectrum of 12 SCFA standards and 5 ISTDs, in order by their retention time (R_T): acetic acid, propionic acid, butyric acid, 2-methylbutyric acid, isovaleric acid, valeric acid, 2-ethylbutyric acid, 2,2-dimethylbutyric acid, 2-methylvaleric acid, 4-methylvaleric acid, hexanoic acid.

4.3. SCFA quantification

SCFAs were quantified based on the calibration curve which was developed by analysing a range of standard dilutions. Some of the calibration curves are shown in Figure 8.

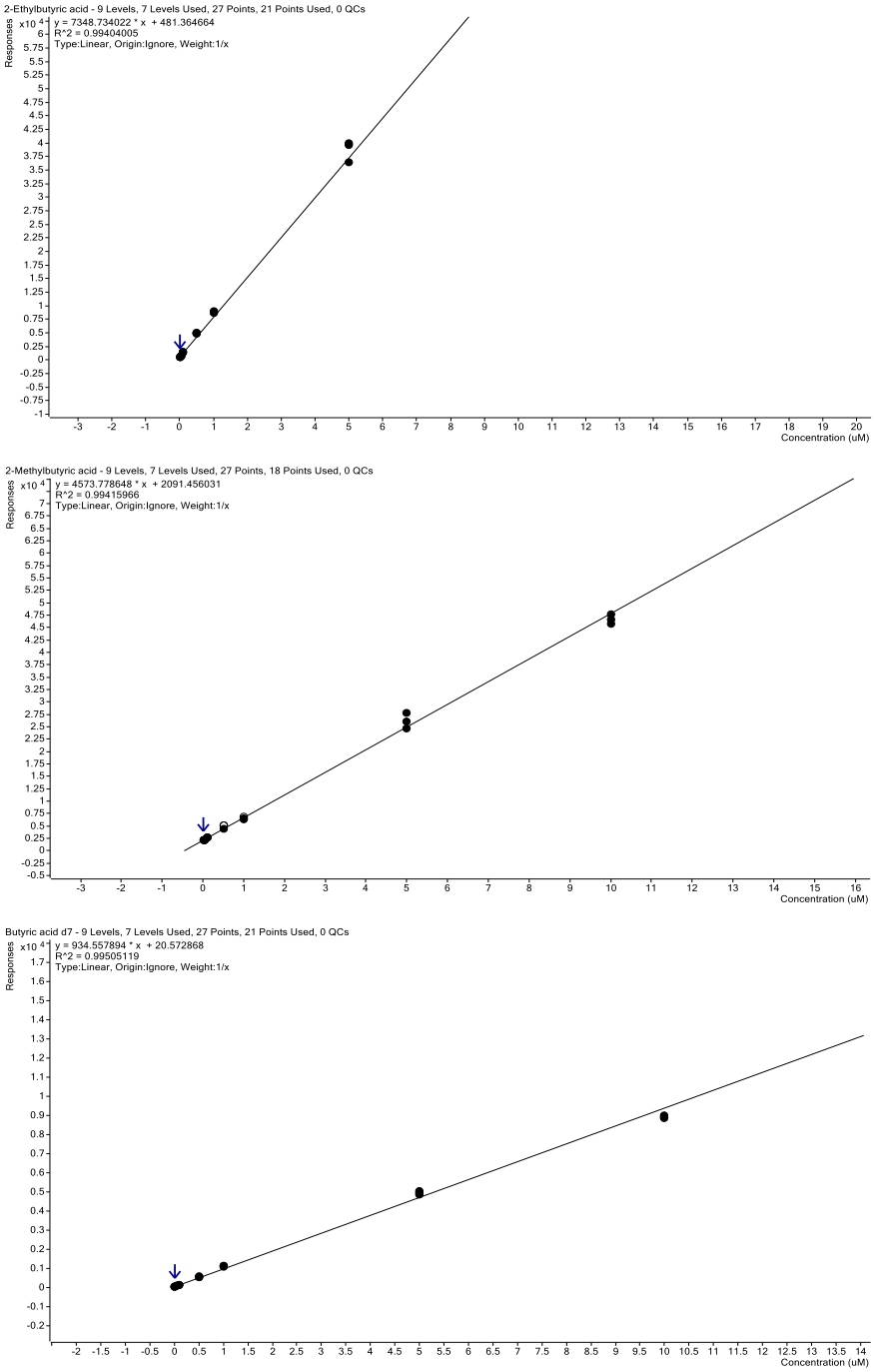


Figure 9. Calibration curves for 2-ethylbutyric acid, 2-methylbutyric acid, and butyric acid d_7.

The concentrations used for the calibration curve ranged: 0.01-30 µg/mL for acetic acid; 0.001-32 µg/mL for acetic acid d_4; 0.001-0.4 µg/mL for propionic and butyric acid; 0.001-0.8 µg/mL for propionic acid d_3; 0.001-0.5 µg/mL for butyric acid d_7; 0.001-1 µg/mL for 2-methylbutyric, valeric, 2,2-dimethylbutyric, 2-ethylbutyric, 2-methylvaleric, 4-methylvaleric acids and hexanoic acid d_{11}; 0.005-1 µg/mL for isovaleric acid; 0.01-1 µg/mL for hexanoic acid; and 0.001-1.1 µg/mL for pentanoic acid d_9. Statistical analysis showed that there is a strong positive correlation coefficient for each of the SCFA standards and ISTDs in the calibration curve as it was higher than R^2=0.99 (Table 5).

SCFA content in rat plasma samples, both SCI and SHAM, was quantified following the calibration curve. Table 8 contains the real measured concentration of ISTDs for each plasma sample, as well as the initial concentration of ISTDs (last column) that was added to each sample for MS analysis. These two values are necessary in calculating the recovery values.

Table 8. Results are shown as concentrations in µg/mL ± SD for each ISTD in 10 examined rat plasma samples (n=5 per group). Last column is the concentration (µg/mL) of ISTDs added to each sample for MS analysis.

	Acetic acid d4	Propionic acid d3	Butyric acid d7	Pentanoic acid d9	Hexanoic acid d11	Concentration of added ISTDs
SCI 1	0,2522 ± 0,2387	0,1155± 0,0200	0,2318± 0,0711	0,6252± 0,0703	0,6333± 0,1542	
SCI 2	0,2534± 0,0827	0,1301± 0,0852	0,2428± 0,0854	0,5198± 0,0276	0,6300± 0,1889	
SCI 3	0,2733± 0,2135	0,1465± 0,0648	0,2657± 0,0594	0,6011± 0,0241	0,6955± 0,1797	
SCI 4	0,2426± 0,3181	0,1277± 0,1045	0,2440± 0,0844	0,5441± 0,0038	0,6167± 0,1513	
SCI 5	0,2610± 0,1125	0,1149± 0,0750	0,2432± 0,0317	0,5740± 0,0253	0,6503± 0,1028	5 µM
SHAM 1	0,2407± 0,1181	0,1169± 0,1688	0,2225± 0,0485	0,5563± 0,0675	0,5751± 0,0431	
SHAM 2	0,2435± 0,1574	0,1244± 0,0746	0,2558± 0,0286	0,5650± 0,1042	0,6443± 0,0586	
SHAM 3	0,2107± 0,1845	0,0962± 0,0797	0,2336± 0,0787	0,5404± 0,0541	0,6236± 0,1488	
SHAM 4	0,2227± 0,0223	0,0932± 0,0541	0,2271± 0,0335	0,5753± 0,0230	0,6282± 0,0462	
SHAM 5	0,2274± 0,1407	0,1068± 0,0377	0,2438± 0,0173	0,5885± 0,0150	0,6309± 0,0436	

To calculate the real concentration of SCFAs in each of the samples (n=5 per group), found in Table 11, the recovery value of the corresponding ISTD was used (Table 9). For further interpretation, the mean recovery of each ISTD per sample group is found in Table 10. Measured SCFA concentrations were expressed in µg/mL.

Table 9. Recovery (%) of each ISTD in SCI and SHAM plasma samples (n=5 per group).

	% recovery				
Sample	**Acetic acid d_4**	**Propionic acid d_3**	**Butyric acid d_7**	**Pentanoic acid d_9**	**Hexanoic acid d_{11}**
SCI 1	79	30	49	112	100
SCI 2	79	34	51	93	99
SCI 3	85	38	56	108	109
SCI 4	76	33	51	98	97
SCI 5	81	30	51	103	102
SHAM 1	75	30	47	100	90
SHAM 2	76	32	54	102	101
SHAM 3	66	25	49	97	98
SHAM 4	70	24	48	103	99
SHAM 5	71	28	51	106	99

Table 10. Mean recovery (%) of each ISTD per sample group as a whole (n=5 per group).

	Σ **recovery (%)**				
Sample group	**Acetic acid d_4**	**Propionic acid d_3**	**Butyric acid d_7**	**Pentanoic acid d_9**	**Hexanoic acid d_{11}**
SCI	80	33	51,6	102,8	101,4
SHAM	71,6	27,8	49,8	101,6	97,4

Table 11. Results are shown as concentrations in μg/mL ± SD in 10 examined rat plasma samples (5 SCI and 5 SHAM).

	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Isovaleric acid	2-Ethylbutyric acid	2-Methylbutyric acid	2-Methylvaleric acid	4-Methylvaleric acid	2,2-Dimethylbutyric acid	Hexanoic acid
SCI1	29,3286 ±10,0291	0,6482 ±1,5212	0,1289 ±0,2799	<LOD	0,0692 ±0,0235	<LOD	0,0444 ±0,0812	0,0056 ±0,0056	<LOD	0,0090 ±0,0190	<LOD
SCI2	23,6213 ±7,6153	0,5858 ±0,5567	0,3319 ±0,1309	<LOD	0,0939 ±0,0320	<LOD	0,0622 ±0,0079	0,0069 ±0,0069	<LOD	0,0067 ±0,0121	<LOD
SCI3	24,9101 ±5,5275	0,6186 ±0,4716	0,3816 ±0,0326	<LOD	0,0716 ±0,0236	<LOD	0,0471 ±0,0476	0,0042 ±0,0042	0,0081 ±0,0070	0,0078 ±0,0094	<LOD
SCI4	26,1776 ±7,9107	0,6195 ±0,7957	0,2198 ±0,0795	<LOD	0,0809 ±0,0290	<LOD	0,0469 ±0,1074	<LOD	<LOD	0,0090 ±0,0067	<LOD
SCI5	34,5693 ±7,5463	0,6781 ±0,7573	0,4183 ±0,1249	<LOD	0,0679 ±0,0137	<LOD	0,0360 ±0,0419	<LOD	0,0067 ±0,0036	0,0064 ±0,0049	<LOD
SHAM1	27,6961 ±7,7251	0,6338 ±0,6365	0,3027 ±0,0857	<LOD	0,0967 ±0,0310	<LOD	0,0782 ±0,0740	<LOD	0,0085 ±0,0083	0,0085 ±0,0083	<LOD
SHAM2	34,5158 ±6,5552	0,6270 ±0,2832	0,2124 ±0,0259	<LOD	0,0862 ±0,0115	<LOD	0,0677 ±0,0102	<LOD	0,0065 ±0,0027	0,0064 ±0,0024	<LOD
SHAM3	33,9591 ±6,9194	0,7383 ±0,7418	0,1358 ±0,1139	<LOD	0,1055 ±0,0554	<LOD	0,0640 ±0,1186	<LOD	0,0042 ±0,0040	0,0040 ±0,0029	<LOD
SHAM4	32,5638 ±7,2883	0,8107 ±0,4862	0,2014 ±0,1213	<LOD	0,0871 ±0,0307	<LOD	0,0595 ±0,0688	<LOD	0,0039 ±0,0038	0,0039 ±0,0035	<LOD
SHAM5	21,8272 ±7,2070	0,5494 ±2,2807	0,1863 ±0,0824	<LOD	0,0908 ±0,0248	<LOD	0,0384 ±0,0271	<LOD	<LOD	0,0053 ±0,0057	<LOD

4.4. Statistical analysis

4.4.1. Principal component analysis

Principal component analysis (PCA) was used to analyse data from all plasma samples (n=5 per group). Figure 10 represents the distribution of plasma samples in the space of principal component 1 (PC-1) and principal component 2 (PC-2). PC-1 and PC-2 represent 35,9% and 22,4% of total variance, respectively (Figure 11a). Variable contribution plot (Figure 11b) showed that acetic, propionic, 2,2-dimethylbutyric and 2-methylvaleric acids contribute the most to the diversity of dimension 1, while butyric, isovaleric and 4-methylvaleric acids contribute the most to the diversity of dimension 2.

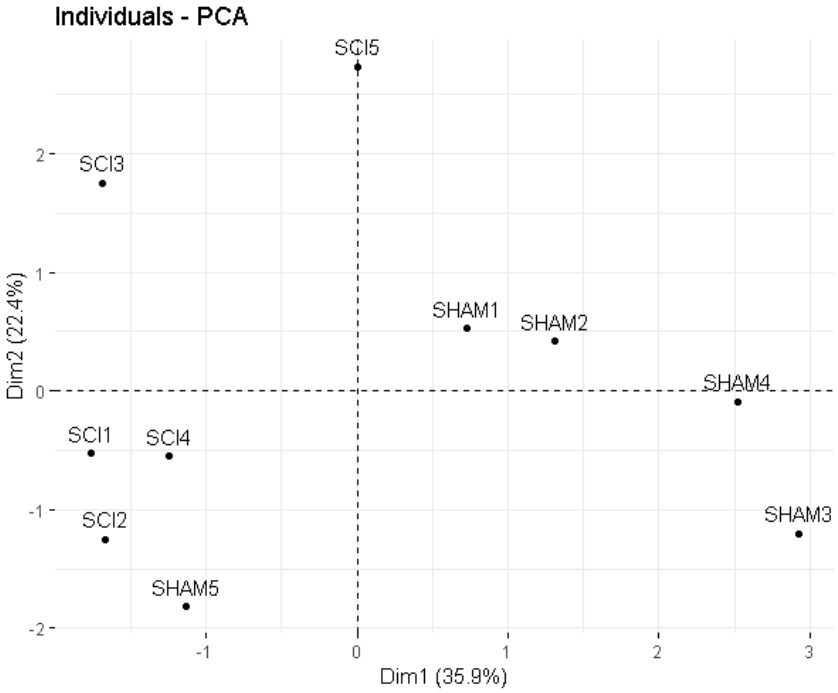


Figure 10: PCA plot analysis of SCI and SHAM samples (n=5 per group), showing PCA-1 and PCA-2 containing 35,9% and 22,4% of variance, respectively.

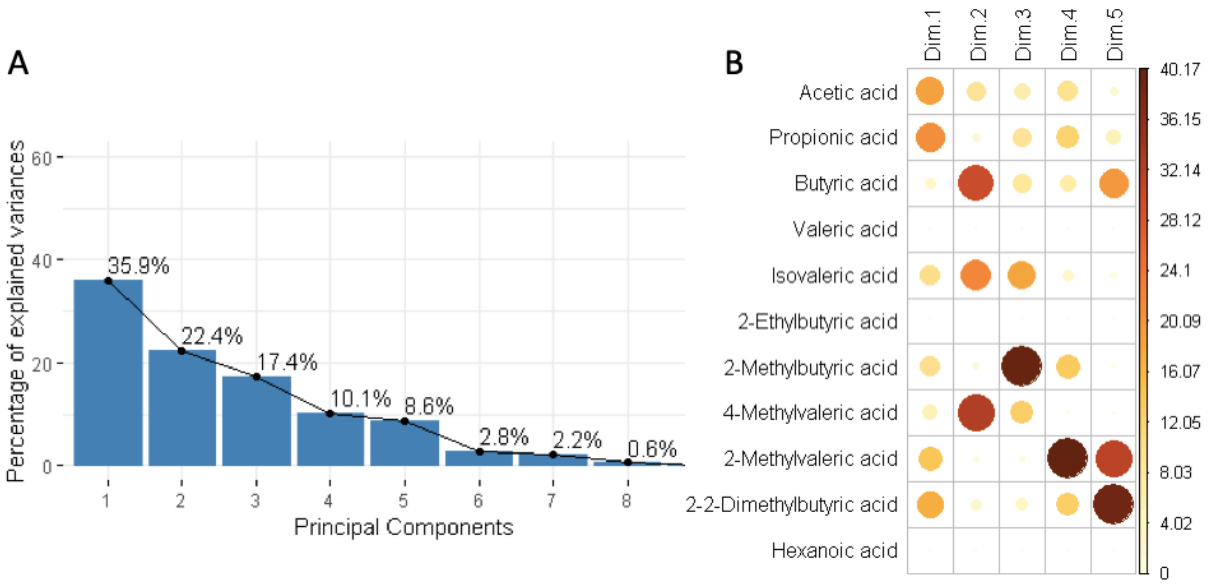


Figure 11. (A) Fraction of total variance described by PC-a through PC-8. (B) Factor map of individual SCFAs contributing to PC-1 through PC-5. SCFAs with bigger and darker dots contribute more to the corresponding principal component.

4.4.2. T-test

A two-tailed unpaired t-test was performed to see whether there is a statistically significant difference in the measured concentration of SCFAs in SCI and SHAM rats. The cut-off or p value was set at 0.1. As seen in Figure 12, t-tests for isovaleric acid (p-value=0.0254), 2-methylvaleric acid (p-value=0.0477), and 2,2-dimethylbutyric acid (p-value=0.0675) have displayed p-values lower than the cut-off value, while the other SCFAs have not. Furthermore, the data for valeric acid, 2-ethylbutyric acid, and hexanoic acid were not tested as all the measured concentrations were below the limit of detection. For the three SCFAs with a p-value below 0.1, it can be said that there are statistically significant differences between SCI and SHAM samples, however, since the cut-off value is 0.1, the evidence for the trend is quite underpowered.

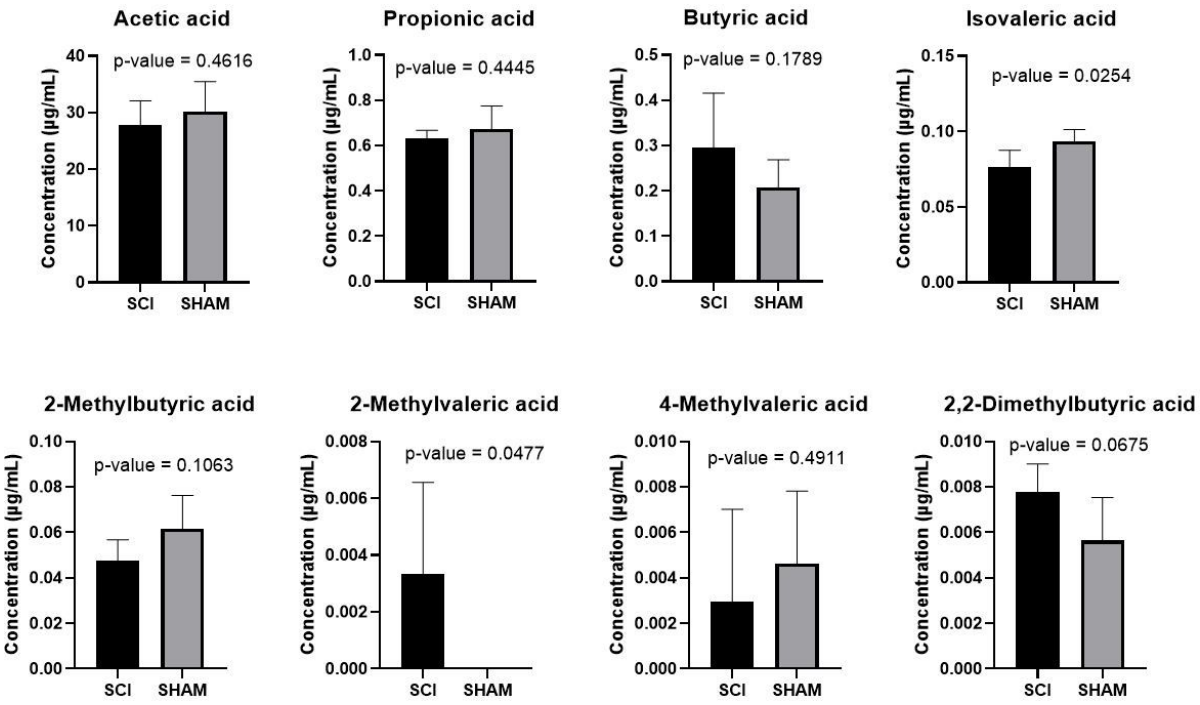


Figure 12. T-test graphs for SCFA concentrations measured in SCI and SHAM samples, where the cut-off value is p=0.1.

5. DISCUSSION

Gut dysbiosis, and changes in the gut's metabolites, are connected to secondary injury and clinical symptoms of SCI. Rodent studies have shown that gut microbiome dysbiosis occurs following SCI, which worsens nerve injury and spinal cord pathology. Kong et al. have conducted an OMICS analysis of the structure of the gut microbiome in SCI patients. They have shown that the gut microbiome of SCI patients differs from the microbiome of healthy individuals as a result of the loss of autonomic nervous system innervation, suggesting that SCI induces gut dysbiosis (31). Acetic, propionic and butyric acids make up 95% of total SCFA content. They are formed at a relatively constant ratio of 60:20:20, respectively (9). In this experiment, the concentration of acetic acid measured for SCI samples ranged between 23.6-34.6 μg/mL, and 21.8-34.5 μg/mL for SHAM samples. The concentration of propionic acid ranged from 0.59-0.68 μg/mL for SCI group, and 0.55-0.81 μg/mL for SHAM group. In SCI samples, the concentration of butyric acid ranged from 0.13-0.42 μg/mL, while it was between 0.14-0.3 μg/mL for SHAM samples.

Kang et al. have shown that microbial richness and diversity are decreased after SCI, and that disturbances of the gut microbiome might contribute to chronic inflammation (32). The quantity of Bacteroidetes, Firmicutes and Actinobacteria, the main phyla producing acetic, propionic and butyric acid, has shown to be reduced in a number of studies done on patients with SCI (33). This would in turn correlate with a reduced production of SCFAs and onset of intestinal diseases as a result of increased gut permeability (31). However, this data varies from study to study due to the differences in the severity of examined SCI and gut microbiome variations among individuals, so it cannot be said with confidence that this is always the case. The main and most consistent reductions occur in the number of butyric acid-producing bacteria (31). SCFAs, particularly butyric acid, have been shown to lead to decreased inflammation by promoting regulatory T cell (T_{reg})

proliferation which then secrete interleukin 10 (IL-10) (34). Acetic acid supplementation can induce the metabolism of acetyl-CoA, which then causes an increase in histone acetylation. This results in well-kept spinal cord lipid content, and attenuates nitric oxide (NO), as well as reactive oxygen species (ROS) production in microglia to alleviate neuroinflammation (9,34). Treatment with butyric acid causes a suppression of demyelination. Furthermore, by inducing oligodendrocyte maturation and differentiation, butyric acid enhances remyelination (9). According to Liu et al., from 7 to 21 days post injury, rats with spinal cord injury who were orally administered SCFAs experienced an enhancement of the hind limb motor and locomotor function (34). SCI recovery is, therefore, ameliorated through the anti-inflammatory influences of SCFAs, and threatened by the lack thereof.

The quantification of SCFAs in serum samples is challenging due to their low concentrations – acetic acid ranges between 50 and 100 μmol/L, while the concentration of propionic and butyric acid ranged between 0,5-10 μmol/L (35). The overall procedure of analysing SCFAs on a mass spectrometer requires the compounds to be derivatised due to their poor ionisation efficiency and susceptibility to water loss during the ionisation of low molecular weight organic acids (24,35). If the procedure were to not include derivatisation, the LC-MS/MS conditions would have had to been highly acidic using 1,5 mM hydrochloric acid (HCl), and even then, the chromatographic separation of SCFAs would be poor because of their hydrophilicity (22). Furthermore, without derivatisation, there would have been numerous interfering peaks from solvents and additives present in the analysed samples. Additional clean-up methods can be developed to eliminate unwanted by-products of analysis and reduce chances of carry-over (23).

As previously mentioned, derivatisation method 1, where AABD-SH was used as the derivatisation agent, was unsuccessful in producing ions needed for the optimisation of MRM parameters. This could be due to the fact that

method 1 includes a drying process in the Speed VAC which likely causes the evaporation of the majority of the samples, as SCFAs are highly volatile. Therefore, the results obtained in the work done by Eun Song et al. could not be reproduced.

Method 2 was chosen as the most suitable SCFA derivatisation protocol because it produced results similar to the ones described in the work done by Dei Cas et al. Using a reactive nucleophile like 3-NPH in the presence of a coupling agent such as EDC, and a base catalyst like pyridine gives more favourable chromatographic and mass spectral characteristics. Most importantly, this method does not include the evaporisation step, which is suspected to lead to loss of a fraction of the samples. Other factors that require considerations are the reaction time (40 mins), and temperature (37°C), which were both proven to be adequate in terms of producing results suitable for the optimisation of MRM parameters.

In terms of MRM parameters, an important observation is the repetition of a few specific *m/z* fragments values for each of the analysed SCFAs, as seen in Table 6. 136.7 ± 0.1, and 151.7 make up two out of three recorded *m/z* fragments for almost all SCFA standards. This is expected because, as explained in Dei Cas et al., these are the *m/z* values of acylhydrazines synthesised as the result of SCFA derivatisation with 3-NPH. In this case, EDC acts as the carboxyl activating agent to couple with primary amines, which yields acylhydrazine. During this, the reaction is catalysed by pyridine.

Due to being very similar in physical properties, SCFAs that share the same number of carbon atoms act very similarly, if not identically on the HPLC column. This results in poor separation and inability to distinguish them one from another. To combat this, and to improve analysis efficiency, dynamic multiple-reaction monitoring (dMRM) is used instead of the traditional multiple-reaction monitoring (MRM) (36). dMRM allows for the allotment of the retention time window, which is listed on the chromatogram of each compound, and is used to adjust the loading cycle time of multiple

compounds (36). When the MS system is given precise instructions at which points in time to look at each compound, it gives a more accurate and disperse chromatographic depiction, which is visible in Figure 6. Although their retention times overlap, SCFAs are clearly separated on the dMRM spectrum.

Internal standards were used to identify the matrix effect commonly occurring in MS analyses of complex compounds. When recovery is near 100, it can be assumed that there is minimal or no matrix effect (20). Values under 100 suggest there is ionisation suppression taking place, while values over 100 indicates ionisation enhancement (20). As seen in Table. the mean recovery values for acetic acid d_4 (80% for SCI and 71,6% for SHAM), propionic acid d_3 (33% for SCI and 27,8% for SHAM), and butyric acid d_7 (51,6 for SCI and 49,8% for SHAM) are very low. This would mean the occurring ionisation suppression is strong and the matrix effect substantially affects the LC-MS/MS data. The mean recovery values for pentanoic acid d_9 (102,8% for SCI and 101,6% for SHAM) show slight ionisation enhancement, but are still close to 100. The values for hexanoic acid d_{11} show slight enhancement in the case for SCI samples (101,4%) and slight suppression for SHAM samples (97,4%). The matrix effect may be reduced with a more thorough extraction protocol in which the samples are more heavily diluted, however, the measured concentrations of SCFAs are already low as it is, so diluting them further may give values that are below limit of detection (LOD). The matrix effect is great due to the fact that all the compounds were directly injected at the same time, which allows them to interfere with one another's ionisations. Another option in reducing the matrix effect would be to add a clean-up method after the samples are eluted from the column, however this gives way to a portion of the samples being lost. Even with these modifications, there is no guarantee the result would be closer to 100. SCFAs, as already mentioned, ionise very poorly and the matrix effect is an expected phenomenon.

Principal component analysis (PCA) shows some grouping occurring for SCI samples and SHAM samples, separately, with SCI 5 and SHAM 5 being the only exceptions (Fig 10). The grouping by differences occurs more significantly in dimension 1 than in dimension 2. Statistical analysis has shown that some SCFA concentrations vary between the SCI and SHAM groups, however, the majority of the concentrations do not show any statistically significant differences. T-tests for isovaleric acid (p-value=0.0254), 2-methylvaleric acid (p-value=0.0477), and 2,2-dimethylbutyric acid (p-value=0.0675) have displayed p-values lower than the cut-off value (0.1), while the other SCFAs have not. It can, therefore, be said that there are statistically significant differences in concentrations of isovaleric, 2-methylvaleric and 2,2-dimethylbutyric acids between SCI and SHAM groups. Furthermore, isovaleric acid is found in significantly lower concentrations in SCI rats, while the concentration of 2-methylvaleric and 2,2-dimethylbutyric acids is significantly higher in SHAM rats. The current research on the meaning of heightened or lowered individual SCFA levels is contradictory. Although the functions of acetic, propionic and butyric acids are vast and becoming increasingly known, the functions of other, less constituted SCFAs like 2-methylvaleric and 2,2-dimethylbutyric acid are not well-reported. Furthermore, the exact role, protective or etiological of each of the compounds, separately, cannot be differentiated (37).

6. CONCLUSION

The experimental work in this thesis was able to successfully modify and apply the derivatisation method using 3-NPH, as described in the work done by Dei Cas et al., to perform LC-MS quantification of SCFAs. Furthermore, the preferred technique of MS-MRM analysis is the dynamic MRM (dMRM) because of its ability to distinguish between isoforms with the same molecular weight, precursor ions, and fragments formed after the compounds are split. Statistical analysis has shown that there are some differences in SCFA concentration between sham animals and those with spinal cord injury, however the sample size is too small to draw any specific and firm conclusions. The experiment would, therefore, have to be repeated on a larger number of samples. Although the functions of acetic, butyric and propionic acids are known, as they make up the vast majority of total SCFA gut content, the functions of other SCFAs cannot be looked at and differentiated separately. Another complication is the fact that some were found in higher concentrations in rats with SCI, and others in SHAM rats. Furthermore, the research on altered SCFA quantity is still contradictory, with some studies reporting detrimental effects of increased SCFA content and others of the opposite, which further confirms that the experiment has to be repeated on a larger sample.

7. WORK CITED

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8. CURICULUM VITAE

Karla Soldatić

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Gender: Female **Date of birth:** 10/04/1999 **Nationality:** Croatian

ABOUT ME

A hard working and dedicated individual who puts maximum effort in all the tasks I take on. At 24, I have lived in 3 countries and am adaptable, internationally minded and easy to communicate with. I like to challenge myself with new experiences which allow me to acquire new skills.

WORK EXPERIENCE

[31/05/2019 – 30/09/2019]

Tourist agency clerk

Lineaverde

City: Opatija

Country: Croatia

Helped organise daily trips planned for groups of over 40 people over the span of 4 months. Managed the company's website, and made flyers for promotion. Coordinated information with 4 other employees, as well as people outside of the company, and made sure deadlines were being timely met.

[03/06/2021 – 17/06/2021]

Intern

Nastavni Zavod za Javno Zdravstvo

City: Rijeka

Country: Croatia

Worked with various analytical tools including mass spectrometry and gas and liquid chromatography to determine the quality of water, food and air samples. Got familiar with and worked on softwares used to process the data. Spent time at the Department of Microbiology where human samples were examined.

EDUCATION AND TRAINING

[29/09/2021 – Current]

Master's Programme in Biotechnology in Medicine

University of Rijeka, Department of Biotechnology

Address: Ulica Radmile Matejčić, 51000, Rijeka, Croatia

[08/2019 – 29/07/2021]

Bachelor's degree in Biotechnology and Drug Research

University of Rijeka, Department of Biotechnology

Address: Ulica Radmile Matječić, 51000, Rijeka, Croatia

LANGUAGE SKILLS

Mother tongue(s): Croatian , English

Other language(s): French - A2

HONOURS AND AWARDS

[2023] **Academic Excellency Scholarship Awarding institution:** County of Matulji, Croatia

Received the academic excellency award for 3 consecutive years from the County of Matulji, Croatia. Only students with an average grade A receive this award.

[2018] **Science and Engineering Excellence Scholarship Awarding institution:** Queen Mary University of London

Received the academic excellence scholarship for the academic year 2018/19 as one of the top 50 students enrolling to Queen Mary University of London

[2018] **Valedictorian award Awarding institution:** Uptown School Dubai

Received the Valedictorian award for academic excellency as the highest achieving student of the generation.

PROJECTS

[05/10/2022 – Current] **Master's Thesis**

Currently performing the experimental work for a master's thesis investigating the circulating concentration of short-chain fatty acids using an LC-MS/MS system. The purpose of the research is to optimise the extraction and quantification method, and to determine whether there are disparities in quantity of short-chain fatty acids between healthy rodents, and those with a spinal cord injury. This determination would be useful in future research as short-chain fatty acids, and the microbiome that synthesises them, are hypothesised to be disrupted in cases of CNS inflammation.

[2021 – 2021] **Bachelor's Thesis**

During the writing of a bachelor's thesis titled "Platelets", the skill set that was obtained included performing extensive research on the chosen topic, to then filter the relevant information. By discussing the findings in a coherent way, the multifaceted nature of cells like platelets was proven. This piece of work received the highest grade.

Link: https://repository.biotech.uniri.hr/islandora/object/biotechri%3A607/datastream/PDF/view