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Development of a novel Ultra Performance Liquid Chromatography Tandem-Mass Spectrometry (UPLC-MS/MS) method to measure L-arginine metabolites in plasma

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ABSTRACT

Introduction: Arginine metabolism is involved in the regulation of several biological processes. Many liquid chromatography tandem-mass spectrometry methods for the determination of arginine and its metabolites have been developed but they are time consuming and imply long pre-analytical procedures. The purpose of this study was to develop a rapid method for the simultaneous determination of arginine, citrulline, ornithine, symmetric and asymmetric dimethylarginine and monomethylarginine in human plasma.

Materials and methods: The pre-analytical procedure consisted in a simple deproteinization. The chromatographic separation was performed using hydrophilic interaction liquid chromatography. Analytes detection was performed with a triple quadrupole equipped with electrospray ion source operating in positive ion mode. Mass spectrometry experiments were conducted in multiple reaction monitoring mode.

Results and conclusions: Recovery ranged from 92.2 to 108.0%. The within-run imprecision and between-run imprecision ranged from 1.5 to 6.8 % and 3.8 to 11.9%, respectively.

Carry over and matrix effect did not affect quantitative analysis. Extraction recovery was between 95 and 105 %. Stability after pre-analytical procedure was tested and all the metabolites were stable after 48 h at 4 °C.

In conclusion, our novel method allow a rapid and easy determination of arginine and its metabolites both for research and clinical routine use.

1. Introduction

Arginine metabolism is involved in many physiological processes, such as immune and endothelial function, reproduction, neurotransmission and tissue integrity [1,2]. The dibasic amino acid arginine may be introduced into the organism through the diet, synthetized endogenously or derived from protein turnover. The endogenous synthesis mainly occurs in the small bowel and along the kidney proximal tubule [3]. Arginine metabolism is regulated by two main metabolizing enzymes, nitric oxide synthase (NOS) and arginase [4]. The metabolic way that leads to nitric oxide (NO) and citrulline synthesis is associated with beneficial effects on immune and vascular health, while the catabolism of arginine to ornithine has been associated with altered immune response and endothelial dysfunction [5,6]. Protein arginine methyltransferase (PRMT) enzymes may transfer methyl groups to arginine residues in proteins [7]. Following proteolysis, methylarginine moieties,

Abbreviations: SPE, solid phase extraction; SDMA, symmetric dimethyl-arginine; ADMA, asymmetric dimethyl-arginine; MMA, monomethylarginine; HILIC, hydrophilic interaction liquid chromatography; ESI, electrospray ionization; MRM, multiple reaction monitoring; NO, nitric oxide; NOS, nitric oxide synthase; PRMT, protein arginine methyltransferase; HPLC, high performance liquid chromatography; CEP, capillary electrophoresis; GC–MS, gas chromatography-mass spectrometry; ELISA, enzyme-linked immuno sorbent assay; LC-MS/MS, liquid chromatography tandem mass spectrometry; UPLC-MS/MS, ultra performance liquid chromatography tandem mass spectrometry; IS, internal standard; CE, collision energy; ULOQ, upper limit of quantification; LLOQ, lower limit of quantification; CV, coefficient of variation; SD, standard deviation; QC, quality control.

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including asymmetric dimethyl-arginine (ADMA), symmetric dimethyl-arginine (SDMA) and monomethylarginine (MMA), are released into the circulation. ADMA, SDMA and MMA may inhibit NOS (Fig. 1) [8]. Higher circulating levels of ADMA and SDMA are associated with lower NO bioavailability and increased risk of cardiovascular events and all-cause mortality [9].

Several methods for the determination of arginine and its derivatives, including those using high performance liquid chromatography (HPLC), capillary electrophoresis (CEP), gas chromatography-mass spectrometry (GC–MS), liquid chromatography coupled to mass spectrometry and enzyme-linked immuno sorbent assay (ELISA), have been developed across different biological matrices [10–14]. The measurement of these molecules represents a considerable analytical challenge. Indeed, their polar nature hampers a good retention in conventional reverse phase chromatography. In addition, some molecules are present in a wide concentration range. Chromatographic separation is also very difficult challenging due to the similar structure of arginine metabolites (e.g. ADMA and SDMA are isomers).

ELISA methods for arginine metabolites assessment are affected by antibody cross-reactivity issues, while GC–MS methods, despite being analytically excellent, require various derivatization steps and, in general very complex sample preparation procedures which makes these methods time-consuming and less suitable for clinical and research use. HPLC methods are certainly preferable, even if those with ultraviolet detection are not very sensitive, while those coupled with fluorescence detectors require sample derivatization as these compounds do not have chromophores, and these are time-consuming and expensive procedures.

All these critical issues can be overcome with the introduction of liquid chromatography tandem mass spectrometry (LC-MS/MS) methods. Many different LC-MS/MS based methods have been developed to date, some with time-consuming SPE clean-up procedures, some with complex derivatization and other with easier sample preparation procedures but lacking of stable isotopically labeled internal standard (IS) for each analyte [15–17].

The purpose of this study was to develop a rapid ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the simultaneous determination of underivatized arginine, citrulline, ornithine, ADMA, SDMA and MMA in human plasma samples.

2. Materials and methods

2.1. Chemicals and reagents

Ammonium formate, ADMA (NG,NG-dimethylarginine dihydrochloride), SDMA [NG,NG'-dimethyl-L-arginine di(p-hydroxyazobenzene-p'-sulfonate) salt], MMA (NG-methyl-L-arginine acetate salt) and L-Arginine were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). L-Citrulline and L-Ornithine dihydrochloride were purchased from Fluka Biochemika (Fluka Chemie, AG, CH). ADMA:HCl: xH_2O (2,3,3,4,4,5,5-d_7) ([$^2H_7$]ADMA, internal standard), L-Arginine:HCl (2,3,3,4,4,5,5-d_7) ([$^2H_7$]Arginine, internal standard), L-Citrulline (4,4,5,5-d_4) ([$^2H_4$]Citrulline, internal standard) and L-Ornithine:HCl (3,3,4,4,5,5-d_6) ([$^2H_6$]Ornithine, internal standard) were purchased from Cambridge Isotope Laboratories (Cambridge Isotope Laboratories, Andover, MA, USA). NG,NG'-Dimethyl-L-arginine-d_6 ([$^2H_6$]SDMA, internal standard) was purchased from Toronto Research Chemicals (Toronto Research Chemicals, Toronto, ON, Canada). Water, Acetonitrile, Methanol and Formic Acid (LC-MS grade) were purchased from Biosolve (Biosolve Chimie, Dieuze, France).

Stock solutions of ADMA (5 mM), SDMA (5 mM), MMA (5 mM), Arginine (0.5 M), Ornithine (0.5 M) and Citrulline (0.2 M), [$^2H_7$]ADMA (5 mM), [$^2H_6$]SDMA (5 mM), [$^2H_7$]Arginine (30 mM), [$^2H_4$]Citrulline (30 mM) and [$^2H_6$]Ornithine (30 mM) were prepared in water and stored at −80 °C.

2.2. Sample and standard preparation.

The analytical procedure requires a deproteinization step, performed by adding 300 μL of methanol containing 0.15 μM of [$^2H_7$] ADMA, 0.15 μM [$^2H_6$] SDMA, 15 μM [$^2H_7$] Arginine, 6 μM [$^2H_4$] Citrulline and 15 μM [$^2H_6$] Ornithine to 50 μL of plasma sample. After vigorous agitation, the sample was centrifuged at 14 000Xg for 5 min at room temperature, and then the supernatant was transferred into an autosampler vial.

Calibration curve was prepared in water with the following concentrations: ADMA and SDMA (0.0, 0.15, 0.25, 0.75, 1.0, 1.5, 5.0 μM),

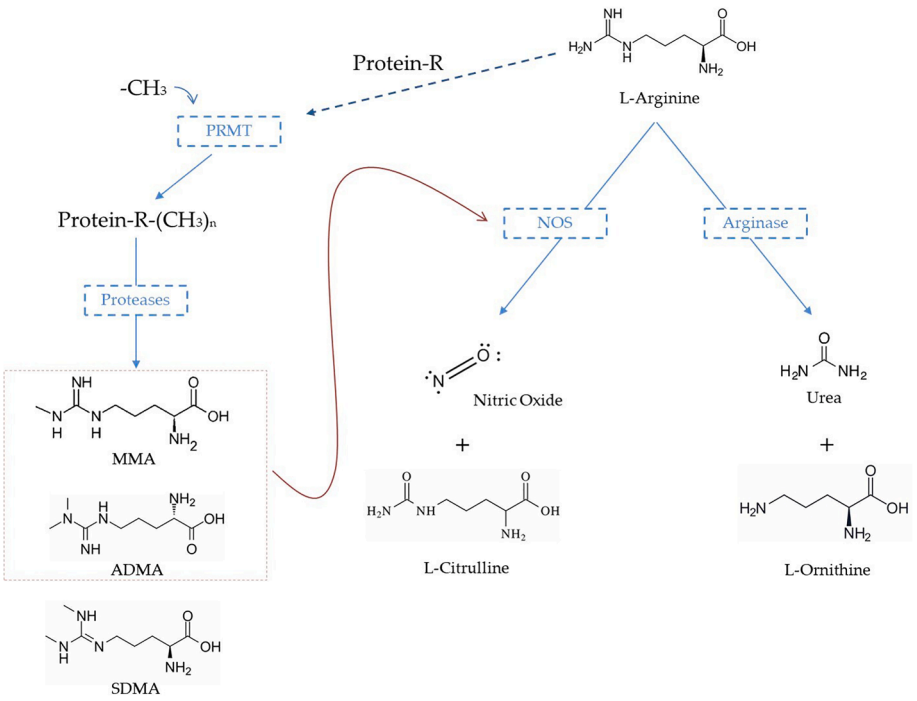

Fig. 1. Arginine metabolism.

Arginine and Ornithine (0, 15, 25, 75, 100, 150, 500 μM), Citrulline (0.0, 6.0, 10.0, 30.0, 40.0, 60.0, 200.0 μM) and MMA (0.0, 0.03, 0.05, 0.15, 0.2, 0.3, 1.0 μM).

Calibration points were treated according to the protocol and calibration curves were constructed by plotting peak area ratio (Analyte/IS) vs nominal concentration. For MMA the calibration curve was constructed by plotting Analyte Area vs nominal concentration.

2.3. Instrumentation

The UPLC-MS/MS system consisted of an UPLC and autosampler Acquity I-Class (Waters corporation, Milford, Massachusetts, USA) and a triple quadrupole mass spectrometer Xevo-TQs Micro (Waters corporation, Milford, Massachusetts, USA) equipped with ESI source.

2.4. Chromatographic conditions

Due to the nature of arginine-related metabolites we used a hydrophilic interaction liquid chromatography (HILIC), which has a complementary selectivity with respect to reverse phase liquid chromatography and allows a good retention of polar molecules. HILIC utilizes a polar stationary phase (polar bonded phase or silica) with an elution solvent composition containing a significant percentage of less polar solvent as compared to the more polar one (water). This is particularly advantageous in mass spectrometry as the high percentage of organic phase promotes the electrospray ionization, ultimately increasing the sensitivity.

The chromatographic separation was performed with a flow rate of 0.45 mL/min using a LUNA HILIC column, 3 μm, 200 Å, 100 × 2.0 mm (Phenomenex, Torrance, California, USA) with a gradient of mobile phase A (H_2O containing 0.1 % formic acid and 20 mM ammonium formate) and mobile phase B (90 % Acetonitrile containing 0.1 % formic acid and 20 mM ammonium formate). The gradient followed this pattern: 0–0.5 min 95 % B, 0.5–3.0 min 80 % B, 3.0–4.0 min 60 % B, 4.0–4.1 min 95 % B, 4.1–5.5 min 95 % B. The oven temperature was set at 40 °C. The injection volume was 2 μL, and the total analysis time was 5.5 min.

In order to keep the system cleaner, the UPLC effluent was directed to ESI for 1.5–3.0 min portion of the run time whereas it was directed to the waste container in the remaining time.

2.5. Mass spectrometer conditions

The ESI source was operating in positive ion mode. The conditions for the detection of all the analytes and labeled internal standards were obtained by direct infusion of standard solutions prepared in water: methanol (1:1 v/v) at different concentrations: 5 μM (ADMA, SDMA, MMA, [$^2H_7$]ADMA, [$^2H_6$]SDMA,) and 500 μM (Arginine, Ornithine, Citrulline, [$^2H_7$]Arginine, [$^2H_4$]Citrulline and [$^2H_6$]Ornithine). The optimized parameters for the ion source were: Desolvatation temperature at 500 °C, Desolvatation gas flow at 1100 L/Hr, Cone gas flow 10 L/Hr, Capillary voltage at 3.0 kV.

Considering that ADMA and SDMA are structural isomers and that their retention time is very close, it was very important to find diagnostic transitions for these two molecules.

The MRM transitions for each analyte, their respective collision energy (CE) and cone voltage values were reported in Table 1.

2.6. Method validation

Method validation experiments were planned following "Clinical and Laboratory Standard Institute" guidelines for diagnostic methods using LC-MS/MS [18–19].

The following parameters were assessed for method validation: upper and lower limit of quantification (ULOQ, LLOQ), carry over, matrix effect, recovery, imprecision within run and between run, extraction recovery and stability.

Table 1
Operating conditions for mass-spectrometric detection of the analytes optimized by direct infusion of standard solutions.

Compound	Q1 Mass (Da)	Q3 Mass (Da) Quantifier	Q3 Mass (Da) Qualifier	CE (volts)	Cone (volts)
ADMA	203.0	45.8	70.0	18	20
[$^2H_7$]ADMA	210.0	46.0	/	21	20
SDMA	203.0	172.0	132.9	18	15
[$^2H_6$]SDMA	209.1	175.1	/	19	15
Arginine	175.0	70.0	60.0	18	20
[$^2H_7$] Arginine	182.3	77.3	/	17	15
Citrulline	176.3	70.0	113.1	11	20
[$^2H_4$] Citrulline	180.2	74.1	/	11	20
Ornithine	133.2	70.0	116.1	13	20
[$_2H_6$] Ornithine	139.2	76.2	/	12	15
MMA	188.9	69.9	172.0	18	25

ULOQ and LLOQ were evaluated by measuring solutions at different concentrations, obtained by diluting standard stock solutions. The concentrations tested for evaluation of LLOQ were: 0.015, 0.075 and 0.15 μM for ADMA and SDMA; 0.003, 0.015 and 0.03 μM for MMA; 1.5, 7.5 and 15.0 μM for Arginine and Ornithine; 0.6, 3.0 and 6.0 μM for Citrulline. Each solution was prepared in duplicate and eight repeated measures were performed for each of them. The LLOQ was defined as the lowest concentration that could be measured with a coefficient of variation (CV) within 20 % and accuracy between 80 and 120 %.

The ULOQ was evaluated by the regression analysis of standards over the concentration range of the calibration curve.

Carry over was assessed by analyzing calibration standards in the following order: zero calibration standard, highest calibration standard and zero calibration standard. Concentration value, following the high concentration standard, should not be > 25 % of the lower calibration standard, corresponding to the LLOQ.

Matrix effect was evaluated comparing the peak areas of each deuterated IS dissolved in water/methanol and each deuterated IS dissolved into the deproteinized plasma matrix.

Results of peak areas of each deuterated IS from plasma matrix and those from water/metha- nol solution (mean ± SD) were compared.

For MMA, internal standard is not commercially available then matrix effect was evaluated using the recovery values.

Recovery and imprecision were evaluated using a plasma pool (QC0), whose analytes concentration had been previously determined, spiked with three different amounts of analytes, as reported in Table 2. The resulting samples were used as quality control samples (QC0 unspiked; QC1; QC2; QC3).

Recovery was evaluated by measuring five independent samples for each QC level assessed in duplicate. Recovery, expressed in %, was calculated as follows: (average of measured values/theoretical value) *100.

The within-run imprecision was evaluated by measuring five independent samples for each QC level in duplicate. Imprecision was calculated as follows: (standard deviation of detected values)/(average of detected values)*100.

Table 2
Spiked concentrations for quality controls.

	ADMA (μM)	SDMA (μM)	Arginine (μM)	Citrulline (μM)	Ornithine (μM)	MMA (μM)
QC0	–	–	–	–	–	–
QC1	+0.2	+0.2	+20	+20	+20	+0.2
QC2	+0.5	+0.5	+50	+40	+50	+0.5
QC3	+1.5	+1.5	+150	+60	+150	+1.0

To assess between-run imprecision, two independent samples for each QC level were processed in duplicate on eight different days over a period of 20 days, using the same formula reported. Imprecision, expressed as the coefficient of variation (CV%), was considered acceptable if not exceeding 15 % for the QC samples.

Extraction recovery was assessed by comparing analytes concentration in samples spiked pre- and post-extraction. A plasma pool was divided into two aliquots. The first one was added with different amounts for each molecule and treated according to the protocol (sample 1). The second one was treated according to the protocol and then enriched with different amounts of each molecules, (sample 2), to achieve the same final concentrations in the two samples. The extraction recovery was calculated for each analyte using the formula $(\text{concentration})_{\text{sample 1}}/(\text{concentration})_{\text{sample 2}} *100$. Extraction recovery was considered acceptable between 60 and 140 %.

The stability of analytes was assessed by analyzing three samples in duplicate, a basal pooled plasma and two spiked, immediately after preparation and after 24 and 48 h storage at 4 °C, −20 °C and −80 °C.

Furthermore, the analytical process was monitored using certified quality controls: two concentration levels (L1 and L2) manufactured by the MCA laboratory of the Queen Beatrix Hospital (The Netherlands) for arginine, ornithine, and citrulline; three concentration levels (L1, L2 and L3) manufactured by Biocrates Life Science (Innsbruck, Austria) for ADMA, SDMA, arginine, ornithine, and citrulline.

Data acquisition and quantitative analysis were performed using MassLynx V4.1 (Waters corporation, Milford, Massachusetts, USA) mass spectrometer software and TargetLynx XS (Waters corporation, Milford, Massachusetts, USA) processing software, respectively.

3. Results

Fig. 2 shows the MRM chromatograms of arginine, ADMA, SDMA, MMA, ornithine and citrulline, and their internal standards for a plasma sample. The Fig. 2 shows adequate chromatographic separation for each analyte without significant interference.

Linearity was obtained for ADMA and SDMA in the range from 0.15 to 5 μM, for MMA from 0.03 to 1 μM, for Arginine and Ornithine from 15 to 500 μM and for Citrulline from 6 to 200 μM.

Negligible carryover was observed, in fact concentrations detected in the zero calibration standard submitted after the high calibrator were lower than 5 % of the LLOQ concentrations for all analytes.

Comparing internal standard signal at the same concentration in solvent and in matrix a slightly enhancement was present in matrix samples, but it was considered irrelevant during method validation.

While the method was validated, eight calibration curves were processed over a period of 20 days and R^2 was always higher then 0.999 for all the analytes.

Recovery and imprecision results are summarized in Table 3. Recovery ranged between 92.2 and 108.0 % for all analytes tested. The within-run imprecision and the between-run imprecision ranged from 1.5 to 6.8 % and 3.8 to 11.9 %, respectively.

Moreover, the results for the commercial quality controls tested were always fully acceptable. Recovery ranges on commercial quality controls are reported in Tables 4 and 5.

Extraction recovery ranged between 95 and 105 %.

Stability, after pre-analytical procedure, was ± 15 % of the nominal concentration for all analytes at 4 °C, −20 °C and −80 °C after 24 and 48 h.

Table 3
Method validation results of imprecision and recovery.

Compound	Level	Recovery	Imprecision	
			Within-run CV(%)	Between-run CV(%)
ADMA	QC0	–	2.2	3.8
	QC1	101.9	1.5	7.2
	QC3	101.6	3.6	4.1
	QC4	107.7	3.3	4.6
SDMA	QC0	–	2.9	8.8
	QC1	104.6	2.2	7.3
	QC3	100.4	6.8	9.0
	QC4	104.8	3.4	9.9
Arginine	QC0	–	2.3	7.0
	QC1	98.7	1.7	8.1
	QC3	97.3	3.5	8.2
	QC4	99.4	3.1	7.2
Citrulline	QC0	–	2.4	6.9
	QC1	93.2	3.6	8.4
	QC3	92.2	1.7	6.5
	QC4	97.4	2.4	7.4
Ornithine	QC0	–	1.9	4.1
	QC1	96.6	1.6	6.2
	QC3	94.1	3.6	5.1
	QC4	96.0	2.6	3.9
MMA	QC0	–	4.3	9.7
	QC1	105.5	3.2	11.2
	QC3	108.0	5.5	4.4
	QC4	103.8	3.5	11.9

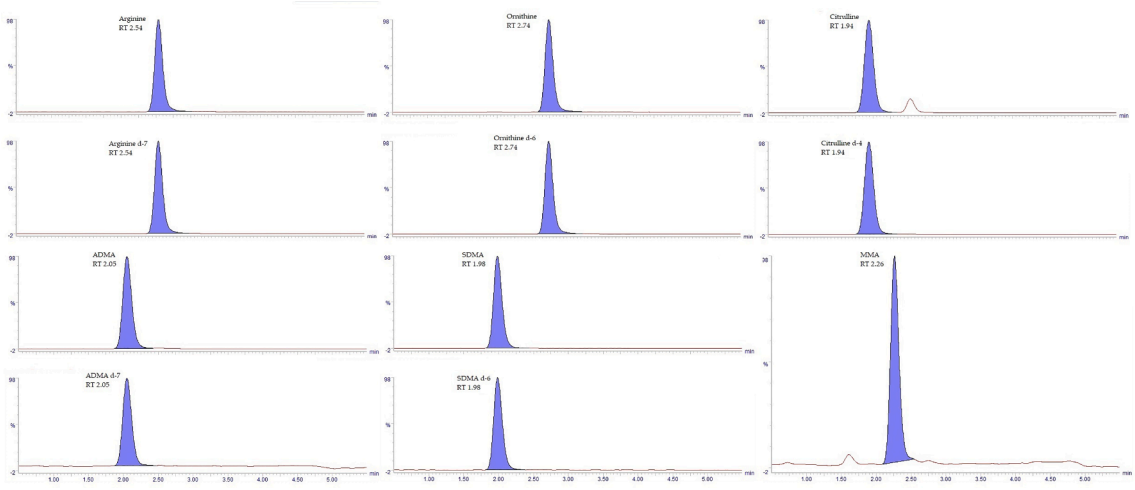

Fig. 2. MRM quantifier transitions chromatograms of arginine, ornithine, citrulline, ADMA, SDMA, MMA and corresponding internal standards in a real plasma sample.

Table 4
Target values and recovery ranges on biocrates life sciences quality controls for adma, sdma, arginine, citrulline, and ornithine.

Compound	Level	Target Value (μmol/L)	Recovery range (%)
ADMA	L1	0.52	93.2–106.7
	L2	1.02	95.6–105.9
	L3	13.52	97.1–103.3
SDMA	L1	0.21	92.5–104.8
	L2	0.42	94.4–101.3
	L3	5.70	94.2–103.6
Arginine	L1	44.3	92.1–105.3
	L2	87.6	95.3–103.6
	L3	240.3	94.8–104.5
Citrulline	L1	43.0	91.4–104.0
	L2	81.5	93.2–103.5
	L3	259.5	95.5–103.2
Ornithine	L1	13.3	96.9–102.8
	L2	25.1	97.2–101.8
	L3	266.9	98.1–104.1

Table 5
Target values and recovery ranges on mca laboratory of the queen beatrix hospital quality controls for arginine, citrulline, and ornithine.

Compound	Level	Target Value (μmol/L)	Recovery range (%)
Arginine	L1	50.0	92.3–104.8
	L2	700.0	93.4–103.4
Citrulline	L1	50.0	91.2–105.4
	L2	700.0	92.5–104.9
Ornithine	L1	50.0	91.7–103.6
	L2	700.0	90.3–102.5

4. Discussion

The determination of circulating levels of arginine metabolites is a useful approach to evaluate NO metabolism and bioavailability.

Our principal purpose was to develop a novel method, for determine a panel of six analytes related to arginine metabolism and synthesis/inhibition of nitric oxide, as rapid and simple as possible without giving up appropriate performance.

The LC-MS/MS based methods are preferable to others for this class of analytes. However, they often suffer from complex sample preparation and lack a stable isotopically labeled internal standard for each analyte.

Our method used isotopically labeled standards for all analytes except MMA, which was not commercially available. During the development of our analytical method several isotopically labeled standard were tested to obtain a reliable MMA quantification. ADMA and SDMA internal standards were selected, due to their similar chemical structure and comparable retention time. The use of labeled internal standards of different molecules did not improve MMA quantification, indeed recovery from MMA QC samples was better using the calibration curve constructed by plotting analyte area vs nominal concentration. This evidence is probably due to the different concentration range and molecule sensitivity to ionization of MMA than those of ADMA and SDMA. Indeed, plotting MMA Area vs ADMA IS Areas we obtained a calibration curve with a very low Area Ratio; lower than 1.0 at the high calibration point. By contrast, despite SDMA had a much higher concentration range than MMA, plotting MMA Area vs SDMA IS Areas, we obtained a calibration curve with a very high Area Ratio. That is due to a very different response to ionization among the two molecules.

Regarding the analytical procedure our understanding was to minimize the time, cost and complexity of the processing. To achieve this, considering the polar nature of the molecules, we decided to use HILIC chromatography, which thanks to its selectivity allowed us to analyze underivatized molecules and reduce the sample preparation to a simple deproteinization. Moreover, HILIC chromatography coupled to ESI LC-MS/MS allows us to have a significant increase in sensitivity.

After testing different mobile phases, with increasing concentration of ammonium formate, we found that the elution condition reported in this method represent the best working option. We obtain an efficient chromatographic separation in a short run time and a not significant carryover for all analytes.

5. Conclusion

In the present investigation, we develop a novel method for the determination of arginine metabolites easy to implement in both research and laboratory routines.

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CRediT authorship contribution statement

Lavinia Santucci: Conceptualization, Methodology, Investigation, Validation, Writing – original draft, Writing – review & editing. **Sara Lomuscio:** Investigation, Validation. **Aniello Primiano:** Methodology, Writing – review & editing. **Riccardo Calvani:** Writing – original draft. **Silvia Persichilli:** Methodology, Writing – review & editing. **Federica Iavarone:** Writing – original draft. **Anna Picca:** Writing – original draft. **Francesca Canu:** Investigation, Validation. **Andrea Urbani:** Conceptualization, Supervision, Writing – original draft. **Jacopo Gervasoni:** Conceptualization, Methodology, Validation, Writing – original draft, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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