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Correction of creatine-creatinine conversion during serum creatinine quantification by two-dimensional liquid chromatography and double-spike isotope dilution tandem mass spectrometry

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ABSTRACT

Background and aims: Development of a candidate reference method based on bidimensional liquid chromatography coupled to ESI-MS/MS and double spike isotope dilution for serum creatinine quantification capable of correcting for creatinine-creatine interconversion during sample pretreatment. Study of the impact of the creatine-creatinine interconversion during the analysis of human serum samples.
Materials and methods: $^{13}C_1$-creatinine and $^{13}C_2$-creatine are added to the serum sample. Separation carried out by bidimensional liquid chromatography combining reversed phase and a strong cation exchange chromatography. The heart cut, containing creatine and creatinine, is automatically transferred to the second dimension. Quantification carried out by double spike isotope dilution tandem MS/MS.
Results: Minimization of spectral interferences and ion suppression due to matrix effects while increasing sample throughput compared to the direct coupling of cation exchange chromatography to the ESI source. Trueness of the method studied with the satisfactory analysis of two certified reference materials. Satisfactory intra- and inter-day precisions obtained analysing a serum pool and control sera. Analysis of 93 serum samples revealed negligible interconversions with no correlation with creatine levels.
Conclusions: The method provides adequate analytical figures of merit for serum creatinine determination according to CSLI guidelines. Negligible creatine-creatinine interconversion is promoted with the applied sample preparation procedure.

1. Introduction

Kidney diseases significantly impact the global population, displaying high rates of morbidity and mortality. The prevalence of kidney diseases is expected to increase in the near future, imposing a significant financial burden on healthcare budgets [1]. Creatinine has been and continues to be a pivotal parameter in the calculation of the glomerular filtration rate (GFR), which is the most widely accepted test for assessing renal function and its associated conditions and diseases [2]. Creatinine is a small inactive biomolecule formed through the nonenzymatic cyclization of creatine during the provision of energy to muscle cells [3]. It is subsequently released into the bloodstream and excreted in urine. Accurately determining creatinine concentration levels in biological samples, especially in serum, is crucial for a precise diagnosis of renal failure [4].

The determination of creatinine in serum samples in routine clinical laboratories is typically carried out using Jaffe or enzymatic automated spectrophotometric assays, as they allow for high throughputs and fast results. Enzymatic methods are considered to offer improved specificity compared to Jaffe-based methods, but they can still be affected by various interferences [5,6]. Consequently, routine clinical methods must be regularly tested against reference measurement procedures or certified reference materials to ensure traceability in the results [7].

Mass spectrometry (MS) offers greater selectivity and sensitivity compared to spectrophotometric and immunogenic assays, and its application is expanding in medical laboratories [8,9]. LC-ESI-MS/MS is considered the most powerful technique for the accurate quantification of clinical biomarkers [10,11]. However, ionization suppression effects in ESI due to matrix effects affect the accuracy and precision of the results [12,13]. The use of isotopically labelled analogues as internal

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standards to apply isotope dilution mass spectrometry (IDMS) is widely regarded as the most efficient standardization strategy to correct for matrix effects [14].

IDMS methods are classified as higher-order measurement procedures and therefore can be considered potential candidates for reference methods. Several reference IDMS based methods/procedures have been approved by the JCTLM for the determination of creatinine in plasma, serum and urine using GC-IDMS or LC-IDMS [15–17]. However, none of these methods account for the possible interconversion between creatine and creatinine during sample treatment. Creatine − creatinine interconversion may explain biased results between samples during CCQM intercomparison exercises as previously reported [18]. Consequently, there is some concern regarding this event that might occur during the sample preparation procedure, leading to significant errors [19]. Such interconversion is expected to be matrix-dependent and depends on pH and temperature [20,21]. However, a detailed study of the occurrence of such interconversions in the analysis of real serum samples has not been carried out thus far.

We have previously developed a methodology able to correct and quantify the potential creatine-creatinine interconversion occurring during the analytical determination of both compounds in human serum samples [22]. The methodology was based on the use of minimally labelled C analogues ($^{13}C_1$-Creatinine and $^{13}C_2$-Creatine) and the measurement of the isotopic distribution of creatine and creatinine by LC-MS/MS. This method employed a cation-exchange chromatographic separation, which unfortunately exhibited significant ionization suppression in the ESI source during creatine elution due to the presence of coeluting matrix components. Consequently, the accurate evaluation of the interconversion factors, and thus the accurate determination of creatine and creatinine, was impeded in some cases. To address this issue, more thorough clean-up protocols can be applied, albeit they typically involve greater time consumption and may introduce additional errors due to sample handling.

The use of bidimensional liquid chromatography (2D-LC) in the Multiple Heart Cutting (MHC) mode provides a valuable alternative to extensive sample clean-up procedures. This technique involves collecting one or several fractions from the first-dimension effluent using a valve equipped with a loop of a specific volume, followed by its subsequent injection into a second-dimension column for further separation [23]. The MHC mode allows for the successful isolation of individual peaks from highly complex matrices within a single chromatographic run. This is achieved through the high chromatographic resolution and increased selectivity obtained by using two different stationary phases and/or mobile phases [24,25], ultimately leading to improved accuracy by reducing ion suppression associated with matrix effects [26]. This approach has already been successfully applied in the IDMS quantification of various biomolecules in human serum and plasma samples [27–30].

This work describes the combination of double spiking IDMS and two-dimensional liquid chromatography operating in the MHC mode for the reliable quantification of creatinine and creatine in human serum. A reversed-phase separation is employed in the first dimension and coupled with a cation exchange chromatography in the second dimension. The online isolation of the single fraction in which creatine and creatinine co-elute from the first dimension enables a quicker chromatographic separation in the second dimension through cation exchange, with fewer matrix effects. By applying the proposed analytical methodology, the occurrence of undesired and matrix-dependent creatine-creatinine interconversion reactions during sample treatment has been investigated for the first time, analysing a total of n = 93 human serum samples.

2. Materials and methods

2.1. Reagents and materials

$^{13}C_1$-labelled creatinine was purchased from Sigma-Aldrich (St. Louis, MO, USA). $^{13}C_2$-labelled creatine was in-house synthesized at the Department of Organic and Inorganic Chemistry of the University of Oviedo [22]. United States Pharmacopeia reference standards of natural abundance creatinine and creatine were purchased from Sigma-Aldrich. Acetonitrile (Optima ™ LC-MS Grade) and methanol (Optima ™ LC-MS Grade) from Fisher Scientific (Waltham, MA, USA), ammonium formate (LiChropur, ≥ 99.0 %) trifluoroacetic acid (Reagent Plus, 99 %) and formic acid (puriss, p.a., >98 %) purchased from Merck were used for the preparation of mobile phases. Liquid Assayed Multiqual Levels 1 and 3 serum controls for creatinine determination were provided by Bio-Rad Laboratories (Hercules, California). Certified reference materials ERM-DA252a and ERM-DA253a (frozen human serum) were purchased from the Laboratory of the Government Chemist (LGC, Teddington, UK).

2.2. Serum samples

A pool of frozen human serum samples from anonymous patients was provided by the Clinical Biochemistry Service of the Central University Hospital of Asturias (HUCA). Venous blood from 93 adult patients were selected randomly from the creatinine levels found in routine testing carried out at the HUCA. Blood samples were extracted at HUCA by venepuncture into test tubes containing EDTA. Most of the samples arose from nephrology patients but samples from haematology and digestive patients were also included. The age range was between 20 and 92 years. The study protocols were approved by the Regional Ethics Committee (Comité Ético de Investigación Clínica del Principado de Asturias) following the tenets of the Declaration of Helsinki (as revised in 2013) with protocols number 178/18 and 149/19.

2.3. Instrumentation

Figure S1 of the Supplementary Material shows the instrumental set-up employed in this work. An Agilent 1290 Infinity 2D-LC system coupled to a triple quadrupole mass spectrometer Agilent 6460 equipped with an electrospray source with a jet stream was used. The 2D-LC system was controlled by OpenLab CDS Chemstation and the triple quadrupole by MassHunter Acquisition software (Agilent Technologies). The first dimension incorporated a 1290 Infinity binary pump connected to an autosampler, thermostated column compartment, and a 1260 Infinity variable wavelength detector with a 10 mm flow cell. The two dimensions were interconnected by a 2-pos/4-port duo valve to which two distinct selector valves including six 80 μL sampling loops were coupled. A vortex mixer FB 15024 (Fisher Scientific) was used for the homogenization of samples and working solutions. All solutions were prepared gravimetrically using an analytical balance model MS205DU (Mettler Toledo, Zurich, Switzerland). A centrifugal vacuum concentrator (Genevac, Suffolk, UK) was used to remove water and organic solvents. Ultra-pure water was obtained from a Purelab Flex 3 water purification system (Elga Labwater, Lane End, UK).

2.4. Sample preparation

A weighed amount of ca. 0.1 g of 8 μg g^{-1} solutions of $^{13}C_1$-creatine and $^{13}C_2$-labelled creatinine in water were added to a weighed amount of ca. 0.2 g of serum sample, certified reference material or serum control. The mixture was vortexed and then 300 μL of methanol were added to the mixture for protein precipitation. Then, the solution was vigorously vortexed for 1 min and then centrifuged at 13000 rpm for 10 min. The supernatant was removed to a clean vial and evaporated to dryness using a centrifugal vacuum concentrator (37 °C). The dried extracts were reconstituted in 500 μL of mobile phase and filtered

through a polypropylene syringe filter (13 mm diameter, 0.22 μm pore size) before being injected into the 2D-UPLC-ESI-MS/MS system.

2.5. Measurements by HPLC-ESI-MSMS

As described in Figure S1 of the Supplementary Material, the UPLC separation on the first dimension was carried out using a reverse phase column Zorbax RRHD Eclipse Plus C18 (3.0 × 50 mm, 1.8 μm particle size, 95 Å pore size from Agilent Technologies. Ultrapure water with 0.1 % TFA (A) and acetonitrile with 0.1 % TFA (B) at 0.4 mL min^{-1} were used as mobile phases. A gradient starting with 1 % B for 2 min, from 1 to 80 % of B until 10 min, 80 % until 12 min, from 80 to 1 until 13 min and 1 % until 18 min was applied. On the second dimension a Zorbax 300-SCX column (2.1 × 50 mm, 5 μm particle size, 300 Å pore size) from Agilent Technologies was used. Mobile phases of the second dimension were ultrapure water with 20 % methanol and 0.1 % formic acid (A) and ultrapure water with 20 % methanol, 10 mM of ammonium formate and 0.1 % formic acid, working at a flow rate of 0.4 mL min^{-1}. The chromatographic gradient on the second dimension started with 10 % B for 1 min, from 10 to 50 % until 12 min, 50 % until 13 min, from 50 % to 10 % until 13.6 min and 10 % until 18 min. The injected volume was 2 μL. The ionization source working conditions are given in Table S1 of the Supplementary Material. For the determination of the isotopologue distribution of creatine and creatinine by SIM mode the *m/z* 130.1 to 135.1 and 112.1 to 117.1, respectively were monitored. Conditions for the measurement of samples and standards by SRM are given in Table S2 and required the monitorization of the transitions 132.1 → 90.1, 133.1 → 91.1, 134.1 → 92.1 and 135.1 → 93.1 with a collision energy of 8 eV for creatine and 114.1 → 86.1, 115.1 → 87.1, 116.1 → 88.1, 117.1 → 89.1 with a collision energy of 7 eV for creatinine.

2.5.1. Calculation of creatine and creatinine concentration by double spike IDMS and multiple linear regression

When applying IDMS and multiple linear regression with tandem MS the experimental isotopologue distribution of a given product ion in the sample A^m, can be assumed to be a linear combination of the isotopologue distributions of the natural abundance product ion, A^s, and those of the isotopically labelled analogues, A^{ti} added to the sample at the beginning of the sample preparation procedure. Due to the differential labelling between creatinine and creatine and the presence of minor impurities of $^{13}C_1$-creatinine in the $^{13}C_2$-creatine spike solution, the matrix calculations both for creatinine and creatine are based on three isotopic patterns and four MRM transitions for each compound. In brief, the isotopologue distribution of the mixture of sample and tracer is deconvoluted by linear regression using Eq. (1), which is applied for both creatinine and creatine chromatographic peaks:

$$\begin{bmatrix} A_1^m \\ A_2^m \\ A_3^m \\ A_4^m \end{bmatrix} = \begin{bmatrix} A_1^s & A_1^{t1} & A_1^{t2} \\ A_2^s & A_2^{t1} & A_2^{t2} \\ A_3^s & A_3^{t1} & A_3^{t2} \\ A_4^s & A_4^{t1} & A_4^{t2} \end{bmatrix} \times \begin{bmatrix} x_s \\ x_{t1} \\ x_{t2} \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \\ e_3 \\ e_4 \end{bmatrix} \quad (1)$$

Where A^m, A^s, A^{t1} and A^{t2} are the relative abundances for four selected MRM transitions for the mixture (*m*), the sample (*s*) and the tracers (*t1* for creatinine and *t2* for creatine respectively) and *e* refers to the error vector. The unknowns x_s, x_{t1} and x_{t2}, the molar fractions of sample and both tracers in the mixture, are calculated applying the equation for each compound (creatinine and creatine independently). Applying the equation to both creatine and creatinine chromatographic peaks, we will obtain six molar fractions, three for each compound. For the evaluation of the possible interconversion of creatinine and creatine during sample preparation/analysis according to the process a double-spike calculation procedure was previously developed in our laboratory [22]:

$$creatinine \underset{F1\rightarrow}{\overset{\leftarrow F2}{}} creatine$$

In this procedure, the molar fractions x_{t2} in the creatinine peak is used to compute the fraction of creatine converted to creatinine during sample preparation (factor *F2*) while the molar fraction x_{t1} in the creatine peak is used to compute the fraction of creatinine converted to creatine (factor *F1*). Using the double-spike approach, we can compute in each sample the corresponding interconversion factors *F1* and *F2* and the degradation-corrected concentrations of creatinine and creatine.

3. Results

3.1. Measurement of isotopologue distributions and calibrator characterization

For a successful application of the double spike IDMS approach using equation (1), the measurement of isotopologue distributions of natural and labelled analogues by MS/MS must be validated. To do so, the experimental values were compared with the theoretical values obtained by specific dedicated software such as IsoPatrn© [31]. Figure S2 of the Supplementary Material shows the comparison of the theoretical and experimental values of the isotopologue distribution measured in MS/MS for the natural abundance and labelled analogues of creatinine and creatine. As can be observed, the experimental isotopologue distributions obtained for both natural and labelled analogues agreed well with the theoretical values. Secondly, the isotopic enrichment of the labelled analogues was calculated as described in a previous publication [32]. The average ^{13}C enrichment of 5 independent replicates and the associated standard deviation obtained were 99.7 ± 0.1 %, 99.4 ± 0.1 % for $^{13}C_2$-creatine and $^{13}C_1$-creatinine, respectively. Finally, concentration of the working solutions of labelled compounds was calculated by reverse isotope dilution using natural abundance United States Pharmacopeia reference standards of Creatine and Creatinine traceable to the International System of units.

3.2. Trueness of the methodology based on double spike isotope dilution and 2D-LC-MS/MS

The trueness of the proposed methodology was evaluated following the CLSI C62 guidelines. For this purpose, two certified reference materials (CRM 252a and CRM 253a) were analysed. Table 1 shows the creatine and creatinine concentrations corrected for interconversion and the interconversion factors F1 (%Creatinine → Creatine) and F2 (% Creatine → Creatinine) for both reference materials. As can be observed, creatinine concentration values in agreement with the certified values were obtained. Concerning the interconversion factors negligible values were obtained for both reactions during the sample preparation procedure employed.

3.3. Intra-day and inter-day variability in the measurement of serum creatinine and creatine by double spike isotope dilution and 2D-LC-ESI-MS/MS

The intra-day (several independent sample preparations and injections in the same day) and inter-day variabilities (repeating the experiment on different days) were evaluated following the CLSI EP15-A2 guidelines. We analysed two serum controls Biorad Liquid Assayed Multiqual Levels 1 and 3 and a pooled plasma. The evaluation was carried out in four different days. In each day 4 independent replicates were analysed, and each replicate was injected 5 times in the 2D-LC-ESI-MS/MS system. The results are given in Table 2. The intraday precision expressed as CV (%) ranged from 0.5 to 1.6 % for creatinine and from 1.3 to 13 % for creatine in the three analysed samples. Interday precision

Table 1
Concentration of creatinine and creatine (μg/g) corrected for interconversions and interconversion factors F1 (%Creatinine → Creatine) and F2 (%Creatine → Creatinine) obtained in the analysis of the certified reference materials ERM-DA252a and ERM-DA253a by double spiking isotope dilution and 2D-LC-ESI-MSMS. Uncertainty of the individual replicates correspond to the standard deviation of n = 3 LC-ESI-MS/MS injections.

Material	Replicate	Creatinine (μg g^{-1})	Creatine (μg g^{-1})	F2 (%Creatine → Creatinine)	F1 (%Creatinine → Creatine)
ERM-DA252a	1	3.09 ± 0.07	0.916 ± 0.001	0.02 ± 0.12	−0.07 ± 0.03
	2	3.12 ± 0.01	0.924 ± 0.013	0.31 ± 0.16	−0.07 ± 0.15
	3	3.15 ± 0.01	0.924 ± 0.009	0.32 ± 0.39	0.01 ± 0.04
	4	3.16 ± 0.01	0.947 ± 0.001	−0.13 ± 0.24	0.01 ± 0.03
	Average ± SD	3.13 ± 0.03	0.928 ± 0.006	0.13 ± 0.22	−0.03 ± 0.05
	Certified value	*3.1 ± 0.5*	*Not certified*		
Material	Replicate	Creatinine (μg g^{-1})	Creatine (μg g^{-1})	F2 (%Creatine → Creatinine)	F1 (%Creatinine → Creatine)
ERM-DA253a	1	51.1 ± 0.2	4.16 ± 0.18	−0.25 ± 0.09	0.09 ± 0.16
	2	51.2 ± 1.0	4.22 ± 0.12	0.08 ± 0.06	0.09 ± 0.12
	3	50.9 ± 0.2	4.11 ± 0.05	−0.03 ± 0.35	0.17 ± 0.04
	4	50.8 ± 0.6	4.23 ± 0.02	−0.25 ± 0.11	0.08 ± 0.11
	Average ± SD	51.0 ± 0.2	4.18 ± 0.06	−0.11 ± 0.17	0.11 ± 0.04
	Certified value	*50 ± 2*	*Not certified*		

Table 2
Intra-day and Inter-day precisions for the detection of creatinine and creatine measured in serum pool and control serum of high and low concentration. Inter-day uncertainty values correspond to the standard deviation of 5 replicates of 4 independent run (n = 20). Intra-day uncertainty values correspond to the standard deviation of 5 replicates of 4 independent run per day for 4 days (n = 80).

Sample	Measurement Day	Replicates	Injections per replicate	Concentration (μg/g)		CV (%)	
				Creatinine	Creatine	Creatinine	Creatine
Low level control serum	1	4	5	0.921 ± 0.014	0.024 ± 0.002	1.6	9.7
	2	4	5	0.896 ± 0.008	0.023 ± 0.002	0.9	8.0
	3	4	5	0.896 ± 0.007	0.021 ± 0.002	0.8	8.1
	4	4	5	0.896 ± 0.004	0.022 ± 0.001	0.5	4.3
	Average			0.902 ± 0.014	0.023 ± 0.002	1.6	8.9
High level Control serum	1	4	5	6.847 ± 0.110	0.104 ± 0.013	1.6	13.0
	2	4	5	6.700 ± 0.060	0.126 ± 0.014	0.9	10.8
	3	4	5	6.608 ± 0.077	0.133 ± 0.011	1.2	8.3
	4	4	5	6.543 ± 0.048	0.124 ± 0.009	0.7	7.0
	Average			6.675 ± 0.138	0.122 ± 0.016	2.1	13.2
Pooled serum Sample	1	4	5	0.859 ± 0.013	0.512 ± 0.009	1.5	1.8
	2	4	5	0.849 ± 0.011	0.536 ± 0.009	1.3	1.7
	3	4	5	0.848 ± 0.010	0.512 ± 0.007	1.2	1.3
	4	4	5	0.831 ± 0.010	0.516 ± 0.007	1.3	1.3
	Average			0.874 ± 0.015	0.519 ± 0.013	1.8	2.4

ranged from 1.6 to 2.1 % and from 2.4 to 13 % for creatinine and creatine, respectively. The higher CV values obtained for creatine were due to its low concentration in the control serum samples. The CV obtained in the pooled plasma sample ranged from 1.3 to 1.5 % and 1.3 to 1.8 % (intraday) while the values of the inter-day precision corresponded to 1.8 % and 2.4 % for creatinine and creatine, respectively. It should be taken int account that, as reported elsewhere, [33] the correction of analytes interconversion by multiple-spiking isotope dilution is possible at the expense of the precision of the initial amount estimates. This would explain a slightly higher CV for interday precision of creatinine compared to previously published LC-MSMS approaches based on single spiking IDMS [3,17].

3.4. Carryover evaluation

In all measurement sessions, carryover was evaluated by injecting the same volume of the initial mobile phase after every fourth sample. We did not observe in any mobile phase injection a detectable signal of the analytes for the concentration range of the samples analyzed in this work: from 3 to 147 μg g^{-1} for creatinine, and from 1 to 67 μg g^{-1} for creatine.

3.5. Detection and quantification limits of the method

Detection and quantification limits were calculated according to the EP17-A CLSI guidelines. Detection limits and quantification limits were calculated as 3 and 10 times the standard deviation of the measurements of 6 independent replicates of PBS performing n = 10 injections per blank. Table 3 shows the results obtained for creatinine and creatine. Detection limits of 0.016 and 0.006 μg g^{-1} for creatinine and creatine, respectively were obtained as 3 times the standard deviations of the blank values. Quantification limits of 0.053 and 0.021 μg g^{-1} for creatine and creatinine, respectively were obtained as 10 times the standard deviation of the blank values.

Table 3
Determination of limit of detection and quantification (μg/g), defined as 3 times and 10 times the standard deviation of the blank, respectively, obtained in the analysis by 2DLC-MSMS of 6 independent replicates of PBS each of them injected n = 10 times. Standard deviation of the blank was thus determined from n = 60 2DLC-MSMS injections.

PBS Blanks	Concentration (μg/g)	
	Creatinine	Creatine
1	0.002 ± 0.006	0.005 ± 0.001
2	0.003 ± 0.009	0.005 ± 0.001
3	0.002 ± 0.007	0.008 ± 0.004
4	0.006 ± 0.002	0.005 ± 0.001
5	0.006 ± 0.001	0.004 ± 0.001
6	0.005 ± 0.001	0.003 ± 0.001
Average	0.004 ± 0.005	0.005 ± 0.002
LOD (3SD)	0.016	0.006
LOQ (10SD)	0.053	0.021

3.6. Analysis of serum samples and evaluation of uncertainty sources

The method was applied to the analysis of 93 serum samples from healthy volunteers and patients with different renal, digestive, or haematological pathologies. The analysis of these samples was carried out to evaluate the occurrence of creatine-creatinine interconversion during the analysis of real serum samples and to characterise the uncertainty sources of the method. Table S3 of the Supplementary Material shows the results obtained for the concentration of both compounds and the associated interconversion factors. The contribution of the individual uncertainty sources to the total uncertainty were evaluated applying the method proposed by Kragten [34]. Table S4 shows the range of the individual contributions (in % of total uncertainty) from the different uncertainty sources considered in the analysis of the 93 serum samples. Briefly, the measurement of the SRM transitions, the concentration of the labelled compounds and the weight of sample and labelled standards were found to be the major contributors.

4. Disscussion

4.1. 2D-Chromatography for the determination of serum creatinine

In a previous work, we carried out the determination of creatinine and creatine in serum samples by strong cation exchange chromatography (SCX) coupled to ESI-MS/MS [30]. This strategy provided a satisfactory chromatographic resolution but showed two main problems when analysing serum samples. First, as observed in Figure S3 of the Supplementary Material, important interferences at the creatine retention time were observed in some serum samples. The creatine-specific SRM transitions of a chromatogram of a serum sample obtained by cation exchange chromatography coupled to an ESI-MS/MS also showed important ionization suppression effects due to matrix constituents. Under these conditions the accurate creatine quantification and therefore the creatine-creatinine conversion determination particularly for low level serum samples is challenging. Secondly, the sample throughput of the SCX column was compromised due to the lower matrix tolerance of the SCX column compared to a reversed phase C18 column.

To avoid these problems, we propose here a 2D-LC strategy based on the use of a reversed phase chromatography (C18) in the first dimension

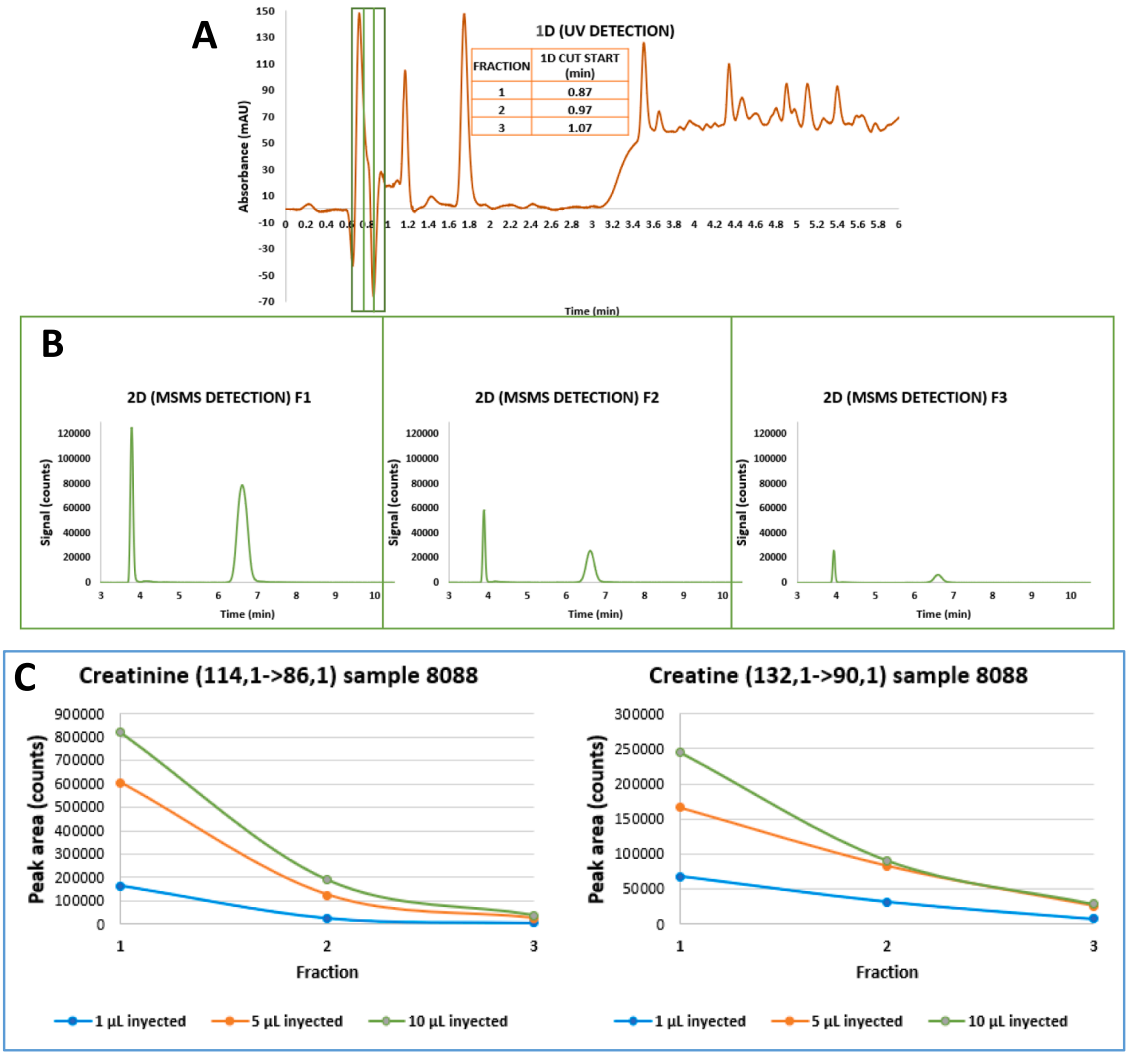

Fig. 1. A) 1D-LC-UV chromatogram of a pooled serum sample obtained by reversed phase chromatography using a C18 column. B) 2D-LC-ESI-MS/MS chromatograms of three consecutive 80 µL heart-cuts (F1, F2 and F3) obtained by cation exchange chromatography C) Peak Area obtained by 2DLC-ESI-MS/MS for creatinine (SRM transition 114.1 → 86.1) and creatine (SRM transition 132.1 → 90.1) In the heart-cuts F1, F2 and F3 when injecting 1, 5 and 10 µL of the same pooled serum sample.

and the SCX chromatography in the second dimension. Highly polar compounds like creatine and creatinine will not show retention in the C18 column but can be simultaneously transferred in a single fraction to the second dimension in which they will be separated by SCX chromatography. In this way, we obtain four potential advantages: i) minimization of the ionization suppression and spectral interferences due to matrix constituents, ii) simultaneous analyte enrichment and sample purification, iii) enhancement of the SCX column life due to a lower introduction of matrix content, iv) the use of mobile phases not compatible with ESI ionization (like TFA containing solutions) in the first dimension to enhance retention of interfering compounds in the reversed phase chromatography.

As shown in Figure S1, the column of the first dimension is coupled to a UV–VIS detector. Thus, the LC-UV chromatogram of the sample can be taken as reference for selecting the fractions to the second dimension. To do so, a high-resolution sampling strategy allows the collection of several consecutive fractions of 1D chromatogram to be transferred to the second dimension. Creatine and creatinine will not show retention in the C18 column. As shown in Fig. 1A, when analysing serum samples, the lack of selectivity of the UV–VIS detector does not allow the identification of the creatine and creatinine retention times due to the presence of interfering matrix constituents. The high-resolution sampling strategy provided by this system allow us to transfer several consecutive 80 μL heart-cuts of the dead volume to the second dimension. Each fraction is then analysed by SCX chromatography and MS/MS detection to ascertain which fraction contains the highest amount of the target analytes. Fig. 1A also shows the time window of the three selected fractions and Fig. 1B show the SCX-MS/MS chromatograms obtained for each fraction. As can be observed in Fig. 1B, the first fraction provided the highest sensitivity for both compounds. To obtain the highest sensitivity without increasing ionization suppression due to matrix effects the comparison of the peak areas of creatine and creatinine in each fraction was carried out using three different injection volumes (1, 5 and 10 μL). Fig. 1C confirms that, due to negligible presence of matrix in the transferred heart-cuts, ionization suppression is barely observed for the three fractions when increasing the injection volume up to 10 μL. Consequently, the first fraction was selected and 10 μL were injected in the analysis of serum samples to obtain the highest sensitivity.

4.2. Analysis of real serum samples and study of creatine-creatinine interconversion

Table S3 shows the results obtained for the n = 93 samples analysed in this work. To have a better comparability with other clinical studies, the concentration values of the serum samples are expressed here in mg dL^{-1} and the interconversions factors are expressed as percentage of creatinine transformed into creatine (F1) and percentage of creatine transformed into creatinine (F2). As can be observed, concentration values for creatinine ranged between 0.30 and 14.74 mg dL^{-1} and between 0.13 and 6.68 mg dL^{-1} for creatine. Fig. 2A shows the representation of the concentration of creatinine vs. creatine in all samples. There seems to be three group of samples: i) those with low concentrations of both creatine and creatinine; ii) those with low concentrations of creatine and increasing concentrations of creatinine; and iii) those with increasing concentrations of both creatine and creatinine. CV (%) for the concentration values are shown in Figure S4. For most of the concentration range measured, CVs are typically below 1 %. Only for very low concentration values the CV values increase clearly above 2 %. Table S4 shows the range of the individual contributions (in % of total uncertainty) from the different uncertainty sources. The main contributors to the total uncertainty were for both compounds the measurement of the isotopologue abundances by SRM and the concentration of the impurity $^{13}C_1$-creatine in the labelled analogue $^{13}C_1$-creatinine and the concentration of the impurity $^{13}C_2$-creatinine in the labelled analogue $^{13}C_2$-creatine.

Finally, Fig. 2B and 2C show the values of the factors F2 and F1, respectively, plotted vs. the concentrations found for both compounds. The error bars indicate the standard deviation of the interconversion factors. As it can be observed, both measured factors (F1 and F2) are negligible and independent from the measured concentrations of both creatine and creatinine. The fact that the average interconversion factor F2is clearly negative: −0.7 ± 0.2 % (SD), could be due to an overcorrection for the impurity content of creatinine in the creatine spike. For F1 the average value (n = 93) is 0.2 ± 0.2 % (SD).

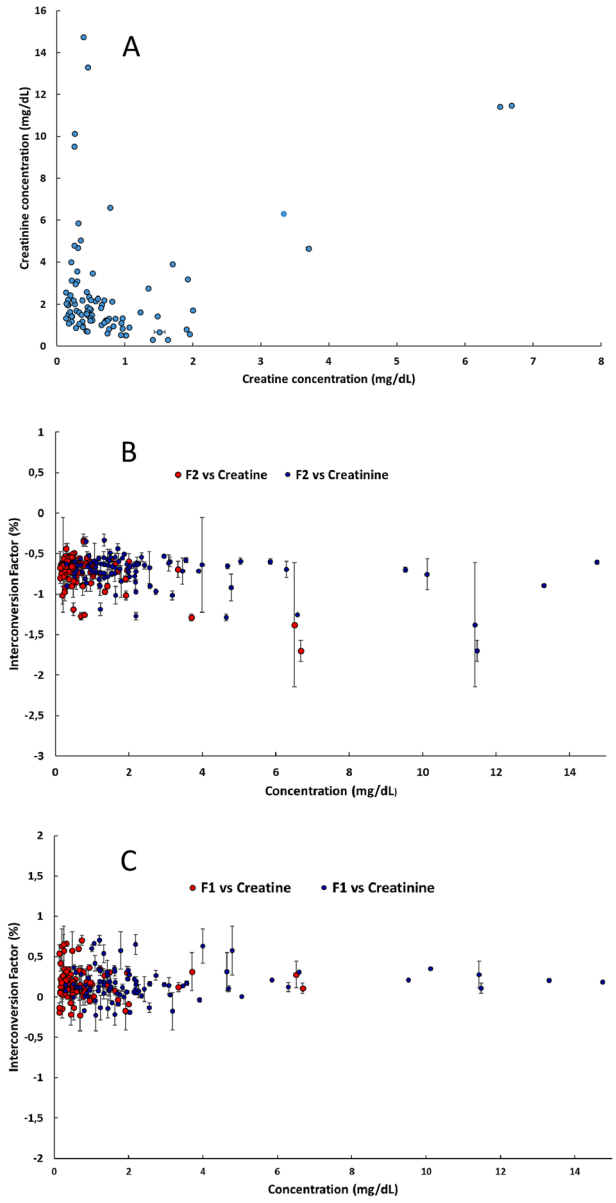

Fig. 2. A) Correlation between the concentration values of creatine and creatinine obtained in the 93 samples analysed in this work B) F2 values obtained (%of Creatine transformed into creatinine) for each creatine and creatinine concentration (mg dL^{-1}) C). F1 values obtained (%of Creatinine transformed into creatine) for each creatine and creatinine concentration (mg dL^{-1}).

5. Conclusions

In this work a candidate reference method for the determination of creatinine has been developed and validated. The proposed methodology involves a 2D-LC strategy which allowed the removal, on the first dimension of most of the matrix components that may affect the

ionization and detection of creatine and creatinine by ESI-MS/MS, avoiding the use of complex and time consuming sample preparation procedures. Moreover, the use of a double spike ($^{13}C_2$-creatine and $^{13}C_1$-creatinine) as tracers for IDMS quantification has allowed to evaluate the potential interconversion between creatine and creatinine under the experimental working conditions. Using the sample preparation procedure proposed in this work, negligible creatine-creatinine interconversion was observed in serum samples. The developed candidate reference method was evaluated for trueness by analysing two certified reference material, providing results within the certified values. Inter and intraday precision were also evaluated showing, for creatinine, CVs below 2.1 % in all cases for low- and high-level serum controls and a pooled serum sample. Creatine provided higher CV values, being below 2.4 % for the pooled serum sample and increasing to around 10 % for the high and low serum controls. Limits of detection and quantification for creatinine were 0.016 and 0.053 μg/g respectively and 0.006 and 0.021 μg/g for creatine. The developed candidate reference method provided adequate analytical characteristics to be employed in the validation of alternative routine methods for the measurement of creatinine and creatine in human serum.

CRediT authorship contribution statement

Daniela Pineda-Cevallos: Data curation, Investigation, Validation, Writing – original draft. **María Funes Menéndez:** . **Adriana González-Gago:** Writing – original draft, Conceptualization, Data curation. **Pablo Rodríguez-González:** Funding acquisition, Methodology, Writing – review & editing, Project administration, Supervision. **J. Ignacio García Alonso:** Funding acquisition, Writing – review & editing, Project administration.

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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2024.117778.

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