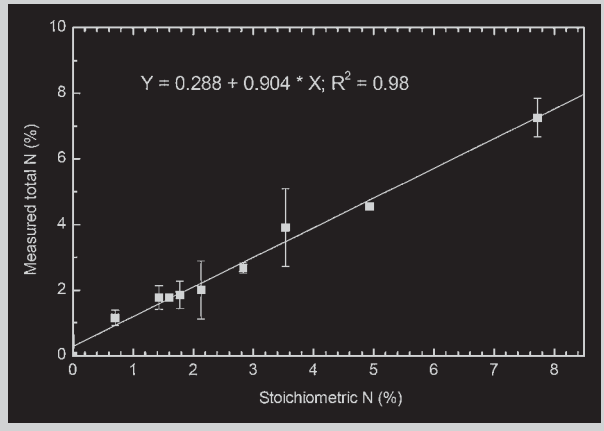
Summary: Accurate determination of chitin and protein contents in crustacean biomass and the intermediate products during the industrial isolation of chitin cannot be made directly from the total nitrogen content, unless the appropriate corrections are applied. This method, however, is affected by the presence of other nitrogen-containing chemical species that are formed endogenously or by the action of microorganisms during the handling of the sample. Therefore, an alternative rapid method to estimate the contents of these components can be very useful both in research and in various fields of application. An original method has been developed to address this problem. The method consists of the development of a set of equations based on the stoichiometric contents of nitrogen of chitin and protein whereby the amounts of each component can be estimated from the value of the total nitrogen content, provided the rest of the proximate composition of the sample is accurately known. In order to validate the procedure, a set of model mixtures of pure chitin and protein concentrate in the solid state, both extracted from shrimp head waste, are used. Excellent agreement between the predicted and real values of chitin and protein are obtained ($R^2 = 0.98$, slope = 0.90). When the proposed method is tested in the analysis of real samples obtained from five different processing protocols of pretreatment of raw shrimp head, it is found that in general the values of protein and chitin contents throughout the various stages of the process vary as expected.



Variation of the measured total nitrogen versus calculated stoichiometric total nitrogen of the chitin-protein mixtures.

Determination of Chitin and Protein Contents During the Isolation of Chitin from Shrimp Waste

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Keywords: chitin; protein; proximate analysis; shrimp head waste; stoichiometric nitrogen

Introduction

Chitin is a structural polysaccharide ubiquitously distributed in nature, particularly in the exoskeleton of arthropods (crustacean and insects), and the cell wall of fungi and other organisms. Chemically, the structure of chitin is comprised of units of *N*-acetyl-2-amino-2-deoxy-D-glucopyranose ($C_8H_{15}O_6N$) bound by β $(1 \rightarrow 4)$ glycosidic linkages (with the loss of a mole of water per glycosidic linkage) (Figure 1). It is the second most abundant natural polymer on earth after cellulose but its rate of turnover in the biosphere is even greater than that of cellulose. Chitin is insoluble in water, dilute mineral acids, and most organic solvents. It is biodegradable and non-toxic in nature.

Commercially, chitin is obtained from shrimp, prawn, krill, crab, and lobster shell waste generated by the fishing

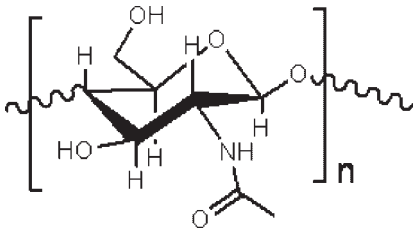

Figure 1. Chitin chemical structure.

industry worldwide. It is mostly used as a raw material to sustain the production of chitosan and glucosamine worldwide of ca. 15 000 ton · year^{-1}.

Several techniques to extract the chitin from different sources have been reported. Most of them, including the main industrial method, rely on chemical processes for the hydrolysis of protein and removal of inorganic matter. Some include a decoloration step to improve the color of the extracted chitin, using solvent extraction or chemical oxidation of the remaining pigments. Generally these methods use large quantities of water and energy, and also often produce corrosive wastes. In addition, the recovery of profitable by-products such as protein hydrolysates and pigments is complicated.[1] A lactic fermentation biotechnological process has been proposed in order to circumvent some of these problems.[2]

The industrial method of isolation of chitin from crustacean shell biomass involves various major steps: 1) grinding, 2) elimination of inorganic matter (calcium carbonate) in dilute acidic medium, and 3) extraction of protein matter in alkaline medium or by an enzyme, and some processes include the mentioned decoloration step. The order of the de-mineralization and de-proteinization operations can be reversed. Boiling or drying operations can also be introduced before the chemical or enzymatic treatments.[1,3]

Crustacean shells consist of compact matrices of chitin fibers interlaced with proteins. These matrices are reinforced through the deposition of mineral salts, mainly those of calcium.[4,5] The shells also contain pigments and other lipid compounds. The quantity and characteristics of these constituents can vary between species and between individuals of the same species as functions of growth stage, gender, feeding, and other environmental conditions. Chitin from animal sources usually occurs in association with protein, which functions as a lower modulus matrix surrounding chitin. X-Ray diffraction and scanning electron microscopy (SEM) studies support the concept that two discrete phases of protein and chitin are linked only at the interface.[6–8] The proteins themselves may be sclerotised, or cross-linked by *o*-dihydric phenols,[9] and this may play a biological role in preventing degradation by chitinases[10] and excessive hydration.[7] It has been demonstrated that >50% of the protein fraction is strongly bound to chitin in a chitin proteoglycan complex, and that aspartic acid and histidine are involved in the chitin-protein covalent link.[11] In chitin samples from different species of crabs, it is found that up to ≈55% of the covalent bonds are of an amide type involving C-1 and C-2 of the *N*-acetylglucosamine units in chitin.[12] The X-ray diffraction patterns of these complexes are consistent with a regular 6_1 helical conformation, whereby a protein chain is linked to every sixth sugar unit along the chain.

The remaining protein fractions are thought to correspond to soluble protein (14%), bound by van der Waal's forces (2%), and hydrogen (25%) and ionic bonds (3%).[13] The existence of chitin–protein complexes associated by virtue of distinct types of bonds, specific linkages, and conformational diversity, makes the use of spectroscopic (FT-IR, Raman, or CD) methods for the precise determination of the protein content in chitinous samples a daunting task. Indeed, these techniques are highly sensitive not only to the overall bulk concentration of protein, but to the modes of interaction and conformational changes of the proteins both free and in chitin complexes.[14–18]

The different biological species used as industrial sources of chitin differ in their structure, and the overall percentages of chitin, protein, and mineral matter in crustacean shell vary between different species within very broad range of each component: chitin 10–20%, protein 13–50%, and mineral matter 15–70%.[19] The proportion of each component, logically, varies through the various steps of the process. Therefore, reliable analytical methods to determine the proximate composition are needed in order to conduct correctly the mass balances of the various unit operations and the yield of the process. In addition, accurate determination of the content of chitin and protein in crustacean shells has practical relevance in the production of other by-products such as protein concentrates and carotenoid pigments (astaxanthin). In addition, estimates of the content of protein and chitin is required for mass and energy flow determinations in environmental process analyses.[21–23]

The analytical determination of protein in biological and inorganic matrices (e.g., biomass, food, manure, biowaste, soil, etc.) is often based in the quantification of total elemental nitrogen, either by wet chemistry methods, such as the Kjeldahl method,[23] by instrumental methods based on the combustion of the sample to release the nitrogen that can be further detected by thermal conductivity in an appropriate equipment,[24] or by near- and mid-IR spectroscopic methods.[25] Other methods commonly used in the biological sciences to this end, such as the Bradford and Lowry methods, are based on the colorimetric reaction of protein with a given reagent.[26,27] Although they are fairly accurate, the biochemical methods are suitable only when proteins are solubilized or dispersed in the analyzed liquid sample (e.g., biological fluids, drinks, etc.) and hence they are not suitable for the quantification of protein confined into an insoluble solid matrix as in the case of crustacean

exoskeleton. Hence, a method based on the determination of protein and chitin in this type of samples must be adequate for samples in solid state.

These difficulties led to the development of alternative methods to determine residual protein in chitin samples based on the hydrolysis of the residual protein followed by the determination of either amino acids produced or the loss in weight of the sample.[28,29] In the method developed by Takiguchi et al.[28] the protein hydrolysis step is carried out in concentrated alkali (10 N NaOH) under reflux. After neutralizing and filtering the reaction mixture, the filtrate is analyzed for its amino acid content using a ninhydrin-hydrintanin solution, and the filtered solid is washed, dried, weighed, and the degree of acetylation measured. The protein content can be estimated from the overall weight loss allowing for the change in the degree of acetylation. The separate determination of amino acid concentration on the filtrate acts as a valuable check on the protein content as measured by the loss in weight. Shimahara and Takiguchi[29] subsequently published a method based solely on the determination of amino acids in the neutralized hydrolysate. However, the protein used as a standard for calculating the factor in the equation used to calculate the protein content was Achilles tendon collagen.[30] Acid hydrolysis has also been used,[31] however, the procedure involves the determination of the amino acid composition by a chromatographic method or with an automatic analyzer. These methods are lengthy and have mostly been tested in chitin samples with low protein contents.[29–31] Therefore, reliable and yet simple methods to determine protein and chitin in chitinous waste biomass are needed so as to develop a standardized method to be used in the assessment of the quality of chitin and by-products.

The problem of determining the protein of samples whose carbohydrate is present primarily in the form of chitin content based on the value of total nitrogen, is that both components contain stoichiometric nitrogen in their chemical structure. Indeed, the nitrogen content in protein varies with amino acid composition, but it amounts roughly to 16%,[32] whereas in chitin it varies with the degree of deacetylation (i.e., 6.89% for fully acetylated chitin). Hence, this work focuses on the development of an original method on how to circumvent this problem and be able to quantify protein and chitin from the total nitrogen content value, provided that the composition of the other components of the sample is accurately known (water, mineral matter, and lipids) and that no other sources of nitrogen, besides protein and chitin, are present.

Ultimately, the proposed method is tested for the analysis of real samples of raw material and of the intermediate products derived from the distinct steps of the processes of isolation of chitin from shrimp head waste under different protocols.

Theoretical Background

Assuming that crustacean shells are grossly composed of chitin, protein, mineral salts, lipids, and water; a set of stoichiometric equations (Equation (1) and (2)) to estimate the chitin (Q) and protein (P) content in crustacean shells is proposed. These equations, which are based on the nitrogen stoichiometric content of chitin and protein, require that the measured percentage values of the total nitrogen (N_t) and the content of the non-nitrogen compounds (K) are determined experimentally. The latter is comprised of the inorganic matter (I), lipid (L), and water (W) content of the sample. Hence, Q and P can be defined as:

$$Q = \frac{(N_t \cdot C_p + K - 100) \cdot C_q}{C_p - C_q} \quad (1)$$

$$P = \frac{(N_t \cdot C_q + K - 100) \cdot C_p}{C_q - C_p} \quad (2)$$

Where K is the sum of the non-nitrogen compounds, as shown in Equation (3).

$$K = I + L + W \quad (3)$$

C_p and C_q are conversion coefficients that relate the mass fraction of nitrogen with protein and chitin, respectively. The usual value for C_p in proteins from marine products is 6.25.[32,33] Whereas Equation (4) defines the value of C_q.

$$C_q = \frac{(\mathrm{GlcNAc} - \mathrm{GlcN}) \cdot F_A + \mathrm{GlcN}}{\mathrm{N}} \quad (4)$$

Where N is the molecular mass of nitrogen (14.007), F_A is the molar fraction of acetylated monomers in the chitin/chitosan polymer chain, GlcNAc is the theoretical molecular mass of a *N*-acetyl glucosamine monomer repeated 'n' times and linked by β, $1 \rightarrow 4$, glycosidic bonds in a chitin/chitosan polymer chain (Equation (5)). The value of GlcNAc varies from 221.21 to 203.19 as 'n' tends to infinity (i.e., as a mol of water is lost per established glycosidic linkage).

$$\mathrm{GlcNAc} = \frac{221.21 \cdot n - 18.02 \cdot (n-1)}{n} \quad (5)$$

GlcN is the theoretical molecular mass of a glucosamine monomer repeated 'n' times and linked by β, $1 \rightarrow 4$, glycosidic bonds in a chitin/chitosan polymer chain (Equation (6)). The value of GlcN varies from 179.17 to 161.15 as 'n' tends to infinity.

$$\mathrm{GlcN} = \frac{179.1724 \cdot n - 18.02 \cdot (n-1)}{n} \quad (6)$$

The proposed set of Equation (1) and (2) depend on the applied nitrogen conversion coefficients (C_p and C_q). These coefficients delimit the total nitrogen content that corresponds to the proportion of nitrogenated compounds (Figure 2a).

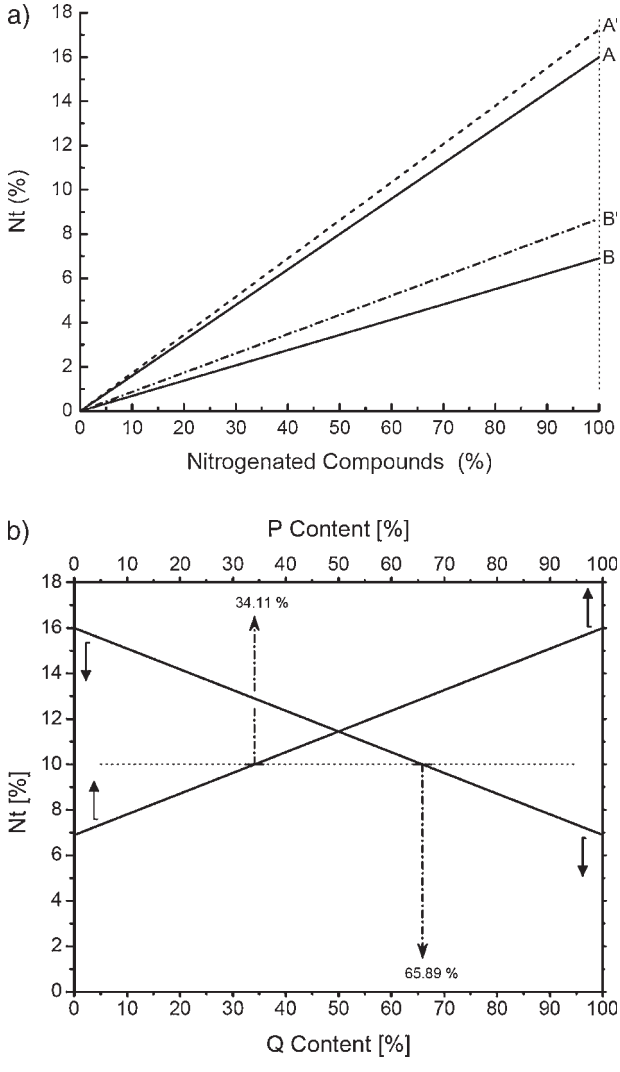

Figure 2. a) Theoretical limits of total nitrogen content (N_t) that correspond to the proportion of nitrogenated compounds ($100 - K$) in crustacean shells and its derivates. Line 0A indicates the values of N_t that may be found when the only source of nitrogen is protein. Line 0B is obtained when chitin is the only nitrogenated compound. Lines 0A′ and 0B′ correspond to possible variations of those limits as a result of divergent C_p and C_q values (as explained in the text). b) Predicted protein (P) and chitin (Q) contents as a function of total nitrogen (N_t) content when $K = 0$. For example, the dotted line indicates an N_t value of 10% that corresponds to 34.1% of protein and 65.9% of chitin.

A value of 6.25 is regularly used as the protein conversion coefficient (C_p), which corresponds to 16% of nitrogen in protein (Figure 2, point A), it is an estimated average for food products proteins. However, there are reports of alternative C_p values, such as 5.8 (Figure 2a, point A′), which was calculated from an average of data determined from bacteria, algae, and aquatic animals.[33] Evidently, an accurate calculation of the C_p based on amino acid analysis of the particular shells in study would improve the P and Q estimations. Error analysis for the estimation of P and Q of the proposed method is included in the Supporting Information section.

Conversely, the chitin conversion coefficient (C_q) is mainly affected by the degree of acetylation. For pure, completely acetylated ($F_A = 1.0$) chitin the calculated C_q is 14.5 (Equation (4)), which corresponds to 6.89% of nitrogen (Figure 2a, point B). The other extreme, completely deacetylated chitosan ($F_A = 0.0$) has 8.69% of nitrogen (Figure 2, point B′). Since both acetylation conditions do not normally occur in nature, an estimate of the degree of acetylation is required. The influence of the degree of polymerization, represented by 'n' as the number of monomers that form the polymeric chain (Equation (5) and (6)), becomes evident when the 'n' value is below 1 000. In addition, the coefficient C_q as determined in Equation (4) could be used in the matrix model, proposed by Vollenweider,[20] to estimate protein, carbohydrates, and lipids from CHN analysis of crustacean and other ecological samples. This model uses a coefficient obtained from chitobiose, as an approach to consider the chitin, as carbohydrate and nitrogen source, contained in the material.

A measured N_t value that falls outside of the limits given in Figure 2a (lines 0A and 0B) may indicate that at least one of the assumptions is not fulfilled and the equations will return meaningless results. Within these limits, the content of protein is inversely proportional to the chitin content, hence, for a given value of N_t there will be only one P value and a corresponding Q one (Figure 2b).

Experimental Part

Materials

Four batches of blue shrimp (*Litopenaeus stylirostris*) head waste were kindly donated by the fishermen at Empalme, Sonora, at the beginning of the shrimp catch season (early September) so as to ensure that the size and species of shrimp was as homogeneous as possible. Chitin used to validate the analytical method proposed was a sample of α-chitin previously isolated from blue shrimp in our laboratory (Lot No. GLD250902) of purity 96.1%, protein 0.6%, ash 1.6%, and lipids 1.8%, and degree of acetylation 95.8%. Chitin was ground through a size 20 mesh (Hammers mill, Metal Works Inc., Hamilton, Mich.) and then washed with mili-Q grade water until the conductivity of the water achieved a steady value. Protein concentrate (84.2% protein, 0.1% ash, and 15.7% lipids) also used in the validation of the analytical method was separated directly from the blue shrimp fresh head waste during the chitin isolation process (see below) and freeze dried. It was defatted with petroleum ether in a Goldfish unit prior to use. All reagents were of analytical grade from Sigma Chemical, Co. (St. Louis MO). KBr was from Merck (Darmstadt, Germany), and it was dried in an oven at 110 °C for ≈12 h.

Mixtures of Protein and Chitin

Pure chitin was mixed with shrimp protein concentrate by accurately weighing the solid powders, and the mixtures were homogenized manually with a spatula. The protein content was adjusted to ≈8.9% in all cases, and chitin content

Table 1. Composition of the model mixtures used to validate the method of determining chitin and protein contents.

Sample	Composition[a)]		
	%		
	Protein	Chitin	KBr
1	8.92	0.0	90.0
2	8.92	2.46	87.5
3	8.92	4.92	85.0
4	8.92	9.84	80.0
5	8.92	19.69	70.0
6	8.92	29.53	60.0
7	8.92	49.22	40.0
8	8.92	88.6	0.0

[a)] Dry weight basis.

was varied from 0 to 90% using KBr as a diluting agent (Table 1).

Isolation Processes of Chitin and Intermediate Products

A batch of raw material of fresh blue shrimp (*Penaeus stylirostrys*) head waste (sample M-L1) was ground in a Comitrol mill (URSCHEL, Freeport, IL) through a mesh #180, centrifuged in a surimi centrifuge (Mexicana de Maquinaria, S.A., Mexico, D.F.), and the separated protein concentrate was collected and subsequently freeze-dried. The waste paste was thoroughly washed with running tap water (sample W-L1). Demineralization was carried out in 1 N HCl at ≈25 °C over 2.5 h (sample DM-L1) and proteins were extracted with 2 N NaOH at 50 °C over 4 h to obtain a batch of chitin (sample Qn-L1). A second batch of raw material (sample M-L2) was pre-treated by boiling it in water during 1 h, ground in an Osterizer kitchen blender, and the chitin was isolated according to the same demineralization protocol (sample DM-L2), while the deproteinization step to isolate chitin was carried out under identical conditions as for M-L1 (sample Qn-L2). The third lot of headwaste (sample M-L3) was pre-treated by drying the whole sample in a convection oven (Lindberg/Blue M., Asheville, NC) at 80 °C over 72 h and chitin was isolated as above (samples DM-L4 and Qn-L3). The fourth batch (sample M-L4) was pre-treated by combining a boiling step followed by an immediate drying pre-treatment before separating the chitin under identical protocols as for the rest of the samples (samples DM-L4 and Qn-L4). The proximate composition of the samples taken from each step of the various processing protocols was analyzed as described below.

Proximate Composition

Official analytical methods were used to determine moisture (Method 991.01, AOAC),[23] mineral matter (Method 942.05; AOAC),[23] and lipids (Method 7.060, AOAC).[23] Total nitrogen was determined using a nitrogen analyzer LECO Model FP-528 (Leco México, S.A. de C.V., México, D.F.) equipment. To this end, ≈500 mg of each sample, previously dried, was incinerated at 850 °C in the equipment and air was used as a blank (Method 990.03 AOAC).[24]

Statistical Analysis

Measurements were conducted in triplicate unless otherwise stated. Kruskall-Wallis one-way ANOVA tests on ranks for mean comparison were performed using a NCSS 2000 statistical software package. The probability value was set to $p < 0.05$ in all tests.

Result and Discussion

Before attempting to test the developed method to determine the chitin and protein contents in a set of real samples of shrimp head shell waste of varying degrees of processing, the experimental validation of the technique is presented. To this end, a series of model mixtures of chitin and protein (Table 1) diluted with KBr are used, in which the protein contents are fixed and progressively greater amounts of chitin are incorporated. The total nitrogen content is determined by the Dumas (nitrogen combustion) method using the LECO nitrogen analyzer. Highly pure chitin and a defatted protein concentrate sample obtained from blue shrimp head waste were utilized to formulate these mixtures. Figure 3a shows the close agreement between the estimated and the real chitin content in the model mixtures. In turn, Figure 3b shows the agreement between estimated and real protein contents of the model mixtures by applying the proposed stoichiometric method. Notice that the series of mixtures are all formulated so that the protein content remains fixed at a value of 8.92%. This is in very close agreement with the mean average and standard error of the estimated protein values of $8.74 \pm 1.3\%$.

In order to assess the validity of the proposed method, a plot for total measured *versus* stoichiometric nitrogen (i.e., calculated from the real chitin and protein contents in the mixture model series) is constructed (Figure 3c). A slope of 0.90 of the linear regression equation ($R^2 = 0.98$) confirms the validity of the method.

In principle, estimation of the chitin and protein content in crustacean shell biomass using the proposed Equation (1) and (2) is simple and may prevent the need for additional measurements. However, the accuracy of these estimates directly depends on the analytical determination of the whole proximate composition of the sample. Inaccuracies in the analysis of N_t or any of the non-nitrogen compounds (I, L, and W) are summed up when the proposed equations are used. In addition, it assumed that the measured values of the non-nitrogen compounds and total nitrogen remain the same throughout the time span of the complete analysis of the sample as a result of environmental and sample handling conditions. In particular, special care must be taken with the moisture content determination as it can easily vary.

Yet another source of uncertainty that affects this method is the presence of nitrogen compounds other

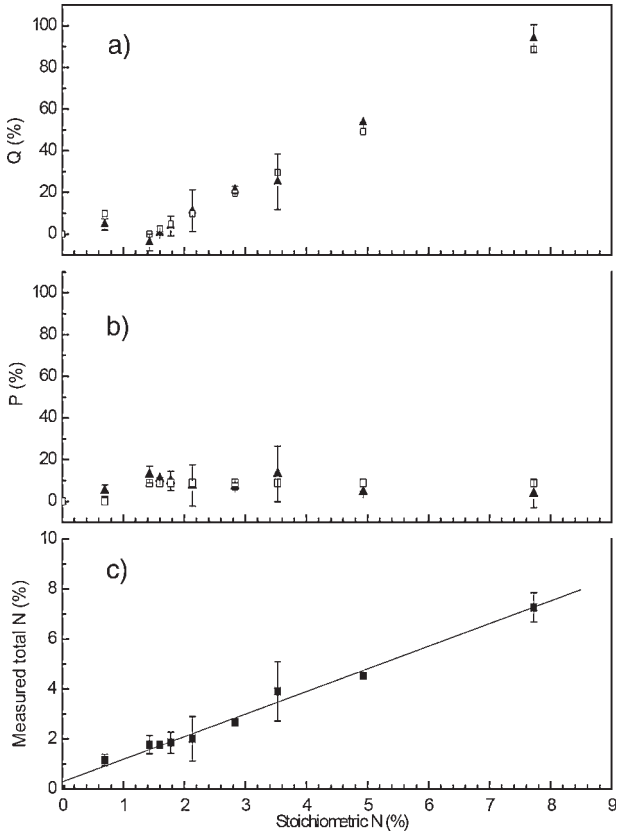

Figure 3. a) Chitin content estimated by the stoichiometric total nitrogen determination method (filled squares) versus real chitin content (empty squares) in model chitin–protein mixtures containing a fixed concentration of protein of 8.9%. b) Protein content estimated by the stoichiometric total nitrogen determination method (filled squares) versus real protein content (empty squares) of the chitin–protein mixtures in model chitin–protein mixtures that contain a fixed concentration of protein of 8.9%. c) Variation of the measured total nitrogen versus calculated stoichiometric total nitrogen of the chitin–protein mixtures. Experimental data in the three frames are mean average values of triplicates ± standard error (error bars).

than chitin and protein, either present as indigenous species (e.g., lipoproteins, free aminoacids such as histidine, trimethylamine oxide, urea, histamine) or generated during spoilage of the sample (e.g., trimethylamine, hypoxanthine, ammonia, etc.).[34,35] Such nitrogen-containing chemical species, each with different stoichiometric amounts of nitrogen in their structure, will undoubtedly also affect the accuracy of the determinations. Indeed, Equation (1) and (2) used for the calculation of chitin and protein contents, consider only the stoichiometric amount of N on chitin and protein, respectively. Hence, the presence of any other source of nitrogen-containing compound bound to contribute to the total nitrogen content in real samples, will interfere with the accuracy of the determination. If any, their contribution of these non-protein nitrogen compounds will be towards a greater protein content (as predicted by Figure 2b). Also, any another source of carbohydrates, besides chitin, can introduce error in the determinations, such as the presence of glycogen,[20] as it is not accounted for in the stoichiometric equations. The need to determine any further components in order to increase the accuracy of the method will limit practical value.

Determination of Chitin and Protein Contents in Real Samples

The method developed in this study for the determination of chitin and protein is tested in the analysis of real samples taken from the various steps of the process of chitin isolation subjected to different pre-treatment protocols.

As explained earlier, the determination of chitin and protein contents implies to determine experimentally the precise amount of the other major components (ash, lipids and moisture), i.e. the value of K in Equation (1) and (2). Hence, Table 2 shows the experimental results for ash, lipids, and total nitrogen contents (on a dry weight basis), along with the estimated protein and chitin contents using the proposed method for a series of shrimp head waste samples processed under various pre-treatments and from each step of the process that leads to chitin isolation.

Inspection of the data shows that the ash content decreases drastically after the demineralization step in all the treatments except for DM-L4 where the value of 12.4% stands out from the rest. Ash contents in chitin products Qn-L1, Qn-L2, and Qn-L3 fluctuate within the range of 1.0–1.4% and are not significantly different among them. Again, sample Qn-L4 exhibits too high a value of ash content of 34.4%, which is significantly larger than the rest of the samples. This unusually high content of mineral matter in sample Qn-L4 is attributed to the boiling and heating pre-treatments to which the raw material was subjected to, which results in the overall reinforcement of the calcium carbonate–chitin matrix. In turn, the lipid contents increase only slightly after the demineralization step in all but the DM-L1 sample, which is consistent with the possibility that a fraction of the lipids in this run may have also been extracted during the removal of calcium salts. As far as the total nitrogen content in the produced chitin samples is concerned, notice that the values of samples Qn-L1, Qn-L2, and Qn-L3 lie close to the theoretical ones (Figure 2a) for samples whose nitrogen is mostly confined in chitin. In turn, the lowest nitrogen value found for sample Qn-L4 is undoubtedly a result of the high residual ash contents.

In order to establish a meaningful assessment of the efficacy of the proposed method it is convenient to consider each stage of the process separately. In the case of the protein content of ground shrimp head waste, it is found that the values estimated are not significantly different among samples M-L1 to M-L4. It is important to emphasize that the method is highly sensitive to the accuracy of the determinations of total nitrogen, ash, lipids, and moisture contents of the sample.

Table 2. Variation of the chemical composition through the various stages of the process of isolation of chitin under different experimental protocols (on a dry weight basis).

Sample code[a)]	Ash[b)]	Lipids[b)]	Total nitrogen[b)]	Protein[b)]	Chitin[b)]
	%	%	%	%	%
M-L1	22.6 ± 0.2*	5.7 ± 0.5***	9.0 ± 0.1*	44.8 ± 0.4**	27.0 ± 0.2*
M-L2	26.4 ± 0.7*	4.4 ± 0.0**	8.6 ± 0.7*	41.9 ± 2.3**	27.2 ± 1.5*
M-L3	24.6 ± 0.1*	6.1 ± 0.2***	9.0 ± 0.0*	46.6 ± 0.1**	22.7 ± 0.1*
M-L4	25.1 ± 0.1*	4.3 ± 0.1**	8.3 ± 0.2*	40.5 ± 1.9**	22.1 ± 0.0*
W-L1	33.7 ± 0.9*	2.3 ± 0.1*	8.1 ± 0.2**	40.7 ± 1.0**	23.3 ± 0.1*
DM-L1	2.5 ± 0.2*	0.8 ± 0.1*	9.0 ± 0.0*	25.6 ± 0.2*	71.2 ± 0.1****
DM-L2	2.7 ± 0.3*	4.1 ± 0.2**	11.7 ± 0.0**	58.0 ± 0.6****	35.1 ± 0.6**
DM-L3	3.3 ± 0.1*	6.6 ± 0.4***	9.4 ± 0.1*	35.1 ± 1.2**	55.0 ± 0.7***
DM-L4	2.4 ± 1.6**	2.0 ± 0.1*	10.2 ± 0.2**	46.5 ± 2.5***	40.1 ± 2.9**
Qn-L1	1.4 ± 0.1*	0.2 ± 0.1*	6.7 ± 0.0**	0	98.7 ± 0.4***
Qn-L2	1.4 ± 0.1*	0.4 ± 0.0*	6.5 ± 0.0**	0	100
Qn-L3	1.0 ± 0.3*	1.3 ± 0.0**	6.2 ± 0.2**	0	100
Qn-L4	34.4 ± 3.0**	0.4 ± 0.2*	4.4 ± 0.1*	0	66.7 ± 4.3*

[a)] Sample codes: M, ground shrimp head waste; W, washed shrimp head paste; DM, demineralized chitin; and Qn, chitin. L1–L4 refer to the various pre-treatments: L1, fresh; L2, boiled in water; L3, heat dried, and L4, boiled-heat dried.

[b)] Mean values (±standard error) within a column and processing step followed by different superscripts are statistically significantly different ($P < 0.05$).

NB. The superscripts denote statistical significant differences among values in Table.

From a comparison of the protein content of the intermediate product W-L1 with the raw material (M-L1) it can be concluded that the washing step brings about a reduction in protein and chitin contents. Further inspection of the data for the de-mineralized samples (DM-L1 to DM-L4) shows that statistically different protein values are found between all the studied samples. Sample DM-L1 appears with the lowest protein contents, which is even lower than that of the washed product (W-L1), even when ≈90% of the mineral matter has been removed during the acid treatment. This seems to indicate that protein is also removed during the acid treatment (i.e., by hydrolysis).

In contrast, sample DM-L2 experiences an increase in protein content. In the case of chitin values for this sample, however, it is worth noticing that the method yields a value only slightly greater than that of the raw materials. Values of protein and chitin in this sample indicate that boiling in water could have brought about the denaturation of protein within the chitin–calcium carbonate matrix, thus effectively retaining the protein much more strongly than for the freshly pre-treated samples.

In sample DM-L3, which is subjected to heat drying during its pre-treatment, protein is lost, while chitin is retained during acid treatment. This is in good keeping with a previous study,[36] in which it was found that when shrimp waste is dried, the protein on the surface of the shell material appears as a compressed powder. Similar relative changes in protein and chitin are observed for sample DM-L4. However, the ash content of this sample is also very high (≈12.4), hence it could have been anticipated that protein (and chitin) are retained.

In the case of the final chitin products it is interesting to notice that the method predicts that no residual protein is left. An explanation offered for this is that the calibration curve (Equation (3)) is constructed on the basis of a fixed amount of protein and up to a maximum chitin/protein ratio of 9.0. It appears that at much greater ratios, the validity of the calibration curve may no longer hold and its usefulness in its present form can only be claimed for samples with a chitin to protein ratio of <9.0.

As a final remark, it is important to draw attention to a very interesting instrumental method worth testing towards the problem addressed in this study, namely solid state ^{15}N NMR. It has been shown that this spectroscopic technique can be used successfully to differentiate between amide and amino residues in chitin and chitosan, respectively.[37] Nitrogen NMR spectroscopy is very attractive for the investigation of biopolymers as chemical shifts of nitrogen are very sensitive to chemical environments.[38,39]

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