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Reliable and simple analytical methods for determination of citrulline and metabolically related amino acids by liquid chromatography after derivatization: comparison between monolithic and core–shell columns

Agustín Acquaviva, Lílian Romero, Cecilia Castells,* Guillermo Ramis-Ramos and José M. Herrero-Martinez

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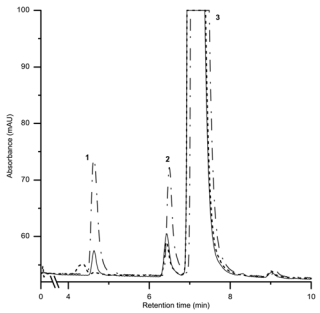

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Analytical Methods

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Reliable and simple analytical methods for determination of citrulline and metabolically related amino acids by liquid chromatography after derivatization: comparison between monolithic and core–shell columns

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We describe the development of a high-performance liquid chromatography (HPLC) method for the determination of citrulline and other amino acids relevant to intestinal diseases. The amino acids were derivatized with 9-fluorenylmethylchloroformate (FMOC-Cl) and their derivatives were separated on two different columns, a core–shell column (Halo C18) and a silica-based monolith (Chromolith Performance RP-18). The derivatization reaction was optimized with respect to pH, buffer concentration and reproducibility. The optimal derivatization conditions were achieved with 0.4 M borate buffer at pH 9.20, a constant ratio of FMOC-Cl/total amino acids (10 : 1) and 75 mM tyramine after 1 min (quenching reaction). The separation conditions with both chromatographic supports were also optimized. The chromatographic performance (peak capacity and global resolution) of these two columns was compared. This proposed HPLC-UV method was satisfactorily applied to the analysis of a real plasma sample.

1. Introduction

Amino acids are involved in the regulation of many metabolic pathways, in protein synthesis, and in biological activities. Thus, the determination of free amino acid concentrations can be used as a biochemical indicator of several pathologic disorders. In particular, citrulline, arginine, and other compounds related to the urea cycle can be used in the clinical diagnosis of several pathologies such as the short-bowel syndrome, and disorders in small-bowel absorptive capacity.[1,2] For this reason, the development of simple, reliable and accurate analytical methods for amino acid determination in biological fluids is of extreme relevance to both gastroenterology and clinical medicine in general.

Amino acids are usually determined by ion-exchange chromatography, HILIC[3,4] or, more commonly, by reversed-phase liquid chromatography coupled with UV,[5] fluorescence[6] and laser induced fluorescence[7] and electrochemical detection.[8] Pre-column derivatization of amino acids not only increases the detectability but usually also enhances the hydrophobicity of highly polar analytes so as to produce adequate retention in HPLC with common columns in the reversed-phase mode.

Several derivatization reagents have been proposesd: dansyl chloride,[9,10] and dabsyl chloride,[11,12] *o*-phthaldialdehyde combined with several thiols,[13,14] 9-fluorenyl methyl-chloroformate,[15–17] phenylisothiocyanate,[18] naphthalene-2,3-dicarboxaldehyde[19] and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate.[20] Derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl) has the advantage of reacting almost immediately and under mild conditions, and both primary and secondary amino groups react to form the corresponding carbamates. These derivatives are quite stable, and detection may be accomplished by either UV absorbance or fluorescence-labelling. Molnár-Perl *et al.*[5,21–23] have published several articles describing a thorough and extensive study of amino acid derivatization conditions using both *o*-phthaldialdehyde/thiols and FMOC-Cl. However, a detailed derivatization study (including aspects such as removal of excess of FMOC-OH and its potential interference and reproducibility of the protocol applied to citrulline and its related amino acids) has not been considered.

In the present work, we focused on the determination of six metabolically related amino acids, especially citrulline, along with other five amino acids (arginine, proline, glutamine, glutamic acid and ornithine) whose plasma levels could be used as an indication of intestinal-absorption anomalies. The

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development of reliable, rapid and simple analytical methods to be transferred to clinical laboratories having different HPLC capabilities is of utmost concern. In this context, the interest of evaluating novel packing designs such as core–shell and monolithic types, which represent promising alternatives to the conventional totally porous packed columns, in order to improve the separation efficiency and analysis time.

In the last few years, several analytical strategies related to column technology have been developed in HPLC, including the use of monolithic supports, packed columns with core–shell particles or with sub-2 μm particles operating at ultra-high pressure (UPLC). Thus, monoliths, in chromatographic terms, are porous rod structures characterized by extremely high permeabilities and column efficiencies compared to sub-5 μm particle-based columns.[24] Other recent column types, introduced to compete with sub-2 μm particle-based packed columns, have a base of core–shell particulate materials. The main feature of these columns is that the particles consist of solid cores surrounded by a porous silica shell, of about 0.5 μm. Therefore, the analyte diffusion paths are shorter than those in conventional totally porous silica particles, which significantly minimizes peak broadening.[25] Both types of chromatographic supports have been applied to the amino acid analysis;[26–29] however, several weaknesses related to both the derivatization protocol (*e.g.* reproducibility) and the separation method (complex gradient elution programs, lack of evaluation and correction of interference arising from the blank reagent, scarce application to human plasma samples, *etc.*) have been found.

In this study, the performance of a monolithic silica column (Chromolith Performance RP-18) and a core–shell column (Halo C18) for the HPLC separation and UV analysis of citrulline and related amino acids (*e.g.*, arginine, proline, glutamine, glutamic acid and ornithine) based on pre-column derivatization by the FMOC-Cl reagent was examined. Prior to this evaluation, optimization of several experimental variables (pH, buffer concentration and reproducibility) of the derivatization reaction protocol was carefully investigated. Then, the separation of amino acid derivatives was achieved by both columns and critically compared in terms of the chromatographic performance. The monolithic column was then used for application of the technique to the analysis of a real human-plasma sample.

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 from BDH (Poole, UK); phenylethylamine hydrochloride (99%, Phea) and tyramine hydrochloride (98%, Tyrn) were from Aldrich (St. Louise, MO, USA); heptane was from Mallinckrodt Chemicals (St. Louis, MO, USA); pentane and anhydrous (glacial) acetic acid were from Merck (Darmstadt, Germany); trichloroacetic acid (TCA), ammonium hydroxide, borax, and formic acid (85% w/w) were from 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). Unless otherwise stated, all chemicals employed were of analytical-reagent grade.

2.2. Standard solutions

Standard solutions of 10 mM of individual amino acids (AAs) were prepared in 0.1 M HCl, stored in a refrigerator and diluted before use. A stock solution of a mixture of the six amino acids (2 mM each) was also prepared. Borate buffer was prepared from 0.2 M borax and adjusted to pH 9.20 (final concentration: 0.4 M buffer solution). The stock solution of FMOC-Cl was prepared immediately before use, by dissolving the appropriate amount of solid in ACN. Solutions of 75 mM of tyramine (Tyrn) and phenylethylamine (Phea) were prepared with distilled water and used without dilution.

2.3. Amino acid derivatization

The optimized derivatization reaction was carried out by mixing the reagents in the following order: 150 μL of borate buffer solution at pH 9.20, 100 μL of AA^T standard solution (where the superscript "*T*" refers to the total concentration of amino acids), 50 μL of the sample after deproteinization and 300 μL of FMOC-Cl solution. This reagent solution was prepared at four different AA^T concentrations: 1.7, 2.8, 7.2 and 14 mM, to keep a constant relationship between the reactants FMOC-Cl and AA^T in the construction of the calibration curve. The mixture was homogenized by vortexing for 1 min at room temperature, the reaction was quenched with 20 μL of a 75 mM Tyrn solution and vortexing for 1 min. All final solutions were filtered through 0.22 μm Nylon membranes (*Micron Separations*, *Inc.*, Westborough, MA, United States). A derivatization reagent blank was also prepared using the same protocol.

2.4. Plasma-sample preparation

Proteins were precipitated by addition of 100 μL of a 30% (w/v) solution of trichloroacetic acid (TCA) per mL of the plasma sample followed by centrifugation at 7000×*g* for 5 min. The resulting supernatant was extracted for derivatization and subsequent chromatographic analysis.

2.5. HPLC separation of derivative amino acids

An Agilent 1100 series LC system (Agilent, CA, USA) equipped with a binary pump, degasser, temperature control column compartment, automatic injector module and diode-array detector was used. Two columns were employed: a Chromolith Performance RP-18 (100 × 4.6 mm) and a Halo C18 (50 × 2.1 mm, 2.7 μm particle size), and both were protected by a guard column provided by each respective manufacturer. Gradient elution was performed by mixing 25 mM formic/ammonium formate buffer at pH 3.75 (solvent A) and ACN (solvent B). A linear gradient from 10 to 80% B in 30 min was performed. Flow rates were set at 2 and 0.25 mL min^{-1} for the monolithic and core–shell columns, respectively. The injection volume was 5 μL and the detection wavelength was set at 260 nm.

3. Results and discussion

3.1. Optimization of derivatization

The systematization and automation of a method to quantify amino acids in biological samples require an adjustment of all variables to achieve suitable reproducibility. For this purpose, several reaction conditions were first investigated (*e.g.*, borate buffer concentration and pH) along with different approaches to minimize the presence of impurities in the blank measurements and alternative ways of quenching the reaction to enhance the protocol reproducibility. Other variables such as temperature, reaction time and FMOC-Cl post-reaction excess were taken from a complete study that had been previously published.[23]

The molar ratio of reactants was chosen according to the previous results critically reviewed by Molnár-Perl.[22] In that review, the author discussed that the reaction efficiency does not change significantly when the ratio of reactants was varied for higher concentrations of FMOC-Cl. However, the author recommended the use of a ratio of 55/1. Here, the absolute concentration of FMOC was varied from 0.15 to 0.72 mM; the ratio between FMOC/AA^T was varied from 2/1 to 55/1 and amino acid peak areas increased until an asymptote was reached for a ratio excess of 10/1. FMOC/AA^Ts at 10/1 and a reaction time of 1 minute were selected for further study. A larger amount of FMOC in the reaction media yielded a wider hydrolyzed peak which interfered with the peaks of interest close to it (Glu and Pro).

Borate buffer was selected since that formulation had been commonly used for derivatization reactions with FMOC-Cl.[22] In the present experiments, the ionic strength was fixed at 0.4 M and the reaction yield was tested at several buffer pHs (8.2, 9.2 and 10.2). Fig. 1 compares the citrulline peak area obtained in the chromatograms resulting from these conditions. The lowest peak signals, when the reaction was conducted at pH 10.2, were attributed to an increased hydrolysis rate of the FMOC-Cl reagent. Another disadvantage at this pH was the presence of an overloaded hydrolyzed FMOC-Cl (FMOC-OH) peak, whose profile hindered the evaluation of the peaks of the adjacent amino acid derivatives. As shown in the figure, the highest peak signal (and consequently the highest yield) was achieved at pH 9.2. At pH 8.2, the peak height was significantly lower indicating that the reaction was incomplete within the reaction time. A similar result was obtained for other amino acids that were investigated (data not shown). A pH of 9.2 was therefore selected for the following studies.

The influence of the ionic strength on this reaction has been previously studied within the range from 0.01 M to 0.4 M.[30,31] We therefore evaluated the reaction yield at higher ionic strengths (0.4–0.8 M). Table 1 presents the results obtained for FMOC-Cit, FMOC-OH and FMOC-Tyrn. The FMOC-Cit peak areas measured were similar over the range of ionic strengths tested, although the peak of FMOC-OH was smaller when the borate buffer concentration was 0.8 M without any increase in the FMOC-Tyrn peak area. These results indicated that borate buffer should be kept at 0.8 M,

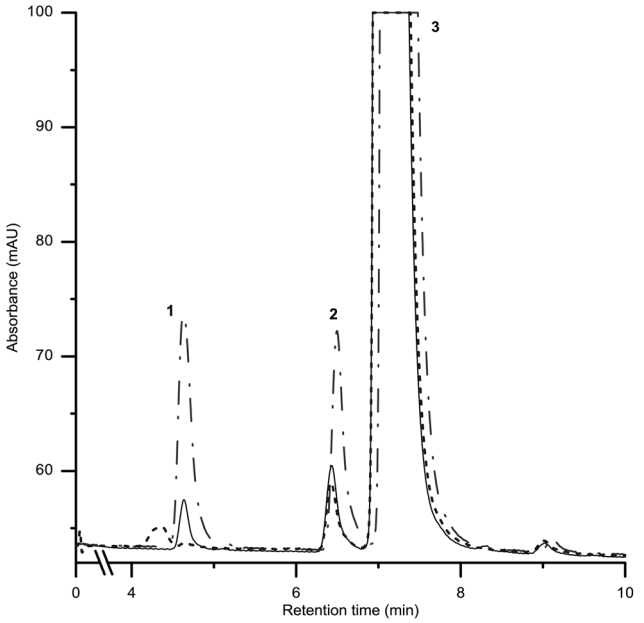

Fig. 1 Effect of buffer pH on the reaction yield. Column: Chromolith Performance RP18 (100 × 4.6 mm i.d.). Elution conditions: linear gradient from 10 to 80% (v/v) ACN at 2% ACN min^{-1}; flow rate, 2 mL min^{-1}; temperature, 25 °C. pH 8.20 (line); pH 9.20 (dash and dot) and pH 10.20 (dash). Peak identification: 1 = FMOC-Cit; 2 = FMOC-impurity; 3 = FMOC-OH. Detection at 260 nm.

but the extremely slow dissolution rate of the borax salt at this high concentration prevented that concentration from being used in practice. Thus, 0.4 M was selected as the buffer concentration.

Since the derivatization reaction is carried out with a high excess of FMOC-Cl, and the carbamate formation with the amino acid competes with the acid chloride hydrolysis at room temperature, the reproducibility of the reaction needed to be assessed. For this purpose, two issues were addressed: (i) an increase in the reproducibility of the reaction (expressed in terms of the reaction yield) and (ii) a reduction in the FMOC-OH peak—it is eluting in the central region of the chromatogram—in order to decrease that interference with the more closely eluting analytes in the run. Three strategies have been reported in the literature to address this particular problem:

(a) The use of immiscible solvents[23,32,33] to stop the reaction, thus extracting the excess of FMOC-OH into the aqueous phase. For this purpose, *n*-pentane and *n*-heptane were selected here as extractants.

Table 1 Peak areas (in arbitrary units) corresponding to reactions performed at different ionic strengths

	Borate buffer		
Selected peaks	0.8 M	0.6 M	0.4 M
FMOC-Cit with Tyrn	12 000	12 250	12 300
FMOC-OH with Tyrn	8500	23 000	20 500
FMOC-Tyrn	800	2500	600

(b) Strong acids such as HCl have been used to reduce the pH of the solution after a fixed reaction time.[31] This approach, however, encountered several drawbacks: the chromatograms presented a very asymmetric FMOC-OH peak, with an area not significantly reduced, and two additional minor peaks being present.

(c) The addition of an excess of amine to produce a secondary reaction aimed at consuming the nonreacted FMOC-Cl. This amine, added after one minute, reacts with the excess FMOC-Cl to generate a new carbamate derivative.[30] Tyramine (Tyrn) and phenylethylamine (Phea) were selected here as reactant amines upon consideration that their resulting FMOC derivatives were much more hydrophobic and would therefore cause no interference within the separation window of the FMOC-amino acids.

Fig. 2 depicts the results of FMOC-Cit and FMOC-OH peak areas after these different treatments to quench the derivatization reaction. The use of the immiscible solvents *n*-pentane and *n*-heptane produced a reduction of *ca.* 30% in the area of the FMOC-OH peak, but the use of the immiscible solvents followed by extraction would be difficult to automatize if the protocol is implemented in an auto-injector. Furthermore, the use of either amine as a quenching agent gave decidedly better results: both produced significant reductions in the area of FMOC-OH, and with Tyrn (the better of the two) the peak areas of citrulline and the chloroformate became equal. A further advantage of Tyrn over the other amine is that the derivative eluted before that corresponding to the FMOC-Orn peak.

The addition of Tyrn led also to an improvement in the precision in the amino acid peak areas. This conclusion was made on the basis of the study of the reproducibility of the reaction performed both with and without a Tyrn standard solution. For this purpose, the derivatization reaction was performed with two solutions (average AA^T concentration of 40 μM), derivatized three times, and each replicate was then injected twice for HPLC analysis ($n = 2 \times 3 \times 2 = 12$). A significantly reduced relative standard deviation (below 12%) for most of the amino acids was obtained when the reaction was quenched with an excess of Tyrn after 1 min (Fig. 3). These precision levels are satisfactory for these relatively low concentration levels.

3.2. Optimization of separation

Chromatographic conditions for attaining a baseline resolution between the amino acid peaks and those arising from the blank impurities present (*i.e.*, the derivatization reagents) were investigated with both columns. Thus, the mobile phase composition, buffer pH, column temperature, and flow rate were optimized. The aim was to evaluate the analysis time, the efficiency, and mainly the simplicity of the whole procedure.

3.2.1. Core–shell column. First, ACN and methanol were tested as eluents under elution gradient conditions. Better peak shapes and efficiencies were observed with ACN, with the elution starting at a composition of 10% (v/v) and increasing by 2% per min up to 80% (v/v) ACN (data not shown). Next, several buffer solutions (*e.g.*, ammonium citrate, acetate and formate) were prepared within the pH range of 2.5–6.4 by keeping the total buffer concentration constant (at 25 mM). Fig. 4 shows the influence of pH on the retention factors and resolutions of six amino acids. When the pH of the mobile phase increased, the decrease in retention observed was attributed to the ionization of the carboxylic group of amino acids at pHs higher than 5. Although the Gln/Cit pair showed a resolution practically

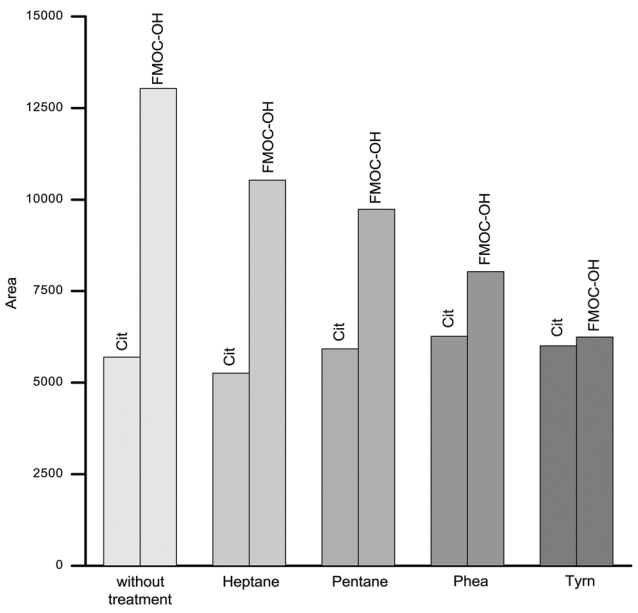

Fig. 2 Peak areas for FMOC-Cit and FMOC-OH with different treatments after derivatization: organic solvent extraction and chemical reaction. Column and gradient elution conditions as in Fig. 1.

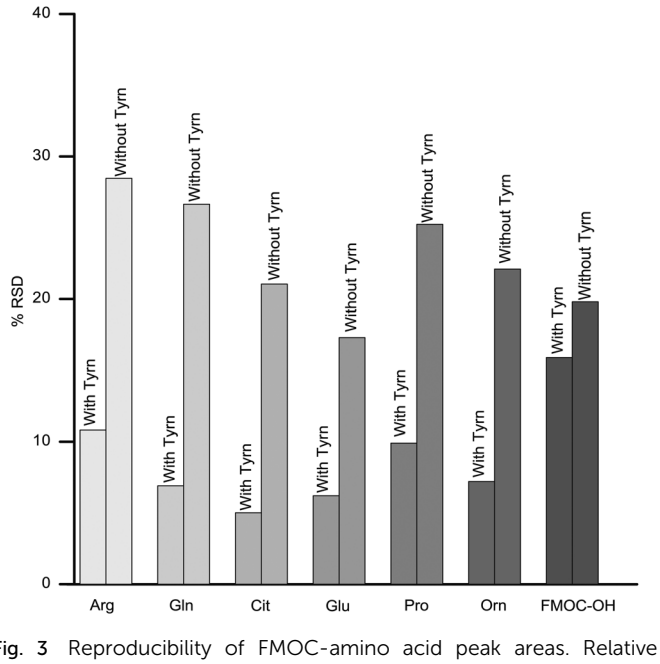

Fig. 3 Reproducibility of FMOC-amino acid peak areas. Relative standard deviation (RSD%) for a series of two injections of five replicates of derivatization reaction ($n = 10$) with and without the addition of Tyrn. Column and gradient elution conditions as in Fig. 1.

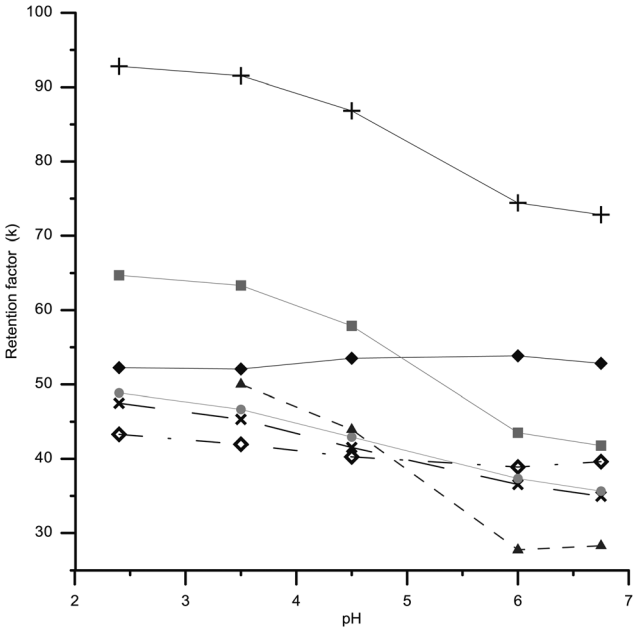

Fig. 4 Retention factor (*k*) *vs.* eluent pH. Column: core–shell C18 (50 × 2.1 mm, 2.7 μm particle size). Gradient elution conditions as in Fig. 1 at a flow rate of 0.25 mL min^{-1}. The aqueous phase was 25 mM citrate buffer at different pHs. Temperature, 45 °C. FMOC-Arg (brown); FMOC-Gln (orange); FMOC-Cit (black); FMOC-Glu (green); FMOC-OH (violet); FMOC-Pro (red) and FMOC-Orn (blue).

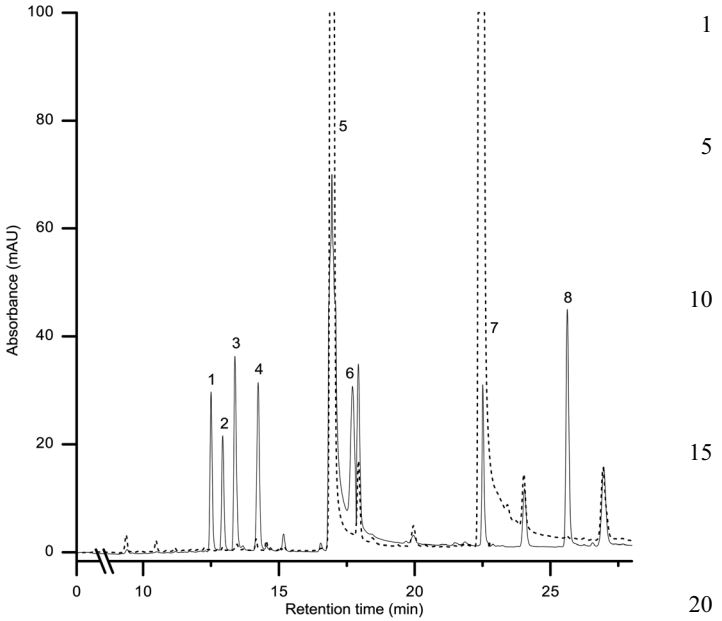

Fig. 5 Chromatograms obtained with the Halo column. Blank profile (dash) with the addition of Tyrn and chromatogram corresponding to a standard solution (line) of FMOC-derivatized amino acids. Gradient elution conditions as in Fig. 1 at a flow rate of 0.25 mL min^{-1}. Temperature, 25 °C. Peak identification: 1 = FMOC-Arg; 2 = FMOC-Gln; 3 = FMOC-Cit; 4 = FMOC-Glu; 5 = FMOC-OH; 6 = FMOC-Pro; 7 = FMOC-Tyrn and 8 = FMOC-Orn. Detection at 260 nm.

independent of the mobile phase pH, most of the peaks exhibited a decrease in separation at increasing pH. On the basis of these results, a mobile phase pH of 3.75 (prepared with ammonium formate) was selected as a reasonable compromise between the resolution and the analysis time. At this pH, the resolution values (R_s) were above 1.4, and no interfering peaks from the blank co-eluted with them. Because of the relatively small bed permeability of this column the drops in pressure developed (at regular linear velocities) were frequently close to the pressure limits handled by any conventional HPLC equipment, so that an increase in temperature would be desirable for decreasing the eluent viscosity and in doing so diminishing the pressure drop. Using the optimal composition, two temperatures were studied: 25 °C (room temperature) and 45 °C (the maximum operational temperature recommended by the manufacturer). Although a lower pressure drop along the gradient was observed at 45 °C, that increase in temperature did not produce significant changes in the analysis time or in the chromatographic efficiency. Therefore, the lower temperature of 25 °C was chosen in order to maintain a simple (and inexpensive) methodology that would be easier to transfer to routine clinical laboratories.

Fig. 5 shows the superposition of a reaction blank chromatogram over that obtained for a mixture of standard amino acids. Small signals (or unidentified peaks) arising from the blank matrix with amino acid peaks can be noted. Nevertheless, no co-elution occurs that would interfere with the quantitation of the amino-acid carbamates in the run.

3.2.2. Silica monolithic column. The separation of amino acids on the silica monolithic bed was also investigated. Using the same gradient the chromatographic performance was first studied by varying the flow rate from 2 and 4 mL min^{-1}, without changing the gradient time or the initial solvent composition. No significant improvements in peak symmetry were achieved in the range of 2–3 mL min^{-1}; however, at 4 mL min^{-1}, the resolution of the critical pair Gln/Cit was reduced, and the pressure drop was close to the highest working pressure recommended by the column manufacturer (*i.e.*, 200 bars). A flow-rate condition of 2 mL min^{-1} was thus selected as a compromise between efficiency, resolution and analysis time. By keeping the gradient at 2 mL min^{-1}, column temperatures of 15, 25, 35 and 45 °C were evaluated. These temperature changes had no strong influence on either the separation or the efficiency, thus, a temperature of 25 °C was selected in order to simplify the chromatographic protocol. Fig. 6 shows the separation of the amino acid standard mixture under the optimal conditions obtained with this monolithic bed. Two unidentified peaks arising from the blank matrix were distinguished, which interfere very slightly with the signals of the Cit and Pro peaks. However, a peak matrix overlapped with the Orn peak. Selectivity was not improved by applying small changes in mobile phase composition.

3.3. Comparison between columns

Peak symmetries for all amino acids analyzed under optimized conditions using these two columns are shown in Fig. 7a. No significant differences are observed in plot 'a'.

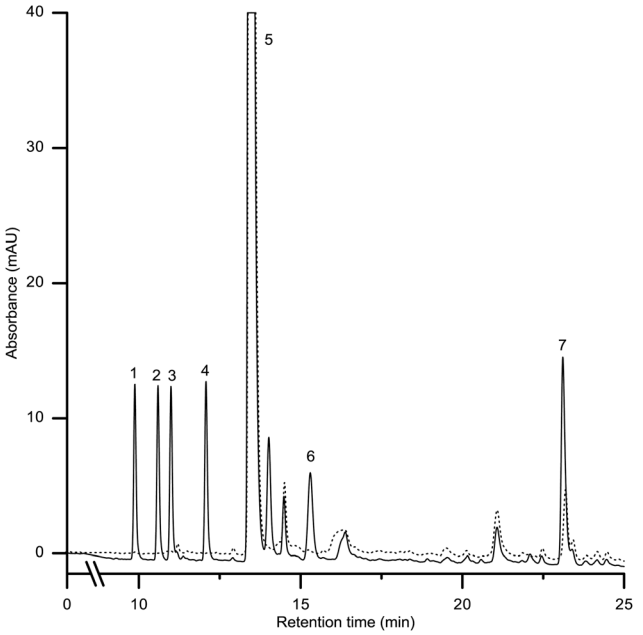

Fig. 6 Chromatograms obtained with the Chromolith column. Blank chromatogram (dash) and chromatogram corresponding to a standard solution (line) of FMOC-derivatized amino acids. Gradient elution conditions as in Fig. 1 at a flow rate of 2 mL min^{-1}. Temperature, 25 °C. Peak identification: 1 = FMOC-Arg; 2 = FMOC-Gln; 3 = FMOC-Cit; 4 = FMOC-Glu; 5 = FMOC-OH; 6 = FMOC-Pro; 7 = FMOC-Orn. Detection at 260 nm.

In order to further evaluate the chromatographic performance of each of the two columns, for comparison, the peak capacity (P_C) and global resolution (R_G) were obtained for the six standard amino acids. P_C was experimentally determined by the well-known equation:[34]

$$P_C = 1 + \left(\frac{t_g}{w}\right) \quad (1)$$

where w is the average peak width at 4σ (13.4% of peak height) in units of time (as measured experimentally) and t_g is the gradient time.

The global resolution, R_G, was also measured as the geometric mean of the resolution between pairs of consecutive peaks. For this calculation, unresolved or unknown pairs of peaks were also included. Fig. 7 shows P_C and R_G values. The latter provided the better chromatographic performance for these particular solutes. The analysis time was also found to be reduced by *ca.* 30% compared to the core–shell column (*cf.* Fig. 5 and 6). Since another additional benefit associated with the use of the monolithic bed was the mild conditions (room temperature and backpressure values below 150 bars), this column was chosen for further studies.

3.4. Application to real samples

The proposed method was evaluated in terms of precision, linearity and sensitivity (limit of detection). Precision was obtained by studying the reproducibility of retention time

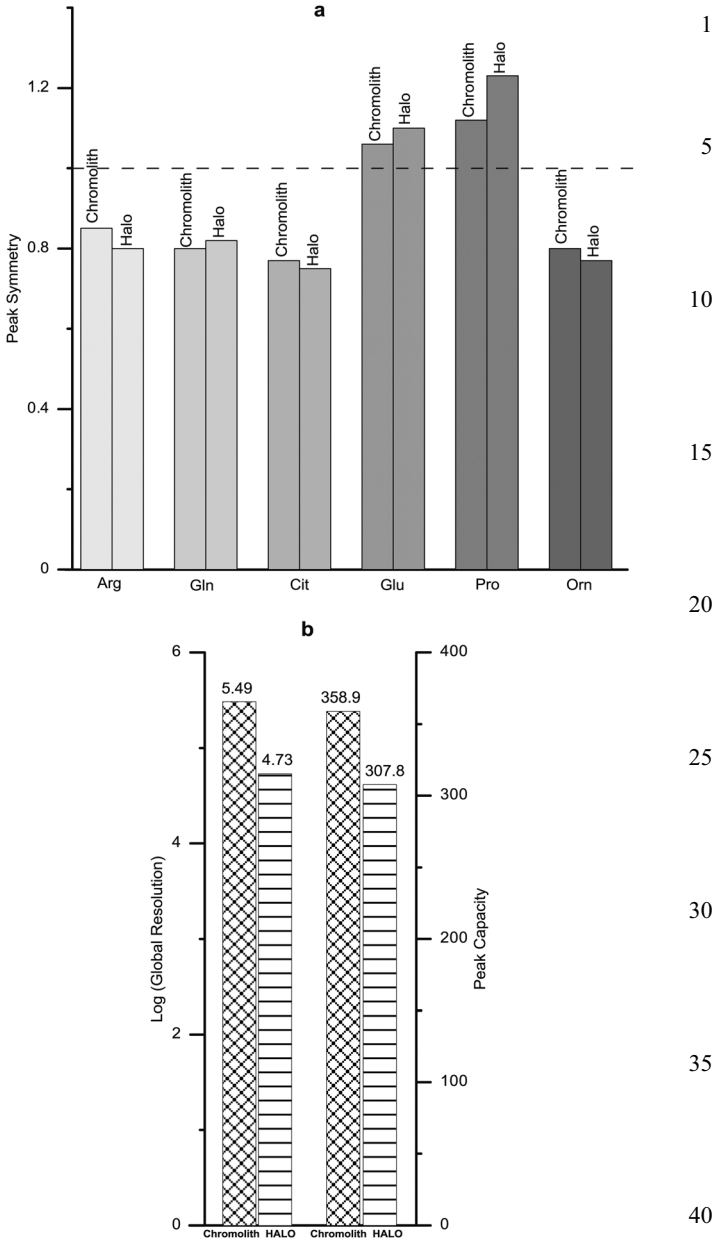

Fig. 7 Evaluation of the separation performance by the Halo and Chromolith columns: (a) peak symmetry and (b) peak capacity and log(global resolution). Elution conditions as indicated in Fig. 5 and 6, respectively.

and peak areas obtained by injecting ($n = 3$) a mixture containing 25 μM of each amino acid. The RSD values for the retention times and peak areas were lower than 5.5 and 7.65, respectively. The limits of detection (LODs) of the solutes were obtained for $S/N = 3$. LODs were comprised between 0.25 μM for Cit and 1.81 μM for Gln. These values were lower than those reported by other authors, working with FMOC amino acid derivatives in conventional RP-C18 columns and UV detection [(4–6 μM)[35]], although these values were higher compared to those found by using fluorescence detection [(picomole range)[23]].

The method developed here was applied to the analysis of citrulline and other related amino acids present in human-plasma samples.

Peak identification of analytes was performed by comparing their retention times with those of the standards, and also by spiking the sample extracts with the standards. Fig. 8 shows the chromatograms of an unfortified plasma sample (solid line) and a sample fortified with the standards of interest (dashed line).

Different studies have reported that the mean amino acid content in a healthy human plasma ranged between 1840 and 3200 μM.[5,36,37] For the purpose of these trials, the approximate mean of this range (2500 μM of AA^T) was considered to be the content in normal-adult plasma. Standard addition calibration curves were also obtained by adding to the plasma extracts from an adult man volunteer, at least five solutions with total amino acid concentrations increasing up to 750 μM of AA^T. The curves were linear with $r > 0.993$, and in most cases (with exception of Orn and Pro), the slopes of calibration curves did not differ significantly from those obtained with the external calibration method. The quantitative results of the six amino acids in this adult-human plasma are shown in Table 2.

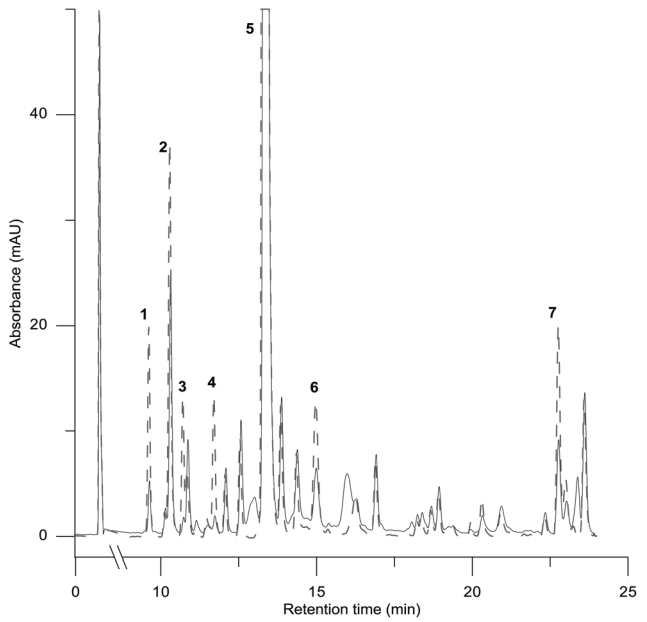

Fig. 8 Chromatograms of the human-plasma sample without (solid line) and with (dashed line) the addition of an amino acid standard solution. Gradient elution conditions as in Fig. 1. Peak identification: 1 = FMOC-Arg; 2 = FMOC-Gln; 3 = FMOC-Cit; 4 = FMOC-Glu; 5 = FMOC-OH; 6 = FMOC-Pro and 7 = FMOC-Orn. Detection at 260 nm.

4. Concluding remarks

A HPLC-UV method for the separation and determination of citrulline and metabolically related amino acids, based on their pre-column derivatization with FMOC-Cl has been developed. The separation of the derivatized amino acids was accomplished in both a Halo core–shell particulate and a Chromolith silica-based monolithic column. The derivatization conditions (including the buffer pH and concentration, the reduction of interfering chromatographic species and other details) were first optimized in order to achieve satisfactory reaction yields and to enhance the reproducibility. Accordingly, the addition of Tyrn, as a quencher of the derivatization reaction, was found to combine with and thus reduce the major interfering component (*i.e.*, the excess hydrolyzed FMOC-OH) and to significantly improve the reproducibility, giving RSD values between 5 and 11% for amino acid contents below 20 μM in the reaction mixture. An optimization of the conditions for the HPLC separation of these amino acids on both commercial chromatographic supports was also performed and the resulting HPLC performance was compared. The Chromolith silica-monolith column showed a better peak capacity and global resolution than the Halo column. The monolithic bed under optimal elution conditions was thus applied to the successful analysis of the amino acids in a real plasma sample. The proposed protocol constitutes a promising and reliable methodology for determining these amino acids and their levels in several human-health disorders.

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Table 2 Calibration curve parameters for each amino acid and their merit figures. Concentrations values (mean ± SD, $n = 3$) found in human plasma (a volunteer adult man)

Amino acids	Intercept ($\pm s_a$)	Slope ($\pm s_b$)	r^2	Linear range (μM added)	Mean in plasma (μM) ($\pm s_x$)
Arg	0.46 (0.01)	7.4 (0.8)	0.9917	0–126.4	169 (2)
Gln	0.34 (0.03)	26 (1)	0.9518	0–124.8	927 (7)
Cit	0.378 (0.009)	2.7 (0.7)	0.9922	0–125.6	88 (2)
Glu	0.37 (0.01)	3.2 (0.8)	0.9866	0–123.7	95 (3)
Pro	0.223 (0.008)	6.8 (0.6)	0.9860	0–124.0	387 (3)
Orn	0.23 (0.01)	11.5 (0.8)	0.9895	0–124.9	375 (5)

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