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(RESEARCH ARTICLE)

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Bio-Circular green economy application for the recovery of high value added compounds, such as astaxanthin and glucosamine, from cephalothorax of Karamote shrimp *Peneaus kerathurus* in Western Greece

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Abstract

Shrimp waste can be used as source of high value added compounds, such as astaxanthin and glucosamine. Astaxanthin (C40H52O4) is a xanthophyll carotenoid and belongs to a larger class of terpenes. Astaxanthin exists in stereoisomers, geometric isomers, free and esterified forms based on the reaction with fatty acid. It is a fat soluble colorful pigment and can be found in aquatic environment in microalgae, salmon, trout, krill, shrimp, crayfish and crustaceans. It has both lipophilic and hydrophilic properties, protect cells, lipids and membrane lipoproteins against oxidative damage.

D-glucosamine (C6H13NO5), an amino sugar (hexosamine), is a part of the structure of two polysaccharides, chitosan and chitin. Naturally shows up in human body and crustacean shells. Glucosamine exists in the form of glucosamine sulphate, glucosamine hydrochloride, or N-acetyl-glucosamine and is extensively used as a dietary supplement in the treatment for osteoarthritis, knee and back pain.

This research aims to optimize by chemical methods the extraction of glucosamine and astaxanthin from cephalothorax of Karamote shrimp *Peneaus* (Melicertus) *kerathurus*. Astaxanthin yield obtained using hexane as extraction solvent. Furthermore, results showed that G-HCl yield obtained with solid/liquid ratio of 1:20, at high hydrolysis reaction temperature and with agitation. Additionally, the low cooling temperature of 5 °C and the use of ethanol support the formation of G-HCl crystals.

Keywords: Astaxanthin; Glucosamine; Shrimp waste; *Peneaus kerathurus*; Chitin

1. Introduction

In recent years great interest has been expressed in isolating components using by-products and wastes. Seafood industries produce significant amounts of discards, consisting mainly of shell, head, bones and amounts of wastewater as process effluents. These discards are associated with the environmental impact on aquatic ecosystems since the release of organic wastes might significantly change the community structure and biodiversity of the benthic assemblages [1-4].

The objective of minimizing fishery discards and to avoid environmental problems can be achieved by establishing alternative solutions to enhance and transform fish wastes as an economic resource (biocircular green economy model). For example, recovering important biomolecules such as proteins (collagen, gelatine), polysaccharides (chitin,

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chitosan), lipids (omega 3) or pigments (astaxanthin or beta-carotene). These biomolecules can further be applied to human and animal consumption in food industry, cosmetics or pharmaceuticals [5-8].

Crustaceans, belonging to the Decapoda order, include prawns, shrimps, lobsters, crayfish and crabs, are regarded as an enriched source of many bioactive substances. *Melicerthus kerathurus* known as karamote prawn is one of the above group. The caramote prawn *Melicertus kerathurus* (FORSK L, 1775) is a native species in the Mediterranean Sea and the East Atlantic, from Portugal to Angola, and lives in sandy-mud bottoms up to depths of about 80 m [9-10].

Shrimp processing produce considerable quantities of solid waste in the form of head and body carapace. Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione), the main pigment found in crustacean which provides the desirable orange color shows higher antioxidant activity when compared to various carotenoids such as lutein, lycopene, and β-carotene. This carotenoid has antioxidant power 10 times greater than *β*-carotene and 500 times higher than vitamin E. Furthermore, presents antitumor properties and protection against free radicals, lipid peroxidation and oxidation of essential polyunsaturated fatty acids [11-16].

D-glucosamine (2-amino-2-deoxy-β-D-glucopyranose), an amino sugar (hexosamine), is part of the structure of chitosan and chitin which compose the exoskeletons of crustaceans. Due to its high aqueous solubility and physical stability is used as a potential solid-dispersion carrier to improve the biopharmaceutical properties of drugs. Glucosamine has anti cancer, anti-inflammatory and antibacterial potential applications in the field of food and medicine [17-24].

This study aims to improve by chemical methods the extraction of glucosamine and astaxanthin from cephalothorax of Karamote shrimp. However, to the best of our knowledge, no studies are available in Greece on extraction of these biomolecules from *Peneaus kerathurus.*

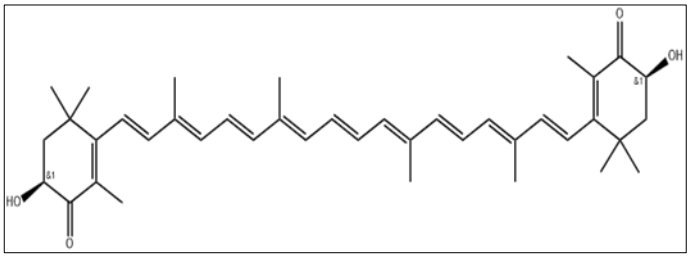

Figure 1 Astaxanthin

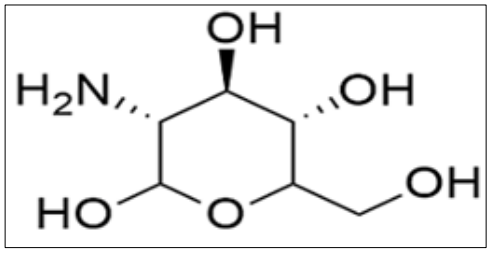

Figure 2 Glucosamine

2. Material and methods

2.1. Description of the fishing area

The experimental fishery of the shrimps was carried out three times from September 2021 to January 2022 in Kastos channel, along the Western Greek coast of the Ionian Sea, near the Kalamos Island (Latitude: 38°33'59.99"N, Longitude: 20°54'59.99"E).

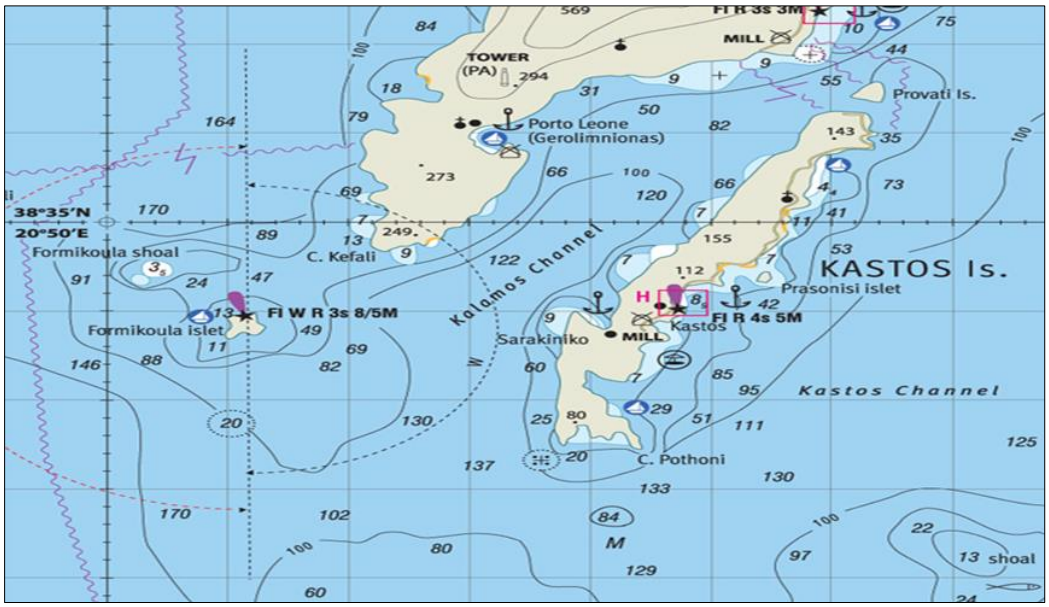

Figure 3 Map of the study area in Kastos channel along the Western Greek coast

2.2. Raw material

A total number of 84 specimens were collected. Samples were taken using circular cast nets with weights distributed around the perimeter. The shells were removed from the animal and secondly the specimens were packed in polyethylene bags, placed on ice, transported to the laboratory and were stored in a freezer at -20 °C until further use.

2.2.1. Reagents

All the chemicals and solvents (NaOH, HCl, acetone, hexane, ethanol, petroleum ether, NaCl, acetic acid) used were purchased from Sigma- Aldrich at the analytical grade or highest level of purity available and used as received. For the preparation of solutions, double distilled water was used.

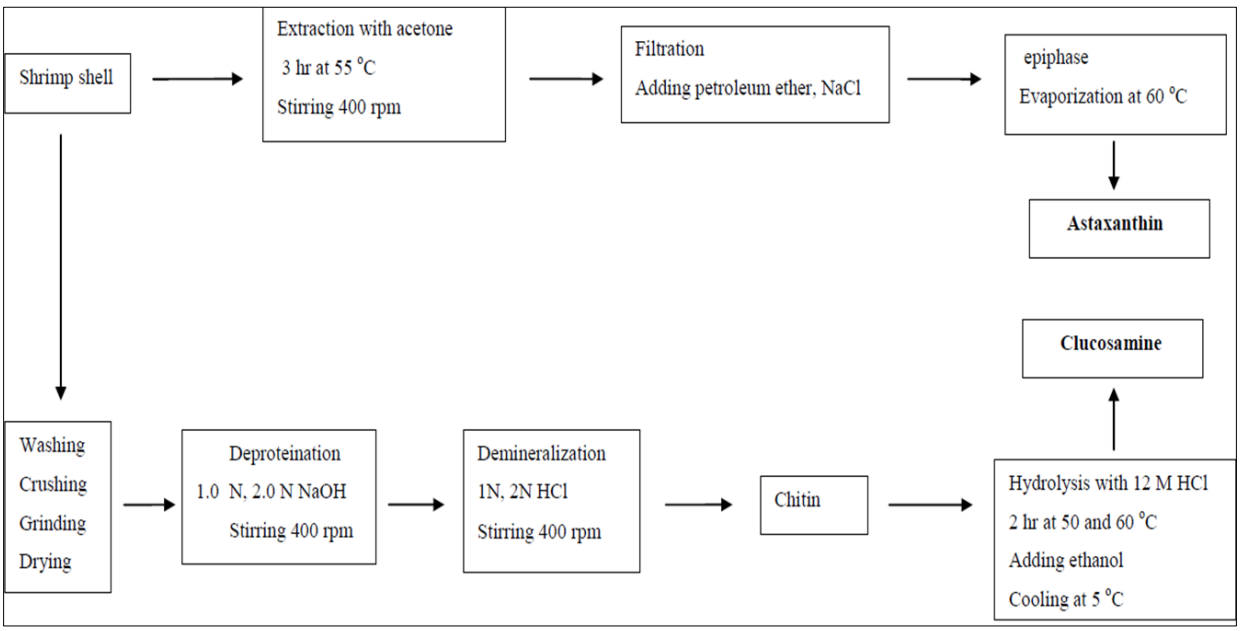

Figure 4 Flow diagram of extraction of chitin, glucosamine and astaxanthin from shells of shrimp *Peneaus kerathurus*

2.3. Methods

Before grinding, the biggest parts of shell samples were crushed and divided in smaller. Drying of samples was obtained by heating in a drying oven (model R. Espinar, S.L.) at 98 °C until constant weight was obtained between two sequential measurements (4-5 h). Drying samples grinded in a mill (System POLYMIX® PX-MFC 90 D) into smaller particles using sieve with 1 mm wide openings [25].

2.3.1. Extraction of chitin by chemical method

Deproteination (Dp)

A total of 6-10 g dry samples of raw shrimp shell waste were treated with 1.0 N and 2.0 N NaOH at solid to solvent ratio 1:5, 1:10, 1:13 and 1:15 (w/v), with constant stirring at 400 rpm for 24 hours at different temperatures, with pH ranged from 11-13. After that, the solution was filtered and the samples were washed with distilled water to neutrality in running tap water. Water from the samples was removed before performing the demineralization process.

Demineralization (Dm)

Samples from deproteination process were treated with 1.3 N and 2.0 N HCl at solid to solvent ratio 1:15, 1:20, 1:40 (w/v), with constant stirring at 400 rpm for 24 hours with pH value ranged pH 1.0-2.5 at room temperature. After that, the solution was filtered and the samples were washed with distilled water to remove acid and calcium chloride. The samples were dried for 3 hours using an oven at 90 °C until constant weight was obtained. The dried sample is now known as chitin.

2.3.2. Chitin yield

The percentage of the yield of chitin was calculated by dividing the weight of extracted chitin to initial dry shrimp shell weight.

Yield was calculated as follows:

$$\text{Yield of chitin (\%)} = (\text{extracted chitin, g})/\text{shrimp shells, g}) * 100$$

2.4. Extraction of glucosamine

Chitin was grinded to fine particles (2 mm), hydrolyzed with 12 M HCl at 50 and 60 °C, then was filtrated by gravity to remove the solids and finally to recover glucosamine addition of ethanol (95 %) at 5 °C was performed. The mixture was cooled for 3 weeks to crystallize and finally the solid crystals were washed with ethanol and dried in an oven at 50 °C for 8 hr.

2.5. Glucosamine yield

The percentage of the recovery of glucosamine was calculated by dividing the weight of extracted glucosamine to initial dry chitin weight. Recovery of glucosamine was calculated as follows: Recovery of glucosamine (%) = (extracted glucosamine, g)/dry chitin, g)*100 [21-23]

2.6. Extraction of astaxanthin

2 g of 5 mm size dry shrimp shell were extracted with 25 ml of solvent (acetone) for three hours at 50-62 °C with constant stirring at 400 rpm. The extract was filtered using whatman filter paper. The pooled extract was collected in a separated conical flask and petroleum ether, NaCl were added. After thorough mixing, the epiphase was collected. The pooled epiphase was kept in water bath at 60 °C for the evaporation of petroleum ether.

2.7. Quantification of Astaxanthin

The extracted astaxanthin again is dissolved in 3 ml of hexane and read at 470 nm in a 1.000-cm cell.

$$AST = (A \times D \times 10^6) / (100 \times G \times d \times E_1)$$ [26- 27]

Where;
AST : concentration in µg/g,
A : absorbance
D : volume of extract in hexane

10^6 : dilution multiple
G : weight of sample in g
d : the cuvette width (1 cm)
E_1 : extinction coefficient: 2100

3. Results

Parameters and details of experiments for the extraction of chitin are demonstrated in Table 1. Some differences can be observed attributed to the ratio of solid to solvent and to the changes of molarity of solutions for deproteination and demineralization. As a result, the percentage of recovery differs in all experiments. The higher values of yield after deproteination were observed in exp IV (68.71%) and II (65.92%) while the lowest value was observed in exp I (55.33%). Also, the percentage of recovery after demineralization (as a ratio to previous step of deproteination), varied from 30.02% to 33.3%. The higher values of yield after demineralization were observed in exp IV (33.3%) and III (32.1%) while the lowest value was observed in exp II (30.02%).

The percentage of yield of chitin varied from 17.38% to 22.9%, the higher values were observed in exp IV (22.9%) and II (19.7%) while the lower value in exp I (17.38%).

Table 1 Experimental details and recovery of chitin

	Experiment			
Parameters	**I**	**II**	**III**	**IV**
Sample (g)	8.00	10.01	6.01	7.00
Molarity of solution for deproteination dp	2 N	1.0 N	1.0 N	1.0 N
Solid to solvent ratio	1:13	1:15	1:10	1:5
Time / temperature	2h / 72 °C	2h / 78 °C	2h / 88 °C	2h / 62 °C
Yield % after deproteination	56.75	65.92	55.33	68.71
Molarity of solution for demineralization dm	2.0 N	2.0 N	1.3 N	1.3 N
Solid to solvent ratio	1:15	1:20	1:40	1:20
Time / temperature	2h / 88 °C	2h / 52 °C	2h / 58 °C	2h / 82 °C
Yield % after demineralization	30.61	30.02	32.1	33.3
Stirring period for dp, dm Rpm	24 h 400	24 h 400	24 h 400	24 h 400
Chitin (g)	1.39	1.97	1.06	1.60
Chitin yield (%)	17.38	19.7	17.7	22.9

Experimental details and recovery of astaxanthin are demonstrated in Table 2. The higher value of recovery was observed in exp II (1.57 μg/g) while the lowest value was observed in exp I (0.21 μg/g).

Table 2 Experimental details and recovery of astaxanthin

	Experiment		
Parameters	**I**	**II**	**III**
Sample weight(g)	2	2	2
sample size (mm)	5	5	5
Solvent (acetone) (ml)	25	25	25
Petroleum ether (ml)	12.5	25	12.5

Evaporation temperature for petroleum ether	60 °C	60 °C	60 °C
NaCl concentration (% w/v)	0.73%	0.73%	0.73%
quantity (ml)	9.4	18.8	9.4
Stirring time (hr)/rpm	2 /400	8/400	8/400
Hexane (ml)	3	3	3
Astaxanthin recovery (µg/g)	0.21	1.57	0.78

Experimental details and recovery % of glucosamine was presented in Table 3. The percentage of yield of glucosamine after acid hydrolysis varied from 45% to 55%. The higher value of recovery was observed in exp III while the lowest value was observed in exp II.

Table 3 Experimental details and recovery % of glucosamine

Exp	**Chitin (g)**	**HCl (ml)**	**Ratio**	**Molarity HCl (M)**	**rpm**	**Time (h) / Temperature (°C)**	**Addition water / ethanol (ml)**	**Glucosamine recovery (%)**
1	1	15	1/15	12 M	400	2/55	15 / 20	48
2	1	10	1:10	12M	400	1.30/50	15 / 30	45
3	1	15	1/15	12 M	400	3/62	15 / 20	55

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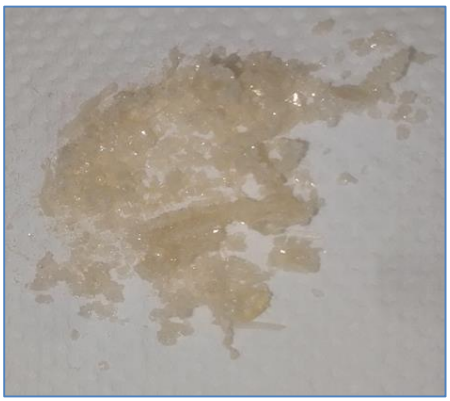

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Figure 5 1) shrimp waste; 2) recovery of glucosamine; 3) recovery of chitin

4. Discussion

Aquaculture has grown to a great extent in the last decades. World-wide aquaculture represents a very important sector capable of supplying huge amounts of animal protein. However, enormous number of discards are generated (bones, shell, head) from seafood industries and are associated with significant environmental impact on aquatic ecosystems [1, 4]

Application of circular bio-economy strategies with the further utilization of wastes would revalorize aquaculture wastes and by-products reducing the environmental impact and offering additional economic benefits. Astaxanthin and glucosamine are two biomolecules which could be isolated from wastes and further be applied in food industry (food additive) as well in cosmetics and pharmaceuticals [5, 7, 28, 29, 30].

In this study our data reveal a variety of the percentage yield of chitin, astaxanthin and glucosamine. In the experiment of chitin, results of 1.0 N solution of HCl for demineralization at a solid to solvent ratio of 1:20 for 2h at 82 °C, 1 N for deproteination at a solid to solvent ratio of 1:5 for 2h at 62 °C with agitation, clearly demonstrate a significant yield of chitin. These values were also correlated with other studies and note the influence of temperature, solid/liquid ratio (g/ml), molarity of solutions (NaOH, KOH, HCl) and agitation on chitin production and acid hydrolysis of chitin [31-39].

The production of glucosamine is also greatly influenced by the experimental conditions [18-24]. In order to increase the crystallization rate, ethanol was added because the recrystallization process was slow at room temperature. The low temperature (5 °C) and the use of this solvent strengthened the formation of glucosamine crystals. The increase in temperature (62 °C instead of 50 °C) combined with the use of agitation dissolved the chitin faster, within half an hour, giving a yellow – brown color in the solution and the yield of hydrolysis in this experiment was more effective. The best yield of glucosamine (55%) was obtained at a solid to liquid ratio of 1/15, using for acid hydrolysis a concentrated solution of 12M HCl for 3 hour at 62 °C with agitation (400 rpm). The lowest value (45 %) of glucosamine was observed in exp. II, when the chitin was hydrolyzed at 50 °C for 1.30 hour due probably to ratio of solid to solvent and water to ethanol.

The astaxanthin contained in the extracts from shrimp species obtained via chemical method was determined by UV–VIS spectrometry at a wavelength of 470 nm. Astaxanthin had relatively high solubility in acetone and acetic acid but was almost insoluble in water. The best yield of astaxanthin was obtained using double amounts of petroleum ether and sodium chloride with agitation for a longer period of time.

5. Conclusion

No studies are available in Greece on extraction of chitin, glucosamine and astaxanthin from Karamote shrimp *Peneaus kerathurus* in Greece. Concerning the parameters of the experiments and the percentage yield, it is believed that the extraction process can be improved applying circular bio-economy strategies. The further utilization of aquaculture waste may reduce the environmental impact and offer additional economic benefits gaining higher yields of these value added compounds.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors have no conflicts of interest to declare

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