Recovery and Encapsualtion of Bioactive Extracts from 
**Haematococcus Pluvialis** and **Phaeodactylum Tricornutum** for 
food Applications

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**Abstract:** In the present work, the microalga Haematococcus pluvialis (HP) and the diatom Phaeodactylum tricornutum (PT) were selected as raw materials for the recovery of multifunctional extracts. Ultrasound assisted extraction (UAE) using coconut oil as solvent was applied under specific optimized conditions. The total carotenoid content for both raw materials were determined using UV-Vis spectrometry. In case of HP astaxanthin content was also determined. Moreover, qualitative and quantitative analysis of polyunsaturated fatty acids in HP and PT extracts took place using Gas Chromatography (GC). The extraction yield focusing on the targeted lipophilic bioactive compounds was high reaching the 80%. Furthermore, the encapsulation of these lipophilic compounds in ulvan nanostructures, a marine derived sulfated polysaccharide, in form of fibrils using the electrohydrodynamic process was examined. The encapsulated forms develop an effective thermodynamical and physical barrier against deteriorative environmental conditions, such as water vapor, oxygen, light, enzymes or pH.

**Keywords:** Antioxidants, anti-inflammatory agents, astaxanthin, carotenoids, electrohydrodynamic process, polyunsaturated fatty acids, ultrasound assisted extraction

I. Introduction

Nowadays consumers apart from taste and organoleptic characteristics of food products are paying great attention to their nutritional value and health claim, they are seeking for functional food products with proved health benefits [1]. In addition European regulation for food additives (1129/2011) intends to restrict or decrease the use of numerous synthetic enhancers and to impose their replacement with natural ingredients [2]. These demands are imposing food producers to search for new ingredients of high nutritional value and biofunctionality, derived from natural sources in a sustainable way. Microalgae, according to newest scientific facts are characterized as a resource for various applications in food industry especially as alternative source of bioactive compounds. The green algae Haematococcus pluvialis is a freshwater species of Chlorophyta from the family Haematococccaceae. Haematococcus pluvialis, during unfavorable growth conditions, initiates carotenogenesis and lipid accumulation while undergoes morphological transformation from green vegetative cells to deep red. Therefore, the red algae Haematococcus pluvialis is one of the most important biological sources of carotenoids. Astaxanthin has, nowadays, gained popularity due to its potential use for the prevention or treatment of heart disorders and circulatory diseases, cancer and inflammation [3]. In addition, astaxanthin is proven to have fat burning activities, as well as, anti-inflammatory and anti-cancer effects [6].

The recovery of bioactive compounds is commonly performed through an extraction method. The most commonly used extraction procedures are mechanical agitation and soxhlet extraction due to the low processing cost and ease of operation. However, these methods use toxic solvents, requiring an energy and time consuming evaporation step for the recovery of solvents. Moreover, the possibility of thermal degradation of bioactive compounds cannot be ignored, due to the high temperatures of the solvents during the long times of extraction [7]. Therefore, conventional extraction techniques tend to be replaced by novel ones such as Ultrasound-Assisted Extraction (UAE) that is an environmentally friendly extraction method and can reach high extraction yields within short time using mild solvent systems, especially in the case of the recovery of sensitive chemical compounds, such as PUFAs and carotenoids [8], [9]. UAE is recognized as an efficient extraction technique that decreases significantly the extraction time, increases the yielding, enhancing in the same time the quality of the extract [10]. Furthermore, for the recovery of sensitive lipophilic compounds addressed to food applications the use of mild food compatible solvent system is needed. Medium-chain triglycerides (MCTs) are promising solvents for the recovery of bioactive compounds, flavours and vitamins because of their polarity and their...
ability to not generate dissonant tastes. Specifically, MCTs are triglycerides whose fatty acids have an aliphatic tail of 6–12 carbon atoms, with most known one the lauric acid that is found in coconut oil [5]. Coconut oil is about 50% lauric acid, making it nature’s richest source of lauric acid. While all the MCTs have known health benefits, lauric acid is the most well-known for its health benefits. Lauric acid is prized around the world as a powerful antimicrobial agent, used in both food preservation as well as in drugs and nutraceuticals [11].

The diet rich in polyunsaturated fatty acids (PUFAs) and carotenoids has been positively correlated with a decreased risk of developing several chronic diseases [12]. However, their bioavailability is often compromised due to incomplete release from the food matrix, poor solubility and potential degradation during digestion. In addition, such lipophilic bioactive compounds in food products are prone to oxidative degradation, not only lowering the nutritional value of the product but also triggering other quality deteriorative changes such as formation of lipid pro-oxidants, development of discolorations or off-flavor defects. The encapsulation of these lipophilic compounds in structurally engineered nano-systems develops an effective thermodynamical and physical barrier against deteriorative environmental conditions, such as water vapor, oxygen, light, enzymes or pH. In addition, nanoencapsulation enhances the bioavailability of lipophilic bioactives, via modulating their release kinetics from the carrier system, solubility and interfacial properties [13], [14]. Polysaccharides are widely used as encapsulation matrix for bioactive components [15], [16]. Polysaccharides of high density, such as marine origin polysaccharide Ulvan, exhibit particular interest because of their satisfactory solubility in aqueous systems. The ulvan derived from marine macroalgae Ulva sp and consists mainly of sulfates, rhamnose, xylose and glucuronic acid. The sulfated polysaccharides used in the formation of nanostructures because of their ionic nature. Their ionic nature permits the formation of complexes with oppositely charged polyelectrolytes, as useful in designing drug carriers since the polyelectrolyte complexes permit binding of drugs in the polymer matrix at a molecular level. Then, the drug is released from the polyelectrolyte complex, or by ion exchange mechanism or by charge interaction and by polymer degradation and dissolution of the complex [17]–[21]. An innovative and promising encapsulation technique of bioactive compounds for food applications is the electrohydrodynamic process known also as electrospinning and electrospaying. During this procedure a viscous solution of bioactive compounds, matrix material and a volatile solvent placed in syringe and charged with a high voltage that creates an electric field causing the polymer to be spun out in thin threads (micro-, nano- fibers/particles) to a collector plate. The comparative advantages of this method compared to the other techniques is the absence of toxic solvents, the operation in room temperature preserving the heat sensitive ingredients, the production of electrospun structures from biopolymers using aqueous solutions - high interest in food and nutraceutical applications, the process stability that permits tunable fiber diameter or particle size with controlled size distribution (10-100 nm) and high encapsulation efficiency [21]–[23]. In this study, the recovery of PUFAs and carotenoids from the microalgae species Haematococcus pluvialis and Phaeodactylum tricornutum using ultrasound assisted extraction and coconut oil as solvent was examined. Furthermore, the encapsulation of the recovered bioactive compounds in an emulsion form through the electrohydrodynamic process using ulvan polysaccharide as matrix was studied.

II. Materials And Methods

2.1 Materials

Haematococcus pluvialis (HP) and Phaeodactylum tricornutum (PT) were delivered in the lab in dried powder form containing 3.02 ± 0.21% and 2.92 ± 0.13% moisture content, respectively. Commercial virgin coconut olive pursued from CHEMCO (GR) was used as solvent. Ulvan polysaccharide was delivered in powder form of 95% purity from MEDBIO SA (GR) with the commercial name Blue Elixir. Boric acid and Calcium chloride were pursued from Sigma Aldrich (UK) in powder form and purity >99.5% and >97%, respectively. Tween 20 was delivered in form of viscous liquid from Sigma Aldrich (UK), with composition lauric acid, ≥40% (balance primarly myristic, palmitic, and stearic acids). Pullulan from Aureobasidium pullulans was pursued in powder form from Sigma Aldrich (UK).

2.2 Extraction

Ultrasound assisted extractions (UAE) were carried out in an ultrasound bath a XO-SM50 Ultrasonic Microwave Reaction System (Nanjing Xianou Instruments Manufacture co., Ltd., Nanjing City, China). Samples of dried microalgae were placed in a beaker with 50 mL in a Solid to Solvent ratio 1:20 w/v dry weight (g) per mL solvent (coconut oil) and extracted while operating at 25 kHz frequency, at 450 Watt and temperature 30 °C for a total duration of 15 min.
2.3 Bioactive content evaluation

2.3.1 Total carotenoids

The total carotenoid content was estimated through Jeffrey 1997 protocol [24], using 90% acetone as solvent. The measurements took place at 510, 480 nm in a Bel photonics UV-Vis 501 spectrometer. Equation (1) was used total carotenoids determination.

\[ \text{Cp (µg/L)} = 7.60*(A480) - 1.49*(A510) \]  

Where, A was the absorbance at 480 and 510 nm.

2.3.2 β-carotene Content

The β-carotene content was estimated through Ben Amotz & Avron 1983 protocol [25], using 80% acetone as solvent. The measurements took place at 480 nm.

\[ C = \frac{A}{d} \text{ (mol/l)} \]  

Where, A was the absorbance at 480 nm, ε was the coefficient (ε = 2273 at 480 nm for β-carotene [25]) and 1 was the distance traveled by the light beam according to the cuvette used.

2.3.3 Astaxanthin Content

Astaxanthin in encysted *Haematococcus pluvialis* cells consists of approximately 70% monoesters, 25% diesters and 5% free form. Therefore, enzymatic hydrolysis should be performed in order to quantify all astaxanthin content. Hydrolysis of astaxanthin esters in crude extract was carried out with cholesterol esterase according to Jacobs et al. (1982) procedure [26]. The recovered from HP biomass extracts were dissolved in acetone using decimal dilutions and directly analyzed on a spectrophotometer UV / visible light. For the determination of astaxanthin standard calibration curve was conducted by standard HP extract containing 10% of astaxanthin obtained by Algatechnologies Ltd. Specifically, for the design of the standard curve serial dilutions of the original extract were used and solutions were measured at 474 nm (maximum absorbance of astaxanthin). The equation that describes the astaxanthin standard curve was:

\[ C ((\mu g \text{ astaxanthin})/(mL \text{ extract})) = (A474-0.028)/0.2146 \times \text{Dilution rate} \]  

Where, A474 was the absorbance at 474 nm.

2.3.4 Fucoxanthin Content

For quantitative determination of fucoxanthin absorbance at 444nm was measured according to equation proposed by Strickland and Parsons [27]:

\[ \text{Fucoxanthin (mg/L)}=1000\times A_{444}/166 \]  

According to literature the Fucoxanthin Specific Absorption Coefficient (L g\(^{-1}\) cm\(^{-1}\)) was 166.

2.3.5 Fatty Acids Methyl Ester profile

Firstly, direct transesterification of HP and PT took place. 5 mL of methanol/ toluene (3:2 (v/v)) and 5 mL of acetyl chloride/ methanol (1:20 (v/v)) were added to an aliquot of 100 µL of the extracts and the mixture was heated at 100 °C for 1 h. After the contents had cooled to room temperature, 5 mL of water and 5 mL of hexane were added to mixtures and the tubes were centrifuged at 3000 rpm for 5 min. The upper phase was selected to test tubes and the samples were infiltrated with sodium sulphate in order to remove moisture. The sample was evaporated to dryness at room temperature. The fatty acid methyl esters were selected using 1 mL of isooctane and stored at 4 °C until injection into the [28]. Fatty acids methyl esters were separated by GC on a Varian Model 3300 gas chromatograph equipped with flame ionisation detector. A flexible fused silica Megabore column (30 m x 0.32 mm, 1 µm film thickness) with bonded stationary phase of CP-WAX was employed. Helium was used as the carrier gas. Injector and the detector temperatures were 250 and 300 °C, respectively. The column temperature was programmed to be initially 140 °C for 5 min and then to increase at a rate of 3 °C to a final temperature of 240 °C. Fatty acids methyl esters were identified by comparison to external standard (SupelcoTM 37 component FAME Mix) and were quantified by internal standard (Nonadecanoic acid (C19:0)), GC data acquisition and handling was carried out by connecting the GC with personal computer and utilizing the Millenium software [28].

2.4 Encapsulation

2.4.1 Preparation of polymer solution enriched with lipophilic compounds for electrospinning

Ulvan and pullulan blends were dissolved in a solution containing 15 mM H\(_2\)BO\(_3\) and 7 mM CaCl\(_2\) in a ratio of 60:40 (v/v). Solutions for ulvan were adjusted at 1.5 wt%, whereas pullulan solutions were at 20 wt%. According to Papadaki et al. 2016, the polymers were blended at the optimum proportion of 30:70 w/w of ulvan and pullulan solutions, respectively [29]. The solution was stirred at room temperature until to ensure a complete dissolution. The direct incorporation of coconut oil enriched with β-carotene and polyunsaturated fatty acids in the ulvan:pollulan solution was made and a stable emulsion using ultrasound sonication was prepared. Specifically a 10% oil in water emulsion was formed using 6.5 % w/v Tween 20 surfactant, the oily phase was consisted of equal parts of HP and PT extracts, while the aqueous phase was consisted of ulvan:pullulan blend as it was described.
2.4.2 Electrohydrodynamic process

The electrospinning experiments were carried out in a Fluidnatek LE-10 (Bioinicia, Spain) electrospinning apparatus at room temperature. The target was placed 15 cm from the capillary tip. The syringe pump delivered polymer solution at a controlled feed rate of 1 mL/h, while the voltage was set at 22 kV in order to form a stable Taylor cone.

2.4.3 Characterization of ulvan-pullulan-lipophilic compounds emulsion and fibers

The conductivity of the emulsions was measured using a Hanna HI 9835 conductivity meter. Surface tension was assessed with the aid of a tensiometer (Krüuss, Easy Dyne). Viscosities of the emulsions were measured using Brookfield DV-II + Pro viscometer at 25 °C with a sample volume of 50 mL operating at 50 rpm using a spindle set of S61, S63 and S64 [30]. The morphology of the electrospun fibers was examined using SEM (FEI Quanta FEG250) after sputtering the samples with a gold–palladium mixture under vacuum. All SEM experiments were carried out at an accelerating voltage of 15 kV. The diameters of the electrospun fibers through the SEM images were measured by Image J software. The Encapsulation Efficiency (EE) of the loaded fibers was determined by measuring the non-entrapped carotenoids and PUFAs according to Moomand and Lim (2014) with some modifications [31]. The amount of encapsulated lipophilic compounds were determined by submerging the electrospun fibres in hexane for 6 hrs to remove the surface compounds. The absorbance of the total carotenoids diluted in hexane was measured at 480 nm, while the PUFAs content was measured according to the procedure described in 2.3.5. The amount of total carotenoids present in the hexane was determined from a calibration curve (R² = 0.99), prepared by spiking hexane with various quantities of b-carotene and with an equivalent of electrospun fibres to account for any matrix effects. The percentage EE values were calculated as:

\[ \text{EE}=(A-B)/A \times 100 \]  

Where A was the total theoretical amount of lipophilic compounds, B was the free amount of lipophilic compounds in collection solution.

III. Results And Discussion

3.1. Characterization of microalgae extracts

In Tables 1-3, the content of coconut oil extracts’ of HP and PT in carotenoids and polyunsaturated fatty acids was presented. According to these results, coconut oil was evaluated as a high selective solvent in bioactive compounds of great interest such as b-carotene, astaxanthin, fucoxanthin, eicosapentaenoic and arachidonic acid. The high selectivity of coconut oil in omega-3, -6 and -9 fatty acids was proven by the final consistency of the extracts in these fatty acids categories was around 76.92 % and 79.97 % of total fatty acids in case of HP and PT, respectively.

<table>
<thead>
<tr>
<th>Carotenoids (mg/mL)</th>
<th>Haematococcus pluvialis</th>
<th>Phaeodactylum tricornutum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total carotenoids</td>
<td>2.53</td>
<td>3.22</td>
</tr>
<tr>
<td>b-carotene (mg/mL)</td>
<td>0.78</td>
<td>0.49</td>
</tr>
<tr>
<td>Astaxanthin (mg/mL)</td>
<td>1.32</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fucoxanthin (mg/ml)</td>
<td>n.d</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Table 2. PUFAs profile of Haematococcus pluvialis extracted by UAE using coconut oil as solvent and ratio (biomass/solvent) at 1:20.

<table>
<thead>
<tr>
<th>PUFAs Profile</th>
<th>mg/ml extract</th>
<th>mg/mg dried biomass</th>
<th>% over Total Fatty Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>ω3</td>
<td>1.89</td>
<td>37.89</td>
<td>33.29</td>
</tr>
<tr>
<td>Of which Eicosapentaenoic</td>
<td>0.08</td>
<td>1.39</td>
<td>1.40</td>
</tr>
<tr>
<td>Linoleic</td>
<td>0.06</td>
<td>0.71</td>
<td>1.03</td>
</tr>
<tr>
<td>ω6</td>
<td>1.10</td>
<td>21.92</td>
<td>19.26</td>
</tr>
<tr>
<td>Of which Arachidonic</td>
<td>0.07</td>
<td>1.32</td>
<td>1.16</td>
</tr>
<tr>
<td>ω9</td>
<td>1.39</td>
<td>27.73</td>
<td>24.37</td>
</tr>
<tr>
<td>Total Fatty Acids</td>
<td>5.69</td>
<td>113.81</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Table 3. PUFAs profile of Phaeodactylum tricornutum extracted by UAE using coconut oil as solvent and ratio (biomass/solvent) at 1:20.

<table>
<thead>
<tr>
<th>PUFAs Profile</th>
<th>mg/ml extract</th>
<th>mg/mg dried biomass</th>
<th>% over Total Fatty Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>ω3</td>
<td>6.11</td>
<td>122.15</td>
<td>62.59</td>
</tr>
<tr>
<td>Of which Eicosapentaenoic</td>
<td>4.57</td>
<td>91.46</td>
<td>46.87</td>
</tr>
<tr>
<td>Docosahexaenoic</td>
<td>0.14</td>
<td>2.88</td>
<td>1.43</td>
</tr>
<tr>
<td>ω6</td>
<td>0.64</td>
<td>12.77</td>
<td>6.54</td>
</tr>
<tr>
<td>ω9</td>
<td>1.06</td>
<td>21.16</td>
<td>10.84</td>
</tr>
<tr>
<td>Total PuFAs</td>
<td>9.76</td>
<td>195.15</td>
<td>100.00</td>
</tr>
</tbody>
</table>
3.2. Characterization of ulvan-pullulan-lipophilic compounds emulsion and encapsulated fibers

In Table 4, the rheological characteristics of ulvan-pullulan water based blend and each raw material separately as well as of the emulsion formatted according to section 2.4.1 is presented.

<table>
<thead>
<tr>
<th>Conductivity (μS/cm)</th>
<th>Surface tension (mN/m)</th>
<th>Viscosity (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulvan 2.23</td>
<td>51.01</td>
<td>7.38</td>
</tr>
<tr>
<td>Pollulan 0.35</td>
<td>69.69</td>
<td>3674</td>
</tr>
<tr>
<td>Blend 30:70</td>
<td>1.07</td>
<td>46.20</td>
</tr>
<tr>
<td>Emulsion solution enriched with lipophilic compounds 0.75</td>
<td>42.64</td>
<td>2606</td>
</tr>
</tbody>
</table>

The emulsion of ulvan-pollulan matrix and coconut oil HP and PT extracts formatted according to procedure described in section 2.4.1, contained 0.27 mg/ml total carotenoids and 0.72 mg/ml polyunsaturated fatty acids based on its consistency and extraction yielding obtained from Tables 1-3. The formatted emulsion shown great stability for over 48 hrs in room temperature. It was an emulsion of great homogeneity where neither particles, insoluble elements, precipitation nor phase separation were observed. According to SEM results (see Fig.1), it was observed that the encapsulation of bioactive compounds was successful as long as no particles stack on the fibers’ surface were detected. All the targeted compounds were entrapped in the fiber. This fact is also quantitatively expressed using the Eq. (5) that presents 94.8% encapsulation efficiency in case of total carotenoids and 88.9% regarding polyunsaturated fatty acids. Using the Image J software to analyze the SEM pictures (Fig. 1), the diameter of encapsulated nanofibers were estimated around 65 nm while the ulvan-pollulan matrice nanofibers diameter before encapsulation step ranged from 20 to 30 nm. This increase in nanofibers diameter is a proven evidence of the successful uniform entrapment of the targeted compounds in the fiber.

![Figure 1. SEM images of electrospun ulvan:pullulan (30:70) blend structures (left) and encapsulated nanofibers (right).](image)

IV. Conclusion

The diet rich in polyunsaturated fatty acids (PUFAs) and carotenoids has been positively correlated with numerous health benefits based on the decreased risk of developing several chronic diseases. The first aim of this study was to select sustainable resources rich in these compounds with proved health claims. The microalgae species of *Haematococcus pluvialis* and *Phaeodactylum tricornutum* were proven an ideal source of omega-3 fatty acids and carotenoids based firstly on the high accumulation of these compounds and secondly on the microalgae cultivation, a process that demands low land occupation and it is characterized from high yielding. In the frames of this study, ultrasound assisted extraction using coconut oil as solvent for the recovery of b-carotene, astaxanthin, fucoxanthin, eicosapentaenoic and arachidonic acid from the selected microalgae species was applied, showing great selectivity and yielding. The incorporation of these lipid extracts rich in carotenoids and PUFAs in food matrices, as well as their preservation and finally the bioavailability of the targeted bioactive compounds to the final consumer arised the need of an encapsulation step. The ability of these lipophilic extracts to be incorporated apart from oily products to water based systems such as juices, beverages, jells etc was considered, in order to broaden the application of these value added compounds. Therefore, an oil in water emulsion was prepared as feed to the electrospinning process from where nanofibers that carried entrapped carotenoids and PUFAs were obtained. The encapsulation process was successful with efficacy around 90% for both carotenoids and PUFAs. Concluding, in this study rich lipid extracts of microalgal origin were obtaining using food grade solvent system and an ecofriendly extraction method. Moreover, the successful encapsulation of these extracts in natural matrices constructed from a polysaccharide water based blend was
achieved. These structurally engineered nano-systems develop an effective barrier against deteriorative environmental conditions, such as water vapor, oxygen, light, enzymes or pH, enhancing the bioavailability of lipophilic bioactives, via modulating their release kinetics from the carrier system, solubility and interfacial properties.

Acknowledgements

This research was financially supported by the IKY Fellowships for Postgraduate Studies in Greece – Siemens Program. The authors would like to thank the Food Chemistry and Technology Laboratory of School of Chemical Engineering at National Technical University of Athens, where the surface tension and viscosity measurements took place.

References


