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Targeted quantitative metabolic profiling of brain-derived cell cultures by semi-automated MEPS and LC-MS/MS

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ABSTRACT

The accurate characterisation of metabolic profiles is an important prerequisite to determine the rate and the efficiency of the metabolic pathways taking place in the cells. Changes in the balance of metabolites involved in vital processes such as glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), as well as in the biochemical pathways related to amino acids, lipids, nucleotides, and their precursors reflect the physiological condition of the cells and may contribute to the development of various human diseases. The feasible and reliable measurement of a wide array of metabolites and biomarkers possesses great potential to elucidate physiological and pathological mechanisms, aid preclinical drug development and highlight potential therapeutic targets. An effective, straightforward, sensitive, and selective liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach was developed for the simultaneous quali-quantitative analysis of 41 compounds in both cell pellet and cell growth medium obtained from brain-derived cell cultures. Sample pretreatment miniaturisation was achieved thanks to the development and optimisation of an original extraction/purification approach based on digitally programmed microextraction by packed sorbent (eVol®-MEPS). MEPS allows satisfactory and reproducible clean-up and preconcentration of both low-volume homogenate cell pellet lysate and cell growth medium with advantages including, but not limited to, minimal sample handling and method sustainability in terms of sample, solvents, and energy consumption. The MEPS-LC-MS/MS method showed good sensitivity, selectivity, linearity, and precision. As a proof of concept, the developed method was successfully applied to the analysis of both cell pellet and cell growth medium obtained from a line of mouse immortalised oligodendrocyte precursor cells (OPCs; Oli-neu cell line), leading to the unambiguous determination of all the considered target analytes. This method is thus expected to be suitable for targeted, quantitative metabolic profiling in most brain cell models, thus allowing accurate investigations on the biochemical pathways that can be altered in central nervous system (CNS) neuropathologies, including e.g., mitochondrial respiration and glycolysis, or use of specific nutrients for growth and proliferation, or lipid, amino acid and nucleotide metabolism.

1. Introduction

Endogenous metabolites play a critical role in brain physiological functions, and their imbalances have been implicated in various central nervous system (CNS) pathologies, from neurodevelopmental disorders to neurodegenerative and demyelinating diseases [1–5]. Therefore, the

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reliable measurement of a wide array of metabolites holds great potential to elucidate physiological and pathological mechanisms [6], to aid in preclinical drug development and to identify potential therapeutic targets [5,7]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a highly sensitive and selective technique for quali-quantitative analysis of small molecules and has been widely used for targeted metabolite profiling in biological matrices [8–10].

This research work was aimed at the design, development, and validation of a bioanalytical methodology for the miniaturised sample treatment and targeted, quantitative LC-MS/MS-based metabolic profiling of brain cell cultures, specifically both cell pellets and culture media. The primary focus was on metabolites that are crucial for cellular bioenergetics since they are involved in biochemical pathways such as tricarboxylic acid (TCA) cycle, malate-aspartate NADH shuttle (MAS), and oxidative phosphorylation (OXPHOS). Furthermore, analysed metabolites are also indicators of the metabolism of amino acids, lipids, and nucleotides, that overall requires active biochemical communication between cytosol and mitochondria in the cell [11–13].

Traditional sample preparation techniques for LC-MS/MS analysis, such as solid-phase extraction (SPE) and protein precipitation, involve extensive sample handling steps and often require large amounts of starting material [14]. To achieve a miniaturised, yet feasible, streamlined, and reliable sample pretreatment protocol, an optimised, original extraction/purification approach based on microextraction by packed sorbent (MEPS) was developed. MEPS is a solid-phase extraction technique that uses a small cartridge packed with sorbent material in a barrel insert and needle (BIN) assembly integrated in a modified syringe, exploited to extract and preconcentrate analytes from the sample. The technique has been widely used for sample preparation in bioanalysis due to its simplicity, speed, and high efficiency [15,16]. However, the manual operation of MEPS can be time-consuming and laborious, leading to a higher risk of sample handling errors [17,18].

To overcome these limitations, eVol® electronical syringe for MEPS was exploited, allowing us to partially automate the MEPS process. The eVol syringe is an electronic device that can be programmed to dispense precise volumes of liquid, making it an excellent tool for sample preparation in bioanalysis [19–21]. The use of eVol-based semi-automated MEPS allows to reduce manual sample handling and enhance method sustainability in terms of sample, solvents, and energy consumption while enabling rapid method development and increasing sample processing throughput [22].

The application of MEPS in combination with LC-MS/MS has shown great potential for metabolic profiling in different biological matrices, e.g., cerebrospinal fluid (CSF) [23] and urine [24], while, on the other hand, examples of MEPS applications to cell cultures and culture media is rather sparse in the scientific literature. One of the main challenges in the analysis of cell cultures is the low abundance of metabolites present in the sample, which requires highly sensitive and selective analytical methods for their detection and quantification and a careful setup and validation of the overall pretreatment and analysis workflow.

For this research, the main aim was to develop a MEPS-LC-MS/MS method, and fully validate it following international bioanalytical guidelines, for the simultaneous quali-quantitative analysis of 41 compounds including amino acids, organic acids, vitamins, nucleotides, neurotransmitters and others in both cell pellets and cell growth medium. These particular analytes have been selected as they are widely recognised to play roles in various metabolic pathways and essential cellular processes. They function as enzymatic substrates or cofactors in pathways like the tricarboxylic acid (TCA) cycle, malate-aspartate NADH shuttle (MAS), oxidative phosphorylation (OXPHOS), amino acid, lipid, or nucleotide metabolism, as well as in the response to oxidative stress. By measuring changes in their intracellular levels or release, either collectively or as crucial compounds in specific pathways, the proposed methodology has the potential to elucidate cellular physiology or offer insights into the mechanisms behind metabolic alterations in pathological conditions.

Both kinds of samples were subjected to carefully optimised sample treatment to obtain satisfactory clean-up and extraction and were coupled to an original targeted quantitative LC-MS/MS method, qualified for this purpose. This work enabled the evaluation, for the first time, of the potential of an automated, miniaturised MEPS pretreatment approach as a viable alternative strategy for quali-quantitative, targeted metabolic profiling of cell cultures. As a proof of concept and secondary aim, the optimised and validated method was applied for the analysis of cell pellet and cell growth medium obtained from murine immortalised oligodendrocytes precursor cells (OPCs; Oli-neu cell line). This is indeed the first report of a miniaturised semi-automated pretreatment protocol based on MEPS applied to microsamples (100 µL) of cell pellet and culture media. The original LC-MS/MS method has been specifically developed and validated (in terms of sensitivity, selectivity, linearity, and precision) for this purpose, leading to the unambiguous determination of all the considered target analytes. Again, for the first time this panel of analytes has been included in a microsample analysis workflow and tested for the first time for absolute quantitation in this kind of cell lines.

2. Materials and methods

2.1. Chemicals, standard solutions, and devices

MS-grade solvents and reagents were purchased from Merck Life Science (Milan, Italy). All target analytes, namely glutamic acid (Glu), glutamine (Gln), aspartic acid (Asp), asparagine (Asn), alanine (Ala), 2-oxoglutaric acid (2OG), succinic acid (SUC), fumaric acid (FUM), malic acid (MAL), citric acid (CIT), pyruvic acid (PYR), lactic acid (LAC), oxaloacetic acid (OXA), N-acetylaspartic acid (NAA), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), glutathione (GSH), glutathione disulphide (GSSG), α-ketoisocaproic acid (KIC), α-ketoisovaleric acid (KIV), leucine (Leu), isoleucine (Ile), valine (Val), tyrosine (Tyr), thiamine (B1), cis-aconitic acid (CAC), aminoadipic acid (AAA), pantothenic acid (B5), γ-aminobutyric acid (GABA), ornithine (Orn), methionine (Met), guanosine (GUO), creatine (CRE), carnitine (Car), folic acid (B9), citrulline (Cit), oxidised nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide (NADH) as well as internal standards (ISs), namely Asp-d3 (IS1), CIT-d4 (IS2), SUC-d4 (IS3), FUM-d4 (IS4), pure powders (all >95% purity), acetonitrile (ACN), methanol (MeOH), and formic acid (FA), (all reagents for mass spectrometry) and other solvents used for sample preparation (all analytical grade) were purchased from Merck Life Science (Milan, Italy).

Ultrapure water (18.2 MΩ•cm) was obtained using a Milli-Q® water purification system from Merck Millipore (Darmstadt, Germany). Analyte and IS stock solutions (1 mg/mL) were prepared by dissolving suitable amounts of pure powders in MeOH and kept at − 20 °C when not in use; the corresponding standard solutions were prepared daily by dilution with a mixture of H_2O and ACN (50:50, v/v) containing 0.25% FA (the dilution mixture was prepared by transferring 125 µL FA to a 50-mL volumetric flask, then bringing to volume with a mixture of equal volumes H_2O and ACN). All solutions were stored protected from light in amber glass vials certified for mass spectrometry from Waters (Milford, MA, USA). Calibration standards were prepared by spiking cell pellet lysate and cell growth medium with appropriate amounts of working standard solutions. The concentration range for each calibration standard was chosen based on the expected physiological range of the target analytes. Quality control (QC) samples at low, medium, and high concentrations with respect to the calibration curves were prepared by spiking samples with appropriate amounts of working standard solutions.

Ten cm ø Petri dishes for cell cultures were purchased from Corning (New York, USA) and were pre-treated with poly-L-lysine (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's

Table 1
Multiple reaction monitoring (MRM) transitions and compound-specific MS/MS parameters.

Analyte	MW (g/mol)	ESI polarity	Q1 (*m/z*)	Q3 (*m/z*)[a]	Collision Energy (eV)
Glu	147.13	-	146.09	128.06	4
Gln	146.14	+	147.08	84.12	16
Asp	133.10	-	132.12	88.10	20
Asn	132.12	+	133.14	74.05	15
Ala	89.09	+	90.08	44.06	11
2OG	146.10	-	145.04	57.10	12
SUC	118.09	-	117.11	73.10	6
FUM	116.07	-	115.12	71.09	2
MAL	134.09	-	133.06	115.06	4
CIT	192.12	-	191.09	111.12	10
PYR	88.06	-	175.09	87.08	4
LAC	90.08	-	89.30	43.50	10
OXA	132.07	-	131.02	87.06	12
NAA	175.14	-	174.09	130.12	14
ATP	507.18	-	506.15	408.20	22
ADP	427.20	-	426.17	328.12	22
AMP	347.22	+	348.20	136.20	21
CTP	483.16	-	482.16	159.09	15
GTP	523.18	-	522.20	159.13	35
GSH	307.33	+	308.30	162.20	17
GSSG	612.60	+	613.60	231.30	34
KIC	130.14	-	129.05	57.07	7
KIV	116.11	-	115.12	70.09	5
Leu	131.17	+	132.11	43.10	25
Ile	131.17	+	132.11	69.10	15
Val	117.15	+	118.09	72.04	15
Tyr	181.19	+	182.20	136.08	14
B1	265.36	+	265.15	122.18	13
CAC	174.11	-	173.15	111.12	10
AAA	161.16	+	162.20	98.20	16
B5	219.23	+	220.19	202.08	22
GABA	103.12	+	104.12	87.09	8
Orn	132.16	+	133.22	70.30	17
Met	149.21	+	150.16	56.08	15
GUO	283.24	+	284.31	152.22	13
CRE	131.13	+	132.15	43.32	17
Car	161.20	+	162.17	103.20	12
B9	441.40	+	442.28	295.19	22
Cit	175.19	+	176.20	70.32	22
NAD+	664.12	-	663.11	273.16	18
NADH	665.13	-	664.12	158.90	24
IS1	136.12	-	135.14	91.12	20
IS2	196.15	-	195.11	115.14	10
IS3	122.11	-	121.15	73.10	6
IS4	120.10	-	119.18	75.13	4

[a] Quantitative product ion

instructions. All components of SATO cell culture medium came from Sigma-Aldrich, except for insulin-transferrin-sodium selenite 100X supplement, Thermo Fisher Scientific (Waltham, Massachusetts, USA). Powders and solvents used for washes and samples collection, PBS (0.9% NaCl in 50 mM phosphate buffer pH 7.4) and 0.01% trypsin – 0.02% EDTA-HBSS were purchased from Sigma-Aldrich.

Cell pellet and growth medium were collected in 1.5-mL microtubes and 15-mL tubes, respectively. All cell counts were performed by using a BLAUBRAND® Neubauer chamber (Sigma-Aldrich).

The eVol hand-held automated analytical syringe (500 µL), and the BIN assembly containing the sorbent materials (C8, C18, and M1 – mixed mode sorbent containing 80% C8 and 20% SCX strong cationic exchange) used for MEPS were purchased from SGE Analytical Science (SGE Europe Ltd., Crook Street, Chester, United Kingdom). A lifetime of 40 pretreatment cycles with the developed protocol was determined on the BIN packed sorbent bed after proper regeneration after every cycle. A Savant SpeedVac SPD 1030 vacuum concentrator from Thermo Fisher Sci (Waltham, MA, US) was exploited to simultaneously evaporate small volume extracts with high throughput.

2.2. LC-MS/MS

The LC-MS/MS analysis was carried out using a Waters Alliance e2695 chromatographic system with an autosampler coupled to a Waters Micromass Quattro Micro triple-quadrupole mass spectrometer that was equipped with an electrospray ion source (ESI). The data were processed with Waters MassLynx 4.1 software. The separations were obtained using a reverse-phase Hypersil Gold pentafluorophenyl (PFP) column (50×2.1 mm; 5.0 µm) from Thermo Fisher Scientific (Waltham, MA, USA), which was maintained at room temperature and equipped with a guard column (PFP, 10×2.1 mm). The mobile phase was a mixture of 0.25% aqueous FA (component A) and 0.25% FA in ACN (component B) that flowed at a constant rate of 0.3 mL/min. The composition gradient was as follows: 0–3.0 min, 2% constant component B; 3.0–4.5 min, linear gradient from 2% to 45% component B; 4.5–8.5 min, 45% constant component B; 8.5–10.0 min, linear gradient from 45% to 90% component B; 10.0–14.0 min, 90% constant component B; 14.0–16.5 min, linear gradient from 90% to 2% component B; and 16.5–19.0 min, 2% constant component B. The total run time was 19 min, including column re-equilibration, and the injection volume was 10 microliters. Multiple reaction monitoring (MRM) transitions were employed, with both positive and negative ionisation (ESI+, ESI-) in polarity switching mode, using two exclusive transitions for each analyte: the most abundant one for quantitative purposes and the second for identity confirmation. The optimised parameters were as follows: ion source voltage, 4.3 kV; ion source temperature, 140 °C; desolvation temperature, 300 °C; desolvation gas flow, 550 L/h (nitrogen as the desolvation gas, argon as the collision gas); dwell time, 300 ms for all compounds. MRM transitions in terms of precursor ions and product ions, and collision energy were optimised and are shown in Table 1.

2.3. Cell culture and sample collection

Immortalised mouse oligodendrocyte precursor cells (Oli-neu cell line; kindly provided by Jacqueline Trotter, University of Mainz, Germany, RRID:CVCL_IZ82) were maintained on poly-L-lysine (Sigma-Aldrich, St Louis, MO, USA) coated 10-cm ø Petri dishes at 37 °C and 5% CO_2 in SATO medium, containing DMEM with 4.5 g/L glucose, 2 mM glutamine, 10 µg/mL insulin, 5.5 µg/mL transferrin, 38.72 nM sodium selenite, 100 µM putrescine, 520 nM, L-thyroxine, 500 nM triiodo-L-thyronine (T3), 200 nM progesterone, 25 µg/mL gentamycin, supplemented with 1% Horse Serum (HS). Once confluent, cells were washed with PBS (0.9% NaCl in 50 mM, pH 7.4 phosphate buffer) and incubated with 0.01% trypsin – 0.02% EDTA-HBSS for 4 min at 37 °C. To inhibit trypsin reaction, an equal volume of DMEM/10% HS was added, and cells collected and centrifugated at 300 ×*g* for 5 min. Oli-neu cells were counted by using BLAUBRAND® Neubauer chamber and 4 × 10^5 cells/10-cm ø Petri dish were plated in complete SATO culture medium and incubated at 37 °C and 5% CO_2.

After 4 Days In Vitro (DIV), growth medium was collected in 15 mL tubes and stored at − 20 °C until analysis. Complete SATO medium was collected as blank. In parallel, confluent cells were washed in PBS and detached with 0.01% trypsin – 0.02% EDTA-HBSS for 4 min at 37 °C. After trypsin inhibition with DMEM/10% HS, cells were centrifuged at 300 ×*g* for 5 min. Supernatant was discarded, and cell pellet resuspended with 1 mL of PBS to proceed with cell count with BLAUBRAND® Neubauer chamber. A mean of 1,538,167 ± 373,704 cells was collected in 1.5 mL microtube, centrifugated 300 ×*g* for 5 min and pellet samples were stored at − 80 °C, until further analysis.

Cells number/pellet sample is a mean ± SD of 3 independent experiments.

2.4. Sample pretreatment

Cell pellet samples were preliminarily disrupted and extracted, then sample clean-up was carried out on cell lysate and culture media by

exploiting an optimised MEPS protocol in order to achieve sample miniaturisation and efficient clean-up and preconcentration of low-volume homogenate cell pellet lysates and culture media. The protocol was based on the use of a semi-automated syringe handling system, employing an M1 sorbent in a BIN assembly.

Cell pellet samples were preliminarily disrupted and extracted by means of an optimised protocol adapted from [25]: 100 µL of an ice-cold MeOH/H_2O mixture (80:20, V/V) containing the ISs was added to the sample vial, and it was sonicated twice at 150 W for 30 s. The cell pellet was vortexed vigorously and transferred to deep freezing temperature (−80 °C) for 1 h, thawed in an ice bath for 10 min, and briefly vortexed. The sample was centrifuged at 4 °C and 3500 ×*g* for 5 min and the supernatant was transferred to a new amber glass vial. This sonication-freeze-thaw-centrifugation cycle was carried out twice in total for complete cell disruption. The two 100-µL aliquots were reunited and subjected to a semi-automated MEPS clean-up procedure. For culture media, a 100-µL sample aliquot was added with 100 µL of a MeOH/H_2O mixture (80:20, V/V) containing the ISs and briefly vortexed before being subjected to the semi-automated MEPS clean-up procedure.

2.4.1. Semi-automated MEPS clean-up

The MEPS protocol was performed by an eVol hand-held automated analytical syringe (500 µL) fitted with a BIN containing 4 mg of M1 sorbent material, enabling MEPS to be semi-automated. Firstly, the sorbent was activated and conditioned by drawing and discharging 200 µL of MeOH followed by 200 µL of ultrapure water with a drawing/discharge speed of 20 µL/s. The loading solution consisting of 200 µL of either cell lysate or culture medium was passed through the MEPS sorbent for 10 draw/discharge cycles without discarding it at 10 µ/s. The cartridge was then washed with 150 µL of H_2O and 150 µL of H_2O:MeOH (95:5, V/V) at 20 µL/s to remove interferences and the sorbent was dried with 3 × 100 µL of air at 20 µL/s. The analytes were then eluted by drawing and discharging 500 µL of MeOH (5 cycles of 100 µL each at 10 µL/s). The eluate was evaporated to dryness with a centrifugal vacuum concentrator, the residue was redissolved in 50 µL of a 50:50 (V/V) mixture of ACN and H_2O containing 0.25% FA, and a 10-µL aliquot was injected into the LC-MS/MS system. For sorbent regeneration, the BIN was rinsed with 2 × 100 µL of H_2O, 2 × 100 µL of 0.1% FA in H_2O and 2 × 100 µL of MeOH (at 20 µL/s) in between sample extractions to maximise the BIN sorbent lifetime up to 40 extraction cycles.

2.5. Method validation

The developed MEPS-LC-MS/MS method for targeted metabolite profiling was assessed in order to fulfil the European Medicines Agency (EMA) guidelines [26]. The tested parameters were linearity (including limit of quantitation, LOQ), selectivity, absolute recovery, precision, matrix effect, stability, and accuracy. Since currently there is no consensus guideline for endogenous substance assay validation [27,28], and the selected analytes are endogenous substances in biological matrices, it is not possible to obtain a completely analyte-free authentic matrix for method development and validation. Thus, in the present study, method development and validation were carried out exploiting the standard addition method by fortifying oligodendroglial precursor cell pellets and fresh culture medium with working standard mixtures of the analytes and ISs at known concentrations. Calibration standards as well as QC samples were prepared by fortifying cell pellet samples (mean cell count 1,538,167 ± 373,704 cells/sample) and fresh culture medium (100 µL) for the assessment of linearity, sensitivity, inter- and intra-day precision and stability.

For linearity, expected concentration ranges for the analytes were deduced from the literature available, from published reports on the same and similar cell lines, and from preliminary assays on the available samples. Starting from these data, calibration ranges were set including expected concentration ranges for each analyte. Samples were spiked with 5 µL of working solutions containing the analytes at seven different concentrations, subjected to the sample pretreatment protocols described above and analysed by LC-MS/MS. The analysis was carried out in triplicate for each concentration. The obtained analyte/IS peak area ratios were plotted as a function of the nominal added concentrations (ng/mL), and the least-squares method was used to obtain calibration curves. A $1/x^2$ weighing factor was applied. LOQ was calculated as the lowest concentration of analyte that could be quantified reliably, with acceptable accuracy (± 20%) and precision (relative standard deviation, RSD < 20%).

Selectivity was assessed by the absence of any interfering signal close to the retention time (t_R) and at the same MRM transition of each analyte. Complete absence of signal was defined as the lack of any signal higher than 3 times the baseline noise, and this was checked as regards peaks partially overlapping those of the analytes.

Absolute recovery was evaluated on both cell pellet and culture medium samples fortified with analyte working solutions at three different concentrations (representative for low, intermediate, and high calibration points for each analyte) and subjected to the previously described MEPS extraction procedure. The obtained analyte peak areas were compared with those obtained by analysing extracts from samples fortified post-extraction with the same nominal concentrations, and absolute recoveries were expressed as percentage. The acceptability criterion was absolute recovery > 80%.

Precision was determined on the same fortified samples: five replicates were analysed on the same day to assess intraday precision and over five different days to assess interday precision, expressed as RSD%. The acceptability criteria were RSD < 10% for intraday precision (<15% for the LOQ) and RSD < 15% for interday precision (<20% for the LOQ).

The IS-corrected matrix effect was evaluated by analysing six sample replicates, fortified post-extraction by adding known analyte concentrations at the same levels as precision assays. The mean analyte/IS peak area ratios for each added concentration was compared with analyte/IS peak area ratios from standard solutions at the same theoretical concentration and the resulting percentage was calculated. Acceptability criterion was a response in the 85–115% range.

Carryover was assessed by the injection of a blank solvent (50:50 water/ACN mixture containing 0.25% FA) after the highest concentration of the calibration curves (n = 3). Carryover was considered acceptable if the signal at the retention time of the target analyte was less than 20% of the lower limit of quantitation (LLOQ) value.

To test analyte stability, fortified cell pellet and culture media samples were stored at − 80 °C and − 20 °C, respectively. At regular intervals (1 week), samples were pretreated and analysed (n = 3). The measured analyte concentrations were compared to those from samples extracted and analysed immediately after fortification (t_0) to assess the percentage of analyte loss. Samples were considered stable until the bias from nominal concentrations was within ± 15%. An additional subset of samples was used to evaluate method accuracy by means of recovery assays: 5 µL of working standard mixtures at low, intermediate, and high concentrations, and fixed amounts of the ISs, were added to cell pellet and culture media sample replicates whose analyte concentrations were already assessed, and the samples were pretreated and analysed. Accuracy, expressed as percent recovery, was calculated by comparing the concentrations obtained from the fortified samples with the concentration in non-fortified samples plus the nominal concentration of the added standard mixture, and the recovery was considered acceptable if > 80%.

3. Results

3.1. Liquid chromatography and mass spectrometry

MS and MS/MS spectra of the analytes and ISs were acquired in the 50–700 *m/z* range by direct infusion in the ESI source of working solutions of the analytes at the concentration of 1 µg/mL in a mixture of

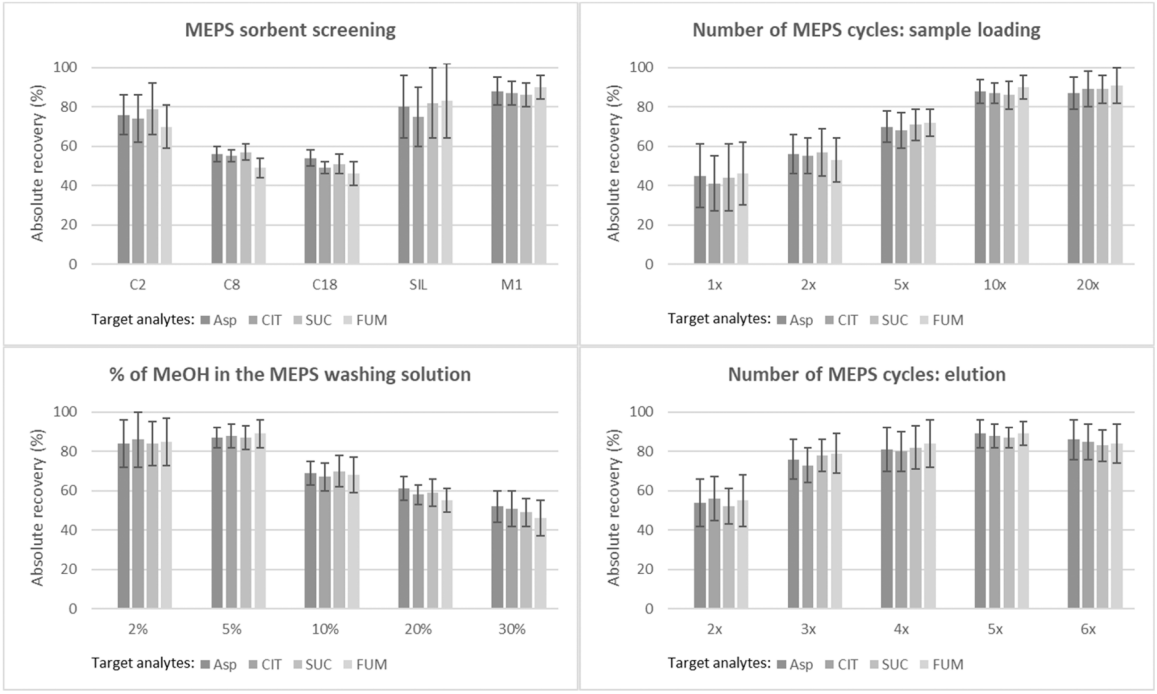

Fig. 1. MEPS protocol development results.

0.25% FA in ACN and 0.25% FA in water (50/50, V/V). All spectra were acquired using both ESI+ and ESI- ionisation modes, in order to choose the best conditions for each analyte and IS. For each analyte, the most abundant signal in the ion scan spectra was selected as the precursor ion, while for product ions, the most abundant fragment ion was used for quantitative purposes, while the second was exploited for identity confirmation. ISs, namely Asp-d3 (IS1), CIT-d4 (IS2), SUC-d4 (IS3), FUM-d4 (IS4), were selected in order to cover the full spectrum of the target analytes from the qualitative and quantitative point of view.

A suitable chromatographic setup was then optimised for the simultaneous analysis of the biomarkers chosen for this study. Various reverse-phase columns and mobile phase conditions were tested, and it was observed that the most hydrophilic compounds could be retained at a higher degree by a PFP sorbent when compared to C8, C18 or cyanopropyl (CN) ones. Therefore, a PFP column was selected for further analysis. The mobile phase was then optimised to improve retention, peak shape, and separation. First, 0.25% FA was chosen as an acidic additive as a suitable compromise for satisfactory retention, peak shape, and MS signal. In order to keep a constant FA concentration and more reproducible MS ionisation throughout a gradient elution, the same amount of FA was added to both water (component A) and ACN (component B). As a final development step, the composition gradient was finely tuned in order to allow satisfactory chromatographic peak resolution and a reasonable analysis run time. Without gradient, the analytes were fully separated within a 2.5-h chromatographic run. Thus, a 10-min linear gradient elution program (from 2% to 90% B) was implemented to shorten run times, hopefully without losing resolution between critical pairs. However, some overlaps were observed in the 0–2 min and 4–7 min zones. For this reason, the gradient was started after 3.0 min, made a bit steeper, stopped at 45% B at 4.5 min, and resumed (again steeper) at 8.5 min. The gradient plateau from 10.0 to 14.0 min was kept. This produced complete resolution for all analytes. The optimised gradient conditions were as follows: 0–3.0 min, 2% B; 3.0–4.5 min, from 2% to 45% B; 4.5–8.5 min, 45% B; 8.5–10.0 min, from 45% to 90% B; 10.0–14.0 min, 90% B; for column re-equilibration, 14.0–16.5 min from 90% to 2% B; 16.5–19.0, 2% B. The flow rate was 0.3 mL/min, and the injection volume was 10 μL.

Under these working conditions, all the target analytes produced sufficiently sharp and symmetric peaks, with a total chromatographic run of 19 min (including column re-equilibration), with sufficient sensitivity and selectivity.

3.2. *Optimisation of semi-automated MEPS sample pretreatment*

MEPS pretreatment technology is based on the general principle of solid phase extraction, but with the sorbent contained within a very small BIN, which constitutes the needle assembly of an electronic syringe which allows to partially automate all the protocol steps. MEPS grants high selectivity and good sample purification and absolute recovery, while being faster, more feasible and using smaller amounts of samples and solvents, when compared to classic SPE. In fact, a typical SPE procedure lasts about 30 min, while an eVol MEPS procedure is usually less time consuming, mainly due to the small volumes involved and the automatic drawing and discharging of solvents. The small volumes would produce a time advantage in fully automated, high-throughput workflows as well, when using automated liquid handling apparatuses. MEPS is also time-sparing in comparison to classical liquid-liquid extraction (LLE) or protein precipitation (PP), since these procedures require lengthy centrifugation, resting and/or cooling phases that are absent from MEPS; solvent usage is obviously much lower in MEPS than in common LLE or PP procedures.

In order to optimise the extraction conditions for the MEPS procedure, several experimental parameters were investigated for their influence on the extraction performance, namely sorbent type, washing and elution solvents and volumes, drawing/discharging speeds and cycles.

Commercially available eVol-MEPS sorbents were evaluated, namely C2, C8, C18, unmodified silica (SIL) and mixed-mode M1 (C8/SCX). The selection of the best sorbent was based on extraction efficiency for all the target analytes, determined by the relative peak area, and reproducibility. Fig. 1 shows MEPS protocol development results related to Asp, CIT, SUC and FUM, chosen as test compounds. Within method development, these analytes were chosen as the test compounds in order to represent a preliminary range of chemical-physical characteristics (e.g., lipophilicity), and optimise the pretreatment protocol to be effective on the panel of selected target compounds. The first MEPS sorbents tested were C18 and C8 ones, but they were not satisfactory in terms of either

Table 2
Absolute recovery, precision and matrix effect results.

Compound	Concentration Level[a]	Intraday Precision (%RSD)[b, c]	Interday Precision (%RSD)[b, c]	Absolute Recovery (%)[b,d]	Matrix Effect (%)[b,d]
Glu	Low	6.8 / 7.1	7.8 / 8.0	89 / 91	91 / 91
	Medium	6.2 / 6.5	7.1 / 7.5	90 / 91	94 / 93
	High	5.6 / 5.9	6.5 / 6.9	92 / 92	95 / 96
Gln	Low	8.0 / 8.1	9.6 / 9.8	86 / 88	87 / 88
	Medium	6.9 / 6.9	8.4 / 8.9	87 / 90	89 / 89
	High	6.4 / 6.6	7.9 / 8.2	92 / 92	91 / 92
Asp	Low	6.6 / 6.7	7.6 / 8.0	88 / 90	90 / 91
	Medium	6.1 / 6.2	7.0 / 7.5	88 / 91	92 / 92
	High	5.4 / 5.7	6.3 / 6.6	90 / 92	94 / 93
Asn	Low	5.8 / 6.0	6.1 / 6.4	88 / 91	90 / 91
	Medium	5.1 / 5.3	5.7 / 5.7	89 / 91	94 / 93
	High	4.9 / 5.1	4.8 / 5.0	92 / 92	94 / 95
Ala	Low	6.0 / 6.2	6.6 / 7.0	86 / 87	90 / 89
	Medium	5.7 / 5.9	5.9 / 5.5	88 / 89	90 / 91
	High	5.1 / 5.3	5.5 / 5.5	88 / 90	94 / 94
2OG	Low	5.8 / 5.8	6.2 / 6.5	86 / 88	88 / 90
	Medium	5.4 / 5.6	5.5 / 5.7	87 / 89	90 / 90
	High	5.1 / 5.4	4.8 / 5.2	90 / 93	92 / 91
SUC	Low	5.7 / 5.7	6.4 / 6.7	88 / 90	91 / 90
	Medium	5.4 / 5.5	5.6 / 5.9	88 / 89	92 / 91
	High	4.9 / 5.0	5.1 / 5.5	89 / 91	94 / 93
FUM	Low	5.7 / 5.7	6.3 / 6.6	90 / 91	91 / 92
	Medium	5.2 / 5.4	5.7 / 6.1	91 / 91	93 / 93
	High	5.1 / 5.4	4.8 / 5.2	94 / 95	95 / 94
MAL	Low	5.5 / 5.7	6.3 / 6.6	88 / 88	93 / 94
	Medium	5.3 / 5.6	5.4 / 5.5	90 / 91	93 / 94
	High	4.8 / 5.1	4.9 / 5.2	92 / 92	95 / 93
CIT	Low	5.9 / 6.0	6.3 / 6.5	87 / 88	90 / 91
	Medium	5.3 / 5.6	5.8 / 5.8	89 / 89	93 / 92
	High	5.0 / 5.2	5.0 / 5.3	91 / 91	94 / 93
PYR	Low	6.9 / 7.1	7.8 / 8.1	89 / 90	92 / 93
	Medium	6.3 / 6.4	7.2 / 7.6	90 / 92	95 / 94
	High	5.7 / 5.8	6.6 / 7.0	92 / 94	97 / 95
LAC	Low	8.1 / 8.2	9.5 / 9.8	86 / 88	88 / 90
	Medium	7.0 / 7.0	8.5 / 9.0	87 / 89	90 / 92
	High	6.5 / 6.5	8.0 / 8.5	92 / 95	91 / 92
OXA	Low	6.7 / 6.7	7.7 / 8.2	88 / 88	89 / 90
	Medium	6.2 / 6.2	7.1 / 7.5	88 / 90	93 / 94
	High	5.5 / 5.7	6.4 / 6.6	90 / 91	95 / 93
NAA	Low	5.9 / 6.4	6.2 / 6.4	88 / 89	90 / 91
	Medium	5.2 / 5.6	5.8 / 6.1	89 / 89	95 / 94
	High	5.0 / 5.2	4.9 / 5.5	92 / 93	95 / 92
ATP	Low	8.1 / 8.3	9.4 / 9.6	86 / 88	86 / 89
	Medium	6.9 / 7.1	8.4 / 8.8	86 / 89	87 / 88
	High	6.5 / 6.5	7.8 / 8.0	90 / 93	90 / 89
ADP	Low	7.1 / 7.2	7.7 / 7.9	86 / 88	90 / 91
	Medium	6.8 / 6.9	7.0 / 7.4	86 / 89	90 / 91
	High	6.2 / 6.3	6.6 / 6.9	88 / 89	95 / 94
AMP	Low	6.9 / 7.0	7.3 / 7.5	86 / 87	88 / 90
	Medium	6.5 / 6.5	6.6 / 6.7	86 / 88	90 / 91
	High	6.2 / 6.3	5.9 / 6.1	90 / 93	92 / 92
CTP	Low	6.8 / 7.2	7.5 / 7.8	88 / 88	91 / 90
	Medium	6.3 / 6.6	6.7 / 7.0	88 / 88	92 / 92
	High	6.3 / 6.5	6.2 / 6.4	89 / 91	94 / 93
GTP	Low	6.8 / 7.3	7.5 / 7.9	90 / 91	89 / 90
	Medium /High	6.4 / 6.8 6.2 / 6.5	6.8 / 7.2 5.9 / 6.6	91 / 92 94 / 94	92 / 92 93 / 90
GSH	Low	8.3 / 8.6	9.3 / 9.8	86 / 86	85 / 87
	Medium	7.1 / 7.5	8.6 / 8.8	87 / 88	86 / 88
	High	6.7 / 6.9	8.0 / 8.5	89 / 88	89 / 92
GSSG	Low	8.3 / 8.6	8.8 / 9.1	86 / 88	89 / 91
	Medium	8.0 / 8.1	8.2 / 8.3	85 / 87	89 / 91
	High	7.4 / 7.7	7.8 / 8.0	87 / 89	94 / 93
KIC	Low	7.1 / 7.2	7.5 / 7.7	84 / 85	87 / 91
	Medium	6.7 / 7.1	6.8 / 6.8	84 / 85	88 / 90
	High	6.4 / 6.6	6.1 / 6.7	89 / 89	91 / 92
KIV	Low	7.0 / 7.2	7.7 / 8.1	87 / 88	87 / 88
	Medium	6.5 / 6.7	6.9 / 7.3	87 / 90	88 / 88
	High	6.5 / 6.6	6.4 / 7.0	88 / 89	91 / 90
Leu	Low	7.0 / 7.4	7.7 / 8.0	89 / 89	88 / 89
	Medium	6.6 / 6.8	7.0 / 7.4	90 / 93	91 / 90
	High	6.4 / 6.5	6.1 / 6.2	93 / 92	92 / 93
Ile	Low	6.7 / 6.8	7.7 / 7.8	87 / 90	91 / 90
	Medium	6.2 / 6.3	7.1 / 7.3	87 / 88	91 / 92
	High	5.5 / 5.8	6.4 / 6.8	89 / 91	93 / 94
Val	Low	6.5 / 6.5	7.5 / 7.6	89 / 89	91 / 90
	Medium	6.0 / 6.3	6.9 / 7.0	89 / 90	93 / 92
	High	5.3 / 5.8	6.2 /6.7	91 / 92	95 / 94
Tyr	Low	6.8 / 7.2	7.8 / 7.9	87 / 88	90 / 91
	Medium	6.3 / 6.6	7.2 / 7.5	87 / 90	91 / 91
	High	5.6 / 5.8	6.5 / 7.0	89 / 92	90 / 93
B1	Low	6.4 / 6.6	7.4 / 7.7	89 / 90	90 / 91
	Medium	5.9 / 6.2	6.8 / 7.1	89 / 92	92 / 92
	High	5.2 / 5.7	6.1 / 6.3	91 / 92	94 / 94
CAC	Low	6.7 / 7.1	7.7 / 7.8	87 / 88	88 / 90
	Medium	6.2 / 6.5	7.1 / 7.5	87 / 89	92 / 91
	High	5.5 / 6.0	6.4 /7.0	89 / 91	92 / 92
AAA	Low	6.5 / 6.6	7.5 / 7.5	89 / 90	90 / 89
	Medium	6.0 / 6.2	6.9 /7.2	89 / 91	89 / 92
	High	5.3 / 5.5	6.2 / 6.5	91 / 92	91 / 93
B5	Low	6.8 / 7.1	7.8 / 8.2	87 / 87	90 / 90
	Medium	6.3 / 6.6	7.2 / 7.5	87 / 89	91 / 90
	High	5.6 / 6.0	6.5 / 6.8	89 / 92	91 / 93
GABA	Low	6.4 / 6.4	7.4 / 7.7	89 / 90	91 / 90
	Medium	5.9 / 6.1	6.8 / 7.2	89 / 92	90 / 89
	High	5.2 / 5.5	6.1 / 6.5	91 / 92	93 / 92
Orn	Low	7.9 / 8.3	9.2 / 9.5	86 / 88	87 / 89
	Medium	6.8 / 7.2	9.0 / 9.2	89 / 88	91 / 90
	High	6.5 / 6.9	7.7 / 8.5	91 / 88	90 / 89
Met	Low	6.9 / 7.2	7.9 / 8.3	86 / 89	90 / 92
	Medium	6.4 / 6.6	7.3 / 7.6	86 / 88	92 / 92
	High	5.7 / 6.0	6.6 / 6.7	88 / 91	94 / 93
GUO	Low	6.6 / 6.8	7.6 / 7.7	90 / 91	91 / 88
	Medium	6.1 / 6.5	7.0 / 7.3	90 / 92	93 / 91
	High	5.4 / 5.7	6.3 / 6.6	92 / 92	95 / 93
CRE	Low	8.0 / 8.2	9.3 / 9.5	86 / 89	88 / 88
	Medium	6.8 / 6.9	8.5 / 8.7	87 / 90	89 / 90
	High	6.5 / 6.7	7.8 / 8.0	91 / 90	92 / 93
Car	Low	6.8 / 7.2	7.8 / 8.3	86 / 88	91 / 91
	Medium	6.3 / 6.6	7.2 / 7.3	86 / 87	93 / 93
	High	5.6 / 6.0	6.5 / 6.6	88 / 89	95 / 93
B9	Low	6.7 / 7.0	7.7 / 8.0	90 / 91	91 / 90
	Medium	6.2 / 6.5	7.1 / 7.5	90 / 92	91 / 91
	High	5.5 / 5.9	6.4 / 6.4	92 / 93	90 / 90
Cit	Low	6.9 / 7.1	7.9 / 7.8	86 / 88	90 / 89
	Medium	6.4 / 6.5	7.3 / 7.5	87 / 89	92 / 88
	High	5.7 / 5.7	6.8 / 7.0	88 / 91	93 / 90
NAD+	Low	8.2 / 8.3	9.5 / 9.3	83 / 86	85 / 87
	Medium	7.3 / 7.6	9.2 / 9.4	85 / 87	88 / 90
	High	6.9 / 7.2	8.9 / 9.0	89 / 89	90 / 89
NADH	Low	8.0 / 8.0	9.1 / 9.2	84 / 85	86 / 89
	Medium	7.0 / 7.2	9.0 / 9.1	85 / 85	90 / 90
	High	6.4 / 6.6	8.0 / 8.3	87 / 88	90 / 93
IS1	\	4.6 / 4.8	4.9 / 5.2	92 / 94	90 / 94
IS2	\	4.8 / 4.8	5.1 / 5.2	90 / 93	92 / 94
IS3	\	4.6 / 4.9	4.7 / 4.9	89 / 93	90 / 92
IS4	\	4.4 / 4.5	4.7 /4.8	91 / 92	93 / 95

[a]With respect to each calibration curve: "Low" = close to the LOQ; "High" = close to the upper linearity limit (ULOQ); "Medium" = average of LOQ and ULOQ.
[b]Cell pellet extracts / culture medium samples
[c]$n = 6$.
[d]$n = 3$.

absolute recovery or sample clean-up. The weakly lipophilic C2 and hydrophilic SIL gave better results in terms of selectivity but were lackin in reproducibility, while M1 provided the best overall results and for this reason it was chosen for further extraction parameter optimisation.

All the main steps of the procedure (loading, washing and elution) were optimised. An aliquot of the sample can be drawn up and down through the MEPS syringe, at a suitable speed, once or several times

Table 3
Linearity and sensitivity data.

Analyte	IS	Linearity range (ng/sample[a] / ng/mL[b])	r^2	LOQ (ng/sample[a] / ng/mL[b])
Glu	IS1	1–500 / 0.5–500	0.9991 / 0.9989	1 / 0.5
Gln	IS1	5–5000 / 1–1000	0.9989 / 0.9990	5 / 1
Asp	IS1	0.5–500 / 0.5–500	0.9992 / 0.9993	0.5 / 0.5
Asn	IS1	0.1–100 / 0.1–100	0.9995 / 0.9993	0.1 / 0.1
Ala	IS2	1–500 / 1–500	0.9989 / 0.9987	1 / 1
2OG	IS3	1–500 / 0.5–500	0.9990 / 0.9988	1 / 0.5
SUC	IS1	1–1000 / 1–1000	0.9988 / 0.9989	1 / 1
FUM	IS4	0.5–500 / 0.5–500	0.9993 / 0.9995	0.5 / 0.5
MAL	IS2	0.5–500 / 0.5–500	0.9991 / 0.9992	0.5 / 0.5
CIT	IS2	0.1–100 / 0.1–100	0.9994 / 0.9992	0.1 / 0.1
PYR	IS3	1–500 / 5–5000	0.9989 / 0.9986	1 / 5
LAC	IS3	0.3–100 / 0.5–500	0.9991 / 0.9993	0.3 / 0.5
OXA	IS3	1–1000 / 1–1000	0.9988 / 0.9990	1 / 1
NAA	IS2	0.3–100 / 0.5–500	0.9990 / 0.9988	0.3 / 0.5
ATP	IS1	10–5000 / 1–1000	0.9985 / 0.9987	10 / 1
ADP	IS1	0.2–100 / 0.2–100	0.9990 / 0.9991	0.2 / 0.2
AMP	IS1	0.1–100 / 0.1–100	0.9988 / 0.9988	0.1 / 0.1
CTP	IS1	1–500 / 0.1–100	0.9989 / 0.9990	1 / 0.1
GTP	IS1	1–500 / 0.1–100	0.9988 / 0.9991	1 / 0.1
GSH	IS1	1–500 / 0.1–100	0.9988 / 0.9989	1 / 0.1
GSSG	IS1	0.5–500 / 0.5–500	0.9986 / 0.9989	0.5 / 0.5
KIC	IS4	1–500 / 0.5–500	0.9992 / 0.9992	1 / 0.5
KIV	IS4	1–500 / 0.5–500	0.9993 / 0.9995	1 / 0.5
Leu	IS2	1–500 / 0.5–500	0.9989 / 0.9990	1 / 0.5
Ile	IS2	1–500 / 0.5–500	0.9988 / 0.9990	1 / 0.5
Val	IS2	1–500 / 0.5–500	0.9990 / 0.9992	1 / 0.5
Tyr	IS1	1–500 / 1–1000	0.9992 / 0.9994	1 / 1
B1	IS4	1–500 / 5–5000	0.9994 / 0.9992	1 / 5
CAC	IS3	1–500 / 0.5–500	0.9990 / 0.9992	1 / 0.5
AAA	IS1	0.5–500 / 0.5–500	0.9988 / 0.9990	0.5 / 0.5
B5	IS2	0.3–100 / 0.5–500	0.9995 / 0.9995	0.3 / 0.5
GABA	IS1	0.1–100 / 0.1–100	0.9988 / 0.9989	0.1 / 0.1
Orn	IS1	10–5000 / 10–5000	0.9986 / 0.9988	10 / 10
Met	IS2	1–500 / 1–1000	0.9992 / 0.9994	1 / 1
GUO	IS2	1–500 / 1–1000	0.9990 / 0.9992	1 / 1
CRE	IS3	5–5000 / 5–5000	0.9987 / 0.9988	5 / 5
Car	IS3	1–500 / 1–1000	0.9990 / 0.9989	1 / 1
B9	IS2	0.5–500 / 1–1000	0.9994 / 0.9992	0.5 / 1
Cit	IS1	0.1–100 / 0.1–100	0.9988 / 0.9990	0.1 / 0.1
NAD+	IS1	10–5000 / 10–5000	0.9985 / 0.9988	10 / 10
NADH	IS1	10–5000 / 10–5000	0.9985 / 0.9987	10 / 10

[a] cell pellet extracts
[b] culture medium samples

(cycles). Thus, in the loading step, the number of cycles and their speed are two of the parameters which affect analyte retention. Different cycles and speeds were tested: retention was satisfactory after 10 draw/discharge cycles of the loading mixtures at a speed of 10 µL/s (mean absolute recovery: <47% with one cycle, <73% with 5 cycles). Good clean-up without excessive analyte losses was obtained by washing the sorbent with 150 µL of water followed by 150 µL of water/MeOH 95:5 (V/V) mixture; an elution step consisting in five cycles with 100 µL of pure MeOH (for a total of 500 µL) proved sufficient for the complete elution (>92%) of all the target analytes (mean absolute recovery: <57% with 2 × 100 µL, <80% with 3 × 100 µL). After each extraction, a sorbent cleaning step was carried out with 200 µL of H_2O, followed by 200 µL of 0.1% FA in H_2O and 200 µL of MeOH. This step avoided carryover effects, but also acted as the conditioning step for the following extraction. The eluate was then evaporated to dryness with a centrifugal vacuum concentrator, the residue was redissolved in 50 µL of a 50:50 (V/V) mixture of ACN and water containing 0.25% FA. Using this MEPS procedure, good absolute recoveries of the target analytes and ISs were reached, while obtaining satisfactory sample clean-up.

3.3. MEPS-LC-MS/MS method validation for targeted biomarker assessment

3.3.1. Absolute recovery, precision, matrix effect and carryover

The absolute recovery of the target analytes by applying the optimised semi-automated MEPS extraction protocol was determined on cell pellet extracts and fresh culture medium fortified at three concentration levels, representative of the calibration curves for each analyte, by comparing pre-extraction and post-extraction fortified samples and expressed as a percentage. High absolute recoveries (> 86%) were obtained for all the analytes (>90% for the ISs) in combination with good reproducibility taking into account three concentrations, always obtaining RSD values lower than 9.8% (<7.9% for the ISs) (Table 2). The standard addition method used to fortify both cell pellet extracts and culture medium considers matrix effects by relying on the assumption that all concentration levels (both endogenous and fortified) are subjected to a proportional ion enhancement or suppression effect because each sample (calibration and QC samples) contains the same amount of co-eluting matrix compounds. For all target analytes, the matrix effect was in the 86–103% range, as reported in Table 2. No carryover was observed for either kind of sample, as no signal higher than the background noise at the retention times and m/z of the analytes was observed when injecting a blank solvent after analysing a sample fortified with the highest concentration of the respective calibration curves (n = 3).

3.3.2. Selectivity, linearity and sensitivity

The developed method was deemed to be selective since chromatographic peaks of all the target analytes were satisfactorily resolved and unambiguously identified by exclusive MRM transitions, and isomeric compounds sharing the same molecular weight were discriminated by

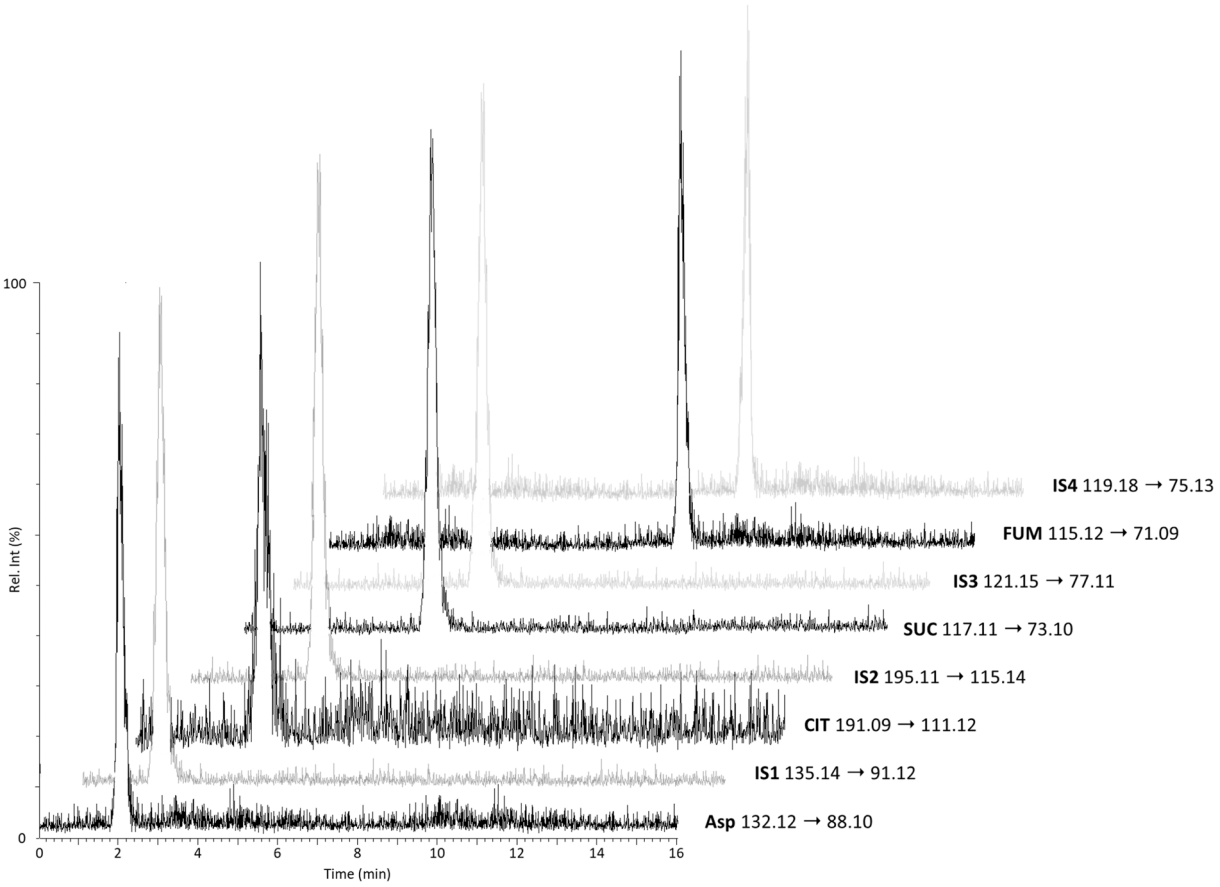

Fig. 2. LC-MS/MS chromatogram of an oligodendrocyte precursor cell pellet lysate sample subjected to the developed MEPS pretreatment protocol. The figure shows a selection of four representative target analyte MRM channels: Asp (27.3 ng/sample), CIT (1.1 ng/sample), SUC (161.6 ng/sample) and FUM (20.9 ng/sample), together with IS MRM channels.

exclusive product ions and/or baseline chromatographic peak separation, thanks to a finely tuned composition gradient in terms of timing and ramping. Moreover, no interfering (i.e., partially overlapping) signal higher than the LOD was detected in any MRM sample chromatogram. Method sensitivity was between 0.4 and 10.0 ng/sample (0.3 and 6.5 ng/million cells) for cell pellet samples and between 0.2 and 5.0 ng/mL for culture medium samples. Method linearity was satisfactory, with correlation coefficients (r^2) always higher than 0.9984 (see Table 3). Linearity assays (carried out in triplicate for each concentration) were assessed on both types of samples, fortified by the standard addition method. Experimental LOQ (and thus linearity range) values assessed by the standard addition method were inherently proportional to the baseline analyte levels observed in the samples used for method development and qualification. As an example, the theoretical LOQ of some of the target analytes would have been way much lower (in the sub-ng/mL range), but LOQ was calculated as the lowest concentration of analyte added to the samples that can be reliably quantified with acceptable accuracy (± 20%) and precision (%RSD < 20%). In addition, stressing method sensitivity for analytes expected at high concentrations would have been outside the scope of the present work.

3.3.3. Stability

In targeted biomarker assessment studies, analyte stability is of the utmost importance in order to assure that observed differences between samples are due to variations in biochemical pathways and not to analyte degradation. In this study, short-term stability as well as benchtop and autosampler stability were tested in cell pellets and culture medium. Stability was assessed in cell pellets stored at − 80 °C and culture medium stored at − 20 °C. The target analytes were stable for at least 14 days (analyte variation < 15%).

The same samples were stable under benchtop and autosampler conditions (RT) for 48 h (analyte variation < 15%).

3.4. Proof-of-concept application to brain-derived cell cultures and accuracy

The developed MEPS-LC-MS/MS methodology was applied to the analysis of the 41 target analytes in cultures of immortalised mouse oligodendrocytes precursor cells (Oli-neu cells). The typical LC-MS/MS chromatograms of a cell pellet sample fortified with the ISs and subjected to cell lysis and MEPS pretreatment are shown in Fig. 2. Concentrations of the target analytes in different cell pellet and culture medium from different oligodendrocytes precursor cells cultures are reported in Table 4 and Fig. 3. Accuracy assays were also performed on real samples, analysing additional replicates after spiking with different analyte concentrations. Very good accuracy was obtained, with absolute recovery values always in the 91–109% range.

4. Discussion

In this study, a novel LC-MS/MS method was developed and optimised for the quantitative analysis of metabolites that play a role in important biochemical processes, where their combined activities or modulation can impact the overall metabolism of a specific cell type. A total of forty-one potential biomarkers were examined for their involvement as enzymatic substrates or cofactors in pathways such as the tricarboxylic acid (TCA) cycle, malate-aspartate NADH shuttle (MAS), oxidative phosphorylation (OXPHOS), amino acid, lipid, or

Table 4
Results from MEPS-LC-MS/MS analysis on real samples.

Analyte	Sample 1		Sample 2		Sample 3	
	Cell pellet concentration[a]	Culture medium concentration[b]	Cell pellet concentration[a]	Culture medium concentration[b]	Cell pellet concentration[a]	Culture medium concentration[b]
Glu	36.4	7.2	26.8	4.3	40.7	3.9
Gln	512.2	80.3	497.8	64.9	480.1	75.2
Asp	27.3	21.9	20.0	16.3	18.1	16.8
Asn	2.9	2.4	6.0	0.8	3.5	1.3
Ala	100.2	57.5	110.1	67.8	112.7	66.3
2OG	126.8	20.2	102.4	31.7	117.9	23.0
SUC	161.6	56.4	140.5	59.2	149.8	44.0
FUM	20.9	15.7	18.7	23.2	30.1	15.3
MAL	15.5	9.3	11.0	4.9	10.2	6.3
CIT	1.1	2.8	0.8	3.1	1.7	1.2
PYR	75.8	98.6	91.0	119.7	83.4	91.2
LAC	14.0	12.1	13.7	14.3	10.2	18.4
OXA	131.7	32.0	111.6	21.4	138.2	23.1
NAA	7.1	5.8	11.5	4.3	8.2	2.4
ATP	883.0	27.2	834.7	33.4	840.4	17.7
ADP	7.4	5.2	8.6	2.4	12.9	3.3
AMP	3.2	2.9	5.7	2.0	3.3	2.1
CTP	59.6	2.3	52.3	3.8	48.5	3.1
GTP	98.1	2.4	83.8	1.2	96.5	0.9
GSH	43.2	3.9	54.6	4.1	47.8	5.4
GSSG	24.0	7.2	26.6	4.3	23.2	3.9
KIC	39.8	5.4	44.6	6.2	33.1	10.4
KIV	27.2	58.0	32.6	45.4	31.3	55.3
Leu	78.5	33.2	71.4	44.7	79.9	34.8
Ile	89.7	43.0	80.4	42.2	83.7	47.9
Val	72.1	109.6	79.2	140.8	64.0	120.1
Tyr	68.5	79.4	72.2	97.7	89.7	81.4
B1	57.3	782.9	70.6	667.9	82.0	859.3
CAC	40.7	21.2	58.0	36.1	49.7	21.6
AAA	15.8	5.4	9.6	6.2	15.9	8.1
B5	11.0	8.1	17.6	9.8	12.4	6.6
GABA	5.1	0.3	3.3	0.1	4.6	0.2
Orn	348.6	293.3	431.5	210.4	390.8	220.6
Met	25.0	74.3	21.7	83.5	28.6	106.8
GUO	29.3	41.6	28.4	59.7	20.0	56.1
CRE	268.2	256.0	292.7	236.9	232.2	216.5
Car	74.3	140.3	63.2	134.6	7.6	161.0
B9	20.9	54.5	16.2	39.0	26.4	39.5
Cit	7.3	7.9	4.1	6.0	2.4	8.7
NAD+	176.0	78.2	167.6	89.5	140.9	77.0
NADH	514.3	190.6	601.6	208.0	552.5	185.4

[a] ng/sample
[b] ng/mL

nucleotide metabolism, as well as oxidative stress response. The quantification of the variations in their intracellular levels or release hold the potential to define the cell physiology or provide insights into the mechanisms underlying metabolic reprogramming in pathological conditions.

In a model of oligodendroglial precursor cell, i.e., Oli-neu cells, a large panel of target biomarkers was simultaneously determined for the first time in cell pellet and culture media samples pretreated by means of an original, miniaturised, and semi-automated MEPS protocol. The extraction and clean-up of such biomarkers was optimised and finally carried out on 200 µL of either cell lysate or culture medium, exploiting an eVol electronical syringe for MEPS, allowing the partial automation of the whole MEPS protocol. The electronic syringe combined with the M1 MEPS BIN assembly was programmed to dispense precise volumes of liquid, allowing to reduce sample handling and enhance method sustainability in terms of sample, solvents, and energy consumption while enabling rapid method development and increasing sample processing throughput.

The method was fully validated according to international guidelines and produced results that were within currently prescribed specifications for all tested parameters (absolute recovery, precision, matrix effect and carryover, selectivity, linearity, sensitivity, stability). In fact, satisfactory recovery values (>86%) were obtained for all analytes after the application of the feasible and effective extraction procedure; correlation coefficient values for the calibration curves were > 0.9984 for all target compounds and LOQ values ranged from 0.4 to 10.0 ng/sample for cell pellets and between 0.2 and 5.0 ng/mL for culture medium. The intra- and inter-day precision values in terms of RSD were $< 9.8\%$. Moreover, the developed method was successfully applied to the analysis of potential biomarkers in murine immortalised oligodendrocytes precursor cell samples, namely cell pellets and culture media, and all the expected compounds were detectable in both types of samples.

To the best of our knowledge, there are no other reported data on the simultaneous quali-quantitative assessment of a large biomarker panel in small-volume samples represented by both cell pellet and culture media pretreated by a miniaturised and semi-automated MEPS pretreatment coupled to LC-MS/MS.

Exploiting this feasible, yet reliable high-throughput analytical approach, the key intermediates or products of several metabolic pathways can be quantified with unambiguous sensitivity and accuracy. As a result, the absolute determination of the levels of these potential biomarkers according to our procedure can give more detailed information about the efficiency and the relative biochemical interactions of vital cellular processes. For example, it can offer insights into the balance between mitochondrial respiration and glycolysis, as well as the cell's

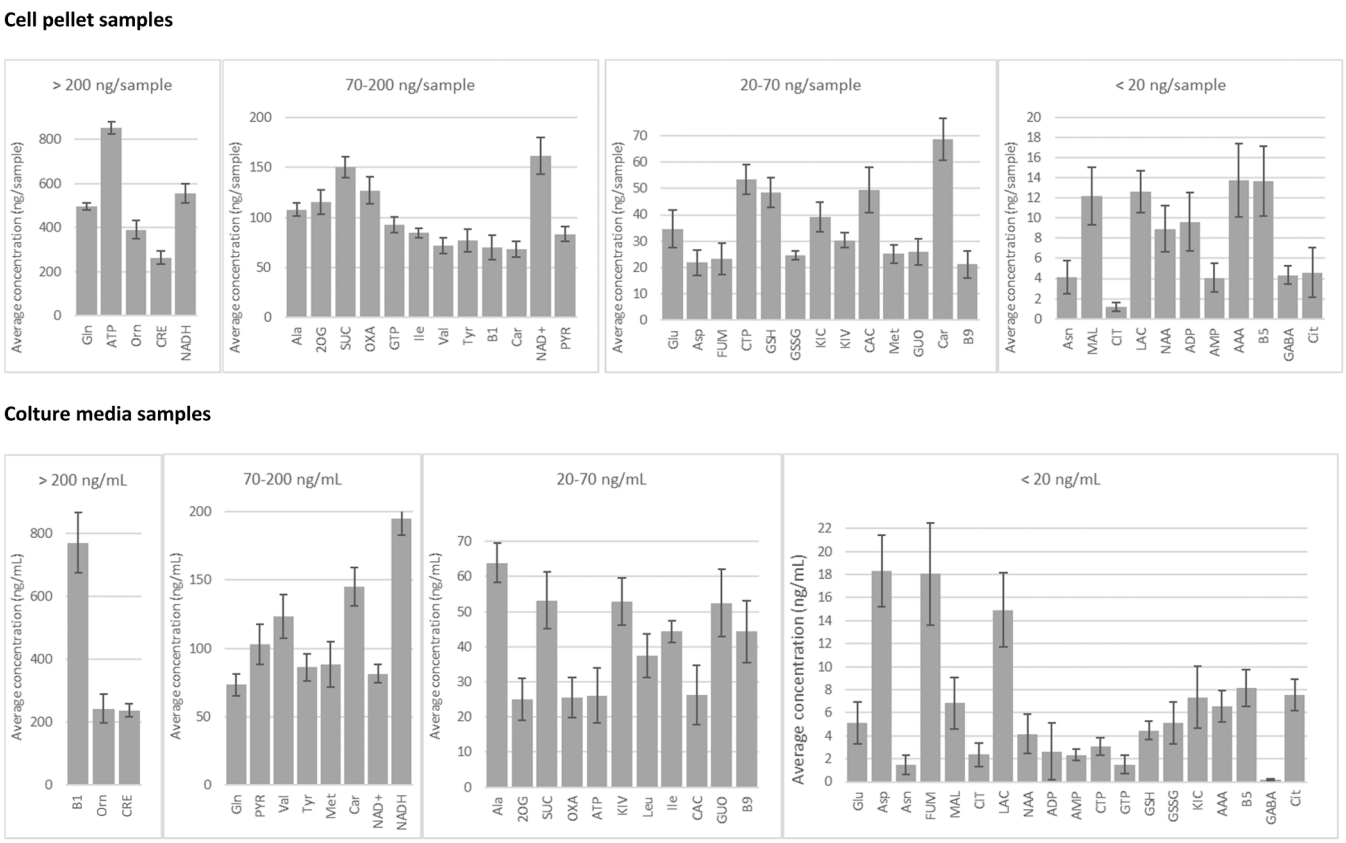

Fig. 3. Overview of the results from MEPS-LC-MS/MS analysis on real samples.

ability to utilise specific nutrients for growth and proliferation, or better shed light on the metabolism of important biological molecules like lipids, amino acids, and nucleotides, thereby defining physio-pathological mechanisms in various cell models.

Such results demonstrate great possibilities for the developed LC-MS/MS method coupled to semi-automated MEPS pretreatment in biomarker and targeted metabolomics studies, to be applied in different research frameworks where a reliable absolute measurement of a wide array of metabolites and biomarkers may hold great potential to elucidate physiological and pathological mechanisms.

5. Conclusions

After extensive fine-tuning and optimisation of the sample pretreatment and LC-MS/MS analysis conditions, an original analytical workflow was validated for the determination of 41 different potential biomarkers in cell pellets and cell culture media. Very satisfactory results were obtained from validation, with all tested parameters (absolute recovery, precision, matrix effect and carryover, selectivity, linearity, sensitivity, stability) within international guideline specifications. The method was then applied as a proof of concept to a model of oligodendroglial precursor cells, obtaining reproducible and reliable results in all cases, with all expected analytes detected and quantified in all samples and in both matrices.

This analytical workflow based on semi-automated MEPS coupled to LC-MS/MS can thus be applied in targeted metabolomic studies to elucidate physiological and pathological mechanisms.

Further studies are in progress to extend the range of detectable and quantifiable analytes and to assess applicability to different cell populations and different culture media.

Author Statement

All authors have seen and approved the final version of the paper being submitted. They warrant that the paper is the original work of the authors, has not received prior publication and is not under consideration for publication elsewhere.

Declaration of Competing Interest

The authors declare no conflict of interest.

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