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MULTIVARIATE ANALYSIS OF CHITIN AND CHITOSAN FROM MARINE WASTE: CLASSIFICATION AND PROPERTY ESTIMATION USING INFRARED SPECTRA

Carla-Cezarina PĂDUREȚU[1*], Raluca Daniela ISOPESCU[2], Ileana RĂU[3]

The infrared spectrum of chitin/chitosan samples could provide valuable information regarding the geographical and/or biological origin of species, and at the same time it can be used for a rapid estimation of the deacetylation degree. As the number of independent variables is very high, principal component analysis (PCA) was applied for dimensionality reduction, while the linear discriminant analysis (LDA) was used for modelling the differences between distinct classes. The deacetylation degree of new chitin/chitosan samples can be rapidly predicted using the infrared spectra by partial least squares (PLS) method.

Keywords: chitosan; chitin; marine waste; infrared spectra; PCA-LDA; PLS

1. Introduction

The most abundant structural material found in the plant kingdom is cellulose, while the most abundant structural and protective material found in the animal kingdom is chitin [1]. Due to high availability from renewable sources, and also due to similar physical, chemical and biological properties with cellulose, chitin is also known as "animal cellulose" [2-4]. The marine organisms (lobster, crab, shrimp etc.) have a higher content of chitin compared to terrestrial organisms [1], including insects such as house cricket [5], or compared to the cell wall of almost all fungi [4]. Deacetylation of chitin leads to chitosan, one of its most important derivatives [6]. Chitosan and chitin are differentiated based on their solubility in acetic acid, chitin being the insoluble part, while chitosan is the soluble part [7], as well as based on the degree of deacetylation [2].

The advanced processing of animal wastes containing chitin is a challenging approach from environmental and technological point of view. The characteristics of chitin containing wastes are decisive for further treatment. As these wastes originate from different geographical zones and from various

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[1] PhD Eng., Department of General Chemistry, University POLITEHNICA of Bucharest, Romania, *e-mail: paduretu.carla@yahoo.com;

[2] Professor, Department of Chemical and Biochemical Engineering, University POLITEHNICA of Bucharest, Romania;

[3] Professor, Department of General Chemistry, University POLITEHNICA of Bucharest, Romania.

biological sources [8], it is very important to correctly describe the raw material that is to be used. When the main properties are expected to depend on the waste material origin, a first attempt is to find out if the raw materials containing chitin can be classified according to their origin. The classification may be performed using multivariate statistical tools such as Principal Component Analysis (PCA), Linear Discriminant Analysis (LDA), Cluster Analysis etc. [9]. In the present work, PCA and LDA were used for sample classification.

PCA is a multivariate statistical method used for reducing the dimensionality of a data set (consisting of a large number of interrelated variables) while retaining the variation in the data set [10]. To achieve this, an orthogonal transformation is used to convert a set of correlated variables into a set of orthogonal and uncorrelated axes named principal components which are ordered so that the first few retain most of the variation present in all of the original variables [10, 11]. PCA provides a reduction of dimensionality, and the orthogonality of the transformation ensures that the principal components can be treated as independent variables in further analysis [10]. The PCA-LDA procedure is used in classification of samples characterized by spectral analysis where the number of initial variables is very high [12, 13].

Partial Least Squares (PLS) is a method known for correlation of a dependent variable Y with a set of independent variable X if the number of independent variables is greater than the number of data samples, and a variable reduction is necessary. PLS technique is applied in the present work to estimate the deacetylation degree of chitin/chitosan samples. Dimzon and Knepper [14] also used this technique for various pre-treatments of infrared spectral data and identified some important bands that are correlated with the deacetylation degree of chitosan.

The aim of the study is to analyse the infrared spectra of chitin and chitosan samples obtained from different marine sources and to set a classification based on the geographical and biological origin using PCA-LDA methods. By potentiometric titration analysis of samples, the deacetylation degree was experimentally measured. Using these data, the infrared spectra was correlated with the deacetylation degree by PLS technique.

2. Materials and methods

2.1. Raw material

The raw material consisted of one shrimp species, *Palaemon elegans* (Rathke, 1837), and four crab species, as follows: *Carcinus mediterraneus* (Czerniavsky, 1884); *Macropipus holsatus* (Fabricius, 1798); *Pachygrapsus marmoratus* (Fabricius, 1787); *Xantho poressa* (Olivi, 1792), which were identified according to Băcescu [15]. All samples were collected as waste from the Romanian Black Sea Coast (Constanța county) and from different restaurants that provide seafood for consuming on the littoral zone of Constanța county. As geographical areas, the waste of *Palaemon elegans* and *Carcinus mediterraneus* species was collected from Midia Port, the waste of *Macropipus holsatus* was collected from Mamaia, the waste of *Pachygrapsus marmoratus* and *Xantho poressa* species was collected from Tuzla. Some of the infrared spectra of chitin/chitosan samples obtained from marine waste were previously used in our studies [16-19], but only for chitosan characterization.

2.2. Extraction and characterization of chitin/chitosan samples

The chemical extraction methods used to obtain all the 71 chitin/chitosan samples from five different marine sources are presented in table 1. For comparison, two samples of chitosan from Sigma-Aldrich were used. All samples were prepared under continuous stirring during the chemical treatments. After each treatment (demineralization, deproteinization, deacetylation), the samples were washed with bi-distilled water until neutral *p*H and dried in oven at 60 °C for 18-20 h. Moreover, after deproteinization step, the samples were additionally washed with acetone and/or ethanol and were rinsed three times with bi-distilled water in order to remove the naturally contained colorants in the exoskeleton of crustacea such as astaxanthin and β-carotene [3].

Table 1

The extraction procedures that were used to obtain the chitin/chitosan samples from five different crustacean wastes [16-19]

Crustacean species	Demineralization	Deproteinization	Deacetylation
	Extraction method (solvent concentration; ratio solid-to-solvent, m/v; contact time, min; temperature, °C)		
Carcinus mediterraneus (4 samples)	4 % HCl; 1:15 (m/v); 1 h; room temperature;	3 % NaOH; 1:20 (m/v); 1.5-2 h; 65 °C;	35-45 % NaOH; 1:20 (m/v); 1 h at room temperature and 1 h at 95 °C;
Palaemon elegans (43 samples)	2-4 % HCl; 1:7-1:13 (m/v); 30-50 min; room temperature;	3-5 % NaOH; 1:10-1:20 (m/v); 2 h; 65 °C;	35-40 % NaOH; 1:15 (m/v); 1 h at room temperature and 1 h at 95 °C;
Macropipus holsatus (15 samples)	4 % HCl; 1:15 (m/v); 1 h; room temperature;	3 % NaOH; 1:20 (m/v); 1.5-2 h; 65 °C;	40-50 % NaOH; 1:10-1:20 (m/v); 1 h at room temperature and 60-100 min at 95-100 °C;
Pachygrapsus marmoratus (4 samples)	4 % HCl; 1:12-1:15 (m/v); 40 min-1 h; room temperature;	3-5 % NaOH; 1:20 (m/v); 1.5-2 h; 65 °C;	35-45 % NaOH; 1:20 (m/v); 1 h at room temperature and 1 h at 95 °C;
Xantho poressa (5 samples)	4 % HCl; 1:8-1:15 (m/v); 40 min-1 h; room temperature;	3-5 % NaOH; 1:15-1:20 (m/v); 1.5-2 h; 65 °C;	35-45 % NaOH; 1:10-1:20 (m/v); 1 h at room temperature and 1 h at 95 °C.

2.3. Infrared characterization

Infrared spectra were recorded in transmittance mode using an instrument from Interspectrum, Interspec 200-X model (Estonia), connected to Essential FTIR ® Spectroscopy Software Toolbox, v.3.50.157. Each sample was dried prior to analysis, and was mixed with previously dried potassium bromide (KBr). The obtained material was pressed in a disc form under a nine tons load using a manual hydraulic press from Specac, GS15011 model. The infrared spectra were registered in the wavenumber range of 4000 to 400 cm^{-1}, at 4 cm^{-1} resolution.

2.4. Statistical analysis and modelling

The spectral data was processed using multivariate statistical analysis consisting of Principal Component Analysis (PCA), Linear Discriminant Analysis (LDA) and Partial Least Squares (PLS) methods. The data was analysed in the frame of MATLAB$^{©}$, v15.0 (The Mathworks, Inc., Natick, Massachussetts, U.S.). The Savitzky-Golay smoothing of spectral data was performed in the frame of GNU Octave, version 5.2.0, John W. Eaton, David Bateman, Søren Hauberg, Rik Wehbring (2019) software. The filtered data were used as input for multivariate analysis.

PCA represents an unsupervised method used for data reduction and possible visualization of grouping tendencies [9]. PCA performs an orthogonal linear transformation to a new coordinate system such that the original signals are decomposed in uncorrelated components which are ordered according to decreasing variance [20]. The PCA transformation matrix $W = [w_1,...,w_n]$ is obtained by performing a general eigenvalue decomposition of the covariance matrix $R = XX^T$, where $w_1,...,w_n$ are n normalized orthogonal eigenvectors of XX^T corresponding to n different eigenvalues λ_1, λ_2,..., λ_n in descending order. The PCA transformation of X is given by equation (1), as shown in Ref. [20]:

$$Y = W^T \cdot X \tag{1}$$

where: Y = transformed signal; W = transformation matrix; X = input signal.

The principal components are calculated so that the first principal component (PC1) accounts for most of the variation in the data set, the second (PC2) accounts for the next largest variation and so on. When significant correlation occurs, the number of useful PCs is much less than the number of original variables [9].

Linear Discriminant Analysis (LDA) is used to separate the classes using a line, a plane or a hyperplane that minimize the interclass variances encoded in the within-class scatter matrix, S_W, while maximizes the distance between the means of the classes encoded in the between-class scatter matrix, S_B [21]. LDA calculates the optimal new direction set by seeking a good projection vector, w^*, that maximizes the criterion function $J(w)$ [21]:

$$J(w)=\frac{w^T\cdot S_{\mathrm{B}}\cdot w}{w^T\cdot S_{\mathrm{W}}\cdot w} \tag{2}$$

Solving the generalized eigenvalue problem, it results [21, 22]: $S_{\mathrm{W}}^{-1}\cdot S_{\mathrm{B}}\cdot w=\lambda\cdot w$, where λ=J(w)=scalar.

PCA-LDA techniques were used to classify the chitin/chitosan samples according to their geographical and biological origin.

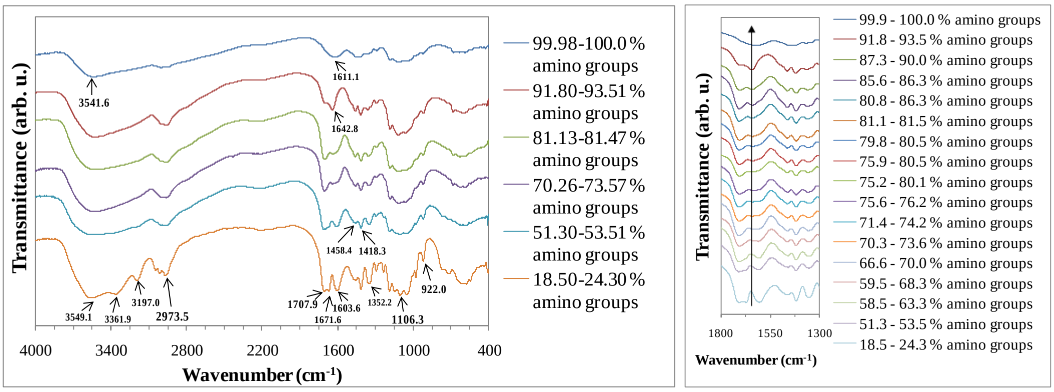
The primary spectral data were smoothened with a Savitzky-Golay filter using a fourth-order polynomial and a frame length of 11 consecutive data points. Therefore, the filtered data was used as input for PCA. The chitin/chitosan samples were assembled in a matrix, *X*, with *m* lines represented by the number of samples (73 samples), and *n* columns represented by the number of variables (3868 wavenumbers). LDA was performed using the first 11 PCs obtained in the PCA (PCA-LDA method).

PLS regression was used to obtain a correlation between the infrared information and the deacetylation degree measured by analytical methods. PLS is a combination of PCA and regression in the sense that a number of principal components are defined and a regression polynomial is obtained using these PCs. Unlike classical PCA, the principal components determined in PLS reflect, in decreasing order, the highest variability in the space of variables *X* and *Y* simultaneously. The infrared samples were divided into training and test sets to validate the obtained regression model.

3. Results and discussion

3.1. Infrared spectral characterization

The infrared spectra allowed the identification of a main peak at 1700 cm^{-1} that corresponds to stretching vibration of C=O bond from the $NHCOCH_3$ group [23, 24]. Also, another important peak was identified around 1650 cm^{-1}, which is usually assigned to bending vibration of N-H bond from the $NHCOCH_3$ group [23, 24]. Using the shrimp waste from *Palaemon elegans* species, samples with different deacetylation degrees (percent of amino groups) were obtained using various methods of extraction [17, 19]. Two, respectively three successive deacetylation treatments were conducted in order to obtain chitosan samples with deacetylation degrees over 90 %. The infrared spectra obtained in KBr disc form are shown in figure 1, along with the deacetylation degree intervals obtained by potentiometric method for each sample. Figure 1b represents a detailed spectral region for the most relevant wavenumbers from 1800 cm^{-1} to 1300 cm^{-1} where the main bands assigned to the structure of chitin/chitosan are found, as also considered by Dimzon and Knepper [14].



(a) (b)

Fig. 1. Infrared spectra of chitin/chitosan samples with different deacetylation degrees: (a) in the 4000-400 cm^{-1} range, respectively (b) in the 1800-1300 cm^{-1} range

In table 2 the main molecular vibrations of the infrared spectra for different samples of extracted chitin and chitosan are presented. Usually, chitin is known as chitosan lower deacetylation degree values, while chitosan is known as chitin with lower acetylation degree values [7].

Table 2

Infrared spectra main assignments for samples of chitin and chitosan

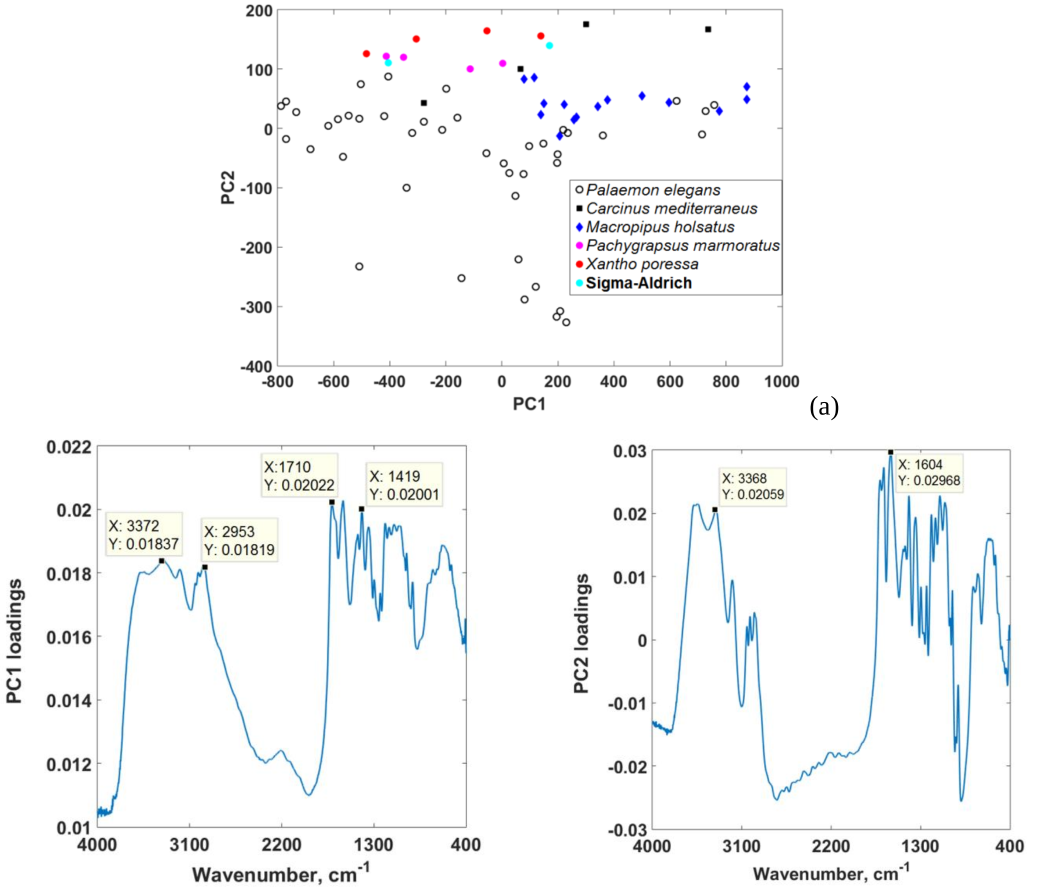
Deacetylation degree (%) / Assignments	18.5 - 24.3	51.3 - 53.5	70.3 - 73.6	81.1 - 81.5	91.8 - 93.5	99.9 - 100.0
	Wavenumber (cm^{-1})					
Stretching vibrations of O-H and N-H groups [25-27]	3582.6 3549.1 3361.9 3197.0	3545.3 3379.6 3206.3	3536.1	3532.3	3533.2	3541.6
Symmetric stretching vibration of CH_3 group and asymmetric stretching vibration of CH_2 group [28] from CH_2OH group; stretching vibrations of CH in pyranose ring [24]	3048.0 3016.4 2973.5	3044.3 3008.0 2962.4	3004.3 2963.3	3001.5 2963.3	3000.5 2963.3	3004.2 2972.6
Stretching vibration of C=O bond in *N*-acetyl group [23, 24]; splitting of Amide I band for chitosan from α-chitin [24]	1707.9 1671.6	1707.9 1681.9	1706.1 1641.8	1705.1	1702.4	-
Bending vibration of NH_2 group and stretching vibration of C-N group in $NHCOCH_3$ group [24]	1603.6	1608.3	1611.1	1640.0	1642.8	1611.1
Bending vibration of CH_2 bond in CH_2OH group [24]; symmetrical deformation of -CH_3 and -CH_2 groups [25]	1458.4	1458.4	1462.1	1462.1	1463.0	1456.5

Deacetylation degree (%) / Assignments	18.5 - 24.3	51.3 - 53.5	70.3 - 73.6	81.1 - 81.5	91.8 - 93.5	99.9 - 100.0
	Wavenumber (cm^{-1})					
In-plane scissoring of CH_3 bond in $NHCOCH_3$ group [24]	1418.3	1418.3	1417.4	1418.3	1420.2	1424.8
Symmetric stretching vibration of $–CH_3$ group and wagging of $–CH_2$ group [24]; deformation vibrations of OH and CH groups [29]; NH deformation vibration and CN stretching vibration [30]	1352.2	1355.0	1358.7	1359.6	1362.4	1365.2
Asymmetric bridge oxygen stretching vibrations; stretching vibrations of CO bonds in primary and secondary OH groups; symmetric and asymmetric stretching vibrations of C–O–C linkages [14, 24, 28]	1296.3 1239.5 1191.1 1149.2 1106.3 1057.0	1298.2 1237.7 1189.3 1106.3 1057.0	1297.3 1186.4 1120.3 1063.5	1295.4 1186.4 1120.3 1063.5	1186.4 1123.1 1063.5	1186.4 1126.8
Ring stretching vibrations [28]; specific vibrations to the structure of saccharides [29]	980.6 922.0	981.6 922.9	922.9	922.9	922.0	923.8

3.2. Chemometric classification

The reduction of dimensionality was applied using PCA to a matrix consisting of 73 samples which were characterized by 3868 wavenumbers (from 4000 cm^{-1} to 400 cm^{-1}). The PCA revealed that over 98 % of the data variability is due to the first three components (89.6 % PC1, 6.7 % PC2 and 2.3 % PC3), while for the first 11 PCs the variability accounts for almost 99.99 % of the data. The chitin/chitosan samples were projected in the PC1-PC2 space, and figure 2a shows that PCA already reveals the tendency of sample grouping according to their biological origin; two distinct groups were noticed: the shrimp samples (*Palaemon elegans*) and the crab samples. Although all species are crustaceans, the projection of chitin/chitosan samples from shrimp waste was mostly found in the lower part of PC1-PC2 representation, while the projection chitin/chitosan samples from crab waste in the upper part.

Figures 2b and 2c reflect the loadings of initial variables (the wavenumbers) in the first two PCs. As it can be noticed, the wavenumber of 1604 cm^{-1} has the highest loadings in PC1 and PC2, showing that the vibration of NH bond from $NHCOCH_3$ group might be the most important in chitosan samples discriminations. Moreover, the stretching vibration of C=O group that was found at 1710 cm^{-1} and is mainly due to the presence of *N*-acetyl group from chitin samples, is also significant. The molecular vibration at 2953 cm^{-1} that could be associated to C–H stretching vibrations from CH and CH_2OH groups [14, 24] has also relatively high loading in PC1. Another important band identified from the infrared spectra around 3370 cm^{-1} is assigned to stretching vibrations of O-H and N-H groups [25] from the structure of chitosan.



(a)

(b) (c)

Fig. 2. The results obtained using: (a) Principal Component Analysis (PCA) in the PC1-PC2 space, respectively the wavenumber loadings in PC1 (b) and in PC2 (c)

For a better classification, LDA was applied using the first 11 components from PCA. The data representation in the first two directions of LDA (figure 3) shows that the infrared spectra could discriminate between the samples based on their biological and geographical origin.

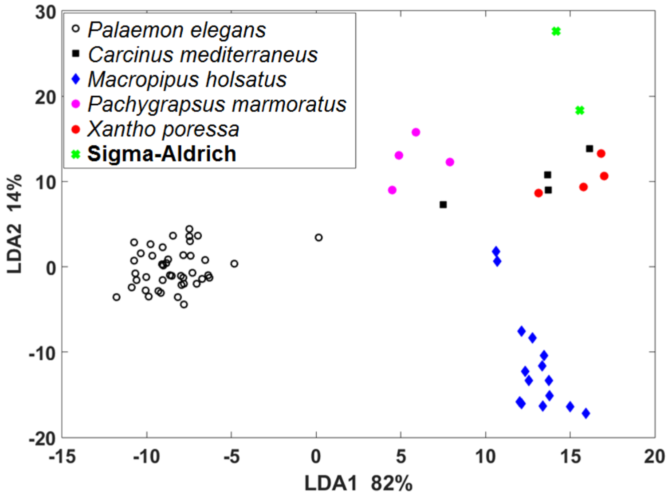


Fig. 3. Linear discrimination analysis (LDA) using the first 11 principal components (PCA-LDA technique) for discrimination of the samples

The results show a clear discrimination of the biological origin of the species: the *Palaemon elegans* samples – the shrimp species – were clearly separated from the crab species samples. This separation is made on the LDA1 direction meaning that biological provenience is the most important in the classification attempt. All four crab species were also separated in distinct classes on LDA2 direction. Moreover, the reference chitosan (Sigma-Aldrich) is derived from crab source, which was found in agreement with the obtained results.

As geographical origin, the *Macropipus holsatus* samples (collected from Mamaia beach – north from Constanța and south from Midia Port), and the *Pachygrapsus marmoratus* and *Xantho poressa* samples (collected from Tuzla – south from Constanța) have been clearly discriminated from each other. As the Romanian Black Sea coast has some differences in terms of geological base (Tuzla has a more rocky like coast than the northern regions) and water salinity (the Black Sea has brackish water, with average salinity of 17-18 ‰, which can suddenly decrease due to sea storms, strong winds and precipitations) noticed for the regions situated at north and south of Constanța [15], the separation of these three crab samples revealed by the infrared spectra may also be influenced by some differences in the environmental characteristics.

The sample differentiation reflected by the chemometric analysis of infrared spectra is also sustained by other physical-chemical measurements, as the ash content (table 3). The ash content was determined at 800 °C, according to F2103 standard [31], for chitosan samples considered as having low and medium deacetylation degree values [32], between 65 and 85 %. The ash content describes the total amount of inorganic material, and the remained samples contain a mixture of salts after combustion.

Table 3

Ash content of the analysed species

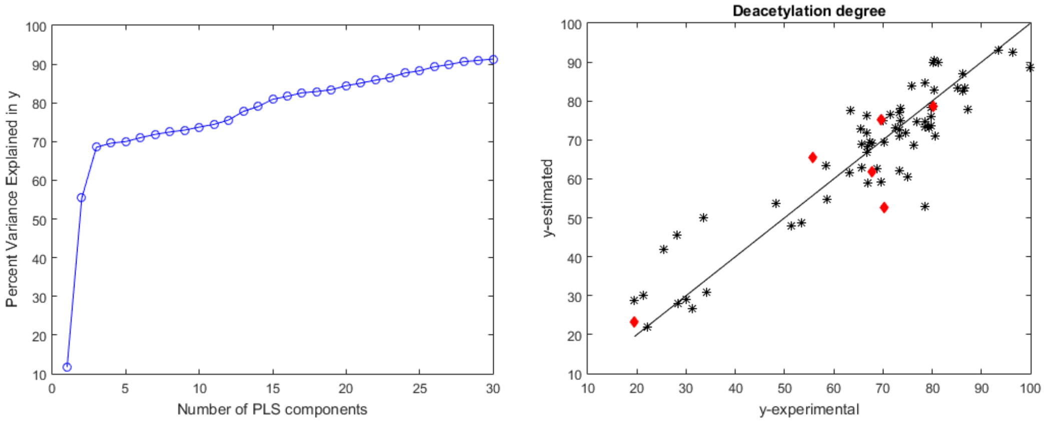
Species	Ash content of carapace (%)	Ash content of chitosan (%)
Palaemon elegans	19.47 ± 0.19	0.17 ± 0.04
Carcinus mediterraneus	37.08 ± 0.22 [16]	0.44 ± 0.31 [16]
Pachygrapsus marmoratus	44.38 ± 0.61 [16]	0.53 ± 0.07 [16]
Macropipus holsatus	52.23 ± 0.50	14.09 ± 1.45
Xantho poressa	47.60 ± 0.36 [16]	1.44 ± 0.37 [16]

As can be noticed from the results in table 3, both the exoskeleton and chitosan obtained from the waste of *Palaemon elegans* shrimp species has a very low ash content compared with all other samples, while the *Macropipus holsatus* crab species has a very high ash content compared with all the other analysed crab species which have in-between values of ash. The *Macropipus holsatus* species lives in waters with relatively high salinity conditions – from 15 ‰ up to 40 ‰ [15, 33], while *Palaemon elegans* species can survive in waters with a wider range of salinity – from 5 ‰ up to 45 ‰ [34]. This differentiation is in good agreement with the sample groups revealed by PCA and LDA of infrared spectra.

3.3. Property modelling

Partial least square regression (PLS) was performed for the smoothed infrared spectra in order to obtain correlation models between the infrared spectra and the deacetylation degree, determined by potentiometric method as presented in Ref. [18]. Unlike Ref. [14], who considered for property correlation only the spectral range between 1800 cm^{-1} and 1500 cm^{-1} (where the main peaks are found), in this study we have used the whole infrared spectra in order to include all possible other influences. In this way, the estimation of deacetylation degree, using the PLS model for new samples, may be used without prior spectral inspection.

All the 73 chitin/chitosan samples from shrimp and crab sources were used in PLS modelling, with 67 samples used for training the PLS model and 6 samples for testing. Unlike PCA, in PLS the principal components are calculated taking into account the common variability of the data and the values needed to be correlated (in this case the deacetylation degree). Depending on the number of principal components considered for calculation, a better correlation could be obtained. As shown in figure 4a, by increasing the number of principal components (PCs) from 1 to 3 an increse up to 70 % of the variability is observed; using 15 PCs the variability is about 80 %, while using 15 up to 30 PCs a slow increase of the variability is noticed (about 90 % of the variability).



(a) (b)

Fig. 4. Data variability depending on the number of principal components (a), respectively (b) correlation of the infrared spectra with the potentiometric results for the deacetylation degree property estimation (* training set; ♦ test samples)

Table 4 and figure 4b present the correlation results for all the 73 samples of chitin/chitosan. The determination coefficient, R^2, was considered a measure of the goodness of fit and also the mean relative error, which was calculated using the relation (3):

$$Err = \frac{1}{n}\sum_{i=1}^{n}\frac{abs\left(y_{\exp} - y_{\text{comp}}\right)}{y_{\exp}} \cdot 100 \qquad (3)$$

where: n is the number of samples in the training and test set, respectively; y_{exp} represents the deacetylation degree evaluated by potentiometric titration; y_{comp} is the value of the deacetylation degree estimated by PLS regression model.

Table 4

PLS modelling results for deacetylation degree estimation using infrared spectra

Number of principal components	Determination coefficient (R^2)	Mean error of training data used for modelling (%)	Mean error of the six tested samples (%)
15	0.810	12.0	9.0
20	0.844	10.7	13.3
30	0.910	7.0	17.0

The best correlation in terms of R^2 was obtained using 30 PCs. But, as can be seen from data in table 4, a very good correlation in the training step would not ensure a resonable estimation of the deacetylation degree for new test samples.

The lowest mean error for the tested samples was obtained for 15 PCs. A trade-off between training and testing steps must be considered. For the investigated samples, the PLS model based on 20 PCs (fig. 4b) may be recommended to predict the deacetylation degree for new samples characterized by infrared spectra.

Similar to the determination of deacetylation degree for chitin/chitosan samples by potentiometric titration, the preparation and measurement of samples in the infrared range could also be highly influenced by various external or internal factors, such as temperature, environmental humidity, equipment parameters or other conditions [35, 36].

6. Conclusions

Chitin and chitosan samples with various deacetylation degrees were obtained by chemical extraction procedure using different marine sources. The entire 4000-400 cm^{-1} spectral range was used to carry out classification based on geographical and biological origin by PCA-LDA method which clearly separated the samples according to their origin processing. Moreover, based on the infrared spectra the main physical-chemical property of chitin/chitosan samples – the deacetylation degree – could be predicted using the PLS regression model developed in this study as it proved to be able to describe the deacetylation degree and recommends the use of infrared spectra for rapid evaluation of this property.

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