Journal of Chromatography B 1222 (2023) 123719

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Structural elucidation of 3-nitrophenylhydrazine derivatives of tricarboxylic acid cycle acids and optimization of their fragmentation to boost sensitivity in liquid chromatography-mass spectrometry

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ARTICLE INFO

Keywords:
Carboxylic acid
Derivatization
Liquid chromatography-mass spectrometry
3-nitrophenylhydrazine

ABSTRACT

Carboxylic acids participate in many metabolic pathways including tricarboxylic acid (TCA) cycle. Therefore, there have been ongoing attempts to develop sensitive liquid chromatography-mass spectrometry methods over the last decades. Derivatization of the carboxylic acids with 3-nitrophenylhydrazine presents a well-established methodology, and yet the derivatized species of polycarboxylic acids and their fragmentation in collision-induced dissociation have not been fully studied before. In our study, we elucidated how annotation of most abundant 3-nitrophenylhydrazine derivatives and optimization of their fragmentation in multiple reaction monitoring can boost the sensitivity, especially for polycarboxylic acids. Finally, the optimized liquid chromatography-tandem mass spectrometry method allowed for low detection limits ranging from 10 pM for 2-oxoglutaric acid to 800 pM for pyruvic acid. All TCA carboxylates were quantified in 20 μL of human plasma and the targeted method was validated in the same matrix. The same methodology with a modified gradient elution was also applied to untargeted screening of fatty acids by using high-resolution mass spectrometry enabling identification of 29 medium- to long-chain fatty acids in human plasma. The TCA carboxylates were also quantified in 10^5 of C2C12 mouse myuotube cells grown under different treatments to proof applicability of the methodology to biological studies in a wider sense. However, unfortunately all the TCA carboxylates were also found in the derivatized blanks in substantial amounts, which prevents from using the methodology for quantification of the carboxylates in less than 10^5 cells.

1. Introduction

Central carbon metabolism (CCM) has been the most studied metabolic pathway in many biological systems with carboxylic acids being one of the most important compounds classes. However, carboxylic acids have always presented a class of polar ionic compounds complicated to analyze, especially with combined liquid chromatography-mass spectrometry (LC-MS) even with the available technology today. Before LC-MS was developed, gas chromatography-mass spectrometry (GC-MS) had been the mostly utilized methodology for analyzing the carboxylic acids in various types of samples, such as water [1], feces [2] or blood [3]. A drawback is that in some applications GC-MS showss low sensitivity and long analysis times [4–6]. Therefore, there have been constant efforts to develop LC-MS/MS methods for quantification of TCA carboxylates; the nature of LC-MS allows avoiding the derivatization and thus accelerates the total analysis time. However, the methods without previous derivatization of carboxylates usually lack sufficient separation and sensitivity thus requiring relatively large amounts of biological material for analysis [7–9]. To reach improved separation and peak symmetry, many ion-paring reagents have been previously used, for example tributylamine [10,11]. On one hand, the ion-pairing additives facilitate separation and improve peak shapes, on the other hand they participate in ionization suppression in electrospray ionization (ESI) and contribute to long-lasting contamination of the LC-MS

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https://doi.org/10.1016/j.jchromb.2023.123719
Received 22 February 2023; Received in revised form 6 April 2023; Accepted 9 April 2023
Available online 11 April 2023

equipment [12].

Still, with the LC-MS instrumentation available today, sensitive quantification of small organic acids remains problematic. However, the sensitivity of quantitation can be enhanced through derivatization of the carboxylic groups with reagents that have originally been used for detection of small organic acids with UV–vis detector such as *o*-benzylhydroxylamine (*O*-BHA) [13], 2-picolyl amine (2-PA) [14], 2,4-dinitrophenylhydrazine (DNPH) [15] and 3-nitrophenylhydrazine (3-NPH) [16]. Substitution of the carboxylic group with a bulky moiety containing the phenyl ring blocks the unwanted ionic interactions of the carboxylic groups with silanol groups in stationary phase and at the same time decreases polarity of the whole molecules thus facilitating separation in reversed-phase liquid chromatography (RPLC), and ionization in electrospray ionization (ESI). 3-NPH derivatization has been utilized in analysis of not only small carboxylic acids [16] including short-chain fatty acids [17,18] but also other important metabolites containing carboxylic, ketone and/or aldehyde groups such as amino acids, sugars and nucleotides [19]. Derivatization of carboxylic groups with 3-NPH yields 3-nitrophenylhydrazides of the respective carboxylic acids. However, aldehydes and ketones combine with 3-NPH to form 3-nitrophenylhydrazones with C═N double bonds, which results in the presence of *cis/trans* isomers. Therefore, LC separation of 3-NPH derivatives of some oxo acids then leads to separation of the isomers resulting in the multiple peaks in the chromatograms and thus accurate quantification requires using isotopically labelled standards. Derivatization of polycarboxylic acids with 3-NPH also yields various derivatized species depending on how many carboxylic groups are derivatized.

In this study, we developed a method for the determination of TCA cycle carboxylic acids using the reversed-phase LC-MS/MS technique and 3-NPH as a derivatization reagent. Derivatization of TCA cycle carboxylates was miniaturized and separation of the derivatives was optimized using HSS T3 and BEH C18 columns. Moreover, derivatized species were studied with high-resolution mass spectrometry (HRMS) to obtain the highest sensitivity possible in the final LC-MS/MS arrangement. The optimized method enabled accurate quantification of all TCA carboxylates except for isocitrate, oxaloacetate and *cis*-aconitate because of unavailability of ^{13}C labelled internal standards in 20 μL of human plasma and 10^5 of C2C12 mouse myotube cells. The same derivatization methodology was applied to the untargeted analysis of human plasma samples, where 29 carboxylic acids were identified by using LC-HRMS and in-house library.

2. Materials and methods

2.1. Chemicals and reagents

Analytical reagent-grade 3-nitrophenylhydrazine hydrochloride (98 %), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC, 97 %), 2,6-di-*tert*-butyl-4-methylphenol (BHT), LC–MS grade acetonitrile and formic acid were purchased from Sigma–Aldrich (MO, USA). Methanol was purchased from Honeywell (Charlotte, NC, USA). ESI-L Low Concentration Tune Mix was purchased from Agilent Technologies (Santa Clara, CA, USA). Deionized water was supplied by MilliQ device from Merck-Millipore (MA, USA). Analytical standards of D,L-isocitric acid (97 %), citric acid (97 %), oxaloacetic acid (97 %), D,L-malic acid (97 %), pyruvic acid (98 %), 2-oxoglutaric acid (98.5 %), fumaric acid (99 %), *cis*-aconitic acid (98 %), succinic acid (99.5 %), L-lactic acid (98 %), succinic acid-D_4 (98 %), pyruvic acid-$^{13}C_3$ (95 %) were purchased from Sigma-Aldrich (MO, USA). Citric acid-D_4 (98 %), 2-oxoglutaric acid-$^{13}C_4$ (97 %), fumaric acid-$^{13}C_4$ (98 %), L-malic acid-$^{13}C_4$ (98 %), L-lactic acid-$^{13}C_3$ (98 %, 20 % w/w in H_2O) were purchased from Cambridge Isotope Laboratories (MA, USA). Pyridine of analytical grade (98–100 %) was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. LC-MS conditions

Quantification of 3-NPH-derivatives of carboxylic acids was achieved on an LC-MS/MS system consisted of an Agilent 1290 UHPLC connected to an Agilent 6490 triple quadrupole (Agilent, CA, USA). The separation was achieved by injecting 5 μL of a sample to an Acquity UPLC HSS-T3 column (100 × 2.1 mm, 1.8 μm, Waters, MA, USA). The mobile phase was delivered on the column by a flow rate of 0.35 mL/min with the following gradient: 0 min (5 % B), 12 min (100 % B), 13 min (100 % B), 14 min (5 % B), 17 min (5 % B). Column and autosampler were thermostated at 30 °C and 4 °C, respectively. Analytes were ionized in an electrospray ion source operated in the negative mode. The source and gas parameters were set as follows: ion spray voltage −3.5 kV, gas temperature 150 °C, drying gas flow 11 L/min, nebulizer pressure 30 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min, fragmentor 380 V. The instrument was operated in dynamic multiple reaction monitoring mode (MRM), and the MRM transitions of derivatized carboxylic acids were optimized by using Agilent MassHunter Optimizer.

The optimized method was compared to a method from the literature [16] using Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm, Waters, MA, USA) at 40 °C and a flow rate of 0.3 mL/min with mobile phase composed of 0.01 % formic acid in water (A) and 0.01 % formic acid in methanol (B) at the following gradient: 0.0 min (18 % B), 9.0 min (90 % B), 9.1 min (100 % B), 10.1 min (100 % B), 10.5 min (18 % B), 13.5 min (18 % B). The data were acquired and processed with Agilent MassHunter software (Santa Clara, USA).

Structures of 3-NPH-derivatives and their most abundant precursor ions were elucidated by an Agilent 1290 UHPLC connected to an Agilent 6546 Q-TOF mass spectrometer. Separation column, mobile phase composition, and gradient were identical to the ones used in quantification of 3-NPH-derivatives with triple quadrupole mass spectrometer. Derivatized carboxylates were ionized in a dual AJS ESI source operated in the negative ionization mode and full scan spectra were collected in *m/z* range from 100 to 1700 at a rate of 4 spectra per second. Source and gas parameters of the Q-TOF mass spectrometer were as follows: gas temperature 150 °C, drying gas flow 5 L/min, nebulizer pressure 20 psi, sheath gas temperature 360 °C, sheet gas flow 12, Vcap 4000 V, nozzle voltage 2000 V, fragmentor 400 V and OctapoleRFPeak 750 V.

Untargeted screening of carboxylic acids in plasma was conducted by using Ultrahigh Performance Liquid Chromatography (UHPLC) system (Agilent 1290 Infinity II) connected to a Bruker timsTOF Pro™ instrument (Bruker, Bremen, Germany). Ions were generated in the negative electrospray ionization (ESI) mode. The samples were randomized and analyzed using reversed-phase ACQUITY UPLC HSS T3 Column, 100 Å, 1.8 μm, 2.1 mm × 100 mm (Waters, Milford, MA). The column and autosampler temperatures were maintained at 40 °C and 10 °C, respectively. Solvent A consisting of 0.1 % formic acid in water and solvent B consisting of 0.1 % formic acid in acetonitrile and propanol (3:1, *v/v*), were used as mobile phases. The injection volume and flow rate were 2 μL and 0.4 mL/min, respectively. The UPLC gradient was programmed as follows: a linear gradient from 0.0 min (3 % B), 9.0 min (100 % B), 14.0 min (100 % B), 14.5 min (3 % B), 17.0 min (3 % B). The ESI source used 10 L/min of drying gas at a temperature of 220 °C. The ESI was set at a 3600 V capillary voltage, and 2.2 bar nebulizer pressure. Detection of the mass/charge ratio (*m/z*) of ions was set from 50 to 1000. Full-scan MS spectra acquisition rates were set to 4 Hz, and the resolution was approximately 60,000. For mass calibration, 50 μL of internal calibrant of 10 mM Na-formate was injected at the beginning of each analysis. Data acquisition was performed with otofControl version 6.0 and Bruker Compass HyStar version 5.0 (Bruker Daltonics, Bremen, Germany) and data processing was performed with Bruker Compass DataAnalysis version 5.2 software.

2.3. Sample preparation

Human plasma was obtained from Biobanken norr (Laboratory Medicine within Region Västerbotten). The informed consent from all human subjects was obtained before submitting their samples into the Biobank. All experiments were performed in accordance with relevant guidelines and regulations. The plasma samples consisted of a pooled plasma from adult male and female donors.

Plasma samples for targeted analysis were prepared as follows: 20 μL of plasma were extracted with 180 μL of methanol containing isotopically labelled internal standards by shaking a sample with a tungsten carbide bead at 30 kHz for 2 min. Proteins were precipitated at −20 °C for 2 h. After precipitation, extracts were centrifuged at 14,000 rpm at 4 °C for 10 min and the supernatant containing polar metabolites was collected in LC vials. Samples were evaporated in a centrifugal concentrator (Thermo Fisher Scientific, Waltham, USA) at room temperature and re-dissolved in 20 μL of 50 % MeOH. Eventually, samples were derivatized and 5 μL were injected.

Plasma samples for untargeted analysis were prepared as follows: 50 μL of plasma were extracted with 200 μL of methanol. Samples were vortexed and proteins were precipitated on ice for 30 min. After precipitation, extracts were centrifuged at 14,000 rpm at 4 °C for 15 min. The supernatant containing the polar metabolites was collected in LC vials. Samples were evaporated with a gentle stream of nitrogen to dryness using a Zipvap 48 position evaporator heater (Terre Haute, IN, USA) at room temperature and re-dissolved with 20 μL of 50 % MeOH. Eventually, samples were derivatized and 2 μL were injected.

C2C12 myoblasts (ATCC, Alexandria, MN, USA) were grown in low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Waltham, USA) supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin/streptomycin, and 2 mM L-glutamine (all from Thermo Fisher Scientific, Waltham, USA) and kept at a temperature of 37 °C and with an atmosphere of 5 % CO_2.

For experiments, myoblasts were seeded at 90–100 % confluency and differentiated into myotubes using low glucose DMEM supplemented with 2 % fetal bovine serum (FBS), 1 % penicillin/streptomycin, and 2 mM L-glutamine, whereby media was changed every second day for 5 days.

Oleic acid (Sigma-Aldrich, MO, USA) was prepared by dissolving in 100 % DMSO (Sigma-Aldrich, MO, USA) at a concentration of 400 mM. The oleic acid was then further diluted in 10 % BSA, DMEM to a working concentration of 400 μM. The myotubes not treated with oleic acid were incubated with 10 % BSA DMEM containing 0.1 % DMSO.

For treatments with the AMPK activator MK-8722, these were administered at the same time as oleate. MK-8722 (Glixx Laboratories, Hopkinton, MA, USA) was dissolved in DMSO (Sigma) at a 30 mM concentration and added to the culture media at a concentration of 10 uM. Myotubes not treated with MK-8722 were treated with 0.033 % DMSO as a control.

Treatments with oleic acid and MK-8722 were initiated at the beginning of day 6 of myotube differentiation and lasted for 24 h. At the end of the treatment the culture media was aspirated, myotubes were washed in ice-cold 1x PBS (phosphate buffered saline) and then incubated in 0.05 % Trypsin-EDTA (Fisher Scientific, Pittsburgh, USA) for 3 min at 37 °C, after which the reaction was stopped by the addition of DMEM with 10 % FBS. The myotubes were collected in a pellet by centrifuging at 200g for 5 min, 4 °C, the supernatant was aspirated and the myotubes were re-suspended in 1 mL of ice-cold 1x PBS. The cells were again centrifuged and re-suspended in 1x PBS, the supernatant aspirated and this re-suspension/centrifugation step was repeated one further time. After removing the supernatant, the cell pellets were flash frozen on liquid nitrogen and stored at −80 °C until the extraction of metabolites.

The cells were then extracted with 300 μL of 90 % methanol containing 3.33 μM isotopically labelled internal standards as follows: the samples were shaken with a tungsten carbide bead at 30 kHz for 3 min and centrifuged at 14,000 rpm at 4 °C for 10 min and the supernatant was collected in LC vials. The extracts were evaporated in a centrifugal concentrator at room temperature and re-dissolved in 20 μL of 50 % MeOH. Eventually, samples were derivatized and 5 μL were injected.

2.4. Derivatization

Dried extracts from the samples were reconstituted in 20 μL of 50 % methanol and derivatized according to a published method [16] with modifications, briefly: 20 μL of 120 mM EDC (dissolved in 6 % pyridine in 50 % methanol) and 20 μL of 200 mM 3-NPH (dissolved in 50 % methanol) were consecutively added to 20 μL of a reconstituted sample. The sample was incubated at room temperature (21 °C) for 60 min, afterwards 40 μL of 0.05 mg/mL BHT (dissolved in pure methanol) were added to the samples and vortexed. In previous experiments, different quenching alternatives were used to test the stability of carboxylic acid derivatives, for example dilution with 0.1 % formic acid in water. However, when the reaction was stopped by only diluting the samples with formic acid in water, the derivative of 2-oxoglutaric acid degraded significantly in the autosampler at 4 °C. On the other hand, the addition of 0.05 mg/mL BHT proved to be the optimal approach for the stability of 3-NPH derivatives. Eventually, 5 μL of a sample were injected on column for a targeted LC-MS/MS analysis and 2 μL were injected on column for untargeted LC-HRMS analysis.

2.5. Preparation of standard solutions

For calibration and optimization purposes, the analytical standards of carboxylic acids were separately dissolved in deionized water to form 1 mg/mL solutions and kept at −20 °C. Afterwards, the stock solutions were diluted to the desired concentration with 50 % methanol and derivatized with 3-NPH.

2.6. Method validation

The method for quantification of derivatized acids on HSS-T3 column was validated through evaluation of parameters such as limit of detection (LOD), limit of quantification (LOQ), carry-over, linearity, matrix effects, accuracy, precision, recovery, repeatability and stability.

Sensitivity of the method was assessed and the LOD values for all carboxylic acids were determined as a concentration corresponding to a signal-to-noise ratio (S/N) of 3 and the LOQ values were determined as the lowest concentration that was quantified with acceptable accuracy (100 ± 20 %). The LOD values for some carboxylic acids (malic acid, citric acid, pyruvic acid, succinic acid, lactic acid) were determined using their isotopically labelled standards because of the presence of these acids in derivatized blank solution. Internal calibration was constructed by using isotopically labelled standards for most carboxylic acids.

Carry-over was assessed in a blank solvent injected on column after repeated analyses ($n = 3$) of the calibration solution of the highest concentration.

Linearity of the method was evaluated through 16-point ($n = 3$) linear regression model yielding the calibration curves constructed by using isotopically labelled standards for most carboxylic acids. Due to the lack of isotopically labelled standards, citric acid-D_4 and 2-oxoglutaric acid-$^{13}C_4$ were used for calibration and quantification of isocitric, and *cis*-aconitic acid and oxaloacetic acid, respectively.

Matrix effects were assessed by using isotopically labelled standards, which were spiked to the samples. To evaluate matrix effects in plasma, the samples were spiked to reach the concentration of 10 μM for internal standards. Peak areas of internal standards spiked to samples ($n = 3$) were compared with the peak areas of pure mixture of internal standards according to the following equation:

$$ME = \frac{IS - S}{IS} \times 100$$

Where *ME* represents matrix effect (%), *IS* is peak area of an internal standard in neat solvent, *S* is peak area of an internal standard spiked into a processed sample.

Accuracy of quantitation was evaluated through analysis of a real sample and the same sample spiked with standard solution at two concentration levels (1 μM and 10 μM standard addition); eventually, concentration in an original sample was subtracted from the total concentration in a spiked sample and accuracy was calculated as [(mean observed concentration)/(spiked concentration)] × 100 %. Accuracy in C2C12 myotube cell extracts was evaluated only at one concentration level (10 μM).

Precision was calculated as RSD of quantitation for 6 repeated measurements of a real sample.

As no surrogate matrix was available, *recoveries* of carboxylates were determined by comparison of the isotopically labelled internal standard peak areas in a processed blank with a fresh blank containing the same concentration of internal standards (1 μM, $n = 6$). Recovery was calculated as [(peak area in processed blank)/(peak area in fresh blank)] × 100 %.

Repeatability in peak areas was determined as RSD of repeated measurements of one of the calibration solutions (1 μM, $n = 6$).

Stability of the 3-NPH derivatized species was assessed at 4 °C and −20 °C after 7 days of storage.

3. Results and discussion

3.1. Optimization of the LC-MS/MS conditions

Collision energies for all MRM transitions of derivatized carboxylic acids (Table S1) were optimized through flow injection analysis of 5 μM of derivatized carboxylic acids. Four MRM transitions were selected and tested in the on-column experiments. The two transitions with the highest signal-to-noise ratio were further used for analysis of real samples. Chromatographic conditions for separation of derivatized carboxylic acids were optimized on an HSS-T3 column (100 × 2.1 mm, 1.7 μm) by using gradient elution of 0.1 % formic acid (*v/v*) in water as mobile phase A and 0.1 % formic acid in acetonitrile as mobile phase B. Methanol was also tested as an organic solvent on HSS-T3 column; however, it did not provide as efficient separation and sensitivity as acetonitrile, especially for citrate and isocitrate derivatives that were not separated with methanol in mobile phase (Fig. S1). Our method was also compared to an already published study [16] using a BEH C18 column (100 × 2.1 mm, 1.7 μm) with 0.01 % formic acid in water (A) and 0.01 % formic acid in methanol (B) as a mobile phase. However, the reported method did not provide separation of citrate and isocitrate isomers. The separation of citrate/isocitrate was also tested on the BEH column using the same conditions as in the optimized method (acetonitrile in mobile phase), which did not provide baseline separation of the isomers of citric and isocitric acids (Fig. S2). Therefore, method was finally validated on the HSS-T3 column with 0.1 % formic acid in acetonitrile as mobile phase B.

Derivatization of ketone and aldehyde functional groups with 3-NPH forms *cis/trans* isomers (Fig. S3) on C═N double bonds [16]. Therefore, the derivatized oxo acids, namely oxaloacetate, 2-oxoglutarate and pyruvate were present as two peaks in the chromatogram measured with the final method (Fig. 1).

Apart from the oxoacids, several other acids, such as *cis*-aconitate (Fig. S4), fumarate, citrate and isocitrate, were also showing multiple peaks in the chromatogram. Presumably *cis*-aconitate and fumarate derivatize into their both tautomeric forms (i) 3-nitrophenylhydrazide (keto form) and (ii) 3-nitrophenylhydrazone (enol form), as described in the literature before [16]. Probably, under our conditions, some of the tautomeric forms are separated, which results in the multiple peaks of

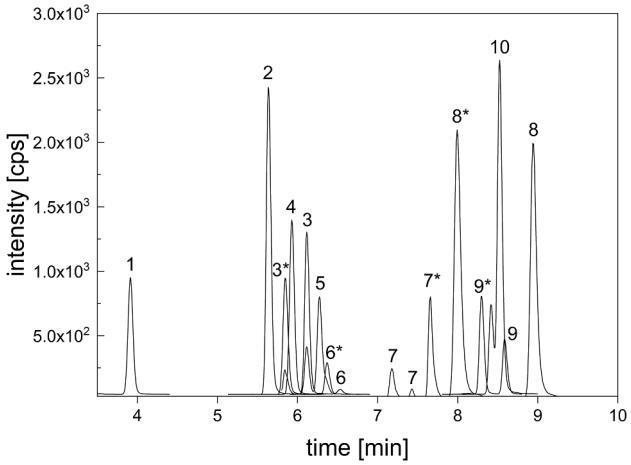

Fig. 1. Dynamic MRM chromatogram of a 100 nM standard mixture of 3-NPH-derivatized carboxylic acids analyzed on an HSS T3 column. Peak assignment: 1 – lactic acid, 2 – malic acid, 3 – isocitric acid, 4 – succinic acid, 5 – citric acid, 6 – fumaric acid, 7 – *cis*-aconitic acid, 8 – pyruvic acid, 9 – oxaloacetic acid, 10 – 2-oxoglutaric acid. Instrumental conditions as described in *LC-MS conditions*. Asterisk denotes an integration peak used for quantification of carboxylates represented by multiple peaks in chromatogram.

fumarate (2 peaks), *cis*-aconitate (3 peaks), isocitrate (2 peaks), and citrate (2 peaks). Doubly derivatized species of isocitrate and citrate were represented by two peaks under the optimized conditions, as shown in Fig. S2. The isocitrate isomers are baseline separated, whilst it is suggested that there are 2 citrate isomers, although not fully separated (Fig. S2, C). The most abundant peak of each acid, providing more than one peak, was used for quantification.

3.2. Mass spectrometry of 3-NPH-derivatized carboxylates

Derivatization of carboxylic acids with 3-NPH forms 3-nitrophenylhydrazine derivatives of the acids (Fig. S3). However, derivatization of dicarboxylic, tricarboxylic and keto acids provides derivatives of various structures. They can either form doubly or fully derivatized species with fully derivatized species being the most abundant ones, except for citric and isocitric acids, where doubly derivatized species show the highest signal as confirmed by LC-QTOF experiments (Fig. S5). The doubly derivatized species were predominant under the derivatization conducted at the room temperature (21 °C) as well as at elevated temperature (40 °C). However, these findings are in contradiction with a recent study [20], where the authors showed that peak area of fully derivatized isocitric acid increases with temperature with an optimum at 25 °C and derivatization time of 30 min. However, under our conditions, the doubly derivatized species were predominant forms of 3-NPH derivatives in standard solutions as well as in human plasma. It is likely that differences in abundance of doubly and fully derivatized species of citrate and isocitrate in the recent literature stems mainly from misinterpretation of mass spectra of 3-NPH derivatives of citrate and isocitrate. Our findings show that the precursors ($m/z = 443$) of citric and isocitric acids were produced via in-source elimination of water during ESI. Subsequently, collision-induced dissociation (CID) in a collision cell provided a product of $m/z = 178$ for citric acid and a predominant product of $m/z = 208$ for isocitric acid that were used for quantification. The proposed fragmentation pathways of citric and isocitric acids are illustrated in Fig. S6 (corresponding fragmentation spectra in Fig. S7). The doubly derivatized species provided signal intensity that is higher than the intensity of the fully derivatized species by factors of 44 and 34 for citric and isocitric acid under our conditions, respectively.

Fragmentation of all studied 3-NPH-derivatized carboxylates provided a selective product ion of $m/z = 137$, which corresponds to an anion of 3-nitroaniline. Fragmentation of all derivatized acids was

characterized by a neutral loss of 3-NPH [$C_6H_7N_3O_2$] of $m/z = 153$, whilst fragmentation of lactic acid provided an anion of 3-NPH $[C_6H_6N_3O_2]^-$ of $m/z = 152$ as the most abundant product. Fragentation of all the derivatized acids also provided a characteristic fragment of $m/z = 178$ $[C_7H_4N_3O_3]^-$.

Additionally, all 3-NPH derivatives were tested in positive ESI as well as negative ESI employing full scan mode, which showed that negative ionization provides higher intensity for all precursors of the acids with average increase in intensity by 62 %.

3.3. Method validation

In contrast with the literature up to now, all the TCA carboxylates were detected in the blank solvent – especially lactate, succinate, malate, and pyruvate – after derivatization with approximate concentration of 5.9 nM for fumarate to 1224 nM for lactate (Table S2). The LC-MS experiments with the derivatized blank solutions were tested at two separated laboratories. Pure solvents (LC-MS grade) and different materials for derivatization (Eppendorf tubes, glass vials, glass test tubes) were also tested. However, the derivatized acids were detected under all above-mentioned conditions. The signals in derivatized blank solutions were also confirmed in HRMS by using the LC-QTOF setup. Identification of the acids in the derivatized blanks based on the MRM transitions, exact m/z in HRMS, and retention times provide sufficient evidence that the signals correspond with these acids. Presumably, the carboxylic acids are present in the laboratory equipment used during the derivatization and/or in the deionized water, methanol and other chemicals. This restricts using the methodology for lower cell count than 10^5, as seen from comparison in Fig. S8. The background levels of the acids also complicated determination of LOD and LOQ values. Therefore, the LODs were determined by using isotopically labelled standards of the respective carboxylates, except for oxaloacetate, isocitrate and *cis*-aconitate for which the isotopically labelled standards were commercially unavailable and thus their estimated concentration in the blanks were considered their LODs. The LOQ values were determined as the lowest concentration with acceptable accuracy of quantitation. Therefore, there were substantial differences between LODs and LOQs, especially for the acids with highest concentration in the blank solvent, namely lactic acid and succinic acid. When comparing LODs, the method proved to be most sensitive for 2-oxoglutaric acid with LOD of 10 pM (1.46 ng/L) and the method showed lowest sensitivity for pyruvic acid with LOD of 800 pM (70.4 ng/L) (Table 1).

Carry-over in % of peak areas in blanks injected after the highest calibration point (100 μM) showed that there were no significant carry-over effects with peak areas in blanks below 0.5 % for all acids. Citric acid and pyruvic acid were the acids with lowest and highest carry-over, respectively.

Linearity of the method was acceptable with R^2 for all acids > 0.9955 with exception of oxaloacetate and *cis*-aconitate that were among the acids lacking the isotopically labelled standards. The lowest calibration point with acceptable accuracy was determined as a starting point for linear calibration, which was limited by the presence of all tested acids in derivatized blank solutions. Fumaric acid showed the widest linear calibration range, which corresponds with the fact that its concentration in the derivatized blank solutions was lowest of all acids.

Endogenous matrix of the samples resulted in ion suppression of all tested acids with exception of citric acid, whose signal was increased through ion enhancement effect [21]. The matrix effects were compensated by using isotopically labelled standards and therefore accuracy of the method was not compromised with exception of oxaloacetate, isocitrate and *cis*-aconitate.

Recovery of the method was evaluated by using isotopically labelled standards and it ranged from 72 % for 2-oxoglutaric acid to 106 % for citric acid (Table S3).

Accuracy in human plasma was evaluated through spiking into plasma at two concentration levels and accuracy was in an acceptable range of 100 ± 15 % for all the acids calibrated with isotopically labelled standards. Accuracy in human plasma was not acceptable for oxaloacetate, isocitrate and *cis*-aconitate because of unavailability of labelled standards.

Accuracy in C2C12 myotube cells was assessed through spiking cell extracts with the standard solution as follows: extracts after reconstitution in 20 μL of 50 % methanol were split in two equal parts, one part was analyzed without standard addition and the other one was analyzed with 10 μM spiked standards. Accuracy in cell extracts was acceptable for all tested acids (100 ± 15 %) with exception of isocitric acid (Table S4).

Precision of the method, expressed as RSD, did not exceed 15 % for all tested acids.

In contrast with precision, *repeatability* of peak areas ranged from 1 % for lactic acid up to 36 % for succinic acid; however by using the isotopically labelled standards, relatively low repeatability in peak areas did not affect precision and/or accuracy (Table 1).

The *stability* of the 3-NPH derivatives was evaluated based on their peak areas measured immediately after derivatization versus peak areas measured after (i) 7 days in the LC autosampler at 4 °C and (ii) 7 days in freezer at −20 °C. Values in Table S5 indicate no significant changes in peak areas for most derivatives except for 2-oxoglutarate, oxaloacetate, and isocitrate, whose peak areas were significantly increased after the 7-day storage at both temperatures. Presumably, the increases in peak ares stem from an ongoing derivatization and fluctuation in MS sensitivity. However, both effects were successfully accounted for by using isotopically labelled standards. Therefore the interday accuracy evaluated on a mixture of standards did not exceed the acceptable range of 100 ± 15 %, except for oxaloacetic acid, which is caused by the lack of its labelled internal standard.

Table 1
Limits of detection (LOD), limits of quantification (LOQ), Accuracy [% of nominal concentration, $n = 10$], precision [%, RSD of concentration, $n = 6$] and repeatability [%, RSD of peak area, $n = 6$] determined in human plasma.

Analyte	LOD [nM]	LOQ [nM]	Accuracy Medium[2]	Accuracy High[2]	Precision	Repeatability
fumaric acid	0.10	8	107.6	106.9	5.8	33
succinic acid	0.05	250	109.8	113.8	2.2	36
malic acid	0.03	15	106.0	110.0	6.9	34
citric acid	0.10	80	96.7	86.5	5.2	22
pyruvic acid	0.80	80	111.5	112.4	9.0	30
2-oxoglutaric acid	0.01	80	105.6	107.5	5.4	20
lactic acid	0.05	3000	111.3	103.3	5.7	1
oxaloacetic acid[1]	<10.0	10	50.9	44.1	14.8	14
isocitric acid[1]	<66.0	70	71.4	56.8	11.1	17
cis-aconitic acid[1]	<32.0	120	96.1	128.4	6.3	2

[1] Isotopically labelled standards unavailable - LOD of oxaloacetic acid, isocitric acid, and *cis*-aconitic acid determined on the basis of the concentration in blank samples, and accuracy not sufficient.

[2] Medium – samples spiked with 1 μM standard solution; high – samples spiked with 10 μM standard solution.

3.4. Proof-of-concept study in C2C12 myotube cells

To test the applicability of this method for *in vitro* conditions, C2C12 myotubes were treated with oleic acid and the AMPK (AMP-activated protein kinase) activator MK-8722, given these are interventions suspected of modulating TCA cycle activity and thus the levels of TCA carboxylates. The final concentrations of carboxylic acids were calculated based on the internal calibration with isotopically labelled standards that were added to the real samples during the cell extraction. The molar amounts in fmol of the carboxylic acids were then normalized to 1,000 cells. Oleic acid is a mono-unsaturated fatty acid found in vegetable and animal fats commonly used in the human diet, which can be broken down by fatty acid oxidation to yield acetyl-CoA which feeds into the TCA cycle. Alternatively, it can be used as a substrate for lipid synthesis when ATP is abundant.

MK-8722 is a potent, highly-specific pharmacological AMPK activator [22]. Activation of AMPK is a key mechanism by which exercise and nutrient deprivation modifies metabolism to promote catabolic pathways fueling ATP synthesis, including the TCA cycle [23]. Acetyl-CoA Carboxylase 1 and 2 (ACC1/2) are a key downstream target of AMPK which promotes a metabolic switch from lipid synthesis towards fatty acid oxidation.

It was therefore surprising to find that MK-8722 has a repressive effect on levels of all the TCA carboxylates measured compared to the control myotubes ($p < 0.05$ for all, one-way ANOVA, Fig. 2), reflecting reduced TCA cycle activity. Additionally, oleic acid also significantly reduced levels of all the TCA carboxylates (again $p < 0.05$ for all, Fig. 2), albeit to a lesser extent than MK-8722, however this effect was not additive when myotubes were co-incubated with oleic acid and MK-8722. It appears likely therefore that after import oleic acid is being funneled into lipid synthesis pathways, rather than being broken down to acetyl-CoA via fatty acid oxidation.

Interestingly, for myotubes co-incubated with both oleic acid and MK-8722, there was no decrease in lactate and pyruvate levels compared to the control myotubes, in contrast to the other carboxylates measured (Fig. 2). Lactate and pyruvate are metabolites yielded from glycolysis, whereby pyruvate fuels the TCA cycle via conversion to acetyl-CoA, whilst lactate is produced from pyruvate during anaerobic glycolysis where glycolysis is uncoupled from the TCA cycle and oxidative phosphorylation. Thus, it appears the combination of oleic acid and MK-8722 upregulates glycolysis compared to the myotubes treated with either oleic acid or MK-8722 alone, with much of the yielded pyruvate being converted to lactate.

3.5. Targeted analysis of human plasma and mouse myotubes

Two sample types were analyzed with the optimized method – human plasma and C2C12 myotubes (Fig. 3). All tested acids were detected and quantified in the human plasma samples. Lactic acid followed by succinic acid represent the two most abundant acids in human

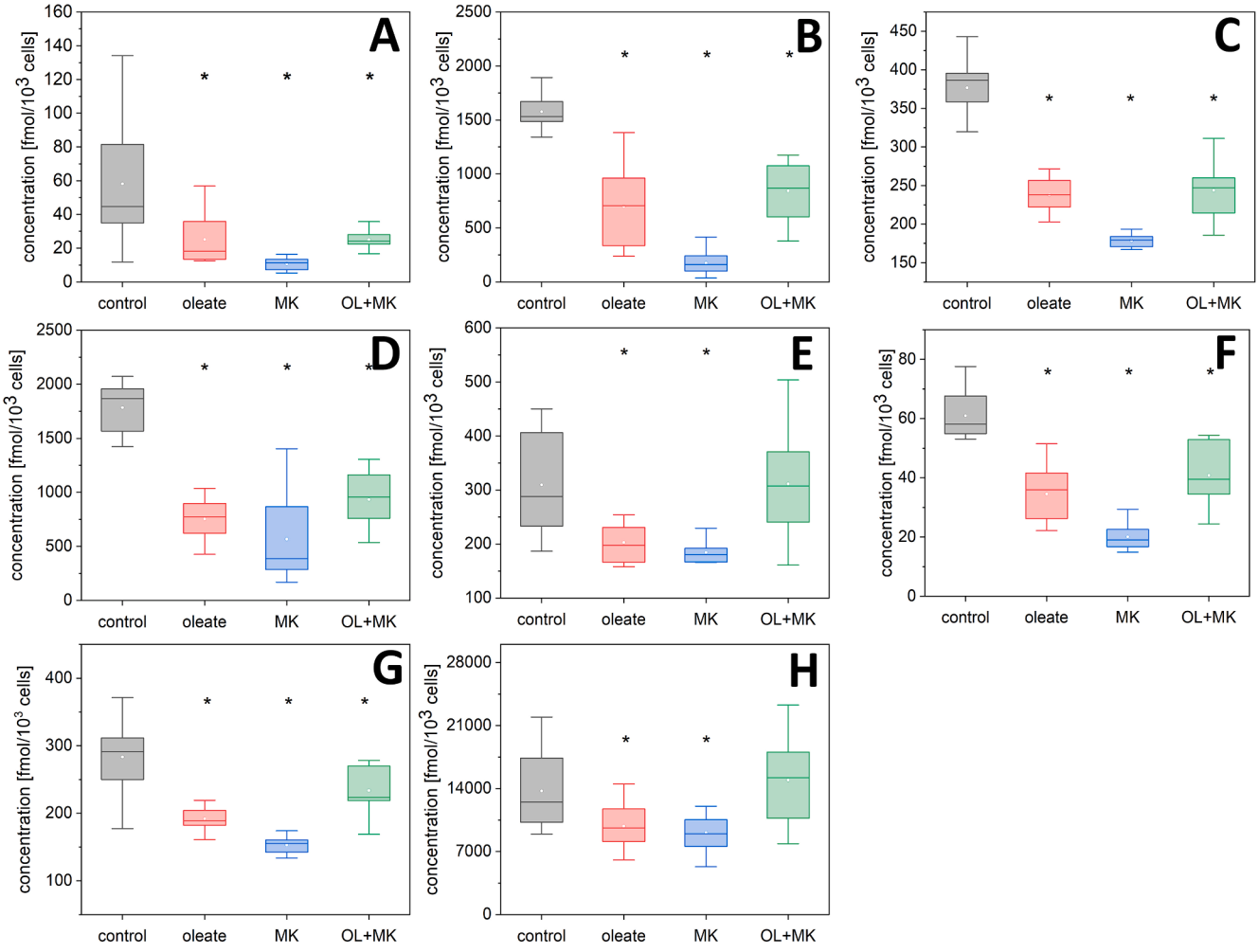

Fig. 2. Normalized levels of carboxylic acids in 10^5 of C2C12 myotubes, control group compared with cells treated with 0.4 mM oleic acid, 10 μM MK (MK-8722), and OL + MK (combination of oleic acid and MK-8722), $n = 10$ for all groups, asterisk denotes statistical significance ($p < 0.05$) in carboxylic acid levels between treated group vs control group. A – 2-oxoglutarate; B – citrate; C – fumarate; D – malate; E – pyruvate; F – isocitrate; G – succinate; H – lactate.

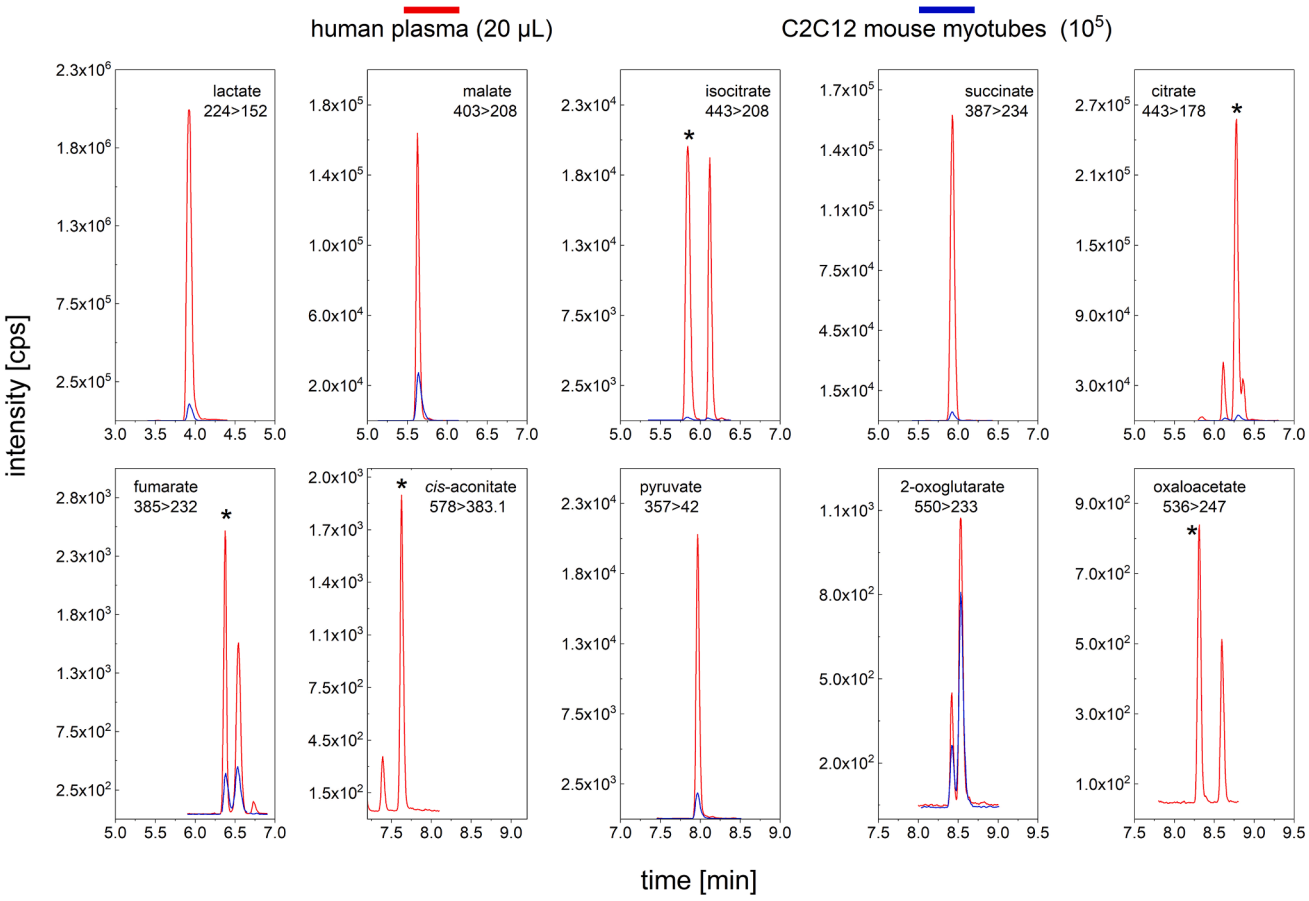

Fig. 3. Extracted ion chromatograms of detected 3-NPH carboxylates in 20 µL of human plasma and in 10^5 of C2C12 myotubes with the optimized LC-MS/MS method. Peaks used for quantification are denoted by an asterisk.

plasma, whereas 2-oxoglutaric acid and fumaric acid presents the acids with lowest concentrations (Table 2). Lactic acid was also the most abundant acid in the C2C12 myotubes, suggesting high levels of anaerobic glycolysis and thus a metabolic profile favouring anaerobic glycolysis over oxidative phosphorylation, which fits with a previous paper which found that C2C12s are highly dependent on anaerobic glycolysis, even under aerobic conditions [24].

The concentration levels of succinate, malate, citrate and lactate in plasma correspond with our previous study, where we quantified the same underivatized TCA acids in 100 µL of human plasma [25]. The concentration levels of the quantified acids in human plasma also correspond with the previously reported values for human serum [7,19] and human plasma [26] in the literature. In some studies that used 3-NPH derivatization, for example [19], the concentration levels for pyruvate and 2-oxoglutarate are incomparable to our results, which implies that the reported values lack sufficient accuracy as the isotopically labelled standards of different carboxylic acids for quantification (nonaoic acid-D_3 as IS for 2-oxoglutarate and citric acid-D_4 for pyruvate) were used in [19]. In our method, absolute quantification of oxaloacetate, isocitrate, and *cis*-aconitate lacked the sufficient accuracy as there were no labelled standards available.

Table 2
Concentration in 20 µL of human plasma ($n = 10$) and in C2C12 myotube cells (10^5, $n = 10$), median ± confidence interval at a confidence level $\alpha = 0.95$ with RSD in parantheses.

Analyte	Concentration in human plasma [µM]	Concentration in cells, control group [fmol/10^3 cells]
fumaric acid	1.20 ± 0.02 (13.4 %)	386.7 ± 29.1 (9.2 %)
succinic acid	11.24 ± 0.15 (8.6 %)	291.2 ± 34.3 (16.7 %)
malic acid	3.71 ± 0.05 (8.5 %)	1868.8 ± 448.4 (29.4 %)
citric acid	96.59 ± 1.36 (9.2 %)	1523.5 ± 340.4 (27.4 %)
pyruvic acid	6.04 ± 0.12 (13.5 %)	314.8 ± 58.5 (24.6 %)
2-oxoglutaric acid	0.59 ± 0.02 (27.8 %)	44.7 ± 17.8 (49 %)
lactic acid	1585.57 ± 3.32 (10.1 %)	12511.7 ± 2609.2 (25.6 %)
oxaloacetic acid*	3.52 ± 0.08 (14.9 %)	<LOQ
isocitric acid*	2.93 ± 0.07 (14.8 %)	55.9 ± 9.9 (21.8 %)
cis-aconitic acid*	2.19 ± 0.04 (12.9 %)	<LOQ

* Inaccurate quantification because of unavailability of isotopically labelled internal standards.

3.6. Untargeted screening of derivatized carboxylic acids using Hr-Ms

Reversed-phase chromatography and mixed-mode chromatography have been the traditional choices for separation and screening of underivatized medium- to long-chain fatty acids in LC-MS due to their low polarity nature [25,27,28]. However, their derivatization to less polar species helps to enhance screening coverage in LC-MS-based applications that require separation of metabolites with wide range of polarities [29,30]. In present study we used 3-NPH-derivatization that is commonly used for analysis of many biological relevant metabolites such as short-chain fatty acids [18,31] and other carbonyl-, carboxyl-, and phosphoryl-containing metabolites [19,20,32]. The derivatization strategy was applied to untargeted analysis of human plasma to identify not only TCA carboxylates but also fatty acids. With a slightly modified method with isopropanol in B mobile phase, we enabled identification of 29 carboxylic acids including several fatty acids in human plasma (Fig. 4, detailed list in Table S6). The acids were identified on the basis of

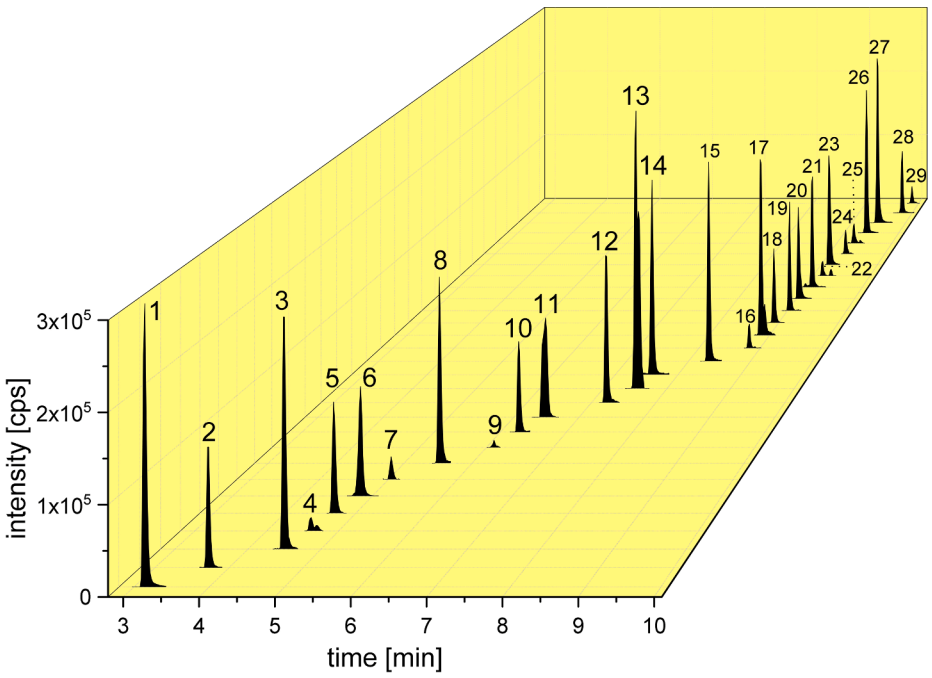

Fig. 4. Extracted ion chromatograms of derivatized acids in human plasma identified by LC-HR-MS by using an HSS T3 column. Peak identification: 1 – lactate (30×), 2 – 2-hydroxybutyrate (25×), 3 – malate, 4 – 2-hydroxyglutarate, 5 – succinate (5×), 6 – citrate (10×), 7 – fumarate, 8 – itaconate, 9 – 2-hydroxyoctanoate, 10 – ketobutyrate, 11 – 2-oxoglutarate, 12 – 2-ketoisovalerate, 13 – 2-ketoisocaproate, 14 – decanoate, 15 – laurate, 16 – eicosapentaenoate, 17 – linolenate, 18 – myristate (5×), 19 – docosahexaenoate, 20 – palmitoleate (5×), 21 – arachidonate, 22 - docosapentaenoate, 23 – linoleate (10×), 24 – 10-heptadecaboate, 25 – dihomolinolenate, 26 – palmitate (10×), 27 – oleate (10×), 28 – stearate (5×), 29 – eicosenoate. Signal of high abundant carboxylates was reduced by a factor stated in parantheses for better perceptibility. Liquid chromatography-mass spectrometry conditions in *LC-MS conditions.*

comparison of the detected features with an in-house mass spectrometry library with exact mass of $[M-H]^-$ and their retention times.

In many screenings of human or animal blood and plasma, reversed-phase chromatography with C18 columns has been used for untargeted screening of biologicacally relevant metabolites including fatty acids [32–34]. However, many of the small carboxylic acids, for example lactic acid and/or tricarboxylic acids such as tricarballylic acid, show very low retention on C18 columns because of their high polarity [35]. Whereas the 3-NPH derivatization allows for untargeted screening of small TCA carboxylates with sufficient retention, as well as medium- to long-chain fatty acids.

4. Concluding remarks

In this study, we optimized an LC-MS/MS method using the 3-NPH derivatization for analysis of TCA carboxylic acids and lactic acid in human plasma and samples containing 10^5 C2C12 myotubes. The derivatization produces various species of singly, doubly and triply derivatized carboxylates. Therefore, the abundance of various species and their product ions was studied using HRMS to confirm the identity of the most abundant precursors. Elucidation of the most abundant derivatized species and optimization of their fragmentation in collision-induced dissociation allowed significantly improved detection; however, all acids were detected in the blank solutions, which hampers the quantification of the acids in small sample amounts. Therefore the LODs were determined by using the corresponding labelled standards when possible and LOQ values were determined as the lowest calibration point with acceptable accuracy. Nevertheless, the method enables quantification of 10 tested carboxylic acids in human plasma. This method was also tested on a proof-of-concept study using 10^5 C2C12 myotubes treated with oleate and/or AMPK activator – MK8722, where it revealed significant changes between control and treated samples in all carboxylic acids. The targeted method enabed quantification of all acids with acceptable accuracy within a 17-minute run with LOQ ranging from 8 nM for fumaric acid to 3 μM for lactic acid. Finally, the derivatization strategy was applied in an untargeted approach to a human plasma sample, where 29 biologically relevant carboxylic acids were identified by using LC-HRMS. The derivatization with 3-NPH offers better chromatographic behaviour of all derivatized carboxylates and low limits of detection but quantification of the derivatized TCA metabolites in small amounts of biological samples is limited by the presence of derivatized TCA carboxylates in the derivatized blank solutions.

CRediT authorship contribution statement

Ondřej Hodek: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **John Henderson:** Methodology, Investigation, Writing – original draft. **Lidia Argemi-Muntadas:** Methodology. **Adnan Khan:** Methodology. **Thomas Moritz:** Resources, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by Novo Nordisk Foundation (grant number NNF18CC0034900), Knut and Alice Wallenberg Foundation (grant number KAW2018.0094 and KAW2014.0279), and Swedish University of Agricultural Sciences. Swedish Metabolomics Centre is acknowledged for the technical support.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2023.123719.

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