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Department of Chemistry

Development of a novel method for the analysis of acyl carnitine profile using liquid chromatography mass spectrometry

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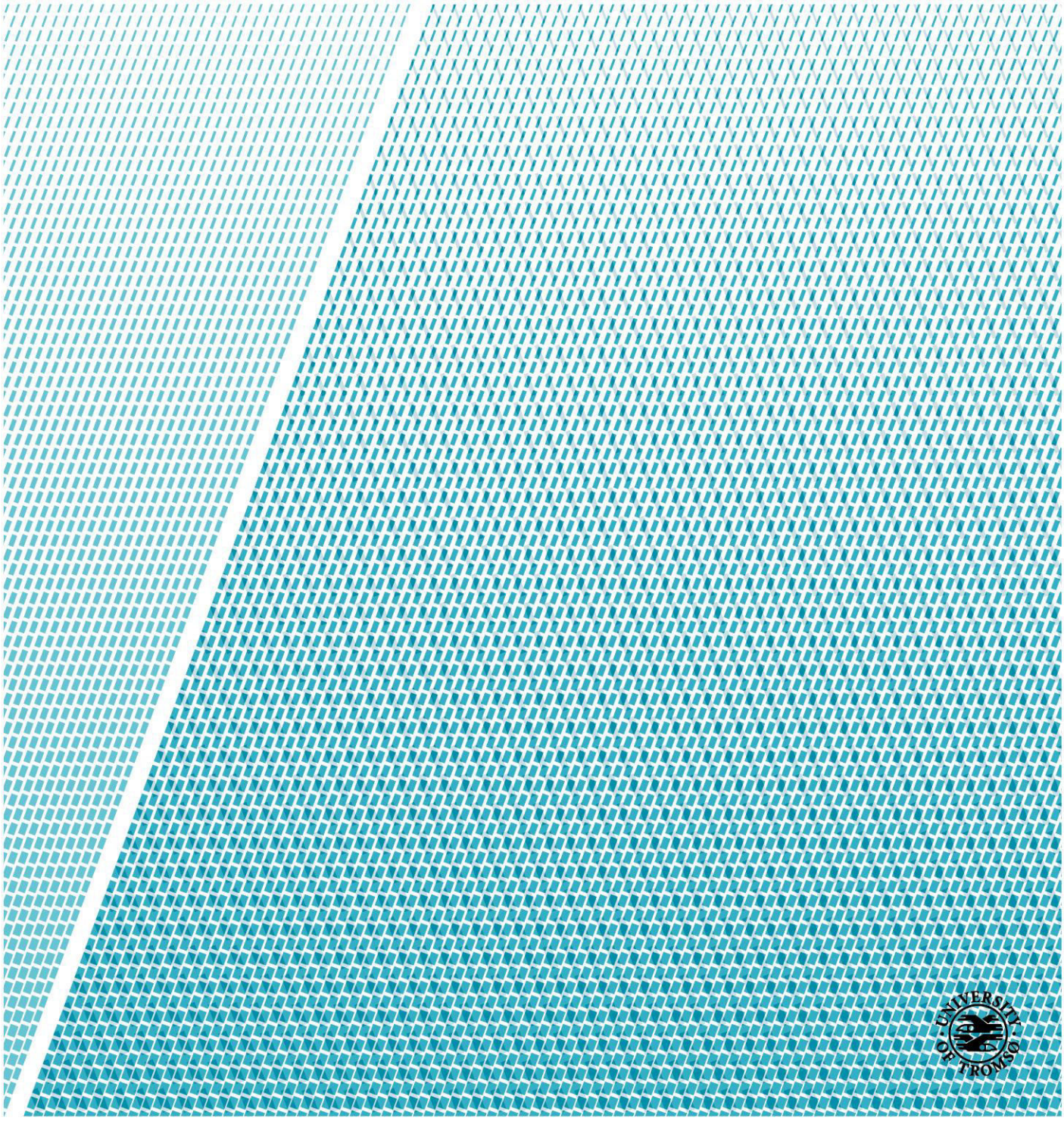
KJE-3900, Master Thesis in Chemistry – May 2019



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Tromsø, May 2019
Hana Spickova

Abbreviations

ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
CE	collision energy
CV	cone voltage
ESI	electrospray ionization
FA	formic acid
FAO	fatty acid oxidation
HSA	hexanesulfonic acid
HFBA	heptafluorobutyric acid
HPLC	high-performance liquid chromatography
IBD	inflammatory bowel disease
IPR	ion-pairing reagent
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of detection
LLOQ	lower limit of quantification
MeOH	methanol
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MRM	multiple reaction monitoring
SIM	selected ion monitoring
S/N	signal to noise ratio
ULOQ	upper limit of quantification
UHPLC	ultra-high-performance liquid chromatography

List of acylcarnitine esters

Name	Acyl-chain	[M+H]+	CAS number
L-carnitine	C0	162	541-15-1
Acetylcarnitine	C2	204	204259-54-1
Propionylcarnitine	C3	218	20064-19-1
Butyrylcarnitine	C4	232	25576-40-3
Isobutyrylcarnitine	C4	232	25518-49-4
2-methyl-butyrylcarnitine	C5	246	256928-75-3
Isovalerylcarnitine	C5	246	31023-24-2
Glutarylcarnitine	C5DC	276	102636-82-8
Hexanoylcarnitine	C6	260	22671-29-0
Octanoylcarnitine	C8	288	25243-95-2
Decanoylcarnitine	C10	316	3992-45-8
Lauroylcarnitine	C12	344	25518-54-1
Myristoylcarnitine	C14	372	25597-07-3
Palmitoylcarnitine	C16	400	2364-67-2
Stearoylcarnitine	C18	428	25597-09-5
Oleoylcarnitine	C18:1	426	13962-05-5

Abstract

Carnitine is a low-molecular-weight compound that has an obligate role in the mitochondrial oxidation of long-chain fatty acids (acyl groups) and energy production. Therefore, acylcarnitines are essential diagnostic markers for complex diseases, fatty acid metabolism disorders, and disorders related to amino acids metabolism. Biochemical screening of the acylcarnitine profile is performed in clinical laboratories using tandem mass spectrometry as the analytical platform.

Analysis of acylcarnitine species can become challenging because several species occur in an isomeric form. A selective and sensitive UHPLC-MS/MS method has been established to analyze an acylcarnitine profile of acylcarnitines with acyl-chain lengths C4-C18. The method includes four isomeric species, two C4 isomers (butyrylcarnitine and isobutyrylcarnitine) and two C5 isomers (2-methylbutyrylcarnitine and isovalerylcarnitine). The analysis was conducted on a Waters Acquity ultra performance liquid chromatograph (UPLC) coupled with a triple quadrupole mass spectrometer operated in electrospray positive mode. Optimization of separation involved testing various parameters such as mobile phase composition, additives, and effect of ion-pairing reagents. The separation efficiency of the C18 column was compared to amide column to investigate the optimal settings for separation of short-chain polar acylcarnitines. The chromatographic separation was achieved using ACQUITY UPLC HSS C18 (1.8 µm, 2.1 x 100 mm) column. Gradient elution was accomplished with a mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. High specificity was obtained with MRM operation mode of MS instrumentation. The process of development MRM method involved a series of testing various parameters such as cone voltage, collision energy and flow rate of the collision gas to achieve the optimal set of conditions. Fragmentation of all acylcarnitines provides a characteristic fragment ion of m/z 85. The limit of detection of the acylcarnitine esters including the isomeric species was 10 pg/ml when measured with the optimized MRM method.

Contents

1 Introduction

1.1 The biological role of carnitine

Carnitine (β-hydroxy-γ-N-trimethylammonium butyrate) is a low-molecular-weight hydrophilic quarternary amine, involved in the lipid metabolism and energy production [1, 2]. The molecule of carnitine is employed as a carrier of activated fatty acids across the inner mitochondrial membrane, where the fatty acids undergo β-oxidation [2]. Basically, it delivers substrate for oxidation which leads to energy production. Carnitine participates in a reversible esterification reaction in which acylcarnitines are generated. Various fatty acyl groups are transferred from coenzyme A to the beta hydroxyl group of L-carnitine which forms acylcarnitines with acyl-chains of different lengths [3].

Figur 1 Molecule of L-carnitine (β-hydroxy-γ-N-trimethylammonium butyrate) 2D (left) and 3D (right). Esterification of L-carnitine on the beta hydroxyl group generates various acyl carnitine esters [3].

Biologically active stereoisomer L-carnitine is present nearly in all the body cells. In a human organism, 75% of carnitine content comes from the food; therefore, its bioavailability varies and depends on the composition of the diet. Animal products, especially red meat, is the most significant food source of carnitine. The bioavailability of carnitine is higher for vegetarians and vegans than for meat eaters since they are adapted to low-carnitine diet [4]. The carnitine content that is not obtained through the diet is synthesized in the liver and kidneys from lysine and methionine, which are two essential amino acids [5]. Free-L-carnitine is transported with bloodstream and extracellular fluids to various tissues. Intracellular transport depends on its uptake capacity. Absorption of carnitines from the diet takes place in the intestine by active

and passive transport across membranes of enterocytes [4]. Carnitine binds activated acyl chains inside the body cells during a metabolic activity where various acylcarnitines are generated. A function of long-chain acyl carnitine esters is a transport of fatty acyl moieties via the mitochondrial membrane into the matrix (see figure 2) where they undergo fatty acid oxidation (FAO). Short-chain and medium-chain acylcarnitines are involved in the removal of organic acids [6]. FAO is a necessary process that generates energy in tissues where the carnitine molecule plays a vital role as a carrier [7].

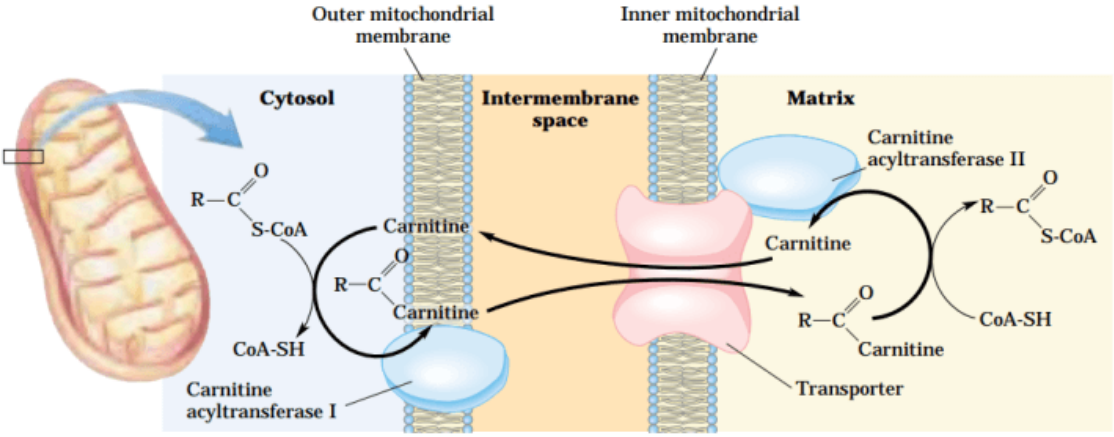


Figure 2 Transfer of free fatty acids from cytosol to mitochondrial matrix [8]

FAO disorders and organic acid metabolism disorders are investigated by screening acyl carnitine profile alterations [2, 7]. The metabolic evaluation includes the analysis of acylcarnitines in various matrices, including plasma, blood, urine or tissues. Obvious deficiencies arise either from a genetic mutation of carnitine transporters, inborn errors of metabolism, or they are associated with other disorders [9]. A particular concentration of carnitine in plasma and tissues is essential for the normal function of metabolism [10]. Extremely low levels of carnitine can result in cardiomyopathy, congestive heart failure, encephalopathy, hepatomegaly, and neuromuscular disorders [3]. Carnitine deficiency has been associated with cirrhosis and liver disorders, obesity, endocrine disorders, diabetes and, inflammatory bowel diseases (IBD) [4, 7].

IBD is a chronical intestinal disorder that includes two principle types: Crohn's disease and ulcerative colitis. An inappropriate inflammatory response to intestinal microbiota is caused

by an interaction of genetic and non-genetic factors. However, the pathogenesis of IBD remains unknown [7, 11]. IBDs such as Crohn's disease and ulcerative colitis are characterized by malabsorption of some nutrients. One of them is carnitine, which is a crucial molecule involved in the energy metabolism of the epithelial cells [12]. L-carnitine has an impact on several factors involved in the outcome of IBD including oxidative stress, the level of activation of the immune cells, and the integrity of the epithelial barrier [13]. Thus, acyl carnitine profile in patients with IBD is investigated, since deficiency of some isoforms of carnitine may play a role in the etiology and pathogenesis of the disease [7]. The development of a selective and sensitive bioanalytical method for analysis of acyl carnitine profile includes several challenges that are progressively eliminated with the modernization of analytical instruments.

1.2 Bioanalysis

Development in analytical technology plays an influential role in the development of new methods. The technology available for bioanalytical purposes significantly improved over the last decades. Progress was achieved with HPLC-UV based techniques and fluorescent detection for LC in the late 1980s [14, 15]. However, the disadvantages of the mentioned technique include a lack of selectivity and a lack of structural information [15]. A revolution in bioanalysis occurred with the possibility to connect the separation ability of UHPLC with high sensitivity detection of MS instrumentation. The possibility to couple a chromatography separation module coupled with the mass spectrometer was achieved using atmospheric pressure ionization (API) [16]. A great selectivity, qualitative and quantitative information and high sensitivity were achieved after an invention of this hyphenated technique which combines liquid chromatography with tandem mass spectrometry (MS/MS) [14]. The development of ultra-high-performance liquid chromatography (UHPLC) allows better separation of complex mixtures in shorter time. Thus – the combination UHPLC-MS/MS provides fast, sensitive and selective analysis of a wide range of organic compounds.

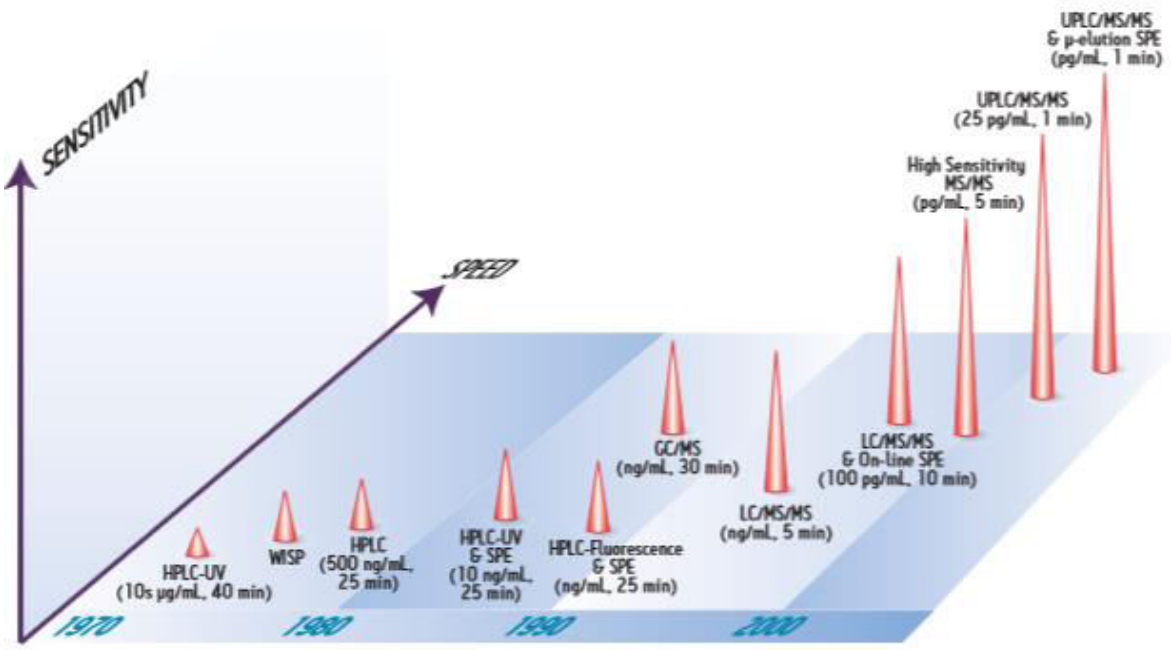


Figure 3 The evolution of analytical technology [14]

1.3 UHPLC-MS/MS

UHPLC-MS/MS is a hyphenated technique that combines a separation ability of UHPLC with a highly sensitive MS/MS detection (figure 4) [17]. This technique is commonly used for bioanalysis due to its selectivity and sensitivity [18], and it was applied for the analysis of the carnitine profile in this study.

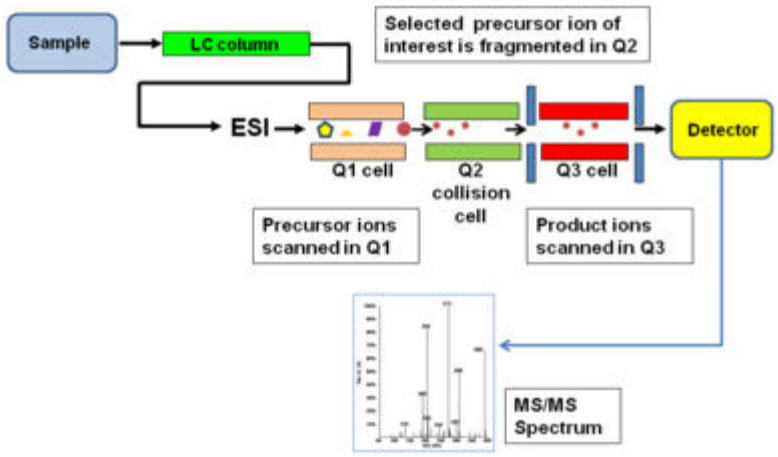


Figure 4 Schema of LC-MS/MS technique [19]. A complex sample is introduced into the LC column where the mixture is separated. Separated sample is consequently ionized in an ion source at the atmospheric pressure (here ESI). Ions are selected by mass filters (here QqQ) and sent to the detector of MS instrumentation.

1.3.1 UHPLC principles

Ultra-high-performance liquid chromatography is a separation technique that pumps a mixture of analytes in the mobile phase under high pressure, through a stationary phase in the chromatographic column [20]. The UHPLC, compared with the classical LC technique, is characterized by small-diameter steel columns packed with small totally porous particles that act as a support for the stationary phase. The mixture of analytes is separated by their different retention times, which depend on the physicochemical properties of the analyte, the stationary phase and the mobile phase [15]. With a given analyte, stationary phase and mobile phase retention time can also be influenced to various degrees as function of column temperature. Chromatographic separation can be enhanced by lowering a band broadening in order to achieve more narrow chromatographic peaks. Band broadening factors such as longitudinal and Eddy diffusion can be reduced. Longitudinal diffusion is a dispersion of analyte molecules in the solvent due to the concentration gradient at the band edges. Its effect can be reduced by using a high linear velocity. Eddy diffusion describes the fact that every analyte molecule can travel on the different trajectories due to nonhomogeneous column packing [21]. As a function of the particle size, it can be reduced by using smaller stationary phase particles. The optimal flow rate depends on the diameter (d_p) of the particles used to pack the column [22]. The optimal flow rate for columns packed with small d_p particles is

higher than for columns packed with larger particles (see figure 4). Using narrow columns filled with small particles at the optimal flow rate increases the back pressure. Modern UHPLC instrumentation has the potential to deliver narrow chromatography peaks with the ability to operate at back pressures up to 18 000 psi [15, 23].

A suitable column for the chromatographic separation is determined by the physical properties of the target analytes. Characteristics that are considered for the selection are hydrophobicity, charge, and size of the molecules. The nature of the stationary phase determines the elution profile [24]. The internal diameter of UHPLC columns is in the range of millimeters. Generally, a smaller internal diameter provides better separation [24].

1.3.2 UHPLC column packing

Most UHPLC columns are made by using spherical, totally porous silica as support for the various stationary phases. The most popular columns are the C-18 (also named RP-18) columns. Here, 18 carbon long alkyl chains are covalently bounded to the surface of the silica particles. The diameter of spherical silica beads in UHPLC is between 1.8 µm and 2.5 µm [24]. Column efficiency increases with decreasing diameter of the beads (figure 5). Bead size is limited due to increasing pressure and reduced flow rate in the column [23]. Packing material with the irregular shape of the particles was used in the first columns to increase the surface area. However, higher efficiency was achieved by spherical porous beads.

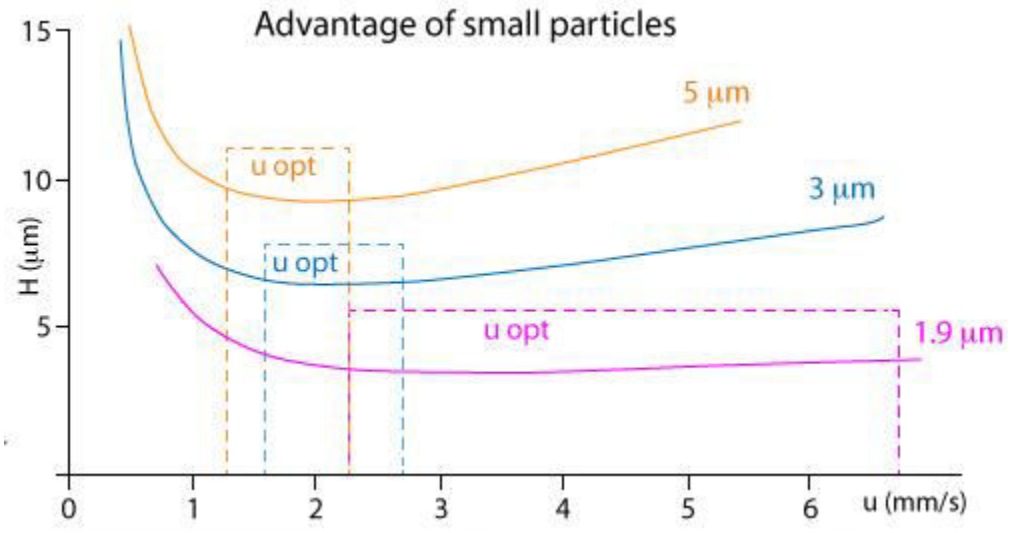


Figure 5 Van Deemter plot. The window for the optimal linear flow rate is wider when using small particles as the column filling [22]

1.3.3 UHPLC column types

UHPLC columns are available in many different lengths, inner diameters, and various stationary phases. Commonly used column types classified by separation mechanism include ion exchange, reverse-phase and normal-phase UHPLC columns [14]. Ion exchange columns contain cationic or anionic stationary phase. Polar analytes carried by a buffer are separated based on their charge. Reversed-phase column is the most commonly used one for a wide range of different types of analytes. Non-polar molecules interact with the hydrophobic stationary phase and retain in the column longer than polar compounds. Non-polar carbon chains are bonded to silica beads which gives the stationary phase hydrophobic properties [25]. Acetonitrile or methanol mixed with water are almost exclusively used as mobile phase components of reversed-phase chromatography. In the analysis of the acyl carnitine profile, reverse-phase C18 column is commonly used. The gradient elution regulates the retention of hydrophilic short-chain acyl carnitine esters. A weak starting mobile phase is linearly increased in order to make the long-chain acylcarnitines elute later. Both isocratic and gradient elution can be used with reverse-phase columns. Normal-phase chromatography is the opposite: the stationary phase is a hydrophilic silica, and the polar molecules retain in the column during the separation process [24].

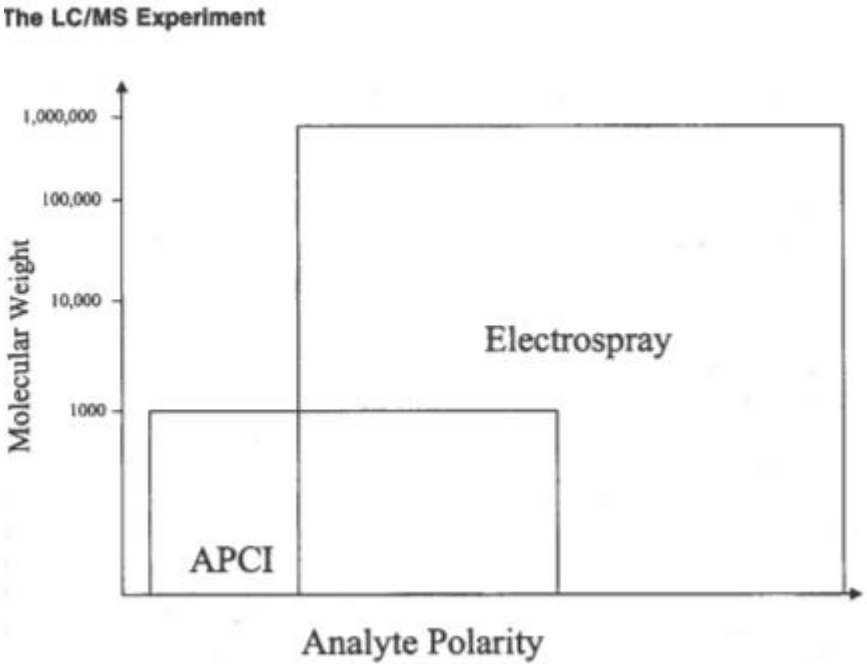
1.3.4 Mass spectrometry instrumentation

The invention of the hyphenated technique of chromatographic separation with MS detection has been revolutionary in bioanalysis due to its high sensitivity and selectivity [16, 17]. Various mass analyzers are used for ion selection, including quadrupole mass filter, time-of-flight (TOF) or ion traps [20]. Multiple reaction monitoring is a commonly used mass spectrometry technique which can quantify compounds within various complex mixtures. This technique applies a triple quadrupole that first identifies ions of interest. Subsequent fragmentation produces daughter ions that can be selected for quantification. Only ions that meet both criteria, specific mother ion of interest and the daughter ion of interest, are isolated. The high sensitivity of this technique is achieved by ignoring the flow of all the other ions and hence increase the signal to noise (S/N) ratio [14]. The MRM technique is used for the quantitative analysis of endogenous and exogenous compounds in plants, environmental samples, blood, serum, plasma, urine, saliva, tissues and other materials.

Mass spectrometry instrumentation includes an ionization source where the molecule of interest is ionized and in most cases also fragmented. Ions are separated according to their mass-to-charge (m/z) ratio by various mass filters. One of the most popular mass filters are the so-called quadrupole instruments. In this work we have used a triple quadrupole instrument. The separation is based on the same deflection of ions with the same m/z ratio [16]. Basically, masses of all the ion and fragments are measured. The mass spectrum displays a plot of the ion signal to the m/z ratio. Known masses and characteristic fragmentation patterns are tools for identification of molecules in the sample [26].

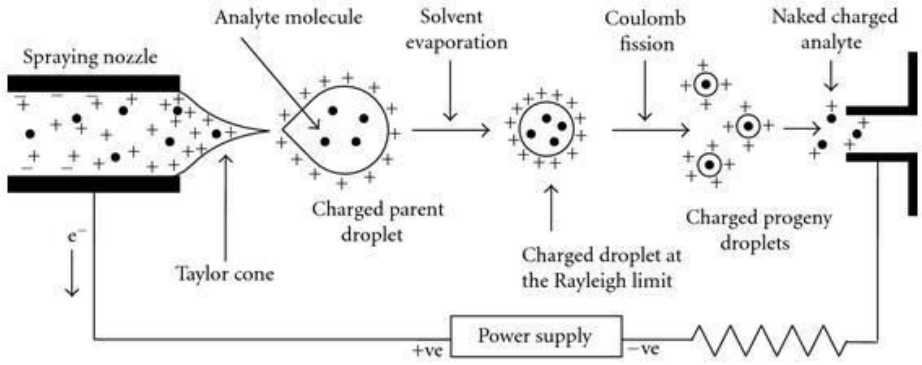
1.3.4.1 Ion sources

Two major types of ionization that are used in LC-MS systems in bioanalytical laboratories are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) [20]. When choosing between APCI and ESI, characteristics such as analyte volatility, analyte polarity, thermal stability and molecular weight should be considered (figure 6) [16].



Figur 6 Applicability of APCI and ESI as a function of a polarity of an analyte and its molecular weight [16].

ESI is a soft ionization source convenient for ionization of thermolabile molecules, where little energy is used in order to achieve a gentle fragmentation. Samples are pumped through a capillary with a specific voltage and nebulized to charged droplets. Rapid evaporation of the charged droplets causes a transfer of the residual electric charge to the analyte (figure 7) [27]. Ions are subsequently transferred to a high vacuum in the mass spectrometer. The ionization source can operate in both positive and negative mode, which allows the selection of positive or negative ions [16]. ESI is widely used when analyzing biological molecules [26]. ESI operating in positive mode was used in this study for the ionization of acylcarnitine molecules.



Figur 7 Schema of ESI ionization source [27]. The Taylor cone emits nebulized liquid drops under high voltage. Solvent evaporation results in charged droplets. Droplet dissociation produce a stream of ions when the charge of the droplet exceeds Rayleigh limit.

When using APCI, a liquid sample is pumped through a capillary and nebulized. Corona discharge causes the ionization of solvent molecules, which transfer their charge to the analyte. APCI is applied for example in lipid analysis [26].

1.3.4.2 Mass analyzers

The selection of ions according to their m/z ratio takes place in an electric and magnetic field, in a vacuum of a mass analyzer. There are several different ways of selecting ions as mentioned previously. A quadrupole mass filter was used during this laboratory work. A quadrupole mass analyzer consists of four parallel conducting rods. Changing voltage on the pairs of opposite rods creates an oscillating electric field in which ions are selected based on their flight trajectories (see figure 8). Mass spectrometer with a single quadrupole can analyze only ions created in the ion source [20]. Single quadrupole can operate in two modes, full scan and selected ion monitoring (SIM). The dominant type of mass analyzer likely to be used in LC-MS systems for quantitative bioanalysis is a triple quadrupole (QqQ). Two mass analyzers are used for tandem mass spectrometry (MS/MS). The triple quadrupole mass analyzer provides better specificity and structural information than a single quadrupole since it involves an extra selectivity step. At the first selectivity step, a precursor ion is selected in the first quadrupole and fragmented in the collision cell (second quadrupole) by collision-induced dissociation. Collisions with an inert gas at a specific collision energy result in fragmentation. The second selectivity step is performed in the third quadrupole. The third

quadrupole selects fragment ions of interest before they reach a detector (see figure 9). Co-eluting compounds might provide ions of the same m/z. However, the probability of two compounds having the same retention times, the same precursor ion and the same fragment ion is very low.

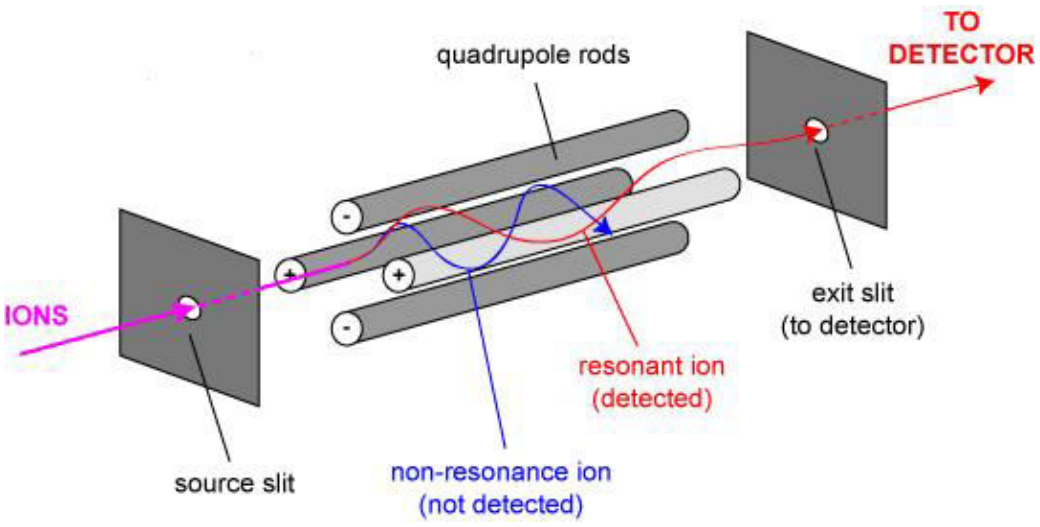


Figure 8 Schema of a quadrupole mass analyzer [28]. Changing voltage on the pairs of opposite rods creates an oscillating electric field in which ions are selected based on their flight trajectories.

A triple quadrupole can operate in several modes [26]:

Table 1 Operation modes of the triple quadrupole mass analyzer

Mode	**Q1**	**Q3**	**Applications**
Parent (precursor) ion scan	Scanned	Fixed	Detection of analytes with a common daughter ion (m/z = 85 for acylcarnitines)
Daughter (product) ion scan	Fixed	Scanned	Scan of fragment ions of one parent ion (determination of MRM transitions)
Neutral loss scan	Scanned	Scanned	Detection of analytes that eliminate a common molecule (glucuronates)
Multiple reaction monitoring (MRM)	Fixed	Fixed	Highly-specific detection, used in this study to detect acylcarnitines

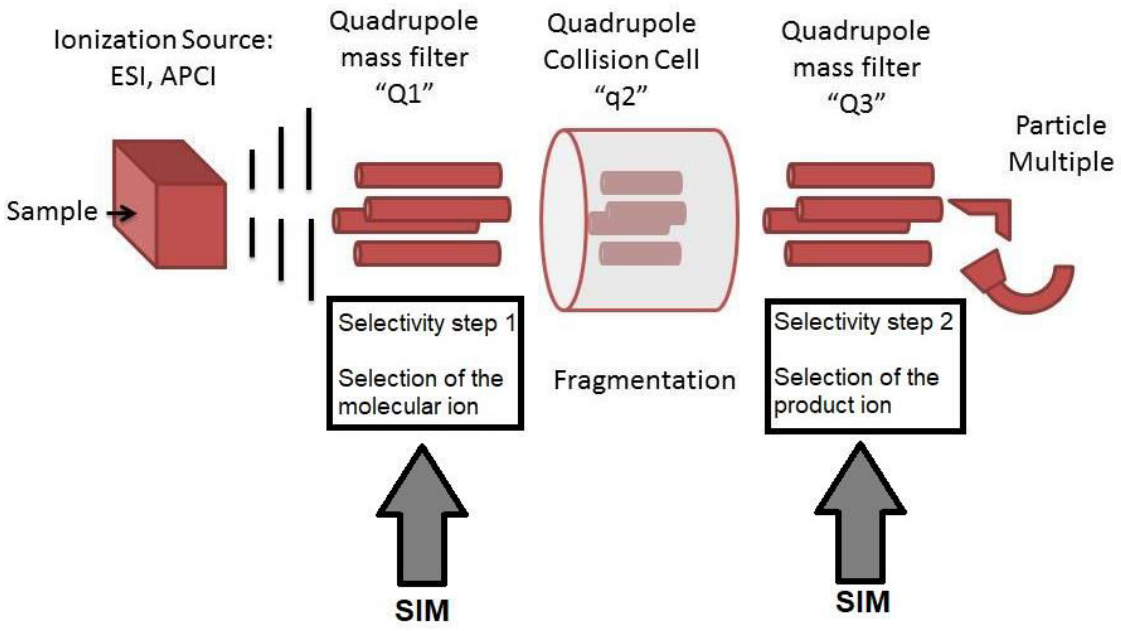


Figure 9 Schema of MRM method. Selectivity step 1: "Q1" selects the molecular ion of interest. Selectivity step 2: "Q2" selects a product ion of the molecular ion of interest [29]

1.4 Analytical challenges in bioanalysis

UHPLC-MS/MS technique used in bioanalysis is the right choice for its selective and sensitive detection ability of analytes of interest in very complex matrices. A bioanalytical method should meet certain requirements and deliver quantitative results with high accuracy and precision. The acylcarnitine profile can be established by direct infusion to ESI-MS/MS. However, this method cannot separate isobaric species and is usually not used in quantitative analysis. Therefore, chromatographic separation coupled with modern sensitive and selective MS detection provide more clinically relevant results [9]. The workflow for the development of a successful bioanalytical UHPLC-MS/MS method requires three main steps: 1) sample preparation, 2) chromatographic parameters and 3) mass spectrometry [14]. All the three steps can influence each other; therefore the method development process is very complex and includes testing and further adjustments to design the optimal set of conditions.

Several challenges must be considered when developing a bioanalytical method. A biological matrix can result in interferences or ion suppression in a mass spectrometry assay. The required level of sensitivity (low levels of detection and quantification) should be achieved to detect the trace amount of the compound of interest in the complex matrix. The time of the analysis must be balanced against all the requirements and considerations. Another important consideration is a resolution of a peak of interest from all other endogenous compounds.

1.5 Metabolomics

Metabolomics is an approach that is based on a comprehensive analysis of metabolites and low-molecular-weight molecules in a biological sample [30]. The field of metabolomics provides several challenges in comparison to other "omics." The approach to analyze the whole metabolome must deal with a wide array of small molecules of various chemical functionalities that contribute to metabolism. Unlike other "omics" fields such as lipidomics or proteomics, metabolomics must focus on specific groups within each metabolome since metabolites have no standard chemical functionality. Relative quantification of metabolites can be achieved with isotope labelling coupled with MS. Stable isotopes have the same chemical formula and structure and behave identically during chromatographic separation. Isotope of a given element contains the same number of protons, but different number of neutrons. An MS can differentiate isotopes by their different m/z ratio. Isotopes exhibit nearly identical chemical behaviour; therefore, the stable isotopes are the best internal standards to enable quantification [31].

Current metabolomics technologies are powerful tools in clinical medicine. Analysis of metabolites provides information for the diagnosis of complex metabolic diseases including inborn errors of metabolism. Metabolomics stands behind the discovery of new therapeutic targets and biomarkers and thus has a significant potential to change a field of clinical chemistry [30].

Methods in the field of metabolomics are continually improving with the development of new analytical techniques and instrumentation. A metabolomic analysis includes both, endogenous metabolites linked to enzymatic activities and metabolites derived from external sources like food and medication. Current technologies enable rapid discoveries, measurement of hundreds of samples with high selectivity, sensitivity, and precision [30, 32]. Metabolomics is

a tool to search for specific markers of diseases as well as metabolite profiles associated with the external environment and their associations with disease risk. Metabolomics studies include for example relationship between diet and disease, search for new cardiovascular biomarkers [33], metabolite profile analysis and risk of developing diabetes [5, 34] or metabolic indicators of cancer [35]. The most recent metabolomics research in the field of clinical medicine introduces an application of metabolomics in viral pneumonia [36], obesity studies [4], Parkinson's disease [32], breast cancer tumours [37], or the role of acyl carnitine profile alterations in inflammatory bowel diseases [7].

2 Aims

The principal objective of this project was to develop an analytical method for the determination of endogenous carnitine and acylcarnitines in colonic mucosa. A set of experimental conditions was investigated to create an analytical method for the determination of acylcarnitines. Several methods are developed for the analysis of acylcarnitines in different tissues by mean of liquid chromatography coupled with mass spectrometry (LC/MS) [6]. These methods require laborious and time-consuming preparation steps such as solid phase extraction and derivatization. Moreover, other methods are restricted to only a few acyl carnitine species and are not able to detect and quantify isomers [38].

Metabolomics analysis on IBD patients' colon biopsies revealed alteration in fatty acid metabolism. Furthermore, changes of acyl carnitine profile in IBD patients' plasma were previously reported [7]. Therefore, comprehensive quantification of acyl carnitine species in colon mucosa is important to establish the link between fatty acid metabolism and IBD occurrence and progression.

The main goal is therefore to develop a simple UHPLC–MS/MS method for the separation and quantification of acylcarnitines including the isomeric forms with a minimal sample pre-treatment. The time-consuming sample pre-treatment processes involve synthesized reagents that can cause unwanted reactions and it can influence the determination of accuracy [38]. This project aims to develop a new UHPLC-MS/MS based analytical method for separation and quantification of acyl carnitines that avoids critical process parameters.

Analytical methods using UHPLC-MS/MS include analysis in the following modes: full scan, selected ion monitoring (SIM), daughter ion scan, parent ion scan and multiple reaction monitoring (MRM) [26, 39]. Chromatographic separation is a physical separation of the acylcarnitines performed prior to the high-tech detection with a highly specific MS instrumentation. The optimization of the chromatographic conditions is a necessary step that includes varying the composition of solvents in the mobile phase, adjusting the pH and optimizing the gradient elution [18].

Full scan monitoring is performed to provide a qualitative picture of the composition of the sample [39]. It involves scanning the actual mass range, in this case, scanning from m/z = 50 to m/z = 450.

In the selected ion monitoring (SIM) mode, data are collected on the selected masses for quantitative studies on the specific ions of interest [39]. Cone voltage (CV) must be investigated to obtain the optimal value that provides the desired fragmentation. SIM is also one of the methods of choice to determine a limit of detection (LOD) and a lower limit of quantification (LLOQ) of individual acylcarnitines. In SIM, minimum 2 ions should be used, one ion as the quantifier ion and one ion as a qualifier ion.

Daughter ion scan mode selects a parent ion, and the scan gives all the daughter ions resulting from fragmentation of that ion [39]. Cone voltage (CV) must be adjusted for this method to provide a high intensity of the molecular ion. Lower fragmentation is usually achieved by lowering down the CV value. Two more parameters must be determined: collision energy (CE) and flow rate of the collision gas, to obtain good fragmentation of the molecular ion, in order to get information about the masses of daughter ions.

Multiple reaction monitoring (MRM), also known as selective reaction monitoring (SRM) is a highly specific and sensitive mass spectrometry technique that can selectively quantify compounds within complex mixtures [39]. Our goal is to obtain all the necessary parameters and data to develop the MRM technique for analysis and quantification of acyl carnitine molecules in the colonic tissue and to find LOD and LLOQ with this technique.

The appropriate validation of the analytical method is essential to demonstrate that the new method is suitable for the analysis of acylcarnitines in colon biopsies. Validation of a method involves using experimental design to prove that the method can produce accurate and precise quantitative results.

A successful analytical method can be a tool for investigating the changes in the mucosal acylcarnitine profile in IBD patients compared to healthy controls.

3 Material and methods

3.1 Chemicals and reference standards

Chemicals and carnitines reference standards used during the laboratory work are summarized in the table 2 and table 3.

Table 2 Chemicals

Chemical	**Abbreviation**	**CAS**	**Producer**
Acetonitrile (UPLC/UHPLC grade)	ACN	75-05-8	VWR Chemicals
Ammonium acetate	NH_4AcO	631-61-8	Sigma Aldrich
Heptafluorobutyric acid	HFBA	375-22-4	Sigma Aldrich
Hexanesulfonic acid natrium salt	HSA	2832-45-3	Sigma Aldrich
Methanol (UPLC/UHPLC grade)	MeOH	67-56-1	VWR Chemicals

Table 3 Acylcarnitine reference standards

Acylcarnitine reference standard (1mg/ml)	**Acyl chain**	**CAS number**	**Producer**
L-carnitine	C0	541-15-1	Sigma Aldrich
Acetylcarnitine	C2	204259-54-1	
Propionylcarnitine	C3	20064-19-1	
Butyrylcarnitine	C4	25576-40-3	
Isobutyrylcarnitine	C4	25518-49-4	
2-methyl-butyrylcarnitine	C5	256928-75-3	
Isovalerylcarnitine	C5	31023-24-2	
Glutarylcarnitine	C5DC	102636-82-8	

Hexanoylcarnitine	C6	22671-29-0	
Octanoylcarnitine	C8	25243-95-2	
Decanoylcarnitine	C10	3992-45-8	
Lauroylcarnitine	C12	25518-54-1	
Myristoylcarnitine	C14	25597-07-3	
Palmitoylcarnitine	C16	2364-67-2	
Stearoylcarnitine	C18	25597-09-5	
Oleoylcarnitine	C18:1	13962-05-5	
Oleoyl-1-^{13}C-L-carnitine hydrochloride			Sigma Aldrich

3.2 LC-MS/MS conditions

The analysis was conducted on a Waters Acquity Ultra Performance Liquid Chromatograph (UPLC) coupled with triple quadrupole mass spectrometer operated in electrospray positive mode. The chromatographic separation was established using ACQUITY UPLC HSS C18 (1.8 µm, 2.1 x 100 mm) column. The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B).

Table 4 Experimental parameters

Experimental parameters	**Conditions**
LC system	**Waters ACQUITY UPLC system**
UPLC columns	ACQUITY UPLC HSS C18 (1.8 µm, 2.1 x 100 mm)
	X Bridge BEH Amide (2.5 µm, 2.1 x 75 mm)
Mobile phases	A1: 0.1% formic acid in water
	B1: 0.1% formic acid in acetonitrile

	(0.1% formic acid in methanol)
	A2: 10 mM ammonium acetate in water
	B2: 10 mM ammonium acetate in acetonitrile (10 mM ammonium acetate in methanol)
Flow rate	0.3 ml/min
Injection volume	300 µl
Column temperature	50°C
Sample temperature	Room temperature
Sample diluents	MeOH:H_2O 1:1 H_2O
MS system	**Waters Tandem Quadrupole Mass Spectrometer**
Ionization mode	ESI+
Capillary voltage	3.0 kV
Source temperature	200°C
Collision gas	Argon
Collision gas flow rate	0.25 ml/min
Dwell time	0.600 s
MS software	MassLynx version 4.1

3.3 Sample preparation

Several methods have been developed to determine acylcarnitines in body liquids and tissues. Most of the methods use tandem mass spectrometry (MS/MS) as a detection tool. These methods are successful, but they do not separate isomers [38]. A method using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been developed in order to separate isomers. However, samples require laborious processing before the analysis which includes time-consuming derivatization. Derivatization is required because the short-chain acylcarnitines do not retain on a reverse phase column due to their hydrophilic properties. Acylcarnitines are often derivatized to their butyl-esters. Alternative derivatization methods include synthesized reagents [2, 38]. Derivatization process can cause unwanted reactions that can influence the determination of the accuracy of the quantifications [2]. Thus, a UHPLC-MS/MS method has been established to separate and identify underivatized compounds [38]. In this project, we have focused on the UHPLC separation of underivatized acylcarnitines with subsequent MS/MS identification.

Acylcarnitine reference standards of concentration 1 mg/ml were purchased from Sigma Aldrich. The reference standards were further diluted using methanol:water (1:1) as a dilution solvent. Dilutions used for each step of the method development are mentioned in each descriptive section of the chapter 3. Diluted reference standards were used in this project for the method development.

3.4 Analytical methods

3.4.1 UHPLC method development

A UHPLC method was developed in order to achieve chromatographic separation. Despite the ability of the MS/MS detector to analyze a mixture of acylcarnitines in the positive ion mode using the multiple reaction monitoring (MRM), chromatographic separation is performed in order to separate isomeric compounds. The separation of isomers is desired for this study due to its clinical importance.

Chromatographic conditions were optimized by testing two chromatographic columns (C18 column and amide column), varying organic solvent composition (methanol and acetonitrile),

pH (ammonium acetate used as a buffer, pH adjusted by formic acid) and ion pairing reagents (heptafluorobutyric acid and hexanesulfonic acid).

3.4.1.1 Acetonitrile as an organic solvent in the mobile phase

The chromatographic analysis was first performed with an ACQUITY UPLC HSS C18 (1.8 µm, 2.1 x 100 mm) column with water and acetonitrile as mobile phases, both containing 0.1% formic acid. Chromatographic conditions of individual acylcarnitines were adjusted by testing gradient elution, column temperature, and flow rate of the mobile phase to achieve an optimal balance between the separation of acylcarnitines and time of the analyses.

Individual acyl carnitine reference standards were diluted 250x in the sample dilution solvent (water:methanol 1:1) prior injection. A volume of 3 µl of the diluted sample was injected at a flow rate of 0.3 ml/min. In the beginning, gradient elution was as follows: 0-15 min linear gradient from 70% A, 30% B to 1% A, 99% B; 15-18 min 1% A, 99% B; 18-19 min 70% A, 30% B. The temperature of the column was set to 50ºC to speed up the separation process. A good separation of the medium-chain and long-chain acylcarnitines (C8-C18) was achieved under the described conditions. Nevertheless, acylcarnitines with the chain-length C0 to C6 eluted too early due to the strong mobile phase composition. Optimization of the chromatographic separation was performed by prolonging the time of the analysis and testing different gradients with the weaker initial mobile phase. Each measurement was performed 3x. The following gradients were tested:

- 0-3 min 90% A, 10 %B; 3-3,5 min 80% A, 20% B; 3,5-20 min linear gradient to 1% A, 99% B; 20-23 min 1% A, 99% B; 23-24 min 90% A, 10% B.

Better separation of the medium-chain acylcarnitines including separation of the C5 isomers was achieved.

A weaker initial mobile phase was tested to prolong retention time of polar short-chain acylcarnitnes in the following gradient elution:

- 0-3 min linear gradient from 99%A, 1% B to 95% A, 5 %B; 3-3,5 min 90% A, 10% B; 3,5-20,5 min linear gradient to 1% A, 99% B; 20,5-23 min 1% A, 99% B; 23-24 min 99% A, 1% B.

The retention time of the short-chain acylcarnitines was prolonged when using only 1% of acetonitrile in the initial mobile phase, however, C0-C4 acylcarnitines co-elute and the polar glutarylcarnitine (C5DC) elutes in the dead volume. A sufficient separation of the medium and long-chain C6 to C18 acylcarnitines can be performed in shorter time (with steeper gradient) to speed up the analysis.

- 0-3 min 95% A, 5 %B; 3-18 min linear gradient to 1% A, 99% B; 18-21 min 1% A, 99% B; 21-22 min 95% A, 5% B.

The problem regarding the low retention of polar compounds persists. However, a partial separation of two isomers with the acyl-chain length C4 and two isomers with the acyl-chain length C5 was achieved. Short-chain acylcarnitines C0, C2 and C3 co-elute in one peak. A sufficient chromatographic separation of the medium-chain and long-chain carnitines was achieved using a steeper gradient.

Another way to improve the chromatographic separation is the optimization of the mobile phase's flow rate. Flow rates of 0.1 ml/min; 0.2 ml/min, 0.3ml/min; 0.4 ml/min and 0.5 ml/min were tested. The flow rate of 0.2 ml/min provides the best result out of the five tested ones and therefore was applied during the first 3 minutes of the analysis when the short-chain polar acylcarnitines and two pairs of isomers co-elute. The flow rate of 0.3 ml/min was satisfactory for the separation of the medium-chain and longer acylcarnitines.

Another separation problem occurred when trying to repeat the analysis. Repeated measurements provided chromatograms showing the long-chain hydrophobic acylcarnitines only. Polar short-chain C0 to C5 carnitines co-eluted (see figure 10). This separation problem occurred due to a short equilibration of the column. The time of the analysis was prolonged for 5 more minutes, which was necessary for the column equilibration with following conditions: 22-28 min 95% A, 5% B with the flow rate of 0.4 ml/min to speed up the process of equilibration (see figure 11).

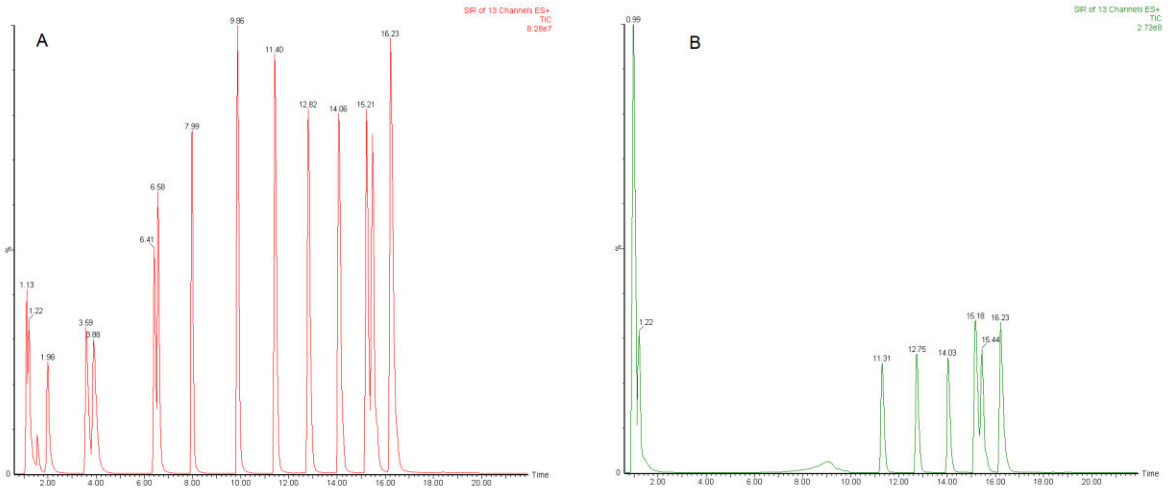


Figure 10 Achieved separation of acylcarnitines (A) and a separation problem in the repeated measurement (B).

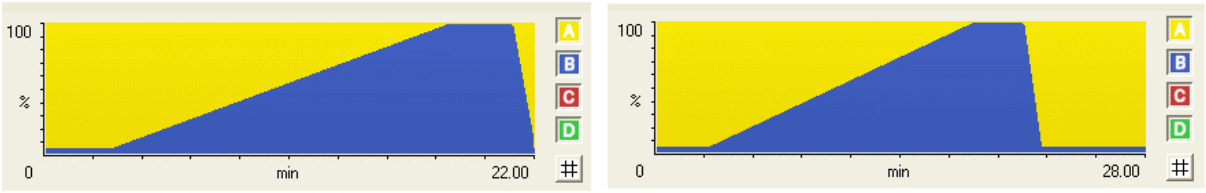


Figure 11 Separation problem was solved by longer equilibration of the column. Five minutes for the column equilibration was added after the gradient elution to avoid co-elution in the repeated measurements. Original gradient elution (left) and new gradient elution with added equilibration time (right)

3.4.1.2 Methanol as an organic solvent in the mobile phase

The optimization of the chromatographic conditions was tested by varying the organic solvent composition. Acetonitrile in the mobile phase was replaced by methanol. Both mobile phases, water (mobile phase A) and methanol (mobile phase B), contained 0.1% of formic acid. Methanol is a weaker mobile phase than acetonitrile, and thus, better separation and longer retention time of the short-chain acylcarnitines was expected. The gradient was adjusted for the weaker mobile phase composition. No significant chromatographic improvement was observed when using methanol instead of acetonitrile (figure 12). A signal of an unknown impurity in the sample (t□18 min) was more dominant than signals of acylcarnitines.

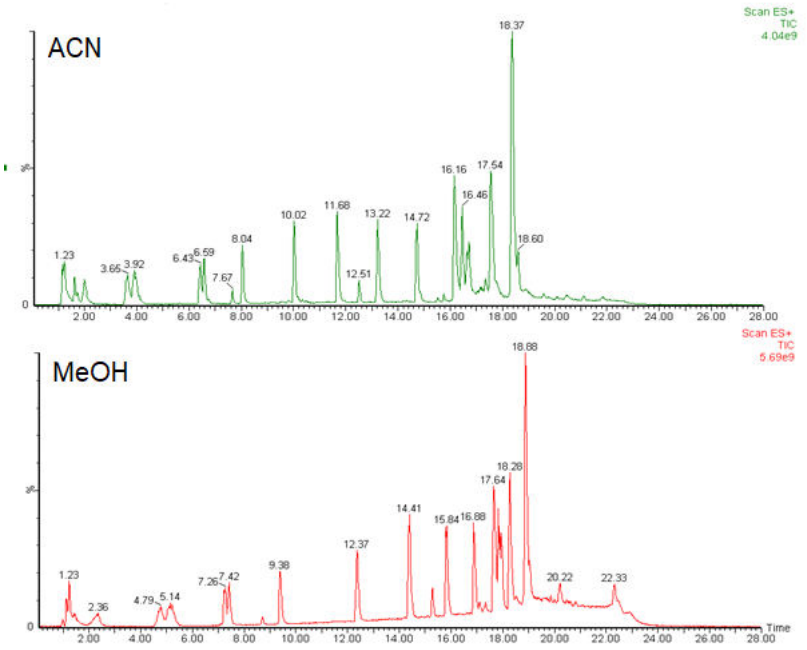


Figure 12 Separation achieved with acetonitrile (ACN, upper chromatogram) in the mobile phase was slightly better than with methanol (MeOH, bottom chromatogram).

3.4.1.3 Ammonium acetate as a buffer

Pieter Giesbertz *et al*. described a method for the analysis of acylcarnitines species including isomeric forms in plasma and liver tissues, where he achieved a chromatographic separation using C18 column, water and acetonitrile as a mobile phase, and ammonium acetate as a buffer [9]. The pH was adjusted with formic acid. Inspired by his method, ammonium acetate was added to both mobile phases, water/acetonitrile, and water/methanol, containing 0.1% formic acid. The concentration of the ammonium acetate used in this experiment was 10mM [6]. There was no significant separation improvement when adding ammonium acetate as a buffer to the two tested mobile phases (figure 13).

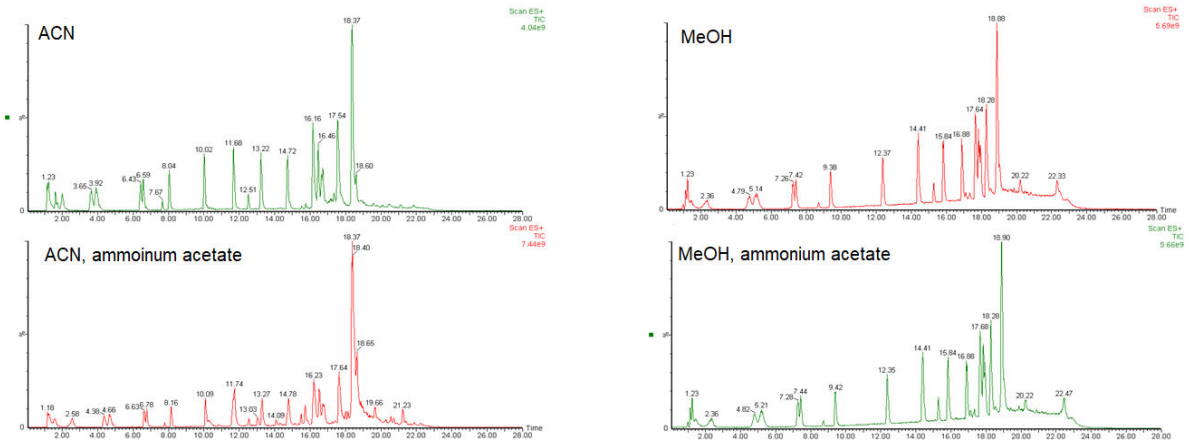


Figure 13 Comparison of the separation achieved with various mobile phases (ACN and MeOH, upper chromatograms) and separation achieved with and without adding ammonium acetate as a buffer (bottom chromatograms).

3.4.1.4 Amide column

Chromatographic separation was tested on an amide column X Bridge BEH Amide (2.5 µm, 2.1 x 75 mm) column XP at column temperature 50°C. The purpose of using amide column was to achieve a better separation of isomers with acyl-chain length C4 and C5 by prolonging a retention time. The amide column is designed to retain analytes and metabolites that are too polar to retain by reversed-phase chromatography. The stationary phase was developed for hydrophilic interaction chromatography to separate extremely polar analytes [40]. Therefore, gradient elution was performed also with a strong initial mobile phase (unlike when using C18 column). Non-polar long-chain acylcarnitines were expected to elute first, followed by the medium-chain acylcarnitines. Polar short-chain acylcarnitines and C4 and C5 isomers were expected to retain in the column and elute with the weaker mobile phase at the end of the gradient elution.

Variables tested when using amide column include solvent composition, the gradient elution, the flow rate of the mobile phase and an effect of ammonium acetate buffer added to the mobile phase.

A mixture of four isomeric species of acylcarnitines was prepared. Two C4 isomers (butyrylcarnitne and isobutyrylcarnitine) and two C5 isomers (2-methylbutyrylcarnitine and

isovalerylcarnitine) were diluted 50x in the dilution solvent (water:methanol 1:1). Each measurement was performed twice. The mobile phase consisted of 0.1% FA in water (mobile phase A) and 0.1% FA in acetonitrile (mobile phase B). The flow rate of the mobile phase during the experiment was 0.3 ml/min.

Various gradient elution conditions were tested in order to obtain a desired separation. First, starting with a weak initial mobile phase (5% and 10% of ACN), the eluents were linearly increased up to 50%, 70%, 90% and 99% of ACN to investigate the optimal steepness of the gradient. Second, the gradient with a decreasing strength of the mobile phase was applied. Starting with a high percentage of ACN (50%, 70%, 90%, 99%) in the mobile phase, the eluents were linearly changed to 5% of ACN. The column was equilibrated for 5 min after each analysis. Isocratic elution with 50% ACN for 10 min was also performed but did not provide any good separation.

The gradient elution that provided the best result out of the tested ones was as follows: 0-7 min 90% A, 10% B to 70% A, 30% B; 7-10min 90%A, 10% B; 10-15min 90% A, 10% B. Using the described gradient elution, acylcarnitines with acyl-chains of the length C4 and C5 were separated; however, their isomers co-eluted. The different gradient elution did not provide sufficient separation of C4 and C5 acylcarnitines. Adjustments of the chromatographic conditions were performed by varying the composition and the flow rate of the mobile phase. Optimal flow rate of 0.3 ml/min was chosen after testing flow rates of 0.2 ml/min, 0.3 ml/min, 0.4 ml/min and 0.5 ml/min.

Variations of the gradient elution described above were also performed with a mobile phase that consisted of 0,1% formic acid in water (mobile phase A) and 0,1% formic acid in methanol (mobile phase B) instead of the acetonitrile. Methanol in the mobile phase provided a slightly improved separation of C4 and C5 acylcarnitines when using the following gradient elution: 0-7 min 90% A, 10% B to 70% A, 30% B; 7-10min 90%A, 10% B; 10-15min 90% A, 10% B. Isomeric forms were not sufficiently separated, though.

Ammonium acetate was added to the tested mobile phases as a buffer. Ammonium acetate (10 mM) in water (eluent A1) and ammonium acetate (10 mM) in acetonitrile (eluent B1), and then ammonium acetate (10 mM) in water (eluent A2) and ammonium acetate (10 mM) in

methanol (eluent B2), were used to create the gradient of the mobile phase (flow rate: 0.3 ml/min). Addition of ammonium acetate as a buffer did not enhance the separation efficiency.

To summarize the experiment, the amide column did not provide any better results than the C18 column regarding the chromatographic separation. The main goal of using amide column was to improve the separation of the isomeric acylcarnitine species. It was a step back instead since the biggest success included only the separation of C4 and C5 acylcarnitines which was achieved already before with the C18 column. Despite similar retention times of the isomeric pairs, their separation was still more significant when using C18 column, since the two pairs of the isomers co-elute on the amide column. The optimization of the method performed on the amide column by varying parameters such as a mobile phase composition, gradient elution, flow rate and adding ammonium acetate as a buffer did not lead to any improvement in the chromatographic separation. Therefore, the C18 column was chosen for further laboratory work in method development of the acylcarnitine profile analysis.

3.4.1.5 Ion-pairing reagents (IPR)

To prolong the retention time of the short-chain acylcarnitines, ion pairing reagents (IPR), such as heptafluorobutyric acid (HFBA) and hexanesulfonic acid (HSA), have been used as additives. The purpose of applying an ion pair reagent is to prolong the retention time of ionic analytes on hydrophobic columns. Ion pairing reagent is an ion species with an opposite charge to the analyte of interest, so an ionic interaction can connect the two molecules in order to form a molecular association [41, 42]. The ionic interaction is subsequently destroyed in the ionization source of MS instrumentation which results in detection of the analyte of the interest (not the molecular association). IPR normally improves separation of polar compounds. Compounds used as IPR are strong organic acids when analyzing amines (acylcarnitines). These organic acids contain hydrophobic alkyl chain which allows binding to the stationary phase in the C18 column. Peng *et al*. described a successful separation of the acylcarnitines using heptafluorobutyric acid as an IPR [38]. However, IPR is not compatible with MS detection. When using a traditional detector, the mobile phase containing an IPR passes through the detector and goes to waste or can be collected for further studies. There are many restrictions concerning the composition of the mobile phase when using an MS

detector. The reason why IPR is not compatible with MS detector is that the eluate from the chromatographic column is deposited in the ion source, and thus non-volatile additives cannot be used. In the traditional procedure, IPR added to the mobile phase is continuously introduced into the mass spectrometer, which can cause its contamination that leads to signal reduction, ion suppression and requires laborious cleaning procedures afterward [42].

Bergman *et al.* developed an LC/MS ion-pairing chromatography for the analysis of catecholamines [42]. This new approach to ion-pairing chromatography was applied to our samples of acylcarnitines that were posing significant challenges to LC-MS/MS method development. In this method, IPR is added directly to the sample vials instead of adding it to the mobile phase. Thus, the amount of IPR introduced to LC-MS/MS is significantly lower; therefore the negative impact on the MS detector is reduced to the minimum.

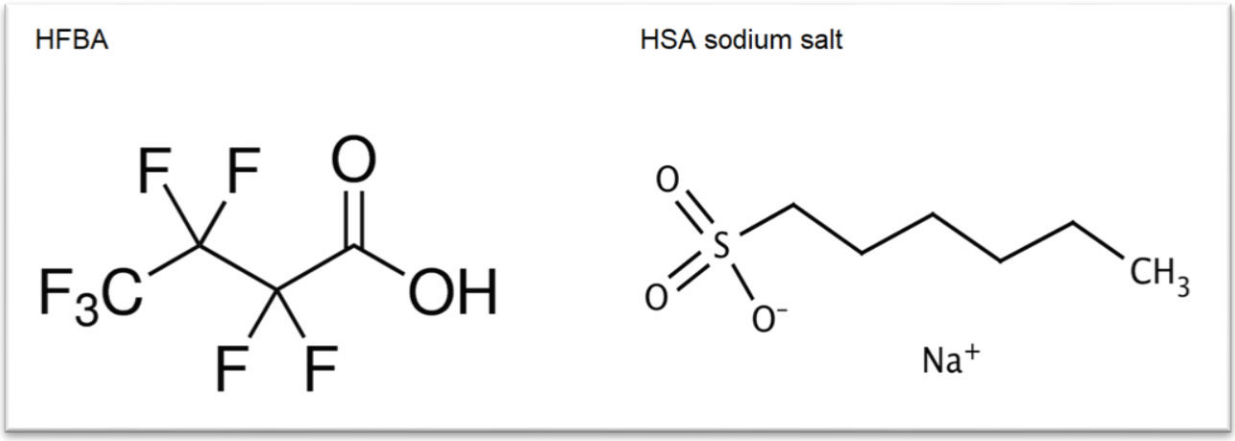


Figure 14 Ion-pairing reagents tested in this study were heptafluorobutyric acid and 1-hexanesulfonic acid sodium salt

Two ion-pairing reagents were chosen for further investigation: heptafluorobutyric acid (HFBA) [41] and 1-hexanesulfonic acid (HSA) [42]. Investigation of the new method with the introduction of IPR into the sample included varying IPR concentrations and gradient elution. A mixture of the short-chain acylcarnitines (of acyl-chain lengths C0, C2, C3, two C4 isomers, two C5 isomers, and C5DC) was diluted 50x in the dilution solvent (methanol:water 1:1). The aim of the experiment with IPR added to our samples was to prolong their retention and enhance the separation of both, the polar compounds and the isomeric species. A mixture of all the acylcarnitines of the acyl-chain lenghts C0-C18 (with the same dilution) was also analyzed with added IPR to see its effect on medium-chain and long-chain acylcarnitines.

Heptafluorobutyric acid

The mixture of C0-C5 was first analyzed without IPR and then with various concentrations of HFBA (6mM, 12mM, 30mM, 45mM). Gradient elution was as optimized as follows: 0-3 min 95% A, 10% B; 3-6 min 40% A, 60% B, 6-9 min 95% A, 5% B, 9-12 min 95%A, 5% B. A full scan analysis of the pure mixture displays all the present acylcarnitines and the persistent separation problem. Figure 15 shows the full scan chromatogram and dispays individual molecular ions of acylcarnitines present in the mixture.

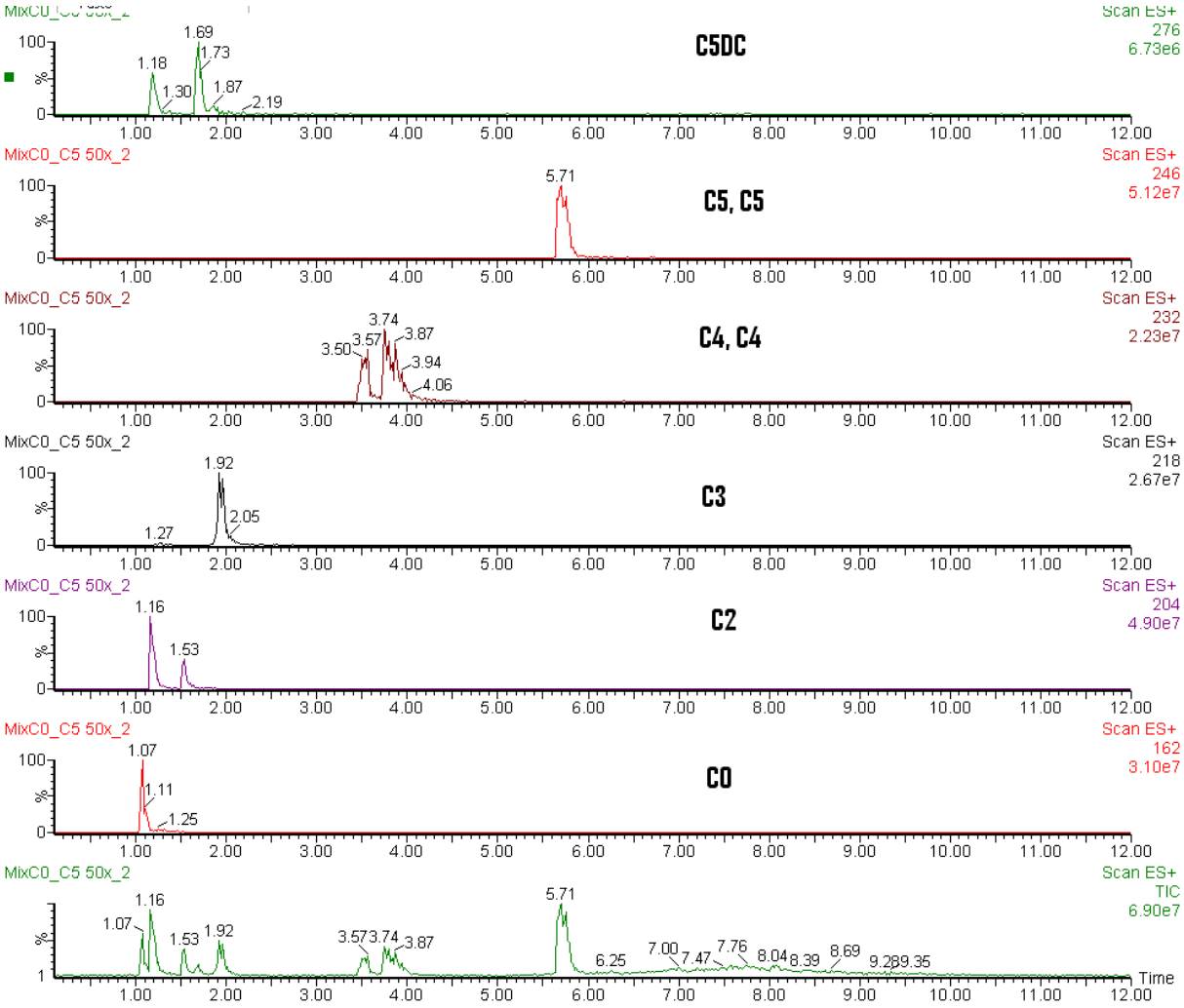


Figure 15 Mix of short-chain acylcarnitines C0 – C5 was separated with following gradient elution: 0-3 min 5% ACN,3-9 min 60% ACN, 9-12 min 5% ACN (bottom chromatogram). The mass of each acyl carnitine in the sample is displayed in upper chromatograms.

After adding HFBA to the sample, the results did not show any peaks in the chromatogram that could indicate the presence of acylcarnitines (figure 16 and 17). Acylcarnitine and IPR form an ionic interaction which should be broken in the ionic source of MS instrumentation. The same m/z values of acylcarnitines were therefore expected in the chromatogram. Apparently, HFBA is too strong organic acid that can cause unwanted side reactions or a strong ion suppression of the acylcarnitine molecules [9]. Two dominant peaks of m/z 171 and 225 are present in all the samples that contain HFBA, but none of them is an acylcarnitine. The gradient elution was adjusted by prolonging the time of equilibration: 0-3 min 95% A, 10% B; 3-6 min 40% A, 60% B, 6-9 min 95% A, 5% B, 9-15 min 95%A, 5% B

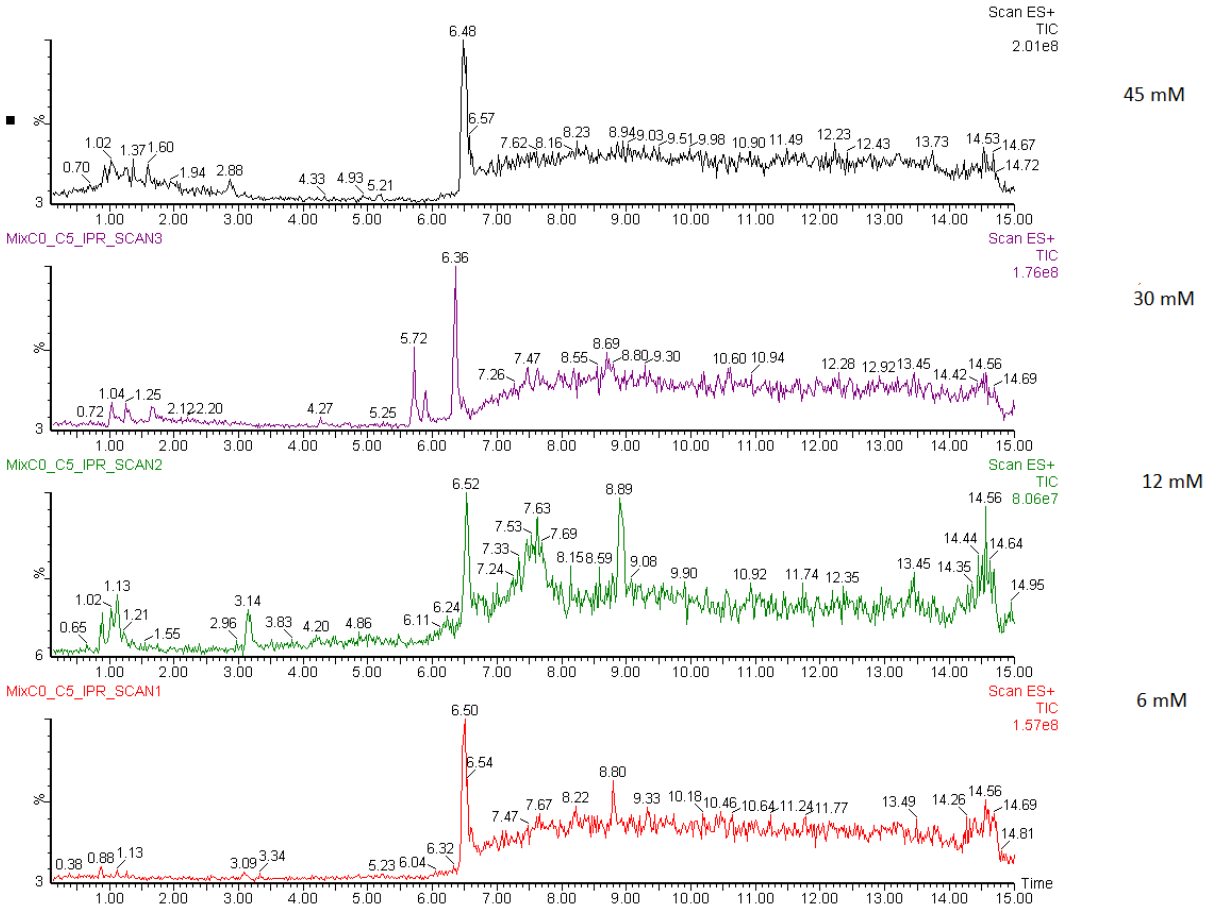


Figure 16 Short-chain acylcarnitines (C0-C5) were not displayed in the chromatograms after adding an increasing amount of HFBA. Unknown dominant peaks appeared in each chromatogram.

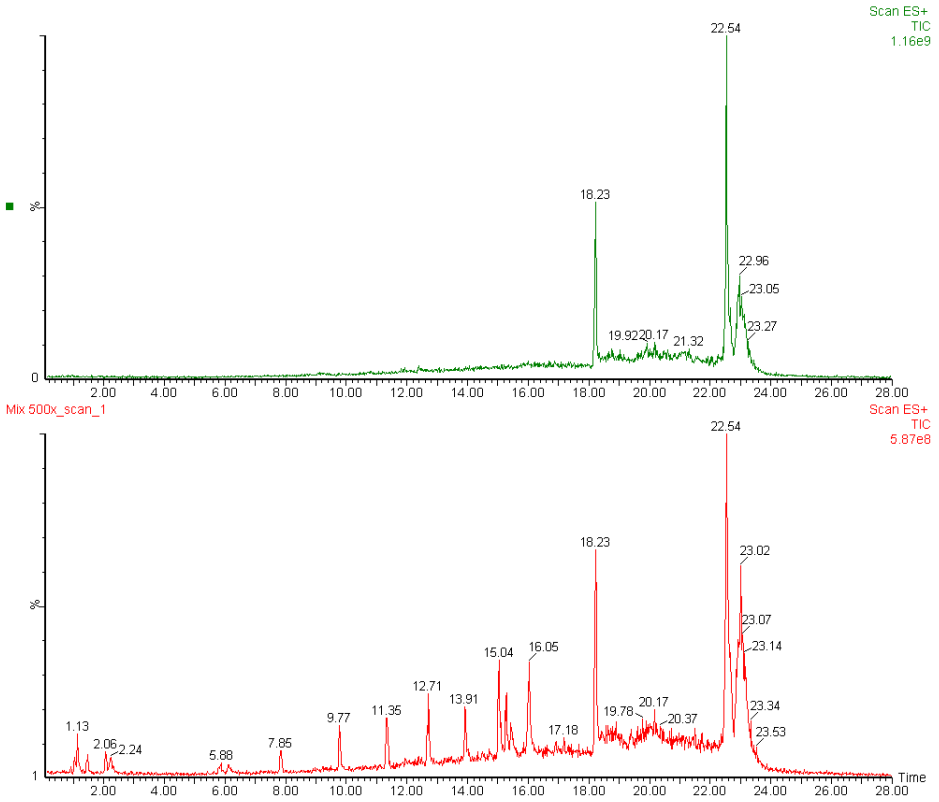


Figure 17 Separation of all the acylcarnitines without adding HFBA (bottom chromatogram) and after adding HFBA (upper chromatogram).

Parent ion scan was applied to the same set of samples. In this mode, a product ion of m/z 85 was selected, and the scan shows all the parent ions which have fragmented to give that a product ion with m/z = 85. All the acylcarnitines contain the product ion of m/z 85, and thus the purpose of using the parent ion scan was to investigate if the carnitines are present in the samples with HFBA. Scanning parent ions of m/z 85 in the pure mixture (without added IPR) clearly show the presence of C0 – C5 acylcarnitines (figure 18). Presence of HFBA in any of the tested concentration caused a significant fragmentation and confirmed our hypothesis that HFBA, as a strong organic acid, may cause unwanted side reactions, a breakdown of the acylcarnitines or strong ion suppression.

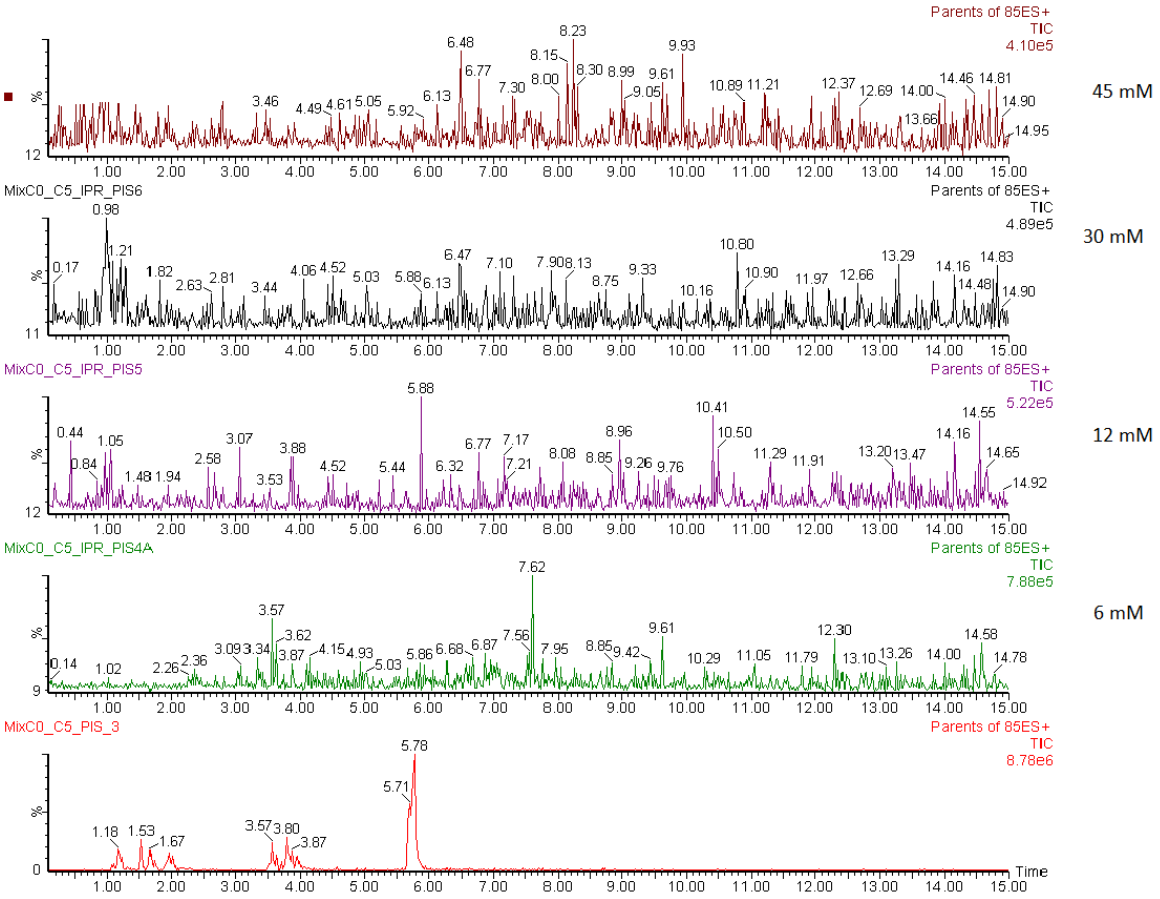


Figure 18 Parent ion scan applied for the analysis of short-chain acylcarnitines (C0-C5) without adding HFBA (bottom chromatogram) and after adding an increasing amount of HFBA (upper chromatograms)

To conclude the experiment, HFBA is not a suitable ion pairing reagent for the analysis of acylcarnitines.

Hexanesulfonic acid

Sulfonic acids were introduced as possible IPR candidates by Bergmann *et al*. in her article about the analysis of catecholamines [42]. A pure mixture of C0-C5 (without added IPR) was analyzed first, and then an analysis of a mixture containing various concentrations of HSA (6mM, 12mM, 30mM, 45mM) was performed. Gradient elution was optimized as follows: 0-3 min 95% A, 10% B; 3-6 min 40% A, 60% B, 6-9 min 95% A, 5% B, 9-12 min 95%A, 5% B. Various concentrations of HSA in the sample caused the same effect as HFBA; the results did not show any peaks in the chromatogram that could indicate the presence of

acylcarnitines. Two unknown peaks of m/z 171 and 225 (t~6.9 and t~8.1 respectively) that are not acylcarnitines, remain after adding HSA in any concentration (see figure 19).

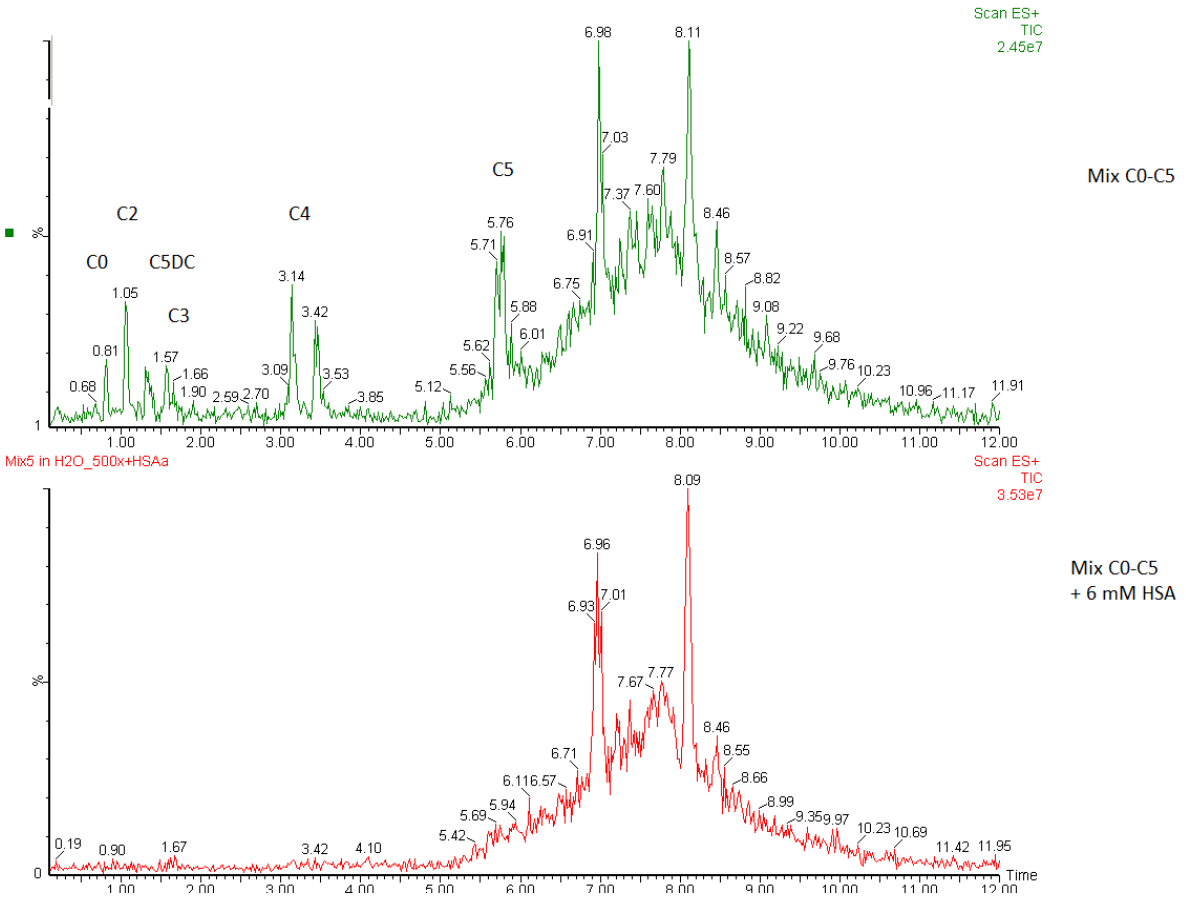


Figure 19 Separation of short-chain acylcarnitines (C0-C5) without adding HSA (upper chromatogram) and after adding HSA (bottom chromatogram)

All the acylcarnitines are readily detectable in the pure mixture without any additives. However, full scan analysis and parent ion scan of samples containing HSA as the additive did not show any peaks in the chromatogram indicating a presence of acylcarnitines. A highly sensitive MRM method was therefore applied. The gradient elution was as follows: 0-3 min 95% A, 10% B; 3-6 min 40% A, 60% B, 6-9 min 95% A, 5% B, 9-12 min 95%A, 5% B. Following MRM transitions were applied:

161 → 85 for L-carnitine (C0)

204 → 85 for acetylcarnitine (C2)

218 → 85 for propionylcarnitine (C3)

232 → 85 for butyrylcarnitine and isobutyryl carnitne (C4)

246 → 85 for 2-methylbutyrylcarnitine and isovalerylcarnitine (C5)

276 → 85 for glutarylcarnitine (C5DC).

Acylcarnitines were detected in the sample with 45 mM HSA, and the retention time of the polar molecules was prolonged as expected. Nevertheless, the signal is nearly 3x weaker (figure 20). Acylcarnitines were detected by MRM method due to its specificity and elimination of the noise. The reduced detector response can be a manifested effect of competition for ionization efficiency in the ionization source between the short-chain acylcarnitines and HSA. Despite the prolonged retention time, the improvement of the chromatographic separation of the isomeric species was not achieved.

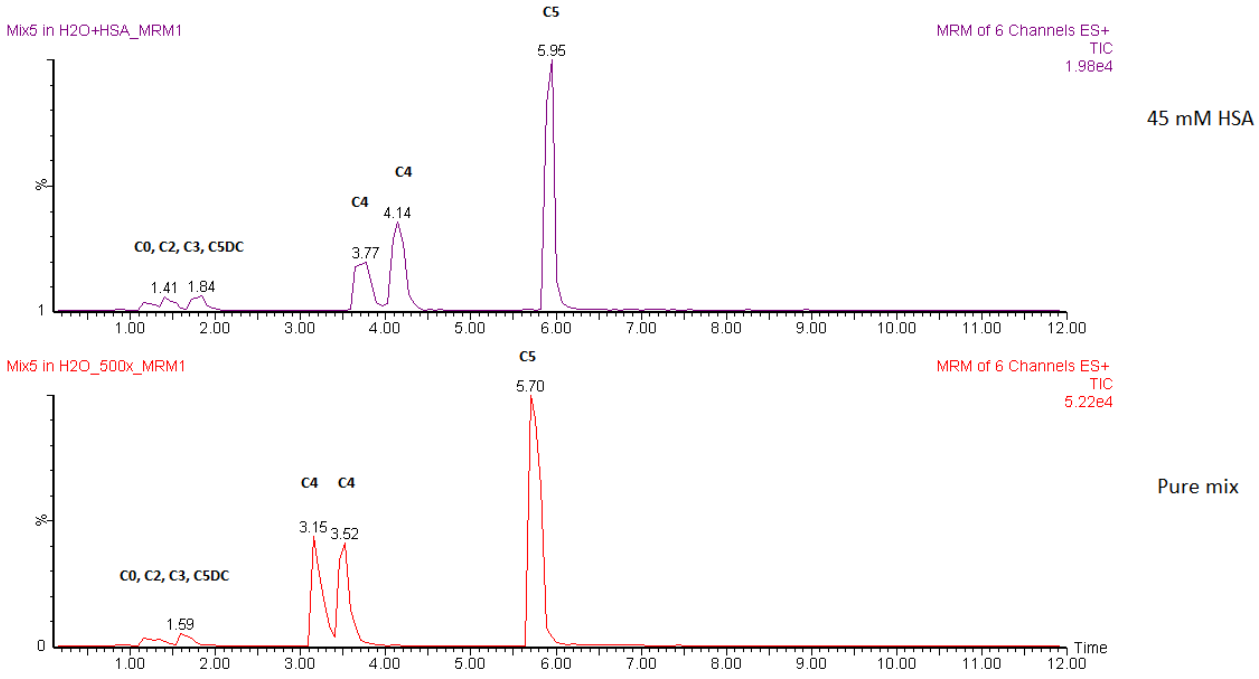
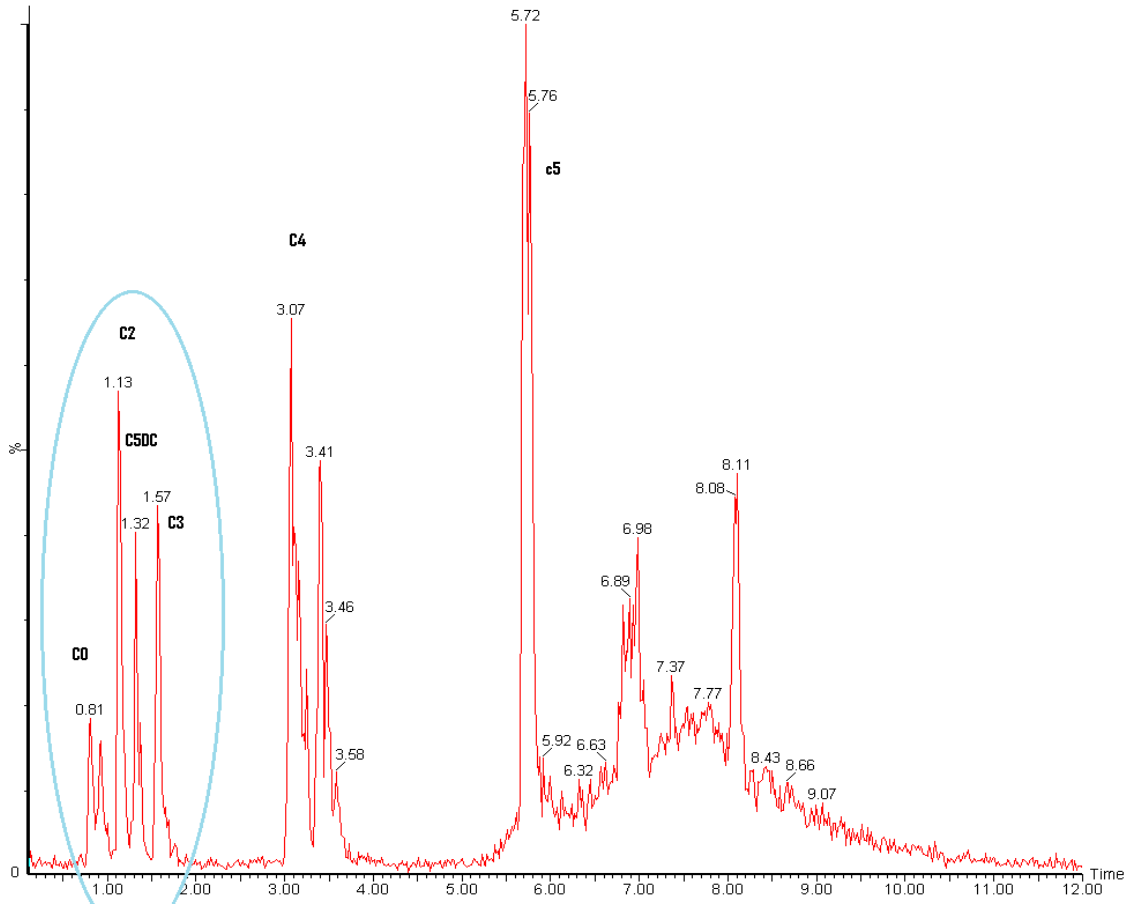


Figure 20 Separation of short-chain acylcarnitines (C0-C5) without adding HSA (bottom chromatogram) and after adding HSA (upper chromatogram) with detection using MRM mode.

To summarize this experiment, ion-pairing reagents are generally successful tools in ion-paring chromatography. In traditional procedures, IPR is added in the mobile phase to make the polar molecules retain in hydrophobic columns. Since IPR in the mobile phase is not compatible with MS/MS detector, a new method that meets LC-MS requirements was developed, where the IPR is added directly to the sample [42]. This method was applied to the analysis of the acylcarnitines in order to improve the separation of polar acylcarnitines and isomeric species. Two IPRs were tested: HFBA and HSA respectively. No matter which IPR was used, a similar tendency was observed. HFBA caused a strong ion suppression and had a negative effect on the separation of acylcarnitines. HSA as an IPR made the polar molecules retain in the column longer. Nevertheless, the ion suppression occurred as well when adding HSA to the sample. For the mentioned reasons, IPR is not used for further laboratory work.

3.4.1.6 Water as a dilution solvent

Several testing runs were performed in order to investigate the effect of the dilution solvent on the sample appearance of the UHPLC-chromatograms. Water as a dilution solvent provides improved separation results for the polar acylcarnitines. Separation of the short-chain polar acylcarnitines of the chain lengths C0, C2, C3 and C5DC was observed when using water as a dilution solvent instead of water:methanol 1:1 (see figure 21).



Figur 21 Separation of the short-chain acylcarnitines was achieved when using water a dilution solvent.

Due to the big challenge to separate polar short-chain acylcarnitines C0, C2, C3 and C5DC, further method development is focused on the acylcarnitines C4 to C18 that retain in the column. The separation of the short-chain acylcarnitines is a subject of another research.

3.4.2 MS/MS method development

Tandem mass spectrometry is a technique of interest in metabolomics due to the strong specificity and high sensitivity, not only in the determination of acylcarnitines. In this study, the technique involves mass selection of the target acyl carnitine ion, fragmentation of the mass-selected ion, and m/z analysis of the fragment ions with m/z 85 which is common to all the acylcarnitines. An MS/MS triple quadrupole utilizes two mass analyzers to enhance the

selectivity through collision-induced dissociation in the collision cell. The first step in the method development is an investigation of the MS response and a development of SIM and MRM method. For the investigation of the MS response, it was necessary to determine if acylcarnitines can be ionized and if they can be detected without any chemical modification. This includes finding the optimal condition in the ion source and in the CE-collision cell.

Both SIM and MRM methods were developed. In the SIM method, the abundances of ions of one or more specific m/z values are recorded. This mode of operation typically results in an increased sensitivity compared to the full scan mode (where the entire mass spectrum is recorded). All ions having target m/z value reach the detector (unlike in full scan mode). Higher selectivity is achieved with MRM method which uses two analyzers in SIM mode to select a quantifier and a qualifier ion of interest.

The process of development of the MS/MS method involves a series of testing various parameters to achieve the optimal set of conditions. Parameters which need to be optimized for an MS detection and quantification of acyl carnitine molecules include MRM transition ions, capillary voltage, cone voltage, ionization mode, source temperature, dwell time and collision energy.

The mode chosen for ionization for detection was an electrospray operating in a positive mode. Dwell time corresponds to sampling rate of the detector. It is calculated from the peak-width at the base. Reproducibility and accuracy is achieved with 10-15 data points across the chromatography peak [43]. Dwell time of 0.600 s calculated for the MRM in this study corresponds to 15 data points. The capillary voltage was 3.0 kV and the source temperature 200ºC.

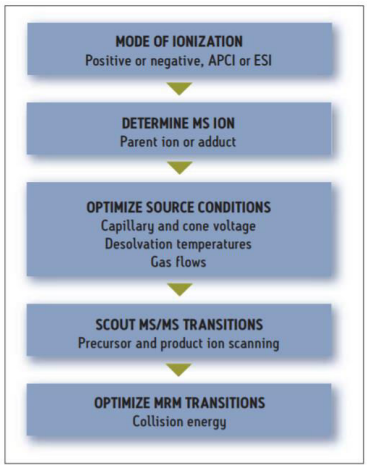


Figure 22 A workflow of MS/MS method development [14]

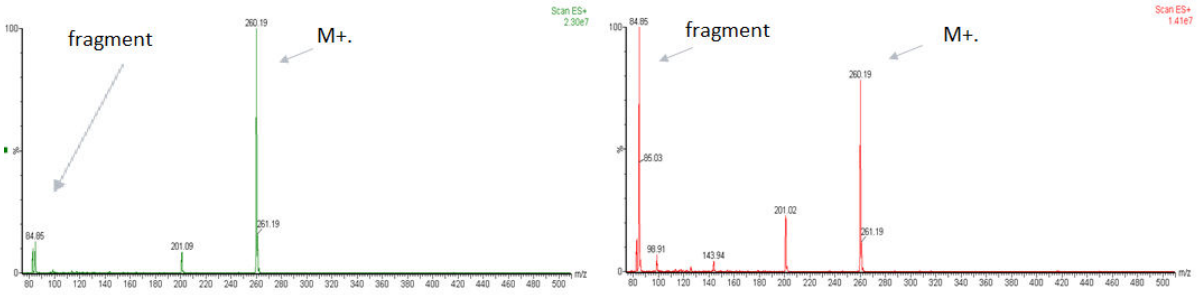
3.4.2.1 Cone voltage

Cone voltage was adjusted for both methods, SIM and MRM. Optimal cone voltage for the SIM method causes fragmentation of the molecular ion and provides both, molecular ion and fragment ions. Ideally, the settings of the ion source should provide high abundance of the molecular ion and high abundance of one or two fragment ions. The molecular ion can be used as the quantifier ion and the fragment ions as qualifier ions in quantitative analysis. Optimal cone voltage was determined for each acyl carnitine ester. The mixture of the acylcarnitines was diluted 50x in the dilution solvent (methanol:water 1:1) and analyzed in full scan mode with various CV (15V, 20V, 25V, 30V, 35V, 40V, 45V, 50V, 55V). Each measurement was performed twice. The spectrum of each acyl carnitine was evaluated, and the optimal CV that provided required fragmentation was chosen.

SIM-based analysis were subsequently performed with the optimal CV, and the limit of detection (LOD) and the lower limit of quantification (LLOQ) were determined for individual acylcarnitines. The detection limit is the lowest quantity of the acyl carnitine that can be distinguished from the noise [44]. Visual determination of the LOD was performed by preparing samples with known concentrations of acylcarnitines and by establishing the level of reliable detection. Signal to noise ratio (S/N) 3:1 is considered acceptable to estimate LOD and was determined by visual evaluation of spectra. S/N 10:1 is considered acceptable to estimate the lower limit of quantification (LLOQ). LOD and LLOQ were determined for

individual acylcarnitines [45]. The LOD determined by the SIM method was in the range of hundreds pg/ml. The LLOQ was calculated as a triple fold of the LOD.

The cone voltage was adjusted for the MRM method. The optimal cone voltage for the MRM method is usually lower than the one for the SIM method in order to obtain a higher yield of the molecular (mother) ion (figure 23). The mother ion selected in the first quadrupole is sent to the collision cell (second quadrupole) where the collision-induced dissociation takes place. A fragment of interest (daughter or product ion) is selected in the third quadrupole. The high specificity is achieved by double selective monitoring of the molecular ion and its product ion. The optimal cone voltage was determined for each acyl carnitine. The area of each chromatography peak of acylcarnitines was calculated by the software. The cone voltage of choice provided a molecular ion with the largest area.



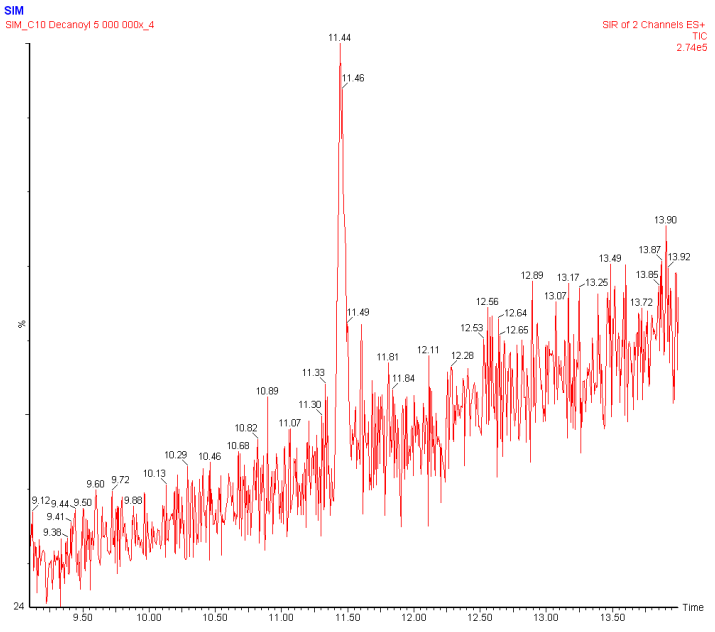
Figur 23 Optimization of CV for MRM (left) and SIM method (right)

3.4.2.2 Collision energy

Collision-induced dissociation is a technique to induce fragmentation of selected ions in the collision cell to obtain their product ions. Molecular ions selected in the first quadrupole are fragmented in the gas phase by collisions with molecules of an inert gas. Product ions are subsequently selected in the third quadrupole before reaching the detector. The efficiency of formation of product ions in MS/MS depends on the collision energy, nature of the collision gas, and flow rate of the collision gas. Argon was used as the collision gas. Product ion scan operational mode was chosen for an investigation of the optimal flow rate of the collision gas and collision energy for each acyl carnitine. The flow rate of the collision gas turned out to be

an important parameter to obtain a good fragmentation. The initial flow rate of 0.3 ml/min was too strong and led to inadequate fragments only. A flow rate of 0.2 ml/min was not enough due to the opposite reasons. Enough fragmentation was achieved with the flow rate of the collision gas of 0.25 ml/min. The molecular ion of each acyl carnitine was selected and various CE values tested. Product ion scan gave all the product ions resulting from the fragmentation of the molecular ion. CE was optimized for each acyl carnitine. The fragmentation of all the acyl carnitine molecules led to a common product ion m/z 85. All the MRM transitions of acylcarnitines contained the common specific product ion of m/z 85. The measured m/z value of the product ion was 84.89 due to an imperfect calibration of the UPLC-MS/MS instrument.

LOD and LLOQ were determined using the MRM method. Samples diluted to the concentration in the range of tens pg/ml showed acceptable S/N ratio 3:1. LOD estimated with MRM method is lower than the LOD determined with SIM method due to the reduced noise. The LLOQ was calculated as a triple fold of the LOD.



Figur 24 Example of the visual evaluation of the limit of detection of decanoylcarnitine with the acceptable signal-to-noise ration 3:1.

3.5 Relative quantification

The quantitative analysis starts with creating a standard curve. Linear dynamic range determines LLOQ and ULOQ. Oleoyl-1-^{13}C-L-carnitine hydrochloride was applied as an isotope-labeled internal standard (IST). Isotope-labeled IST is used for quantification of endogenous compounds such as acylcarnitines [31]. The acylcarnitine of interest is measured relative to the added IST. The choice of IST is based on several requirements such as the same chemical properties as the analyte and possibility to separate IST from the analyte.

Five samples for creating the standard curve were prepared by adding an increasing amount of the acyl carnitine mixture and equal amounts of ^{13}C-isotope-labeled IST. The calibration samples contained acyl carnitine mix of concentrations 20ng/ml, 40 ng/ml, 80 ng/ml, 160 ng/ml and 320 ng/ml. IST was added to each sample in concentration of 80 ng/ml.

When applying ^{13}C-isotope-labeled IST, correct mass spectral data from stable isotope labeling experiments for the distorting effect of naturally occurring stable isotopes must be calculated, because 1,1% of ^{13}C isotope occurs naturally in all molecules [46].

The corrected area of ^{13}C-labeled IST is used to create the standard curve, where y-axis displays a ratio of <area of analyte/area of ^{13}C-labeled IST> and x-axis displays the amount of added analyte.

In relative quantification, changes in a given sample are analyzed relative to another reference sample. Relative quantification was planned to be performed to analyze alterations in the acylcarnitine profile of patients with IBD against healthy control. However, technical failure of the UPLC-MS/MS instrument made it impossible to finish the measurement of calibration samples. The UPLC-MS/MS instrument is currently being replaced and the measurement of the calibration samples is going to be performed on the new instrument.

4 Method Validation

Method validation is performed to demonstrate the reliability of the method and to determine concentration of the molecule of interest in a biological matrix such as plasma, serum, blood, urine, tissue, food or environmental samples. This method was developed to determine the acyl carnitine profile in the colon biopsy. Validation is required whenever a new method is established [47]. The validation parameters of a bioanalytical method are given to ensure the reliability of the final results. Some of the relevant parameters recommended by the regulatory bodies include selectivity, specificity, accuracy, precision, the limit of detection, lower limit of quantification, carry over, calibration curve, matrix effects, robustness and ruggedness [48].

4.1 Selectivity and specificity

Selectivity refers to the extent to which a method can differentiate the analytes of interest and internal standards from components in the matrix or other components present in a sample. A selective method should be able to determine an analyte of the interest without interferences from other molecules present in a sample [48]. Specificity means 100% selectivity when interferences are supposed to occur. One of the approaches to assess selectivity is an analysis of certified reference materials [45]. Specificity (100% selectivity) was achieved with the development of the UHPLC separation method and the specific MRM method. UHPLC coupled with MS/MS detection method involves 3 selective steps; the first selection takes place in the chromatography column, the second and the third selection steps are performed by two mass analyzers in the MS instrumentation. MRM operational mode selects a molecular ion of interest and subsequently a fragment ion of interest which comes from fragmentation of the selected molecular ion. Probability that one compound provide exactly the same molecular and fragment ions is extremely low which makes the MRM technique specific.

4.2 Accuracy

Accuracy indicates a degree of agreement how close is the experimental value to the true accepted reference value [48]. Determining accuracy provides information to which extent

systematic errors affect a method. In bioanalytical methods, accuracy is expected to be better than 15% [47]. Systematic errors include personal errors, errors of the method or errors of the instrument [45]. In theory, they can be minimized during the method development. Two major strategies used to determine accuracy involve measuring an analyte of interest in a reference material and comparing the result with a certified value or determination of the concentration of an analyte in the sample by means of the standard addition technique [45]. Standard addition technique is used when blank samples are not available, such as analysis of endogenous compounds in human samples (acyl carnitine profile alteration in colon biopsies). Due to the instrument failure, experiment to determine accuracy could not be performed.

4.3 Precision

Precision describes how close the repeated individual measurements under specific conditions are. Precision leads to the repeatability and reproducibility in the method validation. A difference between repeatability and reproducibility was explained by P.Araujo in his paper [45]: "*The repeatability ensures that the variability of the results remains constant under identical conditions... The reproducibility makes certain that the variability of the results remains under different conditions and the variation of one or more factors does not contribute significantly to the variability of the results".* Random errors that affect precision can be caused by different uncontrollable variables [45]. The precision is determined by replicating the measurement.

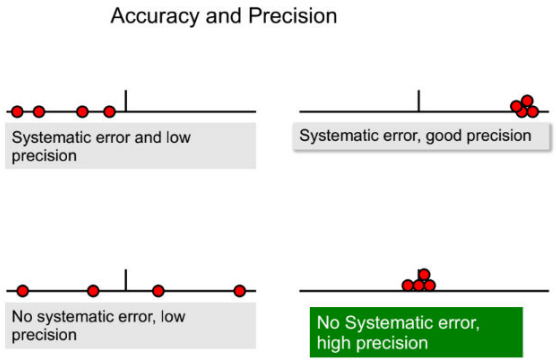


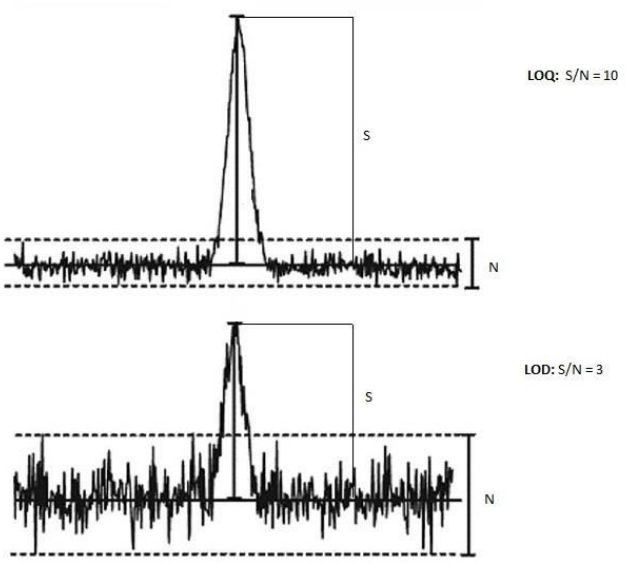
Figure 25 Schematic difference between accuracy and precision (picture taken from lectures of prof.E.Jensen)

4.4 Limit of detection

The limit of detection is defined as the lowest amount of the analyte of interest which is possible to detect with certain reliability. Different approaches are used to determine the LOD, such as visual determination or calculation of the signal to noise relationship. It is mainly based on a minimum signal-to-noise ratio related to a noise baseline. For chromatographic separation techniques, S/N ratio shall be minimum 3:1 [44, 45]. An approach for determination of the LOD used in this study was based on a visual determination of the LOD, performed by preparing samples with the known concentration of the analyte [44].

4.5 Lower limit of quantification

The lower limit of quantification defines the lowest concertation of the analyte that can be quantified and determined with a certain level of precision and accuracy. The LLOQ is evaluated by using similar approaches as for LOD as described in the previous paragraph. LLOQ can be determined based on the visual evaluation of based on the S/N ratio. S/N ratio is normally defined 10:1 for estimating LLOQ [45, 47]. LLOQ is considered being the lowest calibration standard. LLOQ was calculated as the triple fold of the LOD.



Figur 26 Schematic representation of LOQ and LOD. S/N of 10:1 determines LOQ (upper peak) and S/N of 3:1 determines LOD (bottom).

4.6 Calibration curve

Five calibration standards were prepared to perform five-point calibration. For quantification of acyl carnitine concentrations, ^{13}C-isotope-labeled standard 1-^{13}C -oleoyl carnitine was added to the mixture of acylcarnitines. Calibration curves are generated by addition of the increasing amount of acyl carnitine standard mixture and the same amount of the isotope-labeled IST. When using ^{13}C labeled internal standards, a corrected value of the mass spectrometry data must be calculated due to a natural abundance of the heavy stable ^{13}C isotope. The linear range of the calibration curve determines LLOQ and ULOQ [44]. Unfortunately, calibration standards could not be measured due to the failure of the instrument. Therefore, calibration curves are not included in this project.

4.7 Carryover

Carryover is caused by residual analyte from a sample from the previous run. This parameter should be investigated and minimized because it can affect accuracy and precision [49]. Carryover was assessed by injecting a blank sample after analyzing a high-concentration sample. Carryover in the blank sample was not greater than 20% of the LLOQ.

4.8 Stability

Acyl carnitine reference standards were stored in -20°C. Solutions of individual acylcarnitines and acyl carnitine mixtures were stored at 4°C. It was observed that the solutions were stable when stored at room temperature overnight prior analysis.

4.9 Robustness

Robustness is defined as the non-significant reaction of an analytical method to small changes of the internal parameters [45] such as flow rate, column temperature, injection volume, mobile phase composition or cone voltage. This analytical method was robust in terms of mobile phase composition, injection volume, column temperature, and cone voltage. Mobile phase containing methanol/water provided comparable separation as mobile phase containing

acetonitrile/water. Various injection volumes and various column temperatures did not affect the analytical method. Significant variation in fragmentation was observed with variations of the flow rate of the collision gas. The optimal flow rate of the collision gas 0.25 ml/min provided sufficient fragmentation while the flow rate of 0.3 ml/min was already too strong and resulted in tiny fragments only. Collision energy must have been adjusted according to the flow rate of the collision gas. Thus, the robustness of the method was weak in terms of flow rate of the collision gas and collision energy.

4.10 Ruggedness

Ruggedness is defined as a non-significant reaction of an analytical method to external parameters. It evaluates how constant the results when varying factors such as analyst, instruments, laboratories or reagents. Ruggedness determines reproducibility under normal conditions [45]. The method development was performed in a laboratory at IFA using the same UHPLC-MS/MS instrument. I have performed all the measurements, therefore the ruggedness is not validated for this method.

5 Results

5.1 Chromatographic separation

Optimized UHPLC conditions are summarized in the table 5.

Table 5 Optimized UHPLC conditions

Column	ACQUITY UPLC HSS C18 (1.8 µm, 2.1 x 100 mm)
Mobile phase	A: 0.1% FA in water B: 0.1% FA in acetonitrile
Flow rate	0.2 ml/min (0-3 min) 0.3 ml/min (3-22 min) 0.4 ml/min (22-28 min)
Gradient	0-3 min 95% A, 5% B 3-18 min 1% A, 99% B 18-21 min 1% A, 99% B 21-22 min 95% A, 5% B 22-28 min 95% A, 5% B
Injection volume	300 µl
Column temperature	50^0 C
Sample temperature	Room temperature
Dilution solvent	MeOH:H_2O 1:1

Chromatographic separation was obtained using an ACQUITY UPLC HSS C18 (1.8 µm, 2.1 x 100 mm) column at the temperature of 50ºC on the column. A C18 column was selected over an amide column X Bridge BEH Amide (2.5 µm, 2.1 x 75 mm) column XP because of significantly better separation achieved on the C18 column. The separation was achieved with water and acetonitrile as mobile phases, both containing 0.1% FA, at the flow rate of 0.3 ml/min. A flow rate of the mobile phase was adjusted to 0.2 ml/min during the first three minutes of the analysis in order to obtain better separation of the short-chain acylcarnitines and isomers. Column equilibration was performed with the flow rate of 0.4 ml/min to speed up the process of equilibration. Gradient elution was as follows: 0-3 min 95% A, 5% B (flow rate 0.2 ml/min); 3-18 min linear gradient to 1% A, 99% B; 18-21 min 1% A, 99% B; 21-22 min 95% A, 5% B; 22-28 min equilibration of the column with 95% A, 5% B (flow rate 0.4 ml/min).

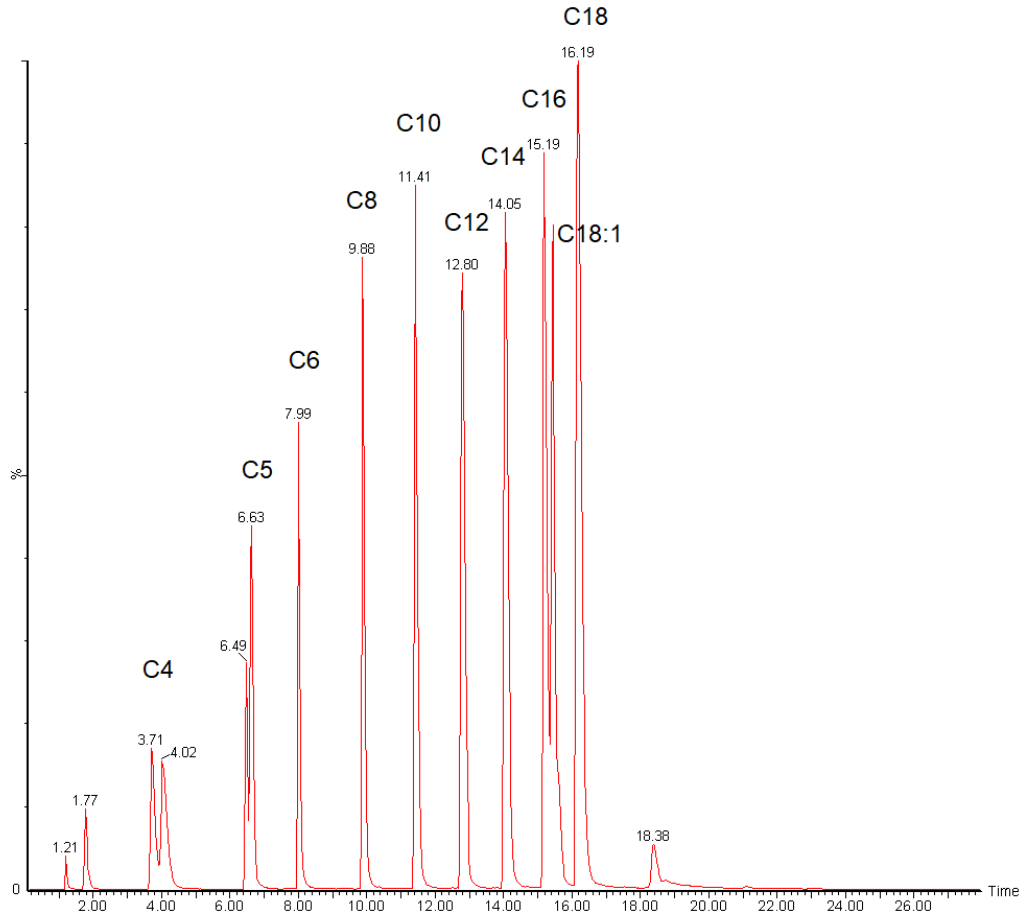
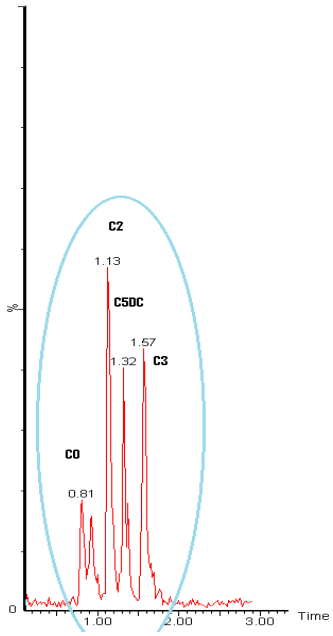


Figure 27 Achieved chromatographic separation of acylcarnitines

A mobile phase consisting of water/acetonitrile with 0.1% FA was favoured over water/methanol with 0.1% FA due to the better ionization ability and the peak shape. Ammonium acetate as a buffer did not result in any significant changes regarding the chromatographic separation.

Ion-pairing reagents were tested as additives in order to change retention time of the polar short-chain acylcarnitines on the hydrophobic C18 column. A new approach of ion pairing chromatography has been used to overcome the incompatibility of IPR with MS detection. IPR was added directly to the sample instead of the mobile phase to avoid the continuous flow of non-volatile IPR into the mass spectrometer [42]. Compounds used as IPR when analyzing acylcarnitines were strong organic acids such as heprafluorobutyric acid (HFBA) and hexanesulfonic acid (HSA). Adding IPR to the samples caused a strong ion suppression. Consequently, IPR was not used further in this study.

The continuous problem with the co-elution and the short retention of the short-chain acylcarnitines was partially solved after exchanging the dilution solvent. Separation of the short-chain polar acylcarnitines of the chain lengths C0, C2, C3 and C5DC was observed when using water as a dilution solvent (instead of water:methanol 1:1). Better separation of short-chain acylcarnitines could have resulted from weaker eluotropic strenght of the dilution solvent. Differences in the solvent strengh might have caused changes in retention behaviour of the polar compounds.



Figur 28 Separation of polar acylcarnitines when using water as a sample diluent

5.2 MS/MS parameters

The process of development of an MS/MS method involves testing a series of various parameters which include ionization mode, MRM transition ions, capillary voltage, cone voltage, source temperature, dwell time, and collision energy. Acylcarnitines were ionized using ESI in positive mode. Optimized MRM conditions are summarized in the table 6.

Table 6 Optimized MRM conditions

Ionization mode	ESI+
Capillary	3.0 kV
Source temperature	200^0 C
Collision gas	Argon
Collision gas flow rate	0.25 ml/min
Dwell time	0.600 s

Cone voltage was adjusted for both, SIM and MRM method. Optimal cone voltage for the SIM method causes fragmentation of the molecular ion and provides both, molecular ion and fragment ions. An increased selectivity was achieved by monitoring the molecular ion and the fragment ion as a quantifier and qualifier ion respectively. The CV was optimized for each acyl carnitine. Subsequently, LOD and LLOQ were determined in the SIM mode. S/N 3:1 was observed with the final dilution 1 000 000x and 5 000 000x which corresponds to the concentration of 1 ng/ml and 200 pg/ml respectively. Optimal CV for the SIM method, LOD and LLOQ are shown in the table 7.

Table 7 Optimal CV for SIM, LOD and LLOQ determined by SIM

Acylcarnitine	**Acyl**	**$[M+H]^+$**	**CV [V]**	**LOD [ng/ml]**	**LLOQ [ng/ml]**
Butyrylcarnitine	C4	232	40	0,2	0,7
Isobutyrylcarnitine	C4	232	40	0,2	0,7
2-methyl-butyrylcarnitine	C5	246	40	1	3
Isovalerylcarnitine	C5	246	40	0,2	0,7
Hexanoylcarnitine	C6	260	40	0,2	0,7
Octanoylcarnitine	C8	288	45	0,2	0,7
Decanoylcarnitine	C10	316	45	0,2	0,7
Lauroylcarnitine	C12	344	50	1	3
Myristoylcarnitine	C14	372	50	1	3
Palmitoylcarnitine	C16	400	50	0,2	0,7
Stearoylcarnitine	C18	428	55	0,2	0,7
Oleoylcarnitine	C18:1	426	55	1	3

The optimal cone voltage for the MRM method was lower than the CV for the SIM method in order to achieve a very gentle fragmentation that yields to the large amount of the molecular ion. The optimal cone voltage was determined for each individual acyl carnitine. Cone voltage of choice for MRM provided the highest yield of molecular ions.

Molecular ion of each acyl carnitine was selected in the first quadrupole and sent to the collision cell (second quadrupole), where the collision-induced dissociation produced fragment ions. Product ion of interest (m/z 85 for all the acylcarnitines) was selected in the third quadrupole. Parameters such as collision energy and flow rate of the collision gas were optimized in order to achieve an optimal production of ions of m/z 85. Optimal parameters were obtained with the daughter ion scan method. The flow rate of the collision gas (argon) was optimized to 0.25 ml/min. The collision energy of individual acylcarnitines was adjusted accordingly to provide sufficient fragmentation.

LOD and LLOQ were determined with the MRM method. LOD was determined by visual evaluation of chromatograms. Estimated LOD with S/N ratio 3:1 corresponds to concentration 10 pg/ml. LLOQ was calculated as a triple fold of the LOD. The LOD and LLOQ determined with MRM method were lower than with SIM due to eliminated noise. Results are shown in the table 8.

Table 8 MRM transitions, optimized CV and CE, LOD and LLOQ determined by MRM

Acylcarnitine	**Acyl**	**MRM (m/z)**	**CV [V]**	**CE [V]**	**LOD [pg/ml]**	**LLOQ [pg/ml]**
Butyrylcarnitine	C4	232 →85	30	25	10	30
Isobutyrylcarnitine	C4	232→85	30	26	10	30
2-methyl-butyrylcarnitine	C5	246→85	30	26	10	30
Isovalerylcarnitine	C5	246→85	30	26	10	30
Hexanoylcarnitine	C6	260→85	30	26	10	30
Octanoylcarnitine	C8	288→85	33	26	10	30
Decanoylcarnitine	C10	316→85	37	26	10	30
Lauroylcarnitine	C12	344→85	38	26	10	30
Myristoylcarnitine	C14	372→85	38	26	10	30
Palmitoylcarnitine	C16	400→85	38	28	10	30
Stearoylcarnitine	C18	428→85	38	30	10	30
Oleoylcarnitine	C18:1	426→85	38	30	10	30

6 Discussion

6.1 Metabolomic studies of carnitine for IBD research

Metabolomics has recently gained considerable interest because it enables system-wide measurements of metabolites and low-weight molecules in biological samples. Current metabolomic technologies are powerful tools in clinical medicine. Metabolomic studies stand behind the discovery of new therapeutic targets and biomarkers and thus has a big potential to add new knowledge to a field of clinical chemistry [30]. Carnitine gained an interest in the field of metabolomics due to its essential role in the energy metabolism and fatty acid transportation. Carnitine is a biomolecule that works as a carrier of activated fatty acids to the matrix via a mitochondrial membrane, where they undergo beta-oxidation [2]. Alterations in the acyl carnitine profile can provide clinically valuable information about metabolic disorders. Carnitine deficiency was associated with various diseases such as cirrhosis and liver disorders, obesity, endocrine disorders, diabetes and recently also with inflammatory bowel diseases (IBD) [4, 7].

IBD is an inflammatory response to intestinal microbiota which is caused by a combination of genetic and non-genetic factors [11]. However, the pathogenesis of IBD remains unknown. Absorption of carnitines from the diet takes place in an intestine by an active and passive transport across membranes of enterocytes. Carnitine as a transporter of fatty acids to mitochondria plays a key role in the energy production for the intestinal tissues. Thus, acylcarnitines are investigated as potential biomarkers of IBD.

Various acylcarnitines occur in isomeric forms or at very low concentrations. Development of a sensitive and selective method is necessary in order to analyze acyl carnitine profile including isomeric species because of their clinical relevance. The invention of hyphenated technique UHPLC-MS/MS was a big step forward in the clinical analysis. The development of the bioanalytical UHPLC-MS/MS method requires optimization of three main steps including a sample preparation, chromatographic parameters and the mass spectrometry [14]. All the three steps can influence each other and require adjustments of several parameters. Bioanalysis of acylcarnitines meets certain challenges. Previously mentioned low abundance of acylcarnitines in complex matrices such as body liquids or tissues requires very sensitive

technique. Acylcarnitines contain acyl-chains of various chain-lengths which brings certain challenges during the separation due to different physicochemical properties of individual acylcarnitines. Here we have described a sensitive and selective method for the analysis of acylcarnitines by UHPLC-MS/MS.

6.2 Chromatographic separation

Modern UHPLC instrumentation has the potential to deliver the desired separation and narrow chromatography peaks. Despite of the modern instrumentation abilities, broader peaks and insufficient separation can appear. Several parameters must be considered before the analysis in order to obtain narrow peaks. Band broadening can be caused by too large injection volume or it can appear due to a wrong choice of the sample solvent [21]. It can occur in the chromatography column or in the tubing connecting an injector and the column or column and a detector. A good choice of the chromatographic and optimization of the chromatographic parameters such as the flow rate of the mobile phase and injection volume are essential in order to achieve desired resolution.

The chromatographic separation was established using ACQUITY UPLC HSS C18 (1.8 µm, 2.1 x 100 mm) column. Separation was achieved with gradient elution. The first problem occurred regarding the repeatability of the measurements. Repeated measurements provided weaker signals of the acylcarnitines and showed co-elution of the short- and medium-chain esters. One hypothesis to explain this problem was sample adsorption to the container. Quaternary ammonium cations and amines has a potential to adsorb to glass vials. Since the separation was very poor also with a freshly prepared mixture, the core of the problem was in an insufficient column equilibration. Longer equilibration of the column was essential to obtain the same chromatograms with repeated measurements. Total running time 28 min included 5 min of equilibration.

The separation problem regarding short retention and co-elution of the short-chain acylcarnitines remained due to their polar properties. Gradient elution with very weak mobile phase consisting of acetonitrile/water prolonged the retention time, however, acylcarnitines with short acyl chains such as C0, C2, C3 and C5DC co-eluted and isomeric species (two C5 isomers and two C4 isomers) were separated with a poor resolution. To achieve separation of

the polar carnitine species, amide column X Bridge BEH Amide (2.5 µm, 2.1 x 75 mm), column XP was applied. A hydrophilic stationary phase of the amide column was designed to retain polar analytes therefore the column was tested in order to solve the separation problem and improve the poor resolution. Optimization of the method performed on the amide column did not lead to any improvement of the chromatographic separation. Therefore, C18 column was chosen for the further laboratory work.

Solvent composition was one of the variables tested in order to achieve sufficient separation. Acetonitrile/water and methanol/water, both mobile phases containing 0.1% FA were applied. FA is added to provide a source of protons. Acetonitrile and methanol are organic solvents commonly used as mobile phases in reversed-phase chromatography. Acetonitrile is 3x more expensive than methanol, however, analytical conditions point at acetonitrile as a solvent of choice for several reasons. Given the same flow rate, acetonitrile-based solutions apply less pressure on the column while the pressure for methanol increases when mixed with water. Elution strength of acetonitrile is generally higher, thus can be used in lower mixture ratio. Elution selectivity differs on each due to different chemical properties of protic (MeOH) and aprotic (ACN) solvents [15]. Since acetonitrile did not provide desired separation, methanol was tested. Chromatographic separation was comparable using the two mentioned solvents as a mobile phase. Selectivity and peak shape were generally better when using acetonitrile, therefore it was used despite its higher costs.

Although analysis of acylcarnitine esters often includes derivatization to enhance sensitivity, analysis of underivatized acylcarnitines was previously described and performed in this study. Derivatization methods include synthesized reagents which can cause unwanted reactions that can influence determination of accuracy [2]. Thus, a UPLC-MS/MS method has been established to separate and identify underivatized acylcarnitines. To improve chromatographic separation, ion pairing reagents were involved. Traditional techniques involve adding an ion pair reagent to the mobile phase to prolong the retention time of ionic analytes on hydrophobic columns. However, IPR is not compatible with MS/MS detection. When using traditional detector, the mobile phase pass through the detector and goes to waste or can be collected for further studies. There are many restrictions with respect to the composition of the mobile phase when using a MS detector, because the eluate from the chromatographic column is deposited in the ion source. In the traditional procedure, IPR is continuously

introduced into the mass spectrometer which can cause its contamination that leads to signal reduction, ion suppression and requires long cleaning procedures afterward [42]. A new approach to ion-pairing chromatography was applied to our samples of acylcarnitines that were posing significant challenges to LC-MS/MS method development [42]. IPR was added directly to the samples instead of adding it to the mobile phase. Continuous introduction of IPR in the mobile phase was avoided and the negative impact on MS/MS minimized. Compounds used as IPR were strong organic acids HFBA and HSA for analysis of acylcarnitines. HSA was favored over HFBA as it made the polar molecules retain in the column longer, nevertheless, the signal was very weak probably due to a strong ion suppression. The chromatogram displayed unknown peaks that could have been caused by a contamination of the sample. Unknown peaks remained also when analyzing a new freshly prepared mixture, which means that IPR might caused unwanted reactions in the sample. For the mentioned reasons, IPR was not used for the further method development for analysis of acylcarnitines. Despite of testing various concentration of IPR, the lowest used concentration might have been still too high. Possibly, incubation was needed to obtain the molecular formation of acylcarnitines and IPR. Further investigation to determine the effect of lower concentrations of IPR added directly in the samples on ion suppression should be performed.

Separation of the short-chain polar acylcarnitines of the chain lengths C0, C2 and C3 was observed when using water as a dilution solvent (instead of water:methanol 1:1). Water probably provided better environment for dissolution of the polar carnitines which enhanced their chromatography separation. Better separation of short-chain acylcarnitines could have resulted from weaker eluotropic strenght of the dilution solvent. Differences in the solvent strengh might improved retention behaviour of the polar compounds.

6.3 Mass spectrometry

ESI in positive mode was applied for ionization. Cone voltage was adjusted for two operational modes separately and for each individual acylcarnitine. Setting the cone voltage for individual carnitines is possible due to programmable time windows. The voltage settings may not be transferable to another instrument. SIM and MRM operational modes require

different levels of fragmentation. Higher cone voltage was applied for SIM mode to obtain both, molecular and fragment ions. MRM method requires lower cone voltage to obtain high yield of a molecular ion which is fragmented later in a collision cell. Inicially, the collision energy was adjusted when using the flow rate of argon 0.3 ml/min.. The flow rate turned to be too harsh since only tiny fragments were obtained undependently on the collision energy. Much lower rates resulted in insufficient fragmentation. The adjustment of the argon flow rate turned out to be an influential parameter for the collision-induced dissociation. The flow rate of 0.25 ml/min provided the desired fragmentation. Collision energy was optimized using the flow rate of 0.25 ml/min. Collision induced dissociation produced the an intense characteristic daughter ion for acylcarnitines of m/z 85. The instrument provided m/z value of 84,89 due to its imperfect calibration.

Dwell time is another parameter which should be adjusted when using MRM method. MS is a digital technique which requires a complete control of the scan speed vs the peak width. The dwell time determines how often the chromatography peak should be sampled by the detector. It is calculated from the peak-width at the base. The optimal dwell time requires at least ten data points across the peak to describe the peak with some accuracy and reproducibility and to avoid introducing both systematic and random errors to the analysis. A dwell time that provides 10 -15 data points across the chromatography peak is optimal for accurate quantification, including low abundant analytes [43]. Dwell time of 0.600 s calculated for the MRM in this study corresponds to 15 data points.

Limit of detection of individual carnitines was estimated by a visual evaluation using both, MRM method and SIM method. Visual evaluation method for determining LOD in the samples of known analyte concentration was favored over calculations based on the S/N ratio. Technical fails of the instrumentation software provided unreliable results of the S/N calculations. The LOD was lower with MRM due the high selectivity of the method and consequent elimination of the noise.

One of the methods for calibration is a stable isotope dilution. Stable isotope dilution is almost exclusively used with mass spectrometry due to its ability to distinguish isotopic species. Known amount of isotopically-labeled substance added to the sample changes the isotopic composition of the analyte. Isotope dilution employs signal ratios to determine the changes in the isotopic composition which enables us to calculate the amount of the analyte of

interest. Labelled and unlabelled compounds are presumed to behave identically due to the same physicochemical properties which means that IST follows the analyte through all the analytical steps in an identicall manner. The last detection step of the analytical assay allows their differentiation based on their different m/z values.

Five calibration samples were prepared with increasing concentration of acylcarnitine mixture and constant amount of isotope-labelled internal standard oleoyl-1-^{13}C-L-carnitine hydrochloride. However, a technical failure of the UPLC-MS/MS instrument made it impossible to finish the measurement of calibration samples. The UPLC-MS/MS instrument is currently being replaced and the measurement of the calibration samples is going to be performed on the new instrument.

To evaluate UHPLC-MS/MS method performance, method was validated as described previously in the chapter 4. This method will be applied to colon biopsies samples.

7 Concluding remarks

The present project described the development of a novel method to separate and identify acylcarnitine esters with acyl-chain length C4-C18 including four structural isomers. Desired chromatographic separation was achieved on a C18 column using a mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. This method does not require prior derivatization which speeds up the whole analysis and eliminates unwanted side reactions. Structural isomers with acyl-chain of C4 such as butyrylcarnitine and isobutyrylcarnitine and acyl-chain of C5 such as 2-methylbutyrylcarnitine and isovalerylcarnine were successfully separated. This project involved a development of a high sensitivity MS/MS assay for the detection of acylcarnitines in the range of pg/ml. Highly-specific MRM method was developed to determine individual acylcarnitine esters including isomeric species in the sample. The ability to separate and identify isomers is desired and clinically relevant for a biochemical screening of FAO and organic acid metabolism disorders. Acylcarnitine profile alterations have been associated with various diseases including IBD. Development of the new rapid and highly-sensitive method for analysis of acylcarnitine esters has a significant value for further clinical research.

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