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EFFECT OF INVASIVE BLUE CRAB CHITOSAN COATING ON EXTENDING THE SHELF LIFE AND QUALITY OF FRESH STRAWBERRY FRUITS

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ABSTRACT

The diversity of marine biomasses is a set of exploitable and renewable resources with application in several sectors. The blue crab was considered as a threat by the Tunisian fishermen in 2014, since it induces several damages on nets but also on captures. In addition, in 2017, the Tunisian State launched a plan to exploit and promote this species of crab. In the context of valuation, the chitin was isolated by a conventional chemical method from the shell of blue crabs. The efficiency of chitin extraction from *Callinectes sapidus (C. sapidus)* was achieved with a yield of 27.6 %.Then; the chitosan was prepared by N-deacetylation of chitin with a yield of 16 %, leading to a degree of acetylation (DA) of 89.9%. For *Portunus segnis (P. segnis)* the yield of chitin was 31.2% while the chitosan yield was 20% and the degree of acetylation was 79.9% After these analyzes, it can be deduced that chitosan has a high degree of purity and that it is considered effective for the preservation of strawberries in favor of its antioxidant and antimicrobial capacity.

Keywords: Invasive species, decapods, blue crab shell, chitosan, antimicrobial and antioxidant activity, Tunisian waters.

1. INTRODUCTION

Crustaceans constitute the second most represented taxon of non-native species in the Mediterranean Sea [1] Biological invasions have been considered worldwide as a major driver of change in Mediterranean marine biodiversity [2] and consumer behaviour. Since their first introduction in the Mediterranean Sea, the blue crabs *Portunus segnis* (an immigrant from the Red Sea) and *Callinectes sapidus* native from the Atlantic Ocean are nowadays very abundant. They are regularly caught and landed by small scale fisheries in Tunisia since 2015 for *Portunus segnis* and later for *Callinectes sapidus*. Tunisia is currently experiencing a concomitant biological explosion by these two species[3].

Firstly, blue crabs were perceived as a disaster due to their severe economic impacts on fisheries [4].Then, this biological explosion turned from a threat to an opportunity for the various stakeholders after the implementation of the National Plan for Promoting Fishing and Marketing Blue Crab which has strengthen scientific knowledge and developed blue crabs value chain. Actually, more than 28 processing units and export companies are implemented in Tunisia and more than 12 millions € exports were recorded in 2019[5]. Furthermore, the development ofinnovative processesnamely aquatic co-products transformation offers outstanding opportunities for exploitation and is nowadays a promoting activity since the ever-increasing demand for high-value natural products worldwide.Also, in the context of sustainable development, the environmentalconcern is actually encouraging industrials to promote thesebio-wastes and to convert theminto high-value bio-compounds like collagen and gelatin [6], bioactive peptides[7], enzymes and specific proteins [8] [9], chitin, chitosan and pigments [10] [11].

Biopolymers and especially polysaccharides are used in widespread sectors such as medical, pharmaceutical, agriculture and agro-food industries due to their biological and physicochemical properties such as biodegradability, biocompatibility, non-toxicity, renewability, and ready availability. Chitin and its major derivative product chitosan are the most important and abundant marine polymers in the world. Due to their

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antimicrobial and antioxidant activities, the applications of chitin and chitosan include food industry, wastewater treatment, agriculture, cosmetics, pharmaceutical, and medical applications, paper production, textiles, etc...

Strawberry is a highly perishable fruit with a short post-harvest life which is mainly due to fungal rot. The shelf life of refrigeratedfresh strawberries is approximately 5 days [12]. In order to extend fruit shelf life, the use of low temperatures combined with modified atmospheres have been investigated [13] [14] [15]. Maintaining quality strawberries can be made in several ways, including using edible packaging [16]. In this article, we will investigate another method of preserving strawberries by applying chitosan and determining its antimicrobial effect.

This work studied the extraction of chitin and chitosan from Tunisian blue crab shells and analyses the effect of chitosan coating on the shelf life and the quality of strawberries marketed in Tunisia.

2. MATERIALS AND METHODS

2.1 Sampling sites

Sampling of *Portunus segnis* and *Callinectes sapidus* was provided from Bizerte (Ghar El Melh, Manzel Abderrahmen)and Tabarka (Malloula)between February and June 2021 (figure 1).

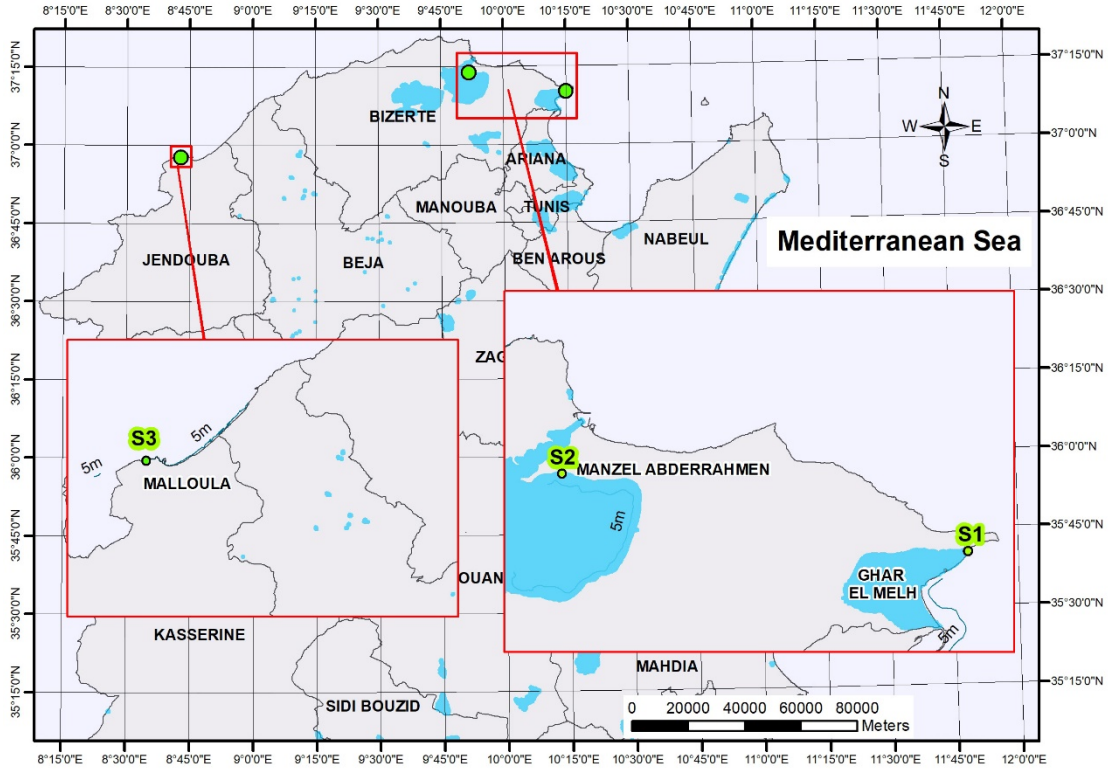

Figure 1. Sampling sites

2.2 Samples preparation:

Samples of blue crabs werewashedwith tap water. Then, shells were manually separated from viscera and muscles and thoroughly washed with cold distilled water. Shells were air dried to remove excess of water. After that, dried samples were ground to a fine powder in an electric mixer.

2.3 Chitin extraction

For chitin and chitosan extraction, the method of [17] was used. The raw powdered sample (25g) was heated to reflux in 250ml 2M HCl solution for 2 hours. Later it was filtered through a filter paper (1mm pore size) and washed several times with distilled water. The sample was dried at 60 C. for 24 h in an oven. The oven-dried sample was refluxed in 2M NaOH solution for 19h. After reflux, the sample was washed with distilled water and deionized until a neutral pH was reached. This treatment deproteinized the sample. The filtered sample of chitin was dried in an oven at 60 C for 12 h then incubated in a mixture of organic solvents (distilled water, methanol and chloroform; in the ratio (4:2: 1) for 1 h for the discoloration and bleaching. Finally, the resulting product was

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filtered, washed with distilled water and dried at 50°Cfor 48 h in an oven, then the percentage of chitin content by dry weight was determined.

2.4 Deacetylation

After the chitin was heated under reflux in 60 % NaOH solution at 120°C for 2 h, it was rinsed several times with distilled water until neutral pH was reached, to obtain pure chitosan. The yield of chitosan was determined from chitin with an analytical balance after drying in an oven at 50 C for 48 h [17].

2.5 Extraction yield of chitin and chitosan determination

After the extraction of the chitin and chitosan, the dry product weight (M1), makes it possible to deduce the mass yield(Ym) of chitin according to the following formula:

$$\mathbf{Ym\ (\%) = ((M1 - M0) \div M0) \times 100}$$

Ym: Chitin/chitosan mass yield; M1:dry weight; M0: wet weight

2.6 Deacetylation degree determination

Before the assay, the samples are prepared according to the following protocol:

- 100 mg ofchitosan (2.1) are placed in a cylindrical vessel to which 3 ml of 0.3 M HCl (2.3) and 40 ml of water (2.2) are added. Stir for 12 hours then:
- Insert the pH electrode of the pH meter as well as the temperature probe into the cylindrical vessel. Check that the pH of the chitosan solution is less than 3, otherwise add 0.3M HCl with the pipette (2.3).
- Neutralize the excess of HCl using 0.1M of NaOH (2.4) in order to obtain a pH of the order of 4.5 corresponding to pKa.
- 2 of the free amine fraction. Let V1 ml be the volume of NaOH poured in
- Continue the addition of NaOH (2.4) to obtain a pH of 8.5 corresponding to pKa +2 of the free amines fraction.Let V2 ml be the volume of NaOHpoured in.

The degree of acetylation ofchitosanis expressed inpercentage.This formula is the ratio between the mass of units of acetylated glucosamine (Ga1) in g in the sample over the mass (Ga2) in g if all the groups were acetylated with:

$$\mathbf{Q = (V\,NaOH \times 0.1) \div (1000\,X\,Mcs)}$$

$$Q = number\,of\,moles\,of\,the\,amino\,fraction\,of\,chitosan\,for\,a\,sample\,of\ 1g$$

$$Mcs: dry\,mass\,of\,chitosan\,in\,the\,test\,sample, in\,g$$

$$\mathbf{VNaOH = V2 - V1}$$
$$\mathbf{= volume\ poured\ in\ ml\ of\ 0.1M\ NaOH\ between\ pH\ 4.5\ and\ pH\ 8.5.}$$

$$\text{Degree of acetylation: } DA = (1 - 162\,XQ) \div (1 + 43\,XQ)$$

2.7 Characterization of materials

2.7.1 Residual fats determination

The determined lipid content is based on the method of According to the method of [18] modified by[19].The sample to be analyzed is boiled in a salt solution (distilled water + NaCl) for 5 minutes to denature the activity of the phospholipases. After draining, the sample is ground in a mortar by gradually adding 10 ml of methanol and 20 ml of chloroform. It is then filtered through filter paper and centrifuged. The lower chloroform phase containing the total lipids is then recovered. After evaporation to dryness under vacuum at 50 ° C. The mass of total lipids in each sample is then estimated. The lipid content is determined according to the following formula:

$$\mathbf{Fat\% = (m3 \div m4) \times 100}$$

With m3: mass of lipids (g)

m4: mass of blue crab shell (g)

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2.7.2 Residual protein contents

Proteins are quantified by Lowry's method. 1 g of the sample to be analyzed is ground in a mortar in the presence of 10 ml of a phosphate buffer (pH=7.2).The solution obtained is subjected to centrifugation for 10 min at 4000 revolutions/min, then the supernatant is recovered.The Lowry method is a colorimetric technique requiring the preparation of a standard range from a solution of serum albumin (BSA) with a concentration equal to 1 mg/ml.

2.7.3 Residual ash contents

The mineral matter content of the samples is determined by incineration of the organic matter according to the official AOAC method (1980) with some modifications established after harmonization between laboratories within the framework of the project. After determining the water content of the samples, crucibles for incineration is placed porcelain in a muffle furnace at a temperature of 550°C up to obtaining a constant mass for 6 hours. After calcination, the temperature is lowered to 200°C before removing the crucibles. The samples are then cooled to room temperature in a desiccator.

2.8 Chitosan coating solution preparation and its application in strawberries

For the preparation of the chitosan coating solution, 0.5 g of every crab chitosan was dissolved in 100 ml of ascorbic acid. Then, 48 pieces of the strawberry of uniform size, shape and color and without any signs of mechanical damage or fungal rot were selected to be coated respectively in both solutions (24 pieces in each chitosan coating solution). After that, all samples were air dried to remove water excess.

2.9 Microbiological analyses

Microorganisms have very different living conditions, so for the detection of each type, we favor its optimal growth conditions.

Table 1 culture media, incubation time and temperature for different microorganisms.

Microorganism	Culture centre	Incubation time	Incubation temperature
Total coliforms	VRB A	24 hours	37 ° C
Fecalcoliform	VRBA	24 hours	44 ° C
Total germs	Agar powder	24 hours	30 ° C
Staphylococcus aureus	Chapman	24 hours	37 ° C
Clostridium sp.	TSN	24 hours	46 ° C

2.10. Determination of the antioxidant power of chitosan in strawberries

The antioxidant activity was evaluated according to the method recommended by [20]. Methanol was added to strawberry juice and shaken in a volumetric flask over bath water for 120 min. Then the solution was concentrated to the volume of 10 ml by rotary evaporator. The methanolic extract (1 ml) with 1 ml of 0.2 mM DPPH was taken into a test tube and mixed on a shaker. The absorbance of the reaction mixture was noted at a wavelength of 571 nm using a spectrophotometer. Methanol was used as a control while DPPH with methanol was used as a control. Antioxidant activity was evaluated using the following formula.

$$A\% = \text{absorbance blanc} - \text{absorbance échantillon absorbance blance} \times 100$$

With A = radical retention activity

2.11. Sensory analysis

According to the standard (ISO 5492 2008), sensory analysis is an analytical methodology whose objective is the determination of the organoleptic properties of a product by the sense organs.

2.11.1 Hedonic test

The evaluation jury was made up of 30 people, chosen at random, from among the students of the ISSPAB. Each taster receives, under the same conditions, for each sample, cups in which the samples coded with random 3-digit numbers are placed. The order of the samples is set at random. The jury fills out an assessment sheet (appendix 3); at which level he mentions, on a given scale, the level of color, appearance, taste, and odor. After each tasting, a glass of water is served to reduce the intensity of adaptation phenomena.

2.11.2 Development of the sensory profile

Three coded samples of the strawberry are presented to the subjects. Each subject must observe, smell and taste the samples and classify them by placing the codes on the intensity scale of the different attributes (2 samples can have the same degree of intensity) i.e. the subject will give a score from 0 to 9 for each criterion.

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3. Statistical analyzes

All the results are expressed as the mean ± standard deviation of the biochemical composition. We performed an analysis of variance (ANOVA) test at 5% significance level using the STATISTICA 10 statistical software.

The results of the sensory analyzes were statistically analyzed based on Principal Component Analysis (PCA) which is a method used to analyze multivariate data using STATISTICA 10 software.

3. RESULT

3.1 The yield of chitin

The chitosan yields of the chitins were determined. Here in this study, the chitin content was found to be 27.6% of the dry weight of *C. sapidus* while the chitosan yield was reported to be 16%. For *P.segnis*, the chitin yield is 31.2% while the chitosan yield is 20%. The values obtained in this study were slightly higher than the values reported by [21] showing that the chitin content was 12.1% and that chitosan was generated at 9.2%.

To conclude, the yield of chitin from *P.segnis* is higher than that of *C.sapidus*, on the other hand the yield of chitosan from *P.segnis* is lower than that in the crabs of *C.sapidus*.

3.2 The degree of deacetylation

The degree of acetylation has been reported for chitosan is from *P. segnis* 89.9% and for *C. sapidus* is 79.4%. These results confirm that the degree of acetylation was found to be high in both species of crab, which explains a high degree of reactive chemical amino groups [22]. Also it can be deduced that the degree of acetylation of chitosan from *P.segnis* is high compared to chitosan from *C.sapidus*.

Both crabs exhibited good chitosan quality ranging from 75 to 98% [24]. The more deacetylated the chitosan, the stronger its antimicrobial activity [24].

3.3 The biochemical composition

The biochemical composition of chitosan is shown in Figure 2.

The average values of the lipid content of the chitosans extracted from *C. sapidus* and *P.segnis*are respectively (0.15% ± 0.02) and (0.12% ± 0.02) and it is noted that these values are not significantly different between the two species.

The mean values of the protein and ash content of chitosans extracted from *C.sapidus* and *P.segnis* are respectively at (0.3% ± 0.02) and (0.2% ± 0.02) and at (0 , 23% ± 0.02) and (0.12% ± 0.02) thus it can be deduced that there is a significant difference in the protein and ash composition in the two species.

From these values we can say that the chitosan extracted from *C.sapidus* is significantly ($p < 0.5$) purer than the chitosan extracted from *P.segnis* since it contains low biochemical composition and lower than the chitosan extracted from *P.segnis*.

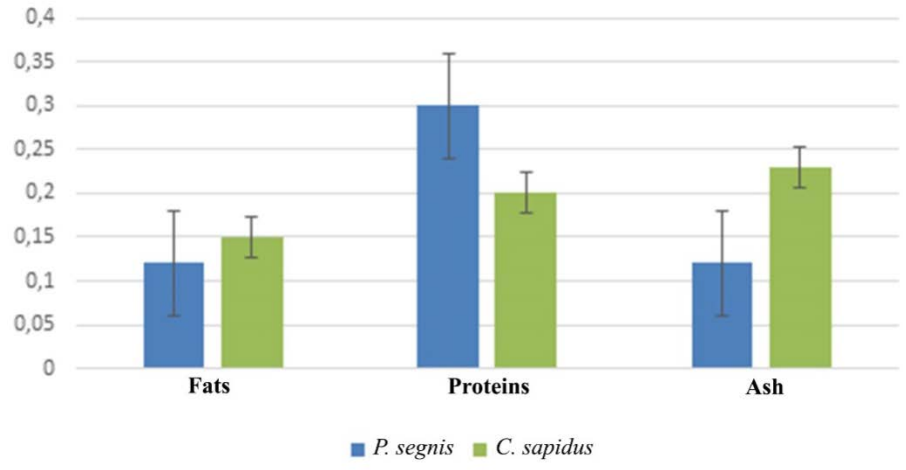

Figure 2: The biochemical composition of chitosan (%)

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3.4. The antioxidant power of chitosan in strawberries

Figure 3 shows the antioxidant activity of the two strawberry samples, one coated with chitosan and the other uncoated (control).

From day 1 to day 3, we observe that the antioxidant activity of the strawberry coated with chitosan is high (81.35) compared to the control (67.72).

On day 3, there was a decrease in the antioxidant activity of the two strawberry samples.

Uncoated fruits showed a significant loss ($p < 0.5$) of antioxidant activity (32.94%) on day 6 compared to coated fruits (48.42%). This decrease continues until the end of the experiment, and uncoated fruits have minimal antioxidant activity (17.25%) compared to coated fruits (26.69%). The coating with chitosan is effective thanks to its antioxidant activity in preserving the quality of strawberries and prolonging their shelf life. [23] showed that chitosan has the ability to prolong the life of fruitstorage due to its ability to modify the internal atmosphere of tissues and its antifungal properties.

To conclude, chitosan is an excellent antioxidant for strawberries. The chitosan coating application has been shown to be a good antioxidant for strawberries.

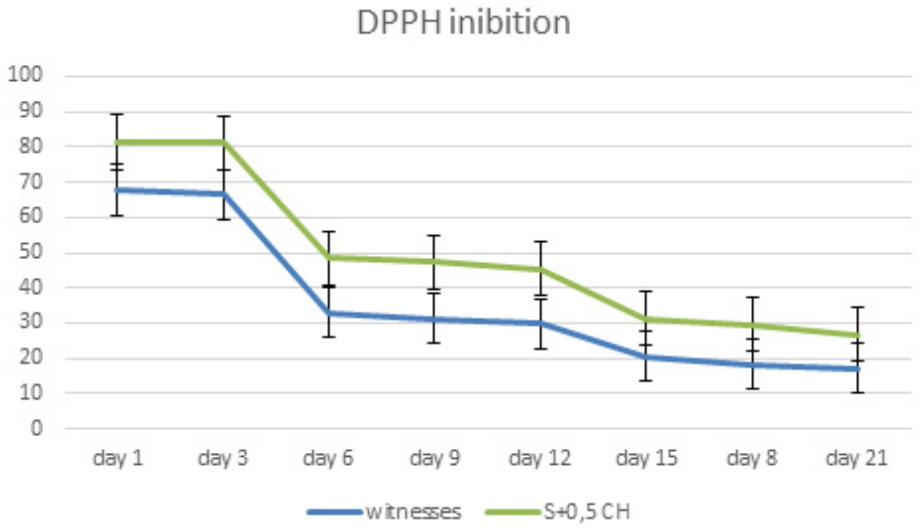

Figure 3: Antioxidant activity of chitosan on strawberries

3.5. Microbiological analysis:

The microbiological results of the strawberry coated with the chitosan solution and of the uncoated strawberry is shown in Table 2.

Table 2T0: the first week of storing the strawberry.T1: the second week of conservation.T2: the third week of conservation. Sample 1: strawberries coated with chitosan from *P.segnis.* Sample 2: strawberries coated with *C. segnis* chitosan. Sample 3: the uncoated strawberry (control).

	Sample 1			**Sample 2**			**Sample 3**			**Reference**
	T0	T1	T2	T0	T1	T2	T0	T 1	T2	
FTAM (UFC)	Abs	< 30	< 30	Abs	Abs	> 300	Abs	> 300	> 300	<10 5 [21]
CT	Abs	Abs	< 30	Abs	Abs	Abs	Abs	10 3	> 300	Absence [21]
CF	Abs	Abs	Abs	Abs	Abs	Abs	Abs	> 300	> 300	Absence [21]
Staphylococcus aureus	Abs	Abs	Abs	Abs	Abs	Abs	Abs	> 300	> 300	CODEX.A absence
Clostridium botulinum	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs	< 30	Absence of NF ISO 7937/05

3.6. Sensory analysis

3.6.1 Principal component analysis (PCA):

Principal Component Analysis (PCA) is a method used to analyze multivariate data. The application of this method to the analytical results found in this work gave us a global view on the recipe most appreciated by consumers, namely for product A and product B. The results of this PCA are illustrated in table (3) which

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presents the contribution of the main factorial axes and figures (4) which respectively show the correlation circle and the projection of individuals on the factorial plane (1: 2).

Table 3: Percentage of the contribution to the explained variance of the main factor axes

	Eigen-value	% Total - variance	Cumulative –Eigen_value	Cumulative - %
1	2,496018	49,92037	2,496018	49,9204
2	1,395142	27,90284	3,891161	77,8232

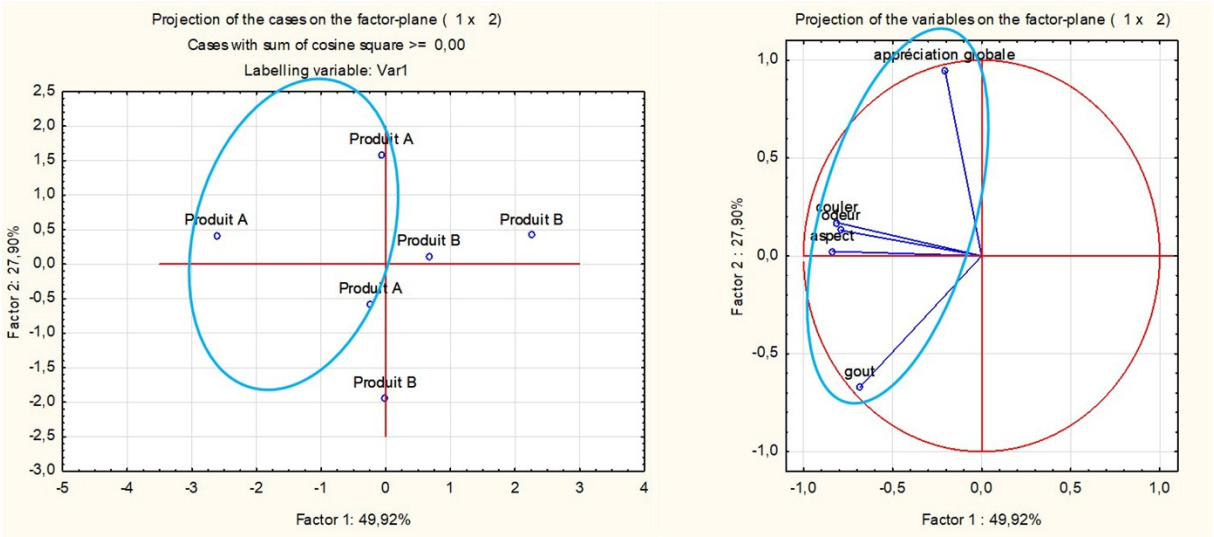

Figure 4: Principal component analysis (Plan1: 2) of products (A and B).

4. DISCUSSION

4.1 The yield of chitin

The studies by [24] on chitosan extracted from shrimp showed different values of the yield such as that the yield of chitin is equal to 13.12% and that of chitosan is 15.4%. From these studies, it can be concluded that the yields of chitin and chitosan extracted from the two species of blue crab are higher and more efficient than in the yields of chitin and chitosan extracted from shrimp and other crabs namely *Libinia dubia [26]*.

4.2 The degree of deacetylation

According to the studies[24] on chitosan extracted from shrimp has a degree of acetylation equal to 79.57%. It is thus observed that the degree of acetylation of chitosan extracted from blue crab (*Portunus segnis*) is higher than the degree of acetylation of chitosan extracted from shrimp, in this context it can be deduced that blue crab chitosan is of good quality and more effective than chitosan extracted from shrimp

4.3 The biochemical composition

The lipid, protein and ash contents are very low which explains the purity of the chitosan which shows that we have chosen the best method of extracting the two crab samples. Studies from [27] have shown different values of proteins, lipids and ash such as 5.4 ± 0.2% protein and 3.7 ± 0.1% ash and the absence of lipid in chitosan extracted from the insect's cuticle.

4.5. Microbiological analysis:

Among the microbiological tests carried out, it can be seen that FTAM represents a low microbial load in sample 1 and 2, on the other hand in sample 3 it represents a high microbial load, this testifies to a slight presence of flora in both first samples, however the latter does not exceed the standards. In addition, there is a total absence of total and fecal coliforms in the strawberrysamples coated with the chitosan solution according to the standards. The same is true for *staphylococci* and sulfite reducing Clostridiums, which are also below the limit. On the other hand, we notice their presence in the third control sample.

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It can be deduced that there is not a significant difference in the microbial load of sample 1 and sample and this explains that there is not a difference between the inhibition of the microbial load of chitosan from *P.segnis* and chitosan from *C.sapidus.*These results are explained by the presence of chitosan in the strawberry samples and its role in inhibiting the bacterial load.

4.6. Sensory analysis

4.6.1 Principal component analysis (PCA):

The results of this analysis showed that the first two axes (1: 2) alone account for 77.82% of the total variance (Table 3). The first axis which comprises alone contributes 49.92% of the observed variability and correlated negatively with the color, taste, odor and appearance tests which have the highest inertias (Figure 4). Nevertheless, the overall assessment is positively correlated with the second axis which explains 27.90% of the observed variability. The projection of the products on the same factorial plane (1: 2) shows a clear separation illustrated by a strong correlation of product A with the determined tests.

5. CONCLUSIONS

Chitin and chitosan have been obtained from blue crab and which has different fields of use depending on the degree of deacetylation. Nanofiber and porous polymers obtained from blue crab could be used effectively in biosensors, filtration (membrane), controlled drug delivery, dental applications and textile areas.Because the blue crab is widely distributed all over the world, and easily cultivated; it is proposed that the shell should be evaluated as an alternative source of chitin as it is a waste after consumption of blue crab as a food source. Samples of chitosan studied can be considered a good source of antimicrobial against the pathogens from which the chitosan is a good food preservative. The use of chitosan nanoparticles as an edible coating can serve as an active packaging and capable of maintaining the quality of food products.

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