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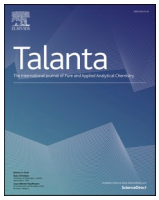

Development and thorough evaluation of a multi-omics sample preparation workflow for comprehensive LC-MS/MS-based metabolomics, lipidomics and proteomics datasets

Lana Brockbals [a,b,*], Maiken Ueland [a,c], Shanlin Fu [a], Matthew P. Padula [c,d]

[a] Centre for Forensic Science, School of Mathematical and Physical Sciences, Faculty of Science, University of Technology Sydney, PO Box 123, Broadway, 2007 NSW, Australia
[b] Department of Forensic Pharmacology and Toxicology, Institute of Forensic Medicine, University of Zurich, Winterthurerstrasse 190/52, 8057 Zurich, Switzerland
[c] Hyphenated Mass Spectrometry Laboratory, Faculty of Science, University of Technology Sydney, PO Box 123, Broadway, 2007 NSW, Australia
[d] School of Life Sciences, Faculty of Science, University of Technology Sydney, PO Box 123, Broadway, 2007 NSW, Australia

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ABSTRACT

The importance of sample preparation selection if often overlooked particularly for untargeted multi-omics approaches that gained popularity in recent years. To minimize issues with sample heterogeneity and additional freeze-thaw cycles during sample splitting, multiple -omics datasets (e.g. metabolomics, lipidomics and proteomics) should ideally be generated from the same set of samples. For sample extraction, commonly biphasic organic solvent systems are used that require extensive multi-step protocols. Individual studies have recently also started to investigate monophasic (all-in-one) extraction procedures. The aim of the current study was to develop and systematically compare ten different mono- and biphasic extraction solvent mixtures for their potential to aid in the most comprehensive metabolomics, lipidomics and proteomics datasets. As the focus was on human postmortem tissue samples (muscle and liver tissue), four tissue homogenization parameters were also evaluated. Untargeted liquid chromatography mass spectrometry-based metabolomics, lipidomic and proteomics methods were utilized along with 1D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and bicinchoninic acid (BCA) assay results. Optimal homogenization was found to be achieved by bead-homogenizing 20 mg of muscle or liver tissue with 200 µL (1:10 ratio) Water:Methanol (1:2) using 3 × 30 s pulses. The supernatant of the homogenate was further extracted. Comprehensive ranking, taking nine different processing parameters into account, showed that the monophasic extraction solvents, overall, showed better scores compared to the biphasic solvent systems, despite their recommendation for one or all of the -omics extractions. The optimal extraction solvent was found to be Methanol:Acetone (9:1), resulting in the most comprehensive metabolomics, lipidomics and proteomics datasets, showing the potential to be automated, hence, allowing for high-throughput analysis of samples and opening the door for comprehensive multi-omics results from routine clinical cases in the future.

1. Introduction

Individual omics studies (e.g. genomics, transcriptomics, proteomics, lipidomics, metabolomics) have been used in the last decade for the study of diseases (including biomarker research and drug discovery) or in fields like agriculture, plant sciences and microbiology [1,2]. In recent years, multi-omics workflows have gained popularity to understand links between genotype and phenotype and to generate a more comprehensive/systemic picture of biological and biochemical processes within an organism [3,4]. Multi-omics has previously been defined by Krassowski et al. as "an approach aiming to improve the understanding of systems regulatory biology, molecular central dogma and genotype-phenotype relationship by combining three or more different omics data" [1]. While the integration of multiple omics

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* Corresponding author. Centre for Forensic Science, School of Mathematical and Physical Sciences, Faculty of Science, University of Technology Sydney, PO Box 123, Broadway 2007 NSW, Australia.
E-mail address: Lana.Brockbals@irm.uzh.ch (L. Brockbals).

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datasets often requires overcoming extensive computational and data processing hurdles (e.g. combining dimensions of very different outputs from each -omics technique), it is crucial to consider multi-omics method integration during study design and sample preparation/extraction [1,4,5]. In general, the importance of sample preparation selection is often overlooked [6]. Depending on the choice of extraction solvent/method, an extraction bias for certain compounds/compound classes can occur. Metabolomics studies by Canelas et al. and Duportet et al. have previously shown that the same samples extracted with different extraction methods/solvents can result in significantly different measurements of metabolite levels and contradictory biological interpretation of active metabolite pathways [7,8]. This stresses the enormous impact that the choice of sample preparation method/-extraction solvent can have on untargeted -omics studies. While a certain level of extraction bias can most likely never be excluded with a simple and rapid extraction workflow, it is crucial to be aware of the limitations of one's sample preparation approach and ideally choose a method that is as unselective and reproducible as possible.

Often for multi-omics studies, collected samples are split into multiple aliquots for -omics specific extraction [9–11]. However, to minimize issues with sample heterogeneity and additional freeze-thaw cycles during sample splitting, multiple -omics datasets should ideally be generated from the same set of samples [5]. For this, frequently biphasic organic solvent systems are used, requiring extensive multi-step protocols to isolate individual compound classes [12–14]. Most common examples of these solvent systems are adaptations of the Bligh-Dyer/Folch- (chloroform, methanol (MeOH) and water) [15,16] or Matyash-extractions (methyl tert-butylether (MTBE), MeOH and water) [17], requiring careful pipetting during phase separations. As a consequence, these workflows are low-throughput and susceptible to sample loss [18]. In general, these extractions usually result in an organic and an aqueous phase along with a protein precipitate, that can be separated out for lipidomics, metabolomics and proteomics analysis, respectively. Historically, however, protein precipitation methods have often been considered unreliable for proteomics analysis and specialized protein extraction was preferred. Nevertheless, due to advancements and more systematic studies, protein precipitation is nowadays well established and one of the most common methods for protein extraction in both bottom-up and top-down proteomic workflows [19–21]. Individual studies have also started to investigate monophasic (all-in-one) extraction procedures, to increase throughput, decrease sample loss and potentially allow automation [18,22].

The aim of the current study was to develop and thoroughly evaluate a multi-omics sample preparation workflow that aids in comprehensive metabolomics, lipidomics and proteomics datasets by comparing and adapting/modifying existing homogenization procedures along with monophasic and biphasic extraction solvent mixtures. The focus was on postmortem human muscle and liver tissue samples. Comparing these two matrices, the aim was also to investigate whether or not a biphasic extraction procedure might be mandatory/advisable to achieve comprehensive lipidomics results for lipid-rich tissue such as liver [23,24].

In total, four homogenization parameters were evaluated along with 10 different mono- or biphasic extraction solvents/mixtures. Selection of the latter were based on adaptations of the classic Bligh-Dyer/Folch and Matyash-extractions along with a variety of all-in-one extraction procedures, such as Boxler et al. [25] and Muehlbauer et al. [18]. For the biphasic solvent mixtures, in addition to the classic 2:1 organic solvent-to-water ratio, a 1:1 mixture (extra water) was trialed, as this was previously suggested to increase the yield of metabolites, while decreasing the amount of lipids extracted within the polar metabolomics phase [23]. Overall, homogenization and extraction parameters were compared and thoroughly evaluated based on untargeted liquid chromatography tandem mass spectrometry (LC-MS/MS)-based metabolomics, lipidomics and proteomics as well as 1D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and bicinchoninic acid (BCA) assay results.

2. Materials and methods

2.1. Homogenization and extraction

Postmortem human tissue samples (thigh muscle and liver samples; recently deceased donors frozen for preservation and thawed for sample collection) were collected at the Anatomical Facility, University of Technology Sydney (UTS) through the Body Donation Program, with donors giving consent in accordance with the New South Wales Anatomy Act (1997). The study was approved by the UTS Human Research Ethics Committee (ETH18-2999; issued July 4, 2019). During method development, several parameters were investigated and optimised focussing on homogenization and extraction. Overall, five different homogenization solvents, five different pulsing frequencies, four different sample amounts and four different sample material-to-homogenization solvent ratios were investigated along with 10 different extraction solvent combinations. All experiments looking at homogenization parameters were conducted using two biological replicates (muscle tissue) and for all experiments optimising the extraction, homogenized pooled samples (one muscle pool sample and one liver pool sample) were used in biological duplicates (the number of technical injection replicates (e.g. n = 3 for metabolomics experiments are specified for each analytical technique below). While Table 1 details the list of changed conditions that were tested throughout the method development phase, the general workflow all samples underwent was as follows: All tissue samples were homogenized using 2 mL microtubes filled with 3 mm zirconium beads on the BeadBug™ benchtop homogenizer (Benchmark Scientific, Sayreville, NJ, US) with addition of phenylalanine-d2 as an internal standard (concentration in homogenate: 2 μg/mL, detectable in positive and negative ionization mode; monitoring extraction variability) and homogenization solvent according to Table 1. The samples were vigorously shaken for 5 min and spun down for 30 s on a benchtop centrifuge. The supernatant (150 μL) was transferred into a fresh 1.5 mL microcentrifuge tube and extracted using the specified solvent (see Table 1). The samples were vigorously shaken for 5 min and centrifuged for 10 min at 14 000 rpm. For miscible monophasic solvent systems, the supernatant was the extract used for both metabolomics and lipidomics mass spectrometric analysis. For biphasic solvent systems (MTBE:Water and dichloromethane (DCM):Water), the aqueous phase was used for metabolomics mass spectrometric analysis, while the organic phase was subsequently analysed for lipids (after evaporation and reconstitution in the same volume of the LC-eluents using the method's starting conditions). Caffeine-d3 (detectable in positive ionization mode) and palmitoleic acid-d2 (detectable in negative ionization mode; concentration in solution each: 5 μg/mL) were added as internal standards to both metabolomics and lipidomics extracts prior to analysis to check for instrument variability. The remaining pellet in all cases (after evaporation of the organic solvent) was further prepared for protein/peptide analysis using 1D gel electrophoresis, BCA assay and/or mass spectrometric analysis, as detailed below.

2.2. Protein content visualisation and quantification (1D SDS-PAGE and BCA assay)

2.2.1. Sample preparation

The protein fraction (protein pellet) was visualized using 1D SDS-PAGE. For samples investigating homogenization parameters (homogenization solvent, pulsing frequency, sample material-to-homogenization solvent ratio and sample amount), the complete protein pellet was resuspended in 10 μL of SDS sample buffer (200 mM Tris-Cl buffer (pH 6.8), 8 % SDS, 40 % glycerol and 0.4 % bromophenyl blue) and water-mixture (1:1), and reduced and alkylated with tris(2carboxyethyl)phosphine (TCEP, 5 mM) and iodoacetamide (IAA, 10 mM). Following heating (95 °C for 5 min) and centrifugation (14 000 rpm for

Table 1
List of conditions tested throughout the method development phase per parameter; variable condition highlighted in italic per parameter; MTBE: methyl-*tert*-butylether, MeOH: methanol, ACN: acetonitrile. DCM: dichloromethane.

Parameter	Sample weight [mg]	Homogenization solvent	Sample material-to-homogenization solvent ratio [mg:uL]	Homogenization pulsing [s] (pause between pulses 1 min on ice)	Extraction solvent
Homogenization solvent	20	*Water*	1:10	3 x 30	MTBE:MeOH (1:1)
	20	*Water:MeOH (1:2)*	1:10	3 x 30	MTBE:Water (1:1)
	20	*Water:ACN (1:2)*	1:10	3 x 30	MTBE:Water (1:1)
	20	*MeOH*	1:10	3 x 30	MTBE:Water (1:1)
	20	*ACN*	1:10	3 x 30	MTBE:Water (1:1)
Pulsing frequency	20	Water:MeOH (1:2)	1:10	*1 x 30*	MTBE:MeOH (1:1)
	20	Water:MeOH (1:2)	1:10	*3 x 30*	MTBE:Water (1:1)
	20	Water:MeOH (1:2)	1:10	*5 x 30*	MTBE:Water (1:1)
	20	Water:MeOH (1:2)	1:10	*1 x 60*	MTBE:Water (1:1)
	20	Water:MeOH (1:2)	1:10	*1 x 90*	MTBE:Water (1:1)
Sample material-to-homogenization solvent-ratio	20	Water:MeOH (1:2)	*1:5*	3 x 30	MTBE:Water (1:1)
	20	Water:MeOH (1:2)	*1:10*	3 x 30	MTBE:Water (1:1)
	20	Water:MeOH (1:2)	*1:20*	3 x 30	MTBE:Water (1:1)
	20	Water:MeOH (1:2)	*1:30*	3 x 30	MTBE:Water (1:1)
Sample amount	*5*	Water:MeOH (1:2)	1:10	3 x 30	MTBE:Water (1:1)
	10	Water:MeOH (1:2)	1:10	3 x 30	MTBE:Water (1:1)
	20	Water:MeOH (1:2)	1:10	3 x 30	MTBE:Water (1:1)
	40	Water:MeOH (1:2)	1:10	3 x 30	MTBE:Water (1:1)
Extraction solvent	20	Water:MeOH (1:2)	1:10	3 x 30	*MTBE:Water (1:1)*
	20	Water:MeOH (1:2)	1:10	3 x 30	*MTBE:Water (2:1)*
	20	Water:MeOH (1:2)	1:10	3 x 30	*DCM:Water (1:1)*
	20	Water:MeOH (1:2)	1:10	3 x 30	*DCM:Water (2:1)*
	20	Water:MeOH (1:2)	1:10	3 x 30	*Isopropanol:Water (1:1)*
	20	Water:MeOH (1:2)	1:10	3 x 30	*Isopropanol:Water (2:1)*
	20	Water:MeOH (1:2)	1:10	3 x 30	*ACN*
	20	Water:MeOH (1:2)	1:10	3 x 30	*MeOH:Acetone (9:1)*
	20	Water:MeOH (1:2)	1:10	3 x 30	*ACN:Water (2:8)*
	20	Water:MeOH (1:2)	1:10	3 x 30	*Butanol:ACN: Water (3:1:1)*

5 min), the samples were loaded onto a gel. The protein pellets of the extraction solvent samples were resuspended in 50 µL of a 1 % sodium deoxycholate (SDC) in HEPES solution that contained TCEP (5 mM) and IAA (10 mM). The samples were heated (95 °C for 5 min), centrifuged (14 000 rpm for 5 min) and incubated (room temperature, 50 min). Five µL of the sample were added to 5 µL of the SDS sample buffer for gel electrophoresis. Another 5 µL were used for a BCA assay as detailed below. Additionally, a 50 µL aliquot of the metabolomics phase, from all extraction solvent samples, was evaporated to dryness, and after reconstitution (10 µL SDS sample buffer:water mixture containing TCEP and IAA), heating and centrifugation, was run with 1D SDS-PAGE to visualize the remaining protein content in the metabolomics fraction.

2.2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For 1D SDS-PAGE, fifteen-well gels were prepared with a 12 % acrylamide separating gel and a 4 % acrylamide stacking gel. Ten µL of sample was loaded onto the gel. Gel electrophoresis was stopped once the dye front started to run off the gel. Proteins were fixed (Water: MeOH:acetic acid, 5:4:1), stained with Coomassie blue G250 and subsequently destained with deionised water. The gels were imaged with the Typhoon™ FLA 9500 biomolecular imager (GE Healthcare Life Sciences, Uppsala, SE) using fluorescence at an excitation wavelength of 685 nm and emission wavelength of 700 nm. The resulting images were manually evaluated in terms of number of protein bands and band intensities.

2.2.3. Protein assay

A Pierce™ BCA protein assay (Thermo Fisher, Waltham, MA, US) was conducted according to the manufacturer's instructions for an estimation of the protein concentration per sample. Bovine serum albumin standards were used as reference standard (0–2000 µg/mL) and all samples were diluted 1:1 with water. Absorbance at 562 nm was measured and protein concentration calculated.

2.3. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis

2.3.1. Metabolomics

The metabolomics fraction of all samples were analysed by an untargeted LC-high resolution (HR) MS method. Data was acquired by data independent acquisition (DIA) using an Acquity M-class LC system (Waters, Milford, MA, US) coupled to a Synapt XS time-of-flight (TOF) MS (Waters, Milford, MS, US). One microliter of sample material was loaded onto a Scherzo SM-C18 column (3 µm particle size, 13 nm pore size, 1 × 150 mm), heated at 40 °C with a flow rate of 75 µL/min. Detailed LC and MS parameters can be found within the supplementary material. All samples were analysed in triplicates in a randomised order. A mix of 27 endogenous reference standards from various compound classes (16 detectable in positive ionization mode, 12 detectable in negative ionization mode, Table S1 and Table S2) was used to monitor system suitability before each run and was the basis for the metabolomics targeted data processing detailed below.

2.3.2. Lipidomics

Only samples comparing the different extraction solvents were analysed with an HRMS-based untargeted lipidomics method. Data was acquired in top-8 data dependent mode (DDA) on an Agilent 1290 Infinity UPLC system (Santa Clara, CA, US) coupled to a Q Exactive Plus orbitrap MS (Thermo Fisher, Waltham, MA, US) with detailed LC-MS parameters summarized within the supplementary material. All samples were analysed once, in randomised order.

2.3.3. Proteomics

In addition to the visualisation and quantification of the protein content using 1D SDS-PAGE and a BCA assay, all samples investigating the extraction solvent, were analysed with an HRMS-based untargeted proteomics method. Based on the quantitative protein results of the BCA assay, 15 µL of sample material (equated to < 10 µg of protein per sample) that was previously resuspended in 50 µL of a 1 % SDC in HEPES solution (containing TCEP and IAA; after heating, centrifugation and incubation detailed above), was incubated with 1 µL trypsin (0.5 µg/µL) at 37 °C for 18 h. The samples were de-salted and cleaned up using STAGE-tips (STop And Go Extraction) following a protocol adapted from Rappsilber et al. as described within the supplementary material [26]. Following evaporation to dryness, the extracts were reconstituted in 25 µL mobile phase A (0.1 % formic acid in water). Data was acquired in DIA mode on an Acquity M-class LC system (Waters, Milford, MA, US), coupled to a Synapt XS time-of-flight (TOF) MS (Waters, Milford, MS, US). Five microliter of sample material was loaded onto a BEH C18 column (1.7 µm particle size, 300 Å pore size, 300 µm × 150 mm) heated at 45 °C with a flow rate of 5 µL/min. Detailed LC and MS parameters adapted from Wang et al. and Distler et al. can be found in the supplementary material [27,28]. All samples were analysed in duplicates/triplicates in a randomised order.

2.4. Data processing

2.4.1. Metabolomics MS analysis– targeted

Skyline (Version 23.1, molecule interface, MacCoss Lab Software) was used for targeted processing of the metabolomics data. For data acquired in positive ionization mode, a target list of 18 analytes (including two internal standards; Table S1) was created within the program including accurate masses $[M+H]^+$ and retention time (RT), based on previously analysed reference standards. Data acquired in negative ionization mode was evaluated for 14 endogenous targets (Table S2, including two internal standards, $[M-H]^-$, RT). Peak area intensities were calculated and variability (relative standard deviation, RSD) between injection replicates compared between conditions (smallest variability was ranked highest; acceptance criteria <20 % analytical variability). For data acquired in negative ionization mode, the number of detected analytes were also taken into account for ranking purposes.

2.4.2. Metabolomics and lipidomics MS analyses– untargeted

The metabolomics and lipidomics samples testing different extraction solvents, were processed with an untargeted processing workflow (for metabolomics samples in addition to the targeted evaluation). For this, peak picking and RT alignment was conducted in the open-source software MS-Dial (Version 4.9, processing parameters detailed in Table S6) [29], all features (global peak list) exported to Sirius (Version 5.8.3) [30] via MS Finder (Version 3.60) to use the chemical formula prediction (Sirius and Zodiac algorithm) [31] as well as Canopus for chemical class prediction (Classyfire algorithm) [32–34]. The number of analytes classified as “organic acids and derivatives” (high number of analytes desired for metabolomics method) or “lipids and lipid-like molecules” (high number of analytes desired for lipidomics method), were compared for the different extraction solvents (zodiac score >0.8 and classyfire probability >70 %). Muscle- and liver tissue samples were processed individually to improve RT alignment. Separate data processing was conducted for data acquired in positive and negative ionization mode.

Additionally, data was processed using the commercially available software Progenesis QI (for metabolomics). Peak picking, RT alignment and lockmass correction were carried out (processing parameters detailed in Table S6). Peak area normalisation using the total ion count was conducted and a filter for normalized abundance >100 was applied. The number of features per extraction condition were compared (mean over technical injection replicates and RSD).

2.4.3. Proteomics MS analysis– untargeted

Progenesis QI for proteomics (Nonlinear Dynamics, Milford, MA, USA) was used for untargeted data processing of the DIA ion mobility-separated proteomics data. Peak picking, lockmass correction, RT alignment and peptide/protein identification were conducted as detailed in Table S6. Peak areas were normalized to total protein abundance. The data was filtered for a positive peptide/protein identification and a normalized abundance >100. The number of features per extraction condition were compared (mean over technical injection replicates and RSD).

2.4.4. Ranking – extraction solvent

To find the most optimal extraction solvent for a multi-omics (metabolomics, lipidomics and proteomics) sample preparation, the following nine parameters were evaluated and ranked as detailed in Table 2. This was conducted independently for muscle tissue (ranked 1–10) and liver tissue samples (ranked 1–7). The highest number was attributed to the best result per category. In cases where two extraction solvents showed the same or very similar results, the conditions were ranked equal. The full list of ranked parameters and decision criteria are listed in Table 3.

3. Results and discussion

3.1. Homogenization experiments

To investigate optimal homogenization parameters, different homogenization solvents, different homogenization pulsing frequencies along with a variety of sample material to solvent ratios and sample amounts were tested.

3.1.1. Homogenization solvent

Homogenization with only MeOH or acetonitrile (ACN) did not yield any phase separation during extraction and no visible protein pellets were formed. Hence, these samples were excluded for the 1D SDS-PAGE analysis and were not visualized in Fig. 1a. During the initial homogenization solvent experiment, samples were centrifuged for 5 min at 14 000 rpm after being vigorously shaken for 5 min and before the supernatant was transferred into a fresh tube for extraction. While this centrifugation step is beneficial to yield clean samples as it favours a clear separation between the homogenized tissue and the supernatant, Fig. 1a shows that a significant proportion of the protein content is left

Table 2
Parameters and corresponding decision criteria for ranking the different extraction solvents/mixures; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis, BCA: bicinchoninic acid, RSD: relative standard deviation.

Parameter	Decision criteria
1D SDS-PAGE – protein pellet	Highest number of protein bands and highest band intensities
1D SDS-PAGE – metabolite fraction	Lowest number of protein bands and lowest band intensities
BCA assay	Highest protein quantification results
Metabolomics – targeted data processing	Lowest variability between technical injection replicates (RSD)
Metabolomics – untargeted data processing, features	Highest number of features and lowest variability between technical injection replicates (RSD)
Metabolomics – untargeted data processing, compound class	Highest number of organic acids and derivatives
Lipidomics – untargeted data processing, features	Highest number of features and lowest variability between technical injection replicates (RSD)
Lipidomics – untargeted data processing, compound class	Highest number of lipids or lipid-like molecules
Proteomics – untargeted data processing	Highest number of identified human peptides/proteins and lowest variability between technical injection replicates (RSD)

Table 3
Number of organic acids and derivatives identified in both positive (pos) and negative (neg) ionization mode in the metabolomics extracts on a superclass level (classyfire probability >70 %, zodiacscore >0.8) using the classyfire algorithm within Sirius (Version 5.8.3); data is based on features with raw peak area >500; mean over replicates (n = 6); MTBE: methyl-*tert*-butylether, DCM: dichloromethane, ACN: acetonitrile, MeOH: methanol; "-" indicates that this experiment was not performed.

Extraction solvent	Number of organic acids and derivatives			
	Muscle		Liver	
	pos	neg	pos	neg
MTBE:Water (1:1)	1153	1899	1216	1812
MTBE:Water (2:1)	1161	2011	–	–
DCM:Water (1:1)	1102	1779	1123	1682
DCM:Water (2:1)	1127	1816	–	–
Isopropanol:Water (1:1)	1143	1970	1196	1850
Isopropanol:Water (2:1)	1143	2000	–	–
ACN	1171	1995	1243	1854
MeOH:Acetone (9:1)	1169	1995	1252	1846
ACN:Water (2:8)	1166	1954	1228	1853
Butanol:ACN:Water (3:1:1)	1144	1949	1237	1823

behind and lost in the homogenization tube. The samples visualized in rows 2.1 to 3.2 (Fig. 1a) were all homogenized with a Water:MeOH-mixture (1:2). However, samples for rows 2.1 and 2.2 underwent 5 min centrifugation, whereas samples for rows 3.1 and 3.2 were only centrifuged down for 30 s with a benchtop centrifuge before transfer of the supernatant. The protein bands in the latter set of samples were a lot more intense and a broader variety of protein bands was visible. This suggests a protein loss after intense centrifugation, potentially leading to a less comprehensive proteomics dataset. To ensure removal of unwanted particles (e.g. homogenized tissue particles), without compromising the proteomics dataset, an intense centrifugation step was included after sample extraction and the proteomics extracts were additionally de-salted and cleaned up using STAGE-tips. Homogenization using Water:ACN (1:2) yielded a very small pellet after extraction, but as seen in rows 4.1 and 4.2, no proteins could be visualized with 1D SDS-PAGE. In parallel to the sample homogenization with MeOH and ACN, it is most likely that protein precipitation and pellet formation already took place in the homogenization tube and no proteins were present in the supernatant transferred into a fresh tube for extraction. ACN is generally considered a stronger protein precipitation agent than MeOH [35], so only the Water:MeOH-mixture (1:2) seemed to be weak enough to prevent extensive premature protein precipitation, while ensuring efficient homogenization. Using water as the homogenization solvent, led to the most intense protein bands on the gel (rows 1.1 and 1.2, Fig. 1a), however, extracts, even after extraction, additional centrifugation steps and STAGE-tip clean-up, contained a big proportion of solid particles. This was particularly evident for the metabolomics extracts that were analysed using an untargeted LC-HRMS method in conjunction with a targeted data processing workflow. On the instrument's curtain plate there was a residue left after a few injections, which would significantly decrease the sensitivity long-term (e.g. with bigger batch sizes). Within the targeted metabolites, both homogenization with Water and Water:MeOH (1:2) gave comparable results in terms of detectability and intensities (data not shown in detail). As a compromise between proteomics and metabolomics results and robustness of the sample preparation for larger batches, homogenization using Water:MeOH (1:2) and benchtop centrifugation was performed for the ensuing experiments, which is in accordance with the original Bligh-Dyer/Folch-method, developed in 1959 and 1957, respectively, for the extraction of lipids [15,16].

3.1.2. Pulsing frequencies

Five different pulsing frequencies for bead homogenization were trialled ranging from 1 × 30 s to 5 × 30 s, both stepwise and continuously. Homogenizing the samples for only 30 s resulted in the weakest protein bands during 1D SDS-PAGE analysis (Fig. 1b–bands 1.1 and 1.2), suggesting that the tissue was not disrupted enough to be able to extract all endogenous molecules. All other pulsing frequencies seemed to be comparable to each other (Fig. 1b). The metabolomics results (untargeted data acquisition, targeted data processing) supported this trend, not showing great differences in detectability or peak area intensity between the remaining 4 tested pulsing frequencies (data not shown in detail). During continuous homogenization (1 × 60 s and 1 × 90 s), the samples were exposed to significant heat due to bead friction. To prevent potential protein degradation due to this heat application, while simultaneously keeping the overall homogenization step time efficient, it was decided to use a 3 × 30 s pulsing frequency, with 1 min on ice in between pulses.Table 4 and 5

3.1.3. Sample material to homogenization solvent ratio and sample amount

Investigating the optimal sample material-to-homogenization solvent-ratio, the most intense and reproducible protein bands were observed for a 1:10 ratio (20 mg tissue sample homogenized with 200 µL solvent; Fig. 1c, bands 2.1/2.2). With less homogenization solvent (1:5 ratio; Fig. 1c, bands 1.1/1.2), less proteins were ultimately extracted, which suggests a saturation effect due to lack of adequate amounts of solvent being used. A higher proportion of solvent (than 1:10) in relation to the amount of tissue did not seem to extract more proteins but instead poses the risk of decreasing the sensitivity of the metabolomics and lipidomics analyses (no solvent evaporation before analysis). Hence, a 1:10 ratio was found to be ideal. The same sample material-to-homogenization solvent-ratio was previously proposed by Yang et al. [36], who developed an optimised extraction protocol for liver tissue samples with subsequent untargeted MS-based metabolomics analysis.

For applications with limited sample material available, it is crucial to be aware of the methods' limitations, e.g. which sample amount leads to reproducible results. Therefore, sample amounts of 5 mg, 10 mg and 40 mg were tested in addition to the previously used 20 mg of tissue material (while keeping a 1:10 sample material-to-homogenization solvent ratio). While the use of 5 and 10 mg of tissue produced slightly less intense protein bands on the 1D SDS-PAGE gel (Fig. 1c, bands 5.1/5.2 and 6.1/6.2), compared to 20 mg (Fig. 1c, bands 2.1/2.2), comprehensive protein coverage was still achieved. This suggests that smaller amounts of tissue could be used for individual cases. However, during interpretation of the data, one needs to be aware and consider that a lower sample size might compromise the reproducibility of the results and reduction in sample amount might not scale linearly with the analyses results; variability of protein band intensities and metabolite intensities observed between replicates (Fig. 1c, bands 5.1/5.2 and 6.1/6.2; metabolomics data not shown in detail). The use of 40 mg tissue

Table 4
Number of peptides identified (human reviewed database) in the proteomics extracts using Progenesis QI (for proteomics); data is based on peptides with normalized peak area >100; mean over biological and injection replicates (n = 5) and corresponding relative standard deviations; MTBE: methyl-*tert*-butylether, DCM: dichloromethane, ACN: acetonitrile, MeOH: methanol; "-" indicates that this experiment was not performed.

Extraction solvent	Number of peptides		RSD [%]	
	Muscle	Liver	Muscle	Liver
MTBE:Water (1:1)	3244	3578	1.6	9.5
MTBE:Water (2:1)	3573	–	2.7	–
DCM:Water (1:1)	3361	3661	3.1	2.1
DCM:Water (2:1)	3423	–	3.8	–
Isopropanol:Water (1:1)	2810	2901	3.4	6.1
Isopropanol:Water (2:1)	3080	–	7.0	–
ACN	3485	3624	2.5	1.8
MeOH:Acetone (9:1)	3447	3636	1.8	1.7
ACN:Water (2:8)	3207	3564	2.3	1.7
Butanol:ACN:Water (3:1:1)	3374	3549	0.5	1.4

Table 5
Results of ranking the individual evaluation criteria for the tested extraction solvents; two ranking sums are presented, "Ranking sum partial" excludes the parameters "Metabolomics untargeted, features" and "Lipidomics untargeted, features" (highlighted in *italic* within the table), "Ranking sum total" includes rankings from all listed evaluation parameters; a) muscle tissue samples; b) liver tissue samples; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis, BCA: bicinchoninic acid, MTBE: methyl-*tert*-butylether, DCM: dichloromethane, ACN: acetonitrile, MeOH: methanol.

	Extraction solvent	1D SDS-PAGE protein pellet	1D SDS-PAGE metabolite fraction	BCA assay	Metabolomics targeted		*Metabolomics untargeted, features*		Metabolomics untargeted, compound class		*Lipidomics untargeted, features*		Lipidomics untargeted, compound class		Proteomics untargeted	Ranking sum partial	Ranking sum total
					pos	neg	*pos*	*neg*	pos	neg	*pos*	*neg*	pos	neg			
a)	**MTBE:Water (1:1)**	3	1	8	10	0.5	*7*	*4*	6	3	*1*	*4*	3	not	6	40.5	56.5
	MTBE:Water (2:1)	10	6	9	1	4	*4*	*6*	7	10	*2*	*8*	4.5	ranked	8	59.5	79.5
	DCM:Water (1:1)	9	3	10	6	4	*4*	*1*	1	1	*3*	*5*	4.5		5	43.5	56.5
	DCM:Water (2:1)	8	4	7	6	4	*5*	*5*	2	2	*6*	*3*	5		4	42	61
	Isopropanol:Water (1:1)	1	2	1	3	4	*4*	*4*	5	6	*4*	*3*	2		2	26	41
	Isopropanol:Water (2:1)	2	5	2	2	5	*4*	*10*	5	9	*6*	*6*	1.5		1	32.5	58.5
	ACN	7	10	4	7	1	*9*	*8*	10	8	*8*	*7*	0.5		10	57.5	89.5
	MeOH:Acetone (9:1)	7	9	4	6	4.5	*9*	*10*	10	8	*10*	*10*	2.5		9	**60**	**99**
	ACN:Water (2:8)	7	7	5	8	1.5	*10*	*4*	8	5	*7*	*3*	1.5		3	46	70
	Butanol:ACN:Water (3:1:1)	7	9	6	10	4	*6*	*8*	5	4	*10*	*9*	4.5		8	57.5	90.5
b)	**MTBE:Water (1:1)**	2	3	2	7	0.5	*6*	*3*	3	2	*1*	*2*	3	not	2	24.5	34.5
	DCM:Water (1:1)	3	3	7	3	1.5	*3*	*1*	1	1	*4*	*5*	3.5	ranked	7	30	36
	Isopropanol:Water (1:1)	1	3	1	2	3.5	*1*	*7*	2	7	*7*	*7*	1.5		1	22	43
	ACN	7	7	6	1	3.5	*6*	*5*	6	7	*5*	*3*	1		7	45.5	57.5
	MeOH:Acetone (9:1)	6	5	5	6	3.5	*6*	*5*	7	4	*6*	*5*	2.5		7	**46**	**61**
	ACN:Water (2:8)	6	6	5	5	2	*7*	*3*	4	7	*3*	*6*	0.5		3	38.5	54.5
	Butanol:ACN:Water (3:1:1)	6	5	5	5	1.5	*3*	*7*	5	3	*2*	*1*	2.5		4	37	46

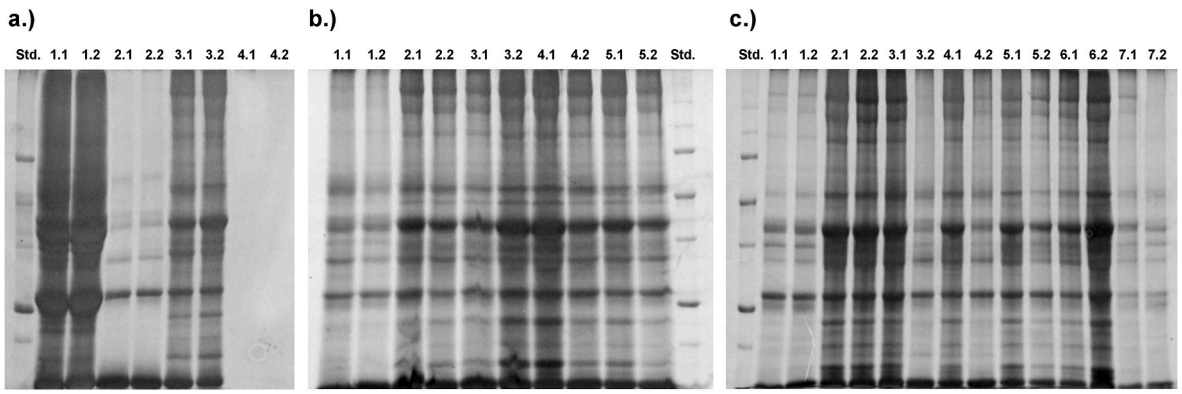

Fig. 1. 1D SDS-PAGE results in duplicate; a) Homogenization solvent, 1: Water; 2: Water:MeOH (1:2) - centrifuged for 5 min at 14 000 rpm before taking supernatant out for extraction; 3: Water:MeOH (1:2) - spun down for 30 s with a benchtop centrifuge before taking supernatant out for extraction; 4: Water:ACN (1:2); b) Pulsing frequencies, 1: 1 × 30 s; 2: 3 × 30 s; 3: 5 × 30 s; 4: 1 × 60 s; 5: 1 × 90 s; c) Sample material to homogenization solvent ratio and sample amount, 1: (1:5); 2: (1:10); 3: (1:20); 4: (1:30); 5: 5 mg; 6: 10 mg; 7: 40 mg.

material reduced the extracted protein content significantly (Fig. 1c, bands 7.1/7.2), which might again indicate a solvent saturation effect. More sample material, therefore, is not always beneficial, but might need to be accounted for (even if keeping a 1:10 sample material-to-homogenization solvent ratio).

3.2. Extraction solvent experiments

Following the optimal homogenization parameters as described above, the experiments investigating the ideal extraction solvent were conducted on homogenized pooled samples. Multiple muscle and liver tissue samples from the same donor, collected at the same time after death, were homogenized individually and subsequently pooled to yield a muscle pool and a liver pool to be able to compare a variety of extraction solvents for two different tissue types. For muscle tissue samples, ten different extraction solvent compositions (mono- and biphasic solvent mixtures) were compared as detailed in the methods section. Due to the availability of limited liver tissue amounts, only seven different extraction solvent compositions were compared for these. As detailed above, previous literature suggests that the use of a 1:1 organic solvent-to-water ratio (instead of a classic 2:1 organic solvent-to-water ratio) could increase the yield of metabolites, while decreasing the amount of lipids extracted within the polar metabolomics phase [23]. As this seemed promising, the focus for the liver sample extraction was on the 1:1-ratios, while the biphasic solvent mixtures in a 2:1-ratio were only evaluated for muscle tissue samples.

3.2.1. 1D SDS-PAGE and BCA assay

The protein content of all proteomics extracts was examined by 1D SDS-PAGE and protein quantification using a BCA assay was conducted. Gel results were visualized in Fig. S1 along with BCA assay results in Table S7. Extraction of the muscle pool sample with MTBE:Water (2:1) and DCM:Water (both 1:1 and 2:1 ratios) resulted in the most intense protein bands, which was supported by the highest quantification results using the BCA assay (5.9–6.1 μg protein/15 μL final extract for the DCM: Water extraction, 4.4–5.4 μg protein/15 μL final extract for the MTBE: Water (2:1) extraction). While a DCM:Water mixture seems to extract proteins most efficiently from the tissue sample, the second replicate of the DCM:Water (2:1) extraction showed significantly less intense bands and only resulted in a BCA quantification result of 2.1 μg protein/15 μL final extract. Such high variability between replicates was most likely caused by the extraction order of the different -omics extracts. Similar to the traditional Chloroform:Water extraction, during extraction with DCM:Water, the protein extract is trapped in the middle between the organic and aqueous phase containing the lipids and the metabolites fraction, respectively. This makes reproducible protein extraction more difficult and hence more variable. In contrast, during the extraction with MTBE:Water, a protein pellet precipitates at the bottom of the tube with the aqueous and organic phases on the top. This favours reproducible protein extraction. Extraction with Isopropanol:Water yielded the least intense protein bands and quantification results indicated very low protein content (0.0–0.2 μg protein/15 μL final extract), suggesting inefficient protein recovery. All tested monophasic extraction solvents (ACN, MeOH:Acetone (9:1), ACN:Water (2:8) and Butanol:ACN:Water (3:1:1)) showed similar visual and protein quantification results to each other, ranging between 1.5 and 3.5 μg protein/15 μL final extract. Extraction of the liver pool samples resulted in similar findings (Fig. S1b and Table S7).

Following the described results, DCM:Water and MTBE:Water extraction solvents received the highest rankings for both the visual 1D SDS-PAGE findings as well as for the protein quantifications by BCA assay, followed by the monophasic extraction solvents. Isopropanol: Water was ranked to be the weakest protein extraction solvent (Table 5).

While aiming for the highest protein content in the proteomics fraction, it is also important to consider that proteins or large peptides might remain in the (aqueous) solvent phase, depending on the precipitation strength of the extraction mixture. In the long term, this can have a negative impact on chromatographic performance and metabolomics column lifetime, and therefore overall robustness of the analytical method. Hence, following recommendations from Bruce et al. [37], the metabolomics fraction of each extraction was also run on an 1D SDS-PAGE gel to visualize the residual proteins or large peptides (Fig. S2). The least intense protein bands for both muscle and liver tissue extracts were found for all monophasic extraction solvents. After tissue extraction with MTBE:Water, DCM:Water and Isopropanol:Water, a high proportion of proteins seem to remain in the metabolomics extracts. As this is an undesirable effect, during ranking, the mentioned biphasic extraction solvent mixtures were scored lower than the monophasic solvents.

3.2.2. Metabolomics

3.2.2.1. Targeted. To evaluate the extraction efficiency of the different solvent mixtures with respect to metabolites, a set of 16 endogenous metabolites in positive ionization mode and 12 metabolites in negative ionization mode were investigated for peak area reproducibility across replicates (n = 6). RSD values per analyte per extraction solvent mixture are listed in Tables S8 and S9 (positive ionization, muscle and liver tissue results, respectively) and Table S10 and Table S11 (negative ionization, muscle and liver tissue results, respectively). Four positively ionized analytes (caffeine, cortisone, nicotinic acid and proline) were not detected in any of the analysed muscle and liver tissue samples. All

targeted analytes have previously been confirmed by reference standards (RT and accurate mass) during the initial MS method development phase. Additionally, their natural occurrence in at least one of the studied matrices (skeletal muscle tissue and/or liver tissue) has been reported in the literature. However, targeted analyte concentrations in these cases could be below the method's limit of detection, so these were excluded for evaluation. Overall, extraction of the muscle pool sample with MTBE:Water (1:1) and Butanol:ACN:Water (3:1:1) showed the best reproducibility between replicates (hence ranked highest), while MTBE:Water (2:1) extraction lead to the greatest variability between replicates for the targeted analytes in positive ionization mode (therefore ranked lowest). Extra water (a 1:1-ratio) compared to the original Bligh-Dyer/Folch-extraction method (Chloroform:Water in a 2:1-ratio) was previously found to increase the yield of metabolites, while decreasing the amount of lipids extracted in the polar metabolomics phase [23]. As the extraction with MTBE:Water follows a similar extraction mechanism, this, in turn, could explain higher peak area responses leading to less variability between replicates with a higher proportion of water. Generally, it is to be expected that the use of different solvents favours the extraction of different compound classes. Uracil was not detected in the muscle pool samples, when extracted with ACN or ACN:Water (2:8) and those two extraction solvents also lead to the weakest peak area responses for the liver tissue samples. Hence, this seems to be systematic and might also impact other structurally similar analytes, e.g. small pyrimidine derivates. The concept of selective extraction will be further investigated within the untargeted data processing results. Overall, the targeted analytes (positive ionization) show a similar extraction behaviour for both muscle and liver tissue samples, with MTBE:Water (1:1) also exhibiting the least variability between replicates when extracting liver tissue samples.

In negative ionization mode, not all of the 12 targeted analytes could be detected with any of the used extraction solvent mixtures. Only seven targeted analytes (chenodeoxycholic acid, deoxycholic acid, phenylalanine, raffinose, tryptophan, uric acid and uridine) were successfully extracted with one or more of the solvent mixtures in either muscle or liver tissue samples or both matrices. However, variability between replicates (particularly for injection replicates) was extremely high and, with the exception of chenodeoxycholic acid (liver sample) and phenylalanine (muscle and liver sample) extracted with Isopropanol:Water (1:1 or 2:1), all other RSD values exceeded the set 20 % acceptance criteria (Tables S10 and S11). Low sensitivity and analytical variability with repeated polarity switching could be the cause (between batches, no polarity switching within batches). For the ranking, the number of detected analytes per extraction solvent mixture was also taken into account besides variability across replicates and overall, only half the maximum points were given for this parameter in order to prevent artificial bias towards these weak results. Following this, Isopropanol:Water (2:1) was ranked with 5 points for the muscle samples and Isopropanol:Water (1:1), ACN and MeOH:Acetone (9:1) were awarded 3.5 points for the liver sample extraction. MTBE:Water (1:1) extraction resulted in the highest variability between replicates for both muscle and liver tissue samples, hence was only ranked with 0.5 points for this parameter (Table 5).

3.2.2.2. Untargeted. The targeted evaluation of the metabolomics dataset was followed by an untargeted workflow. It has previously been discussed in the literature that the "total number of features" is neither a good quality indicator for an untargeted metabolomics method nor is it a reflection of true positive hits [38]. As it is still one of the most commonly employed parameters for the evaluation of different sample preparation methods and software processing workflows, the total number of features per extraction solvent mixture was still assessed (also taking variability between replicates into account) in this study. This was complemented with an investigation into the number of organic acid compounds extracted per solvent mixture, which was based on feature identification on the compound class level (level 3 identifications according to the metabolomics standards initiative proposed minimum reporting standards [39]).

Similar to the targeted processing approach, the variability between replicates was first assessed. Fig. 2a shows the principal component analysis results of all muscle sample replicates (positive ionization) per trialled extraction solvent mixture (n = 6). High variability can be observed for all extraction solvents except for DCM:Water (1:1 and 2:1) and MTBE:Water (1:1). It is striking that for all other extraction solvents, the first technical injection replicate seems to be different to the two remaining injection replicates for each biological sample. A similar behaviour is also visible for the negatively ionized dataset (muscle samples, Fig. S3a) and for the liver samples (both ionization techniques, Figs. S4a and S5a). Although the exact reason for this observed phenomenon is unknown, this could be caused by evaporation effects after piercing the vial lid during the first injection. For the current experiment all three injection replicate injections were carried out from the same vial, however, based on the results it is recommended to use individual aliquots for each technical replicate injection and/or change the pierced lid after injection to prevent extensive solvent evaporation. Cooling of the autosampler tray to 4 °C did not significantly improve this issue. For the monophasic extraction solvents containing only, or a very high proportion of, organic solvents, this phenomenon was expected. This can be alleviated by evaporating the extracts to dryness (e.g. using N_2 or a vacuum centrifuge) and reconstituting them in a more aqueous solvent (e.g. the LC method's starting conditions). Particularly for untargeted metabolomics studies, however, sample evaporation always poses the risk of analyte loss, which is why the extracts were directly injected. To look at similarities in extraction behaviour of the tested solvent mixtures, a principal component analysis of the first technical injection replicate was carried out (Fig. 2b; muscle tissues sample, positive ionization mode). Both DCM:Water mixtures (1:1 and 2:1) show a similar extraction behaviour to MTBE:Water (1:1). This indicates that, except for the muscle tissue extraction with MTBE:Water (2:1), all other biphasic solvent mixtures seem to extract similar metabolite patterns, leading to clustering. MTBE:Water (2:1) seems to be more similar to Isopropanol:Water (1:1 and 2:1) and Butanol:ACN:Water (3:1:1) in terms of metabolite extraction. The classic protein precipitation solvents like MeOH:Acetone (9:1), ACN and ACN:Water (2:8) also cluster together indicating similarities in their untargeted metabolomics profile. The general trend for principal component separation between monophasic and biphasic extraction solvents was also clearly visible in negative ionization mode (muscle tissue) and for both ionization modes when extracting liver tissue samples (Figure S3b, S4b and S5b). This could also be caused by the fact that the extracts from the monophasic extractions contain both the metabolomics and the lipidomics fraction for analysis. Although the LC-column used for the metabolomics method favours retention and hence analysis of polar metabolites, the multimode column properties might also facilitate analysis of some lipids. In an untargeted data processing workflow, features could therefore also be lipids that contribute to the visual extraction solvent separation. This is also reflected by the number of features extracted. As visualized in Fig. 3a and b and detailed in Table S12, ACN, MeOH:Acetone (9:1), ACN:Water (2:8) and Butanol:ACN:Water (3:1:1) lead to the highest number of metabolomics features extracted in positive ionization mode (both muscle and liver tissue samples; normalized peak area of individual replicates >100). In negative ionization mode, MTBE:Water (2:1) and Isopropanol:Water (1:1 and 2:1) both showed a similar number of features compared to the aforementioned monophasic extraction solvents, with DCM:Water mixtures (1:1 and 2:1) consistently (both ionization modes) extracting the least number of features and hence scoring last for this evaluation parameter (Fig. 3a and b, Table S12). The proposition by Wu et al. [23] that a biphasic extraction might be mandatory for reproducible extraction of lipid-rich tissue such as liver cannot be supported as the monophasic extraction solvents show better results in terms of reproducibility and variety of extracted compounds. While it

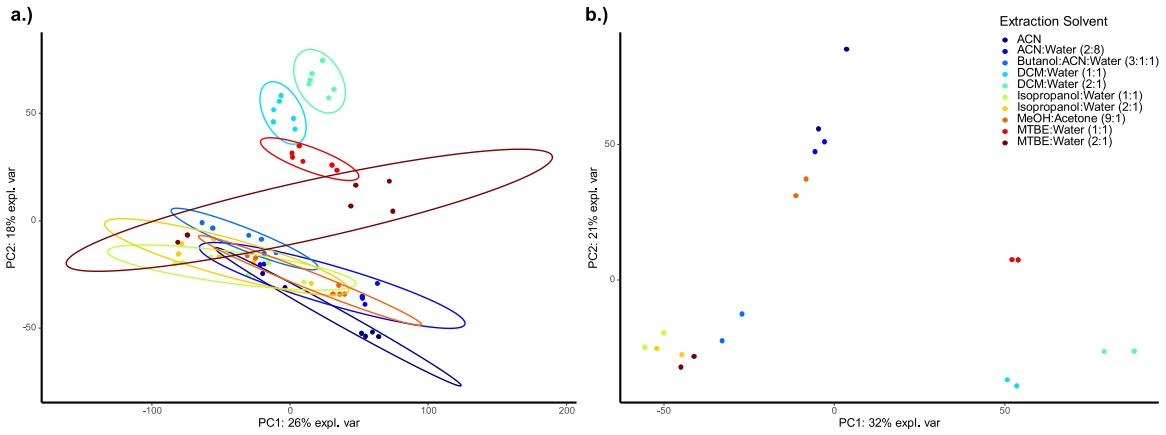

Fig. 2. Principal component analysis of the untargeted metabolomics data (positive ionization) from muscle samples; underlying data includes all features (raw abundance) with a retention time >1 min and <19 min; a) all biological (n = 2) and injection (n = 3; technical) replicates; b) only injection replicate 1 of both biological replicates; MTBE: methyl-*tert*-butylether, DCM: dichloromethane, ACN: acetonitrile, MeOH: methanol; PC: principal component.

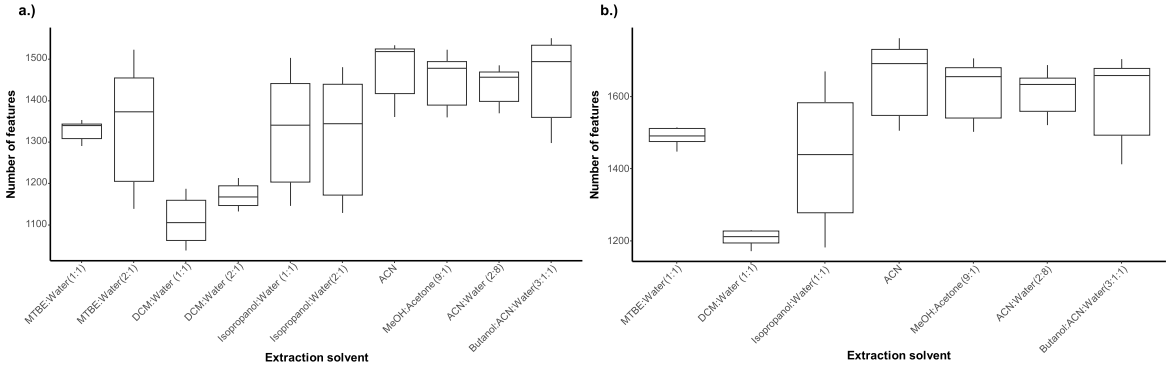

Fig. 3. Boxplots visualizing the number of features extracted with the untargeted metabolomics workflow in positive ionization mode; n = 6 per extraction solvent (2 biological replicates and 3 replicate injections (technical replicates) of these); all relative standard deviations between replicates are <15 %; a) muscle tissue samples; b) liver tissue samples; MTBE: methyl-*tert*-butylether, DCM: dichloromethane, ACN: acetonitrile, MeOH: methanol.

was already discussed that the total number of features in an untargeted metabolomics experiment is not necessarily a reflector of true positive hits [38], DCM:Water (1:1 and 2:1) was also found to lead to the least number of organic acids and derivatives identified on the compound class level (Table 3). For muscle tissue samples, the top 3 extraction solvents for a comprehensive metabolomics dataset (positive ionization) were MeOH:Acetone (9:1), ACN and ACN:Water (2:8) using both the total number of features and the compound class identification as evaluation parameters. In negative ionization mode, additionally to these extraction solvents, MTBE:Water (2:1), Isopropanol:Water (2:1) and as well as Butanol:ACN:Water (3:1:1) also performed well for either or both evaluated parameters. In the light of the findings by Wu et al. [23] it is surprising that the 2:1 organic solvent-to-water mixtures performed worse than the extraction solvents with a 1:1-ratio for our untargeted metabolomics evaluation. The addition of extra water was previously found to increase the yield of metabolites, which cannot be supported with our current findings. Overall, looking at the compound class identifications seems to be a good complementary evaluation criterion for evaluating extraction solvent differences during method development.

3.2.3. *Lipidomics*

For analysis of the lipidomics samples, both biological replicates per extraction solvent were injected once, so there were no technical injection replicates of the injections due to instrument availability. Examining the total number of features extracted from muscle tissue with each solvent (mean over biological replicates; positive and negative ionization mode), the most features were calculated for Butanol:ACN:Water (3:1:1) (Table S13). Isopropanol:Water (1:1 and 2:1), ACN, MeOH:Acetone (9:1) and ACN:Water (2:8) show a slightly lower number of extracted features. Extraction of both the muscle and liver tissue (both ionization modes) with the two biphasic extraction solvents (DCM:Water (1:1 and 2:1) and MTBE:Water (1:1 and 2:1)), lead to the least number of processed features. For the liver tissue extraction, MeOH:Acetone (9:1) showed the highest number of total features, with all other monophasic solvents having a slightly lower number of features extracted (Table S13). Similar to the untargeted metabolomics results, a high number of total features is not necessarily a reflector of true positive hits and features extracted with the monophasic solvents could be the result of metabolite retention within the lipidomics method. Overall, the number of features calculated for the untargeted lipidomics dataset is much higher than for the metabolomics dataset. Although processing was conducted with the same software (Progenesis QI for metabolomics), analytical flowrates, acquisition modes and hence general instrument parameters were different (microflow DIA for metabolomics data, highflow DDA for lipidomics data). This can have a significant impact on the method's sensitivity and hence data processing results. With the compound class identification workflow, only a small fraction of the total number of features were classified as lipid and lipid-like molecules, particularly in negative ionization mode (Table S14). Although the utilized Classyfire algorithm (within Sirius [34]) includes all major lipid classes (e.g. glycerophospholipids, sphingolipids and

phosphatidylcholines) and was closely aligned with the LIPID MAPS classification scheme [34,40], the very few lipid identifications might be a result of lacking MS/MS spectra (less than 10 % of the features have corresponding MS/MS spectra). Attempted identification of the untargeted lipidomics data processed through Progenesis QI for metabolomics using the LipidBlast database [41], also suffered from weak and hence unreliable identification scoring through the lack of MS/MS data (data not shown in detail). Following this, compound class specific identification for lipidomics data acquired in negative ionization mode was not considered for the final ranking at all and scoring for the lipidomics data in positive ionization mode was divided by 2 to prevent artificial bias. Here, DCM:Water (1:1 for liver tissue extraction and 2:1 for muscle tissue extraction) ranked highest for this parameter along with MTBE:Water (1:1 for liver tissue extraction and 2:1 for muscle tissue extraction) and Butanol:ACN:Water (3:1:1). It is not surprising that the highest number of lipid and lipid-like molecules were found for the biphasic extraction solvents, where the organic fraction is specifically utilized to extract lipids. However, the difference in extracted lipids compared to the other solvent mixtures is not very high, also indicating good lipid recovery with monophasic solvents. Combining the two described parameters it is again supported that compound class identification (lipids and lipid-like molecules) seems to be a suitable complementary evaluation criterion for method comparison purposes.

3.2.4. Proteomics

The proteomics dataset was already assessed visually and quantitatively on the protein level as described in a previous section ("1D SDS-PAGE and BCA assay"). However, these results cannot distinguish between different proteoforms or proteins of a very similar size. To overcome this, a peptide-centric, untargeted LC-MS/MS method was used to evaluate the comprehensiveness of the proteomics data. The number of peptides identified using the reviewed human proteome (normalized abundance >100) was compared along with the reproducibility between replicates (n = 5). The number of extracted and positively identified peptides in muscle samples was highest using MTBE:Water (2:1) as an extraction solvent, closely followed by ACN, MeOH:Acetone (9:1) and DCM:Water (2:1) (Table 4). Similarly, for the extraction of liver samples, DCM:Water (1:1), MeOH:Acetone (9:1) and ACN showed the highest number of positively identified peptides. For both muscle and liver samples, extraction with Isopropanol:Water (1:1) resulted in the lowest number of peptides. In general, variability between replicates tended to be slightly higher for the biphasic extraction solvents (Table 4) as also visualized within the PCA plot (Fig. 4a). The ranking results take both number of peptides and variability between replicates into account. Hence, ACN, MeOH:Acetone (9:1) received the highest ranks for this parameter for both muscle and liver samples (complemented with Butanol:ACN:Water (3:1:1) for muscle samples and DCM:Water (1:1) for liver sample extraction). The high rankings for a number of monophasic extraction solvents seems surprising, as biphasic solvent systems e.g. with MeOH:Chloroform are among the most commonly used extraction solvents for proteomics workflows [19,21]. This shows the importance to regularly review common practices and develop state-of-the-art sample preparation methods. As an efficient monophasic proteomics extraction solvent with consistent recovery, acetone protein precipitation has previously been proposed and optimised, putting a particular emphasis on a high salt concentration and high incubation temperature for high protein recovery [42]. A pure acetone precipitation or acetone as the main precipitation solvent, however, has also been shown by Bruce et al. [37] to lead to poorly reproducible metabolomics results. As a minor component within a solvent mixture it showed promising results, though. Hence, as a compromise for the current multi-omics study, only a MeOH:Acetone (9:1) mixture was included as an extraction solvent and indeed resulted in one of the highest ranks for the untargeted proteomics dataset as detailed above. Based on the principal component analysis plots (Fig. 4), the monophasic extraction solvents ACN, ACN:Water (2:8) and Butanol:ACN:Water (3:1:1) show a very similar peptide profile after extraction (strong overlapping clustering), suggesting the potential for comprehensive proteomics data with these extraction solvents as well. While the overall number of peptides identified (Table 4) is below the expected number of identifications for a modern shotgun proteomics approach, it needs to be realized that the postmortem samples utilized for this study have likely undergone an unknown level of postmortem protein degradation (e.g. creating non-tryptic peptides containing uncharacterized amino acid (post-translational) modifications) as previously described elsewhere [43].

3.2.5. Ranking

Considering the ranking results across all 9 investigated parameters (Table 5), MeOH:Acetone (9:1) as an extraction solvent shows the best results with a ranking score of 99 for muscle tissue samples and 61 for liver tissue samples. Including the two categories that were capped at the half maximum points, the highest possible ranking score would have been 120 for muscle and 84 for liver samples. While MeOH:Acetone (9:1) does not reach these top scores, it seems to achieve the best compromise in extracting a comprehensive targeted and untargeted metabolomics, lipidomics and proteomics dataset. Good ranking results were also seen for ACN-extraction (muscle and liver tissue) and for extracting muscle tissue with Butanol:ACN:Water (3:1:1) or liver tissue with ACN:Water (2:8). Overall, the monophasic extraction solvents show better scores compared to the biphasic solvent systems, despite

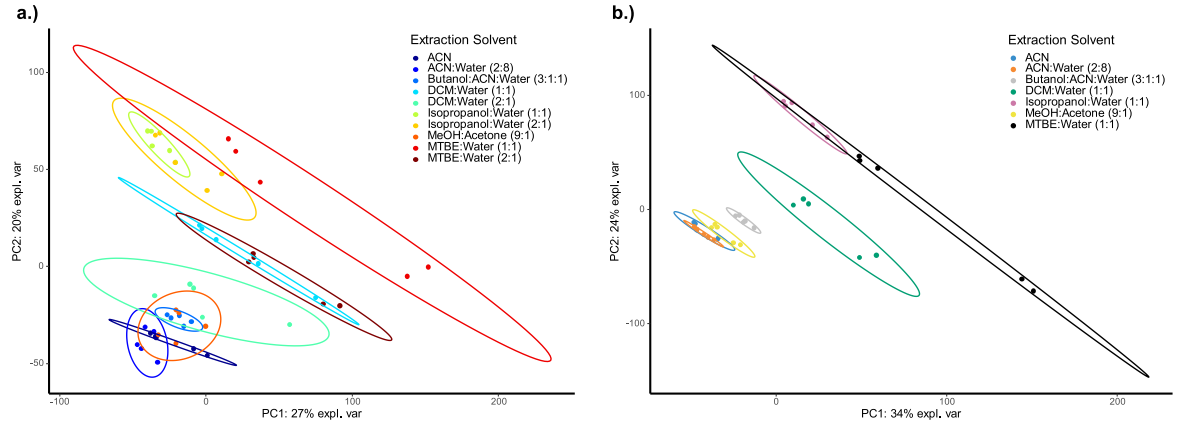

Fig. 4. Principal component analysis of the untargeted proteomics data; underlying data includes all features (normalized abundance) with a positive peptide identification; a) muscle tissue samples; b) liver tissue samples; MTBE: methyl-*tert*-butylether, DCM: dichloromethane, ACN: acetonitrile, MeOH: methanol, PC: principal component.

their recommendation for one or all of the -omics extractions. Based on the current results, if a biphasic extraction solvent is desired for a specific purpose, it would be recommended to extract muscle tissue samples with MTBE:Water (2:1). As detailed above, it is suggested in the literature that the "total number of features" is neither a good quality indicator for an untargeted metabolomics/lipidomics method nor is it a reflector of true positive hits [38]. It was therefore also tested if the ranking results might change if this parameter was not considered. For this, the untargeted datasets (number of features) for both metabolomics and lipidomics (positive and negative ionization) were excluded for the ranking (Table 5, column "Ranking sum partial"; excluded parameters highlighted in italic). Overall, MeOH:Acetone (9:1) was still found to have the highest ranking for both muscle and liver tissue samples (although ranking results are less variable in this case).

4. Conclusions

Based on the tested and evaluated homogenization and extraction solvent parameters, it was found to be optimal to homogenize 20 mg of muscle or liver tissue with 200 µL (1:10 ratio) Water:MeOH (1:2), using 3 × 30 s pulses. After transfer of the supernatant, optimal extraction solvent was found to be MeOH:Acetone (9:1), resulting in the most comprehensive targeted and untargeted metabolomics, lipidomics and proteomics datasets. While it has to be stressed that a specialized sample preparation workflow, solely optimised for either of the three -omics techniques, might result in even greater data depth, the currently proposed workflow is suggested in cases where low sample amounts are available and/or multi-omics data integration is aimed for. Special emphasis of the current study was on fresh postmortem human tissue samples, with the view to analyse longitudinally collected human postmortem biopsy tissue samples to investigate the postmortem interval. While this method can serve as a basis for multi-omics studies in other matrices, the results would need to be confirmed. To counteract issues with the reproducibility of metabolomics/lipidomics technical injection replicates based on the pure organic nature of the extracts, it is recommended to use a separate vial for each technical replicate/injection (due to risk of evaporation through pierced lids). It was shown, that even for lipid-rich tissue such as liver tissue, monophasic extraction solvents seem to be suitable, not supporting previous propositions that biphasic extraction solvents are mandatory. In addition to the proposed sample preparation workflow, the current study has also shown that compound class identification seems to be a suitable evaluation criterion for metabolomics and lipidomics method comparison purposes, complementing classic evaluation parameters like "total number of features". The use of MeOH:Acetone (9:1) as a monophasic extraction solvent system has the potential to be automated, allowing for high-throughput analysis of samples. Monophasic extraction solvents like MeOH:Acetone (9:1) are very frequently used as protein precipitation agent for example in routine clinical settings (e.g. detection of xenobiotics). This potentially opens the door for comprehensive multi-omics results from routine case samples in the future, massively extending possible clinical study cohorts.

CRediT authorship contribution statement

Lana Brockbals: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Maiken Ueland:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition. **Shanlin Fu:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Matthew P. Padula:** Writing – review & editing, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Data statement

Raw research data can be obtained from the corresponding author upon reasonable request. Data cannot be uploaded to a repository, as untargeted research data is based on human postmortem samples that could be processed to extract sensitive information.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2024.127442.

Data availability

Data will be made available on request.

References

[1] M. Krassowski, V. Das, S.K. Sahu, B.B. Misra, State of the field in multi-omics research: from computational needs to data mining and sharing, Front. Genet. 11 (2020) 610798.
[2] A.E. Steuer, L. Brockbals, T. Kraemer, Untargeted metabolomics approaches to improve casework in clinical and forensic toxicology—"Where are we standing and where are we heading? WIREs Forensic Science 4 (4) (2022) e1449.
[3] B. Zhang, B. Kuster, Proteomics is not an island: Multi-omics integration is the key to understanding biological systems, Mol Cell Proteomics 18(8, Supplement 1) (2019) S1–S4.
[4] S. Canzler, J. Schor, W. Busch, K. Schubert, U.E. Rolle-Kampczyk, H. Seitz, H. Kamp, M. von Bergen, R. Buesen, J. Hackermüller, Prospects and challenges of multi-omics data integration in toxicology, Archives of Toxicology 94(2) (2020) 371–388.
[5] F.R. Pinu, D.J. Beale, A.M. Paten, K. Kouremenos, S. Swarup, H.J. Schirra, D. Wishart, Systems biology and multi-omics integration: viewpoints from the metabolomics research community, Metabolites 9 (4) (2019) 76.
[6] D. Vuckovic, Current trends and challenges in sample preparation for global metabolomics using liquid chromatography-mass spectrometry, Anal Bioanal Chem 403(6) (2012) 1523–1548.
[7] A.B. Canelas, A. ten Pierick, C. Ras, R.M. Seifar, J.C. van Dam, W.M. van Gulik, J. J. Heijnen, Quantitative evaluation of intracellular metabolite extraction techniques for yeast metabolomics, Anal Chem 81(17) (2009) 7379–7389.
[8] X. Duportet, R.B.M. Aggio, S. Carneiro, S.G. Villas-Bôas, The biological interpretation of metabolomic data can be misled by the extraction method used, Metabolomics 8(3) (2012) 410–421.
[9] X. Li, X. Zhou, T. Teng, L. Fan, X. Liu, Y. Xiang, Y. Jiang, P. Xie, D. Zhu, Multi-omics analysis of the amygdala in a rat chronic unpredictable mild stress model of depression, Neuroscience 463 (2021) 174–183.
[10] J.A. Stefely, N.W. Kwiecien, E.C. Freiberger, A.L. Richards, A. Jochem, M.J. P. Rush, A. Ulbrich, K.P. Robinson, P.D. Hutchins, M.T. Veling, X. Guo, Z. A. Kemmerer, K.J. Connors, E.A. Trujillo, J. Sokol, H. Marx, M.S. Westphall, A. S. Hebert, D.J. Pagliarini, J.J. Coon, Mitochondrial protein functions elucidated by multi-omic mass spectrometry profiling, Nat Biotechnol 34(11) (2016) 1191–1197.
[11] K.A. Overmyer, E. Shishkova, I.J. Miller, J. Balnis, M.N. Bernstein, T.M. Peters-Clarke, J.G. Meyer, Q. Quan, L.K. Muehlbauer, E.A. Trujillo, Y. He, A. Chopra, H. C. Chieng, A. Tiwari, M.A. Judson, B. Paulson, D.R. Brademan, Y. Zhu, L. R. Serrano, V. Linke, L.A. Drake, A.P. Adam, B.S. Schwartz, H.A. Singer,

S. Swanson, D.F. Mosher, R. Stewart, J.J. Coon, A. Jaitovich, Large-scale multi-omic analysis of COVID-19 severity, Cell Syst 12(1) (2021) 23–40.
[12] L. Xu, Q. Zhao, J. Luo, W. Ma, Y. Jin, C. Li, Y. Hou, M. Feng, Y. Wang, J. Chen, J. Zhao, Y. Zheng, D. Yu, Integration of proteomics, lipidomics, and metabolomics reveals novel metabolic mechanisms underlying N, N-dimethylformamide induced hepatotoxicity, Ecotoxicol. Environ. Saf. 205 (2020) 111166.
[13] E. Rampler, D. Egger, H. Schoeny, M. Rusz, M.P. Pacheco, G. Marino, C. Kasper, T. Naegele, G. Koellensperger, The power of LC-MS based multiomics: exploring adipogenic differentiation of human mesenchymal stem/stromal cells, Molecules 24 (19) (2019) 3615.
[14] J. Kang, L. David, Y. Li, J. Cang, S. Chen, Three-in-one simultaneous extraction of proteins, metabolites and lipids for multi-omics, Front. Genet. 12 (2021) 635971.
[15] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can J Biochem Phys 37(8) (1959) 911–917.
[16] J. Folch, M. Lees, G.H.S. Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J Biol Chem 226(1) (1957) 497–509.
[17] V. Matyash, G. Liebisch, T.V. Kurzchalia, A. Shevchenko, D. Schwudke, Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics, J Lipid Res 49(5) (2008) 1137–1146.
[18] L.K. Muehlbauer, A. Jen, Y. Zhu, Y. He, E. Shishkova, K.A. Overmyer, J.J. Coon, Rapid multi-omics sample preparation for mass spectrometry, Anal Chem 95(2) (2023) 659–667.
[19] D.P. Donnelly, C.M. Rawlins, C.J. DeHart, L. Fornelli, L.F. Schachner, Z. Lin, J. L. Lippens, K.C. Aluri, R. Sarin, B. Chen, C. Lantz, W. Jung, K.R. Johnson, A. Koller, J.J. Wolff, I.D.G. Campuzano, J.R. Auclair, A.R. Ivanov, J.P. Whitelegge, L. Paša-Tolić, J. Chamot-Rooke, P.O. Danis, L.M. Smith, Y.O. Tsybin, J.A. Loo, Y. Ge, N. L. Kelleher, J.N. Agar, Best practices and benchmarks for intact protein analysis for top-down mass spectrometry, Nat Methods 16(7) (2019) 587–594.
[20] V.-A. Duong, H. Lee, Bottom-up proteomics: advancements in sample preparation, Int J Mol Sci 24(6) (2023) 5350.
[21] J.L. Nickerson, V. Baghalabadi, Z. Dang, V.A. Miller, S.L. Little, A.A. Doucette, Organic solvent-based protein precipitation for robust proteome purification ahead of mass spectrometry, J. Vis. Exp. 180 (2022) e63503.
[22] K.A. Overmyer, T.W. Rhoads, A.E. Merrill, Z. Ye, M.S. Westphall, A. Acharya, S. K. Shukla, J.J. Coon, Proteomics, lipidomics, metabolomics, and 16S DNA sequencing of dental plaque from patients with diabetes and periodontal disease, Mol. Cell. Proteomics 20 (2021) 100126.
[23] H. Wu, A.D. Southam, A. Hines, M.R. Viant, High-throughput tissue extraction protocol for NMR- and MS-based metabolomics, Anal Biochem 372(2) (2008) 204–212.
[24] C.Y. Lin, H. Wu, R.S. Tjeerdema, M.R. Viant, Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics, Metabolomics 3(1) (2007) 55–67.
[25] M.I. Boxler, T.D. Schneider, T. Kraemer, A.E. Steuer, Analytical considerations for (un)-targeted metabolomic studies with special focus on forensic applications, Drug Test Anal 11(5) (2019) 678–696.
[26] J. Rappsilber, M. Mann, Y. Ishihama, Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips, Nat Protoc 2(8) (2007) 1896–1906.
[27] Z. Wang, M. Mülleder, I. Batruch, A. Chelur, K. Textoris-Taube, T. Schwecke, J. Hartl, J. Causon, J. Castro-Perez, V. Demichev, S. Tate, M. Ralser, High-throughput proteomics of nanogram-scale samples with Zeno SWATH MS, Elife 11 (2022).
[28] U. Distler, J. Kuharev, P. Navarro, S. Tenzer, Label-free quantification in ion mobility-enhanced data-independent acquisition proteomics, Nat Protoc 11(4) (2016) 795–812.
[29] H. Tsugawa, K. Ikeda, M. Takahashi, A. Satoh, Y. Mori, H. Uchino, N. Okahashi, Y. Yamada, I. Tada, P. Bonini, Y. Higashi, Y. Okazaki, Z. Zhou, Z.J. Zhu, J. Koelmel, T. Cajka, O. Fiehn, K. Saito, M. Arita, M. Arita, A lipidome atlas in MS-DIAL 4, Nat Biotechnol 38(10) (2020) 1159–1163.
[30] K. Dührkop, M. Fleischauer, M. Ludwig, A.A. Aksenov, A.V. Melnik, M. Meusel, P. C. Dorrestein, J. Rousu, S. Böcker, Sirius 4: a rapid tool for turning tandem mass spectra into metabolite structure information, Nat Methods 16(4) (2019) 299–302.
[31] M. Ludwig, L.-F. Nothias, K. Dührkop, I. Koester, M. Fleischauer, M.A. Hoffmann, D. Petras, F. Vargas, M. Morsy, L. Aluwihare, P.C. Dorrestein, S. Böcker, Database-independent molecular formula annotation using Gibbs sampling through ZODIAC, Nat Mach Intell 2(10) (2020) 629–641.
[32] K. Dührkop, L.-F. Nothias, M. Fleischauer, R. Reher, M. Ludwig, M.A. Hoffmann, D. Petras, W.H. Gerwick, J. Rousu, P.C. Dorrestein, S. Böcker, Systematic classification of unknown metabolites using high-resolution fragmentation mass spectra, Nat Biotechnol 39(4) (2021) 462–471.
[33] H.W. Kim, M. Wang, C.A. Leber, L.F. Nothias, R. Reher, K.B. Kang, J.J.J. van der Hooft, P.C. Dorrestein, W.H. Gerwick, G.W. Cottrell, NPClassifier: a deep neural network-based structural classification tool for natural products, J Nat Prod 84(11) (2021) 2795–2807.
[34] Y. Djoumbou Feunang, R. Eisner, C. Knox, L. Chepelev, J. Hastings, G. Owen, E. Fahy, C. Steinbeck, S. Subramanian, E. Bolton, R. Greiner, D.S. Wishart, ClassyFire: automated chemical classification with a comprehensive, computable taxonomy, J Cheminform 8 (2016) 61.
[35] C. Zeki Ö, C.C. Eylem, T. Reçber, S. Kır, E. Nemutlu, Integration of GC-MS and LC-MS for untargeted metabolomics profiling, J. Pharm. Biomed. Anal. 190 (2020) 113509.
[36] R. Yang, Q. Zhao, D.-D. Hu, X.-R. Xiao, F. Li, Optimization of extraction and analytical protocol for mass spectrometry-based metabolomics analysis of hepatotoxicity, Biomed. Chromatogr. 32 (12) (2018) e4359.
[37] S.J. Bruce, I. Tavazzi, V. Parisod, S. Rezzi, S. Kochhar, P.A. Guy, Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/mass spectrometry, Anal Chem 81 (9) (2009) 3285–3296.
[38] Y. Wartmann, M.I. Boxler, T. Kraemer, A.E. Steuer, Impact of three different peak picking software tools on the quality of untargeted metabolomics data, J. Pharm. Biomed. Anal. 248 (2024) 116302.
[39] L.W. Sumner, A. Amberg, D. Barrett, M.H. Beale, R. Beger, C.A. Daykin, T.W. Fan, O. Fiehn, R. Goodacre, J.L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A.N. Lane, J.C. Lindon, P. Marriott, A.W. Nicholls, M.D. Reily, J. J. Thaden, M.R. Viant, Proposed minimum reporting standards for chemical analysis chemical analysis working group (CAWG) metabolomics standards initiative (MSI), Metabolomics 3(3) (2007) 211–221.
[40] E. Fahy, S. Subramaniam, R.C. Murphy, M. Nishijima, C.R. Raetz, T. Shimizu, F. Spener, G. van Meer, M.J. Wakelam, E.A. Dennis, Update of the LIPID MAPS comprehensive classification system for lipids, J Lipid Res 50 Suppl (2009) S9–S14.
[41] T. Kind, K.H. Liu, D.Y. Lee, B. DeFelice, J.K. Meissen, O. Fiehn, LipidBlast in silico tandem mass spectrometry database for lipid identification, Nat Methods 10(8) (2013) 755–758.
[42] J.L. Nickerson, A.A. Doucette, Rapid and quantitative protein precipitation for proteome analysis by mass spectrometry, J Proteome Res 19(5) (2020) 2035–2042.
[43] L. Brockbals, S. Garrett-Rickman, S. Fu, M. Ueland, D. McNevin, M.P. Padula, Estimating the time of human decomposition based on skeletal muscle biopsy samples utilizing an untargeted LC–MS/MS-based proteomics approach, Anal Bioanal Chem 415(22) (2023) 5487–5498.