Bioactive compounds derived from marine alien species in the Mediterranean for cosmeceutical applications.

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Keywords: Eastern Mediterranean; *Lagocephalus sceleratus*; *Pterois Miles*; *Fistularia Commersonii*; Bioactive compounds; Cosmeceutical applications
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Abstract

In the present study, the exploitation of marine alien species *Lagocephalus sceleratus, Pterois miles* and *Fistularia commersonii* was examined through the recovery and valorization of added value bioactive compounds with potential application on cosmetic products, with the aim of contributing to the control of their population and consolidation of Mediterranean basin. In particular, polyunsaturated fatty acids, collagen and naturally occurring fish toxins (i.e. tetradotoxin) were extracted from the flesh, skin, bones and internal organs of the three studied species. The efficient recovery of the pre-mentioned marine derived components was achieved through the optimization of proper protocols and application of state of the art extraction techniques. Moreover, analytical techniques were used in order to fully characterize the produced extracts. Finally, in order to cover the unpleasant odor of fish origin bioactive compounds and to protect them from adverse environmental conditions, their encapsulation in polymeric matrices is necessary. For the encapsulation the innovative electrohydrodynamic process was used and specifically electrospraying.

Introduction

The cosmetics and personal care industry represents a multi-million dollar sector (expected to garner about $430 billion by 2022) [1] and its solid growth is supported by consumers who increasingly expect more and more innovative products with bioactive compounds of high quality and efficacy. Sustainability is one of the macrotrends that is shaping today's industries, and the cosmetics sector is no exception. "Sustainable" cosmetics are defined as cosmetic products using natural ingredients ideally produced from renewable raw materials [2] [3].

The consumer awareness towards innovative, sustainable, and efficacious products to produce new cosmetic formulations has brought natural marine resources into cosmetic industry as a new, valuable, trendy component [4]. Marine-based cosmetic formulations vary in their composition and properties, species of animal, age, and catching origin [5]. So, a good characterization practice and assessment of quality is important to choose the right component for each formulation.

Towards sustainability, the valorization of underexploit marine sources such as invasive species is crucial. The invasion of these species induces negative effects both in socioeconomic aspects and in the marine ecosystem making their exploitation vital as a measure towards the management of their population. The present study aims to find a way to valorize in cosmetic industry the three of the most important invasive alien species of the Mediterranean basin, the *Lagocephalus sceleratus* (Gmelin, 1789), the *Pterois miles* (Bennett, 1828) and the *Fistularia commersonii* (Rüppell, 1838). In this study, it is proposed to carry out an evaluation on highly commercial substances with cosmeceutical interest of particular species, such as

collagen and omega-3 fatty acids, as well as the development of methods of their processing and utilization in cosmetology.

F. commersonii is a source of polyunsaturated fatty acids (PUFAs) which appear to have significant health benefits, especially, DHA and EPA. Particularly, omega-3 fatty acids have beneficial properties for skin, protecting it from photo-aging process, skin carcinogenesis, dermatitis and also reveal a positive effect on the healing of skin wounds [6]. *P. miles* is a rich in marine collagen source which has the ability to slow down the formation of free radicals by preventing skin aging, as well as fortifying skin repair and regeneration [7] [8]. Furthermore, there are several examples of potent natural toxins being used as drugs in human medicine; for example, botulinum toxin, from the bacterium *Clostridium botulinum*, employed in situations where excessive muscle contraction is observed, or even with cosmetic purposes. Naturally occurring fish toxins can be studied as sustainable and more economic alternative. Specifically, tetradotoxin (TTX) found in *L. sceleratus* acts by inhibiting the mechanism of sodium ion transport in the sodium-potassium pump in nerve and muscle cells. So far, this toxin has been used as a drug in patients with migraine headaches, heroin addiction and cancer. In the field of cosmetics, one possible use of TTX could be the development of injectable products such as Botox, to treat skin relaxation [9].

In the cosmetic industry, three are the key factors to a successful formula: stability, safety and efficacy and they are all linked to how its active ingredients are preserved. Moreover, in order to cover the unpleasant odor of fish origin fatty acids and collagen and to protect them from adverse environmental conditions, their encapsulation in polymeric matrices is necessary. For the encapsulation electrospraying technique was applied. This technique based on the application of a high-voltage electric field, which creates a jet of charged polymer, forming the Taylor cone on the jet, thereby drying the solvent and producing fiber. Encapsulation is an efficient process for the entrapment of an active ingredient (core material) within another substance, usually a polymeric matrix (wall material). This way, the active agent is completely covered and isolated from its external environment so its biological activity is preserved, allowing its release at a controlled rate and under the conditions desired [11] [12]. As this polymeric barrier starts to decompose, the active agent, little by little, penetrates the skin to deeper layers [13]. This way the formula works for a longer period and without a lot of activity released at once.

Moreover, the particle size of the encapsulated compound is very important as it determines the skin layer-target that active agents can reach. Micro-and Nanoencapsulation produce very small vehicles (1-1000 μm and 10-1000 nm respectively) with a large surface area that is available for sites of adsorption and desorption, chemical reaction etc. In particular, nanoencapsulation, due to the smaller size of encapsulates, is more effective than microencapsulation, improving controlled release and providing more precise targeting of the bioactive compound [14]. This way, active agents can be delivered deeper into the skin more effectively, with no pain or danger for the living tissues [15].

In the present study, cyclodextrin was selected as wall material for the formulation of final products. The name cyclodextrin refers to cyclic, water-soluble oligosaccharides with α-D-glucopyranose building blocks arranged in a circular ring. The most common molecules are α-, β- and γ-cyclodextrins formed from six, seven and eight glycopyranose units respectively.

Cyclodextrin is a substance often used as a matrix material for the inclusion of bioactive substances in the cosmetics industry and in the food industry. Some of the main reasons for the widespread use of cyclodextrin by industries are:

• semi-natural product produced by simple enzymatic treatment from a renewable abundant source, starch,

• non-toxic, not absorbed from the gastrointestinal tract and fully metabolized by the colon microflora, and

• its price is low enough (less than $ 10 / kg) so that it can be used for most industrial products.

The cyclodextrins have a truncated cone shape where the hydrogen atoms of the C-H groups are located outside the cavity of the molecule and the polar hydroxyl groups of the sugars are also located outside the

cone giving a hydrophilic character to the outer surfaces of the cyclodextrin facades. Due to these polar groups, cyclodextrins are soluble in water. In contrast, the inside of the pit is hydrophobic as its polarity is lower than that of water. This dual nature results in cyclodextrins forming soluble, reversible inclusion products with low water solubility compounds [16].

Materials and Methodology

Extraction of Tetradotoxin (TTX)

Samples of *L.sceleratus* were dissected and the visceral organs were removed and separated from the muscle and skin. The intestines and muscle were separately crushed and 1% acetic acid in methanol was added in order to be homogenized using a BagMixer. The homogenized sample was then put in an ultrasonic bath (Dentsply Neytech Ultrasonic 28H) for 10 mins and freezed at -18°C for additional 10mins. The solution was centrifuged at room temperature at 4000 rpm for 20 min in a ROTOFIX 32A-Hettich and the supernatant was freezed until further analysis.

In order to quantify TTX in LC-MS / MS, the sample was purified by solid phase extraction (SPE). The adsorbent in the SPE microcillars (10mg) consisted of a polymer strong cation exchange material. For the elution of TTX and its analogues from the microcillars aqueous solution of 0.1N HCl was used. The samples were then diluted with water in a ratio of 1:20 and analyzed using liquid chromatography-twin mass spectrometry (LC-MS / MS, Agilent 1260 Infinity binary HPLC in combination with Agilent 6460C Triple Quadrupolr Mass Spectrometer).

The identification and quantification of TTX compounds was performed with external standard method using the Certified Reference Material (CRM-03-TTXs) of the company CIFGA.

Extraction of Lipid Content

According to Bligh and Dyer method, fish samples of muscle and skin from *F.commersonii* were homogenized using Cloroform/Methanol (1:2 v/v) using a bagmixer and magnetic stirrer (SELECTA MULTIMATIC -5N). The chloroform phase of the filtrate was separated using a separation funnel and evaporated using a BUCHI Vacuum V-800. The sample was dried at 42°C for 2hrs and weighed. Total lipid content (TL) was gravimetrically determined according to the equation:

$$\%TL = \frac{W(g)oil}{W(g)biomass} * 100$$

Following the determination of TL, preparation of fatty acid methyl esters (FAMEs) was followed by the redissolution of lipids into chloroform in order to be analyzed. FAMEs were identified using a GC-MS (GAS) system (CHROMATOGRAPH SHIMADZU GC-17A) with Varian 450 analytical instrument equipped with a column DB5. The carrier gas was helium and its flow rate was equal to 1mL / min. The injection temperature was adjusted to 270 °C, the carrier gas flow was 1 mL / min and its column temperature is increased from 125 °C to 300 °C within 35 min at a rate of 5 °C / min. The PUFAs were identified by comparison with external standards and quantified using calibration curves.

Extraction of Collagen

Collagen content from *P.miles* was determined using the pepsin soluble collagen (PSC) method. For the extraction, samples were treated with NaOH 0.1M in a magnetic stirrer for 5hrs with the replacement of NaOH every 2hrs, in order to remove non-collagen proteins. After the removal of NaOH and washing of the biomass, 0.5 M acetic acid at a ratio of 1:5 w/v and 1.5% (w/v) pepsin were added and the sample was continuously stirred for 20Hrs. The extracts were then centrifuged and the supernatants were separated. The samples were spectrophotometrically (UV-VIS SPECTROPHOTOMETER, UV-M51, BEL PHOTONICS) determined according to collagen calibration curve at 650 nm.

Encapsulation of bioactive compounds

A cyclodextrin solution was used in order to dilute collagen and fatty acids using continuous stirring before the sample was put at the electrospraying nozzle. The encapsulation experiments were carried out in a Fluidnatek LE-10 (Bioinicia, Spain) electrospraying apparatus at room temperature. The parameters of distance (cm) between the target and the capillary tip, the feed rate (μL/h) of the syringe pump delivering the solution and the voltage (kV) in order to form a stable Taylor cone, were studied and are shown in Tables 1-3.

Results

The extraction procedure of TTX in *L. Sceleratus* was investigated for both flesh tissue and internal organs of the fish, using a 1% solution of acetic acid in methanol. The internal organs were found to contain an increased concentration of TTX compared to the flesh, as shown by the comparison of the samples in Figure 1. According to LC/MS analysis, the estimated TTX of *L. sceleratus* visceral organs and flesh was 14.10 μg and 1.77 μg per gr of tissue respectively.

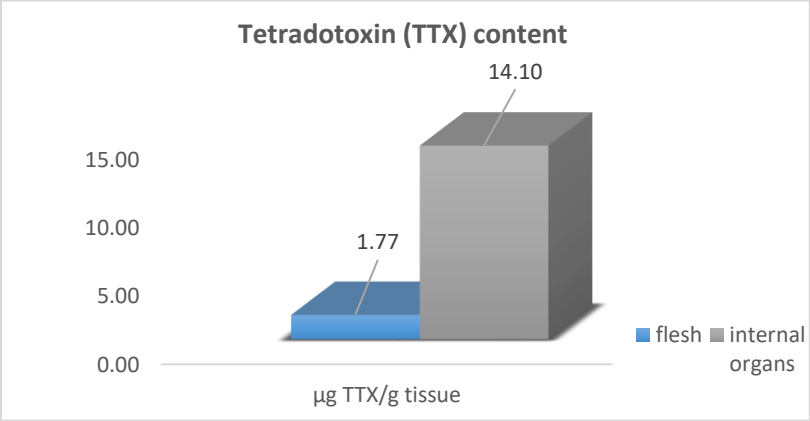

Figure 1 Comparison of TTX concentration of flesh and visceral organs of L.sceleratus

In order to determine the amount of collagen contained in both bones and flesh of *P. miles*, the calibration curve is first constructed using a standard collagen solution in various dilutions. Based on the equation of the collagen calibration curve, the initial amount of collagen in each sample is calculated. Figure 2 illustrates that lionfish has a higher amount of collagen in the bones than in the skin, 8.9mg and 6.9mg per gr of tissue respectively. On the other hand, in order to investigate the concentration of collagen as far as the comparison to other species is concerned, the common salmon *Salmo salar* was chosen to determine collagen content using the same protocol described in this study. Results (Fig.2) showed that bone tissue of the two species was in close proximity, 44.6 mg/gr for lionfish and 41,5 mg/gr for salmon. On the contrary, skin tissue of lionfish was found to be significantly higher at 34.8 mg/gr, than that of salmon which was only 2.6 mg/gr.

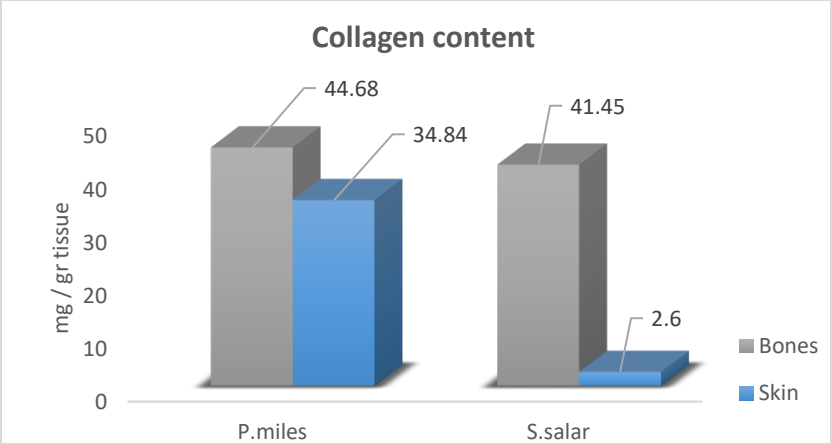

Figure 2 Comparison of collagen concentration of bones and skin in lionfish P.miles versus salmon S.salar

In order to determine the amount of total lipids present in *F. commersonii* flesh and skin samples, the Bligh and Dyer method was followed using Chloroform/ methanol 1:2 v/v. The results of Figure 3A indicate that a higher content of total lipids was observed for skin samples. The total amount of fatty acid methyl esters (FAMEs) in each sample is shown in Figure 3B, according to which, a higher amount is observed in the skin of *F. commersonii.*

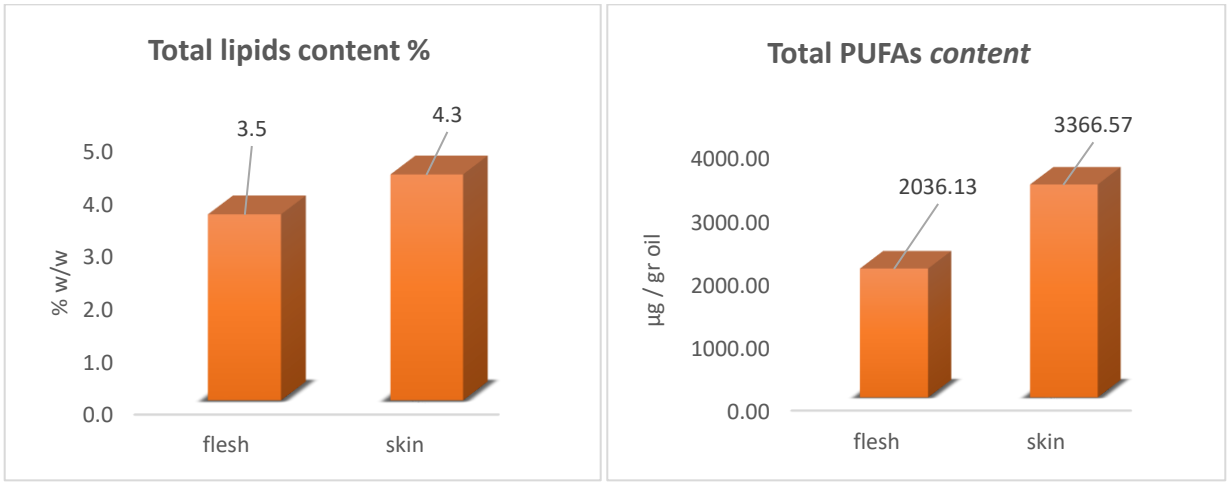

Figure 3 A) Total lipids content (%) of F. commersonii of flesh and skin samples B) Total concentration of FAMEs (µg/gr fishoil) in F.commersonii samples of flesh and skin

PUFAs are identified by comparison with external standards and quantified using a callibration curves. Results of FAMEs analysis of flesh and skin of *F. commersonii* are shown in Table 1 and Figures 4 and 5, which present the GC chromatograms and FAMEs quantification respectively. The FAMEs identified were Myristic acid C14.0, Palmitic acid C16.0, Stearic acid C18.0, Oleic acid C18.1 and Eicosidexaenoic acid (DHA) C22.6. FAMEs concentration of both flesh and skin seem to follow the same trend (Fig.5) : Palmitic acid > Stearic acid > DHA > Oleic acid > Myristic acid.

Table 1 Quantification of identified FAMEs in F.commersonii using GC-MS (GAS) chromatography

C:D	Common name	Elution time (min)	% w/w in *F. commersonii* Flesh	% w/w in *F. commersonii* Skin
C14:0	Myristic acid	11,86	3,32	3,85
C16:0	Palmitic acid	15,79	33,70	35,21
C18:0	Stearic acid	19,52	25,79	26,31
C18:1	Oleic acid	19,12	15,91	16,23
C22:6	Eicosidexaenoic acid (DHA)	25,71	21,28	18,41
Total			100	100

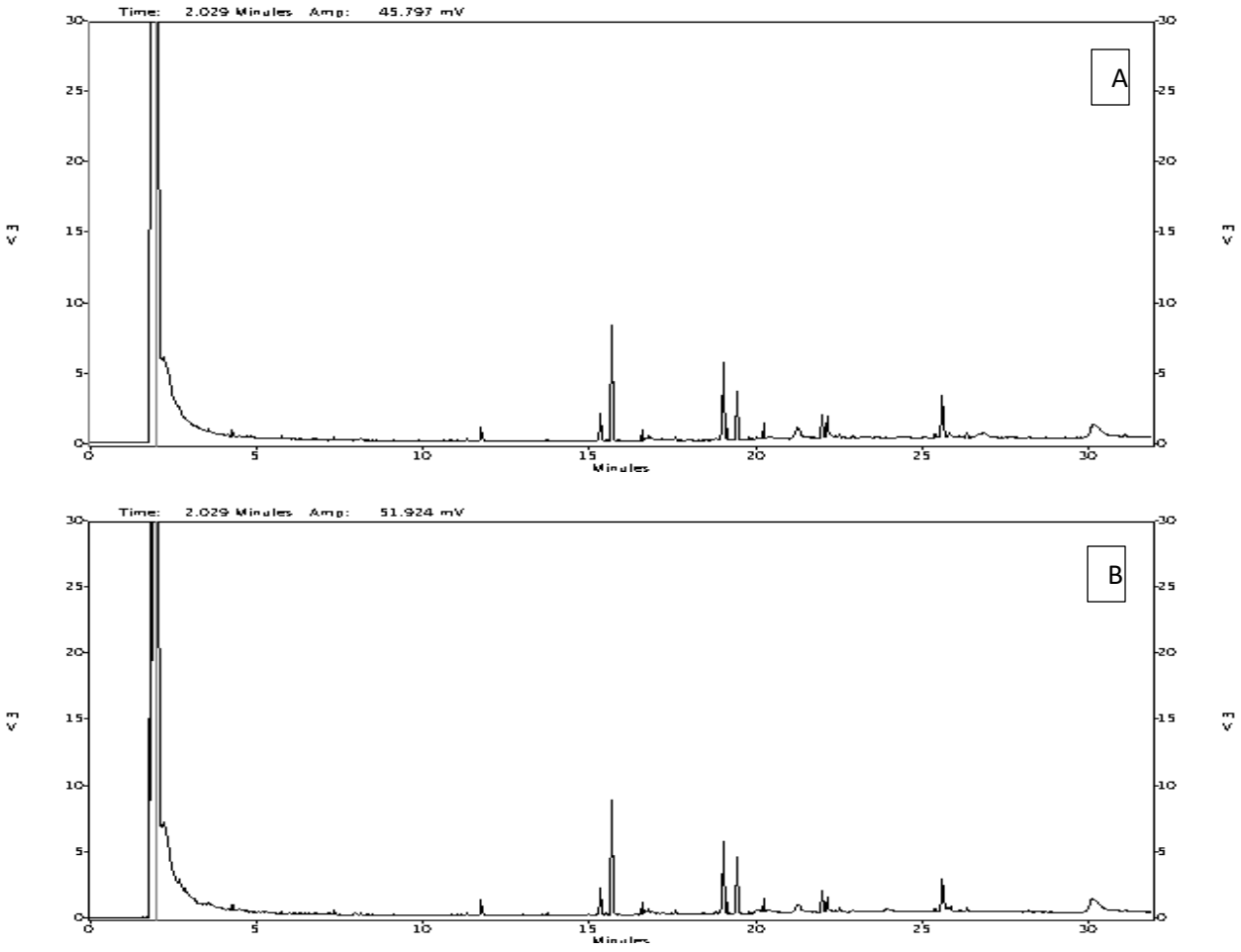

Figure 4 GC-MS (GAS) chromatography of F.commersonii FAMEs from fish flesh (A) and skin (B)

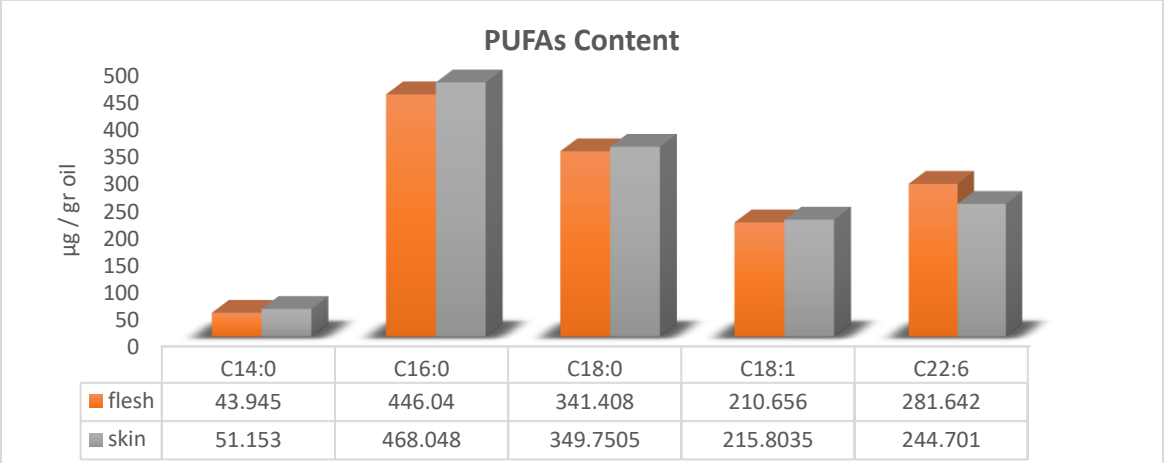

Figure 5 FAMEs quantification of flesh and skin in F. commersonii identified as Myristic acid C14.0, Palmitic acid C16.0, Stearic acid C18.0, Oleic acid C18.1 and Eicosidexaenoic acid (DHA) C22.6

Table 2 Dissolution of 1g omega-3 fatty acids and 1g collagen in a final volume of cyclodextrin solution 10mL. Parameters of Feed rate (µL/h), Distance (cm) and Voltage (kV) used for the process optimization. Results of encapsulation criteria: Normal flow without droplets, Stable Taylor cone formation and Uniformity of collected nanocapsules marked as √ when successful.

Feed rate (µL/h)	Distance (cm)	Voltage (kV)	Optimum encapsulation Criteria		
			Normal flow without droplets	Stable Taylor cone formation	Uniformity of collected nanocapsules
600	15	22.0-23.5	×	×	×
600	15	24.5-28.0	√	×	×
700	15	24.5-28.5	√	√	×
800	15	24.5-27.5	√	√	×
900	12	24.0-28.5	√	√	
900	15	24.5-28.0	√	√	×
1000	12	25.0-28.5	×	×	×
1000	15	24.5-28.0	√	×	×
1100	12	25.0-28.1	√	×	×
1100	15	24.5-27.5	√	×	×
1200	12	28.1	×	×	×
1200	13	27.0-28.3	√	×	×
1200	15	24.5-27.5	√	×	×
1300	13	27.0-28.5	√	×	×
1300	15	24.5-28.0	√	×	×
1400	13	27.0-28.0	√	×	×
1400	15	24.5-28.0	√	×	×
1500	13	27.0-28.0	√	×	×
1500	15	24.0-28.5	√	×	×

Following the extraction of bioactive ingredients from the three invasive species, the next step was the application of these substances in cosmetology products. For this purpose, the optimal conditions for inclusion of omega-3 fatty acids and collagen in a cyclodextrin matrix through electrohydrodynamic process were investigated. The electrohydrodynamic process parameters that were optimized were the feed rate, the distance from the feeder to the collector as well as the applied voltage. The effect of the pre-mentioned parameter tuning was evaluated using three basic criteria. Firstly, the achievement of normal flow without the formation of droplets, a crucial factor for the homogenicity of the final product as well as the size of the produced nanocapsules. Secondly, the formation of stable Taylor cone another fact that affects the size of the produced nanocapsules and finally the uniformity of the collected encapsulated material. The operating conditions tested and the proportion of bioactive substances in the cyclodextrin solution are shown in the following tables (Tables 2 - 4).

Table 3 Dissolution of 0.5g omega-3 fatty acids and 0.5g collagen in a final volume of cyclodextrin solution 10mL. Parameters of Feed rate (μL/h), Distance (cm) and Voltage (kV) used for the process optimization. Results of encapsulation criteria: Normal flow without droplets, Stable Taylor cone formation and Uniformity of collected nanocapsules marked as √ when successful.

			Optimum encapsulation Criteria		
Feed rate (μL/h)	Distance (cm)	Voltage (kV)	Normal flow without droplets	Stable Taylor cone formation (≥ 5mns)	Uniformity of collected nanocapsules
700	15	28.9	√	×	×
800	15	27.0-29.2	×	×	×
1000	10	25.0-28.6	√	×	×
1000	12	26.0-28.0	×	√	×
1000	13	26.0-28.1	×	√	×
1000	14	28.5	√	√	√
1000	15	25.5-28.5	×	×	√
1050	15	25.5-28.5	×	√	×
1100	13	26.0-28.2	×	×	×
1100	15	27.0-29.4	×	√	×
1150	15	28.5	×	×	×
1200	13	25.0-27.0	×	×	×
1200	15	23.5-28.5	×	×	×
1250	15	23.7-28.2	×	×	×
1300	13	27.0	√	×	×
1300	15	28.2	×	√	×
1500	15	22.3-28	×	×	×

Table 4 Dissolution of 0.7g omega-3 fatty acids and 0.3g collagen in a final volume of cyclodextrin solution 10mL. Parameters of Feed rate (μL/h), Distance (cm) and Voltage (kV) used for the process optimization.

Results of encapsulation criteria: Normal flow without droplets, Stable Taylor cone formation and Uniformity of collected nanocapsules marked as √ when successful.

Feed rate (μL/h)	Distance (cm)	Voltage (kV)	Optimum encapsulation Criteria		
			Normal flow without droplets	Stable Taylor cone formation (≥ 5mns)	Uniformity of collected nanocapsules
600	13	23.5-28.0	×	×	×
700	13	26.0-28.0	×	×	×
1000	10	25.0-28.0	√	×	×
1000	11	25.7-28.0	√	×	×
1000	12	25.5-28.5	√	×	×
1000	13	26.5-28.0	√	√	√
1000	14	24.5-28.5	√	√	×
1000	15	25.0-29.5	×	×	×
1200	15	25.0-28.5	×	×	×
1300	15	25.0-28.5	×	×	×
1300	15	24.5-28.5	×	×	×
1500	15	24.5-28.5	×	×	×

Discussion

Changes in the levels of TTX in *L.scleratus* have been shown to be a function of season and sex, where most toxic fishes were found to be female in the summer season [17] [18]. According to the same research the highest TTX level in internal organs of female fish reached an amount of 52.1 while muscle tissue was reported at 2.83μg/g during the winter but was otherwise below the toxic limit. In accordance to these results, the TTX level of internal organs and muscle tissue in our study reached 14.10 μg/g and 1.77 μg/g respectively.

For the cosmetic industry, marine collagen has been previously obtained from coldwater fish skins, such as cod, haddock and salmon [19]. According to literature salmon collagen shows skin antiageing and systemic redox effects [20], therefore it was selected as reference material to be compared with the recovery yields of collagen from *P. miles* skin and bones. Based on our research, both species show similar amount of collagen content in bones, whereas *P. miles* contained a significantly higher amount of collagen in skin samples. Specifically, the collagen content in *P. miles* skin samples were found to be thirteen times higher than those of *S. salar* (Figure 2).

The lipid content of *F. commersonii* in both flesh and skin tissue samples was found to be 3.5 and 4.3% respectively. Although, the total lipid content of skin tissue samples was 22% higher than those of flesh, the PUFA content in lipid fraction of skin was found to be 65% higher. At this point it is essential to mention that in cosmetic industry there has been increasing interest in the relationship of fish oil with skin protection and homeostasis, especially with respect to the omega-3 polyunsaturated fatty acids (PUFAs) and particularly docosahexaenoic acid (DHA) [21]. According to our study, DHA was found to be in escess in the lipid fraction of both flesh and skin tissue samples of *F. commersonii* with 21,28 and 18,41% of PUFAs content respectively. This fact posing *F. commersonii* as an ideal source of lipids for application in cosmetic industry.

In order to sufficiently incorporate in cosmetic products, the fish collagen and omega-3 fatty acids isolated in this study, the encapsulation electrospraying process was evaluated aiming at the efficient odour

covering, enhancement of bioactive compounds' protection and bioavailability. The process functional parameters were optimized based on the uniformity of the final encapsulated powder product as shown in Tables 2-4. The results obtained from the investigation, showed that the best operating conditions of electrospraying are achieved for feed rate equal to 1000μL / h, voltage equal to 27kV and distance of the collection surface from the nozzle equal to 13cm, for cyclodextrin solution with a content of 7% omega-3 fatty acids and 3% collagen.

Conclusion

The present study can turn the current problem of invasive alien species into a "Win-Win" solution for both Mediterranean fisheries and cosmetology companies. In addition to the innovation brought to the cosmetics sector due to the opening of a new market of innovative formulations based on unique products origin, the invasive species' exploitation is expected to create a new type of fishery that will bring economic benefits to professional fishermen themselves. In addition, it will contribute to the reduction of invasive fish stocks in the Mediterranean and consequently in the control of the ecological and economic impact on marine life, fisheries, human well-being and health.

According to our research, new cosmetic markets will open up based on natural and sustainable ingredients of unique origin. In addition, the integration of new technologies and know-how in the Mediterranean cosmetic industry will give a significant advantage over its competitors in the international market. The utilization of bioactive ingredients from marine alien species will have a significant financial impact since easily applicable to industry and cost-effective processes are proposed for the valorization of an unexploited and in excess source. The use of bioactive ingredients obtained in the context of this study will lead to the development and production of innovative end products for cosmetology, enriched with genuine natural substances that will have significant social and health benefits.

Acknowledgments

This presented work is part of the project EXPLIAS funded by the Fisheries and Maritime Operational Program 2014 – 2020 of the Greek Ministry of Agricultural Development and Food, and the European Maritime and Fisheries Fund.

Conflict of Interest Statement

The authors declare no conflict of interests

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