MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

"Sample preparation methods for the analysis of the muscle metabolome"

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2023 / Vienna 2023

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 862
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Chemie
Betreut von / Supervisor:	Univ.-Prof. Dr. Gunda Köllensperger

Acknowledgement

First of all, I would like to express my gratitude to every single member of the Koellensperger lab for their willingness to help. They have been a great support, as I could approach them at any time when faced with challenging questions for which I could not find answers on my own. I would also like to thank them for welcoming me to activities outside of work and for allowing me to exchange conversations with them on a friendly level. Also, big thanks to Cristina Coman and Katharina Kaser for lending and explaining me the instruments I needed for this work. Above all, I would like to extend my gratitude to Harald Schöny for his excellent teaching, mentoring and guidance in establishing the work steps and with the handling of new software and equipment. I have gained an enormous amount of valuable knowledge under his tuition within the past six months. I also wish to thank Prof. Gunda Koellensperger for providing me with the opportunity to work on my thesis in her working group. I am glad that I ultimately chose this group after considerable contemplation because I felt genuinely welcomed and well taken care of by everyone. I was also astonished by the knowledge and wisdom of the individuals here. They were all an inspiration and motivation for me. Furthermore, I appreciate Prof. Koellensperger for giving me the opportunity to participate in conferences, enabling me to broaden my horizons in the world of science.

Lastly, I wish to express my gratitude to my parents. My father, who works hard to provide for our family, and my mother, who was willing to make sacrifices and leave everything behind to give her children the opportunity to grow up in a place where the personal development of an individual takes priority over performance pressure. Without their support, I would not have been able to progress this far in all aspects of my life.

Abstract

Malignant hyperthermia (MH) is a genetic disease triggered primarily by inhaled anesthetics. While *in vivo* genetic testing can be used to detect the disease in individuals, this testing method is not very sensitive. An alternative and more reliable option is an *in vitro* diagnosis test known as the halothane/caffeine contracture test (CHCT). However, this test requires a muscle biopsy to access the muscle strips needed. This can lead to various consequential damages for patients, including limited mobility and potential infections. To develop a gentler and less invasive diagnostic method, the potential of metabolomics is currently explored in an international project. The ultimate goal is the discovery of a specific marker molecule (biomarker) for MH on whose detection the new diagnostic method will be based. In order to study the metabolome of muscle tissue, a robust sample preparation method must first be developed.

The purpose of this work is to investigate various sample preparation methods for the analysis of a muscle metabolome, particularly the homogenization step. Liquid chromatography-mass spectrometry (LC-MS) was used for data acquisition. The reproducibility, the ability of the methods to maintain analyte stability in the samples, and the overall method performance were investigated using a selected set of muscle metabolites and lipids based on a targeted analysis.

The investigations indicated that a using pure organic solvent, either in combination with the gold standard method (mortar and pestle) or a high-performance bead homogenizer, yielded the most excellent results. Furthermore, it was observed that maintaining a low temperature throughout the pre-analytical steps is a critical point that must be considered.

Zusammenfassung

Die Maligne Hyperthermie (MH) ist eine genetisch bedingte Krankheit, die hauptsächlich durch inhalative Anästhetika ausgelöst werden kann. Man kann die Krankheit durch *in vivo* Gentests nachweisen, jedoch ist diese Testmethode nicht sehr sensitiv. Eine weitere und zuverlässigere Möglichkeit ist eine *in vitro* Diagnose, der sogenannte Halothan/Coffein-Kontraktionstest (CHCT). Dieser Test erfordert eine Muskelbiopsie, um die für den Test benötigten Muskelstreifen zu entnehmen. Für die Patienten bedeutet dies, dass sie mit möglichen Folgeschäden rechnen müssen, angefangen bei eingeschränkter Mobilität bis hin zu infektiösen Komplikationen. Um eine schonendere Diagnosemethode zu entwickeln, wird das Potenzial von Metabolomics erforscht. Das Ziel ist es, ein für die MH spezifisches Marker-Molekül (Biomarker) zu finden, auf dessen Detektion die neue Diagnosemethode basieren soll. Um das Metabolom von Muskelgewebe zu untersuchen, muss zunächst eine robuste Probenvorbereitungsmethode entwickelt werden, was mich zu dem Ziel dieser Arbeit führt.

In dieser Arbeit sollen Probenvorbereitungsmethoden für die Analyse eines Muskelmetaboloms, insbesondere die Methoden zur Homogenisierung, untersucht werden. Zur Datenerzeugung wurde die Flüssigchromatographie-Massenspektrometrie (LC-MS) angewendet. Die Reproduzierbarkeit, die Fähigkeit der Methoden, die Analyten in den Proben stabil zu halten, und die allgemeine Leistung der gewählten Methode wurden anhand einer definierten Menge an Muskelmetaboliten und -lipiden in einer gezielten Analyse untersucht.

Die Untersuchungen zeigten, dass reines organisches Lösungsmittel in Kombination mit der Goldstandard-Methode (Mörser und Pistill) oder reines organisches Lösungsmittel in Kombination mit einem Hochleistungs-Bead-Homogenisator die besten Ergebnisse zeigten. Darüber hinaus wurde beobachtet, dass die Aufrechterhaltung niedriger Temperaturen während der prä-analytischen Schritte ein kritischer Punkt ist, der unbedingt berücksichtigt werden muss.

List of Abbreviation

Acetonitrile	ACN
Adaptive focused acoustics	AFA
Ammonium formate	AF
Caffeine/halothane contracture test	CHCT
Chloroform	$CHCl_3$
Diacylglycerol	DAG
Electrospray ionization	ESI
External standard	ESTD
Gas chromatography	GC
Hydrophilic interaction liquid chromatography	HILIC
Internal standard	ISTD
Isopropanol	IPA
Liquid chromatography	LC
Liquid-liquid extractions	LLE
Lysophosphatidylcholine	LPC
Lysophosphatidylethanolamine	LPE
Malignant Hyperthermia	MH
Malignant Hyperthermia Association of the United States	MHAUS
Malignant Hyperthermia Negative	MHN
Malignant Hyperthermia Susceptibility	MHS
Mass spectrometry	MS
Mass-to-charge ratios	m/z

Methanol	MeOH
Methyl-tert-butyl ether	MTBE
Microliter	µL
Milliliter	mL
Nitrogen	N_2
North American Malignant Hyperthermia Registry	NAMHR
Nuclear Magnetic Resonance	NMR
Octanol/water partition coefficients	XlogP
Phosphatidic acid	PA
Phosphatidylcholine	PC
Phosphatidylethanolamine	PE
Phosphatidylglycerol	PG
Phosphatidylinositol	PI
Phosphatidylserine	PS
Quality control	QC
Relative standard deviation	RSD
Reversed-phase chromatography	RP
Simultaneous Metabolite, Protein, Lipid Extraction	SIMPLEX
Sphingomyelin	SM
Sphingosine-1-phosphate	S1P
Triacylglycerol	TAG
Ultra high-performance liquid chromatography	UHPLC
Water	H_2O

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1 INTRODUCTION

1.1 Malignant Hyperthermia

The first reported incident of malignant hyperthermia (MH) was documented by Denborough *et al*. in 1962.[1,2] MH is an exceedingly rare and life-threatening genetic disorder, described as an uncontrolled hypermetabolic response of the skeletal muscles to volatile anesthetics such as halothane, desflurane, or sevoflurane, as well as the muscle relaxant succinylcholine. In some cases, it can also be triggered by other factors like extreme stress, excessive heat, or intense physical activity.[3] When MH is triggered by the aforementioned factors, it leads to an uncontrolled release of calcium from the sarcoplasmic reticulum into the cytoplasm of the muscle cells. As a second messenger, calcium ions cause the interlocking of actin and myosin filaments, leading to muscle fiber contraction. In addition, glycogenolysis and cell metabolism are activated, resulting in an overproduction of heat and lactate. The activation of the oxidative cycle leads to heightened oxygen consumption as well as increased carbon dioxide production.[4] Further symptoms include rapid and inconsistent heart rate, raised blood pressure, increased body temperature, masseter muscle spasm, muscle rigidity, acute kidney failure, and release of catecholamines. In the event of an MH episode, anesthetic gas administration needs to be stopped instantly and anesthesia is continued using intravenous agents. Administration of dantrolene, which inhibits the release of calcium from the sarcoplasmic reticulum, at a concentration of 2 mg/kg should be initiated as rapidly as possible and repeated in 5-minute intervals until the patient's cardiac and respiratory systems have returned to a normal and stable state.[5] While the prevalence of genetic predisposition to MH may be as high as 1 in 3,000 individuals, the incidence of an MH reaction during anesthesia is much lower, ranging from 1:5,000 to 1:100,000 cases.[3]

There are two diagnostic methods to test for Malignant Hyperthermia Susceptibility (MHS). The first option is a genetic test, although these tests are not very sensitive. Even if the test yields a negative result, it does not necessarily rule out the presence of MHS. The second method is the caffeine/halothane contracture test (CHCT), a standardized protocol established by the Malignant Hyperthermia Association of the United States (MHAUS), which provides more reliable results.[4] The principle of this diagnostic method is explained in more detail in section 2.1 (p.14).

Allen *et al*. reported in a study that the CHCT exhibits excellent sensitivity of 84-100% and a specificity of 69-97%.[6,7] However, it is worth noting that undergoing a muscle biopsy is a physically challenging procedure for the patients. Most of them cannot walk properly for a decent amount of time after the biopsy. Furthermore, there is a risk of infection, venous thrombosis, or nerve injury, just to name a few, that come with undergoing an invasive surgical biopsy. In addition, there are only a few specialized centers worldwide, in which the test can be performed.[8] Hence, there is a demand for a minimally-invasive diagnostic method for MH screening - a requirement that patients and clinicians are actively seeking to fulfill. This novel method aims to be established by examining specific metabolic features that differentiate Malignant Hyperthermia Susceptibility (MHS) patients from those who are not susceptible to MH (MHN).

1.2 Metabolomics - from its historical evolution to its current understanding

Metabolites are defined as small molecules in biological organisms.[9] They are important components or intermediates in signaling and biochemical pathways as well as key molecules in energy sources. When a set of metabolites synthesized by a biological system is considered, it is referred to as the 'metabolome'.[10] Their concentrations give information on the biological functions of the observed organism and define its phenotypes in response to genetic or environmental changes.[11,12] Therefore, numerous metabolites are used by clinicians as diagnostic markers for biological conditions and responses to chemical treatment to track the patient's health status.[12]

Roger Williams first suggested the idea of the 'metabolic profile' in the late 1940s. He introduced the concept that individual people had individual spectra of metabolites.[13] Through the technological evolution during the late 20th century and thus, the progression of analytical techniques, it was eventually possible to detect metabolites in a quantitative manner. Thus, Hornig *et al*. manifested the idea of the metabolic profile into an analytical discipline 'metabolic profiling' in 1971.[14] It was only in 1998 that the expression 'metabolomics' was introduced by Steven Oliver in a review about the functional genomics of yeast.[15,16] Gonzalez *et al*. defined the term as the systematic quantification and identification of all metabolites in a biological system or in a biological sample.[17] Both endogenous and exogenous metabolites are evaluated and assessed. In other words, metabolomics is a comprehensive analytical method to gather

information from the metabolome of a biological sample.[18] Usually, biofluids, cells, or tissues of the organism under investigation are used for this purpose.[19]

A major challenge in the analysis of the metabolome is the immense diversity of the metabolites in their physicochemical properties (e.g.: molecular weight, volatility, stability, and hydrophobicity), as well as in the wide concentration range.[20] Developing a reliable, robust, and reproducible experimental design demands a substantial amount of knowledge and careful consideration. Compounds of the metabolome include inorganic species to hydrophilic carbohydrates, alcohols, ketones, amino acids, nucleotides, etc., and also lipids. Therefore, it is impossible to comprehensively identify and quantify the entire metabolome in only one analytical run. This also means efficient sample preparation in combination with multiple analytical methods is of outmost importance to extract as much information as possible.[21]

1.3 Importance of sample preparation – there is no universal method

The sample preparation of the biological samples plays a pivotal role in the overall analytical process and has a great impact on the quality of analysis. Besides its importance, sample preparation can be often challenging for the experimenter. In most cases, it revolves around extracting as many metabolites as possible within it.[22] However, this is easier said than done due to their immense diversity. As already mentioned above, the versatile physicochemical properties of the analytes, as well as the wide range of concentrations across several orders of magnitude pose significant challenges in sample preparation. This is also the reason why most errors tend to occur in the pre-analytical phase.[23] It is also worth noting that there is no universal method for covering the full metabolic profile.[22] Hence, exquisite experimental planning is a crucial part of the pre-analytical process.

In **Figure 1**, a very simplified form of a typical workflow for the sample preparation of skeletal muscle tissue is represented. It involves (1) collecting the samples and immediate quenching, (2) homogenizing and powdering the muscle tissue while maintaining a low temperature and (3) extracting the metabolites with the desired method.[24]

Figure 1: Typical workflow of the sample preparation of muscle tissue in a nutshell

The first step, sampling the muscle tissues, is a very critical and the most demanding step. The enzymes in the freshly excised muscle have to be inhibited as fast as possible in order to avoid turnover of the metabolites, which can happen within a second or even less.[21] The inactivation of metabolism can be achieved by either directly placing the sample in pre-cooled organic solvents, such as methanol or chloroform, or by immediate extreme temperature withdrawal through snap freezing the tissue in liquid nitrogen (N_2) at -196 °C.[24] The rapid change in temperature is the most reasonable method to inhibit enzyme activity, according to Fiehn *et al*.[21] Before collecting and quenching the samples, it is essential to determine the approach for normalizing the data acquired from the measurement. For tissue samples, the mass or protein content is usually taken. If the decision is made to employ wet mass for normalization, then the muscles must be weighed before quenching.

In the second step, the samples have to be homogenized. The gold standard for this procedure is to grind the samples in a mortar under liquid N_2 with a pestle.[24] This method not only requires big amounts of sample material but is also very time-consuming. For this reason, there is a growing trend of more and more people turning to automated methods, such as bead homogenizers (high throughput homogenizers) or ultrasonic homogenization techniques. These offer advantages in terms of timesaving, with durations of only a few minutes, thus boosting throughput and providing a way in which cell-lysis and homogenization can be done within one single procedure.[25]

Mortar and pestle: The mortar and pestle is a widely applied method for homogenizing frozen tissue samples. In this process, the samples are ground and pulverized through the friction generated by sliding two hard surfaces against each other, effectively breaking down and ripping the samples. It is important to note that friction forces can generate heat, therefore cryogenic grinding is often employed by grinding the samples under liquid nitrogen. This

method is simple and cost-effective when compared to advanced homogenization instruments. Grinding with the mortar and pestle is a well-established method for single experiments. However, when dealing with a larger number of samples, it has limitations in terms of throughput. Additionally, it is not suitable for small sample sizes, as they can be easily lost while grinding on the surface, leading to complications in sample recovery. Furthermore, contamination is another factor that should be considered.[26,27]

High throughput homogenizers: High throughput homogenizers are a result of the application of bead beating methods to a larger format. Samples and beads of corresponding sizes are placed in vessels (vials, well plates, or tubes) that are locked into a device that rapidly moves the vessels up and down, causing the beads to rupture and grind the samples. This method can process a wide range of samples in terms of hardness and size with high throughput and efficiency. It offers the advantage of no cleaning and eliminates the risk of contamination since the tubes and beads are single-use and disposable. However, high-throughput homogenizers come with a substantial cost.[26,27]

Sonication: During sonication, samples are ruptured using a probe that vibrates at high frequencies due to the piezoelectric effect. When current is applied to the piezoelectric material, contraction occurs and vice versa, leading to the generation of small shock waves by the application of rapid current oscillations. To focus the energy of these shock waves, the piezoelectric material is attached to a metal probe. This focused energy emitted by the probe is quite intense. When the probe contracts, it creates a negative pressure, causing the liquid to flow upwards with the probe. When it expands, the liquid is pushed away. Repeating this process generates microscopic shock waves. However, liquids cannot vibrate as rapidly as piezoelectric materials. Therefore, small vacuum cavities form during the contraction of the probe. As the probe expands, these cavities implode, generating microscopic shock waves. This process is known as ‘cavitation’. It can become extremely powerful when the energies of all the imploding cavities are combined. This method is highly effective for processing cell suspensions, microorganisms, and, in general, small particles like homogenized tissue. Nevertheless, sonication is not quite suitable for the homogenization of solid tissue. Furthermore, it generates great amounts of heat. Therefore, when heat labile analytes are of interest, an additional strategy to cool the samples is crucial. Moreover, the throughput is relatively limited.[26,27] There are modern sonicators that are designed to circumvent the limitations, by providing an additional cooling system or enhancing the sonication techniques

to enable the homogenization of solid tissues. For instance, the company Covaris produces a variety of ultra-sonication instruments that can be applied from cell substrates to solid tissues.

Other homogenizing methods, for instance, would include those that are based on shearing forces, such as the 'Dounce homogenizer', named after its founder Alexander Dounce. They are great options for cell cultures with acceptable efficiency and low prices, but not suitable for the homogenization of solid tissues.[28]

The last step of sample preparation is the extraction of the metabolites from the homogenized sample. In **Figure 2** the octanol/water partition coefficients (XlogP) of some common metabolites and the polarities of the mostly used extraction solvents are represented.[22] XlogP serves as a measure of the relationship between lipophilicity and hydrophilicity of a substance.[29] As can be seen, extracting all components with the use of only one solvent is not feasible. Therefore, two-phase liquid-liquid extractions (LLE), as shown in the figure, with solvents of different polarities are often practiced, especially to capture as many metabolites as possible and particularly, when lipids are also of interest. If only metabolites are relevant for the experiment, a simple extraction with a methanol/water mixture (4:1, v/v) is often satisfactory.[30]

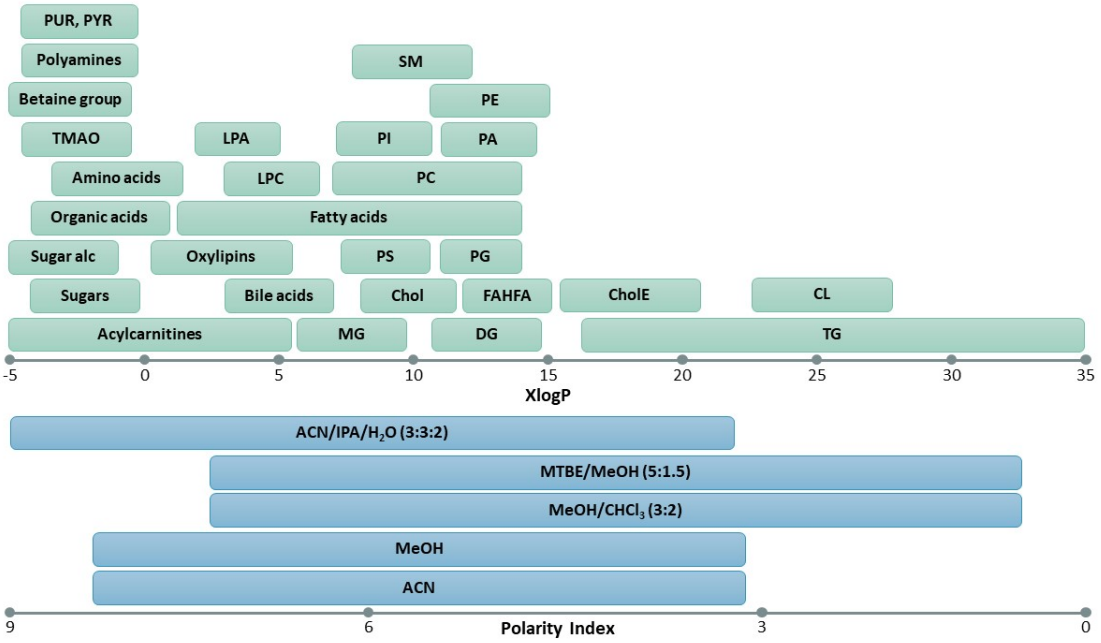

Figure 2: XlogP and polarity index of common metabolites and extraction solvents (adapted from Fiehn *et al.*, 2015)[22]

The blue bars represent various solvent mixtures and their corresponding polarity index windows. A higher value indicates a more polar nature of the solvent mixture. For example, the mixture ACN/IPA/H2O (3:3:2) has a higher polarity than pure MeOH. The green bars represent different metabolite and lipid classes and their respective octanol/water partition coefficients. A higher coefficient suggests that the substances are more lipophilic in nature.

It is important to consider that the extraction solvent should avoid further chemical or physical alterations of the metabolites and should be suitable for the subsequent analytical process.[21] After extraction, the samples can undergo filtration to remove larger compounds. Moreover, the extraction solvents can be evaporated to reconstitute the analytes in a solvent suitable for LC-MS analysis.

There are three well-established LLE protocols that are commonly applied in lipid research: the method according to (1) Folch, (2) Bligh-Dyer, and (3) Matyash.[31] All three were originally established to isolate lipids from the sample matrix, with an organic phase to isolate less-polar substances (lipids), along with a more polar mixed phase of methanol and water to enrich the polar substances (other metabolites). **Table 1** shows the three mentioned methods along with their corresponding solvents and ratios respectively.[32–34]

Table 1: Commonly used two-phase extraction methods

Method	Solvents	Volumetric ratio
Folch	Chloroform/methanol/water	8:4:3 (v/v/v)
Bligh-Dyer	Chloroform/methanol/water	2:2:1.8 (v/v/v)
Matyash	Methyl-*tert*-butyl ether (MTBE)/methanol/water	10:3:2.5 (v/v/v)

The reason for the varying ratios between the Folch and the Bligh-Dyer protocols is the origin of their formation. The Folch method was designed for the extraction of brain tissue[32], whereas the Bligh-Dyer extraction was applied to fish muscles.[33] The protocol according to Matyash was employed on human blood plasma, *Escherichia coli* (NA-22 strain), mouse brain tissue, and the eggs of the *daf-22* strain of *Caenorhabditis elegans*, a roundworm species.[34] The Matyash method offers the advantage of not relying on the use of toxic chloroform. In addition, it is particularly attractive for lipidomics analysis because the MTBE is in the upper phase making it easy to pipette and isolate the lipid-rich phase. In contrast, chloroform is found in the lower phase, and access to the lipid-enriched phase is only achieved by passing through the upper phase first. This can lead to contamination and is generally not very practical.[22] Therefore, numerous laboratories are taking advantage of the Matyash method and adapting the protocol to their specific needs. For instance, Coman *et al*. introduced the 'Simultaneous Metabolite, Protein, Lipid Extraction' (SIMPLEX) in 2016.[35] In contrast to the methods mentioned above,

the SIMPLEX protocol also makes use of the phase in which more polar substances are enriched.[35] In Folch, Bligh-Dyer, and Matyash, this phase was originally discarded, as the primary aim was to isolate lipids from the remaining components of the sample for analysis.[31] Additionally, the SIMPLEX method retains the protein pellet, allowing for protein analysis as well. Therefore, the SIMPLEX method. Accordingly, the SIMPLEX method enables the analysis of metabolites, lipids, and proteins with one extraction protocol.[35]

Once a method has been chosen, the next step is to determine the ratio in which the samples will be extracted in the corresponding solutions. The following section provides the ratios that the authors of the aforementioned LLEs used for their experiments.

Folch assumed that one gram of brain tissue is equivalent to one milliliter of tissue volume. For his extraction, he used 20 mL of the extraction mixture for one milliliter of tissue. Thus, for 1 mL of tissue, 10.6 mL of chloroform ($CHCl_3$), 5.3 mL of methanol (MeOH), and 4 mL of water (H_2O) were employed.[32] In the Bligh-Dyer protocol, 100 g of the muscle sample were treated with 200 mL $CHCl_3$, 200 mL MeOH, and 180 mL H_2O.[33] Matyash utilized 200 µL of each sample material for extraction with 1.5 mL MeOH, 5 mL MTBE, and 1.25 mL H_2O. The sample materials were as follows: For the *Escherichia coli* bacteria, there were approximately 54,000,000,000 cells in the 200 µL suspension in an aqueous 0.1% ammonium acetate solution. The mouse brain tissue was homogenized in an aqueous solution with 0.1% ammonium acetate and 200 µL of the homogenate was subjected to extraction. Furthermore, approximately 900 eggs of *Caenorhabditis elegans* were suspended in 200 µL of water, and for human plasma, only 20 µL were extracted with the above-mentioned amounts of solvents.[34]

After the samples have been extracted, taking into account the above criteria, the extracts of the analytes can now be investigated for their concentration and species using appropriate instrumentation.

1.4 Instrumentation – a quick overview of the commonly used techniques

The instruments used for the metabolome analysis of a given sample should allow the detection of a multitude of diverse metabolites. The most widely used strategies for the examination of metabolites include Nuclear Magnetic Resonance (NMR) spectroscopy along with Mass

spectrometry (MS).[12] The advantage of NMR is that it is a non-destructive method that requires only minimal sample preparation, but it demands at least metabolite concentrations up to micromolar ranges in samples and has only moderate sensitivity as well as narrow dynamic range.[11,36] In the contrary, the MS permits the detection of much lower concentrations of nanomol or even picomol. Thus, its superior sensitivity and broad dynamic range make it a more advantageous method.[11,19] The use of chromatographic separation methods is commonly applied before the mass spectrometer to boost the coverage and adequately resolve low abundant components.[37] For example, it is possible to perform Gas Chromatography-Mass Spectrometry (GC-MS), but this method only works for volatile components. Analytes that are non-volatile need to undergo derivatization in order to convert them into a volatile form and to transfer them into the gas phase, which is a very time-consuming process. Therefore, the utilization of Liquid Chromatography-Mass Spectrometry (LC-MS) has become prominent today.[20]

1.4.1 Liquid chromatography

Chromatography is a method that is applied to separate mixtures of substances and subsequently determine them both qualitatively and quantitatively. Liquid chromatography is based on the separation of various analytes present or transported in the liquid mobile phase based on their interactions with the functional groups of the firm material of the stationary phase (mostly packed in a column). These interactions are mainly found to be physical adsorption or absorption mechanisms.[38] The better the analyte can interact with one or the other phase, the faster or slower it moves to the other end of the stationary phase. In column chromatography, a hollow body (usually column-shaped or hollow wire-like) is packed with the solid material that functions as the stationary phase. Depending on the composition of the stationary phase, substances with certain polarities can be separated more efficiently.[39] Therefore, the selection of the chromatography methods is highly dependent on the properties of the metabolites under investigation. In metabolomics, methods like hydrophilic interaction liquid chromatography (HILIC) for polar substances and reversed-phase chromatography (RP) for less polar components are mostly used.[22,40] Ultra high-performance liquid chromatography (UHPLC) is an extended form of LC. It has the advantage that the column can be loaded with much smaller particle sizes which offers better separation of the substance components within a shorter time span and is therefore gladly used by many scientists.[41]

To increase the coverage of analytes, another approach involves combining two chromatographic columns with different separation capabilities, as presented by the group of Prof. Koellensperger in 2019 (**Figure 3**). This method, known as the 'Dual setup', is constructed in such a way that a HILIC column and a RP column are switched in sequence. Two 2-position, 6-port post-column valves were installed, for both the HILIC and the RP method, followed by a T-piece before the sample initiation into the ionization source of the MS. This allows the successive orthogonal separation.

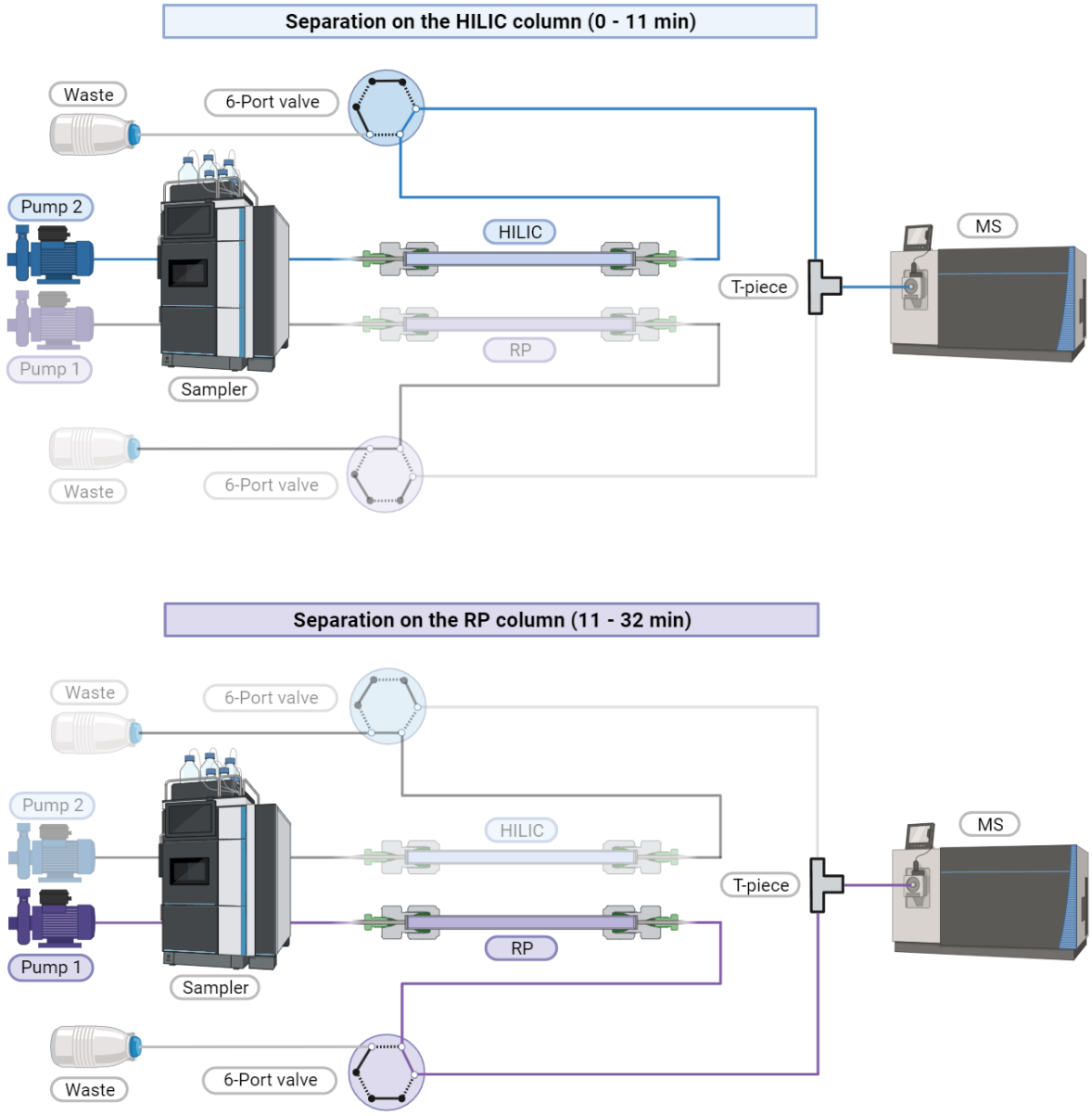

Figure 3: Dual setup chromatography (adapted from Schwaiger *et al.*, 2019)[42]

First, sample extracts pass through the HILIC column, where polar substances are separated and introduced to the MS, while the eluent of the RP column is directed into the waste. Whilst the HILIC column is undergoing re-equilibration for the next cycle, another sample extract is injected into the RP column and the less-polar substances are separated and eventually analyzed in the MS. To enable this transition, the 6-port valves are switched, directing the eluates from the RP to the MS and eluents of the HILIC to the waste. Subsequently, the valve is switched again and while the HILIC column is already separating molecules of the next injection, the RP column can equilibrate. Hence, the dual setup not only offers the advantage of enhanced coverage but also shortens the time by enabling one column's equilibration while simultaneously separating on the other.[42]

To increase separation efficiency, the mobile phase composition can be designed as a gradient system. Increasing amounts of solvent B are gradually added to solvent A with B being the solvent of greater elution efficiency.[43] Commonly used solvents in RP and HILIC are acetonitrile (ACN), isopropanol (IPA), methanol (MeOH), and water (H_2O) with 0,1 % formic acid (FA).[22] In order to build up a suitable method for the scientific inquiry, numerous parameters can be further refined, such as the column temperature, pH value, or the injection volume, just to name a few.[43]

1.4.2 Mass spectrometry

In mass spectrometry, it is not the masses of the analyte molecules that are measured, despite what the name suggests. Instead, the mass-to-charge (m/z) ratios are determined. Thus, a mass spectrum is essentially a graph of ion intensity plotted against the respective m/z values.[44] Both qualitative (molecular structure) and quantitative (molecular concentration) data can be obtained using MS.[45] The unit of measurement is Daltons per unit charge. A typical mass spectrometer is roughly composed of three key elements: (1) the ionization source, (2) the mass analyzer, and (3) the detector.[44] The ionization source is the initial component of the mass spectrometer through which the analytes pass. It ionizes the molecules, converting them into positively or negatively charged ions, and subsequently transfers them into the gas phase. These ions pass through the mass analyzer and arrive at the detector at distinct locations depending on their m/z. The location information is recorded by computer systems and eventually transformed into signals for analysis.[45]

1.5 The two main analytical approaches of metabolomics

Metabolomics studies are traditionally performed either using a targeted or an untargeted approach.[46] Furthermore, both approaches, tandem-MS (MS2 or MS/MS) in combination with LC can be applied to enhance selectivity, sensitivity, coverage, and reproducibility.[47] In tandem MS, ions are selected according to a specific m/z in the first analyzer (MS1). These 'precursor ions' are then fragmented in a collision cell, and the resulting 'product ions' are isolated once more in the second mass analyzer (MS2) based on a certain m/z. The ensuing spectrum contains the reaction products of the precursor ions. Tandem-MS is a powerful technique for structure elucidation of the original molecule of unknown compounds in complex mixtures based on the examination of their fragment spectra.[48,49]

1.5.1 Untargeted approach

The focus of untargeted metabolomics is to measure, compare, and then identify as many metabolites as possible within a biological sample. The interest is in identifying metabolites that are not yet known.[50] In order to measure as many metabolites as possible in a sample, effective extraction is the first challenge and a very important step. By screening the entire metabolome of a sample, hypotheses are made and possible candidate biomarkers of a certain disease are identified.[40,46] Usually, it is important to use a high-resolution instrument, as high mass accuracy plays a key role in resolving isotopic peaks with high precision and thus, proper annotation and statistical analysis with reduced errors. In the untargeted approach, the main mode of operation lies in the generation of fragment spectra. The experimentally generated fragment spectra of the unknown metabolites are compared with the predicted spectra of all possible candidates with databases of choice. The candidate whose predicted spectrum has the best match with the spectrum of the sample is the molecule in demand.[46] However, identification by means of databases is limited. Not all detected signals can be identified because the databases are still very incomplete. In the absence of a determined molecule, the experimental spectrum is mapped to the predicted spectrum from the next best match, ultimately resulting in an incorrect identification. Therefore, identifications from the untargeted analysis always need to be validated by the targeted approach using reference materials.[46,51]

1.5.2 Targeted approach

In the targeted approach, the focus lies not in exploring as many molecules as possible, but rather in the accurate quantification of a predetermined list of metabolites. These metabolites are chosen based on their relevance to the scientific question.[46] This method requires a high level of sensitivity, as well as specificity, and throughput. Therefore, information obtained through targeted approaches is more reliable. Biomarkers initially discovered through the untargeted approach are subsequently validated by targeted metabolomics.[40] Hence, it is a great method to find answers to specific biochemical questions or hypotheses.[11] To enhance accuracy, internal standards (ISTDs), such as ^{13}C labeled metabolites of interest, are spiked to the samples during the early stages of sample preparation. This practice allows the correction of errors that may occur during the pre-analytical steps.[52] Ideally, standard dilution series are also measured in order to calculate the absolute concentrations of the analytes, using a calibration curve.[40] However, obtaining standard materials for all the relevant metabolites can be difficult. This is primarily due to their high cost and the limited availability of commercial sources. Therefore, the coverage of metabolites that can be correctly analyzed is limited.[19]

1.6 Objectives

This master's thesis serves as a preliminary work for a larger project in which the muscle metabolome of patients suffering from malignant hyperthermia will be examined. With the current state of medicine, the most accurate and confidential diagnosis method is by performing a biopsy, which is time-consuming, and some patients even suffer from consequential damage. A less invasive test is needed and in a current international project, the use of metabolomics is explored. It is necessary to evaluate the efficiency and limitations of the sample preparation and develop an optimized workflow for quenching, homogenization, and extraction.

The focus of this master's thesis revolves around diverse homogenizing techniques for the preparation of muscle tissue samples. Four different homogenization methods and two homogenization solvents will be investigated regarding their reproducibility and ability to maintain sample stability. Using pork from the butcher to spare the precious clinical samples, the ideal workflow was developed and preliminary targeted metabolomics results show the applicability of the method.

2 MATERIALS AND METHODS

2.1 Caffeine/Halothane Contracture Test (CHCT)

CHCT is the only reliable method of testing for malignant hyperthermia susceptibility in the current state of medicine. For this test, muscle fibers with a mass of approximately 100-150 mg are biopsied from the patient's thigh. These muscle fibers are then attached between two electrodes (**Figure 4**) in a chamber filled with a Krebs-Ringer buffer solution (**Table 2**) and connected to a balance (**Figure 5**). In the chamber, the muscles are gassed with oxygen and carbon dioxide to prevent premature muscle cell death. After the muscles have equilibrated in the buffer solution, which typically takes 15 to 20 minutes, they are exposed to halothane gas or a caffeine solution within the chamber. As the muscles contract, they pull on the scale and the contraction capacity can be determined by the amount of grams indicated.[6]

Figure 4: Electrodes for the CHCT

The electrodes to which the muscles are attached with threads for the CHCT are indicated by white arrows.

Figure 5: Balance and chamber for the CHCT

In this graph, the balance, indicated by the yellow arrow, and the chamber, marked with the white arrow, used for the CHCT are shown.

Table 3 shows the guidelines for diagnosis according to the North American Malignant Hyperthermia Registry (NAMHR).[4,53] An example of what the result of a CHCT looks like is described in more detail in section 3.1.1 (p.26).

Table 2: Composition of the Krebs-Ringer bicarbonate buffer solution (adapted from Kirby-Smith *et al.*, 2023)[54]

Components	g/L
D-Glucose	1.8
Magnesium Chloride (anhydrous)	0.0468
Potassium Chloride	0.34
Sodium Chloride	7.0
Sodium Phosphate Dibasic (anhydrous)	0.1
Sodium Phosphate Monobasic (anhydrous)	0.18

Table 3: Protocols and guideline for the diagnosis of Malignant Hyperthermia (adapted from Rosenberg *et al.*, 2020)[4]

Classification	North American Protocol
MH susceptible	≥ 0.7 g contracture to 3 % halothane or ≥ 0.3 g contracture to 2.0 mM caffeine
MH susceptible	Contracture to only halothane or only caffeine
MH negative	No contracture or < 0.7 g contracture to halothane or < 0.3 g contracture to 2 mM caffeine

2.2 Solvents and standards

All solvents for the extraction as well as for the LC were of LC-MS grades. Acetonitrile, isopropanol, methanol, *tert*-Butyl methyl ether and water were purchased from Honeywell. Formic acid was ordered from VWR and ammonium formate from Sigma-Aldrich.

Two internal standard mixtures were used for this experiment. The Mouse Splash standard was purchased from Avanti Polar Lipids with the following lipid species: 15:0-18:1 (d7) Phosphatidylcholine (PC), 15:0-18:1 (d7) Phosphatidylethanolamin (PE), 15:0-18:1 (d7) Phosphatidylserin (PS), 15:0-18:1 (d7) Phosphatidylglycerol (PG), 15:0-18:1 (d7) Phosphatidylinositol (PI), 15:0-18:1 (d7) Phosphatidic acid (PA), 18:1 (d7) Lysophosphatidylcholine (LPC), 18:1 (d7) Lysophosphatidylethanolamin (LPE), 18:1 (d7)

Cholesteryl ester, C18 (Plasm)-18:1 (d9) PC, 15:0-18:1 (d7) Diacylglycerol (DAG), 15:0-18:1 (d7)-15:0 Triacylglycerol (TAG), d18:1-18:1 (d9) Sphingomyelin (SM) and C18(Plasm)-18:1 (d9) PE.[55] Gerrit Herrmann from ISOtopic solutions provided the ISO1 standard mixture, a fully ^{13}C labelled yeast extract of the species *Komagataella phaffii*. The metabolites identified in the extract are given in **Table 4**.[56]

Table 4: Routinely identified metabolites of the Komagataella phaffii (ISO1) (taken from the website of ISOtopic solution)[56]

Amino Acids and Derivatives		
S-Adenosyl-homocysteine	Glutamate	Ornithine
3-Methyl-2-oxovalerate	Glutamine	Phenylalanine
Alanine	Glycine	Proline
Arginine	Guanidine acetate	Sarcosine
Argininosuccinate	Histidine	Serine
Asparagine	Homoserine	Threonine
Aspartate	Isoleucine	Tryptophan
Betaine	Kynurenine	Tyrosine
Citrulline	Leucine	Valine
Cystathionine	Lysine	α-Aminoadipate
Dihydroxy-isovalerate	Methionine	α-Ketoisovalerate
Nucleobases Nucleosides and Nucleotides		
5'-Deoxy-5'-Methylthioadenosine	Cyclic guanosine monophosphate	Guanosine triphosphate
5-Methyluridine	Cytidine monophosphate	Inosine
Adenine	Cytidine triphosphate	Inosine monophosphate
Adenosine	Deoxyadenosine monophosphate	Pseudouridine
Adenosine diphosphate	Guanine	Uridine
Adenosine monophosphate	Guanosine	Uridine diphosphate
Adenosine triphosphate	Guanosine diphosphate	Uridine monophosphate
Cyclic adenosine monophosphate	Guanosine monophosphate	Uridine triphosphate

Continuation of Table 4: Metabolites of the Komagataella phaffii (ISO1)

Organic Acids		
cis-Aconitate	Gluconate	Pyruvate
Citrate	Isocitrate	Succinate
Hydroxyglutarate	Lactate	α-Ketoglutarate
Fumerate	Malate	
Sugar and Sugar Phosphates		
Dihydroxyacetone phosphate	Fructose-6-phosphate	Mannose-6-phosphate
2-Phosphoglycerate	Galactose	Ribose
6-Phosphogluconate	Glucose	Ribose-5-phosphate
Erythritol	Glucose-6-phosphate	Sedoheptulose-7-phosphate
Fructose	Mannitol	Trehalose
Fructose-16-bisphosphate	Mannose	
Vitamins and Coenzymes		
Nicotinamide adenine dinucleotide oxidized (NAD+)		Nicotinamide adenine dinucleotide reduced (NADH)
Nicotinamide adenine dinucleotide phosphate oxidized (NADP+)		Choline Nicotinamide
Other Small Molecules		
Glutamylcysteine	Glutathione reduced (GSH)	Mevalonate
Glutathione oxidized (GSSG)		

2.3 Sample preparation

In the following section, the experimental procedure of the pre-analytical steps spanning from collecting the samples to the extraction is described in detail. For a better overview, a brief summary of the entire workflow is shown graphically in **Figure 6**. The solvents used for all sample replicates, along with their respective volumes, can be found in the attachment (p. 48) in **Table S 1**.

2.3.1 Sample collection and storage

Pork meat from the abdominal region, taken from an 8-month-old pig, was obtained from the butcher and transported on ice to the laboratory, which took approximately 2 hours. In the laboratory, the meat was sectioned into pieces weighing around 10-30 mg (CPA225D Semi-Micro Monolithic Weighing System from Sartorius). They were then immediately snap-frozen and stored at -20 °C for further analysis after noting the exact mass for data normalization.

2.3.2 Homogenization

Four different homogenization tools were investigated: an ultra-sonicator (Covaris), two bead homogenizers (Minilys and Precellys), and the mortar and pestle, which is considered the gold standard. Furthermore, two homogenizing solvent systems were examined: pure MeOH and a mixture of MeOH/H_2O (1:1, v/v). The solvents were cooled the day before extraction: MeOH to -20 °C and H_2O to 4 °C. For all homogenizing methods, a solvent-to-sample size ratio of 20 µL/mg was employed and 10 µL of ISO1, as well as 5 µL of Mouse Splash, were spiked into all samples.

Bead homogenization (Minilys and Precellys): Two distinct instruments from Bertin Technologies were applied. The first homogenizer is the Minilys personal homogenizer, which does not provide a cooling system. 2 mL Tubes (polypropylene co-polymered) with 1.4 mm zirconium oxide beads from Peqlab Technologies were utilized. The second device is the Precellys 24 tissue homogenizer, a more advanced gadget that can be connected to a cooling system. Again, 2 mL Tubes (polypropylene co-polymered) were used, but for this method, a zirconium oxide bead size of 2.8 mm was chosen. For the sake of simplicity, the two methods will be referred to as the ‘Minilys’ and ‘Precellys’ methods based on the instrument’s names.

Pre-cooled MeOH or MeOH/H_2O (1:1, v/v) was placed in the homogenizing cup with the ceramic beads according to the respective sample size-to-homogenizing solvent volume ratio (20 µL/mg). Additionally, 10 µL of ISO1 and 5 µL of Mouse Splash were spiked into the solvent as ISTDs. Afterward, the samples (cooled in dry ice after taking them out of the -80°C freezer) were placed into the homogenization tubes as fast as possible, to avoid preliminary sample thawing, by snipping the Eppendorf tube, in which the samples were stored. The cups with their contents were then homogenized in the bead homogenizer. For the Minilys, it was

important to cool the samples on ice between each homogenization step. One cycle comprised 10 seconds of homogenization at 5000 rpm, followed by a 60-second pause on ice. The Precellys was connected to a liquid N_2 reservoir to cool the system down to 4 °C by regulating the N_2 flow. One cycle included 20 seconds of homogenization at 6800 rpm, followed by a 60-second pause to avoid sample heating due to frictional force. For both methods, four cycles were performed in total. After homogenizing the sample, the homogenate was transferred into a 2 mL Eppendorf to isolate the beads from the mixture.

Ultrasonication (Covaris): The S220 Focused-ultrasonicator was obtained from Covaris together with the MM-7M Benchtop Chiller with Centrifugal Pump from PolyScience. This approach will be called the 'Covaris' method throughout this work.

The snap-frozen samples were placed into the Covaris glass tube (Covaris), in which the precooled homogenizing solvent was prepared before, together with small Teflon pieces to stir the homogenate during the sonication process. The appropriate volumes of the ISTDs were spiked into the sample. A Peak power of 260 W, a duty factor of 10 %, a cycle per burst of 200, and a treatment time of 360 seconds were chosen for the homogenization. Furthermore, the process was performed under cool conditions at 4 °C. The finished homogenate was transferred into a 2 mL Eppendorf tube subsequently.

Mortar and pestle: The frozen tissues were placed in the precooled mortar and carefully ground into a homogeneous powder under liquid N_2. The ground sample was aliquoted into separate Eppendorf tubes with a pre-cooled spatula and the exact mass was noted. This was a critical step, where precision and speed were of great importance to avoid sample thawing and water condensation, which could lead to inaccurate mass measurements. The respective amount of the two distinct homogenization solvent systems and the ISTDs were added simultaneously to the sample eventually. After vortexing the suspensions for about 20 seconds, they were sonicated in an ultrasonic bath, filled with water and ice, for 10 minutes and incubated in liquid N2 for one minute to induce cell lysis. These two steps were repeated three times to induce the lysis of the muscle cells.

2.3.3 Sequential extraction

For this experiment, the SIMPLEX extraction protocol according to Coman *et al*. (2016) was applied and adapted to obtain both, lipids and metabolites.[35] The extraction mixture for lipid isolation consisted of MeOH/MTBE/H_2O in a ratio of 1.2:4:1 (v/v/v/), with the homogenization solvent already containing the methanol or a portion of methanol and water respectively. After enrichment of the lipids into the MTBE phase and transferring them to a separate tube, additional volumes of methanol were introduced for protein precipitation and isolation of the remaining metabolites, so that the MeOH/H_2O mixture had a final ratio of 4:1 (v/v).

Extraction and phase separation: The appropriate amount of pre-cooled MTBE (4 °C) was added to the sample. After vortexing for 10-20 seconds, the sample was incubated in the shaker (ThermoMixer F by Eppendorf) for 2 hours at 1000 rpm and 4 °C.

To maintain the temperature, the shaker block was covered with aluminum foil. This was done because it was observed that the temperature was not stable and tended to rise, possibly due to the summer conditions in the laboratory. After incubation, the corresponding volumes of water with 0.1% ammonium formate were added and vortexed to induce phase separation. Eventually, the extracts were centrifuged for 15 minutes at 14,000 g and 4 °C (Z 445 K Universal Centrifuge from Hermle).

Preparation of the lipid fraction for LC-MS analysis: The MTBE phase (upper phase) was removed and aliquoted into 200 µL fractions. Those aliquots were dried under vacuum with the GeneVac EZ-2.3 ELITE Centrifugal Evaporator from SP Scientific and stored in the freezer at -20 °C until analysis for two days. The dried pellets were reconstituted in 100 µL pure IPA and centrifuged at 14,000 g for 15 minutes to remove any debris prior to analysis. Finally, the extracts were transferred into HPLC vials for LC separation and MS measurement. Sample aliquots not further used for MS analysis were stored at -80 °C as dried pellets.

Preparation of the metabolite fraction for LC-MS analysis: For the precipitation of proteins, cold MeOH was added additionally to the lower phase, to obtain a MeOH/H2O ratio of 4:1 (v/v). The samples were then incubated at -20 °C overnight. After centrifugation at 14,000 g and 4 °C for 30 minutes, the supernatant was transferred and divided into 200 µL aliquots., The

fractions were dried under vacuum and reconstituted in 100 µL 70 % ACN subsequently and stored at -20 °C for one day until analysis. Lastly, the extracts were moved to HPLC vials for chromatographic separation and MS analysis. Aliquots not subjected to further analysis were stored at -80 °C as dried pellets.

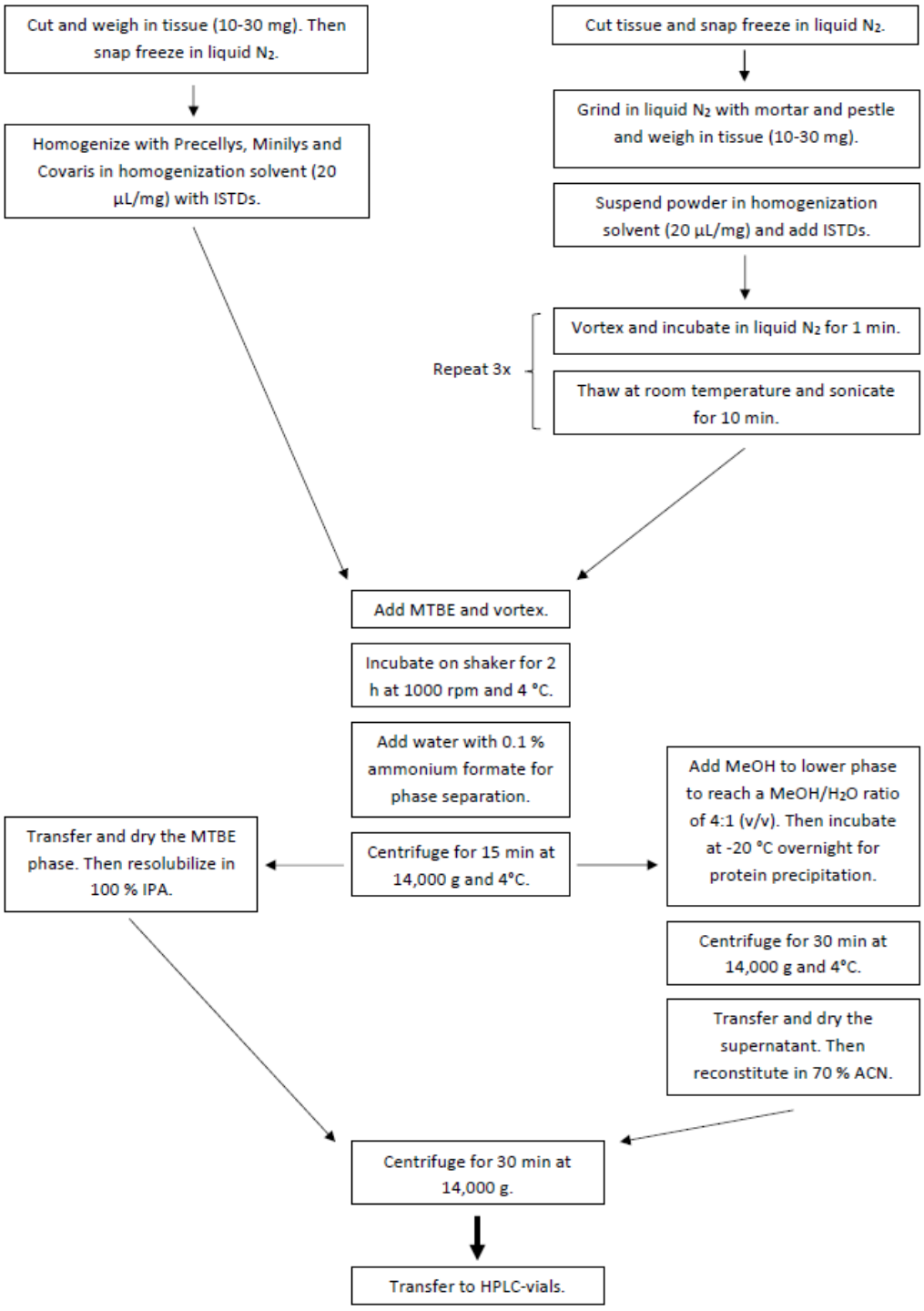

Figure 6: Applied workflow for the pre-analytical steps

2.3.4 External Calibration

For the external calibration, standard dilution series for the lipids as well as for the metabolites were created with an in-house mix of reference standard materials (ESTDs). For both series, 5 µL of Mouse Splash and 10 µL of ISO1 were added, respectively. The dried ESTD stock solutions were reconstituted in 200 µL of IPA or H_2O for a final concentration of 50 µM. In total, the stock solutions were diluted into 14 distinct concentrations: 25, 10, 5, 1, 0.75, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025, 0.01, 0.050, and 0.001 µM. Additionally, a blank with only the corresponding ISTD was prepared. The molecule compounds of the ESTDs are given in the attachment (p.50) in **Table S 2** and **Table S 3**.

2.4 Data acquisition

2.4.1 Liquid chromatography

Hydrophilic interaction liquid chromatography: The Aquity UPLC-BEH-Amide column (150 mm x 2.1 mm; 1.7 µm) and the Acquity UPLC-BEH-Amide VanGuard precolumn (5 mm x 2.1 mm; 1.7 µm) were purchased from Waters for HILIC chromatography. The column oven was set to 40 °C and the flow rate for separation was 0.4 ml/min. Solvent A was made of water and solvent B consisted of ACN/H_2O (95:5, v/v). In both solvents, 10 mM ammonium formate and 0.125 % formic acid were included. The gradient applied is represented in **Table 5**, as well as in **Figure 7**.

Reversed phase chromatography: For RP separation, the Acquity HSS T3 column (150 mm x 2.1 mm; 1.8 µm) with the Acquity VanGuard precolumn (5 mm x 2.1 mm; 100 Å; 1.8 mM) from Waters was applied. The temperature in the column oven was set to 45 °C and the flow rate to 0.25 mL/min. Solvent A was composed of ACN/H_2O (6:4, v/v), while solvent B contained IPA/ACN (9:1, v/v). Both solvents included 10 mM ammonium formate and 0.125% formic acid. The gradient is depicted in **Table 6** and is also graphically represented in **Figure 8**.

Table 5: HILIC solvent gradient with the corresponding flow rate in mL/min

Time [min]	B [%]	Flow [mL/min]
0	100	0.4
2	100	0.4
7.7	70	0.4
9.5	40	0.4
10.25	30	0.4
12.75	100	0.4
14	100	0.4
14	100	0.05
28	100	0.05
28	100	0.4
30.5	100	0.4
31	100	0.4

Table 6: RP solvent gradient of with the corresponding flow rate in mL/min

Time [min]	B [%]	Flow [mL/min]
0	55	0.05
8	55	0.05
8	55	0.25
11	55	0.25
19	65	0.25
24	85	0.25
26	100	0.25
30.5	100	0.25
30.5	55	0.25
31	55	0.25

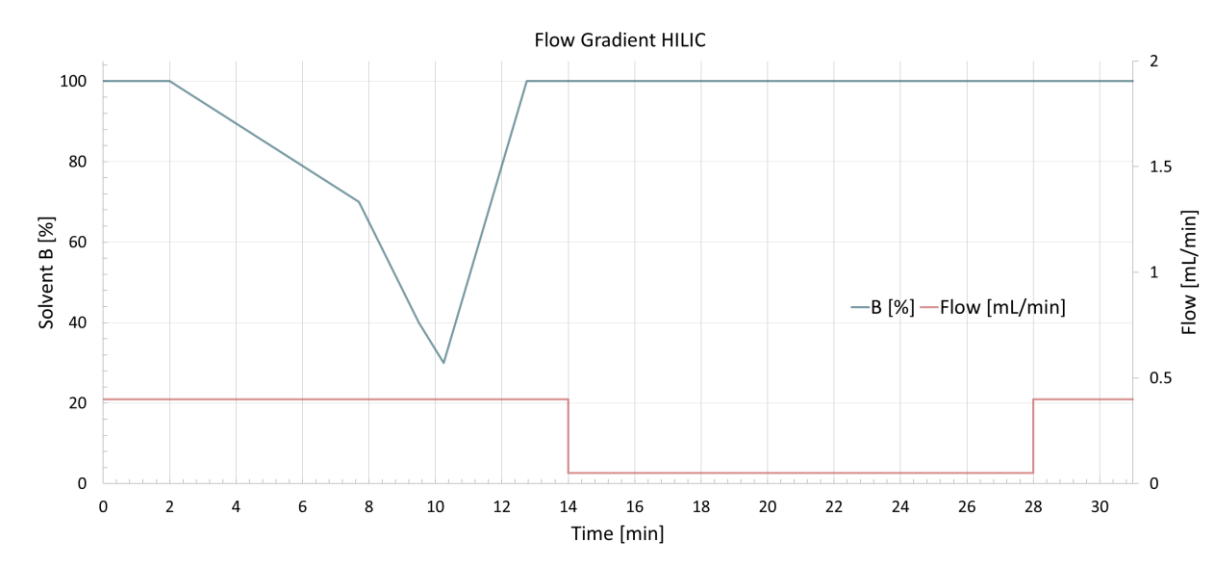

Figure 7: Gradient and flow rate of the BEH Amide HILIC separation in the dual system

The gradient is shown in blue following the relative composition of B. The flow rate in mL/min is shown in red. Lower flow rates have been applied during the separational run of the RP-LC.

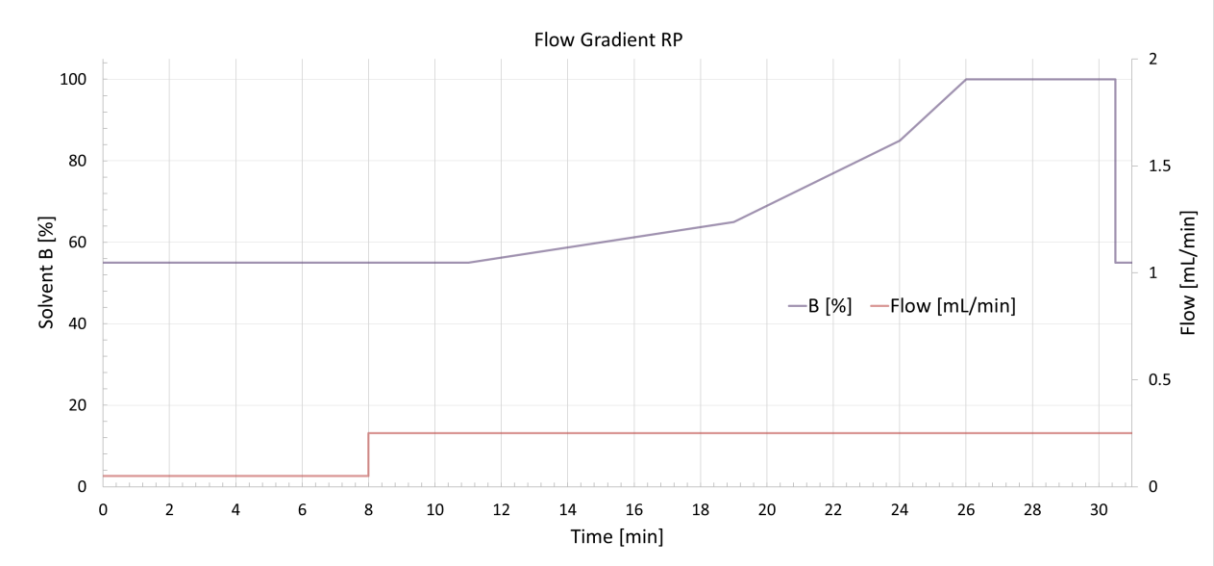

Figure 8: Visual representation of the RP gradient with the respective flow rate

The gradient is represented in blue, corresponding to the relative composition of B, while the flow rate is depicted in red in mL/L. Lower flow rates were employed during the separation process in the HILIC-LC.

Setup for the orthogonal separation of HILIC and RP liquid chromatography: The LC strategy used for this work was based on the dual separation method (HILIC-RP) according to Schwaiger *et al*. (2019).[42] For the analysis of both extracts, the metabolite and the lipid fractions, the autosampler temperature was set to 4 °C. Additionally, two column departments were used, to achieve different column temperatures for the respective columns. HILIC separation occurred up to minute 10, followed by RP chromatography starting at minute 11. The entire method had a duration of 31 minutes.

2.4.2 Mass spectrometry

The Q Exactive HF Orbitrap mass spectrometer from Thermo Fisher Scientific, a high-resolution instrument, with an ESI source, was used for data acquisition. The ionization source parameters were set as follows: sheath gas 60, auxiliary gas 25, sweep gas 2, spray voltage 3.1 kV in negative and positive mode, capillary temperature 300 °C, auxiliary gas heater 370 °C, and S-Lens RF level 30. The automatic gain control was 1,000,000. A full mass scan in negative, as well as in positive mode, was acquired in polarity switching mode at a resolution of 120,000. For the HILIC separation, a mass range of 60 – 900 m/z, and for the RP 250 – 2000 m/z was scanned.

2.4.3 Quality control and randomization

To track the performance of the methods and instruments (LC and MS), a quality control (QC) sample was integrated into the data measurement process. The QC sample was identical to one of the ESTD solutions with a concentration of 1 µM, which was derived from the serial dilution series used for external calibration. It was introduced into the LC-MS system at a frequency of every 10^{th} injection of other samples. Furthermore, the samples were analyzed in a randomized order with three solvent blank injections at the beginning and at the end of the sequence.

2.5 Data Processing

The raw data (.raw) was converted into mzML files via MSConvert 3.0 (Proteowizard) with the following parameters: Binary encoding precision was 64-bit, Write index, zlib compression, and TPP compatibility were selected, and a peak picking filter with a vendor ms-Level of 1 was applied. The mzML files were then imported into Skyline 22.2 (MacCoss Lab) and peak integration was carried out manually. The integrated peaks were subsequently exported to a CSV file and underwent further data processing using Microsoft Excel and RStudio 2021.09.2+382. The concentrations of the detectable compounds were inspected, as well as their relative standard deviations (RSDs) for each homogenization method. Moreover, the formation of sphingosine-1-phosphate (S1P) was investigated to monitor sample stability for every strategy, and instrument stability was observed through the signals of the QC samples.

3 RESULTS AND DISCUSSION

The purpose of this master's thesis is to evaluate different homogenization methods for the preparation of muscle tissue regarding their reproducibility and ability to maintain sample stability, using pork meat from the butcher. Targeted metabolomics with a dual-LC-MS technique and targeted data analysis were performed to gain insight into the applicability of the investigated methods. Four homogenization techniques, Minilys, Precellys, Covaris, and mortar and pestle, and two distinct homogenization solvent systems, pure MeOH and a mixture of MeOH/H2O (1:1, v/v), were examined. The results will be applied to a larger study that aims to explore the muscle metabolome in patients with malignant hyperthermia, for which the only reliable diagnostic method includes a muscle biopsy. Through this project, a less invasive diagnostic test based on metabolomics is to be established.

The focus of reproducibility investigations primarily centered on metabolite data rather than lipids. However, a detailed examination of sample stability was conducted for the lipid species S1P. Additionally, the stability of the instrument was evaluated by tracking signal areas from QC samples for both metabolites and lipids.

3.1.1 Example of a Caffeine/Halothane Contracture Test

In **Figure 9**, the CHCT results from a MHS patient are exemplarily shown. The yellow and purple colored lines are the muscle's reaction signals to halothane. The dark blue and pink colored lines are those from the exposure to caffeine. The graph is meant to be read from right to left. The big jumps of the amplitudes (marked with green) result from the electrical stimulation of the muscles to verify their vitality and suitability for the test. The first dark blue signal (marked with orange) has failed and can be ignored. Presumably, it was decided to run this caffeine test later again on another muscle, because it fell out of the thread through which it is attached to the scale. After the electrical stimulation, there is an extended phase during which the curves remain relatively flat. During this time, the muscles are allowed to equilibrate in their new environment. At the bottom and the top margin, the added amounts of the respective substances at the corresponding time are indicated in red. In the case of halothane, a clear increase can be seen in the purple curve at a concentration of 2% (marked with white). The yellow curve also displays a slight increase. In the caffeine tests, there is no observable increase.

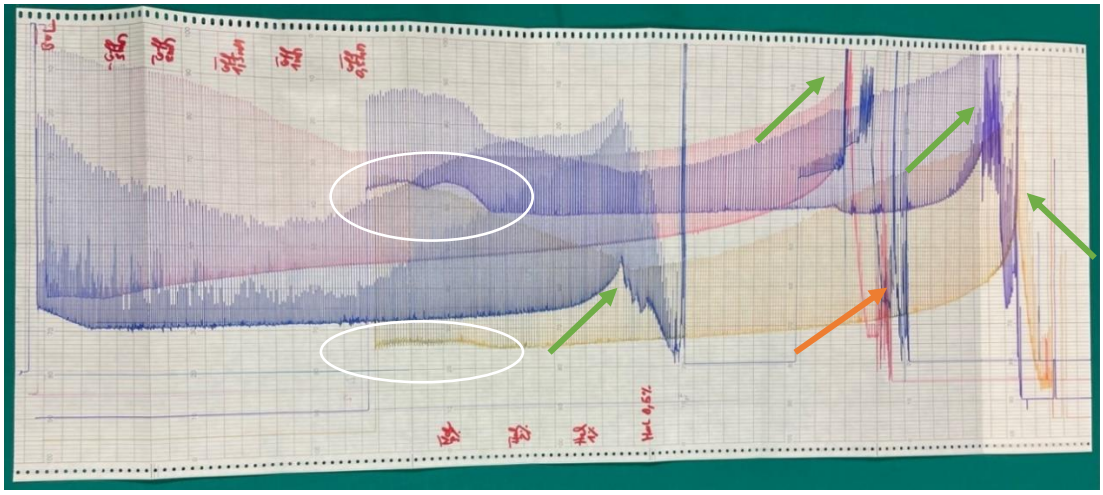

Figure 9: Example of a CHCT result

Nevertheless, according to the North American guidelines (**Table 3**), this CHCT would be classified as positive, indicating that the patient in question is MHS.

3.1.2 Quality Control

To ensure that the acquired data is reliable, a quality control (QC) process was employed.[57] Typically, QC processes occur during and after data collection and involve activities such as monitoring temperature, instrument stability and sensitivity, and method performance, just to name a few. Internal or external standards such as technical replicates or solvent blanks are commonly used as QC samples.[58] Randomizing the samples is also an important criterion to be considered to guarantee that each experimental sample is exposed to the same environmental conditions.[57]

For this work, an ESTD solution at a concentration of 1 µM spiked with internal standards, including both lipids and metabolites, was used as the QC sample. It was injected six times after every 10th injection of other samples throughout the measurement. Furthermore, the experimental samples were injected in a randomized order.

In **Figure 10** and **Figure 11**, signals of the QC samples are represented with respect to their order of measurement. For the sake of simplicity, only amino acid compounds for the metabolite

QCs and phosphatidylcholines for the lipid QCs were included in the plots. Significant fluctuations in QC samples would indicate that the produced data might lack reliability.

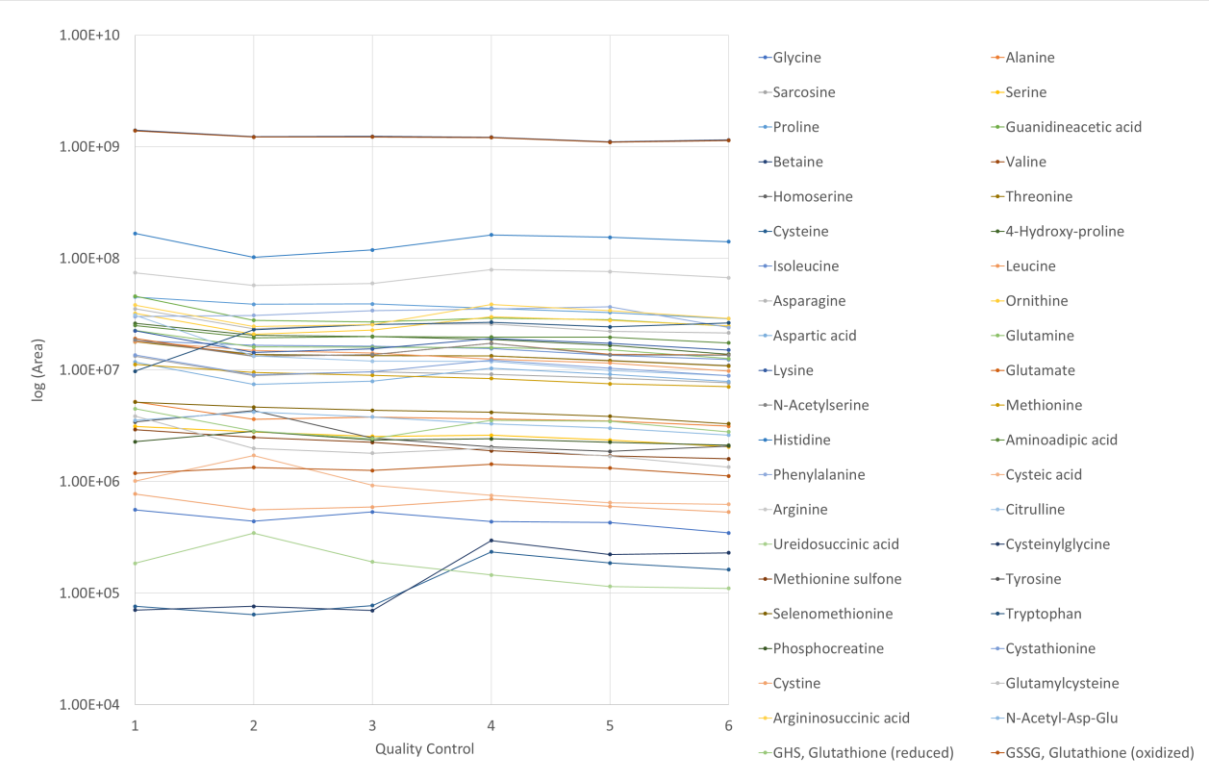

Figure 10: Peak areas of amino acids from metabolite QC samples

The areas are plotted in logarithmic form against the QC samples 1-6 following the order of their injection. The first QC sample was therefore injected relatively at the beginning of the measurement and the last towards the end.

For the metabolites in this measurement (**Figure 10**), the signals are mostly stable with a few exceptions. A stable signal was also observed for other metabolite categories. It seems that the chosen method remains suitable and can be applied to metabolite analysis. However, in the lipid QC samples (**Figure 11**), there is a recognizable decrease in the signal areas of the selected phosphatidylcholines. This phenomenon was also observed for other lipid classes. This could potentially occur due to lipid auto-oxidation or degradation within the sample, as the complete measurement process took approximately 34 hours. However, the most plausible explanation is the formation of micelles that may have occurred due to the temperature setting of 4 °C in the autosampler. Typically, a temperature of 10 °C is employed to prevent micelle formation in lipid analysis.[59] The low temperature may have caused the lipids in the HPLC vial to precipitate over time, leading to the adsorption of micelles on the vial walls. Conducting further

experiments with the appropriate temperature setting is necessary to derive accurate conclusions.

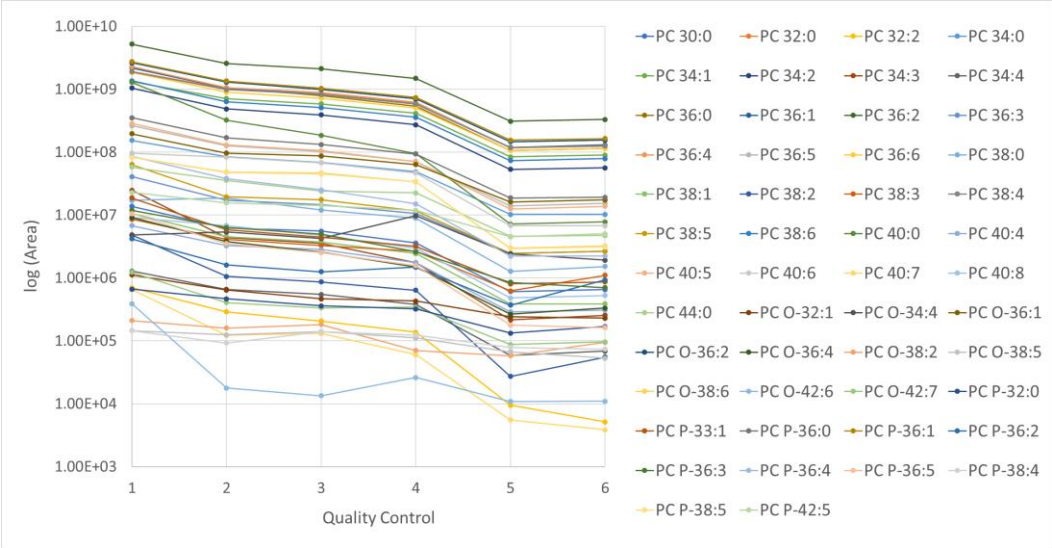

Figure 11: Peak areas of phosphatidylcholines from lipid QC samples

The areas are presented in logarithmic scale and plotted against the corresponding QC samples, revealing a signal drift over time.

Furthermore, the retention times of the two QC samples were also examined in more detail. It is evident that, with only a few exceptions, they exhibit consistent behavior across all QC samples, for both metabolites and lipids (**Figure 12** and **Figure 13**).

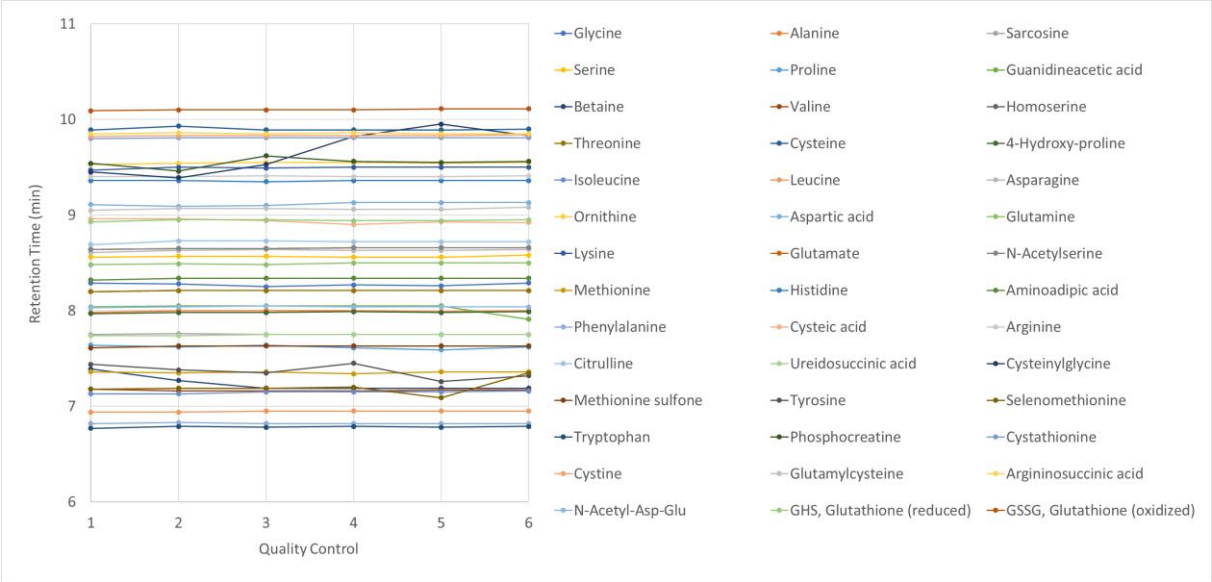

Figure 12: Retention times of amino acids in the metabolite QC samples.

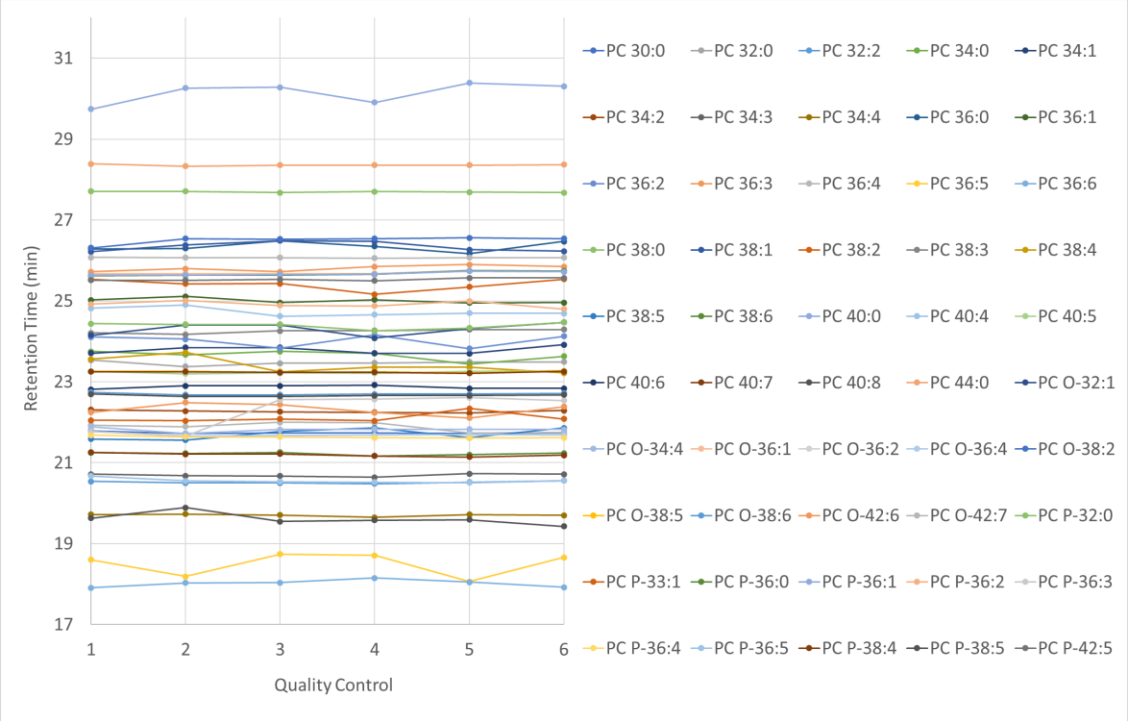

Figure 13: Retention times of phosphatidylcholines in the lipid QC samples.

Despite the observed decrease in lipid QC sample intensities, it is assumed that the stability of MS is likely maintained, as metabolite signals remained stable. The reduction in signal intensity is probably attributed to micelle formation due to the low autosampler temperature. Confirmation of chromatographic stability for both lipids and metabolites was achieved by assessing retention times. Hence, it can be concluded that the method employed and the generated data uphold a certain degree of reliability, allowing for additional data evaluation.

3.1.3 Summary of the employed homogenization methods

In **Table 7**, the different homogenization methods tested for this work are summarized. The Minilys method, while enabling the homogenization of three samples in parallel and being somewhat time-efficient, posed some labor-intensive challenges. Moving samples in and out for cooling after each cycle required constant attention, and manual time tracking was less practical. In contrast, the Precellys method proved to be the fastest and most convenient. Once the parameters and homogenization program were set, the process was entirely automated. Its ability to homogenize up to 24 samples simultaneously made it the most efficient in terms of workload. The Covaris method, surprisingly exhibited excellent performance. However, it

could only handle one sample at a time, requiring continuous presence during the process to load the next sample into the device after each homogenization. Homogenization using a mortar and pestle is straightforward but has some limitations. It demands larger sample quantities and a substantial amount of liquid nitrogen. Homogenizing larger tissue samples can be challenging because they become extremely hard due to snap freezing in liquid nitrogen. Furthermore, this method is the most time-consuming, as the cell lysis cycle following homogenization, including 1 minute of incubation in liquid nitrogen and 10 minutes of ultrasonic treatment, has to be considered.

Table 7: An overview of the applied homogenization techniques

Method	Parameters/Procedure	Cooling	Category
Minilys	10 seconds homogenization at 5000 rpm 60 seconds pause on ice 4 cycles	Only between cycles at 4 °C	Bead homgenizer
Precellys	4 °C	4 °C	Bead homgenizer
Covaris	Peak power 260 W Duty factor 10 % Cycle per burst 200 Treatment time 360 seconds	4 °C	Ultra-sonication
Mortar and pestle	The samples were ground under liquid nitrogen until they were finely pulverized. Powder aliquots were suspended in the homogenization solvent and cell lysis was performed: Incubation under liquid nitrogen for 1 minute Sonication for 10 minutes in a sonication bath filled with ice water Repeated for 3 x	-196 °C for grinding, then sonication in ice water	Grinding

3.1.4 Reproducibility – observation through relative standard deviations

Through the observation of the relative standard deviation (RSD), the variability of a measurement can be determined. RSD values reflect the reproducibility of the acquired datasets. Lower RSDs of detected peaks indicate better reproducibility and reliability.[60]

To demonstrate these two factors in the experiments conducted in this study, a plot was created to display the number of molecules falling within the RSD ranges of > 10%, 10-30%, 40-50%, and > 50% as colored segments of bar graphs (**Figure 14**). In general, two trends emerge from this graph: (1) more molecules fall within the two low RSD ranges (< 10 % and 10-30 %) when pure MeOH is used as the homogenization solvent, and (2) the mortar and pestle method yielded the best results regardless of the solvent choice.

For MeOH as a solvent, most of the molecules fall within the RSD range of 30-50 % for the Minilys method and almost none is observed in the lowest RSD range (< 10 %). For the Precellys, and the Covaris method, as well as the mortar and pestle method, the primary distribution is in the RSD range of 10-30 %. However, there is a noticeable difference for the Covaris method, as a significant number of molecules also fall into the lowest RSD range, similar to the results obtained with the mortar and pestle method. These observations agree well with the fact that the Minilys homogenizer lacks the ability to cool the system and therefore gave less advantageous results. Presumably, during this sample preparation phase, more transformation products were generated compared to other methods, since the enzyme activity could only be quenched by the organic solvent. In contrast, the homogenization methods, which were able to maintain a low temperature in addition to the use of the organic solvent, have the advantage that the enzymes can be inactivated even more efficiently. In addition, sample shredding or crushing with the Minilys bead homogenizer may be less efficient in general than with other methods. For instance, when compared to the Precellys, which is also a bead homogenizer, the Minilys could only achieve a maximum speed of 5000 rpm, whereas the Precellys reached up to 6800 rpm. Moreover, for the Minilys only 10-second homogenization cycles were performed due to the critical issue of sample heating and the need for manual cooling. Thus, there are more transformation reactions of certain metabolites in the samples, resulting in wider variations in the concentrations and therefore in higher RSDs for the Minilys, compared to the homogenization with the Precellys, Covaris, and the mortar and pestle. In the case of the Precellys method, sample heating could have occurred during the shaking interval

due to frictional forces, resulting in a lower number of exhibiting the lowest RSD range (< 10 %) compared to the results obtained with Covaris or the mortar and pestle homogenization method.

With the mortar and pestle, a significant majority of the molecules fall within the two low RSD regions compared to the others. This observation confirms that the gold standard, the mortar and pestle, is indeed the most effective technique for homogenizing tissue samples. However, it is time-consuming and requires considerable sample amounts, making it not always compatible for studies with a limited number of samples.

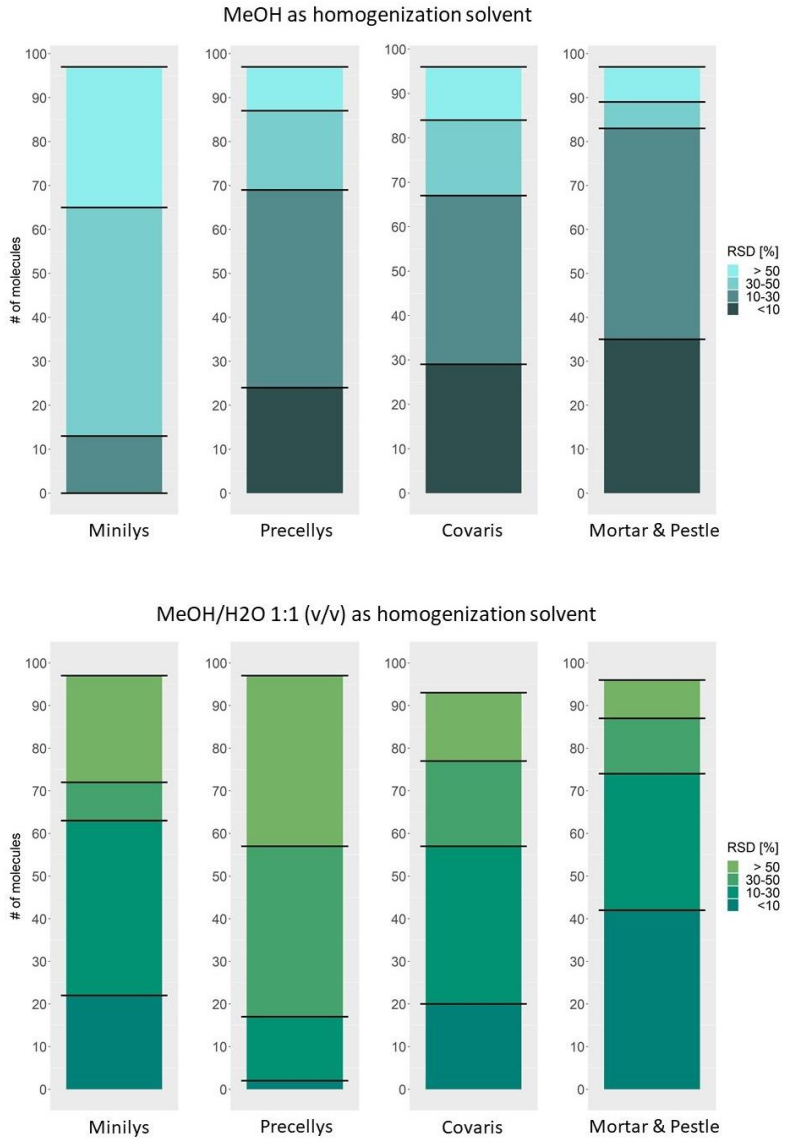

Figure 14: Number of molecules that fall within the corresponding RSD windows

The blue bars show the results from the use of pure MeOH as the homogenization solvent, whereas the green bars represent the findings from the homogenization with a mixture of MeOH/H_2O (1:1, v/v). Moreover, the individual homogenization methods, Minilys, Precellys, Covaris, and Mortar, and Pestle are listed from left to right. The darker the shading of the respective RSD range, the lower value is indicated. These RSD domains are further marked by black lines.

The same observations occurred for the approach with the solvent mixture (MeOH/H_2O), represented through the green-colored bars. The trend is similar to the approach with pure MeOH concerning the number of molecules within the RSD ranges for the 4 homogenization methods. However, there is a difference, as there are fewer molecules found within the lower RSD ranges. Therefore, homogenization with pure MeOH appears to be more advantageous as the homogenization solvent. However, it is important to note that the pure MeOH solvent had a lower temperature compared to the solvent mixture. MeOH was pre-cooled at -20 °C, while water could only be cooled to 4 °C to prevent freezing. As mentioned earlier, lower temperatures generally result in better quenching quality, as enzymes can be inhibited more effectively in cooler environments.

Furthermore, it was surprising that the bead-based homogenization methods behave in the opposite manner. In this context, the Minilys, when used with MeOH/H_2O as the solvent seem to outperform the Precellys with more analytes falling in the lower RSD ranges.

In order to provide a more concise and straightforward representation of the RSDs, a boxplot was created to compare the performance of the two solvent systems, pure MeOH and MeOH/H_2O, for each homogenization method (**Figure 15**).

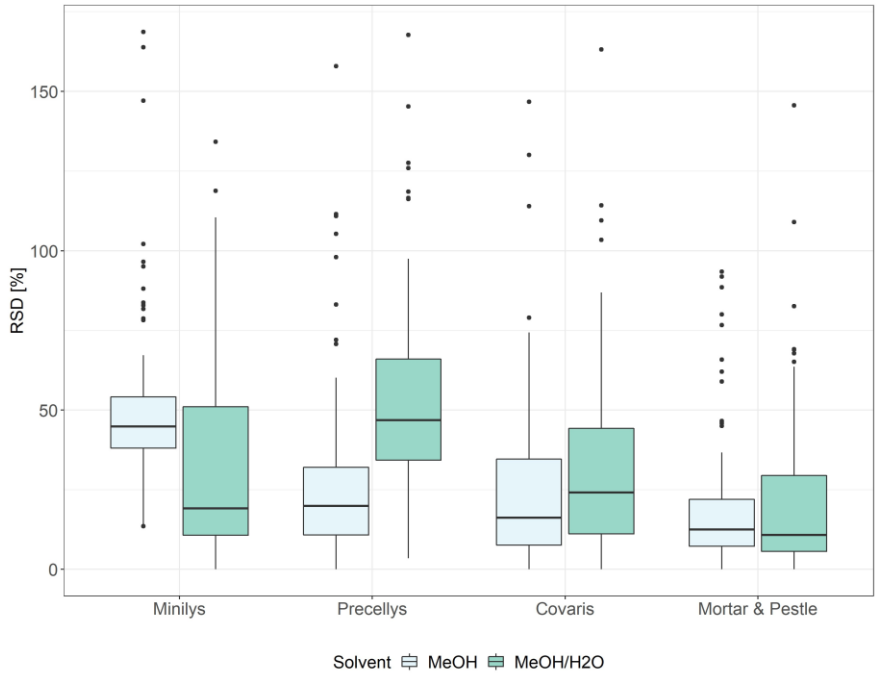

Figure 15: Boxplot of the four investigated homogenization methods

The green color corresponds to the solvent mixture, whereas blue stands for the pure organic solvent.

It can be seen at a glance that even here the mortar and pestle is superior to the other methods, regardless of the solvent choice. Additionally, it is evident that pure MeOH as a solvent outperforms the MeOH/H_2O mixture for almost every homogenization method. The RSD distributions for the methanol homogenizations are of lower values and narrower in their ranges for most methods, with the exception of the Minilys method. The Minilys appear to perform better when the solvent mixture is used. Further experiments are planned to validate this finding.

Moreover, a contrasting behavior of the RSDs with respect to the solvent composition is observed with the two bead homogenizers, as already discussed earlier. The examination indicates that homogenization based on mechanical forces is highly dependent on the solvent choice. However, it remains unexplained why the RSDs exhibit contrasting behavior in response to solvent variations for the bead homogenization methods. This could be attributed to an undetected experimental error, issues with the data analysis, or even the presence of an outlier. Further investigation is needed to delve into these aspects.

For homogenization with a mortar and pestle, the medians for both solvent systems hardly differ from each other. It seems that the solvent composition does not exert a significant impact on the molecules during cell lysis in the ultrasonic bath. It is necessary to mention that during homogenization by grinding in the mortar, the samples are not yet exposed to the solvent at this point, unlike other methods where the samples are homogenized directly in the solvent. This factor, combined with the fact that the RSDs are only minimally influenced by the solvent composition during sonication, might explain the similar RSD distribution.

Moreover, the minimal effect of solvent choice during sonication would also explain why the Covaris method shows less deviated and relatively low RSDs compared to the bead homogenization methods. The S220 focused ultrasound device is based on the Adaptive Focused Acoustics (AFA) developed and patented by the company Covaris. AFA provides a precise, accurate, and controlled delivery of energy to the samples. When exposed to these waves, small bubbles form between the sample tissue, leading to tissue dissociation as they explode, down to the individual cell level. During this process, cell walls are ruptured, leading to cell lysis.[61] The minor variations in the RSD medians for the different solvents might be attributed to the fact that, in the case of the Covaris method, both homogenization and cell lysis were carried out in a single step. In contrast, with the mortar and pestle, the tissue was already well pulverized when undergoing cell lysis in the sonication bath. This enhancement in

accessibility to the sample surface resulted in even more efficient homogenization within the given time frame.

In summary, based on the observations made in this section, the following conclusions can be drawn: (1) Employing MeOH as the homogenization solvent enhances reproducibility, (2) the gold standard method gave the best results in this respect, and (3) ultrasound-based methods indicated less dependence on the solvent choice compared to the bead homogenizers.

3.1.5 Sample stability – observation through sphingosine-1-phsosphate

To assess sample stability, a more detailed examination of the lipid species sphingosine-1-phosphate (S1P), both falling into the class of lysophospholipids, was conducted.[62] Together with the species LPC 18:2, it serves as a marker to track the stability of the sample molecules throughout the pre-analytical steps.[63] During sample collection and preparation, lipids are prone to undergo oxidation, hydrolysis, and turnover due to enzymatic processes or varying temperatures and, the presence of oxygen and/or water. All these criteria lead to lipid degradation, as well as transformation of other metabolites, within a given sample.[63]

For instance, long-term storage of samples at room temperature leads to an increase in the quantity of LPCs, LPEs, and FAs, while the amount of PEs and PCs decreases. This change may be attributed to the cleavage of the ester bonds within these phospholipids.[64] A similar concept applies to S1P: the cleavage of FA and choline residues leads to the formation of the lipid species S1P from sphingomyelin. In a study, it was reported that long-term storage of samples at room temperature results in an increased concentration of S1P.[65]

For the purpose of this work, only the formation of the species S1P, arising from the cleavage of certain lipids, was observed to examine the sample stability. The area signals of S1P were normalized and plotted against selected lipid species included in the ISTD. The same color code as for the RSDs was applied in these graphs with respect to the homogenization solvents. The color blue represents pure MeOH as the solvent and green stands for the mixture MeOH/H_2O. **Figure 16** shows the signal of S1P normalized against the lipid species sphingomyelin (SM 36:2). A higher amount of S1P indicates elevated degradation of the molecules within the sample, due to enzymatic or chemical processes.

The Minilys method revealed the highest detection of S1P molecules, indicating a notable decrease in sample stability. This result was expected since the instrument does not have a built-in cooling capability. The Covaris method yielded the second-worst outcomes. Thus, homogenization via ultrasound appeared to provide a relatively reproductive method according to the RSD observations, but it is apparently associated with significant analyte degradation. The mortar and pestle method ranked second in terms of sample stability, generating only half the amount of S1P compared to the Minilys method. Once again, the mortar and pestle, considered as the gold standard, showed outstanding performance. The method that was able to keep the samples most stable was the Precellys method, although strong solvent dependence occurred, as observed with the RSDs. As previously described in the observations regarding the RSDs, further in-depth investigation is required to gain a more precise understanding of this significant solvent dependency.

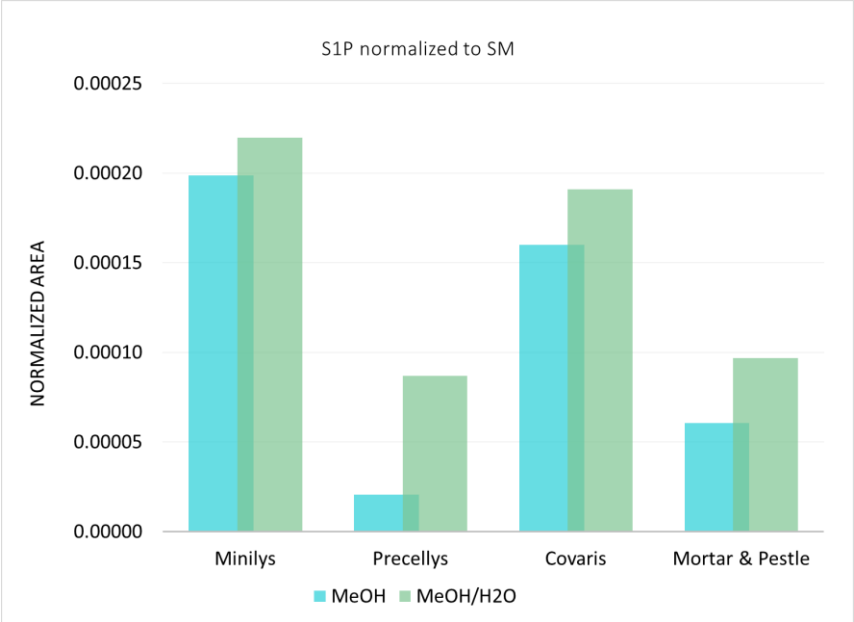

Figure 16: S1P normalized to sphingomyelin

The blue colored bars represent the results from the homogenization with pure methanol as the homogenization solvent, whereas the green colored ones depict observations from the utilization of a MeOH/H_2O (1:1, v/v) solvent mixture.

The use of pure MeOH as solvent showed better sample stability across all four homogenization methods. Hence, it is advisable to avoid aqueous solutions during sample homogenization.

Nevertheless, it is essential to acknowledge that pure methanol was maintained at a considerably lower temperature (-20 °C) than the solvent mixture MeOH/H_2O (-20 °C and 4 °C). This is likely to have a substantial impact on sample stability. The ability to cool the solvent to sub-zero temperatures makes the use of pure methanol more advantageous. The Precellys method in combination with pure MeOH was clearly superior to all other methods in terms of sample stability.

To validate these observations, an additional plot was created in which the S1P signals were normalized against those of lysophosphatidylcholine (LPC 18:1) (**Figure 17a**), and almost the same conclusions were reaffirmed with the exception of the Minilys method. In this case, a higher concentration of S1P was found for the Minilys method when the sample was homogenized in pure MeOH. As an additional control, another plot was constructed where the S1P areas were normalized against those of cholesterol (**Figure 17b**). Once more, the use of pure methanol with the Minilys method resulted in an increased formation of S1P, while all other methods were consistent with the previous plots. However, the observed signals are not derived from absolute quantification using standard materials. Therefore, these statements are only conjectures for the time being. To draw definitive conclusions, future experiments are needed to investigate the absolute concentrations of S1P.

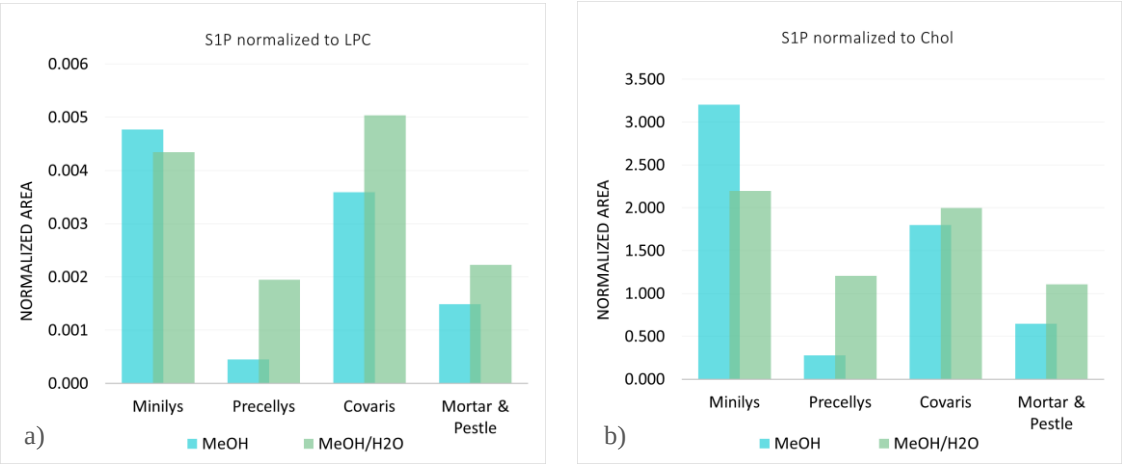

Figure 17: S1P normalized to sphingomyelin and lysophosphatidylcholine

In this Figure, the area of S1P normalized to a) lysophosphatidylcholine and b) cholesterol is depicted. The blue color represents the homogenization with pure MeOH and the green bars stand for the homogenization with MeOH/H_2O (1:1, v/v) as the homogenization solvent.

According to the observations, we can draw the following conclusions: (1) in the case of most homogenization methods, it is preferable to use pure MeOH as the homogenization solvent that provides lower temperatures, (2), the Precellys method demonstrates the ability to maintain the analytes most stable, and (3) the combination of pure MeOH as a solvent with the Precellys homogenization method yielded the best results in terms of sample stability.

4 CONCLUSION

In this work, we explored four different homogenization methods: Minilys, Precellys, Covaris, and mortar and pestle, along with two homogenization solvents, pure MeOH and a MeOH/H_2O mixture (1:1, v/v). Since the aim of this work was to evaluate the applied homogenization strategies, the examination was limited to the reproducibility and sample stability of each method. Moreover, investigations about reproducibility predominantly emphasized data evaluation of the metabolites rather than the lipids. Only the lipid species S1P was further investigated to gain insight into sample stability concerning each parameter under examination – homogenization solvents and methods. Furthermore, the instrument's stability was assessed by monitoring the signal areas from the QC samples for both metabolites and lipids.

The gold standard method, which involves homogenization with mortar and pestle, yielded the best results in terms of reproducibility. Moreover, this method exhibited minimal dependence on the solvent used. However, the actual homogenization step was conducted without solvent. In contrast, bead homogenizers were particularly influenced by the solvent choice. In the case of the Precellys method, similar reproducibility to the gold standard was achievable when using methanol, but high RSD values were observed when employing the MeOH/H_2O mixture. Surprisingly, the Minilys method showed the opposite behavior. Nevertheless, it is crucial to note that the solvents were also at different temperatures. This introduces another variable that warrants further investigation. The inquiry extends to whether the observed differences are solely temperature-related or if the solvent's position has a direct impact on the outcomes. The Covaris method indicated less dependence on the solvent, but homogenization with pure methanol still performed slightly better than using the MeOH/H_2O mixture.

In terms of sample stability, the gold standard method also performed well, coming in second. However, the combination of the Precellys method with pure methanol as the solvent proved to be superior to all other methods. Nevertheless, it is worth noting that the signals from the S1P marker were only normalized against standard substances due to the unavailability of reference material for S1P. Thus, performing absolute quantification of the marker itself is another important task to be addressed and will validate these findings.

From the examination of instrument stability, it was recognizable that reliable data was generated for metabolites with the chosen method. However, for lipids, a more detailed investigation is necessary due to the observed decrease in signal intensity as the measurement progressed.

Considering that the aim of this study is to apply the findings and conclusions to the primary project, which involves investigating the muscle metabolome of human muscle samples from biopsies, the preference would be for the Precellys method with pure methanol as the homogenization solvent. This choice is driven by the following considerations: (1) it yielded excellent results in terms of reproducibility and sample stability, (2) it is a time-efficient method providing high throughputs, and (3) it is suitable for homogenizing smaller sample quantities, allowing for more economical preparation of precious samples. This method will be applied in the second part of the project. Currently, the clinical samples are being collected.

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6 ATTACHEMENT

Table S 1: Masses and volumes for the respective homogenization methods and subsequent extraction

Minilys					
Pork (mg)	MeOH [µL] (20 µL/mg)	MeOH /H_2O [µL] (1:1)	MTBE [µL]	Water + AF [µL]	Addition MeOH [µL] (MeOH/H_2O 4:1)
19.57		195.7/195.7	1305	327	1112
16.52		165.2/165.2	1101	276	939
19.15		191.5/191.5	1277	320	1089
14.82	296		988	248	842
11.13	223		742	186	633
17.28	346		1152	289	982
Precellys					
Pork (mg)	MeOH (20 µL/mg)	MeOH/H_2O (1:1)	MTBE (µL)	Water + AF (µL)	Addition MeOH (MeOH:water 4:1)
20.62		206.2/206.2	1375	345	1172
16.09		160.9/160.9	1073	269	915
18.19		181.9/181.9	1213	304	1034
11.8	236		787	197	671
14.39	288		959	240	818
21.68	434		1445	362	1232
13.61	272		907	227	774

Continuation of Table S 1: Masses and volumes for the respective homogenization methods and subsequent extraction

Covaris					
Pork (mg)	MeOH (20 µL/mg)	MeOH/H_2O (1:1)	MTBE (µL)	Water + AF (µL)	Addition MeOH (MeOH:water 4:1)
19.43		194.3/194.3	1295	325	1104
16.1		161.0/161.0	1073	269	915
18.57		185.7/185.7	1238	310	1056
22.36	447		1491	374	1271
14.41	288		961	241	819
14.81	296		987	247	842
Mortar & Pestle					
Pork (mg)	MeOH (20 µL/mg)	MeOH/H_2O (1:1)	MTBE (µL)	Water + AF (µL)	Addition MeOH (MeOH:water 4:1)
19.56		195.6/195.6	1304	327	1112
21.13		211.3/211.3	1409	353	1201
21.16		211.6/211.6	1411	354	1203
15.07	301		1005	252	857
17.02	340		1135	284	967
18.78	376		1252	314	1068

Table S 2: Metabolites in the in-house ESTD mixture

No.	Molecule Name	Sum Formula	Molecular weight g/mol
1	1-Methylhydantoin	C4H6N2O2	114.10
2	2-(Carbamoylamino)butanedioic acid	C5H8N2O5	131.13
3	4-Hydroxy-proline	C5H9NO3	131.13
4	Adenine	C5H5N5	135.13
5	alpha-Aminoadipic acid	C6H11NO4	161.16
6	Biotin	C10H16N2O3S	244.31
7	Choline chloride	C5H13NO	139.62
8	Cytosine	C4H5N3O	111.10
9	Fructose	C6H12O6	180.16
10	Fumarate	C4H4O4	116.10
11	Glyoxylic acid	C2H2O3	92.05
12	Guanidineacetic acid	C3H7N3O2	117.11
13	Guanine	C5H5N5O	151.12
14	Homocysteine	C4H9NO2S	135.18
15	Mannitol	C6H14O6	182.17
16	Methionine sulfone	C5H11NO4S	181.21
17	N-Acetyl-L-aspartic acid	C6H9NO5	175.14
18	Pyruvate	C3H4O3	110.04
19	Seleno-methionine	C5H11NO2Se	196.11
20	Thymine	C5H6N2O2	126.12
21	Uracil	C4H4N2O2	112.09
22	Urea	CH4N2O	60.06
23	2′-Deoxycytidine	C9H13N3O4	227.22
24	3-Methyl-2-oxovaleric acid	C6H10O3	152.12
25	3-Methylcytidine	C10H15N3O5	369.35
26	5'-Deoxy-5'-Methylthioadenosine	C11H15N5O3S	297.33
27	5-Methyluridine	C10H14N2O6	258.23
28	Adenosine	C10H13N5O4	267.24
29	Asparagine	C4H8N2O3	132.12
30	Carnitine	C7H15NO3	197.66
31	Cysteic acid	C3H7NO5S	187.17
32	Cytidine	C9H13N3O5	243.22
33	Fructose-6-phosphate	C6H13O9P	336.32
34	Galactose	C6H12O6	180.16
35	Glutamine	C5H10N2O3	146.14

Continuation of Table S 2: Metabolites in the in-house ESTD mixture

No.	Molecule Name	Sum Formula	Molecular weight g/mol
36	Guanosine	C10H13N5O5	283.24
37	Hydroxyglutaric acid	C5H8O5	192.08
38	Inosine	C10H12N4O5	268.23
39	Isoguanosine	C10H13N5O5	301.26
40	Malate	C4H6O5	134.09
41	Mannitol 1-phosphate	C6H15O9P	262.15
42	N-Acetyl-Asp-Glu	C11H16N2O8	304.25
43	Sarcosine	C3H7NO2	89.09
44	Thymidine	C10H14N2O5	242.23
45	Tryptophan	C11H12N2O2	204.23
46	Uridine	C9H12N2O6	244.20
47	alpha-Ketoglutarate	C5H6O5	168.08
48	AMP	C10H14N5O7P	347.20
49	CMP	C9H14N3O8P	367.16
50	Cysteine	C3H7NO2S	121.16
51	Cysteinyl-glycine	C5H10N2O3S	178.21
52	Dihydroxyacetonephosphate	C3H7O6P	181.90
53	Flavinadenin dinucleotide	C27H33P2N9O15	829.52
54	Glucose-1-phosphate	C6H13O9P	304.10
55	GMP	C10H14N5O8P	407.18
56	Inosine 5'-monophosphate	C10H13N4O8P	392.17
57	Ketoisovalerate	C5H7NaO3	138.10
58	Lactate	C3H6O3	112.06
59	Mannose	C6H12O6	180.16
60	Mevalonic acid	C6H12O4	155.10
61	N4-Acetylcytidine	C11H15N3O6	285.25
62	N-Acetyl-serine	C5H9NO4	147.13
63	L-Ornithine	C5H12N2O2	168.62
64	Oxaloacetic acid	C4H4O5	132.07
65	Palmitic acid	C16H32O2	256.42
66	Phosphocreatine	C4H10N3O5P	255.08
67	Ribose-5-phosphate	C5H11O8P	310.10
68	Succinate	C4H6O4	270.14
69	Thymidine 5′-monophosphate	C10H15N2O8P	366.17
70	UMP	C9H13N2O9P	368.15
71	2-Phosphoglycerate	C3H7O7P	252.00
72	1-Methylnicotinamide	C7H8N2O	172.61
73	6-Phosphogluconate	C6H13O10P	342.00

Continuation of Table S 2: Metabolites in the in-house ESTD mixture

No.	Molecule Name	Sum Formula	Molecular weight g/mol
74	Adenosine diphosphate	C10H15N5O10P2	427.20
75	Argininosuccinic acid	C10H18N4O6	334.24
76	Betaine	C5H11NO2	117.15
77	L-Citrulline	C6H13N3O3	175.19
78	Erythrol	C4H10O4	122.12
79	Gluconate	C6H12O7	218.14
80	Glucose-6-phosphate	C6H13O9P	282.12
81	Glutamyl-cysteine	C8H14N2O5S	250.27
82	Inositol	C6H12O6	180.20
83	Isocitrate	C6H8O7	258.07
84	Ribulose-5-phosphate	C5H11O8P	274.07
85	S-(Adenosyl)-methionine	C15H22N6O5S	507.82
86	Sedoheptulose-7-phosphate	C7H15O10P	290.16
87	Uridine 5′-diphosphate	C9H14N2O12P2	550.09
88	3-Phosphoglycerate	C3H7O7P	230.02
89	Adenosine 5′-triphosphate	C10H16N5O13P3	551.14
90	Citrate	C6H8O7	210.14
91	Cytidine 5′-triphosphate	C9H16N3O14P3	527.12
92	2′-Deoxyadenosine 5′-monophosphate	C10H14N5O6P	331.22
93	2-Deoxycytidine 5'-Monophosphate	C9H14N3O7P	351.16
94	Guanosine 5′-triphosphate	C10H16N5O14P3	523.18
95	Homoserine	C4H9NO3	119.20
96	Kynurenine	C10H12N2O3	208.21
97	Melatonine	C13H16N2O2	232.28
98	NAD+	C21H27N7O14P2	685.41
99	NADH	C21H29N7O14P2	709.40
100	NADP+	C21H28N7O17P3	787.34
101	NADPH	C21H30N7O17P3	833.35
102	Octopamine	C8H11NO2	189.64
103	Propionyl-L-carnitine	C10H19NO4	217.26
104	Ribose	C5H10O5	150.13
105	Serotonine	C10H12N2O	212.68
106	Spermidine	C7H19N3	145.25
107	Spermine	C10H26N4	202.34
108	Thiamine hydrochloride	C12H17N4OS	337.27
109	Uridine 5′-triphosphate	C9H15N2O15P3	550.09
110	2'-Deoxyuridine	C9H12N2O5	228.20
111	3'AMP	C10H14N5O7P	347.22

Continuation of Table S 2: Metabolites in the in-house ESTD mixture

No.	Molecule Name	Sum Formula	Molecular weight g/mol
112	Adenosine 3′,5′-cyclic monophosphate	C10H12N5O6P	329.21
113	Guanosine 3′,5′-cyclic monophosphate	C10H12N5O7P	345.21
114	cis-Aconitate	C6H6O6	174.11
115	L-Cystathionine	C7H14N2O4S	222.26
116	Dihydroxyisovalerate	C5H10O4	134.13
117	Erythrose-4-phosphate	C4H9O7P	222.10
118	Fructose-1,6-bisphosphate	C6H14O12P2	406.06
119	Glucose	C6H12O6	180.16
120	Glutathione, oxidized	C20H32N6O12S2	612.64
121	Glutathione, reduced	C10H17N3O6S	307.32
122	Nicotinamide	C6H6N2O	122.12
123	Pseudouridine	C9H12N2O6	244.20
124	Trehalose	C12H22O11	378.33
125	Xanthine	C5H4N4O2	152.11
126	Xylose	C5H10O5	150.13

Table S 3: Lipid species in the in-house ESTD mixture

No.	Molecule Name	Sum Formula	Molecular weight g/mol
1	AcCa 18:1	C25H47NO4	426.65
2	AcCa 6:0	C13H25NO4	259.34
3	Cer 34:1;2	C34H67NO3	537.90
4	Cer 36:1;2	C36H71NO3	565.96
5	Cer 42:0;2	C42H85NO3	652.13
6	Cer 42:1;2	C42H83NO3	650.12
7	Cer 42:1;3	C42H83NO4	666.12
8	Cer 42:2;2	C42H81NO3	648.10
9	CL 64:4	C73H134O17P2	1223.84
10	CL 72:4	C81H150O17P2	1321.94
11	Co Q10	C59H90O4	863.35
12	DG 32:0	C35H68O5	568.91
13	DG 34:1	C37H70O5	594.95
14	DG 36:2	C39H72O5	620.99
15	DG 38:4	C41H72O5	645.01
16	DG 40:6	C43H72O5	669.03
17	FA 16:0	C16H32O2	256.42
18	FA 18:0	C18H36O2	284.48

Continuation of Table S 3: Lipid species in the in-house ESTD mixture

No.	Molecule Name	Sum Formula	Molecular weight g/mol
19	FA 20:1	C20H38O2	310.52
20	FA 22:0	C22H44O2	340.58
21	Hex2Cer 34:1;2	C46H87NO13	862.18
22	HexCer 34:1;2	C40H77NO8	700.04
23	HexCer 36:1;2	C42H81NO8	511.90
24	HexCer 42:2;2	C48H91NO8	570.02
25	LPC 16:0	C24H50NO7P	495.63
26	LPC 18:0	C26H54NO7P	523.68
27	LPE 18:0	C23H48NO7P	481.60
28	MG 16:0	C19H38O4	258.44
29	PA 34:1	C37H71O8P	674.93
30	PA 36:2	C39H73O8P	700.97
31	PC 32:0	C40H80NO8P	734.04
32	PC 34:0	C42H84NO8P	762.09
33	PC 34:1	C42H82NO8P	760.08
34	PC 34:2	C42H80NO8P	758.06
35	PC 36:0	C44H88NO8P	790.15
36	PC 36:1	C44H86NO8P	788.13
37	PC 36:2-1	C44H84NO8P	786.12
38	PC 36:2-2	C44H84NO8P	786.12
39	PC 36:4-1	C44H80NO8P	782.08
40	PC 36:4-2	C44H80NO8P	782.08
41	PC 36:6	C44H76NO8P	778.05
42	PC 38:4	C46H84NO8P	810.14
43	PC 38:6	C46H80NO8P	806.11
44	PC 40:0	C48H96NO8P	846.25
45	PC 40:6	C48H84NO8P	834.16
46	PC 44:0	C52H104NO8P	902.36
47	PC 48:0	C56H112NO8P	958.47
48	PC P-36:1	C44H86NO7P	772.13
49	PE 34:1	C39H76NO8P	718.00
50	PE 36:2	C41H78NO8P	753.11
51	PG 34:1	C40H77O10P	749.01
52	PG 36:2	C42H79O10P	775.05
53	Carotine	C40H56	536.88
54	Tocopherol	C29H50O2	406.69
55	PS 34:1	C40H76NO10P	762.01
56	PS 36:2	C42H78NO10P	788.04

Continuation of Table S 3: Lipid species in the in-house ESTD mixture

No.	Molecule Name	Sum Formula	Molecular weight g/mol
57	SM 36:2;2	C41H81N2O6P	715.06
58	SPB 18:1;2	C18H37NO2	299.49
59	CE 16:0	C43H76O2	625.07
60	CE 18:0	C45H80O2	653.12
61	CE 18:2	C45H76O2	649.09
62	Cholesterol	C27H46O	386.66
63	Ergosterol	C28H44O	396.65
64	Stigmasterol	C29H48O	412.69
65	TG 42:0	C45H86O6	723.16
66	TG 48:0	C51H98O6	807.32
67	TG 48:3	C51H92O6	801.28
68	TG 54:0	C57H110O6	891.48
69	TG 54:3	C57H104O6	885.44
70	TG 54:6	C57H98O6	879.39
71	TG 54:9	C57H92O6	873.34
72	TG 60:3	C63H116O6	969.60
73	TG 66:3	C69H128O6	1053.76