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Simultaneous determination of cysteine, cystine and 18 other amino acids in various matrices by high-performance liquid chromatography[1]

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Abstract

An extraction protocol and a reversed-phase high-performance liquid chromatographic method for the simultaneous determination of cysteine, cystine and 18 other amino acids in biological and vegetable samples are described. Among the different methods proposed for amino acid determination, phenylisothiocyanate was used as the reagent for derivatization. Chromatograms obtained in the analysis of standard solutions and actual samples are reported, together with regression equation, correlation coefficient (>0.999 for all), limit of detection and recoveries (between 86 and 102% for all the examined matrices) for each amino acid. Practical protocol and method applications in normal patients and patients affected by different pathologies, and in algal products are discussed. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Cysteine; Cystine; Amino acids

1. Introduction

The separation and determination of the amino acid content of biological samples is very important in biochemistry and clinical chemistry. The analyses are considered an important starting point in the attempt to find a correlation between the amino acids and some pathologies [1]. In fact, recent theories attempt to correlate altered levels of some amino acids (i.e., cysteine) in blood and tissue to the development of pathologies such as diabetes, AIDS and cancer. The analysis of amino acids is also important in the food industry where nutritional analysis of foods for human consumption has great economic importance, particularly for the third world countries which need an inexpensive food source. In these two different fields of interest, the analytical procedure requires different approaches: the clinical interest is focused on the amount of free amino acids in biological fluids and/or tissues while the nutritional interest is focused on the amount of total amino acids. Among the several methods suitable for the determination of amino acids in numerous matrices, reversed-phase high-performance liquid chromatography (RP-HPLC) is currently the commonest meth-

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od of analysis, with fluorescence detection [1–8] and UV detection [9–16] according the involved derivatization method.

The quantitative, pre-column derivatization of amino acids with phenylisothiocyanate (PITC) to obtain phenylthiocarbamyl (PTC) derivatives was introduced in the last decade [9–11] to overcome problems associated with the ion-exchange procedure (such as poor derivative stability, less than quantitative reaction yields, interference from reagent peaks and a lack of reactivity with secondary amino acids).

Although the PTC-AA analysis with UV detection is a well-know (and studied) problem, it is still not trivial because of the complexity of such matrices. In particular, the simultaneous determination of cysteine and cystine without a previous oxidation/reduction step, along with the determination of other amino acids, has not yet been reported in the literature, since cysteine easily oxidizes to cystine [12]. To avoid this drawback, cysteine must undergo a pre-treatment, e.g. with iodioacetic acid (IA) to obtain a S-carboxymethyl-derivative before derivatizing the sample with PTC, but this treatment introduces supplemental problems, so in many papers the determination of cysteine/cystine is not considered [17]. Another non-trivial task in amino acid analysis is tryptophan determination in protein hydrolyzates [18]. Under acidic conditions, tryptophan is totally destroyed. An alternative derivatization method was performed to determine only this amino acid using methanesulphonic acid [19,20].

In this paper a simple, accurate, and reproducible method for extraction and simultaneous determination of cysteine and cystine along with 18 other amino acids in biological fluids (plasma, serum, and urine) and in tissue homogenates (thyroid gland), both from normal patients and from patients affected by pathologies, and in foodstuff (natural and commercial products of Cuban origin) is described. Special attention is devoted to the applicability and limitations of this method with regard to the determinations mentioned above.

2. Experimental

2.1. Solvents and reagents

HPLC grade acetonitrile was used while analytical grade sodium acetate buffer, ethanol and acetic acid were used (Carlo Erba, Milan, Italy). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare standard solutions and sodium acetate buffer. HCl (6 *M*), triethylamine (TEA), methanesulphonic acid and PITC, all sequanal grade, and ''amino acid standard H'' containing Arg, His, Ile, Leu, Lys, Met, Phe, Tyr, Thr, Val, Ala, Asp, Glu, Gly, Pro, Ser and $(Cys)_2$ (see Table 1) [2.50 μmol/ml each except $(Cys)_2$, 1.25 μmol/ml] were purchased from Pierce (Rockford, IL, USA). Individual amino acids, including Cys and S-carboxymethylcysteine (S-CMC), sulphosalicylic acid (SSA), sodium deoxycholic acid (DOC) and trichloroacetic acid (TCA) (0.37 *M*) were purchased from Sigma (St. Louis, MO, USA).

2.2. Extraction and derivatization procedures

2.2.1. Preparation of standard solutions

For amino acid identification, single standard solutions (10^{-3} *M*) in 0.1 *M* HCl were prepared by weighing amount of each amino acid. For quantitative analysis, a standard solution (obtained by adding Asn, Trp, Cys and Nle, 2.50 μmol/ml each, to the amino acid standard H hydrolysate) containing 20 amino acids, was used.

2.2.2. Preparation of samples

Venous blood and urine were collected simultaneously between 8 and 9 a.m. from eight healthy patients and eight patients affected by diabetes (from those latter only venous blood was collected), both of them on an empty stomach. All the samples were processed within 1 h of collection: in particular, venous blood was treated with an anticoagulant, heparin, to obtain plasma (after centrifugation). The thyroid gland tissues were supplied from Policlinico Umberto I of University of Rome and stored at −25°C until ready to use. One milliliter samples of plasma or serum were deprotenized by adding an equal volume of 6% SSA, mixing on Vortex for 1.5 min, and centrifuging at 3000 *g* for 15 min. The supernatant liquid was stored at −25°C. The same procedure was followed for urine samples using 30% SSA. Fifty milligrams of powdered tissue (i.e. thyroid gland) were deprotenized by adding five volumes of 3% SSA, vortexing and centrifuging at

3000 *g* for 20 min. The supernatant was filtered through a 0.45-μm filter (Whatman, UK).

A 150-mg amount of powdered alga (i.e. *Spirulina platensis*) was added to 2 ml of 0.37 *M* TCA, and vortexed for 1.5 min. A 0.2-ml sample of 3.6 m*M* DOC was added to assist the protein precipitation [21] and after 10 min two centrifugations were performed. The first one at 3000 *g* for 15 min, the second one at 4500 *g* for 1 h.

All the supernatants were processed as described below for the determination of free amino acids. In the case of alga, total amino acids were also determined by hot acidic (and basic) hydrolysis extraction. To a 15 mg sample of powdered alga in a 5 ml vial, 1 ml of 6 *M* HCl (or 6 *M* methanesulphonic acid) was added under nitrogen flow. The vial was then sealed and placed in an oven at 110°C for 22 h. After cooling at room temperature, the hyrdrolysate was filtered on a 0.45-μm Whatman filter. The solution was processed as described below.

2.2.3. Drying and derivatization

The derivatization procedure is based on the well-known reaction between PITC and amino acids [9]. The supernatant (free amino acids) from the previous steps (100 μl for alga, 50 μl for plasma, serum, urine and thyroid gland) or the solution obtained from the hot acid (and basic) hydrolysis of alga (25 μl) (total amino acids) was added to 25 μl of internal standard (I.S.) (Nle, 10^{-3} *M*). The solution was dried at 35°C by rotary evaporator (Büchs, Switzerland). After, 50 μl of a solution of ethanol–water–TEA (7:1:1) were added and the solution was vortexed for a few seconds and redried 10 min at 35°C.

A 25-μl volume of the derivatizing solution (ethanol–water–TEA–PITC) (7:1:1:1) prepared immediately prior the use, was added to the solution coming from the vortexing. The solution was then allowed to stand for 20 min. The excess reagent was removed by rotary evaporation (50 min at 35°C). The dried sample was redissolved in 250–500 μl of the chromatographic mobile phase A. For the cysteine analysis, 200 μl of sample were previously added to 25 μl of I.S. and 25 μl of 100 m*M* IA. After standing for 15 min the solution was dried with 50 μl of ethanol–water–TEA (7:1:1) added. The procedure described above was then followed. A 50-μl aliquot was injected into the chromatographic system for sample analysis.

2.3. Chromatographic equipment

The HPLC equipment was a Varian Chromatograph 9012 (Varian, CA, USA), equipped with a UV–Vis detector set at λ=254 nm. A Model 330 heater module (Alltech Deerfield, IL, USA), was used to keep the column temperature constant at 45±1°C. Separations were carried out on a LC-18 DB Supelcosil, 25×0.46 cm I.D. column, 5 μm. A laboratory-made C_{18} guard column was used to protect the analytical column. The sample was introduced with a Rheodyne valve equipped a 50-μl external loop. Mobile phase flow-rate was 1 ml/min and a ternary gradient was employed where mobile phase A was 0.7 *M* sodium acetate containing 2.5 ml adjusted pH to 6.4 with acetic acid, mobile phase B was water and mobile phase C was acetonitrile–water (80:20). The gradient was from A–B–C (20:75:5) to A–B–C (20:30:50) in the first 25 min, then from that to A–B–C (10:10:80) in 1 min, and was then held constant for 4 min. A period of 10 min was required to re-equilibrate the initial column condition.

3. Results and discussion

The ability to reproduce retention times is a critical factor in the analysis of free amino acids in a physiological sample. Using an I.S. ensured chromatographic stability: the retention times, either between runs or between changes of matrices, varied less than 3% when the column temperature was kept constant at 45°C. Also, the reproducibility of the method ranged <5% intra-day and <8% inter-day for all amino acids.

Fig. 1A illustrates that Cys at 10 nmol injected can be determined as PTC-S-CMC (peak 3) together with the other PTC-amino acids (at 5 nmol injected level of each amino acid) in samples where the determination of Cys and $(Cys)_2$ is required. However, while no problem exists at these levels of amino acids, determination at low levels (≤500 pmol injected) should be carried out using a separate aliquot, because impurities present in the IA (used for the carboxymethylation) or products formed during the PTC derivatization may significantly interfere (Fig. 1B).

Table 1 shows the linearity range, regression

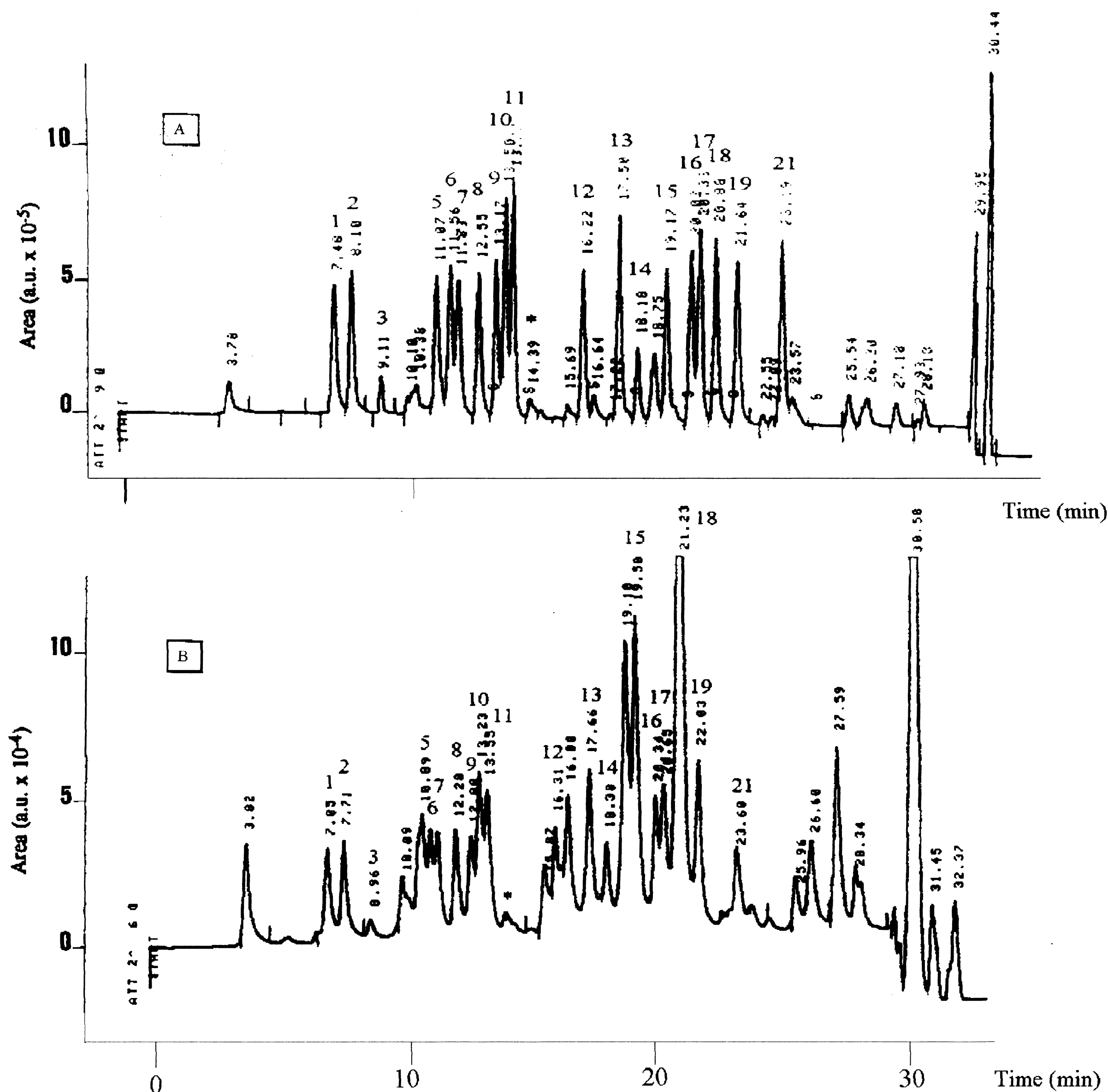

Fig. 1. Chromatograms of a standard solution of PTC-amino acids containing Cys, where the Cys carboxymethylation was carried out in situ before PITC-derivatization at high level of amino acids (injection: 5 nmol of each amino acid and 10 nmol of Cys) (A), and at low level of amino acids (injection: 500 pmol of each amino acid and 1nmol of Cys) (B). The marked with * between peaks 11 and 12 is unidentified and it also present during a blank run. For peak identification see Table 1, for chromatographic conditions see the text.

equation, correlation coefficient (*r*), and limit of detection (LOD) for each amino acid except Asn and Trp. Trp is still under investigation because of overlapping with ornithine. Varying amounts from 300 pmol to 20 nmol were derivatized and the correlation coefficients exceeded 0.999 for all samples. At 0.001 a.u.f.s. and for a signal-to-noise ratio of 3, the LOD ranged between 0.02 pmol/μl injected for $(Cys)_2$ and 0.17 pmol/μl injected for Asp. (Cys) presents the poorest linearity range and LOD (0.30

Table 1
Linearity range and limit of detection (LOD, s/n=3/1) for PTC-amino acids

Amino acid	Concentration range (pmol/μl)	Regression equation	Correlation coefficient (r)	LOD (pmol/μl)
1 Aspartic acid (Asp)	6–400	$y=27555x+3562$	0.99943	0.17
2 Glutamic acid (Glu)	6–400	$y=28968x+3035$	0.99926	0.16
3 Cysteine (Cys)	40–500	$y=22342x-9170$	0.99946	0.30
4 Asparagine (Asn)				
5 Serine (Ser)	6–400	$y=27524x+5978$	0.99931	0.14
6 Glycine (Gly)	6–400	$y=23477x+6576$	0.99912	0.15
7 Histidine (His)	6–400	$y=33749x+4027$	0.99965	0.16
8 Arginine (Arg)	6–400	$y=28160x+4031$	0.99961	0.11
9 Threonine (Thr)	6–400	$y=27175x+4116$	0.99935	0.12
10 Alanine (Ala)	6–400	$y=27465x+5523$	0.99950	0.13
11 Proline (Pro)	6–400	$y=34698x+6124$	0.99960	0.09
12 Tyrosine (Tyr)	6–400	$y=30176x+783$	0.99962	0.10
13 Valine (Val)	6–400	$y=30006x+2099$	0.99963	0.10
14 Methionine (Met)	6–400	$y=30097x-1054$	0.99976	0.11
15 Cystine ($(Cys)_2$)	3–200	$y=25204x-5201$	0.99926	0.02
16 Isoleucine (Ile)	6–400	$y=27167x+545$	0.99985	0.10
17 Leucine (Leu)	6–400	$y=33271x-500$	0.99974	0.10
19 Phenylalanine (Phe)	6–400	$y=29587x-4234$	0.99923	0.11
20 Tryptophan (Trp)				
21 Lysine (Lys)	6–400	$y=55296x-7996$	0.99935	0.08

pmol/μl). These LODs are in excellent agreement with those of Bidlingmeyer et al. [10], but are in contrast to most previous papers [6,9] where a 50 pmol LOD was reported.

Table 2 shows that good recoveries are obtained from all the matrices examined. For plasma and serum, the recovery values (%) of each amino acid are essentially equivalent except for Tyr, Met, and Leu. In urine and thyroid gland, the recoveries ranged from 86 to 102%. In algal samples, the

Table 2
Recovery (%) of amino acids from plasma, serum, urine, thyroid gland and natural (Nat.) and commercial (Comm.) algae

Amino acid	Plasma	Serum	Urine	Thyroid gland	Nat. alga	Comm. alga
Asp	93±3	94±3	99±3	95±3	96±1	94±2
Glu	94±3	94±2	98±1	100±2	94±2	92±1
Cys	96±2	95±2	94±2	97±3		
Ser	89±2	82±2	86±2	100±2	95±2	97±2
Gly	82±2	82±3	89±3	96±3	99±1	98±3
His	94±2	99±4	100±3	95±2	89±3	86±4
Arg	96±2	98±2	100±2	100±2	98±1	102±3
Thr	81±1	78±2	91±1	92±1	95±1	98±1
Ala	90±2	85±1	89±3	97±2	101±2	98±2
Pro	86±4	89±2	91±2	97±2	98±2	99±1
Tyr	90±2	104±7	101±2	86±2	89±2	88±4
Val	96±1	96±4	99±1	98±1	95±4	93±1
Met	88±1	98±8	86±1	102±4	95±1	96±1
$(Cys)_2$	102±3	98±2	102±4	93±3	97±1	96±2
Ile	93±1	100±6	99±1	95±1	98±1	95±1
Leu	88±3	99±6	100±3	99±2	99±1	101±3
Phe	102±4	102±4	98±3	99±4	97±3	98±2
Trp	95±2	97±3	99±2	96±4	96±2	95±3
Lys	91±2	96±5	92±4	99±4	92±3	95±4

recovery values ranged from 87 to 102%. For all of the recoveries, the low standard deviation values associated with each amino acid show that the two different extracting schemes yield similar results and the derivatization method is independent from the analyzed matrices, either between biological samples or between vegetable products.

Fig. 2 shows typical chromatograms obtained from the PTC-amino acid analysis in thyroid gland (A) sample, and cysteine (B). The large peak at 4 min is due to the deproteinizing solvent (SSA) but does not influence the first peak elution (aspartic and glutamic acids).

In Table 3 the amino acid levels in plasma, serum and urine of normal subjects are reported. The amino acid values of our study in plasma, serum and urine

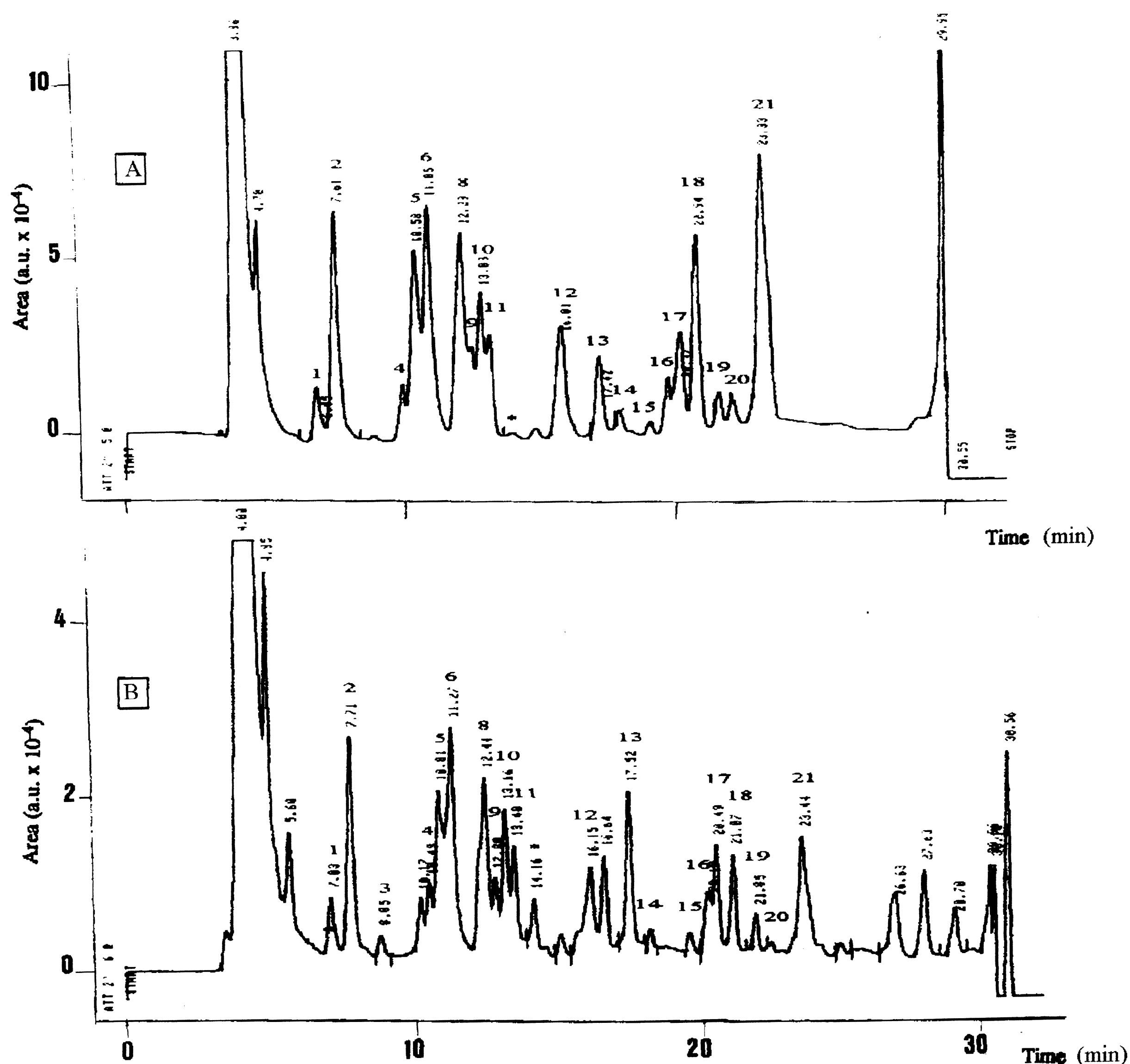

Fig. 2. Typical chromatograms obtained for the PTC-amino acids in thyroid gland (A) sample and for Cys (B) in thyroid gland sample. For peak identification see Table 1, for chromatographic conditions see text.

Table 3
Average levels (nmol/ml) of amino acids in the plasma, serum and urine of eight healthy patients

Amino acid	Plasma	Serum	Urine
Asp	2.5±0.2	5.4±0.2	38±4
Glu	23±3	37±4	88±7
Cys	4.6±0.5	5.2±0.7	9.2±1.4
Asn	40±4	50±5	180±7
Ser	528±33	495±13	389±11
Gly	196±4	214±17	1634±55
His	52±3	60±4	1129±90
Arg	63±5	75±2	N.d.
Thr	107±6	115±6	532±11
Ala	449±16	331±11	94±2
Pro	153±9	207±20	468±19
Tyr	87±2	83±3	420±6
Val	324±5	224±14	47±1
Met	21±2	23±2	5.6±0.4
$(Cys)_2$	18±1	28±2	25±2
Ile	60±7	92±3	13±1
Leu	152±12	130±10	26±1
Phe	71±5	567±14	37±3
Trp	107±6	26±2	28±3
Lys	179±8	131±7	338±15

N.d.: not detected.

are close to those obtained by other authors with similar [13,22,23] or significantly different methods [1,3,4,24,25]. The differences observed in plasma amino acid reference values regard only Asp and Ser [3], whereas, very high values are shown by Gly, His, Tyr and Lys in urine samples. The Trp value should be considered an estimated value because of the reason mentioned above. This is the first time that values obtained from the simultaneous analysis of 20 amino acids including cysteine and cystine are reported.

Table 4 reports the values obtained for the plasma of healthy and diabetic patients and for the thyroid gland of healthy patients and those affected by thyroid cancer. In the first case, marked differences are observed for Asp, Lys, Val, Cys and $(Cys)_2$. In the second case, a general decrease of amino acid levels (with the exception of six compounds) is observed.

Finally, in Table 5 the amino acid profile together with the total amount of amino acid present in both algal products are reported. There is good agreement between our values and of those found in the

Table 4
Amino acids level in the plasma (nmol/ml) of eight healthy and diabetic patients (a and b) and in the thyroid gland (nmol/mg) of three healthy patients and three patients affected by thyroid cancer (c and d)

Amino acid	Plasma		Thyroid gland	
	(a) Healthy	(b) Diabetics	(c) Healthy	(d) Cancer
Asp	2.5±0.2	16±1	2.6±0.2	1.5±0.1
Glu	23±3	28±1	8.4±0.8	5.9±0.5
Cys	4.6±0.5	3.1±0.4	0.54±0.07	−
Asn	40±4	46±4	1.4±0.2	1.2±0.1
Ser	528±33	488±10	7.3±0.5	7.2±0.4
Gly	196±4	146±12	7.1±0.1	8.2±0.6
His	52±3	45±4	1.1±0.3	−
Arg	63±5	92±2	6.8±0.7	9.2±1.0
Thr	107±6	88±5	1.1±0.1	0.41±0.03
Ala	449±16	321±12	3.9±0.5	2.6±0.4
Pro	153±9	199±14	2.3±0.1	2.2±0.2
Tyr	87±2	81±44	7.2±0.3	3.8±0.2
Val	324±5	168±11	2.5±0.1	1.6±0.3
Met	21±2	17±1	0.86±0.05	0.87±0.07
$(Cys)_2$	18±1	11±1	0.14±0.01	0.16±0.01
Ile	60±7	85±3	3.7±0.3	0.68±0.07
Leu	152±12	121±14	3.1±0.5	1.8±0.3
Phe	71±5	54±10	1.8±0.2	0.77±0.13
Trp	107±6	147±9	0.94±0.08	0.48±0.03
Lys	179±8	331±19	5.7±0.4	4.9±0.3

Table 5
Total (A, B) and free (C, D) amino acids in the algae *Spirulina platensis*

Amino acid	(A) Natural (mg/g)	(B) Commercial (mg/g)	Reference (mg/g)			(C) Natural (mg/g)	(D) Commercial (mg/g)	Reference (mg/g)	
			E	F	G			F	G
Asp	73±4	84±5	61	24	26	0.93±0.17	3.3±0.7	0.16	0.06
Glu	120±3	108±7	91	50	28	6.01±0.5	1.9±0.4	1.61	0.41
Ser	31±3	27±2	32	13	9.9	0.17±0.02	0.24±0.15	0.10	0.08
Gly	4.3±0.2	3.5±0.3	32	22	16	0.31±0.02	0.07±0.01	0.15	0.14
His	12±1	11±1	10	3.4	2.8	−	−	0.04	0
Arg	106±11	99±8	43	20	15	0.47±0.03	1.0±0.1	0.08	0.02
Thr	36±2	30±3	32	13	9.2	0.48±0.04	0.58±0.05	0.15	0.05
Ala	13±1	11±1	47	34	23	0.42±0.02	0.33±0.03	0.96	0.43
Pro	25±3	20±2	27	11	9.0	2.2±0.1	2.4±0.1	0.28	1.0
Tyr	25±1	25±1	30	7.3	5.6	0.19±0.02	1.5±0.2	0.42	0.06
Val	36±2	31±3	40	24	14	0.53±0.04	0.70±0.05	0.29	0.10
Met	15±1	14±1	14	2.8	11	−	−	0.07	0
$(Cys)_2$	7±1	6±1	6	2.4	0.8	−	−	0	0
Ile	50±4	46±5	35	10	7.3	0.31±0.03	0.79±0.04	0.22	0.06
Leu	65±3	58±5	54	25	18	0.22±0.02	0.66±0.07	0.24	0.13
Phe	36±1	34±2	28	21	12	−	0.53±0.08	0.53	0.08
Trp	1.0±0.1	0.9±0.1	9.0	0.2	0	−	−	0.23	0
Lys	38±2	39±3	29	16	9.0	0.20±0.03	0.69±0.15	0.09	0
Total	693	648	626	300	217	12.4	14.7	5.62	2.62

A and C: natural product from Cuba. B and D: commercial product from Cuba. E: *Spirulina platensis* [26]. F and G: Mediterranean green algae [29]. −: absent.

literature [26] for the total amount of amino acids and amino acid profile except the Gly, Ala and Arg levels. Further, it should be noted that the Met and Cys values are in agreement with the literature data. This means that the acid hydrolysis does not cause problems in this determination, in contrast with previous paper [5,27]. The free amino acid profile was also determined with the relative data shown in Table 5.

We conclude that the extraction scheme involving SSA is valid and can be applied to other biological matrices. PTC-method is reliable to determinate simultaneously Cys, $(Cys)_2$ and 18 other amino acids in widely different matrices but it shows also some limitations when the amino acid levels are close to their LODs. The authors are developing a OPA-method (with fluorescence detection) [28] to cross-check data and a procedure to analyze very low levels (pmol/μl) in various matrices. Finally, considering the economic importance of amino acid levels for feed formulation and the absence of a protocol to analyze amino acids in foodstuff, the procedure involved here can be applied to other different products routinely.

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References

[1] G.A. Qureshi, A.R. Qureshi, J. Chromatogr. 491 (1989) 281.
[2] Y. Ishida, T. Fujita, K. Asai, J. Chromatogr. 204 (1981) 143.
[3] G. Blundell, W.G. Brydon, Clin. Chim. Acta 170 (1987) 79.
[4] G.A. Qureshi, M.S. Baig, J. Chromatogr. 459 (1988) 237.

[5] A. Cubedo Fernández-Trapiella, J. Assoc. Off. Anal. Chem. 73 (1990) 935.
[6] P. Fürst, L. Pollack, T.A. Graser, H. Godel, P. Stehle, J. Chromatogr. 499 (1990) 557.
[7] A. Engström, E. Per, B. Andersson, Josefsson, W.D. Pfeffer, Anal. Chem. 67 (1995) 3018.
[8] R.C. Dorresteijn, L.G. Berwald, G. Zomer, C.D. de Gooijer, G. Wieten, E.C. Beuvery, J. Chromatogr. A 724 (1996) 159.
[9] R.L. Heinrikson, S.C. Meredith, Anal. Biochem. 136 (1984) 65.
[10] B.A. Bidlingmeyer, S.A. Cohen, T.L. Tarvin, J. Chromatogr. 336 (1984) 93.
[11] S.A. Cohen, B.A. Bidlingmeyer, T.L. Tarvin, Nature 320 (1986) 769.
[12] P.S. Janssen, J.W. Van Nispen, P.A.T.A. Melgers, H.W.M. Van Den Bogaart, R.L.A.E. Hamelinck, B.C. Goverde, Chromatographia 22 (1986) 345.
[13] G. Sarwar, H.G. Botting, J. Assoc. Off. Anal. Chem. 73 (1990) 470.
[14] A.S. Feste, J. Chromatogr. 574 (1992) 23.
[15] S.F. Shang, H. Wang, Chromatographia 43 (1996) 309.
[16] N. Naulet, P. Tesson, N. Bouler, G.J. Martin, Analusis 25 (1997) 42.
[17] I. Molnár-Perl, J. Chromatogr. A 661 (1994) 43.
[18] I. Molnár-Perl, J. Chromatogr. A 763 (1997) 1.
[19] R.J. Simpson, M.R. Neuberger, T.-Y. Liu, J. Biol. Chem. 251 (1976) 1936.
[20] S.H. Chiou, K.T. Wang, J. Chromatogr. 448 (1988) 404.
[21] J.R. Clayton, Q. Dortch, S.S. Thoresen, S.I. Ahmed, J. Plankton Res. 10 (1988) 341.
[22] V. Fierabracci, P. Masiello, M. Novelli, E. Bergamini, J. Chromatogr. 570 (1991) 285.
[23] B.L. Rosenlund, J. Chromatogr. 529 (1990) 258.
[24] G.A. Qureshi, L. Fohlin, J. Bergström, J. Chromatogr. 297 (1984) 91.
[25] J. Le Boucher, C. Charret, C. Coudray-Lucas, J. Giboudeau, L. Cynober, Clin. Chem. 43 (1997) 1421.
[26] R. Henrikson, Earth Food Spirulina. How this Remarkable Blue-green Algae can Transform your Health and our Planet, Ronore Enterprises, Laguna Beach, CA, 1989, p. 160.
[27] M. Spindler, R. Stadler, H. Tanner, J. Agric. Food Chem. 32 (1984) 1366.
[28] L. Campanella, G. Crescentini, P. Avino, in preparation.
[29] C. Paradessi, G. Lotti, F. Marchini, C. Benelli, Agrochimica XXXVII (1993) 187.