Microchemical Journal 129 (2016) 29–35

Contents lists available at ScienceDirect

Microchemical Journal

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

Analysis of citrulline and metabolic related amino acids in plasma by derivatization and RPLC. Application of the extrapolative internal standard calibration method

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ARTICLE INFO

Article history:
Received 31 March 2016
Received in revised form 1 June 2016
Accepted 3 June 2016
Available online 04 June 2016

Keywords:
Extrapolative internal standard method
Citrulline
RP-HPLC
Pre-column derivatization

ABSTRACT

We propose to apply the extrapolative internal standard calibration method (EISM) for quantitative analysis of citrulline and other metabolically related amino acids in plasma by pre-column derivatization, liquid chromatography and UV detection. Whenever derivatization is required and an amino acid-free matrix is nonexistent, the analytical challenge is to detect and correct matrix effects and, also to control the experimental conditions of the chemical reaction, mainly the concentration of the derivatizing reagents.

Plasma samples contain free amino acids, non-peptidic amino acids and other amino compounds, such as biogenic amines, that would react with most derivatizing reagents and interfere in the analysis. In this particular work, and considering the scope of the intended application, chromatographic conditions were chosen for the analysis of citrulline, arginine, glutamine, glutamic acid, proline and ornithine by precolumn reaction with FMOC-Cl and RP-HPLC-UV analysis.

Linear least square regression of analyte to internal standard (IS) signal ratio versus the amount of spiked standard amino acid were established and linear regression parameters for each curve were calculated. The information provides by an IS, which is also derivatized, can be used to detect matrix effects due to the chemical reaction. This calibration method was validated and the figures of merit were estimated.

1. Introduction

Many quantitation methods are based on the acquisition of a unique analytical signal after a given chemical reaction [1]. The specific reagents are chosen according to the analyte chemical nature and to the type of detection instruments. These reactions, such as derivatization or complexation, are intended to obtain an specific and unique pure product and, ideally, in a 100% yield, which fulfill relevant analytical requirements of: i) producing the enhancement of the analyte signal and also avoiding interference of this signal under the detection conditions or; ii) obtaining a derivative product with the physical properties appropriate to be submitted to the analytical measurement.

Besides the increment in the laboriousness due to the steps included within the sample pretreatment, the introduction of a chemical reaction before the analytical measurement has many consequences on the quality of the analytical results. A very obvious one is the introduction of one or more reagents to the samples and, thus, potentially interferences would be incorporated. This issue would affect the signal uncertainty although, theoretically, would be taken into account when the reagent blanks are evaluated. Eventually, the potential systematic errors can be corrected during the calibration procedures. Other expectable consequence is the probable decrease in repeatability because of the increment in the manipulation steps and, therefore, the increment in the uncertainty of the final result. However, one more subtle is the need of the reaction control to yield a constant product amount. Protocols of derivatization usually provide many relevant details about the specific reaction. It is quite common, however, to check and optimize the chemical and physical conditions under those procedure guidance and to introduce minor adjustments in order to improve the attributes of the analytical response according to the specificity of the sample matrix or to the available laboratory auxiliary instruments. Among the most relevant variables affecting the yields, the concentration of the derivatization reagents must be fixed in order to guarantee the reproducibility of those reactions. Usually these optimization steps are carried out using standard solutions of the analytes.

Accurate quantitative information of the concentration of citrulline, arginine, and amino acids related to the urea cycle can be used as biochemical indicator for clinical diagnosis of specific pathologies. Recent studies suggested that citrulline concentration in plasma would be an index of physiological disorders [2–4]. The analysis of amino acids using a cation-exchange separation column followed by post-column derivatization with ninhydrin, remains the established standard in clinical routine [5]. However, this method requires dedicated instruments

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http://dx.doi.org/10.1016/j.microc.2016.06.005
0026-265X/

Table 1
Amino acids concentration levels and final reactant ratio for the three calibration methods.

EM[a]			SAM[b]			EISM[c]		
Level	Stock solution [AA^T solution] μM	[FMOC-Cl]/[AA]^{Td}	Level	Stock solution [AA^T solution] μM	[FMOC-Cl]/[AA]^T	Level	Stock solution [AA^T solution] μM	I.S. [1.2 mM] [μL]
1	0.05	166.7	0	0	[e]	0	0	5
2	0.10	55.6	1	0.08	100.0	1	0.08	5
3	0.26	30.3	2	0.16	50.0	2	0.16	5
4	0.36	22.2	3	0.32	25.0	3	0.32	5
5	0.60	13.3	4	0.60	13.3	4	0.60	5
6	0.96	8.3	5	0.80	10.0	5	0.80	5
7	1.56	5.1	6	0.91	8.7	6	0.91	5
8	2.04	3.9	–	–	–	–	–	–
9	2.40	3.3	–	–	–	–	–	–

[a] External standard method.
[b] Standard addition method.
[c] Extrapolative internal standard method.
[d] All [FMOC-Cl]/[AA]^T is fixed with the total amino acid standard solution added.
[e] Unknown ratio due to the background concentration.

and long run times. A lot of effort has been spent on the development of reversed phase HPLC methods based on pre-column derivatization of the amino groups with UV or fluorescence detection [6–12]. More recently, many other reagents to enhance the signals in analysis of amino acids by LC/ESI/MS were specifically designed [13–16]. Generally applied reagents for amino acids and polyamines are o-phthaldehyde (OPA) and 9-fluorenyl methyl chloroformate (FMOC-Cl). Both reagents yield products that are detectable at low concentrations, involve rapid reactions occurring in aqueous solutions and at room temperature. Those are four highly desirable conditions to simplify the routine analysis.

Reactions using FMOC-Cl have two additional advantages: greater stability of the formed carbamates as compared to OPA-derivatives, and feasibility of reaction of primary and secondary amino groups, such as proline. Several researchers have extensively studied the conditions that affect the reaction between amino acids and FMOC-Cl with the aim of maximizing the yield of carbamate derivatives [9,17,18].

There is a number of different calibration methods used in chemical analysis that can be classified in three categories: interpolative, such as, external standard method (EM) or internal standard method (IM); extrapolative, the most known is the standard addition method (SAM); and indicative method, which include all titration techniques [19]. A type of extrapolative method, is named extrapolative internal standard method (EISM) [20], in which the standard analyte is added directly to the sample along with a constant amount of an internal standard. The calibration plot is constructed from the signal relative to an internal standard as a function of either, the concentration of standard solutions added to the sample or the ratio between concentrations of spiked standard respect to the IS.

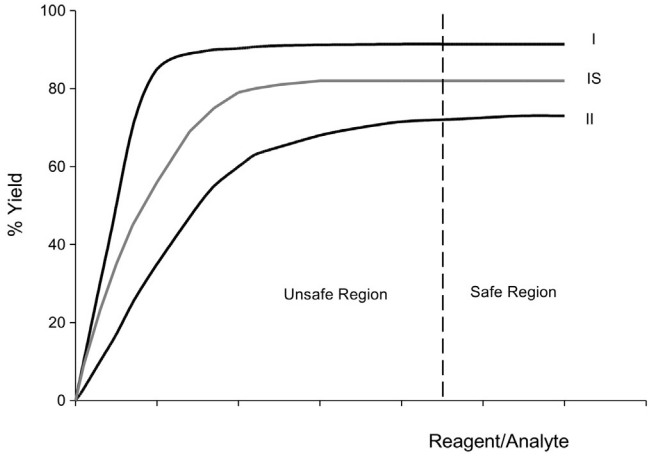

Fig. 1. Hypothetical dependence between reaction yields and reagent/analyte ratio for two solutes and for the IS.

In this study, the EISM was tested with a very specific aim, i.e., to evaluate if the concentration of derivatizing reagent could be a limiting variable affecting the reaction yield. This is a very important issue that must be assessed whenever pre-column derivatization is required and the analyte amounts (along with all other reactive components) present in the real sample are completely unknown. The EISM was applied to the determination of citrulline, arginine, glutamine, glutamic acid, proline and ornithine present in plasma samples. Although, our interest was focused on these metabolically related analytes, human plasma is a very complex matrix, i.e., can contain different amino acid concentrations depending on age and even between healthy and unhealthy patients. Moreover, other amino compounds, such as biogenic amines, do react with most derivatizing reagents. Under such a circumstance, not only the matrix effects due to the sample itself must be assessed but also, the reaction conditions and the independence in the relationship between reactant and analyte concentrations must be checked to avoid bias in the analytical results.

2. Experimental

2.1. Reagents

9-Fluorenylmethyl chloroformate (FMOC-Cl) and citric acid were obtained from Fluka (Buchs, Switzerland); arginine (Arg), citrulline (Cit), glutamic acid (Glu), proline (Pro), ornithine (Orn) and glutamine (Gln) standards were provided by BDH (Poole, UK); tyramine hydrochloride (98%, Tyrn) and aminobutyric acid were from Aldrich (St. Louise, MO, USA). The FMOC-Arg and FMOC-Cit were purchased to Sigma-Aldrich (St. Louise, MO, USA). Trichloroacetic acid (TCA), ammonium hydroxide, borax, and formic acid (85% w/w) were from Anedra (Anedra, Argentine) and HPLC-grade acetonitrile (ACN) was from Baker (Mexico City, Mexico). Water was purified by means of a Milli-Q Purification System (Simplicity, Millipore, MA, USA).

2.2. HPLC instrument and gradient composition

An Agilent 1100 series LC system (Agilent, CA, USA) equipped with a binary pump, degasser, temperature-controlled column compartment, automatic injector module and diode-array detector was used. A Chromolith Performance RP-18 (100 × 4.6 mm) column, protected by a guard column provided by the manufacturer, was used. Separations were carried out by gradient elution. The eluent was varied linearly from A-B (90–10%) to A-B (20–80%) at 2%B·min^{-1}, A = 25 mM formate buffer pH 3.75, and B = ACN. Temperature was 25 °C. Flow rate was 2 mL·min^{-1}.

2.3. Standard solutions

Amino acid (AA) standard solutions 10 mM were prepared in 0.1 M HCl and diluted before use. Since glutamine gradually degrades during storage, the solution was kept frozen for no more than one week. Aminobutyric acid 1.2 mM (internal standard) was prepared in water. Borate buffer solution was prepared from 0.2 M borax and adjusted to pH 9.20. The stock solution of FMOC-Cl was prepared immediately before reaction by dissolving the solid in ACN to a final concentration of 4 mM. Highly concentrated aqueous solutions of Tyrn (75 mM), used to deplete the FMOC-Cl reagent, were prepared and used without dilution.

2.4. Sample preparation

Heparinized blood samples, obtained from healthy male volunteers from our laboratory, were collected and immediately centrifugated at 8000*g* during 10 min. The proteins were precipitated by addition of 100 μL of a 30% w/v solution of TCA per mL of the plasma sample followed by centrifugation at 7000*g* for 5 min. The resulting supernatant was frozen at −18 °C. Before analysis, samples were thawed, and aliquots of 20 μL of plasma were mixed with standard solutions prior derivatization and subsequent chromatographic analysis.

2.5. Amino acid derivatization

Briefly, 130 μL of buffer solution was added to the amino acid standard solution, AA^T (where the superscript "T" refers to the total concentration of amino acids), 20 μL of deproteinized plasma sample and 300 μL of FMOC-Cl solution. The final reaction solvent was 50:50 ACN:water and final volume was 600 μL. The mixture was homogenized by vortexing for 1 min at room temperature, then, the reaction was quenched with 20 μL of a 75 mM Tyrn solution followed by vortexing for 1 min. All final solutions were filtered through 0.22 μm Nylon membranes (Micron Separations, Inc., Westborough, MA, USA).

2.6. Calibration

External calibration was constructed by dilution of the standard solutions from 2 to 100 μM of each amino acid (nine concentrations), followed by derivatization. Additionally, a blank containing 0.1 M HC1 was prepared by following the same procedure than amino acids standard solutions. Triplicates of each level were carried out in order to check the linearity.

SAM and EISM were carried out by addition of standard solutions (0–40 μM of each amino acid) to plasma samples (20 μL). In EISM, 5 μL of internal standard was added to the mixture. The analysis was performed at seven concentration levels in triplicates. Linear least square regression of signal against the spiked amino acid concentration was calculated for SAM. Regression of the signals ratio between each analyte and the internal standard versus amino acid concentration were computed for the EISM. Table 1 gathers the information about of standard solutions concentration and the ratios between FMOC and total amino acids.

3. Results and discussion

Fig. 1 shows the hypothetical dependence between reaction yield and derivatization reagent concentration (or derivatization reagent/amount of analyte) for two substances, I and II, with different kinetics and maximum yields. The dotted line at a given ratio separates two regions: on the right, all changes in concentrations do not affect the corresponding reaction yields, i.e., samples with a small amounts of standard solutions added will provoke not go beyond the safe zone. As a consequence, SAM and EISM calibration equations will be correlated. On the other hand, assuming that the original samples contain an abnormally large amount of reactive analytes (unknown) and the addition of standards during calibration makes the ratio cross the border towards an unsafe region (left side) for one analyte, the SAM slope will be significantly smaller than that obtained within the safe region for analyte II, although the unawareness of the sample content makes the issue unnoticed. In such a circumstances, however, the use of an EISM calibration curve would make noticeable the fact that the reaction yield was affected due to the depletion of the derivatizing reagent.

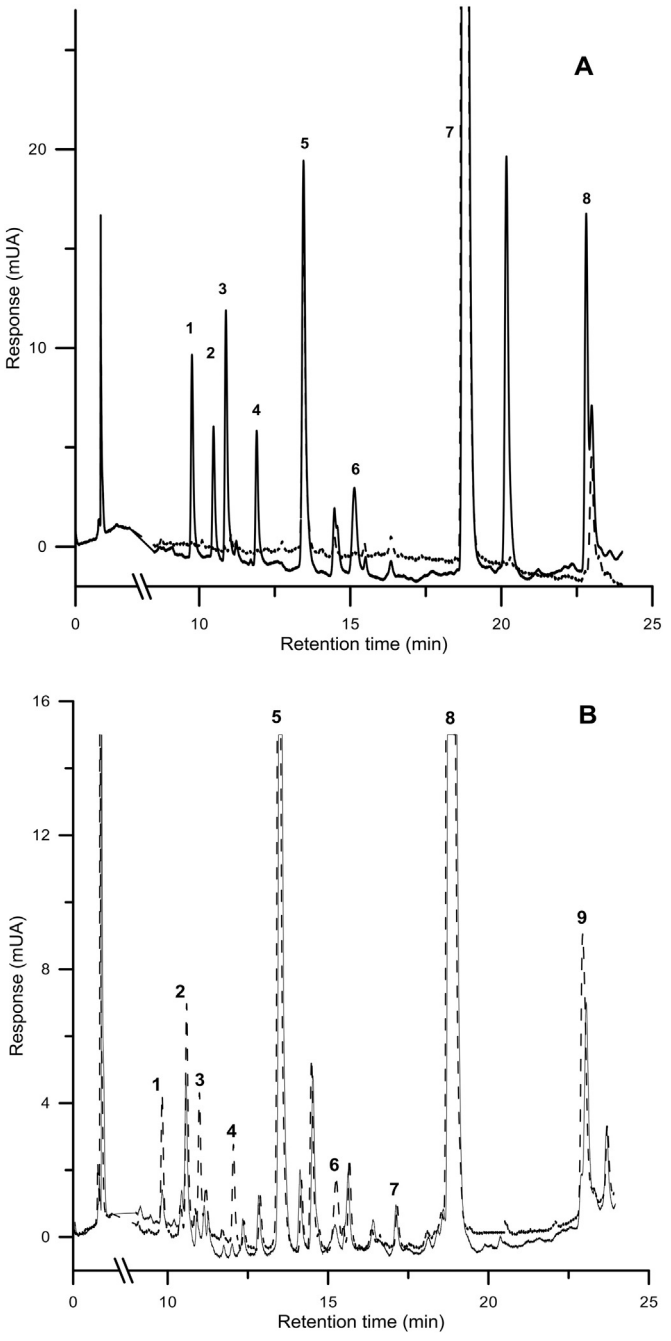

Fig. 2. Representative RP-HPLC chromatograms of FMOC-derivatives. Column: Chromolith 100 × 4.6 mm, flow rate: 2 mL·min^{-1}. Mobile phase: solvent A: formate buffer 25 mM pH 3.75/solvent B: ACN. The eluent was varied linearly from A–B (90–10%) to A–B (20–80%) at 2%B·min^{-1}, Temperature: 25 °C. Wavelength: 260 nm. (A) Chromatograms obtained from a standard solution containing 40 μM of each amino acid (full line) and reagent blank (dashed line). Peak identification: 1: Arg, 2: Gln, 3: Cit, 4: Glu, 5: FMOC-OH, 6: Pro, 7: Tyrn, 8: Orn. (B) Chromatograms of a normal human plasma sample with IS. Full line: without standard addition; dashed line: spiked with 13.3 μM of each amino acid. Peaks: 1: Arg, 2: Gln, 3: Cit, 4: Glu, 5: FMOC-OH, 6: Pro, 7: IS, 8: Tyrn, 9: Orn.

Table 2
Least square regression and lack-of-fit results.

	Gln		Arg		Cit		Glu		Pro		Orn	
	EM	SAM	EM	SAM	EM	SAM	EM	SAM	EM	SAM	EM	SAM
$S^2_{x/y}$	0.248	0.061	0.390	0.061	0.689	0.273	0.166	0.035	0.053	0.020	0.612	0.610
b ($\pm s_b$)	0.169 (0.004)	0.233 (0.004)	0.242 (0.004)	0.281 (0.004)	0.280 (0.006)	0.329 (0.004)	0.152 (0.003)	0.224 (0.003)	0.105 (0.002)	0.122 (0.002)	0.451 (0.009)	0.56 (0.02)
a ($\pm s_a$)	0.02 (0.02)	4.34 (0.09)	0.5 (0.2)	0.78 (0.09)	1.1 (0.3)	0.29 0.09)	0.2 (0.1)	0.35 (0.06)	0.22 (0.07)	0.72 (0.05)	0.6 (0.3)	1.4 (0.4)
n	17	18	24	19	21	20	19	20	21	20	15	14
$F_{calculated}$	0.187	0.066	0.008	0.075	0.002	0.056	0.028	0.145	0.0002	0.00004	0.08	0.025
$t_{calculated}$	0.16		1.79		0.78		2.4		0.96		1.69	

$S^2_{x/y}$ is residual variances of regression; b and a are slope and intercept of the regression; $F_{calculated}$, coefficient of variances for a confidence level of 95% and $t_{calculated}$, statistical parameter for slope comparison.

Chromatographic results obtained for the amino acid standards and samples eluted from a monolithic C18 column are shown in Fig. 2. Plots A and B correspond to the chromatograms of the standards and of human plasma, respectively. Good resolutions between FMOC-amino acids respect to unidentified peaks from the reaction (secondary products and/or impurities) and from the main peaks FMOC-OH and FMOC-Tyrn were obtained.

The pre-column derivatization of citrulline and related amino acids followed by chromatographic separation had been studied [21]. Optimization of reaction conditions included: i. buffer type, concentration and pH, ii. reaction time and temperature, iii. concentration of FMOC-Cl and FMOC-Cl/amino acid ratios, and iv. conditions to stop the reaction at a fixed time using either, non-polar extracting solvents, addition of strong acids to cause an abrupt change in pH of the reaction mixture, or the addition of a highly concentrated competing reactant to scavenge the FMOC excess. This last option was assayed with Tyrn and it was chosen because the product did not interfere in the chromatogram and high reproducibility values were achieved. The reaction yield under the aforementioned conditions, was evaluated for citrulline and arginine. The peak areas obtained after reaction corresponded to 93 and 95% as compared with the peak area of standard solutions prepared from pure commercial FMOC-Cit and FMOC-Arg, respectively. Those areas did not change after 1, 5, 10 and 20 min of incubation. Not all the analytes (amino acids in this example), however, have the same chemical kinetics [18,22] neither similar final yields at the maximum. Several researchers have stressed the importance of maintaining the molar ratio of FMOC/amino acids within a certain range [23–25], although, these data do not show conclusive results [17]. As reaction rates are proportional to the reactant concentrations, the derivatizing reagent concentration must be kept constant. However, as many components of the sample will react during mixture incubation, in addition to the competence of water molecules (FMOC-OH generation), one key issue is to provide an excess of FMOC-Cl to prevent a decrease in its concentration which can affect the reaction rates.

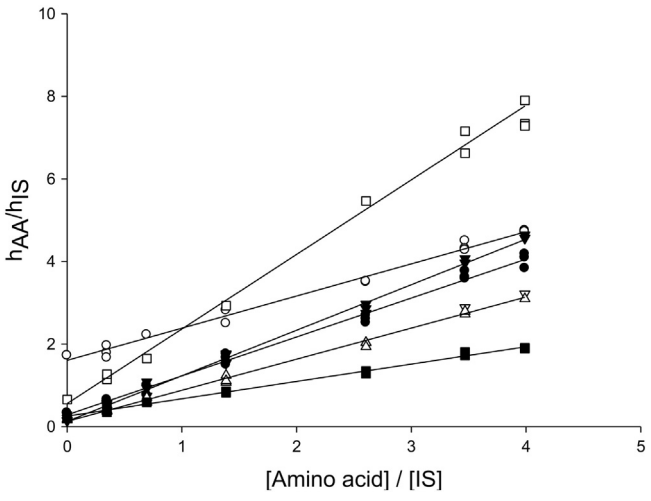

Fig. 3. EISM curves of Arg (●), Gln (°), Cit (▼), Glu (◇), Pro (■) and Orn (□).

3.1. Method validation

The condition of homoscedasticity has been evaluated before determination of figures of merit, since this condition is certainly not always fulfilled, and assuming homoscedasticity can lead to wrong limits of predictions from the calibration curves. The standard deviation of the signals for each amino acid measured at all concentration levels were randomly scattered (data not shown) indicating the homoscedasticity of the data across the tested concentration range, i.e., the independence of dispersion in the peak area as referred to the injected analyte quantity. This can be attributed to the relatively narrow concentration range taken in this study.

3.1.1. Linearity

Different studies reported that the total amount of free amino acids in healthy human plasma is in the range between 1840 to 3200 μM. Specifically for citrulline, a significant variability with the age has been observed for healthy people [18,26,27], and the concentration can be up to two orders higher for some pathologies such as citrullinemia. In order to plan the standard addition calibration, an average of that range of AA^T was considered.

The lack-of-fit test was applied to check the linearity of the calibration according to recommendations of the Analytical Method Committee [28]. Linearity should be evaluated for three replicates with a minimum of five levels of concentration [29]. In this study, seven levels made in triplicate, and duplicate injections were carried out. Table 2 shows the statistical parameters obtained after fitting by least square linear regression analysis of external and standard addition responses for each amino acid, together with the calculated *F* values for the six amino acid derivatives. The results indicate no evidence of lack-of-fit to a linear model for any of the amino acids within the studied concentration ranges (EM: 0–600 μM and SAM:

Table 3
Least square regression values from the EISM calibration curve, LOD, LOQ and found concentration (C_x).

EISM						
	Arg	Gln	Cit	Glu	Pro	Orn
a ($\pm s_a$)	0.28 (0.05)	1.60 (0.05)	0.13 (0.04)	0.13 (0.03)	0.25 (0.02)	0.5 (0.2)
b ($\pm s_b$)	0.95 (0.02)	0.78 (0.02)	1.10 (0.02)	0.75 (0.01)	0.42 (0.01)	1.80 (0.07)
$S_{x/y}$	0.126	0.128	0.117	0.074	0.046	0.367
r^2	0.994	0.992	0.991	0.994	0.995	0.995
C_x [μM] ($\pm s$)	87 (1)	593.3 (0.6)	33.4 (0.3)	48.8 (0.3)	174.4 (0.4)	88.6 (0.7)
LOD [μM]	0.8	5.4	0.9	0.6	0.6	2.5
LOQ [μM]	1.6	17.2	2.7	1.8	1.6	7.7

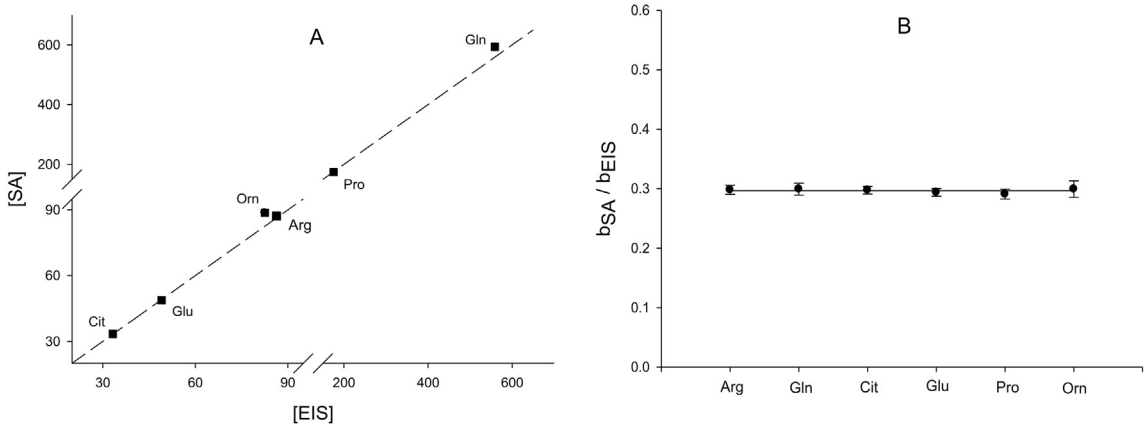

Fig. 4. A) Amino acids concentration estimated from SAM vs. EISM calibration curves. B) Slope ratio between methods for each amino acid (b_{SAM}/b_{EISM})·Standard deviations are represented by vertical bars and straight line corresponds to the average (0.2965).

0–150 μM). The highest value ($F_{exp} = 0.187$) corresponded to the EM curve of FMOC-Gln and it was well below the critical *F* with a 95% of confidence for 5 and 14 degrees of freedom ($F_{crit} = 3.289$). The linear model appears to be adequate for both calibration methods, and it is expected that the linear range extends beyond these limits.

The intercepts of the EM calibration for all amino acids, which are an estimation of the contribution to the signal due to the reaction blanks, were statistically zero. The chromatogram obtained by derivatization of an amino acids-free solution (Fig. 2A) confirms that no signal due to the chemical reaction appears at the elution times of the target FMOC-amino acids.

The possibility of matrix effect can seriously affect the accuracy of the analytical results [30]. Proportional bias was estimated by comparing the slopes of the straight lines of EM and SAM methods. If these slopes are statistically similar, then proportional bias is not involved. To check the similarity of both slopes, a Student's *t*-test was applied after checking that the variances of both linear calibrations were statistically similar [28]. The calculated *t*-values, gathered in Table 2 are lower than $t_{0.05,35} = 2.58$. Similarities in the observed slopes suggest that no proportional errors are present for this specific matrix, although this could not be a generic conclusion applicable to other plasma samples.

3.1.2. Extrapolative internal standard method (EISM)

The EISM was rarely used for quantification of amino compounds [20,22,31,32]. However, it is very common to add an IS as a probe for recovery studies. A non-essential amino acid, aminobutyric acid, was employed as internal standard (IS). The IS was added in a constant amount for all levels of the calibration curve (Table 1), and all the measurements were carried out by triplicates (n = 3).

Fig. 3 shows the EISM calibration curves for the six amino acids analyzed in human plasma and Table 3 summarizes the least square regression results. In Fig. 4, the found concentration of these amino acids calculated from both, EISM and SAM, calibration methods were plotted. Points are scattered closed to the line with slope 1 and intercepts zero, indicating no significant differences in the concentration estimated for any of these amino acids. These results demonstrate that all the amino acids reached the maximum derivatization yield. Under this situation, no difference between both calibration methods are expected. The same do not apply if the chemical reaction of one or more analytes would not reach the "safe zone" described in Fig. 1. Fig. 4B shows the slope ratios between SAM and EISM calibration curves. Error bars were estimated from the standard deviations of both slopes by classical error propagation rules. A constant value for the six amino acids is indicative that there were no differences in the chemical reaction between different amino acids even when the relationship between derivatizing reagent and total amino acids was varied from 100/1 (24/1) to 8/1 (6/1). The ratios within brackets are calculated considering an average amount of amino acids present in plasma of health adults.

3.1.3. LOD and LOQ

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated using the IUPAC convention [33], as 3.28 and $10S_0$, respectively, where S_0 is calculated from the corresponding calibration curve as:

$$S_0 = \frac{S_{x/y}}{b}\left\{\frac{1}{n}+\frac{1}{m}+\frac{\bar{x}^2}{Q_{xx}}\right\}^{1/2} \quad (1)$$

where $S_{x/y}$ is the standard deviation of the regression, *b* the slope, *n* the replicates, *m* the number of samples, $\bar{X}$ is the concentration mean of the calibration, and Q_{xx} is the sum of squares of *x*. The estimated LOD and LOQ are summarized in Table 3. LOD ranged between 0.6 μM for Glu and Pro to 5.4 μM for Gln. The obtained limits were somewhat lower than those reported by other authors using FMOC-Cl as derivatization reagent, employing conventional columns of C-18 and UV detection (4–6 μM) [24].

3.1.4. Precision and accuracy

The results for intra-day (n = 5) and inter-day (n = 6) variations were evaluated by analyzing 40 μM solutions within a day and then duplicates were determined at three and ten days from the former. Results, expressed as % RSD, are summarized in Table 4. Intra-day RSD were not higher than 9.6%, and intermediate precision (inter-day RSD) for all compounds ranged from 6.7 to 10.5% (glutamic acid). These values are reasonable considering that the analytical precision depends on the reproducibility of the sample pretreatment which includes the whole derivatization reaction.

Table 4
Precision and recovery factors.

	Arg	Gln	Cit	Glu	Pro	Orn
Inter-day (n = 3)	8.0	10.4	9.4	10.5	6.7	7.0
Intra-day (n = 5)	6.5	8.6	7.1	6.7	9.3	9.6
% recovery (s)	72 (6)	91 (4)	70 (10)	76 (10)	69 (6)	66 (8)

Standard deviation is shown between brackets.

Accuracy can be evaluated through the recovery test. The procedure for recovery test is similar to that for SAM, except that in the standard calibration the analyte standard solution is added to a sample immediately before the measurements, whereas for the recovery test the sample is spiked at the beginning of the pretreatment to evaluate the whole analytical procedure, i.e., before protein precipitation [34].

In this validation, the accuracy of the method was verified adding known amounts of analyte standard solutions to a known amount of human plasma and subjecting the mixture to the proposed deproteinization followed by the derivatization procedure. Spiked samples were prepared in triplicate at three concentration levels: 41.7; 208.3 and 416.7 μM of total amino acids and recoveries (%R) were calculated according to IUPAC convention [35]. Statistical analysis performed with recoveries at three levels showed no significant differences (for a confidence level of 95%) between values. The calculated mean recoveries are reported in Table 4.

4. Conclusions

The extrapolative internal standard method (EISM) has been applied to the determination of citrulline and metabolically related amino acids in plasma after their pre-column derivatization with FMOC-Cl, followed by HPLC separation and UV detection. Figures of merit for the determination of citrulline, arginine, ornithine, glutamine, glutamic acid and proline in human plasma have been determined.

The implementation of the described method was aimed to overcome the analytical problems related to matrix with endogenous presence of unknown amount of reactive compounds along with the need of derivatization. Since the derivatizing reagent is mixed directly with the sample, reaction efficiency (yield) is easily influenced by sample matrix and by coexisting reactive components.

The comparison between the EISM and the SAM calibration method allowed to assess that the chemical derivatization reaction was under the corresponding maximum yield for every analyzed amino acid. It was observed that the responses were reproducible at all standard levels within the linear range of the curve, regardless of the total amino acids to FMOC-Cl ratios. As a summary, the EISM has advantages over the SAM method for analytical measurements based on a signal produced after a given reaction, since it can be used to be sure that the chemical derivatization reaction is not affecting the quantitative results.

Acknowledgement

The authors kindly acknowledge Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP2013-2015), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT3597) and Universidad Nacional de La Plata (UNLP, 11X/696) for financial support.

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