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Determination of free amino acids in pig plasma by precolumn derivatization with 6-*N*-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for measuring amino acids in pig plasma has been developed by using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as a precolumn derivatization reagent. With the method presented here it was possible to separate 24 amino acids in pig plasma within one single run in 45 min, while only 18 amino acids were properly separated with the original AccQ.Tag conditions used for analysis of hydrolysate samples. The recovery was above 90% for most amino acids, and the mean coefficient of variation of the retention times below 0.30%. The within- and between-assay reproducibility for the determination of plasma amino acids showed C.V.s below 2.2% and 4.5%, respectively, for most amino acids analyzed. In the present work, most of the plasma amino acids were assayed with high sensitivity, accuracy and good reproducibility in a relatively short time and on very small amounts of sample. © 1997 Elsevier Science B.V.

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1. Introduction

An accurate and precise determination of free amino acids in plasma samples, together with its quantification in feeds and digesta, is needed to improve our knowledge of amino acid metabolism in farm animals. This determination is usually accomplished by precolumn derivatization and reversed-phase HPLC which gives short analysis times and increased sensitivity.

The derivatizing reagents most frequently used for free amino acid determination in blood are 9-fluorenylmethyl-chloroformate (FMOC-Cl) [1,2], *o*-phthaldehyde (OPA) [3,4] and phenylisothiocyanate (PITC) [5–8], but none of these methods are perfect.

One problem encountered with FMOC-Cl is the need to remove, by extraction, excess reagent with pentane in order to minimize the formation of the hydrolysis product. This step is tedious and results in poor precision since some amino acids are also extracted by pentane.

The limitation of the OPA method is that OPA reacts only with primary amino acids and produces

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unstable derivatives, mainly with glycine, alanine, lysine and ornithine [9].

PITC forms stable derivatives with both primary and secondary amino acids [10]. However, this method is very time-consuming since the excess of PITC must be removed in order to avoid interfering peaks. Other problems inherent in the PITC method are the poor stability of the mobile phase, poor resolution of arginine, threonine, alanine and proline, and baseline instability [11].

The method described in the present paper, AccQ.Tag, developed by Cohen and Michaud [12], has been shown to be very sensitive, quantitative and reproducible for protein and peptide amino acid analysis [12–17] and also for feed samples [15,16,18] with high correlation coefficients and excellent linearity for standard mixtures ranging from 25–500 μM [15].

AccQ.Tag provides an important advance in the amino acid analysis compared with other techniques reported. The 6-aminoquinolyl-*N*-succinimidyl carbamate (AQC) reagent reacts with primary and secondary amines and very stable derivatives are produced in a few seconds. The derivatization process is very simple and the detection limits are below 1 pmol. Unwanted side reactions are prevented because the excess reagent is hydrolysed to 6-aminoquinoline (AMQ) in less than 2 min.

The main objective of this work was to improve the analysis of free amino acids in porcine plasma using a precolumn derivatization with a new reagent, AQC, followed by reversed-phase separation, which so far has been used only for proteins, peptides and feed hydrolysate samples.

2. Experimental

2.1. Chemicals

The derivatization reagent AQC (Waters AccQ.Fluor Reagent), borate buffer (Waters AccQ.Fluor Borate Buffer), eluent A (Waters AccQ.Tag Eluent A, concentrate) and amino acid standards (Pierce H) were obtained as a kit from Waters Assoc. (Mildford, MA, USA). Acetonitrile (HPLC grade) was from Labkemi (Stockholm, Sweden) and sodium dihydrogen phosphate monohy-

drate from Merck (Darmstadt, Germany). Sulphosalicylic acid, DL- α -amino-*n*-butyric acid and the other standard amino acids L-OH-proline, L-asparagine, L-glutamine, L-aurine, L-citrulline, L-ornithine and L-tryptophan were purchased from Sigma (St. Louis, MO, USA). High-purity water was supplied by a Milli-QTM purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

A Pharmacia LKB HPLC system (Uppsala, Sweden) was used and included two HPLC pumps 2248, high pressure mixer, autosampler 2157, column oven 2155 and a UV variable-wavelength detector Model 2141. The fluorescence detector was from Shimadzu, Model RF-551 (Shimadzu, Kyoto, Japan). A 386 computer was used to control the HPLC system (HPLC-manager, Pharmacia) and to evaluate the data (Nelson data). Separation was achieved by using a 4 μm AccQ.Tag C_{18} column (150 \times 3.9 mm I.D.) with a guard column, packed with Nova-Pak C_{18} (Waters).

2.3. Preparation of samples

2.3.1. Standard samples

A stock solution of amino acid standards (representative of a physiological plasma sample) containing 24 amino acids was made by adding OH-proline, asparagine, glutamine, taurine, citrulline, ornithine and tryptophan (previously dissolved in 0.1 *M* HCl) to the amino acid standard kit purchased from Waters, containing 17 amino acids. The concentration of each amino acid in the stock solution was 2273 pmol μl^{-1} .

An amino acid standard mixture for calibration runs was prepared by diluting the stock solution 25 times with water, and by adding the internal standard α -aminobutyric acid (ABA) (100 pmol μl^{-1} in 0.1 *M* HCl). The final concentration of ABA was 50 pmol μl^{-1} and for the other amino acids 45.45 pmol μl^{-1} . Before derivatization, 15 μl of this amino acid mixture was transferred to a vial containing 70 μl of the AccQ.Fluor borate buffer (pH 8.7).

2.3.2. Plasma samples

The blood sample was collected from a Yorkshire×Landrace pig into a heparinized tube and centrifuged at 3500 *g* for 10 min at 4°C. The plasma sample was kept at –20°C until analysis.

Aliquots of 100 μl of plasma were diluted with 100 μl of HCl (0.1 *M*) and deproteinized by the addition of 20 μl of cold (4°C) sulphosalicylic acid (SSA) (30%) and centrifuged at 2500 *g* at 4°C for 30 min. In order to obtain an optimal pH for derivatization (between 8.2 to 10.0), 140 μl of deproteinized plasma was diluted with 800 μl of the AccQ.Fluor borate buffer. Before derivatization, 10 μl of internal standard (100 pmol μl^{-1}) were added.

2.3.3. Spiked plasma samples

Spiked samples were prepared by adding different volumes of the standard amino acid stock solution to the plasma.

Aliquots of 100 μl of plasma were mixed with 100, 50 and 10 μl of the standard stock solution (2273 pmol μl^{-1}). HCl (0.1 *M*) was used to fill up to a final volume of 200 μl . The samples were deproteinized and treated as described in Section 2.3.2.

2.4. Derivatization reagent and procedure

2.4.1. Plasma samples

A plasma solution sample (deproteinized plasma diluted with the borate buffer) of 70 μl was pipetted into a 6×50 mm tube and mixed with 10 μl of internal standard (ABA). The derivatization reaction was then initiated by addition of 27 μl of AQC-reagent. After 1 min incubation at room temperature, the content was transferred to an autosampler vial and capped with a silicone-lined septum. The vial was placed in a heating block for 10 min at 55°C in order to finish the derivatization procedure. After that, the samples were ready for injection.

2.4.2. Spiked samples

Samples of 10, 20 and 60 μl spiked plasma solutions were mixed with 10 μl of ABA as internal standard. The derivatization was carried out by the addition of 50 μl of AQC-reagent to each sample and followed the same procedure as described for plasma samples. Samples containing more than 400

pmol, the first two samples, were diluted with eluent A just before injection.

2.5. Chromatographic analysis

Separations were carried out on a (150×3.9 mm I.D. AccQ.Tag C₁₈) reversed-phase column, with a flow-rate of 1.0 ml min⁻¹ and performed at 37°C.

New and original conditions were set up for running the chromatographic analysis.

The new conditions performed, namely modified eluent conditions (ME), were established based on a substitution of the eluents and the optimization of the gradient applied to the original method described by Waters [19].

The gradient profile and composition of the mobile phase is shown in Table 1.

For regeneration, the column was washed for 10 min with 100% of eluent B and then equilibrated for 9 min with 100% of eluent A. The original eluent conditions (OE) were adjusted following the method described by Waters [19] with time elongation at the end (Table 2).

For column regeneration the same procedure as described before was used.

A fluorescence detector was used to quantify the content of all amino acids. When OE conditions were used, the ultraviolet absorbance detector was used simultaneously in order to obtain a better quantification of tryptophan.

All standards and plasma samples were run with the new (ME) and the original (OE) Waters conditions and the results were compared.

Table 1
Gradient profile for the new conditions (ME)

Time (min)	A ^a (%)	B ^a (%)
Initial	100	0
0.5	98	2
15	93	7
19	90	10
26	68	32
35	60	40
50	60	40
51	0	100
56	0	100

^a Eluent A=12.5 mM sodium phosphate pH 6.3–acetonitrile (100:1, v/v). Eluent B=12.5 mM sodium phosphate pH 6.3–acetonitrile (70:30, v/v).

Table 2
Gradient profile for the original conditions (OE)

Time (min)	A ^a (%)	B ^a (%)
Initial	100	0
0.5	98	2
15.0	93	7
19.0	90	10
32.0	67	33
35.0 ^b	67	33
36.0 ^c	0	100
41.0	0	100

^a Eluent A=Waters AccQ.Tag Eluent A concentrate, pH 5.02.
Eluent B=acetonitrile–water (60:40, v/v).

^b 33: Original elution time from Waters Assoc.

^c 34: Original elution time from Waters Assoc.

3. Results and discussion

By using the modified chromatographic conditions (ME), it was possible to separate 23 of the total of 24 standard amino acids plus the internal standard (ABA) within 45 min (Fig. 1). The only amino acid which was not possible to identify was tryptophan.

The separation of pig plasma and spiked plasma samples was good for most of the amino acids when ME conditions were used. Only the group of amino acids taurine, threonine, histidine, citrulline and alanine showed a less satisfactory resolution (Fig. 2). While a 15-cm long column was used in the present report, a better resolution of these amino acids might be achieved by using a longer (25–30 cm) column.

The amino acid levels obtained with the ME conditions were within the range found in normal pig plasma [20,21].

Fluorescent derivatives were obtained after a few seconds and remained stable for up to one week at room temperature. Excess of reagent was hydrolysed to AMQ, which appeared in the chromatograms together with ammonia without interfering with the amino acid quantification.

The total time, including chromatographic separation and column re-equilibration, was 64 min. As mentioned in descriptions of many other methods, it was impossible to detect tryptophan in these chromatographic runs. It should be emphasized, however, that tryptophan was detected without any problem using the original conditions (OE) and a UV absorbance detector.

By using the OE conditions, only 17 of 24 amino

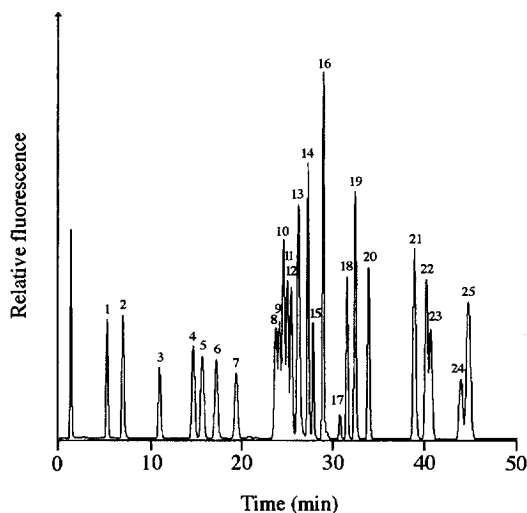


Fig. 1. Elution profile of an AQC-derivatized physiological amino acid standard (39 pmol per amino acid). Column: AccQ.Tag (150×3.9 mm I.D., 4 μm); flow-rate: 1.0 ml min⁻¹; eluents: sodium phosphate–acetonitrile, ME conditions (Table 1). Non-standard abbreviations used: AMQ=6-aminoquinoline (hydrolysed reagent), α-ABA=α-aminobutyric acid. Peaks: 1=aspartic acid; 2=glutamic acid; 3=OH-proline; 4=serine; 5=asparagine; 6=glycine; 7=glutamine; 8=taurine; 9=threonine; 10=histidine; 11=citrulline; 12=alanine; 13=ammonium+AMQ; 14=arginine; 15=proline; 16=ABA(I.S.); 17=cystine; 18=tyrosine; 19=valine; 20=methionine; 21=isoleucine; 22=leucine; 23=ornithine; 24=lysine; 25=phenylalanine.

acids plus the internal standard (ABA) were properly separated (Fig. 3). It was not possible to achieve separation of hydroxyproline, serine, asparagine, histidine, glutamine, citrulline and threonine, all of which were resolved by using the ME conditions.

Sarwar and Botting [5] reported separation of 23 amino acids in human plasma in 21 min using the PITC method. Besides the disadvantages that represent the use of a UV absorbance detector in the PITC method (where the sensitivity is low compared with that of the other methods), the inferior linearity with cystine, the lengthy sample preparation, as well as unsatisfactory resolution of Asn–Ser–Gln–Gly was obtained.

The precision of the AccQ.Tag method was assessed by evaluating the coefficient of variation (C.V.) of both retention times and peak areas in various analyses.

Irrespective of the conditions used, or whether the samples were taken from the same vial or analyzed

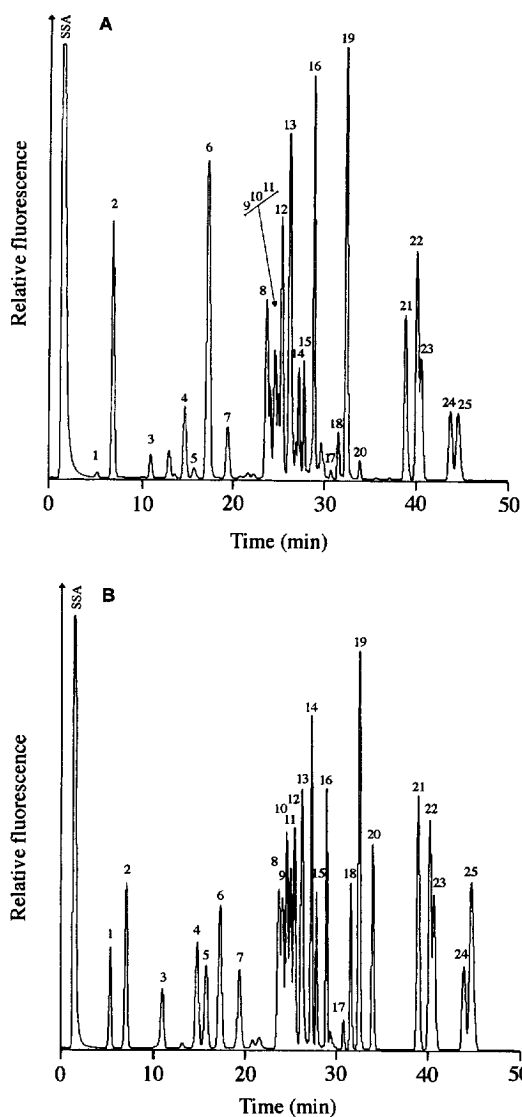


Fig. 2. (A) Elution profile of an AQC-derivatized pig plasma sample and (B) spiked pig plasma sample, under identical experimental conditions as in Fig. 1. Non-standard abbreviations used: SSA=sulphosalicylic acid, AMQ=6-aminoquinoline (hydrolysed reagent), α -ABA= α -aminobutyric acid. For amino acid peaks abbreviations see Fig. 1.

within the same day, the C.V.s for retention times were never higher than 1%.

Retention times were constant, giving C.V. values similar to those of the other methods published [7,10,22].

Peak area reproducibility using ME conditions,

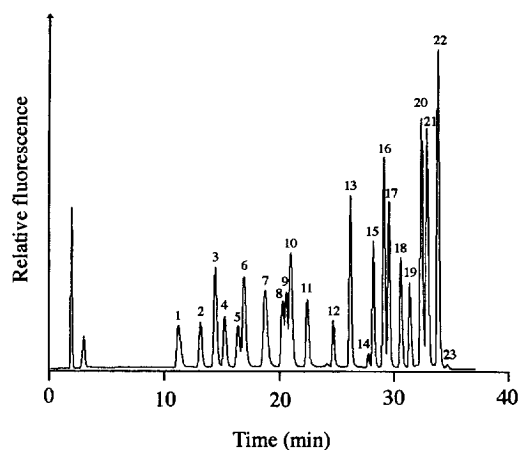


Fig. 3. Elution profile of an AQC-derivatized physiological amino acid standard (39 pmol per amino acid). Column: AccQ.Tag (150 \times 3.9 mm I.D., 4 μ m); flow-rate: 1.0 ml min⁻¹; eluents: eluent A=acetonitrile, OE conditions (Table 2). Non-standard abbreviations used: AMQ=6-aminoquinoline (hydrolysed reagent), α -ABA= α -aminobutyric acid. Peaks: 1=AMQ+OH-proline; 2=aspartic acid; 3=serine+asparagine; 4=glutamic acid; 5=glycine; 6=histidine+glutamine; 7=ammonium; 8=taurine; 9=arginine; 10=threonine+citrulline; 11=alanine; 12=proline; 13=ABA(I.S.); 14=cystine; 15=tyrosine; 16=valine; 17=methionine; 18=ornithine; 19=lysine; 20=isoleucine; 21=leucine; 22=phenylalanine; 23=tryptophan.

expressed as the C.V. ($n=5$) ranged from 0.7% to 4.5% between-runs and from 0.4% to 2.2% within runs for most amino acids. Only arginine (7.1%) and aspartic acid (16.2%) showed higher variation between-run and aspartic acid (11.2%) in within-run samples (Table 3).

With OE conditions, the between-run precision of plasma amino acid concentration ranged from 3.9% to 7.1% ($n=5$) for most amino acids, with the exception of aspartic acid 11.4% and tryptophan 36% (when using fluorescence detection). The within-run variability with OE conditions ranged from 1.2% to 7.0% ($n=5$), with the exception of tryptophan 19% (detected by fluorescence) (Table 3).

It should be noticed that, when comparing the values recorded in Table 3, the intra- and between-assay precision was improved when the new ME chromatographic conditions were used.

The results obtained show the high reproducibility of this method for determination of free amino acids according to the values published by Graser et al. [3] and others [7,22–25], and much better than other

Table 3

Reproducibilities of peak areas using chromatographic conditions ME and OE: Between- and within-run precision of concentrations of AQC amino acid derivatives in pig plasma samples ($n=5$)

Amino acid	Between-run (C.V., %)		Within-run (C.V., %)	
	ME	OE	ME	OE
Aspartic	16.2	11.4	11.2	7.0
Glutamic	1.1	4.6	0.6	3.2
OH-Proline	2.6	nd	1.7	nd
Serine	2.3	nd	0.5	nd
Asparagine	4.5	nd	1.6	nd
Glycine	1.2	4.7	0.5	3.5
Glutamine	0.7	nd	0.8	nd
Taurine	1.4	nd	0.4	nd
Threonine	1.9	nd	1.4	nd
Histidine	2.3	nd	1.5	nd
Citrulline	2.4	nd	1.8	nd
Alanine	2.7	4.8	0.4	2.6
Arginine	7.1	nd	0.9	nd
Proline	4.2	3.9	1.7	2.9
Cystine	2.9	7.1	2.1	1.8
Tyrosine	1.8	5.4	0.7	3.5
Valine	2.0	4.8	0.4	2.9
Methionine	2.3	6.0	1.2	3.2
Isoleucine	1.2	5.0	1.7	2.8
Leucine	1.1	5.2	1.1	2.8
Ornithine	2.2	5.1	1.4	3.6
Lysine	1.4	5.2	2.2	2.6
Phenylalanine	1.9	5.3	2.0	2.8
Tryptophan ^a	nd	36	nd	19
Tryptophan ^b	nd	4.1	nd	1.2

nd: not determined

^a Tryptophan detected by fluorescence.

^b Tryptophan detected by UV absorbance.

data [6,26,27], where peak areas of most of the amino acids (especially for aspartic acid, serine, hydroxyproline, histidine and methionine) had high C.V. values.

Only aspartic acid showed an imprecision higher than 10% in most of the chromatographic runs. High C.V.s for this amino acid have also been reported [10,26,28–30], giving support to the contention that aspartic acid would be difficult to quantify. The relatively high coefficient of variation of aspartic acid, might be explained by the presence of only small amounts of this amino acid in plasma.

With regard to tryptophan, the determination of this amino acid using OE condition and a UV absorbance detector was highly satisfactory compared with other methods with which its quantifica-

tion is not possible or poor reproducibility, difficult detection or overestimation of its concentration are encountered [23,24]. Even though it is believed that the low blood concentration and also the SSA deproteinization of the sample may result in variability of tryptophan values [31], it was possible to achieve C.V.s lower than 2% in all chromatographic runs by using the AccQ.Tag method.

The recovery was evaluated by using ABA as internal standard. The recovery of amino acids was calculated by analyzing replicate spiked plasma samples and comparing the peak areas with those obtained by injection of unspiked plasma.

The overall recovery was very good for most of the amino acids, with mean values of 91, 100 and 92%, respectively, for the three amounts of standard

amino acids added to plasma samples (Table 4). Using ME conditions, only three amino acids were shown to have lower recoveries.

The recoveries of histidine and citrulline were around 85% and 87%, respectively. Tyrosine showed recoveries between 80–88% irrespective of the conditions used.

The relatively low recovery of histidine and citrulline might have been due to insufficient separation (Fig. 2). For tyrosine, it is difficult to give a simple explanation of the relatively lower recovery.

The AccQ.Tag method was checked for linearity and all amino acids were linear in the range of 227–2273 pmol, with the exception of aspartic acid, glutamic acid, tryptophan (only Trp detected by

fluorescence), citrulline and alanine. Those amino acids were linear from 227 to 1136 pmol.

The recovery varied significantly in the high concentration range investigated (adding 2273 pmol of standard to 1 μ l of plasma), the recovery was not satisfactory for the amino acids mentioned above as these were not linear at such high concentrations. The increase was around 15–20 times the physiological amino acid concentrations, giving values impossible to obtain even under pathological conditions.

The HPLC AccQ.Tag method described by Cohen and Michaud [12] has been proved to be an excellent method for analyses of hydrolysate proteins and feed amino acids [12–18].

Table 4
Recovery (%) of amino acid standards added to pig plasma samples^a

Amino acid	Amount added					
	2273 pm		1136 pm		227 pm	
	ME	OE	ME	OE	ME	OE
Aspartic	49	49	101	88	89	90
Glutamic	54	55	101	94	94	91
OH-Proline	90	nd	100	nd	90	nd
Serine	91	nd	101	nd	91	nd
Asparagine	92	nd	101	nd	89	nd
Glycine	85	95	104	104	96	96
Histidine	89	nd	80	nd	85	nd
Glutamine	94	nd	108	nd	93	nd
Arginine	90	nd	100	nd	90	nd
Taurine	91	nd	102	nd	95	nd
Threonine	89	nd	102	nd	92	nd
Citrulline	76	nd	94	nd	87	nd
Alanine	77	97	101	108	92	95
Proline	91	94	105	103	100	97
Cystine	86	92	100	100	98	88
Tyrosine	88	88	84	80	86	84
Valine	94	95	106	101	92	92
Methionine	95	94	102	101	90	90
Isoleucine	97	94	103	100	93	91
Leucine	94	94	103	100	94	93
Ornithine	94	90	106	100	94	90
Lysine	89	91	104	102	95	90
Phenylalanine	88	92	99	95	90	90
Tryptophan ^b	nd	73	nd	94	nd	100
Tryptophan ^c	nd	85	nd	89	nd	82

nd: Not determined. ME, OE: new and old Waters conditions, respectively.

^a Mean value of 5 replications.

^b Recovery of tryptophan using the fluorescens detector.

^c Recovery of tryptophan using the UV absorbance detector.

By using the new ME conditions, we have demonstrated the suitability of the AccQ.Tag method for routine analyses of primary and secondary free amino acids in plasma, giving the possibility of using the same method for feeds and plasma amino acid analysis, which represents an important advantage in animal nutrition studies.

In conclusion, using a modification of the eluents and a gradient optimization, the liquid chromatographic AccQ.Tag method reported in the present study can be used for simple, sensitive, accurate and reproducible determination of most nutritionally important amino acids in plasma, using very small volumes of sample and performed in a relatively short time. However, more work is needed to improve the resolution between the group of amino acids taurine–threonine–histidine–citrulline and alanine in order to increase the accuracy of this method.

In addition, it should be noticed that α -aminobutyric acid can be a normal constituent of plasma samples (as shown in humans and rats). Therefore, the use of another internal standard (norvaline or norleucine) should be evaluated before the present method is applied in routine amino acid analysis.

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