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# Derivatization of the tricarboxylic acid cycle intermediates and analysis by online solid-phase extraction-liquid chromatography–mass spectrometry with positive-ion electrospray ionization

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ABSTRACT

The analysis of cellular metabolic processes is of fundamental biological interest. Cellular metabolites, such as the intermediates of the tricarboxylic acid (TCA) cycle, provide essential information about the metabolic state of the cell. Not only is the TCA cycle a key factor in the energy regulation within aerobic cells, it possibly also plays a role in cell signaling. This paper describes a novel derivatization strategy, using the empirically selected *N*-methyl-2-phenylethanamine as derivatization reagent with a carbodiimide as co-reagent, for the selective derivatization of carboxylic acids, such as the di- and tri-carboxylic acids of the TCA cycle. Optimization of the derivatization protocol is described. This procedure enables analysis of the derivatives using on-line solid-phase extraction and reversed-phase liquid chromatography in combination with sensitive positive-ion electrospray ionization mass spectrometry. The complete procedure, involving the use of core–shell silica column material, allows the rapid analysis of TCA cycle intermediates in sample matrices, here shown for pig heart tissue extracts, with a good linearity over 3–4 orders of magnitude. Detection limits range from 12 to 1000 nM, depending on the analyte.


## 1. Introduction

The analysis of cellular metabolites in order to unravel metabolic processes is of fundamental biological interest [1,2]. Determination of the concentration and the flux of cellular metabolites, such as the intermediates of the tricarboxylic acid (TCA) cycle, in many instances provides relevant information for the understanding of biological processes. The TCA cycle not only is a key factor in the energy regulation within aerobic cells, but TCA cycle intermediates also play an important role in various signaling events. As such, they are frequently used by system biologists as a tool in the evaluation of pathological events [2,3].

For the analysis of TCA cycle intermediates various analytical techniques have been applied [1,2,4–6]. The most prominent one is gas chromatography mass spectrometry (GC–MS), following derivatization of the analytes with either *N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MtBSTFA) [6], or *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA) [7]. However, these derivatization reactions have to be performed in non-aqueous conditions, whereas the analytes of interest are present in aqueous (cellular) systems. The necessary phase transfer involves evaporation to dryness and reconstitution, which is a time consuming process with the risk of loss of analytes [6]. Alternatively, capillary electrophoresis (CE) is readily amenable to aqueous samples, but the compatibility of CE buffer systems with MS [8] and the poor separation of carboxylic acids without having a cation-coated capillary with reversed polarity, which is challenging [9], limit the applicability of CE–MS in this field. Therefore, liquid chromatography mass spectrometry (LC–MS) appears to be the method of choice.

Several LC–MS/MS based methods for the analysis of TCA cycle intermediates have been described. Methods based on the use of ion-pair reversed-phase chromatography suffer from ionization suppression caused by the ion-pairing agents [10]. Moreover, this technique limits the versatility of instrumentation which has been used with ion-pairing agents, because of memory effects. Alternatively, LC–MS based on hydrophilic interaction chromatography (HILIC) has been proposed for the analysis of TCA cycle intermediates. However, co-elution of the highly polar analytes with endogenous salts may cause severe ionization suppression when

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using HILIC [11]. Also, the necessity of high organic solvent as starting conditions in order to obtain optimal peak shapes again asks for a similar phase transfer as for GC–MS.

Besides all these separation related issues, the detection sensitivity of small carboxylic acids in negative-ion electrospray ionization (ESI−) is often problematic. Recent publications report ESI− detection limits (LODs) in the micromolar range, being 8 μM [11] and 1 μM [12] for succinic acid (SA), whereas LODs in the low micromolar-high nanomolar range, e.g., 1.3 μM for isocitrate (ICA) [6], have been reported for (surprisingly) ESI− after derivatization with aniline as derivatization reagent.

This paper describes an alternative strategy for the selective and sensitive analysis of TCA cycle intermediates to overcome the aforementioned drawbacks. The method is based on reversed-phase LC separation and sensitive positive ion electrospray ionization (ESI+) MS after employing a novel derivatization strategy. The LC–MS method includes an on-line solid-phase extraction (SPE) clean-up procedure and the incorporation of a Kinetex Core–Shell $C_{18}$ column to accelerate sample throughput. We report on the design of the label, the optimization of the derivatization protocol, and the development and validation of the analytical method. Application of the method to the analysis of the TCA cycle intermediates in heart tissue extract is demonstrated, e.g., in comparing heart tissue samples with and without previous $^{13}$C-acetate labeling in order to enable $^{13}$C-flux analysis using a quadrupole time of flight (QTOF) MS.

## 2. Experimental

### 2.1. Chemicals and reagents

3-(Ethyliminomethyleneamino)-*N*,*N*-dimethyl-propan-1-amine hydrochloride salt (EDC) (>99.9%), *N*-methyl-2-phenylethanamine (MPEA) (99%), phenylethanamine (PEA) (≥99.0%), citric acid (CA) (≥99.0%), isocitric acid (ICA), alpha-keto-glutaric acid (αKG) (≥99.0%), succinic acid (SA) (≥99.0%), succinic acid $D_4$ ($SAD_4$) (98 atom% D), fumaric acid (FUA) (≥99.0%), malic acid (MA) (≥98%) and tartaric acid (TA) (≥99%) were all purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Acetonitrile (ACN) (LC–MS grade) and formic acid (FA) (99%) were purchased from Biosolve (Valkenswaard, The Netherlands). Water was purified by a Millipore (Amsterdam, The Netherlands) Milli-Q unit. Hamster hearts for initial studies were obtained from a sacrifice with other purposes (LUMC, The Netherlands) and the $^{13}$C-treated heart tissue was obtained from sacrificed stroke induced pigs, also used in other studies.

### 2.2. Instrumentation and protocols

#### 2.2.1. Online SPE

Samples were analyzed on an online SPE LC–MS system [13]. The online SPE clean-up step was achieved by using a Shimadzu LC-10AD pump (Shimadzu, 's Hertogenbosch, The Netherlands) continuously pumping 20% ACN, 80% $H_2O$ and 0.1% FA with a flow rate of 500 μL/min, using a $C_8$ SPE cartridge (4 mm × 2 mm, 5 μm) (Phenomenex, Aschaffenburg, Germany). The switch-valve used was from Spark Holland (Emmen, The Netherlands) and triggered to switch 0.5 min after starting the system.

#### 2.2.2. HPLC

The HPLC system consisted of a Shimadzu LC-20AB quaternary pump, a Spark Holland column oven set to 30 °C, and a Gilson Model 234 auto-injector (Gilson, Villiers le Bel, France), equipped with a 100 μL injection loop. The column used was a Kinetex Core–Shell $C_{18}$ reversed-phase column (100 × 2.1 mm i.d., 2.6 μm particles) (Phenomenex). A flow rate of 300 μL/min was used throughout all experiments. The pump supplied a gradient with the following settings: 0 min, 70% mobile phase A (98% $H_2O$, 2% ACN and 0.1% FA), 30% mobile phase B (98% ACN, 2% $H_2O$ and 0.1% FA), maintained for 0.5 min. Subsequently, the concentration of mobile phase B was increased to reach 90% at 6.5 min, held constant for 1 min. Re-equilibration time was 2 min.

#### 2.2.3. LC–MS conditions

All measurements were performed on a Bruker MicrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany). The source was operated in ESI+ mode. Source performance and transfer settings were optimized by direct infusion of derivatized SA in 50% ACN, 50% $H_2O$ and 0.1% FA. Mass spectra were recorded in an $m/z$ range from 200 to 700. The data acquisition time was set to 1.5 Hz. Capillary voltage was set to 4.5 kV. Nitrogen (99.9990%) was used as nebulizer gas at 1.6 bar and as drying gas at 8.0 L/min (200 °C). Argon (99.9995%) was used as collision gas. Collision energy was 4.0 eV. All measurements were performed in $MS^1$-mode only, unless stated otherwise.

#### 2.2.4. Optimization of reaction conditions

To investigate the influence of the EDC concentration, the excess of the label, the water percentage in the reaction mixture, and the reaction temperature, 25 μL of a 5 μM SA solution in 50% ACN, 50% $H_2O$ (v/v) was transferred into a plastic test tube. To these solutions, 25 μL of an EDC solution in 50% ACN, 50% $H_2O$ (v/v), and 50 μL of an MPEA solution in ACN were added. The mixture was incubated overnight at room temperature or the chosen reaction temperature and diluted with water to 1.0 mL prior to SPE-LC–MS analysis.

The influence of EDC was determined by using 100, 250, 500 and 1 M EDC solutions (50% ACN, 50% $H_2O$ (v/v)) and a label solution containing 20 mM of MPEA. To investigate the influence of label excess, 50 μL of a 1, 2.5, 5, 10, 20, 50 or 100 mM solution of MPEA was added to the test tubes containing a 100 mM EDC solution. The water content of the final derivatization mixture was varied among 5, 10, 15, 20, 25, 30, 40 and 50% by changing the initial water content of the SA and the EDC solutions added (with 100 mM EDC and 10 mM MPEA). Temperature influence was examined at room temperature (ca. 25 °C), 40 °C and 60 °C (with 100 mM EDC solution and 10 mM MPEA solution).

#### 2.2.5. Kinetic curves

The kinetics of the derivatization reaction was investigated by derivatizing CA according to the optimized protocol at different temperatures. After mixing all chemicals, a sample was taken every 30 min and diluted with water to 1.0 mL prior to SPE-LC–MS analysis. Kinetics was determined at room temperature (ca. 25 °C), at 40 °C and at 60 °C.

#### 2.2.6. Optimized derivatization procedure

The following optimized conditions were used in real sample analysis: 12.5 μL of aqueous sample, 25 μL 1 M EDC in 10% $H_2O$, 90% ACN (v/v), 50 μL 10 mM MPEA in ACN, and 12.5 μL of ACN are mixed and placed in a water bath at 60 °C for 45 min. For experiments including PEA, the same protocol was used by substituting MPEA with PEA. The resulting mixture was diluted with water to 1.0 mL prior to SPE-LC–MS analysis.

#### 2.2.7. Synthesis of $N^1$,$N^4$-dimethyl-$N^1$,$N^4$-diphenethylsuccinamide and $D_4$-$N^1$,$N^4$-dimethyl-$N^1$,$N^4$-diphenethylsuccinamide.

To learn more about the derivatized acids and their behavior, SA and $SAD_4$ were derivatized and isolated at a preparative scale by using the following protocol. Two hundred mg of SA or $SAD_4$ was dissolved in 1 mL water. Then, 2 g of EDC dissolved in 1 mL water and 8 mL of a 1.0 M solution of MPEA in ACN was added. The whole

mixture was transferred into a flask and placed on a magnetic stirrer while being heated to 60 °C overnight. The reaction mixture was diluted four times with water and thereafter injected onto a preparative LC system consisting of two Shimadzu LC10 AD pumps, a Purospher Star column (250 mm × 10 mm i.d., 5 μm) (Merck, Darmstadt, Germany), a manual injector with a 250 μL injection loop, and an Applied Biosystems 759A Absorbance Detector, operated at 281 nm. The pumps supplied a gradient at a flow rate of 4 mL/min. The gradient started at 70% mobile phase A (98% $H_2O$, 2% ACN and 0.1% FA), 30% mobile phase B (98% ACN, 2% $H_2O$ and 0.1% FA) and the percentage of mobile phase B was increased to reach 90% at 10 min, held constant for 1 min. Re-equilibration time was 2 min.

The fraction containing the derivatized SA or $SAD_4$ was collected manually and dried under a gentle stream of nitrogen. The residue was redissolved in 100% ACN and 1.0 g of magnesium sulfate was added to dry the solvent. This solution was filtrated. The filtrate was dried in a desiccator under vacuum. The reaction yielded white substances, the products were dissolved in ACN to give 40 mM stock solutions of both $N^1$,$N^4$-dimethyl-$N^1$,$N^4$-diphenethylsuccinamide (MPES) and $D_4$-$N^1$,$N^4$-dimethyl-$N^1$,$N^4$-diphenethylsuccinamide ($MPESD_4$). These were the basis for further characterization. The identity of the prepared substances was proven by direct infusion of a 5 μM solution (50% ACN) into the above described high-resolution MS system.

*2.2.8. Extraction of heart tissue*

$^{13}C$-labeled pig heart samples were generated as follows: after 5.5 min infusion of $^{13}C$-labeled acetate into the animal, part of the left ventricular free wall of the heart muscle was quickly freeze-clamped in situ between an aluminium clamp precooled to the temperature of liquid nitrogen, the heart was cut out and placed into liquid nitrogen. The tissue was stored at −80 °C until further analysis.

Extraction of the TCA cycle metabolites from the heart tissue was carried out as follows: prior to analysis, the tissue was freeze-dried (Modulyo freeze-dryer; Edwards, Dordrecht, The Netherlands) for at least 48 h. Then, it was cut into small pieces representing the sub-epicardial, mid-myocardial and sub-endocardial layers. After weighing the tissue, it was homogenized in 4 mL of 0.6 M ice-cold perchloric acid under rigorous mixing for 1 min. Next, the whole mixture was centrifuged for 10 min at 4000 × *g*. The supernatant was taken and adjusted to pH 7.0 with buffer containing 3 M KOH and 0.3 M imidazole. The sample was centrifuged again and the supernatant was freeze-dried for 48 h. Freeze-dried supernatants were dissolved in 50% ACN, 50% $H_2O$ (v/v) prior to derivatization. In case of hamster heart samples, adjustment to pH 7.0 was done with 3 M KOH.

*2.2.9. Matrix effects*

Possible ionization suppression was evaluated using the post-column infusion method [14]. A derivatized heart sample was injected while applying a constant post-column infusion of 10 μM $MPESD_4$ in 50% ACN, 50% $H_2O$ (v/v) (6 μL/min) via a T-piece inserted in the transfer tubing between the LC column and the MS.

*2.2.10. Linear range and limits of detection/quantification*

A seven-level calibration line (25, 50, 250, 500, $2.5 \times 10^3$, $5.0 \times 10^3$ and $2.5 \times 10^4$ nM) was prepared employing 25 μL of a hamster heart tissue extract in 50% ACN, 50% $H_2O$ (v/v) spiked with 10 mg/mL of all relevant acids as well as 5.0 μM TA and 5.0 μM $SAD_4$ as internal standards (IS) in each sample. The exact same procedure was carried out by replacing the heart tissue extract with blank solvent 50% ACN, 50% $H_2O$ (v/v). To determine the limits of detection (LOD) and limits of quantification (LOQ), the signal-to-noise (S/N) ratio at the lowest concentration measured was used to estimate the concentration levels yielding three and ten times the S/N-ratio, respectively. This is a widely used method to determine LOD and LOQ in the field of metabolomics [5,15–17].

*2.2.11. Stability*

Intermediates from the TCA cycle (5 μM) (CA, ICA, αKG, SA, FUA and MA) were derivatized according to the optimized derivatization protocol. A sample was taken from the reaction mixture every hour for 10 h and was injected into the LC–MS. From the same mixture, a sample was taken after 24, 48 and 168 h as well and measured in duplicate.

## 3. Results and discussion

In order to allow a high sample throughput, we aimed at a rapid chromatographic separation. Therefore, we incorporated a core–shell based Kinetex $C_{18}$ column in our analytical system, which was coupled to an on-line SPE clean up step. This allows full automation of the analysis as well as reduction of secondary effects, possibly caused by reagent excess, thereby also diminishing contamination of the ESI source. The choice for a core–shell particle column was initially based on the fact that such columns can be a good alternative to sub-2 μm particle columns. While core–shell columns are compatible with conventional HPLC equipment, ultra high pressure LC (UHPLC) demands special equipment [18]. The chromatographic performance of the developed system was satisfactory. The asymmetry factors for all analytes were between 0.8 (MA) and 1.2 (TA), achieved plate numbers (*N*) ranged from 7000 for FUA to 22,000 for ICA. The resolution for the most critical peak pair (FUA:SUC) was 1.3.

### 3.1. Selection of the derivatization label

Earlier studies in our group have already shown the feasibility of labeling aldehydes and carboxylic acids with aniline based derivatization reagents [16,17,19]. Deprotonation of the acid and activation of the carboxylate function by means of a carbodiimide, such as EDC, results in the formation of an *O*-acylisourea [20]. This intermediate can be attacked by an amine to form a stable amide (Fig. 1) [21]. The reaction was previously applied by us for the labeling of mono-carboxylic acids such as short-chain carboxylic acids, prostanoids and non-steroidal anti-inflammatory drugs using our newly developed aniline-based labels 4-(2-(trimethylammonio) ethoxy)benzeneaminium dibromide (4-APC) and 4-(2-((4-bromophenethyl)dimethylammonio)ethoxy)benzenaminium dibromide (4-APEBA) [16,17,19]. These labels show favorable ESI+ characteristics, as they carry a permanent charge of a quaternary ammonium function. Initially, the 4-APEBA label was tested in the derivatization of the TCA cycle intermediates. Unfortunately, this led to the formation of mixtures of derivatives for individual acids. LC–MS analysis demonstrated that such mixtures contained mono-, di-, and tri-labeled species of the analytes and complete tri-labeling could not be achieved, even with longer derivatization times. Moreover, cyclisation reactions to cyclic imides (i.e. succinimides), occurring after the first amide-label had been introduced, were observed (vide infra). Further evaluation of these data indicated that the problems experienced with the 4-APEBA labeling can partly be ascribed to steric hindrance and Coulomb repulsion. Both 4-APC and 4-APEBA carry their reactive amine in close proximity to a relatively large aromatic ring. In the derivatization of small di- and tri-carboxylic acids, such as CA, this leads to steric hindrance. In addition, after introducing the first label, the permanent charge incorporated in 4-APEBA may cause Coulomb repulsion rendering the derivatization of the second and third carboxylic acid function more difficult. Based on these observations, it was evident that the challenging small polyacids required an alternative label.

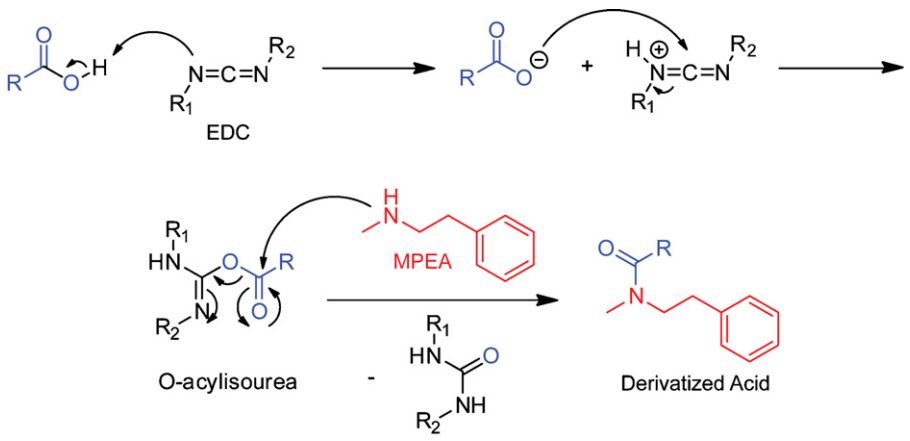


**Fig. 1.** Reaction mechanism for the coupling of MPEA to a carboxylic acid (blue), induced by EDC (carbodiimide) via an *O*-acylisourea intermediate. The label is emphasized in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In this design, we had to take in account that small multifunctional acids have to be derivatized. Some of the design characteristics of the 4-APC and 4-APEBA labels were maintained, such as the use of a phenyl group for sufficient retention in reversed-phase LC. Also the reaction should lead to one final derivatized product per target compound, with preferably all acid functions being derivatized. Initial tests, using simply aniline, as reported by Yang et al. [5], did not provide satisfying results, perhaps still due to steric hindrance. Therefore, it was decided that a spacer group should be incorporated between the phenyl ring and amine group to overcome steric effects. The p$K_a$ of the reactive amine will increase due to this spacer insertion because it is no longer in conjugation with an aromatic ring. The resulting higher nucleophilicity should enhance the attack of the amine onto the $\delta^+$ carbon atom of the *O*-acylisourea intermediate, although one should bear in mind that the higher basicity also means that lower amounts of free amines may be available in aqueous systems. To test our initial design, the commercially available PEA was selected as label. Unfortunately, this primary amine still led to unwanted side products. For instance, in the labeling of CA, next to the desired triply labeled derivative with a protonated molecule $[M+H]^+$ with *m*/*z* 502.27 other derivatives were observed, e.g., an apparently doubly labeled derivative ($[M+H]^+$ with *m*/*z* 381.17) (Fig. 2A). Accurate-mass determination of the latter derivative is consistent with intramolecular succinimide formation: in CA, doubly labeled at the carbons 1 and 5, the remaining free carboxylic acid function at carbon 3 can undergo activation by EDC and a subsequent intramolecular attack by a neighboring amide to give the succinimide. This observation led to a simple but crucial modification of the label: MPEA, having an *N*-methyl group rather than an N–H group as in PEA, was selected in order to block the succinimide formation (Fig. 2B). Accurate-mass determination shows that the triply labeled CA ($C_{33}H_{42}N_3O_4^+$) is indeed formed: $[M+H]^+$ with experimental *m*/*z* 544.3124 and theoretical *m*/*z* 544.3170 (mass error 8.4 ppm). A further advantage of the secondary amine as a label is the enhanced ESI + ionization efficiency related to the formation of a tertiary amine derivative. Conveniently MPEA does not have to be synthesized as it is readily available in good quality.

### 3.2. Optimization of reaction conditions

After selecting an appropriate label for the derivatization of the TCA cycle intermediates, optimization of the reaction conditions was investigated by studying the influence of various experimental parameters: the EDC concentration, the excess of the label, the water percentage in the reaction mixture, the reaction temperature, and the reaction time.

#### 3.2.1. Optimization of label excess

It was found that the extent of derivatization was dependent on all tested parameters. From Fig. 3, it can be derived that the optimum concentration of label before mixing is between 2.5 and

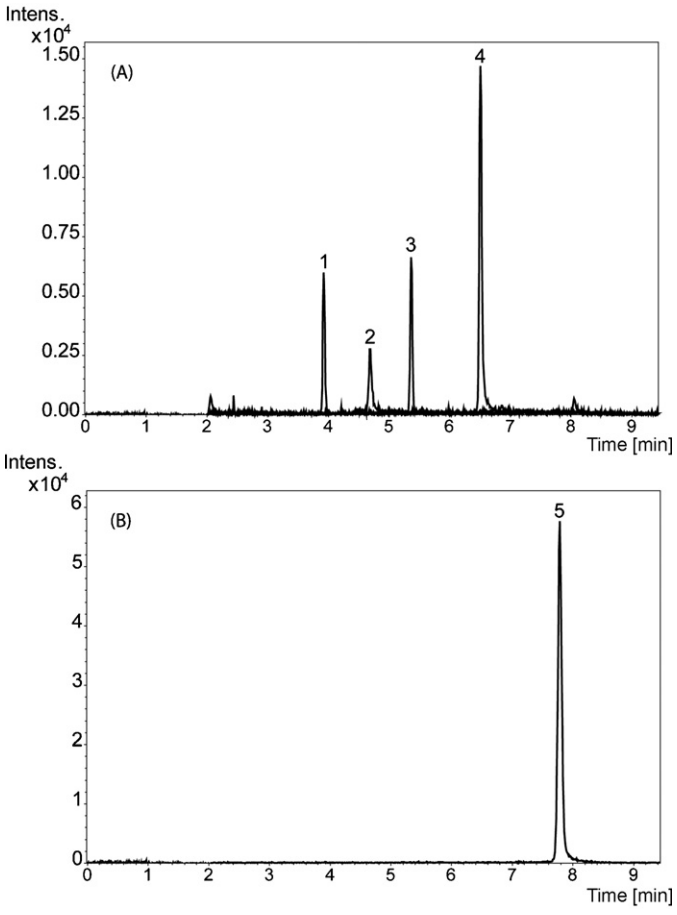


**Fig. 2.** (A) Overlaid extracted ion chromatograms of (1) the singly PEA-labeled CA with *m*/*z* 296.1, (2) the doubly PEA-labeled CA with *m*/*z* 399.2, (3) the doubly PEA-labeled CA with apparent ring-formation with *m*/*z* 381.2 and (4) the triply PEA-labeled CA with *m*/*z* 502.3 (10 μM CA was used). (B) Extracted ion chromatogram of (5) the triply MPEA-labeled CA (10 μM). No other products containing CA were found.

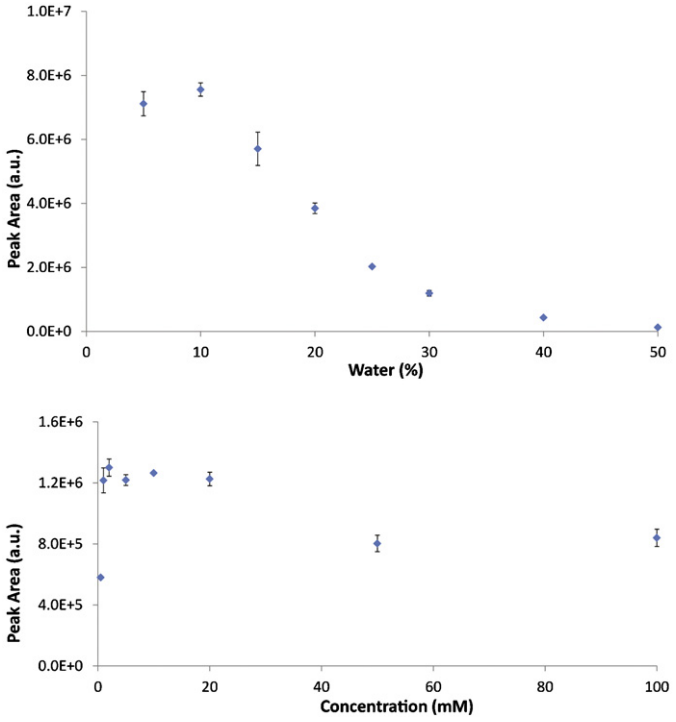


**Fig. 3.** Optimization of the reaction conditions to derivatize SA ($n = 3$). The upper graph shows an optimal final concentration of water around 15%. Lower graph indicates that the MPEA concentration before mixing is optimal between 2.5 and 20 mM.

20.0 mM. It is believed that the high p$K_a$ of the secondary amine influences the pH of the otherwise unbuffered reaction mixture when present in too high concentrations. However, when the amount of label was too low, an insufficient excess caused a non-optimal yield.

### 3.2.2. Optimization of water percentage

The percentage of water in the complete reaction mixture also proven to have a significant influence on the reaction. A plausible explanation could be that EDC hydrolyses in the presence of water into 1-(3-(dimethylamino)propyl)-3-ethylurea, effectively lowering the available amount of EDC for derivatization [22]. An optimum was found to be around 10% water in the reaction mixture. Some water is necessary in this coupling reaction in order to dissolve the analytes and the water-soluble EDC. Moreover, as stated by Yang et al. [5], the hydrolysis of EDC increases dramatically when the reaction is performed while heated (60 °C). This results in a lower derivatization yield. Lowering the percentage of water in the reaction by using ACN as an additional solvent was found to diminish this effect and to enhance the yield.

### 3.2.3. Reaction kinetics and temperature effects

The optimized conditions were used to inspect the kinetics of the derivatization. Different temperatures greatly affect the time needed for an optimal labeling. From Fig. 4 it can be derived that 60 °C resulted in the fastest labeling of the tri-carboxylic acid CA, as was expected. Moreover, higher overall reaction yields were obtained at higher temperatures. Therefore, 60 °C was selected as the temperature of choice. Temperatures higher than 60 °C were not tested given the boiling point of ACN.

### 3.3. Validation of the method

To correct for any possible variation in the derivatization yield and for other influences that can affect the obtained signal, two

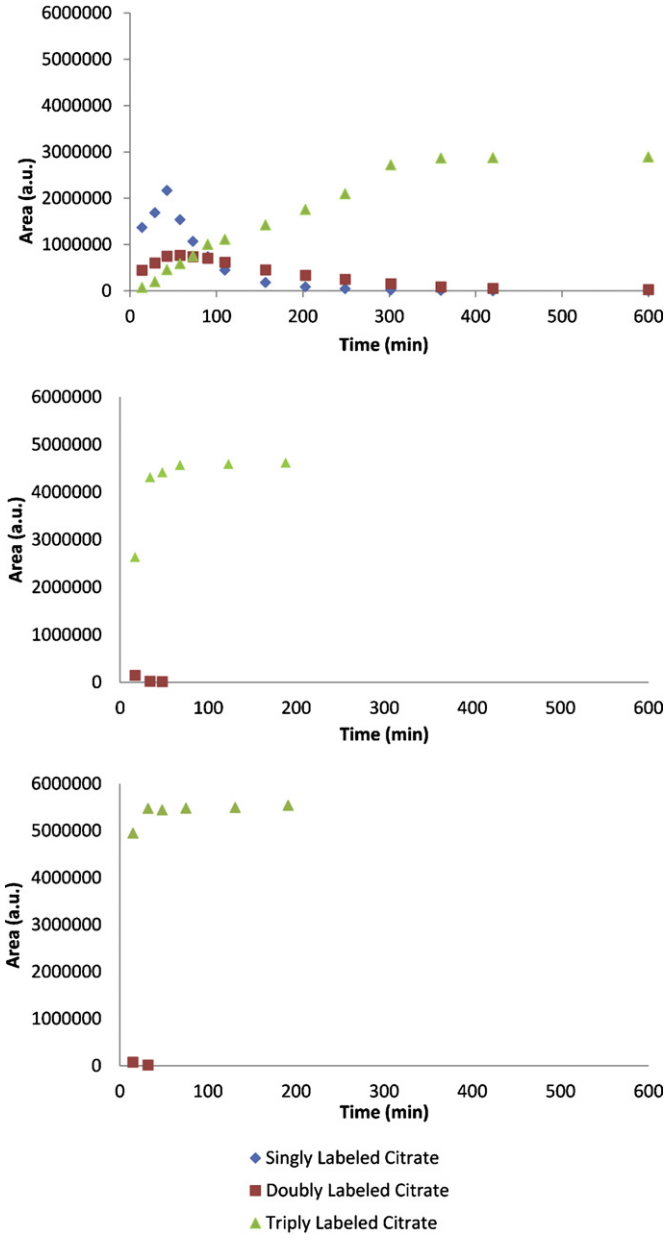


**Fig. 4.** Kinetic curves of the reaction between CA (10 μM) and MPEA; from top to bottom: room temperature, 40 °C, 60 °C. In the latter two, singly labeled CA is not shown as it was found to be below the LOD.

internal standards were tested. At first, $SAD_4$ was chosen as an isotopically labeled internal standard for all analytes. After mixing and labeling 5 μM $SAD_4$ together with different concentrations of SA, mutual ionization suppression between SA and $SAD_4$ occurred as a function of concentration (data not shown). After correction for $SAD_4$, good linearity is obtained for SA. However, due to the suppression effects on SA, $SAD_4$ was found not to be an appropriate internal standard for the other analytes. Because isotopically labeled internal standards were not available to us for the other carboxylic acids, TA was examined as an analogue internal standard for all other analytes except SA. TA does not co-elute with any other compounds tested and therefore is not suffering from ionization suppression caused by any of the other acids. It was found that TA was indeed an appropriate analogue internal standard for all other acids in both standard solutions and biological matrices (data not shown).

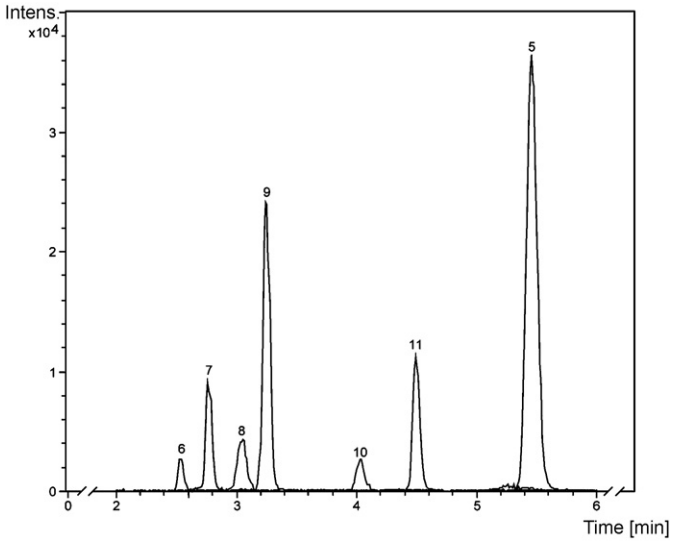


**Fig. 5.** Overlaid extracted ion chromatogram from MPEA derivatives (5 μM) of (6) TA *m/z* 385.21, (7) MA *m/z* 369.22, (8) FUA *m/z* 351.20, (9) SA *m/z* 353.22, (10) αKG *m/z* 381.22, (11) ICA *m/z* 544.31, (5) CA *m/z* 544.31.

### 3.3.1. Calibration, linear dynamic range

After correction by the labeled TA and $SAD_4$ (IS), linearity was observed for all labeled acids. For CA, linearity ($R^2$ = 0.998) ranged between 4 orders of magnitude between 40 nM and 250 μM, while all other analytes showed an acceptable linear range ($R^2 \geq 0.981$) of at least 3 orders of magnitude between 1 μM or less and 250 μM (higher concentrations were not tested). Both inter- and intraday reproducibility ($n$ = 3) were found to be within 5% RSD. The LODs for all analytes were better than 60 nM, except for αKG (1000 nM) and FUA (200 nM). In the best case, these LODs are almost 100-times lower than the ones reported for label-free ESI− methodologies based on selected reaction monitoring in triple quadrupole instruments [11,12]. The LOD of 1 μM found for αKG is still comparable with other methods, e.g., Bajad et al. [11] describing an LOD of 100 ng/mL = 0.7 μM. Compared to an earlier study employing aniline as derivatization reagent [5], the achieved LODs for most of the analytes were approximately 10- to 20-times lower. A standard chromatogram with all analytes is shown in Fig. 5.

### 3.3.2. Stability of the derivatives

After successful derivatization of the aforementioned acids, the stability of the derivatives was evaluated by carrying out an analysis at different time points. The results showed that the formed derivatives were stable, showing a less than 4% signal decrease over a time period of one week (168 h). Indeed, amides are known to be stable under normal conditions [23]. This stability enables the possibility to prepare larger sample sets simultaneously.

### 3.3.3. MS/MS

MS/MS experiments conducted for SA showed a preferred neutral loss of 135.1 u at medium collision energies (10 eV). Under the above stated low collision energy settings (4 eV), all acids besides αKG already showed this behavior to some extent. The MS/MS characteristics for SA are depicted in Supplementary material S1. This fragmentation pattern offers the possibility of neutral-loss analysis to selectively detecting MPEA derivatives only, and as the analyte structure is conserved in the formed fragment, the ability to further characterize the analyte itself by subsequent $MS^n$ experiments.

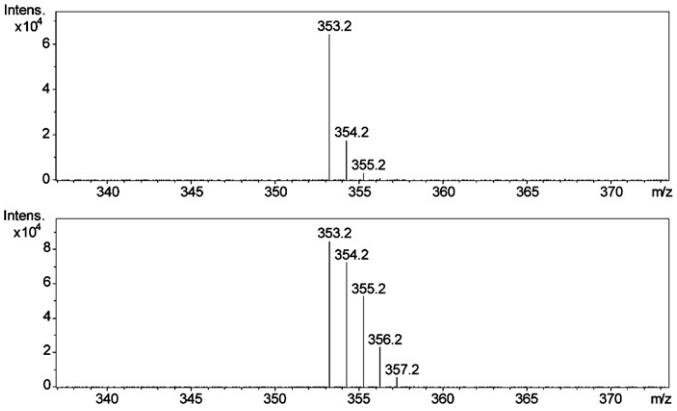


**Fig. 6.** Upper spectrum shows a natural isotopic pattern, where the lower spectrum indicates implementation of $^{13}$C-acetate into SA.

## 3.4. Application

### 3.4.1. Matrix effects

Post-column infusion of $MPESD_4$ resulted in no significant decrease in the response for $MPESD_4$ when injecting a hamster heart tissue extract on the online SPE LC–MS system (see Supplementary material S2). This indicates that endogenous compounds do not generate notable ionization suppression effects. As is shown in Table 1, also no significant deterioration in precision (%RSD) was observed when the acids were analyzed in a pig heart tissue matrix.

The incorporation of an online SPE step is beneficial when dealing with biological matrices as this provides a clean-up step to wash off any salts that could interfere with the separation or contaminate the ion source of the MS instrument.

### 3.4.2. $^{13}$C-labeled pig heart tissue

To illustrate the use of the developed protocol in metabolomic studies, we analyzed $^{13}$C-enriched pig heart tissue. The analyzed heart tissue extract originated from an experiment focused on the study of metabolic fluxes within the tissue. Thus, after the infusion of $^{13}$C-labeled acetate, incorporation of the $^{13}$C isotope in TCA cycle intermediates within the biological system of the animal could be determined and traced back. This type of information can be extremely useful to investigate particular metabolic pathways. Such studies especially benefit from the here described methodology when the labeling strategy is combined with high resolution time-of-flight mass spectrometry, allowing the highly sensitive determination of minute amounts of TCA cycle intermediates and accurate assessment of their corresponding isotopic patterns. This is demonstrated for SA in Fig. 6: by comparison of the isotope pattern for the heart tissue sample with that of an analytical standard, the implementation of $^{13}$C atoms in SA and other TCA cycle intermediates can be followed. This proves the applicability of the method.

### 3.4.3. Considerations of instrument type

An obvious instrument choice for targeted quantitative analysis would be a triple quadrupole mass spectrometer (QqQ MS), providing the highly selective and thereby sensitive selected reaction monitoring (SRM) data acquisition mode. However, this generally requires a quite effective separation of analytes prior to introduction to the MS instrument as (partially) overlapping of peaks may result in mutual ionization suppression, as observed in this study for SA and $SAD_4$. This would complicate quantitative analysis. If next to known carboxylic acids also unknown or less common acids need to be studied, the neutral loss analysis mode would be an appropriate choice when the described method of derivatizing

**Table 1**
Validation data.

| Compound | Slope[a] ($n=3$) | Intercept[a] ($n=3$) | $R^2$ in solvent standards | $R^2$ in heart tissue | Standards RSD (%) ($n=3$) | Matrix RSD (%) ($n=3$) | LOD[c] (S/N = 3) (nM) |
|---|---|---|---|---|---|---|---|
| MA | $7 \times 10^{-4} \pm 2.8 \times 10^{-5}$ | 1.92 ± 0.08 | 0.981 | 0.999 | 4.0 | 4.6 | 60 |
| FUA | $5 \times 10^{-4} \pm 2.0 \times 10^{-5}$ | 0.79 ± 0.03 | 0.990 | 0.990 | 4.0 | 4.7 | 200 |
| SA[b] | $4 \times 10^{-3} \pm 9.6 \times 10^{-5}$ | −0.35 ± 0.01 | 0.999 | 0.995 | 2.4 | 1.4 | 12 |
| αKG | $4 \times 10^{-4} \pm 1.3 \times 10^{-5}$ | −2.09 ± 0.07 | 0.991 | 0.996 | 3.2 | 1.8 | 1000 |
| ICA | $1.4 \times 10^{-3} \pm 6.6 \times 10^{-5}$ | −1.47 ± 0.07 | 0.998 | 0.996 | 4.7 | 2.7 | 50 |
| CA | $4 \times 10^{-3} \pm 1.8 \times 10^{-4}$ | 4.85 ± 0.22 | 0.998 | 0.988 | 4.5 | 1.9 | 40 |

Relative standard deviation (RSD) (intraday).
[a] Values referring to matrix calibration ($n=3$), $R^2$-values are calculated after correcting for IS TA.
[b] SA was corrected for $SAD_4$.
[c] Values calculated from aqueous calibration, concentrations referring to the sample concentration.

carboxylic acids is used, because a specific loss of the label ($m/z$ 135.1) can be observed in most cases. However, this technique is not very sensitive and additionally some derivatives will potentially not show the loss to the same degree as others, e.g., αKG versus SA. In this study, a MicrOTOF-Q was used for MS analysis, which has the benefit of being able to acquire data over a wide $m/z$ range with high speed, high mass accuracy, and accurate determination of the (possibly enriched) isotope patterns. This enables the detection of known derivatives without a specific neutral loss, while still allowing observation of neutral losses of the derivatives if they do show this behavior. Additionally, Q-TOF instruments are less sensitive in the lower mass range. The derivatization increases the mass of the analytes and thereby improves the sensitivity for such.

## 4. Conclusions and perspectives

The described procedure for the derivatization of clinically relevant carboxylic acids in biological matrices with MPEA enabled successful analysis at low concentrations. The SPE-LC-method aids to a fast and automated sample clean-up, reducing the introduction of salts and other contaminants into the MS ion source, thus prolonging the reliable use of the instrument. Without the need for special ultra-high-pressure pumps, the use of the core–shell column material allows short analysis times of less than 10 min, including separation and re-equilibration, and resulting in sharp peaks with widths down to 10 s at the base. Furthermore, the new approach resulted in LODs which were low enough to determine the analytes of interest in relevant matrices down to 12 nM, where other methodologies show LODs in the micromolar ranges. Additionally, the technique proved to potentially be a valuable tool for analysis of metabolic fluxes, when $^{13}C$-labeling is used. The complete method could become even more useful when an isotopically labeled variant of MPEA would be synthesized. This would enable a group specific internal standard technology [5] approach, allowing more analytes to be quantified without needing expensive isotopically labeled analytes to be used as internal standards. Automation of the derivatization, e.g., by means of a liquid handler could further improve reproducibility and enable a less labor intense sample preparation. A study of carboxylic acids in other biofluids such as urine, using this procedure, is presently ongoing.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.07.095.

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