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Protein and amino acid contents in the crab, *Chionoecetes opilio*

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Abstract

Total protein, by the Kjeldahl method, and amino acid content, by HPLC, were determined in crab samples. Proteins were determined in the shell and in the discard meat, separately.

An HPLC method was used to analyse the amino acids in dried crab shells after derivatization with phenylisothiocyanate. The resulting amino acids were chromatographed on an ODS2 column with UV detection at 254 nm. The mobile phase was a mixture of 0.14 M sodium acetate buffer, pH 6.2, containing 0.05% triethylamine and 60:40 (v/v) acetonitrile–water, at a flow rate of 0.9 ml min^{-1}. The amino acids found at highest concentrations were arginine, lysine, glutamic acid and serine.

Keywords: Protein; Amino acid; Kjeldahl; HPLC; Crab shell; Digestion blocks; Phenylisothiocyanate

1. Introduction

The Snow crab, *Chionoecetes opilio* (Brachyura: Majidae), is an important commercial species in the northwest Atlantic, especially in areas of Newfoundland and Labrador (Squires & Dawe, 2003). Although it is also found in the northern Pacific, the Bering Sea, the Arctic Ocean and the Sea of Japan, in the Atlantic Ocean, *C. opilio* is the only species present. In the Atlantic, snow crabs commonly live on sandy or muddy bottoms at temperatures ranging from −1 to 5 °C and at depths varying between 20 and 420 m (Plante, 2001).

Generally, fish and shellfish meat is considered to be highly nutritious, owing to its content of essential amino acids and proteins. In addition to their dietary importance, proteins affect food texture, as also do small peptides, and amino acids contribute to food flavour (De la Cruz-García, López-Hernández, González-Castro, Rodríguez-Bernaldo De Quirós, & Simal-Lozano, 2000). Although the amino acid composition of a variety of foods has been known for many years, relatively little information has been collected on the amino acid composition of fish and shellfish (Thompson & Farragut, 1966).

Marine invertebrates are widely used as food and feed supplements throughout the world. Crabs, among many other invertebrates, are considered to be important shell fishery products (Gókoolu & Yerlikaya, 2003).

Proteins have high molecular weights; they have colloidal effects when soluble, they have amphoteric properties and their complete hydrolysis produces a mixture of amino acids (Calmet, 1987). Proteins are present in almost all foods, although often in very small amounts. The principal nutritional value of proteins is as a source of essential amino acids. Currently, there is great interest in developing rapid, reliable and precise analytical methods for evaluating food quality in terms of amino acid content (Sánchez-Machado, López-Cervantes, López-Hernández, Paseiro-Losada, & Simal-Lozano, 2003). Some crustacean cuticular proteins have been partially or completely sequenced; they were derived from either solid, calcified exoskeleton or arthrodial membranes (Ditzel, Andersen, & Højrup, 2003).

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Amino acids in foods are generally determined after derivatization. The preferred method is usually reversed-phase HPLC with precolumn derivatization, which has the advantages of short analysis time, simple instrumentation and low cost (Sánchez-Machado et al., 2003). In order to effectively detect amino acids, however, it is first necessary to chemically modify them, which usually involves converting them into derivatives that absorb or fluoresce in the ultraviolet–visible (uv–vis) wavelength range. Several reagents have been developed for this purpose. Typical derivatization reagents include 9-fluorenylmethyl chloroformate (FMOC-Cl) (Fabiani, Versari, Parpinello, Castellari, & Galassi, 2002), *ortho*-phthalaldehyde (OPA) (Herbert, Barros, Ratola, & Alves, 2000; Calull, Fábregas, Marcéb, & Borrull, 1991) and phenyl isothiocyanate (PITC) (González-Castro, López-Hernández, Simal-Lozano, & Oruña-Concha, 1997). PITC, FMOC-Cl and OPA are widely used for analysis of amino acids, but PITC derivatization methods are less sensitive than are methods based on fluorimetric detection (Sánchez-Machado et al., 2003).

In the present study, the crab species, *C. opilio*, was investigated to determine its protein content by the Kjeldahl method and amino acids content by high-performance liquid chromatography after derivatization with phenylisothiocyanate. The aim of this study was to demonstrate the nutritional value of *C. opilio* shells for use as feed in fish farms.

2. Materials and methods

2.1. Standards and reagents

Kjeldahl catalyst (6.25% $CuSO_4 \cdot 5H_2O$) and acetanilide were purchased from J.P. Selecta, s.a. (manufactured by Panreac Quimica S.A.). Sulphuric acid (95–97%), sodium hydroxide (35%), hydrochloric acid (fuming, 37%), boric acid, methylene blue and methyl red were from Merck (Darmstadt, Germany). Purified water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Amino acid standards (L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-hydroxyproline, L-proline, L-serine, L-threonine, L-tyrosine and L-valine), the derivatizing reagent, PITC, and sodium phosphate (Na_2HPO_4) were from Sigma Chemical (Madrid, Spain). Triethylamine (TEA) was from Carlo Erba (Milan, Italy). HPLC-grade acetonitrile and methanol were from Merck (Darmstadt, Germany). Sodium acetate was purchased from Panreac (Montplet & Esteban s.a., Barcelona-Madrid) and glacial acetic acid was from Vorquimica, S.L. (Vigo, Spain). Filter papers were from Whatman (Maidstone, England) and MFS-13, membrane PTFE (pore: 0.45 μm, diameter: 13 mm) were from Advantec MFS/Inc (CA, USA).

2.2. Samples (harvest and preparation)

Snow crabs were collected in the north Atlantic, between Greenland and Canada (65° 40′ N, 54° 30′ W) in October, 2003. Generally, meat from the body and claw portions of crabs is collected manually and sold as a food product. For the purposes of this study, shells were frozen in the ship and transferred to our laboratory where they were later defrosted.

After defrosting, the length and the width of the crab shells were measured. The mean width and length were 11.38 ± 0.96 cm and 11.9 ± 0.94 cm. Afterward, shells were weighed to determine percentage of moisture by draining for 4 h, drying at 50 °C for 48 h, and further drying in a vacuum oven for 4 h until a constant weight was obtained. Finally, samples were ground with a coffee mill and stored in the dark.

Proteins were determined in the shells and in the discard meat inside shells separately, while determination of amino acids in shell and discard meat were done jointly.

2.3. Direct method for protein determination

2.3.1. Principle

The method consisted of mineralizing the sample with concentrated sulphuric acid and alkalizing with sodium hydroxide solution. The ammonium liberated was collected by distillation and recovered in boric acid solution. Subsequent titration with hydrochloric acid made it possible to calculate the initial amount of ammonium present in sample.

2.3.2. Digestion (Bloc-digest, J.P. Selecta, s.a., Spain)

Nearly 0.3 g of sample was accurately weighed, ground and homogenized on nitrogen-free paper and placed in the digestion tube. 4 g of Kjeldahl catalyst were added to the sample tube, as also were 25 ml of sulphuric acid 96% ($d = 1.84$) and a few glass beads.

Digestion tubes containing samples were put into a bloc-digest unit with the fume extractor on. The digestion was carried out with a programme set at 125 °C for 30 min, 200 °C for 30 min, and 400 °C for 120 min.

At the end of the process, the liquid obtained was a transparent blue in colour. The sample was cooled to ambient temperature. Distillate water (50 ml) was dosed slowly into each sample tube. The sample was then cooled at ambient temperature again. Although this did not occur in our case, if precipitate appears, the sample should be shaken or heated slightly.

2.3.3. Distillation (distilling unit, J.P. Selecta, s.a.,Spain)

Fifty millilitre of boric acid solution 4% (p/v) and some drops of mixed indicator were placed in an Erlenmeyer flask. The Erlenmeyer flask was placed under the refrigerant, making sure that the output tube was immersed in the boric acid. Afterward, 50 ml of NaOH 35% were introduced and the distillation was carried out until a minimum

of 150 ml of distilled solution were collected. The process took approximately 10 min.

2.3.4. Blank

After the sample distillation, a blank test with the described method was done, using 5 ml of distilled water instead of sample.

2.3.5. Titration

The distillate obtained was titrated with 0.1 N hydrochloric acid until the solution changed in colour from green to purple.

2.3.6. Detected nitrogen

Nitrogen and protein contents of the sample were obtained as follows:

$$\% \text{ Nitrogen} = [1.4 \times N \times (V_1 - V_0) \times f]/P,$$

N being hydrochloric acid normality, V_1, hydrochloric acid volume used in the titration (ml), V_0, hydrochloric acid volume used in the blank test (ml), f, correction factor of 0.1 N HCl and P, sample weight in g.

$$\% \text{ Protein} = \% \text{ Nitrogen} \times F,$$

F being the % Nitrogen to % Protein conversion factor. A value of 6.25 is commonly used for crude protein. Depending on the nature of the sample, other conversion factors can be used for improved accuracy.

2.4. Checking of nitrogen determination (recovery measure with acetanilide)

2.4.1. Principle

This test made it possible to check the whole Kjeldahl nitrogen determination process, including digestion and distillation.

The test consisted of the digestion and distillation of an acetanilide sample with a known nitrogen content. The quantity of detected nitrogen was calculated after distillation. A proper process has a recovery of over 99%.

2.4.2. Preparing the sample

0.3 g of acetanilide was weighed with a precision of ±0.1 mg on nitrogen-free paper and was introduced into a digestion tube with 4 g of Kjeldahl catalyst, as well as sulphuric acid and a few grains of treated pumice stone.

2.4.3. Digestion, distillation, white test and titration

Digestion, distillation, white test and titration included the same chemicals and steps as in the direct method for protein determination.

2.5. Preparation of samples and standards in determination of amino acids

Hundred milligrams of each sample were placed in screw-cap tubes (16 mm × 125 mm) and hydrochloric acid (6 M, 10 ml) containing 1% phenol was added. The tubes were then closed under nitrogen, and placed in an electric oven at 110 °C for 24 h. After hydrolysis, tube contents were vacuum-filtered through Whatman no. 41 paper to remove solids. The filtrate was diluted to 25 ml with ultrapure water in a volumetric flask and 1 ml of the resulting liquid was filtered through a 0.45 μm pore-size membrane.

A standard solution, containing 1.25 μmol/ml of each amino acid in 0.1 N hydrochloric acid, was obtained.

2.6. Derivatization with PITC

The derivatization procedure was a modification of the method used by (González-Castro et al., 1997). Amino acid standard solution or hydrolysed sample (20 μl) was placed in a vial and dried in a vacuum oven for 2 h at 65 °C. After this, 30 μl of methanol–water–TEA (2:2:1) were added to the residue and the resulting solution was vacuum-dried for a further10 min at 65 °C. Next, 30 μl of the derivatizing reagent – methanol–water–TEA-PITC (7:1:1:1 [v/v]) – were added, and the vials were vortex-mixed for 30 s and left to stand at room temperature for 20 min. Finally, the resulting solution was vacuum-dried for 15 min at 65 °C. Prior to injection, 150 μl of diluent, consisting of 5 mM sodium phosphate (Na_2HPO_4) with 5% acetonitrile, brought to pH 7.43 with phosphoric acid, were added to each vial with vortex-mixing for 15 s.

2.7. HPLC equipment and conditions

HPLC was performed with a Spectra-Physics (San José, CA, USA) chromatograph equipped with a PV-1580 quaternary pump (Jasco, Tokio, Japan), a 20 μl injection loop (Rheodyne, Cotati, CA, USA), and a Spectra Focus UV–visible forward optical scanning detector. Data processing was carried out with the software programme, Jasco ChromPass Chromatography Data System for Windows® (version 1.7.403.1). Compounds were separated on a 25 cm × 4 cm i.d., 5 μm particle size, ODS2 reversed-phase column (Teknokroma, Barcelona, Spain). A column header (Spectra-Physics model SP8792) was used to keep the column temperature at 27 ± 0.1 °C.

The mobile phase was a gradient prepared from two solutions, A and B. Solution A was 0.14 M sodium acetate

Table 1
Gradient programme employed for the separation of amino acids

Time (min)	Flow rate (ml/min)	% Eluent A	% Eluent B
0	0.9	90	10
8.0	0.9	90	10
10.0	0.9	70	30
14.0	0.9	70	30
20.0	0.9	52	48
22.0	0.9	0	100
25.0	0.9	0	100
28.0	0.9	90	10
35.0	0.9	90	10

buffer containing 0.05% (v/v) TEA (pH adjusted to 6.2 with glacial acetic acid). Solution B was 60:40 acetonitrile:water. The flow rate was 0.9 ml min^{-1} and the detection wavelength 254 nm.The gradient programme is shown in Table 1.

3. Results and discussion

3.1. Protein contents

We performed assays with varying amounts of sample (2, 1, 0.5 and 0.3 g), Kjeldahl catalyst (8 g = one tablet and 4 g = half tablet), sulphuric acid (25, 20 and 10 ml) and sodium hydroxide (100, 75 and 50 ml). The concentration of hydrochloric acid was also modified from 0.25 N to 0.1 N. After testing, optimum digestion and distillation were obtained with 0.3 g sample, 4 g Kjeldahl catalyst, 10 ml of sulphuric acid (95–97%), 50 ml of sodium hydroxide (35%) and 0.1 N hydrochloric acid. In addition, samples were subjected to a 3-step programme, controlling time and temperature that had also been tested to obtain optimum results (30 min at 125 °C, 30 min at 200 °C, and 120 min at 400 °C).

Protein contents ranged from 35.5 ± 3.16 g/100 g dry weight in shell to 33.0 ± 13.76 g/100 g dry weight in the discard content. These results were not comparable to those found by other authors, because previous studies had not separated discard meat from shell. Furthermore, the procedures for the determination of total proteins were not the same.

Some authors sampled different parts of crabs, such as green crab, blue crab and swim crab (Skonberg & Perkins, 2002; Gókoolu & Yerlikaya, 2003), and different sites, to determine nutritional composition in terms of proteins (Naczk, Williams, Brennan, Liyanapathirana, & Shahidi, 2004) and others did so according to the season of the year (Rosa & Nunes, 2003).

With regard to reported percentage of crude protein obtained, study values were $32.2\% \pm 2.44$ for freshwater crayfish waste meal (Lovell, Lafleur, & Hoskins, 1968) and 83.5–80.6% and 7.06–4.31% for other types of crab, such as the green crab, depending on raw meat or shell discards and where they were captured (Naczk et al., 2004).

3.2. Amino acid contents

Seventeen amino acids were analysed in our study, with an injection time of 35 min, preceded by a derivatization step lasting about 180 min, of which 20 min were for reaction between the amino acids and PITC. In comparison with other derivatization methods for HPLC analysis of amino acids, PITC derivatization required relatively long times (Fabiani et al., 2002). Trials indicated that the derivatized amino acids were stable for 48 h when stored dry under refrigeration in glass tubes with a screw-cap. The various amino acids were identified by comparing retention times with those obtained from amino acid standard solutions and from individual standard amino acid solutions. Only one of the seventeen amino acid tested, hydroxyproline, was undetected.

Regression equations for the calibration plots for each amino acid are listed in Table 2, all plots were based on analyses of at least four dilutions of the corresponding amino acid standard. Relationships between concentration and peak area were always linear, with coefficients of determination reaching 0.99 or better.

There are few studies on determination of amino acids by derivatization with phenylisothiocianate (González-Castro et al., 1997; Sánchez-Machado et al., 2003; De la Cruz-García et al., 2000), or with other derivatization agents (Fabiani et al., 2002; Calull et al., 1991). However, many studies involving the separation of amino acids use an amino acid analyzer instead of derivatization (Thompson & Farragut, 1966; Naczk et al., 2004; Gallagher & Brown, 1975; Min-Soo Min-Soo, Jin-Soo, & Shahidi, 2003).

We performed assays with varying amounts of sample (50, 100 and 150 mg) and with varying amounts of 6 M HCl (5, 10 and 15 ml). The best results for hydrolysis were obtained with 100 mg of sample and 10 ml of 6 M HCl, at a temperature of 100 °C for 24 h, as was also reported by others authors (González-Castro et al., 1997; Sánchez-Machado et al., 2003).

Testing was carried out with ammonium acetate and sodium acetate buffers at varying pH levels (5.9, 6.2, 6.25 and 6.4) for optimization of elution conditions. In the end, the sodium acetate buffer, at a pH of 6.2, provided the best conditions. A mobile phase consisting of two solutions, A and B was also tested. Solution A was unmodified acetonitrile:water (v/v; 60:40), while solution B was modified to obtain good separation of alanine (Ala) and proline (Pro). After testing to find the best column temperature

Table 2
Calibration relationships and retention times for the different amino acids considered

Amino acid	Calibration equation	r^2	Retention time (min)
Asp	$y = 0.1153x + 0.5125$	0.9962	3.31
Glu	$y = 0.1259x - 0.125$	0.9999	3.85
Hyp	$y = 0.1465x + 0.35$	1	4.43
Ser	$y = 0.2022x - 0.1$	0.9975	6.55
Gly	$y = 0.3057x + 1.2$	1	7.38
His	$y = 0.0481x + 2.875$	0.9049	10.56
Arg	$y = 0.1027x - 0.175$	0.9842	11.04
Thr	$y = 0.2087x - 2.8375$	0.9984	12.35
Ala	$y = 0.1885x - 1.75$	0.9957	12.97
Pro	$y = 0.2203x + 0.35$	0.998	13.50
Tyr	$y = 0.1244x - 0.525$	0.9997	16.75
Val	$y = 0.1793x + 0.1375$	0.9988	17.07
Met	$y = 0.141x - 0.425$	1	18.29
Ileu	$y = 0.1497x - 0.675$	0.9995	20.67
Leu	$y = 0.1752x - 0.2625$	1	20.90
Phe	$y = 0.1225x + 0.4875$	0.9998	22.56
Lys	$y = 0.143x - 0.5625$	0.9891	24.15

and flow rate, 25 °C appeared better than 27 °C and 0.9 ml min^{-1} was better than 1 and 1.1 ml min^{-1}.

An example of HPLC separation of the amino acids from a dry sample of snow crab with discard content is shown in Figs. 1 and 2 shows the HPLC separation of a standard solution, indicating that the samples were well resolved.

Table 3, shows mean and standard deviation (g/100 g of dry weight) for the amino acid contents of thirty snow crab samples (carried out in duplicate). The highest contents were found for arginine (2.35 g/100 g of dry weight) and lysine (2.07 g/100 g of dry weight). Other amino acids, such as glutamic acid and serine, were also important. Some amino acids (tryptophan, asparagine and glutamine) were destroyed by acid hydrolysis and were not determined. Cysteine was not determined because of the rapid oxidation of this compound to form cysteic acid. (González-Castro et al., 1997). Jaswal (1990) reported substantially higher

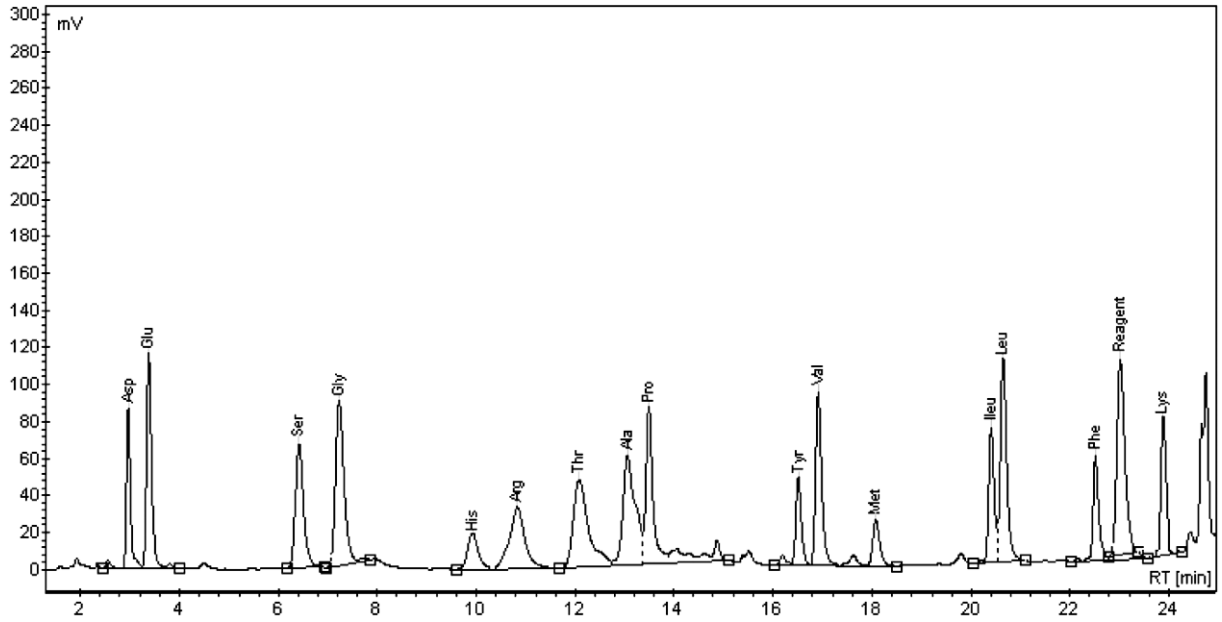

Fig. 1. A typical chromatogram showing the amino acids detected in a Snow crab sample (shell and discard meat).

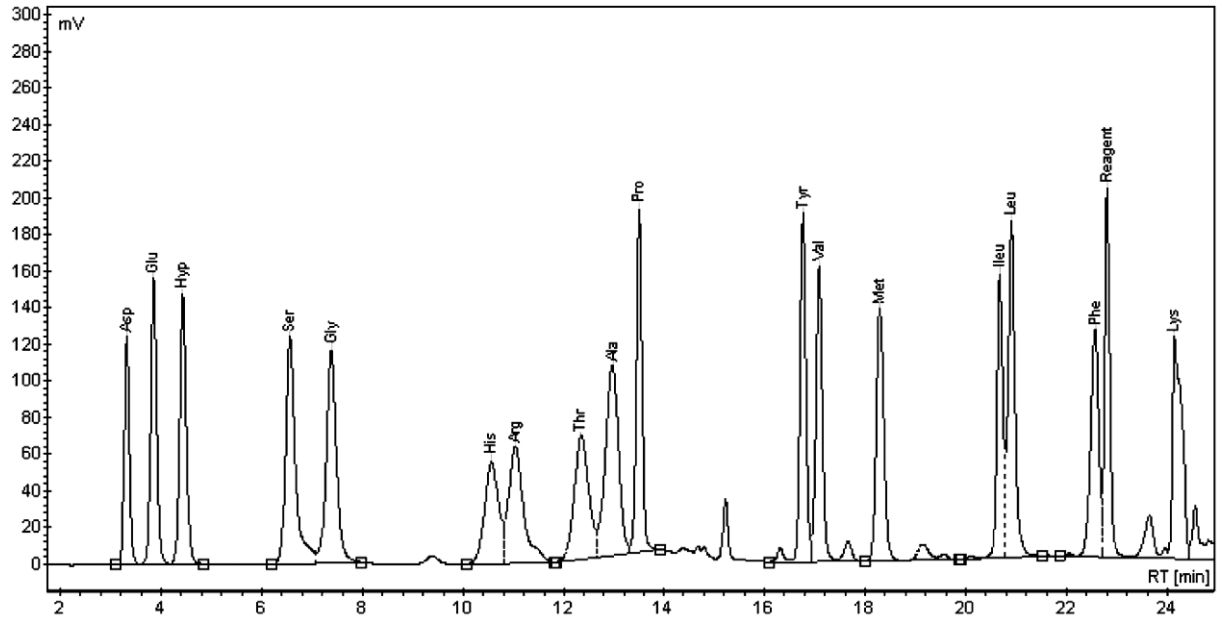

Fig. 2. Chromatogram of an amino acid standard.

Table 3
Amino acid contents (g/100 g d.w.) of crabs analysed (shell and discard meat)

Amino acid	Mean[a] ± SD[b]
Asp	1.23 ± 0.43
Glu	1.97 ± 0.48
Ser	1.64 ± 0.26
Gly	1.30 ± 0.28
His	1.45 ± 0.25
Arg	2.35 ± 0.54
Thr	1.58 ± 0.44
Ala	1.46 ± 0.35
Pro	0.91 ± 0.29
Tyr	0.98 ± 0.17
Val	1.38 ± 0.24
Met	0.48 ± 0.13
Ileu	0.96 ± 0.27
Leu	1.58 ± 0.3
Phe	1.3 ± 0.29
Lys	2.07 ± 0.76

[a] Mean g AA/100 g dry sample.
[b] Standard deviation.

results for these amino acids in the same type of crab; of the sixteen amino acids analysed, only five were below the values in our study. In a study involving green crabs, Naczk et al. (2004) found higher amino acid contents than those found here with the snow crab; nevertheless, it is noteworthy that these authors analysed meat from the body and claw portions, while we analysed meat content and shell content, jointly.

Some studies have preferentially analysed different tissues in specific types of crab. In one such example, involving the Dungeness Crab (*Cancer magister*), Allen (1971) analysed gonads, exoskeleton, hepatopancreas, visceras, muscle and hemolymph, and distinguished between male and female. The result was great variety in the reported values.

Comparing the snow crab results to those for other types of shellfish, we can see that, in a study on sea urchin, De la Cruz-García et al. (2000), report a total amino acid content which is twice as high as ours, for both raw gonad samples and canned gonad samples. In fact, our crab samples were only higher for four amino acids, while threonine, alanine and proline were not separated in the sea urchin study.

With respect to the quantity of amino acids in vegetables, only aspartic acid is noteworthy (González-Castro et al., 1997) and this is the only amino acid that exceeds our values for snow crab.

4. Conclusions

Protein analysis by the Kjeldahl method provides several advantages: it makes it possible to work under appropriate conditions, to carry out other types of analysis at the same time, and to work with several samples. Although the digestion of proteins requires almost 3 h of analysis, distillation is very fast.

The HPLC method presented here enables simultaneous analysis of seventeen amino acids in snow crab shells and discard meat, jointly. Only one amino acid was undetectable. The results of the present paper suggest that reversed-phase HPLC separation and UV detection of PITC derivatives of amino acids are useful for the determination of amino acids in this type of crab. Furthermore, this method provides rapid results, short analysis times, simple instrumentation, and low cost. The only small drawback is that the derivatization stage requires more time before proceeding to the analysis.

In conclusion, snow crabs are a nutritive resource with useful by-products that may have commercial value. This study shows that crabs caught in the northwest Atlantic have high protein levels (35.5 ± 3.16 to 33.0 ± 13.8 g/100 g dry weight in shells and discard content, respectively). Furthermore, amino acid content is also high, especially for arginine (2.35 g/100 g of dry weight) and lysine (2.07 g/100 g of dry weight). These results indicate that snow crabs may be useful as feed in fish farms.

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