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FREE AND TOTAL AMINO ACID COMPOSITION IN BLUE-GREEN ALGAE

Luigi CAMPANELLA[1] (°), Mario Vincenzo RUSSO[2], Pasquale AVINO[3]

[1] Dipartimento di Chimica, Università di Roma "La Sapienza", Piazzale Aldo Moro 5, 00185, Rome, Italy
[2] Facoltà di Agraria (DiSTAAM), Università del Molise, Via De Sanctis, 86100, Campobasso (CB), Italy
[3] DIPIA-ISPESL, Via Fontana Candida 1, 00040 Monte Porzio Catone (Rome), Italy

Summary - A simple, accurate and reproducible analytical method is described for the extraction and the simultaneous determination of 18 amino acids in different for geographical origin Spirulina alga samples using phenylisothiocianate as derivatizating agent in natural feed. The best experimental hydrolysis conditions have been studied varying the temperature, the time and the hydrolyzing reagent. The separation and the quantitative analysis of the by-products have been carried out by HPLC analysis and UV detection. An amino acid pattern is compared with that proposed by the Food Agriculture Organization (FAO) for an ideal protein and with those of some traditional feed.

Riassunto - Viene descritto un metodo analitico semplice, accurato e riproducibilità per l'estrazione e la determinazione con una sola analisi di 18 amminoacidi di campioni di alga Spirulina provenienti da differenti zone geografiche. Sono state studiate le condizioni sperimentali più idonee per l'idrolisi, variando la temperatura, i tempi ed i reattivi idrolizzanti. La separazione e l'analisi quantitativa dei derivati è stata effettuata con l'HPLC a fasi inverse con eluizione a gradiente e con rivelatore UV. Una distribuzione amminoacidica viene confrontata con quella proposta dalla Food Agriculture Organization (FAO) per una proteina ideale e con quelle di alcuni alimenti tradizionali.

INTRODUCTION

An important research line of World Health Organization (WHO) and Food Agriculture Organization (FAO) concerns the alternative production of proteins from foods different from the

(°) Author for correspondence; Fax: (+39) –(0)6 490631; e-mail: luigi.campanella@uniorma1.it

traditional ones, of both animal and vegetal origin. In this view, Spirulina is an alimentary source potentially excellent both from the energetic and protein content point of view. Spirulina, photosynthetic and multicellular organism, belongs to the blue-green algae and lives in stagnant waters, expecially in Africa, organized in characteristic spiral shaped, very regular colonies. It imparts culture medium a green-blue color due to the presence of chlorophyll pigment and of the accessory phycocyanin pigment[1]. More than twenty five are the known species of Spirulina but only two can be used as a food: Spirulina platensis, growing in Ciad, Kenya and Ethiopia, and Spirulina maxima, along the shores of Texcoco Lake in Mexico.

The chemical dry biomass composition points out the high protein composition of Spirulina which is advantageous compared with other food sources. In fact, from a nutritional point of view[2] Spirulina commercialized in the form of pills or tablets is recommended by suppliers mainly as a health food, a protein source, vitamin supplement, diet pill. New cultivation techniques may change the chemical composition as well the commercialization processes of the alga biomass (processing, packaging and distribution) may introduce environmental pollution or variation of the chemical product composition.

In this paper the amino acid (AA) profile of Spirulina products of both natural and commercial origin is reported. The free and total AA levels have been investigated and compared with other products based on Spirulina. A frame of this work is dedicated to the investigation on the time-temperature parameters of the hydrolysis. Finally, according to the FAO reference four AAs like lysine, sulphorate (cysteine+methionine), threonine, tryptophane, are taken into account to evaluate the nutritional sample value.

MATERIALS AND METHODS

Materials, equipment and HPLC analysis

HPLC grade acetonitrile is used while anhydrous sodium acetate, ethanol, perchloric acid and nitric acid are of analytical grade from Carlo Erba (Milan, Italy). HCl (6 N), triethylamine (TEA), and phenylisothiocianate (PITC) are “sequanal grade” chemicals from Pierce (Rockford, IL, USA). Individual AAs (“Kit D-L Amino acids”), trichloroacetic acid (TCA) and sodium deoxicolate (DOC) are purchased from Sigma (St. Louis, MO, USA). A Milli-Q water purification system (Millipore, Bedford, MA, USA) is used to prepare mobile phases, standard solutions and buffers.

The Spirulina samples are both commercial and natural products coming from different countries. In Tab. 1 the main characteristics of the sample very similar each other are reported. Basically, their aspect is like a pill or a powder and the expiration time is about 2-3 years if they are stored at room temperature.

A Vaschetti & Grosso (Turin, Italy) conventional oven, a Büchi (Büchi, Switzerland) rotavapor and an Edwards (Edwards, England) high vacuum pump were also involved in the present work. Finally, 5 mL-, 10 mL- and 20-mL volume vials were purchased from Supelco (Bellefonte, PA, USA).

A Varian (Palo Alto, CA) 9012 liquid chromatograph equipped with a Varian 9050 UV-Vis detector and a Rheodyne 7125 injection loop, was used. The column was a Supelcosil LC18-DB, 25×0.46 cm, 5μm, kept at 45°C by thermostatic module Alltech mod.330. A ternary gradient was involved and the mobile phase flow was 1.0 mL/min. The three eluents were: A: 0.7 M CH_3COONa + 2.5 mL/L TEA + CH_3COOH to pH 6.4; B: water; C: CH_3CN-H_2O (80+20, v/v). The PTC-AAs were monitored at λ= 254nm. The gradient program was the following: from 20% A, 75%B, 5% C to 20% A, 30% B, 50% C in 25 min then to 10% A, 10% B, 80% C in 1 min and hold for 4 min.

TABLE 1. Characteristics and origin of the samples investigated in this study.

Sample	*Organism*	*Country of origin*	*Product, presentation*	*Recommended doses (g day^{-1})*	*Expiration date (years)*	*Notes*
1)Spirulina	*Sp. platensis*	Cuba	Dried seaweed, scales	-	-	Natural
2)SpirAll	*Sp. platensis*	Cuba	Plastic bottles, pills	0.8-1.6	3	Commercial
3)Spirulina maxima	*Sp. maxima*	Italy	Details sale, dry powder	Not indicated	2	Commercial
4)Spirulina Messicana	*Spirulina*	Mexico	Plastic bottles, pills	1.2-2.4	3	Commercial. Mix of different *Sp.*
5)Spirulina Health	*Sp. sosa + texcoco*	U.S.A.	Plastic bottles, pills	1.8-3.7	3	*Sp.* (60% *sosa tex.* + 40% *proteus*) + excipients
6) Alga Marina	*Spirulina*	U.S.A.	Plastic bottles, pills	1-2	3	30% different *Sp.* species + 70% excipients

Methods

Extraction of total AAs The process is based on the acid hydrolysis of the proteins and following solubilization of the present free AAs[3-6]. Into a vial 10 mg of dry algal biomass are transported with one mL of hydrolyzing solution (HCl 6M or $HClO_4$ 8M). Then, the vial was sealed and placed in a conventional oven. The adopted four hydrolyses conditions were at different temperature and time: (A) 150°C for 2h, (B) 140°C for 4h, (C) 120°C for 8h and (D) 110°C for 22h. After cooling at room temperature, all the hydrolysates were filtered on a 0.45-μm Whatman filter (Deerfield, IL, USA). Each solution was processed as described below.

Extraction of free AAs A 150-mg amount of powdered alga was added to 1 mL of 0.37M TCA, and vortexed for 1.5 min. A 0.2-mL sample of 3.6 mM DOC was added to assist the protein precipitation 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: the supernatant was processed as described below.

Drying and derivatization The derivatization procedure between PITC and AAs with formation of a substituted thiourea to be detected at λ= 254 nm, was previously described[7]. Shortly, 25 μL of the extract and 25 μL of Nle 10^{-3} M (I.S.) are derivatized with 25 μL of a mixture ethanol-H_2O-TEA-PITC (7+1+1+1 v/ v). After the reaction, the sample is dried and 250 μL of mobile phase A are added. After filtration, 50 μL of the solution has analyzed by means of HPLC.

RESULTS AND DISCUSSION

The research for the optimal hydrolysis conditions was articled into two steps, the first devoted to the hydrolysis study and the second to the quali- quantitative analysis of the obtained AAs. The latter, performed on a proteic standard[7], foresees that: 1) the acid is removed from the reaction mixture in order to prevent the presence of an unknown peak to be directly related to it and eluted with the other AAs; 2) the removal of ammonium ion too eventually present in the sample as it

could interfere with the derivatization procedure; (3) the derivatization occurs rapidly and quantitatively at room temperature by means of volatile reagents and solvents; (4) the quantitative AA analysis by HPLC. It must be underlined that the acid hydrolysis destroys tryptophane that so cannot be quantitatively determined: it should be necessary an alkaline hydrolysis. As concern the quantitative amino acid recovery after the hydrolysis, the comparison has been carried out using the internal standard (I.S.), Norleucine: in fact, after derivatization and analysis and after hydrolysis, derivatization and analysis, the ratio between the Norleucine peak areas are almost equal. Therefore, since the amino acids behave equally, the hydrolysis doesn't cause losses of substance.

The attention was therefore paid to the study of the best conditions of acid hydrolysis by means alternatively of two mineral acids, i.e. HCl “sequanal grade” reagent 6M and $HClO_4$ 8M, and an oven treatment.

Study of the optimal hydrolysis conditions

The influence of the extraction time (2, 4, 8, 22 h) directly correlated with temperature (150°C, 140°C, 120°C and 110°C, respectively), was investigated on maintaining constant the dry algal biomass (10 mg). All the experimental tests able to help us in individuating the best analytical conditions were performed on Sample 1. This since its picking up from the natural ecosystem in Caribbean Sea, that in an uncontaminated site, was considered as reference sample among the considered ones. Successively, the other samples will be compared with it under chemical composition and nutritional capacity point of views.

Fig. 1 reports a chromatogram relative to PTC-derivatives obtained from the total AAs present in algae after thermal hydrolysis with $HClO_4$. The tryptophane peak is absent as the compound is degraded during hydrolysis[9]. As it concerns the hydrolysis with HCl, the bubbling effect was studied: it was applied for about 1 min and finalized to prevent the possible oxidation of the stable AAs due to the atmospheric oxygen.

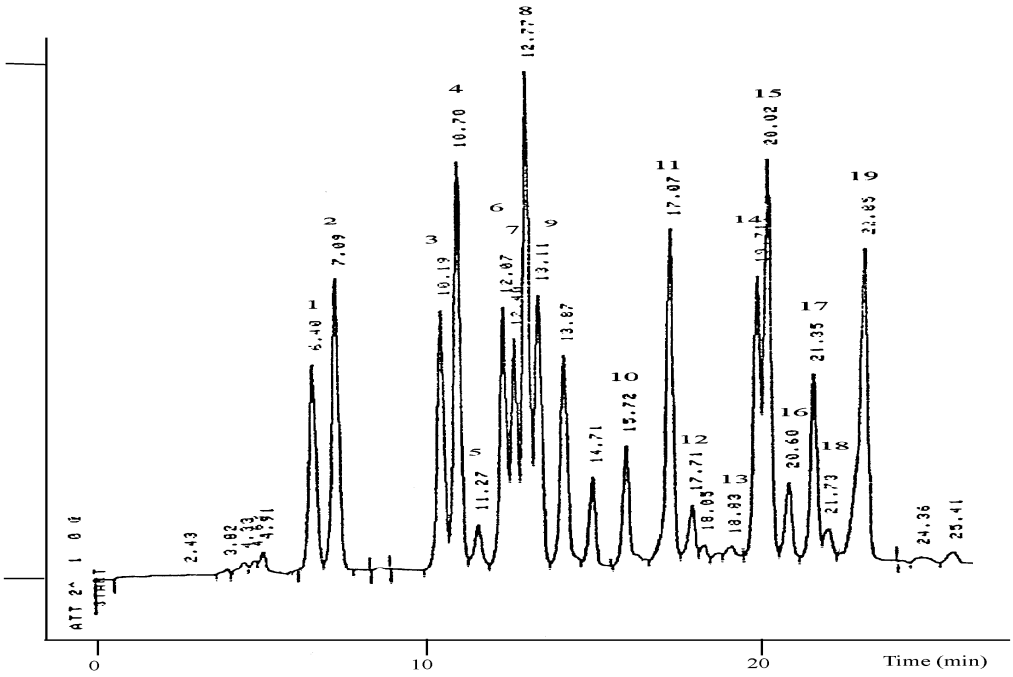

FIGURE 1. Typical chromatogram of total AA extraction from Spirulina platensis. Hydrolysis condition: $HClO_4$ with nitrogen bubbling, 22h and 110°C. For peak identification: see Table 2. For chromatographic conditions: see Material & Methods.

The chromatogram obtained without bubbling (Fig. 2) has the typical trend of the PTC derivatives with well shaped, well separated, not overlapped peaks, with no differences from the chromatogram of a sample bubbled with nitrogen.[8]

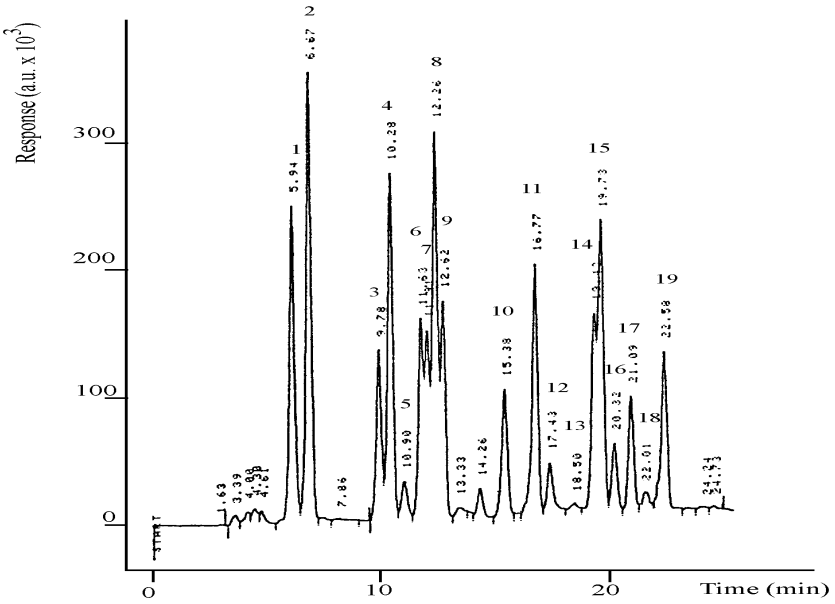

FIGURE 2. Typical chromatogram of total AA extraction from Spirulina platensis. Hydrolysis condition: HCl without nitrogen bubbling, 22h and 110°C. For peak identification: see Table 2. For chromatographic conditions: see Material & Methods.

Tab. 2 reports the AA levels (mg/g) obtained under the four different hydrolysis conditions to optimize the process for an established dry biomass amount (10 mg). The results of each hydrolysis refers to five different extraction procedures. The % variational coefficient not reported ranges from 2% (for aspartic acid) to 5% (for threonine) and yields information exclusively on the operating procedure protocol (extraction process, derivatization, chromatographic analysis): the general low value is a confirmation of the efficiency of the protocol used in the AA determination in so complex matrices as the vegetal ones.

It is observed that the total AA recovery is greater in the hydrolysis condition D (22h, 110°C) passing from 358 mg/g of dry biomass to 690 mg/g. Probably, larger contact times and lower temperatures permit a more efficient hydrolysis while in opposite conditions a faster hydrolysis reaction and a possible AA degradation can occur. This behaviour takes for each AA. Fig. 3 graphically summarizes the situation where some AAs (glutamic acid, histidine, tyrosine, valine, isoleucina, phenylalanine) were chosen on the basis of their positions in the chromatogram. It is possible to observe that the extracted AA amount increases with the hydrolysis time and tends to the value corresponding to a hydrolysis time of 22 h. A longer time of the process (22 h) and a lower temperature (110°C) permits a more efficient hydrolysis of the proteins and a total solubilization of free and bound AAs. Further investigations on increasing the dry algal biomass (18 mg), have yielded lower recoveries of total AAs but generally comparable.

As it concerns the used acids, in the case of $HClO_4$ hydrolysis the chromatograms are those ones typical of the PTC derivatives and the results obtained for each one of the AAs are very similar to those ones obtained with HCl: as hydrolising reagent $HClO_4$ is a valid alternative to HCl

"sequanal grade". Preliminary studies performed with other acids such as HNO_3 11M and H_2SO_4 9M, gave no satisfactory results.

TABLE 2. Amino acid levels (mg/g of dry biomass) obtained comparing the different acid hydrolysis conditions for 10 mg of dry biomass of the Sample 1.

Amino acid	*2h, 150°C*	*4h, 140°C*	*8h, 120°C*	*22h, 110°C*
Aspartic acid	38.5	41.2	53.2	73.1
Glutamic acid	50.2	64.2	67.8	90.6
Serine	16.8	26.2	31.3	34.8
Glycine	19.7	27.9	34.3	51.3
Histidine	6.1	4.8	8.8	6.9
Arginine	28.3	40.3	43.8	56.2
Threonine	17.8	28.3	33.2	36.7
Alanine	33.5	41.6	46.1	48.6
Proline	20.0	25.3	33.9	35.2
Tyrosine	18.8	21.3	22.7	25.0
Valine	22.4	28.5	34.0	36.7
Methionine	4.5	7.2	11.9	15.0
Cystine	1.5	1.8	3.0	5.2
Isoleucine	22.8	20.2	33.9	41.0
Leucine	31.2	49.8	58.0	65.0
Phenylalanine	15.1	22.7	30.7	36.0
Tryptophane	-	-	-	-
Lysine	10.6	16.3	22.7	33.0
Total	*357.9*	*467.5*	*569.3*	*690.3*

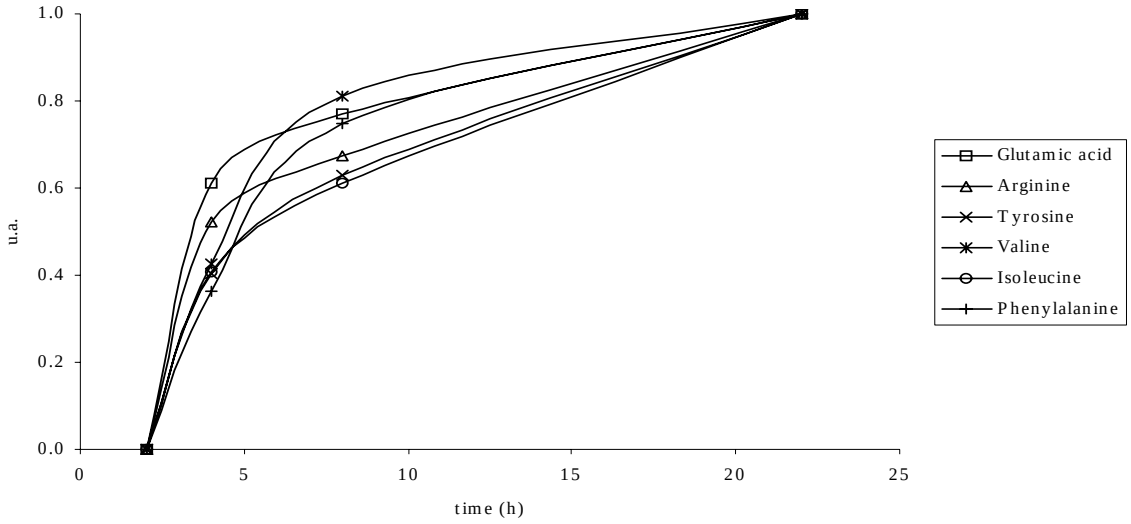

FIGURE 3. Some AA trends related with the thermal hydrolysis parameters (time and temperature).

Finally, as it concerns the chemical treatment, some authors[9] state that the hydrolysis in

conventional oven at 22 h and 110°C yields accurate results while satisfying results with a microwave oven[10] are also obtained, but this reveals to be unsatisfying as, although time is saved and recovery for some AAs is good, not all the PTC derivatives are eluted; particularly: in each analysis methionine and proline are absent, this probably being due to the morphological characteristics of Spirulina alga, in contrast to bibliographic evidence.[10]

Analysis of the samples

The best conditions for a significant and reproducible hydrolysis are based on the use of $HClO_4$ 8M (cheaper than HCl 6M "sequanal grade") for 22 h at110°C with an algal dry biomass sample of 10 mg: these conditions have been adopted to analyze each commercial sample of Spirulina. Basically, recovery values for the total AAs in the natural and commercial products are good, falling in the range between 87% (histidine) and 102% (arginine).

In Tab. 3 the total AA contents, as mg/g, in each algal sample are reported. Sample 1 has the highest AA concentrations (687 mg/g, corresponding to about 70% of the dry biomass). Among the Spirulina-based products, Samples 2 and 3 are similar enough to the pending one (638 mg/g and 631 mg/g, respectively), while Sample 4 is characterized by a lower content (486 mg/g). As it concerns the Sample 5, the total composition is 541 mg/g (80% of that one of Sample 1, natural product); very anomalous is the composition of Sample 6 (3.8 mg/g).

TABLE 3. Total AA content (mg/g ± s of dry biomass) in analyzed alga samples (a= absent).

Amino acid	*Sample 1*	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*	*Sample 6*
Aspartic acid	73±3	64±5	64±3	48±5	54±5	0.178±0.011
Glutamic acid	90±3	89±7	96±5	90±6	86±5	0.282±0.022
Serine	34±3	23±2	23±3	19±2	25±3	0.224±0.001
Glycine	51±1	39±1	39±1	32±2	28±4	a
Histidine	7±1	6±1	7±1	7±2	6±1	a
Arginine	56±11	48±8	48±7	41±1	38±4	0.121±0.008
Threonine	36±2	30±3	29±2	21±1	24±1	0.099±0.007
Alanine	49±1	51±1	47±1	35±2	43±4	0.189±0.016
Proline	35±3	22±2	21±3	19±1	20±1	0.115±0.018
Tyrosine	25±1	30±1	29±2	18±1	12±1	0.091±0.012
Valine	36±2	54±3	52±2	41±3	43±5	0.152±0.016
Methionine	15±1	16±1	17±2	7±1	9±1	0.241±0.061
Cyst(e)ine	5±0	5±0	4±0	2±0	3±0	a
Isoleucine	41±2	41±1	39±3	19±1	31±3	0.285±0.016
Leucine	65±3	56±5	53±4	34±3	51±1	0.181±0.017
Phenylalanine	36±1	29±2	27±2	24±1	29±1	a
Tryptophane	-	-	-	-	-	-
Lysine	33±2	35±3	36±2	27±2	39±1	1.44±0.05
Total	*687*	*638*	*631*	*486*	*541*	*3.75*

The different yields for some AAs can be related to the different excipients, present at different concentrations in the dry biomass of each sample: this heterogeneity produces differences in the recovery data of the total AAs, which enough systematically reflect on the content of each AA in

the Samples 2-5. For instance, aspartic acid, present in the natural product at concentrations value of 73 mg/g, is present at the same level in Sample 2 and 3, while it is about 70% in the Sample 4 and 20% on the Sample 5. The only case of homogeneous behavior is that one of histidine, present in all the samples at a concentration ranging between 6 and 7 mg/g. As it concerns Sample 6, the correlation with the analogous AAs of Sample 1 is less systematic varying between 0.3 and 0.8% in the case of serine and methionine, and 4% in the case of lysine.

Also the analysis for the determination of the free AAs was performed (Tab. 4) but the measured concentrations (1-2%) are very low, so confirming the literature data[11]. Particularly, the free AA determination in the Sample 6 is rather problematic so that it cannot be reported as reliable results: only some AAs result to be separated and quantified, i.e. aspartic acid, glutamic acid, alanine, valine and lysine while the other ones, even if identified, are affected by rather marked errors.

TABLE 4. Free AA content (mg/g±s of dry biomass) of the analyzed alga samples (a= absent).

Amino acid	*Sample 1*	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*
Aspartic acid	0.93±0.17	3.3±0.7	0.85±0.06	a	0.76±0.09
Glutamic acid	6.0±0.5	1.9±0.4	2.8±0.6	3.9±0.2	0.96±0.10
Serine	a	a	a	0.05	0.07±0.00
Glycine	0.17±0.02	0.24±0.15	0.04±0.00	0.05±0.00	0.07±0.01
Histidine	0.31±0.02	0.07±0.01	0.11±0.02	0.11±0.02	0.02±0.00
Arginine	a	a	a	0.07±0.00	0.03±0.00
Threonine	0.47±0.03	1.0±0.1	0.04±0.00	0.23±0.05	0.03±0.00
Alanine	0.48±0.04	0.58±0.05	0.20±0.03	0.04±0.00	0.02±0.00
Proline	0.42±0.02	0.33±0.03	0.85±0.03	0.46±0.09	0.14±0.02
Tyrosine	2.2±0.1	2.4±0.1	0.89±0.02	a	0.01±0.00
Valine	0.19±0.02	1.5±0.2	0.06±0.00	0.04±0.00	0.01
Methionine	0.53±0.04	0.70±0.05	0.30±0.06	0.11±0.02	0.04±0.01
Cyst(e)ine	a	a	a	a	a
Isoleucine	a	a	a	a	0.02±0.00
Leucine	0.31±0.01	0.79±0.05	0.07±0.00	0.03±0.00	0.03±0.00
Phenylalanine	0.22±0.02	0.66±0.07	0.05±0.00	0.04±0.00	0.01±0.00
Tryptophane	a	0.53±0.08	a	0.03±0.00	0.02±0.00
Lysine	0.20±0.03	0.69±0.15	0.06±0.00	0.15±0.04	0.23±0.02
Total	*12.43*	*14.69*	*6.35*	*5.35*	*2.37*

In the other samples the situation is quite different. The free AAs in the Sample 1 and 2 are 2% of total: histidine, methionine, cystine, phenylalanine, tryptophane are absent, as index of their presence in the sample as proteic AAs. As it concerns Sample 3 and 4 the free AAs are at a concentration of 1% of the total and analogously Sample 5 behaves, even if in this case the spectrum is more complete being present histidine, methionine and cystine.

In Tab. 5 the variability of some AAs for Sample 1-5, compared with the level of the analogous AAs in an ideal protein proposed by FAO and in other products such as egg, casein and soy flour. FAO Commission[12] really proposed a correlation between AA composition of proteins and their nutritional value. Only four essential AAs have been considered as able to limit the proteic quality

of the human diet, and so to act as indices of the proteic quality of a food: lysine, sulphurate AAs (methionine+cysteine, threonine, tryptophane), which constitute the so called FAO aminoacidic pattern.

TABLE 5. Variability of the FAO pattern, as mg/g of protein, (lysine, sulphurate AAs, threonine and tryptophane) for the Samples 1-5 and comparison with the level of the analogous AAs in an ideal protein proposed by the FAO and in other products, like egg, casein and soy flour.

	Lysine (mg/g)	*Sulphur AAs (mg/g)*	*Threonine (mg/g)*	*Tryptophane (mg/g)*
Pattern FAO	16	17	9	5
Sample 1-5	26-39	8-21	21-33	5-12
Egg	61	31	51	16
Casein	69	21	43	14
Soy flour	58	20	40	16

It can be observed that Spirulina samples contain AA amounts, with the only exception of lysine, of the same order as the FAO ideal protein. Interesting is the high level of lysine (about the double than in the pattern), which could let consider Spirulina as a powerful proteic integrator of cereals. Finally, it is noted that sulphurate AAs represent the limiting AAs in Spirulina: they are at about 14 mg/g level, from which a chemical index of 80% compared to FAO pattern, as measure of the proteic quality, can be deduced.

CONCLUSIONS

It is well known that different sea algae could constitute new feed sources and/or products with eco-compatible technologies at low cost to satisfy the increasing feed problem of the world population. Particularly, Spirulina, present in seas and in oceans and in proximity of many countries of the third world and characterized by a high nutritional power and by the presence of a wide complex of vitamins, mineral, fat acids, etc., could result a food economically convenient resource for many country populations.

In this paper a study to identify and quantify the AAs in Spirulina samples is reported. The results underline that the best experimental conditions to obtain elevated AA recoveries are the followings: i) 10 mg of alga, ii) temperature of 110°C for 22 h and iii) $HClO_4$ as hydrolyzing reactive.

From the nutritional point of view, the Spirulina presents an elevated total AA level, around the 70% of the dry biomass, and only the Sample 6 shows a low level of total AAs, i.e. less of the 4% of the dry biomass. A main point has resulted the comparison between the FAO's "AA pattern" and the AAs of Spirulina, where it results that the sulphur AAs are the limiting AA instead the top level of lysine makes Spirulina like an ideal cereals integrator.

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