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Improving liquid chromatography-tandem mass spectrometry determination of polycarboxylic acids in human urine by chemical derivatization. Comparison of *o*-benzyl hydroxylamine and 2-picolyl amine.

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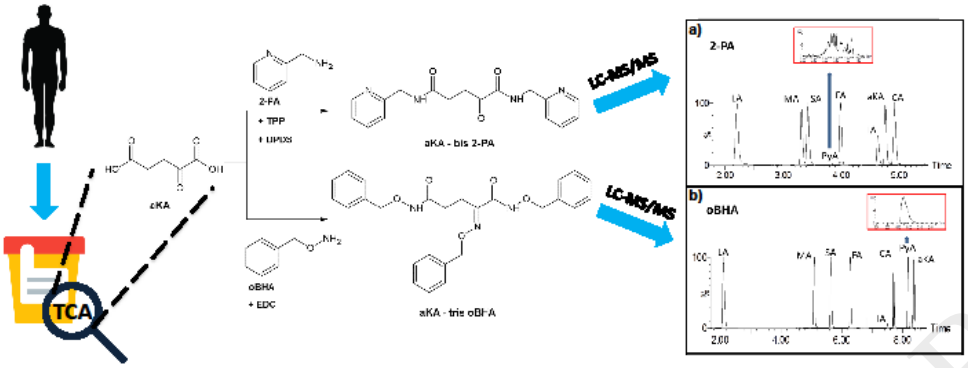
Graphical_Abstract



Highlights:

- Chemical derivatization is able to improve LC-MS/MS analytical performance.
- oBHA and 2-PA have been described as derivatizing agents for carboxylic acids.
- Their performance for the quantification of polycarboxylic acids is compared.
- oBHA provides better chromatographic behavior, sensitivity and robustness.
- oBHA is preferred for the determination of urinary polycarboxylic acids.

Abstract

Due to its high sensitivity and specificity, liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) could be considered as the gold-standard in targeted metabolomics. Although LC-MS/MS allows for the direct detection of a large number of molecules, the proper quantification of highly polar compounds such as poly-carboxylic acids in complex matrices like urine is still a challenge. Chemical derivatization offers a suitable way to improve chromatographic behavior and sensitivity for these compounds. Several derivatizing agents have been proposed for the LC-MS/MS determination of carboxylic acids but studies dealing with their comparison in challenging scenarios are scarce. Here we present the evaluation of two different derivatization agents; *o*-benzylhydroxyl amine (oBHA) and 2-picolyl amine (2-PA);

for the quantification of the (poly)-carboxylic acids belonging to the tricarboxylic acid cycle in urine. The suitability of both derivatizating agents was compared by validation of the two approaches. Derivatization with oBHA showed important advantages against 2-PA derivatization such as (i) providing better sensitivity, (ii) more stable derivatives and (iii) allowing for the proper validation of a larger number of analytes. Moreover, while 2-PA derivatization failed in the determination of the target analytes in some stored urine samples, oBHA derivatization successfully allowed for their appropriate determination in the same samples. A comparison between the concentrations obtained using oBHA derivatization and those provided by an external laboratory using UV and GC-MS detection revealed a satisfactory agreement between both results. Additionally, the concentrations obtained by the oBHA method for a set of 38 urines are in agreement with those previously reported in the literature. As a conclusion, our results show that the use of oBHA is preferred against 2-PA for the detection and quantification of (poly)-carboxylic acids in urine.

1. Introduction:

Metabolomics aims to the comprehensive study of the metabolites present in a biological system [1]. During the last decade, the use of metabolomics strategies has substantially grown being currently a common approach in a broad number of application fields [2–5]. Metabolomics approaches can be divided into targeted and untargeted. Untargeted metabolomics deals with the detection of the largest number of metabolites and is ideal for the detection of unexpected changes in metabolite levels. On the other hand, targeted metabolomics aims the accurate determination of a relatively small and specific number of metabolites being the best choice in order to obtain comprehensive and accurate information of a predefined pathway.

Liquid chromatography coupled to (tandem) mass spectrometry (LC-MS(/MS)) can be currently considered the gold standard in metabolomics studies due to its intrinsic selectivity, specificity, sensitivity and the capability of performing multiple compound analyses in single runs [6,7]. Whereas high resolution instruments are commonly used in untargeted approaches, the use of triple quadrupole instruments is preferred for targeted strategies due to their higher sensitivity. Additionally to this sensitivity, MS/MS based methods also provides an outstanding specificity based on the selection of specific transitions in selected reaction monitoring (SRM) methods [8,9]. In addition to this specificity, chromatographic separation also allows for distinguishing between isobaric metabolites and for overcoming some undesirable effects such as ionization suppression. Although this separation can be obtained by gas chromatography (GC) [10], liquid chromatography (LC) is the preferred alternative for metabolomics approaches [11,12].

Since targeted metabolomics methods aim for the quantitative determination of a limited number of metabolites, the whole analytical workflow (sample collection, clean up, extraction, chromatography and detection) should be carefully optimized for the determination of the targeted analytes. Among the different alternatives, chemical derivatization is useful for many reasons. Firstly, derivatizing agents can transform poorly ionizable or non-ionizable compounds into easily detectable ones [13,14]. Secondly, the addition of extra groups in the molecule might change the physico-chemical properties of the metabolite favoring a proper chromatographic behavior. Finally, the chromatographic changes induced by the derivatization can help to reduce the ion suppression produced in the MS ion source [11]. For these reasons, derivatization has become a common strategy in targeted metabolomics methods dealing with the detection of both poorly ionizable and poorly chromatographed analytes [15]. Poly-carboxylic acids are among that last category of analytes.

A broad variety of derivatizing agents are used in LC-MS/MS. For instance, Girard's reagents and hydroxylamine are used for derivatizing ketones [16,17], dansyl chloride is used for aliphatic alcohols [18] phenols and amines [19], 2-picolyl amine (2-PA) and N-methyl-2-phenylethanamine are used for carboxylic acids [20–23] and *o*-benzylhydroxyl amine (oBHA) [24,25] is used for the derivatization of both ketones and carboxylic acids.

This study is focused on the development of targeted metabolomics approaches for the determination of poly-carboxylic acids in urine testing both oBHA and 2-PA as derivatizing agents. On the one hand, 2-PA can react with carboxylic acids resulting in the formation of

amides (**Fig 1**). On the other hand, oBHA reacts not only with carboxylic acids but also with ketones for the formation of oxyamides and oximes respectively (**Fig 1**). Tricarboxylic acid cycle (TCA) (**Fig 2**) has been selected as a model of a metabolic pathway of poly-carboxylic acids. TCA has the particularity of the simultaneous existence of monocarboxylic acids (pyruvic and lactic acid), dicarboxylic acids (α-ketoglutaric, succinic, fumaric, malic and oxaloacetic acid) and tricarboxylic acids (citric, cis-aconitic and isocitric acid). Moreover, urine has been chosen as matrix model for method development. The suitability of urine in testing different approaches for TCA determination lies not only in its high relevance as a marker of several imbalances [26–32] but also in the great complexity of the matrix, the potential bacterial degradation of the samples and the high abundance of carboxylic acids which would hamper the proper derivatization of the analytes.

Previous work performed by our research group demonstrated the usefulness of 2-PA for the determination of monocarboxylic acids in complex matrices such as urine [21,22] although its suitability for derivatization of polycarboxylic acids in complex matrices remains unexplored. On the other hand, the usefulness of oBHA for the derivatization of polycarboxylic acids has been demonstrated [24,25] although its suitability for the determination of these metabolites in complex matrices has not been proved. Thus, the aim of this study is to compare 2-PA and oBHA derivatizing agents for the determination of poly-carboxylic acids in human urine. The study is focused on the comparison of both approaches in terms of sensitivity, precision, accuracy and matrix effect. The application to real samples is used as additional factor to conclude which of the two tested approaches is preferred for the quantification of TCA metabolites in urine.

2. Materials and methods:

2.1. Chemicals

Citric acid (CA), citric acid-2,2,4,4-d4 (CA-d4), isocitric acid (IA), α-ketoglutaric acid (aKA), succinic acid (SA), succinic acid-2,2,3,3-d4 (SA-d4), fumaric acid (FA), fumaric acid-^{13}C4 (FA-^{13}C4), malic acid (MA), pyruvic acid (PyA), lactic acid (LA), lactic acid-^{13}C3 (LA-^{13}C3), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *o*-benzyl hydroxylamine (oBHA), triphenylphosphine (TPP), 2,2-dipyridyl disulfide (DPDS), 2-picolylamine (2-PA), creatinine and ammonium formate (HPLC grade) were purchased from Sigma Aldrich (St. Louis, MO, USA). Malic acid-d3 (MA-d3) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Formic acid (LC/MS grade), acetonitrile, methanol, pyridine, hydrochloric acid and ethyl acetate were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.2. Preparation of standard solutions

Stock solutions (1 mg/mL) for each analyte were independently prepared by diluting adequate amounts of standards in ultrapure water. The working solutions were prepared by mixture of the stock solutions and dilution in ultrapure water. Generally the set of standard solutions ranged from 10 ng/mL to 800 µg/mL. Working internal standard (ISTD) consisted in a mixture containing 100 ng/mL for SA-d4 and FA-^{13}C4, 1.2 µg/mL for LA-^{13}C3 and 10 µg/mL for MA-d3 and CA-d4 in ultrapure water.

2.3. Preparation of TCA-free urine

Due to the endogenous nature of the TCA analytes, an artificial TCA-free matrix was prepared in order to estimate both the LLOQ and the ULOQ. For this purpose, six different matrices were pooled and 1 mL of the pool was passed through a C18 cartridge (Sep-Pak C18 cartridges, 500 mg, Waters) previously conditioned with 2 mL of methanol and 2 mL of water. After washing with 2 mL of water, urinary components were eluted with 2 mL of methanol. The organic extract was evaporated and reconstituted with 2 mL of saturated NaCl.

2.4. Sample preparation and derivatization

2.4.1. oBHA

An aliquot of 5 µL of urine (or standard) was mixed with 30 µL of ISTD solution. Then, oBHA derivatization was performed based on the strategy previously described by Tan *et al.* [24]. Briefly, freshly EDC 1 M was prepared in pyridine buffer (5.4 mL of fuming HCl, 8.6 mL of pyridine and 86 mL of ultrapure water at pH 5.0) and freshly oBHA 1 M solution was prepared

in acetonitrile:ultrapure water (3:2). To assure the proper homogeneity of the derivatization reagents, both 1 M EDC and 1 M oBHA solutions were mixed and sonicated. For sample derivatization, 100 µL of the oBHA:EDC mixture was added in the sample vial. After 60 minutes of reaction at room temperature, 1 mL of ultrapure water was added and the mixture was extracted with 4 mL of ethyl acetate by shaking in a vortex at maximum power during 1 minute. After centrifugation (3800 rpm, 5 minutes), the organic layer was separated and dried under a nitrogen stream in a water bath at 40 ºC and 15 psi. The extracts were reconstituted in 150 µL of ultrapure water:methanol (1:1) resulting in a final 30 fold dilution of the sample. Finally, 10 µL of the mixture was injected into the LC-MS/MS system.

2.4.2. 2-PA

An aliquot of 5 µL of urine (or standard) was mixed with 30 µL of ISTD mixture. The solution was then derivatized with 2-PA following the strategy proposed by Higashi *et al.* [20]. Concisely, the derivatization was performed in acetonitrile medium by the addition of 750 µL of freshly prepared TPP 2.6 mg/mL, 750 µL of freshly prepared DPDS 2.2 mg/mL and 750 µL of 1 mg/mL of 2-PA (stored at 4 ºC) in the sample. The mixture was left to react during 10 minutes at 60 ºC. After cooling at room temperature, the mixture was evaporated under a nitrogen stream in a water bath at 48 ºC and 15 psi. The extracts were reconstituted in 150 µL of ultrapure water:methanol (9:1) resulting in a final 30 fold dilution of the sample. Finally, 10 µL of the mixture was injected into the LC-MS/MS system.

2.5. LC-MS/MS conditions

The chromatographic separation and detection of the analytes was performed using an Acquity UPLC system (Waters Associates, Milford, MA, USA) coupled to a triple quadrupole (Quattro Premier) mass spectrometer provided with an orthogonal Z-spray-electrospray interface (ESI) (Waters Associates).

The LC separation was carried out at 55ºC using an Acquity BEH C18 column (100 mm x 2.1 mm i.d., 1.7 µm) (Waters Associates) at a flow rate of 300 µL/min. Water-ammonium formate (1 mM)-formic acid (0.01 %) and methanol-ammonium formate (1 mM)-formic acid (0.01 %) were selected as mobile phases.

The MS used nitrogen as drying gas and nebulizing gas. The desolvation gas flow was set at 1200 L/h and the cone gas 50 L/h. The selected capillary voltage was 3 kV in positive ionization mode. The nitrogen desolvation temperature was 450 ºC and the source temperature 120 ºC. The collision gas was argon at a flow of 0.21 mL/min.

The detection of the derivatized analytes was performed by the selected reaction monitoring (SRM) methods described in Table 1. Both methods included 2 transitions for each analyte and the most specific one was selected for the quantification. In the case of oBHA derivatization, a gradient program was used the percentage of organic solvent linearly changed as follows: 0 min, 30 %; 1 min, 30 %; 6 min, 55%; 6.8 min, 80%; 8.3 min, 99%; 9 min, 99%; 9.01 min, 30%; 10

min, 30%. For PA derivatization the percentage of organic solvent linearly changed as follows: 0 min, 5 %; 1 min, 5 %; 7.5 min, 50%; 8 min, 99%; 10 min, 99%; 10.1 min, 5%; 11.5 min, 5%.

2.6. Validation

The method validation was performed taking into account the impossibility to obtain a blank matrix due to the endogenous presence of the analytes. The following parameters were evaluated:

2.6.1. Linearity

Due to the endogenous nature of the analytes, linearity was evaluated in ultrapure water. Calibration standards were prepared in triplicate at seven concentration levels for each analyte in the expected concentrations obtained after a pre-analysis of real samples. Calibration standards were analyzed and calibration curves were estimated by least-squares linear regression using a weighting factor of 1/x. Based on EMA regulations [33], the back calculated concentrations of at least 75% of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. In case replicates are used, the criteria (within ±15% or ±20% for LLOQ) should also be fulfilled for at least 50% of the calibration standards tested per concentration level.

2.6.2. Estimation of the LLOQ and the ULOQ

The estimation of the LLOQ and ULOQ was performed by spiking 6 replicates of the TCA-free urine (see section 2.3) with known amounts of the analytes at two levels. Levels were selected based on the results obtained in the analysis of real samples. For LLOQ the following levels were selected: 6.7 µg/mL (CA), 1.3 µg/mL (IA), 0.6 µg/mL (aKA), 0.3 µg/mL (SA), 0.03 µg/mL (FA), 0.05 µg/mL (MA), 0.5 µg/mL (PyA) and 4 µg/mL (LA). ULOQ was tested at the following levels: 2500 µg/mL (CA), 500 µg/mL (IA), 250 µg/mL (aKA), 100 µg/mL (SA), 10 µg/mL (FA), 10 µg/mL (MA), 90 µg/mL (PyA) and 150 µg/mL (LA). Based on EMA regulations [33] LLOQ was validated if accuracies between 80% and 120% and RSD < 20% were obtained. ULOQ was validated if accuracies between 85% and 115% and RSD < 15% were obtained.

2.6.3. Calculation of concentrations using the standard addition method

For calculating sample concentrations, each urine sample containing an unknown concentration of endogenous analytes was divided into 10 individual aliquots: three of them were not spiked whereas the remaining were spiked with known and increasing amounts of analytes with the same concentration levels of the calibration curve. In this way, a standard curve could be created for every analyte in exactly the same matrix in which the analysis was performed. A solvent calibration curve was also injected in the same batch. Two

concentrations were obtained: the “calculated concentration” and the “real concentration”. A visual representation of both is shown in **Supplementary Figure 1**.

Firstly, the “calculated concentration” of the sample was obtained by the interpolation of the response (analyte/internal standard) obtained for the three non-spiked aliquots in the solvent standard curve.

Secondly, for establishing the “real concentration” a standard curve was generated for every analyte in every tested sample. Four of the multiple added concentrations points were selected for performing the standard addition curve: the corresponding at 0 (average response of the instrument of the three non-spiked samples), the added concentration similar to half of the “calculated concentration” (X/2), the added concentration similar to the “calculated concentration” (X), and the added concentration similar to twice the “calculated concentration” (2X). The rest of the points were discarded for the analysis. Then, the “real concentration” in the sample was determined by the extrapolation of the regression line as the negative X-intercept of the calibration curve prepared for that particular analyte (**Supplementary Figure 1**)

2.6.4. Intra- and inter-assay accuracy and precision

Intra- and inter-assay accuracy and precision were evaluated by the standard addition approach in 12 different urine samples. Accuracy values for every analyte in each matrix were calculated by dividing the “calculated concentration” by the “real concentration” (see subheading 2.6.2) and it was expressed as a percentage.

The 12 urine samples were analyzed in three different experimental days (six matrices on the first assay, and three matrices on the second and third assays). Intra-day accuracy was calculated as the average of the 6 individual accuracy values of the 6 matrices analyzed on the first validation assay. Inter-day accuracy of the method was calculated as the average of the 12 individual accuracy values of the 12 matrices analyzed over the three validation assays.

Intra-day precision was calculated as the relative standard deviation (RSD) of the 6 accuracy values obtained on the first validation assay. Inter-day precision was calculated as the RSD of the 12 matrices analyzed over the validation assays. The method was considered accurate and precise if accuracies were in the range 80%-120% and standard errors were below 20%.

2.6.5. Limit of detection (LOD) and sensitivity comparison

The LOD was defined as the lowest concentrations with a value of the single/noise ratio (S/N) of 3. The calculation of LOD in solvent was performed by estimating the S/N from the chromatogram of TCA intermediates at the lowest calibrator’s concentration assayed.

Relative sensitivity was used for comparison of the two derivatization procedures in matrix. For this purpose, the average of the S/N values for the basal real samples used at the validation procedure was calculated for each analyte and derivatization strategy.

2.6.6. Matrix effect

The matrix effect was estimated by comparison between the result of the response of the analyte (and ISTD) in the urine samples spiked at the highest concentration (subtracting the contribution of the unspiked urine samples) and the one of the standard prepared in solvent at the same concentration. For the matrix effect evaluation, both unnormalized data and results normalized by ISTD were tested.

2.6.7. Stability and carry-over

Short-term stability of urinary TCA analytes was checked before and after storing samples at -20 ºC and 4 ºC during 72 h. Moreover, freeze-thaw cycle stability was also tested by comparison between fresh samples and 3 times freeze-thaw urines (n=3). Based on the EMA criteria [33],analytes were considered stable if the mean concentrations were within ±15% of the concentrations found after analysis of the fresh sample.

The carry-over was determined from the injection of different blank samples after the injection of high concentration standard. The absence of interference was accepted when the response was less than 20% of the lower calibrator concentration and 5% in the ISTD [33].

2.7. Urine samples

Six spot urine samples collected from six healthy volunteers were used for method validation. In order to minimize degradation of CA, 200 µL of HCl 6 N were added to 10 mL of sample after collection [34].

The applicability of the method was tested by the analysis of spot urine samples collected from 20 healthy volunteers (10 females, 7-57 years old and 10 males, 8-59 years-old). Sample collection was performed in the frame of a clinical study approved by the Local Ethical Committee (CEIC-IMAS, Institut Municipal d'Assistència Sanitària, Barcelona, Spain, project number 2014/5553/I). From the 20 samples, 12 were freshly collected and 8 stored at -20 ºC in periods ranging from 6 to 24 months.

For the comparison of the results with the obtained in external analysis, a set of 12 spot urine samples was collected from healthy volunteers (5 males, 26-44 years old, 7 females 24-45 years old) and divided into three aliquots. Two of them were externally analyzed (see section 2.8) and the other one was analyzed by the developed method.

In order to compare the results with the normal ranges previously reported in the literature, results of the 38 samples previously described were normalized by creatinine (µmol

analyte/mmol creatinine) determined by standard methods using Infinite M200 equipment (Tecan Trading AG, Switzerland). 2.8. External analysis

Two of the aliquots (10 mL each) of the set of 12 samples selected for comparison were frozen and submitted to an external laboratory (Laboratorios Echevarne; wwws.echevarne.com) which has in the portfolio the determination of four of the selected analytes (CA, LA, aKA and FA). Urinary concentration of CA was measured by UV whereas LA, aKA and FA were determined by GC-MS.

3. Results and discussion

3.1. Method optimization

3.1.1. Sample storage stability

The proper determination of some acids in urine can be affected by storage conditions [34,35]. Therefore, the stability of the analytes in urine (n=6) at two temperatures (4 ºC and -20 ºC) was evaluated (see **Supplementary Table 1**). In accordance with previous findings [34], the only compound that showed significant differences in concentration after 72 h storage was CA, whose levels decreased down to around 20% of the initial value after 72 h at 4 ºC. These differences were not observed after short-time storage at -20 ºC. The reduction of the pH by the addition of HCl (200 µL of HCl 6 N per 10 mL of sample) into samples diminish CA degradation in line with previous results [34,35]. The effect of HCl has been reported to be related with avoiding CA hydrogenation [36]. Therefore, results indicate that it is recommended either to analyze the sample briefly after collection or to immediately freeze it. If possible, the addition of HCl as soon as possible is also advisable in order to avoid CA degradation.

3.1.2. Derivatization

The main structural feature of the analytes from TCA cycle is the presence of several carboxylic acids. Thus, different species can be potentially obtained for one analyte, i.e. mono-derivative, bis-derivative and tris-derivative, depending on the reaction yield. Therefore, optimization of the derivatization process is a critical step of the method development.

One of the factors that might alter the derivatization yield in the analysis of urine is the presence of large amounts of compounds with carboxylic acids which can compete with the analytes for the derivatizating agent. Thus, both sample and derivatizating agent volumes used might be critical in obtaining a proper yield.

The influence of the sample volume used in the analysis was tested for both derivatizing agents. Volumes ranging from 5 µL to 100 µL were examined. The optimal volume was found to be 5 µL of urine samples for both oBHA and 2-PA derivatizing agents. The use of higher volumes started to decrease the derivatization yield increasing the presence of partially-derivatized analytes.

Furthermore, other factors such as derivatizing agents' volume, reaction temperature and derivatization time was optimized for both oBHA and 2-PA. Regarding oBHA, the addition of 50 µL of EDC 1 M and oBHA 1 M in standards and samples and the reaction time of 1 hour at room temperature previously reported for other matrices [24,25] were found to be the optimal conditions also in urine. The addition of larger volumes, larger reaction times or higher temperatures did not improve reaction yield (**Supplementary Figure 2**).

In the case of 2-PA, initial tested conditions were conducted by adding 50 µL of TPP (10 mM), DPDS (10 mM) and 2-PA (10 mM) during 10 minutes at 60ºC as previously reported in pretreated saliva [20]. No increases in reaction yield were found by varying temperature and time conditions. However, the increase on addition derivatizing agent (addition of 750 µL instead of 50 µL) improved the reaction yield in urine, especially for tricarboxylic compounds e.g. CA.

3.1.3. Chromatography

Accurate chromatographic resolution is important for the determination of TCA metabolites. Firstly, it is required for the separation of isobaric analytes sharing the same SRM transitions (e.g. CA and IA; **Table 1**). Secondly, the molecular mass of some TCA metabolites only differ in 2 Da (**Table 1**). In these cases, the ^{18}O isotopes of the lightest analyte can interfere in the detection of the heaviest one. That is especially important when both analytes are present at different concentration ranges. Specifically, we observed a substantial interference of FA-$^{13}C_4$ with SA. Thus, the separation of these analytes is required.

In a recent publication [37], the direct detection of TCA analytes by LC-MS/MS was explored although the poor retention of the analytes in a C18 column hampered the required separation. We tried to improve the chromatographic behaviour by using HILIC columns but the results were not satisfactory regarding peak shape. The reaction of TCA analytes with both oBHA and 2-PA promotes the conversion of high polar compounds into medium to low polar analytes increasing their retention in the C18 column. Since 2-PA derivatives are commonly more polar than oBHA ones, two different gradients were optimized. For 2-PA derivatives it was required to start the gradient with a low percentage of organic solvent (5%). In contrast, a proper chromatographic behavior could be obtained for oBHA derivatives by starting in a 30% of organic solvent allowing for a softer slope of the gradient. Both methods allowed for the chromatographic separation of all analytes in around 10 min (**Fig 3**).

Regarding chromatographic behavior of the two selected derivatizations strategies, both 2-PA and oBHA derivatives have similar patterns for most of the target analytes. The only exception was found to be PyA. Whereas PyA-oBHA shows a suitable peak shape (**Fig 3a**), derivatization with 2-PA provided an inappropriate chromatographic behavior which can induce to PyA misidentification (**Fig 3b**). These unsatisfactory results were not improved by addition of increased amounts of salts into the mobile phase.

3.1.4. MS

The formation of amides in 2-PA derivatives and the formation of oxyamides and oximes in oBHA derivatives imply the addition of nitrogen atoms in the analytes. The presence of nitrogen atoms in both derivatives promote the ionization of analytes as $[M+H]^+$ (**Table 1**). No abundant adducts were observed in the scan mode.

The collision induced dissociation of the $[M+H]^+$ precursor ions was established for each analyte at three collision energies. A common fragmentation pattern was observed depending on the derivatizing agent. On the one hand, oBHA derivatives commonly showed the product ion at m/z 91 and a less sensitive one at m/z 124 (**Fig 4**). Moreover, a neutral loss of 123 Da was also observed. On the other hand, 2-PA derivatives commonly showed product ions at m/z 92, 109 and the neutral loss of 108 Da. The proposed fragmentation pathways are shown in **Fig 5** with aKA as example. As can be seen in the figure, the presence of the derivatizating agent guided the fragmentation and all common ions are related with the moiety added with the derivatization.

Two SRM methods were developed for the determination of oBHA and 2-PA derivatives respectively (**Table 1**). Two transitions were selected for each analyte based on their specificity and sensitivity. For several analytes, the most abundant transition -of both derivatization agents- did not always corresponded to the most specific one. In these cases, the most specific transition was selected for quantification being the most abundant one used for qualitative purposes. In general, transitions involving the neutral loss of 123 Da and 108 Da were selected for quantification of oBHA and 2-PA derivatives, respectively. Transitions for oBHA derivatives were similar to those previously published [25].

As stated above, low reaction yields can occur during the derivatization procedure. Tricarboxylic acids such as CA are more prone to suffer from incomplete derivatization. Although this undesirable effect has not been reported in culture cell [25], it is a potential limitation in more complex matrices like urine. Since reaction yield can differ sample to sample, it was monitored by selection of a transition for the bis-derivatized form (**Table 1**). The formation of multiple derivatives might be problematic for some analytical purposes mainly related with the sensitivity and precision of the method. In the studied scenario, the large amounts of CA found in urine minimize the effect of the formation of multiple derivatives on sensitivity. Additionally, the use of CA-d4 in the ISTD mixture corrects potential moderate-low variations in derivatization efficiencies and therefore minimizes the effect of the multiple derivatives on precision. In any case, monitoring CA-bis-derivatives was selected as an adequate marker for testing global derivatization process.

3.2. Method validation

Two methods for the quantification of the analytes (based on 2-PA and oBHA derivatization, respectively) were validated in human urine.

3.2.1. Linearity

Real analyte-free matrix for method validation was unavailable due the endogenous nature of TCA analytes in urine. As previously reported by our laboratory for the validation of other endogenous metabolites [12], calibrators were prepared in ultrapure water.

The calibration range and the correlation coefficient obtained for each analyte (**Table 2**) was established according to the expected concentration in human urine, that were estimated from a set of different spot urines collected from healthy volunteers. In the case of oBHA derivatives, the method was found to be linear for all analytes with back calculated concentrations within the established variations of the nominal value in more than 80 % of the standards. Remarkably, two of the analytes (aKA and PyA) were more accurately quantified without ISTD correction than with the correction by any of the labeled ISTD used. These two analytes show a keto function in alpha position to the carboxylic acid whereas none of the selected labeled ISTD had this function. This structural consideration can be behind the observed behavior for these analytes. It is expected that the addition of at least one ISTD with these characteristic can help in the proper determination of these compounds. Regarding the other analyte without its own labeled ISTD (IA), it was successfully corrected with CA-d4 since both have a similar structure.

A similar behavior was observed for 2-PA derivatization. Thus, the method was found to be linear for all analytes with back calculated concentrations within the established variations of the nominal value in more than 75 % of the standards. Regarding ISTD, a proper correction was only achieved in those analytes with their labeled counterpart. Thus, the best results for IA and aKA were obtained without ISTD correction. As previously stated in section 3.1.3, the inappropriate peak shape of the 2-PA derivative of PyA hampered the evaluation of the linearity for this analyte.

3.2.2. Estimation of the LLOQ and ULOQ

The use of artificial matrices for the whole validation process was rejected since they do not contain an important part of the matrix and therefore they would not reflect the problems associated with the urinary interferences and the aforementioned low derivatization yields. However, spiking an artificial TCA-free matrix was the only way to estimate both LLOQ and ULOQ. The selected concentrations were adjusted to the expected concentration ranges for each analyte. Results are summarized in **Table 2**.

The derivatization with oBHA allowed for the proper quantification at the LLOQ for all analytes with recoveries ranging from 96% to 113% and RSD < 15 %. Similarly, the ULOQ could be satisfactorily estimated sine recoveries between 91% and 110 % and RSD < 12% were obtained.

In contrast with the fully satisfactory results with oBHA derivatization, the approach based on 2-PA derivatization failed in the proper quantification of some of the analytes. Thus, although most of the analytes fulfilled the requirements at both the LLOQ and the ULOQ, IA showed a RSD > 20 % at the LLOQ (27%) and aKA showed a RSD > than 15 % at the ULOQ (19%). Remarkably, none of the internal standards used in the approach was found to be adequate for the correction of these analytes. The absence of adequate internal standards may be behind the inadequate RSD obtained for these analytes.

3.2.3. Accuracy and precision

Both LLOQ and ULOQ were estimated by using an artificial matrix. Therefore, the effect of matrix interferences (not present in TCA-free urine but present in real urine) on the quantification of the analytes cannot be properly evaluated. For that reason, we used the standard addition approach for the evaluation of accuracy and precision in real matrix. Standard additions were performed in six different urine samples. Endogenous levels of the analytes (**Table 3**) in these samples covered a wide range of the estimated concentrations.

Substantial differences on accuracy and precision were observed depending on the derivatizing agent. Thus, when using oBHA derivatization, both intra- and inter-day accuracies were found to be suitable with recoveries ranging from 80 to 120% for all analytes (**Table 3).** RSD ≤ 20% were obtained for all analytes in both intra- and inter-day precision experiments.

In contrast, poor accuracy and precision values were obtained after 2-PA derivatization. Thus, 2-PA derivatization yields highly depended on chemical structure, being essential to quantify target compounds by using labeled ISTD. Therefore, only those metabolites with labeled internal standards showed recoveries ranging between 80 and 120% and RSD ≤ 20% (**Table 3**). Unlike results obtained after oBHA derivatization, unacceptable inter- and intra-day precision (RSD ranging from 38% to 68%) were obtained for IA and aKA, although unsuitable results may be corrected by using an appropriate labeled internal standards.

3.2.4. Sensitivity

Because high amounts of the target analytes in urine are present in urine, sensitivity was not critical in this biological matrix. However, sensitivity can be important when transferring the methodology to other matrices, such as plasma or saliva. In this study, it was found that oBHA derivatization generally allowed for obtaining either similar or lower LODs than 2-PA in solvent (**Table 2**).

Relative sensitivity in matrix (expressed as S/N) also depends on matrix effects and therefore, their comparison reflects better the method sensitivity for a specific matrix. According to this, 2-PA derivatives are more sensitive in urine samples for LA and FA, while oBHA derivatization resulted in a more sensitive approach for CA, aKA, MA and especially for PyA and IA.

3.2.5. Matrix effect

Matrix effect may be relevant in both, the derivatization step and the inhibition of the ionization in the electrospray source produced by matrix interferences. Besides the absolute matrix effect (expressed as recovery), the consistency of the effect with the matrix was evaluated by calculating the RSD of the effect in several matrices (n=6). The requirement of ISTD correction was evaluated by testing both unnormalized and normalized matrix effect. Matrix effect was evaluated using six different urine samples.

Regarding oBHA derivatization no substantial matrix effects were observed for most compounds even without correction by ISTD. The main exception of the tricarboxylic acids (CA, CA-d4 and IA) for which recoveries close to 200% with a RSD > 100% were obtained without normalization by ISTD (**Table 3**). These effects could be associated with the difficulties found in the derivatization process for tricarboxylic acids. The correction of CA and IA data by CA-d4 allowed for obtaining appropriate recoveries (89 %) and RSD (< 15%) for both analytes. These results showed that the use of the labeled ISTD (CA-d4) is critical for the proper quantification of tricarboxylic acids.

Similar results for tricarboxylic acids were obtained after 2-PA derivatization (**Table 3**). Additionally, poor recoveries and RSD values were found for α-keto-carboxylic acids (PyA and aKA) without correction by the ISTD. As stated before, the absence of a labeled ISTD with α-keto-carboxylic acid hampered the proper correction of the matrix effects even after normalization. Therefore we could conclude that the use of 2-PA as derivatizing agent is not a suitable approach for PyA and aKA quantification.

3.2.6. Stability and carry-over

Short-term stability of the analytes at different storage conditions was confirmed as described in section 3.1.1. Regarding the stability of both oBHA and 2-PA derivatized analytes in the extracts, it was evaluated during 3 days at 10 ºC (sample manager temperature). Thus, oBHA derivatized analytes, were found to be stable up to 3 days after derivatization in agreement with previous reports [24]. In contrast, it was not possible to appropriately quantify 2-PA derivatized analytes at day 3, as sensitivity decreased significantly.

Regarding freeze-thaw stability, mean concentrations were in the range 93%-107% for all analytes after three freeze-thaw cycles. Finally, no important carry-over was observed after the injection of the most concentrated standard.

3.3. Method application

As a final step of the comparison between the two derivatization approaches, the applicability of the two methods was evaluated by the analysis of 20 spot urine samples (12 fresh samples and 8 stored samples). Although reliable results were obtained for both derivatizing agents in the analysis of freshly-collected urine samples (n=12), performance observed in fresh urine samples was not reproduced in stored ones. Thus, whereas oBHA derivatization succeeded in the quantification of all the TCA metabolites, 2-PA failed in the determination of all analytes (including the ISTDs) in 4 out of 8 stored samples (**Fig 6**). Reanalysis of the same samples confirmed the poor performance of 2-PA derivatization for some of the stored samples. Therefore oBHA derivatization was selected for the determination of TCA metabolites in human urine.

A set of 12 urine samples was simultaneously analyzed in an external laboratory for four of the analytes (CA, aKA, LA and FA). The comparison of the results is summarized in **Fig 7**. Excellent

correlation was found for the results of CA and aKA with a slope close to 1 and a correlation coefficient higher than 0.99. Satisfactory results were obtained for LA although in that case the dispersion of the results was higher and the correlation coefficient lower (r =0.97). In the case of FA, the external laboratory only reported quantitative results for two out of the 12 samples being the rest below their LLOQ. In contrast, the presented method was able to determine the FA in all the samples. Remarkably, the two reported values were the most concentrated ones and reported concentrations were in agreement with the ones obtained by oBHA derivatization (concentrations 83% and 117% of the external concentrations).

Finally, the results obtained after oBHA derivatization for the 38 samples analyzed (6 samples for validation, 20 for comparison between derivatizating agents and 12 for comparison with external results) were compared with those reported in the literature. As it can be seen in **Table 4**, the method was able to properly quantify the endogenous values presented in all samples analyzed. Additionally, after correction by creatinine the results obtained by the developed method based on oBHA derivatization were in agreement with the previously reported reference values. Thus, it confirmed the suitability of the quantification of TCA analytes performed by the developed method.

Conclusion

The potential of oBHA and 2-PA as derivatizing agents for polycarboxylic acids has been evaluated. The determination of TCA analytes in human urine has been selected as a case of study. Although derivatization with 2-PA was less time-consuming and consequently preferable in terms of throughput, oBHA derivatization showed several critical advantages that make it the strategy of choice for urinary TCA determination. Firstly, PyA was only properly detectable after oBHA derivatization. Additionally, oBHA derivatization allowed for the validation of 8 TCA metabolites whereas only 5 could be validated with 2-PA. Finally oBHA derivatization could be successfully applied to both freshly collected and stored urine samples whereas 2-PA derivatization failed in some of the stored samples. Thus, results indicated that derivatization with oBHA is preferred against 2-PA derivatization in the determination of poly-carboxylic acids.

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Tables

Table 1. Molecular weight (MW) and SRM conditions for the selected analytes.

		oBHA						2-PA					
Analytes	**MW (g/mol)**	**RT (min)**	**Precursor m/z**	**Product m/z**	**Cone voltage (V)**	**Collision voltage (eV)**	**Specie**	**RT (min)**	**Precursor m/z**	**Product m/z**	**Cone voltage (V)**	**Collision voltage (eV)**	**Specie**
SA	118	5.79	329	91 206[a]	20	25 10	Bis	3.47	299	92 109 191[a]	15	30 10 20	Bis
FA	116	6.42	327	91 204[a]	15	20 10	Bis	3.98	297	92 189[a]	15	30 20	Bis
MA	134	5.22	345	91	15	20	Bis	3.35	315	109	15	20	Bis

				222[a]		10				207[a]			
CA	192	6.27/ 7.81	385/508	91/91 181/385[a] 233	15	20/25 20/10 10	Bis/ Tris	2.89/ 4.91	463/373	109/109 229/265 355[a]	15	30 20	Bis/ Tris
IA	192	7.59	508	91 385[a]	15	25 10	Tris	4.57	463	229 355[a]	15	30 20	Tris
aKA	146	8.47	462	91 339[a]	15	20 5	Tris	4.84	327	173 201[a]	15	30 20	Bis
LA	90	2.22	196	91 124[a]	15	10 5	Mono	2.24	181	92 109[a]	15	20 10	Mono
PyA	88	8.28	299	91 181[a]	15	20 10	Bis	3.85	179	92 109[a]	15	25 15	Mono

[a] Transition used for quantification.

Table 2. Analytes, internal standard used for each analyte, linearity and LOD for the studied analytes. LLOQ: Lowest Limit of Quantification, ULOQ: Upper Limit of Quantification, Rec: Recovery.

	oBHA								2-PA							
Analytes	ISTD	Linearity		LOD (µg/mL)	LLOQ*		ULOQ*		ISTD	Linearity		LOD (µg /mL)	LLOQ*		ULOQ*	
		Range (µg/mL)	r		Rec. (%)	RSD (%)	Rec. (%)	RSD (%)		Range (µg/mL)	r		Rec. (%)	RSD (%)	Rec. (%)	RSD (%)
CA	CA - d_4	1.2 – 2500	0.997	0.5	112	9	105	8	CA - d_4	4 – 2500	0.990	0.5	116	12	96	6
IA	CA - d_4	0.4 – 800	0.998	0.1	106	15	91	12	-	30 - 260	0.996	1	116	27	80	14
aKA	-	0.3 – 200	0.998	0.2	109	6	100	9	-	7.5 - 200	0.953	1	84	14	108	19
SA	SA - d_4	0.27 – 100	0.997	0.15	96	8	96	7	SA - d_4	1.2 – 100	0.989	0.15	114	3	91	3
FA	FA - $^{13}C_4$	0.03 – 20	0.995	0.005	103	14	91	5	FA - $^{13}C_4$	0.08 - 20	0.992	0.01	94	5	94	3
MA	MA - d_3	0.04 – 10	0.994	0.01	97	8	101	8	MA - d_3	1.1 – 10	0.992	0.25	114	18	94	7
PyA	-	0.5 – 110	0.995	0.5	111	6	110	10	-	-	-	-	-	-	-	-
LA	LA - d_3	0.6 - 150	0.998	0.5	113	14	98	6	LA - d_3	5.5 - 150	0.990	0.5	103	19	103	5

* For concentration values of the estimated LLOQ and ULOQ see section 2.6.2.

Table 3. Accuracy, precision and matrix effect. Endog: Endogenous, Rec: Recovery.

Analytes	Endog. range of validation samples (µg/mL)	oBHA								2-PA							
		Intraday (n=6)		Interday (n=12)		Unnormalized matrix effect (n=6)		Normalized matrix effect (n=6)		Intraday (n=6)		Interday (n=12)		Unnormalized matrix effect (n=6)		Normalized matrix effect (n=6)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)
CA	264 - 745	116	11	114	15	179	121	89	12	116	20	110	18	173	173	107	17
IA	39 – 155	110	19	110	18	205	116	89	15	192	68	200	62	120	51	103	28
aKA	18 – 52	88	6	95	18	81	8	81	8	115	38	152	56	140	33	140	33
SA	0.57 – 4.8	105	12	104	13	100	6	95	6	108	12	105	11	70	20	111	20
FA	0.30 – 0.67	101	11	104	18	92	5	97	9	108	15	105	15	92	18	109	9
MA	0.87 – 2.3	90	10	100	20	107	14	93	8	110	14	114	12	95	29	125	16
PyA	24 – 82	88	12	94	18	83	6	83	6	-	-	-	-	-	-	-	-
LA	4.1 – 79	83	9	84	18	66	11	104	15	112	9	117	12	43	22	120	20
CA - d_4	-	-	-	-	-	196	98	-	-	-	-	-	-	135	75	-	-
SA - d_4	-	-	-	-	-	106	6	-	-	-	-	-	-	73	18	-	-
FA - $^{13}C_4$	-	-	-	-	-	94	6	-	-	-	-	-	-	95	14	-	-
MA - d_3	-	-	-	-	-	113	9	-	-	-	-	-	-	82	24	-	-
LA -$^{13}C_3$	-	-	-	-	-	96	7	-	-	-	-	-	-	38	6	-	-

Table 4. Concentration ranges (n=38, 30 fresh urine samples and 8 stored urine samples) obtained in this study for selected analytes using oBHA derivatization compared with normal ranges previously reported.

Compounds	Calculated concentration range (µg/mL)	Calculated concentration range (µmol/mmol creatinine)	Concentration range from literature (µmol/mmol creatinine)
CA	42 – 1513	49-874	49-611 [38–40]
IA	27 – 307	15-133	5.3-119 [38,40,41]
aKA	0.6 – 72	0.37-55	0.18-150 [38,40,42–44]
SA	0.3 – 56	0.34-37	0.3-72 [38,40,45]
FA	0.12 – 2.7	0.14-1.32	0.1-10.3 [40,46]
MA	0.3 – 6.4	0.23-3.56	0.41-5.3 [38,42,47]
LA	1.9 – 79	3.4-78	3.5-250 [38,40,42,48]
PyA	0.9 – 82	0.9-232	0.8-70 [38,40,48,49]

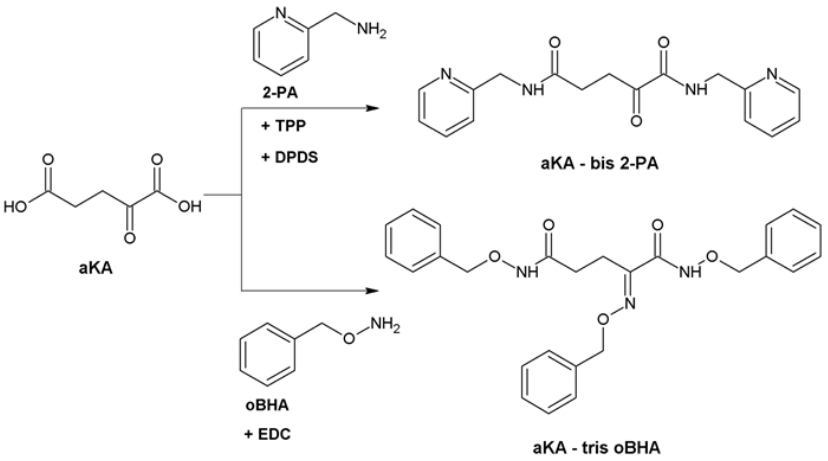

Figure 1. 2-PA and oBHA derivatization reactions. Example of the derivatives obtained with α-ketoglutaric acid. aKA: α-ketoglutaric acid, 2-PA: 2-picolyl amine, TPP: Triphenylphosphine, DPDS: 2,2-dipyridyl disulfide, oBHA: *o*-benzylhydroxylamine, EDC: *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride.

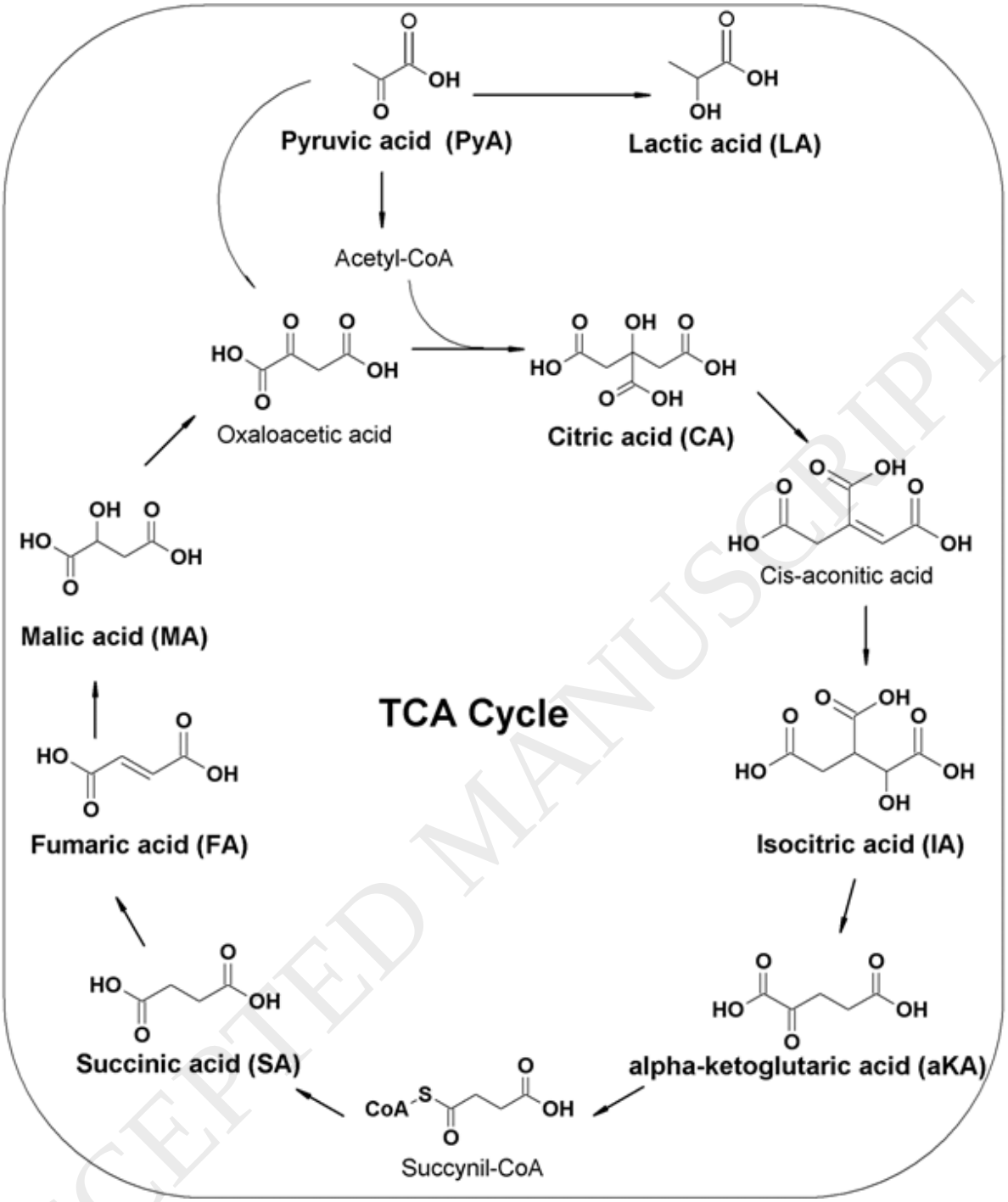

Figure 2. Compounds of the tricarboxylic acids (TCA) cycle. In bold the analytes selected for this study.

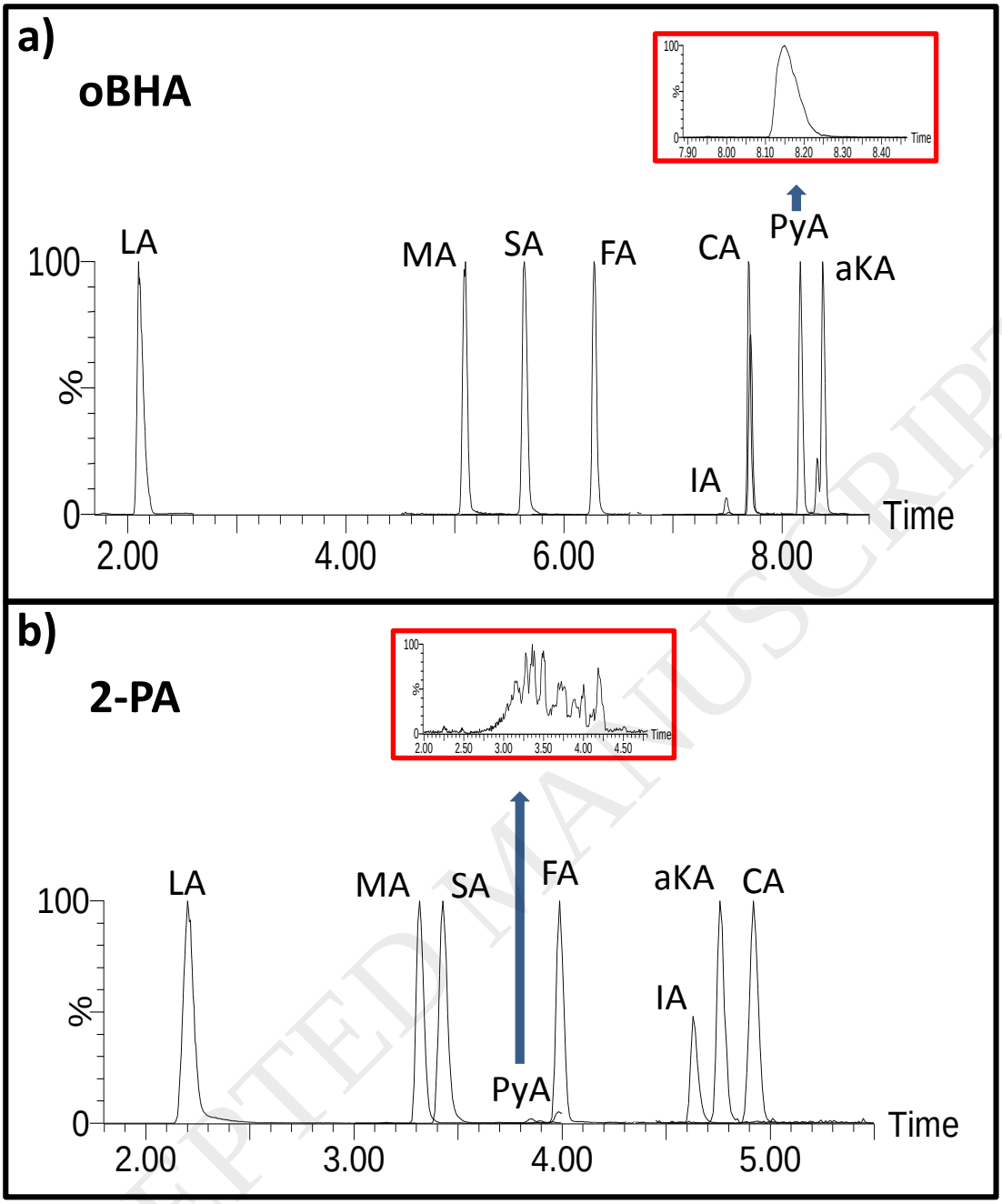

Figure 3. General LC-MS/MS chromatogram overview of a) oBHA and b) 2-PA derivatized species. LA: lactic acid, MA: malic acid, SA: succinic acid, FA: fumaric acid, IA: isocitric acid, CA: citric acid, PyA: pyruvic acid and aKA: α-ketoglutaric acid. Highlighted from the general chromatogram the chromatographic peak shape obtained for PyA under a) oBHA derivatization and b) 2-PA derivatization.

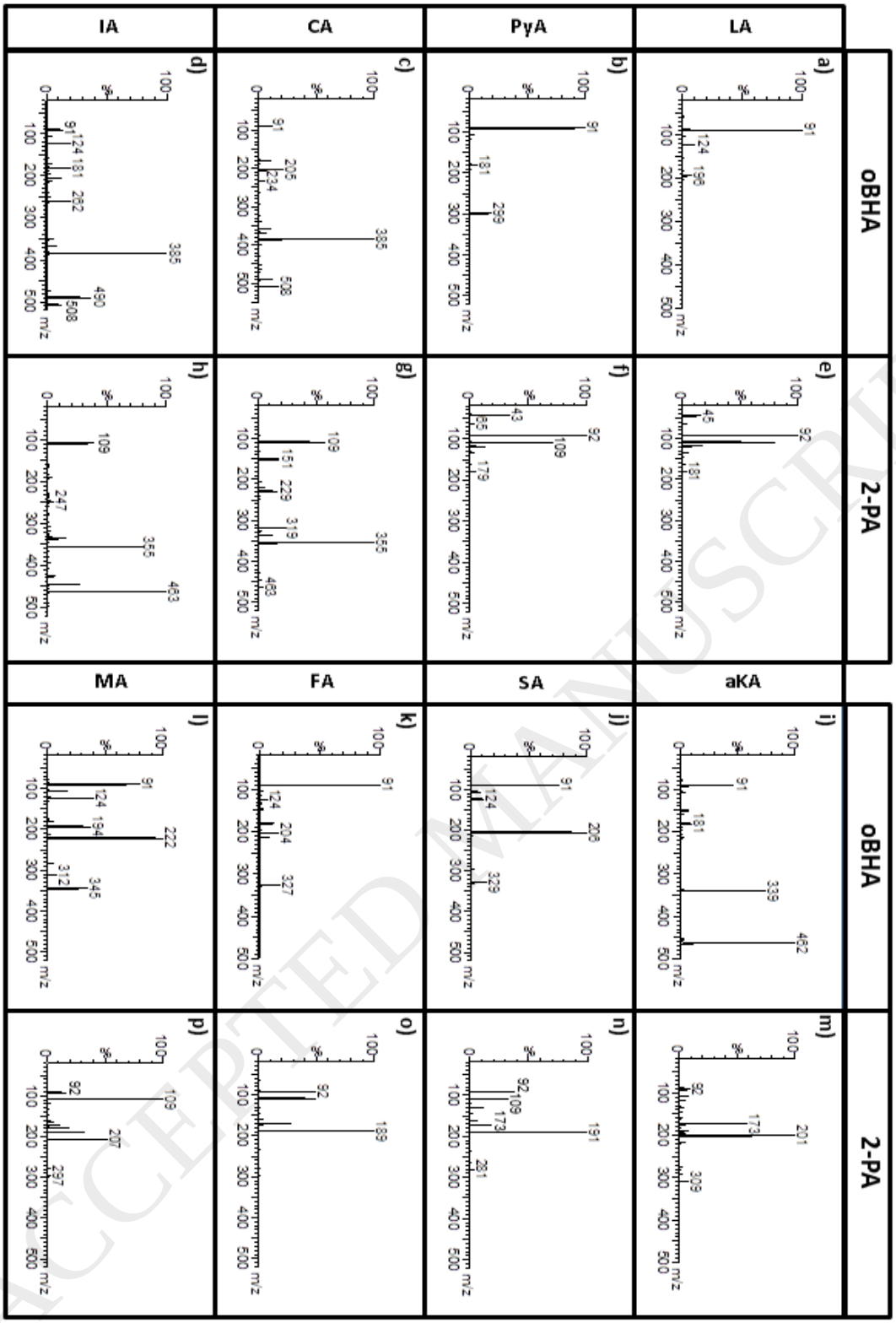

Figure 4. ESI-MS/MS spectra of oBHA-derivatives of (a) lactic acid, (b) pyruvic acid, (c) citric acid, (d) isocitric acid, (i) α-ketoglutaric acid, (j) succinic acid, (k) fumaric acid and (l) malic acid. ESI-MS/MS spectra of 2-PA-derivatives of (e) lactic acid, (f) pyruvic acid, (g) citric acid, (h) isocitric acid, (m) α-ketoglutaric acid, (n) succinic acid, (o) fumaric acid and (p) malic acid The MS/MS spectra were recorded by the collisional activation of $[M+H]^+$ of the respective derivatives at 20 eV of collision energy.

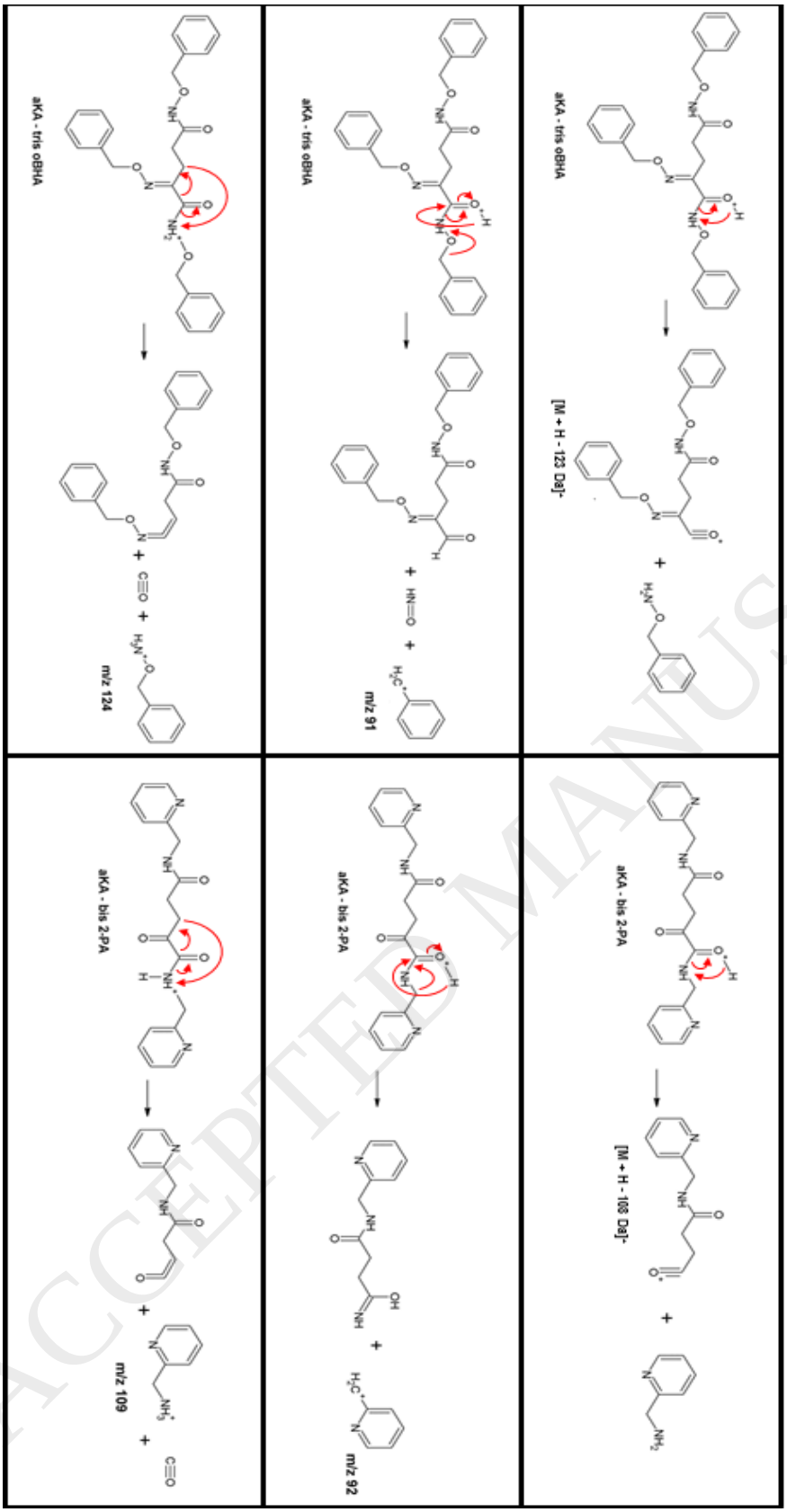

Figure 5. Common CID pathways postulated for oBHA and 2-PA derivatized compounds. Fragmentation pathways for α-ketoglutaric acid are shown as example.

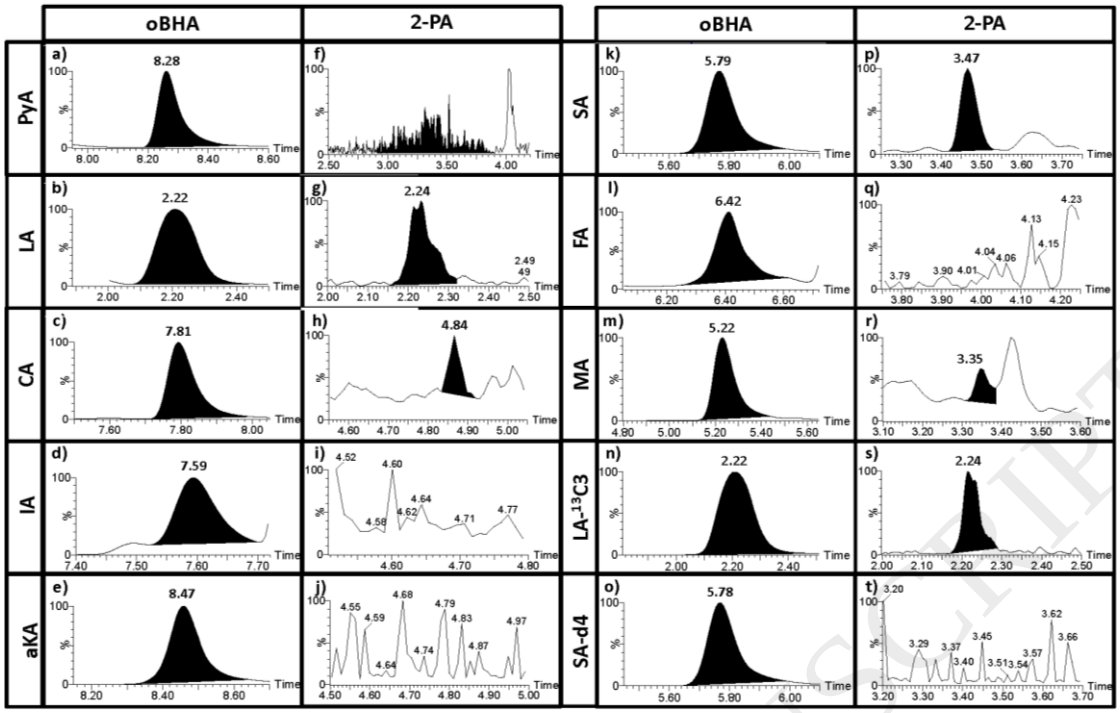

Figure 6. LC-MS/MS chromatograms of a selected stored urine sample. All and two internal standards (LA-$^{13}C_3$ and SA-d4) are shown as example of inadequate 2-PA derivatization compared with oBHA. Analytes concentrations are: PyA 3.6 µg/mL; LA 2.3 µg/mL; CA 237 µg/mL; IA 36 µg/mL; aKA 12 µg/mL; SA 0.49 µg/mL; FA 0.17 µg/mL; MA 0.48 µg/mL.

Figure 7. Correlation between the results obtained by the developed method based on oBHA derivatization and the ones routinely used in an external laboratory. (A) CA, (B) aKA and (C) LA.