# ULTRASOUND AND MICROWAVE ASSISTED EXTRACTION OF DOCOSAHEXAENOIC ACID (DHA) FROM *CRYPTHECODINIUM COHNII* MICROALGA

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## ABSTRACT
*Crypthecodinium cohnii* is a heterotrophic marine dinoflagellate and represents a major source of omega- 3 fatty acids, and especially docosahexaenoic acid (DHA), a long chain polyunsaturated fatty acid (PUFA) with significant health benefits. With the current increasing demand for oils rich in DHA from natural sources, attention has been focused on the cultivation and extraction of *C. cohnii*. Thus, the development of efficient and environmentally sustainable extraction processes, such as ultrasound and microwave assisted extractions, for the recovery of high-quality oil is an important challenge. The objective of the present study was the recovery of DHA for food applications from *C. cohnii*. Therefore, non-polar, non-toxic and food grade solvents were used and the extraction conditions were gentle in order to protect the heat-sensitive components.
*C. cohnii* biomass, grown on acetic acid, was freeze-dried and the samples were extracted for a total duration of 10 min under ultrasounds or under microwaves. Hexane, hexane: isopropanol (2:3) and 2-butanol were tested to compare the influence of organic and food grade solvents on the DHA extraction. The extraction yields were calculated and the DHA content was evaluated through Gas Chromatography.
The results showed that ultrasound assisted extraction (UAE) using hexane: isopropanol (2:3) was the best combination. For this reason, these two systems were further examined, while changing the extraction time, the solvent/biomass ratio and the ultrasound power.
The results of the extraction parameters scanning demonstrated that the extraction power did not significantly affect the yield, while the extraction time and the solvent/biomass ratio were found to be more crucial parameters. The most effective solvent/biomass ratio was 20, while the best extraction time was 15 min. In conclusion, UAE using hexane: isopropanol with the suitable conditions significantly improved the yields and preserved the high quality of DHA.

## INTRODUCTION
Polyunsaturated fatty acids (PUFAs) are long chain fatty acids with many double bonds [1]. Omega-3 (ω-3) PUFAs represent a specific category of PUFAs, where the first double bond is located between the third and fourth carbon atom counting from the methyl end of the fatty acid [2]. The best-known omega-3 PUFAs include the α-linolenic acid (ALA 18:3 ω-3), eicosapentaenoic acid (EPA, 20:5 ω-3), docosapentaenoic acid (22:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3). These long chain ω-3 PUFA are subject of intensive research since their consumption is related to important health benefits and has beneficial effects against many diseases [3].
Especially, DHA has numerous positive effects on health and, thus, a wide range of pharmaceutical applications [4]. The particular importance of DHA for the proper visual and neurological development of infants is recognised by the World Health Organisation (WHO) and many governmental agencies, who recommend the inclusion of DHA in infant formulae [1], [4]–[6].
The principal dietary source of DHA is fish oil. However, the massive exploitation of fish leads to serious environmental consequences and depletion of fish stocks. Furthermore, the use of fish oil is limited due to its typical fishy and undesirable smell and taste, and poor oxidative stability. Moreover, fish accumulate lipid-soluble environmental pollutants and the purification of specific fatty acids is difficult. Therefore, the development of alternative approaches for DHA production is of great interest [2], [4], [5], [7].

It has been indicated that ω-3 PUFAs in fish are actually derived from zooplankton consuming ω3 PUFA-synthesising microalgae through the marine food chain [4],[5]. Microalgae are a heterogeneous group of photosynthetic organisms. Lately, the consumers growing demand for natural bioactive compounds has motivated researchers and industries to investigate microalgae as a potential source of ω-3 fatty acids [3], [8].

Microalgae biomass presents various advantages as a source of PUFAs, and more specifically DHA, since is particularly suitable for extraction and purification of individual PUFA due to its stable lipid composition. The synthesis of great amounts of ω-3 PUFA in microalgae biomass, the absence of cholesterol and contaminants in their produced oils, such as heavy metals, and their satisfactory taste represent additional advantages [2], [4], [5].

Among the various microalgae, the heterotrophic marine dinoflagellate *Crypthecodinium cohnii* represents one of the major DHA sources thanks to its unique fatty acid composition and fast growth rates [3], [7]. *C. cohnii* can accumulate high amounts of lipids with 30–60% DHA fraction of the fatty acids and only small traces of other fatty acids, such as eicosapentaenoic acid (EPA) [5], [7].

Solvent extraction techniques are widely employed methods for the recovery of lipids from microalgae biomass [9]. An efficient extraction requires the full penetration of the solvent into the cell biomass and the match of the polarity of the solvent and targeted compound. Ultrasound-assisted extraction (UAE) and microwave assisted extraction (MAE) represent environmentally benign techniques, which significantly improve extraction of microalgae, having higher efficiency, reduced extraction times and increased yields, as well as low to moderate costs and negligible added toxicity. Through the phenomenon of cavitation during UAE, the release of PUFAs from the microalga cells is promoted by the disruption of cell walls and the facilitation of solvent access to the cell content. On the other hand, MAE is based on the principle that microwave dielectric heating is selective, and releases negligible amounts of heat to the environment [8], [10]. The most common solvents for lipid extraction are chloroform–methanol, hexane, hexane–isopropanol and butanol [3].

During extraction, moisture can prove problematic, acting as a barrier between solvent and cells, and thus, restricting the solvent access to cells [8]. Dehydration represents an essential treatment prior to extraction, as it achieves the production of stable biomass, the extension of storage time and the avoidance of microbial, chemical and sensory deterioration [11], [12]. Drying time, as well as drying temperature, represent the most crucial parameters affecting not only the final nutritional value of the microalgal oil, but also the performance of the next step of extraction [12]. Despite the high energy demands, freeze-drying is the gentlest dehydration method and achieves efficient disintegration of the tough microalgae cell wall [13].

## MATERIALS AND METHODS

### *Chemicals and strains*

Solvents and reagents used in the extraction experiments were of analytical grade and purchased from Sigma–Aldrich. *C. cohnii* ATCC® 30772TM was obtained from the American Type Culture Collection (ATCC).

### *Drying Treatment*

*C. cohnii* was delivered wet and was stored at −30°C. Afterwards, the samples were dehydrated for 8 h using a laboratory freeze-dryer (Leybold-Heraeus GT 2A, Koln, Germany) under the effect of high vacuum (3 mbar).

### *Ultrasound Assisted Extraction (UAE)*

UAE was performed in a XO-SM50 Ultrasonic Microwave Reaction System (Nanjing Xianou Instruments Manufacture co., Ltd., Nanjing City, China) operating at 25 kHz frequency, at 450 W and 45°C for a total duration of 10 min. The samples were placed in a beaker with a solid to

solvent ratio 1:20 g dry weight per mL solvent. The solvents, which were selected based on the bioactive content of *C. cohnii*, were hexane, hexane: isopropanol (2:3) and 2-butanol. The best performing solvent system was further examined, while changing other extraction parameters, and specifically:

- the extraction time: 5, 10 and 15 min,
- the solvent to solid ratio: 10, 20 and 30 and
- the ultrasound power: 150 W (17%), 450 W (50%) and 750 W (83%)

***Microwave assisted extraction (MAE)***

MAE was conducted in a XO-SM50 Ultrasonic Microwave Reaction System (Nanjing Xianou Instruments Manufacture co., Ltd., Nanjing City, China) operating at 250 W, 40°C with a stirrer speed of 200 rpm for a total duration of 10 min. The solid to solvent ratio was 1:20 g dry weight per mL solvent. The used solvents were hexane, hexane: isopropanol (2:3) and 2-butanol.

***Soxhlet extraction***

The Soxhlet extraction was applied for 8 h, using hexane: isopropanol (2:3) as solvent in a solid to solvent ratio: 1/30 and it was assumed that it recovered the maximum quantity of oil, and thus the maximum yield.

***Determination of extraction yield***

After the extraction and centrifugation, all the extracts were evaporated under reduced pressure (rotary evaporator) for the removal of the solvents and the calculation of the mass of the obtained oil. The extraction yields of the extracts were calculated as follows:

$$EY\% = [(g\ obtained\ oil / g\ dry\ biomass) / MAX\ Soxhlet\ yield] \times 100\% \quad (1)$$

***Identification and quantification of PUFAs***

Identification and quantification of the PUFAs was based on their chromatographic behavior on Gas Chromatography (GC). Before the GC analysis, transesterification was performed. Specifically, the extracted lipids were esterified as follows: 1 mL of the extract was mixed with 2.5 mL of a mixture of 92:8 Methanol: HCl. The reaction took place at 60$^{o}$C for 15 min. The resulting solution was left to cool after the addition of 2.5 mL of 5% (w/v) $Ca_2Cl$ solution. The fatty acid methyl esters (FAMEs) were extracted using 5 mL of hexane. The lipid content of the alga as FAMEs was analyzed in GC-MS with a Varian 450 analytical instrument equipped with a DB5 column. The carrier gas was helium and its flow rate was equal to 1 mL/min. 1 mL of the esterified extracts was injected at the temperature of 270$^{o}$C with a thermal profile starting at 125$^{o}$C and finishing at 300$^{o}$C within 35 min with a constant pace of 5$^{o}$C/min. PUFAs were identified by comparison to external standards and were quantified by use of standard curve that was formed using solutions of pure PUFAs diluted to different concentrations.

**RESULTS AND DISCUSSION**

The extraction yields, as calculated by Equation 1, are presented in the next Table.

***Table 1.*** *Extraction Yields of UAE and MAE extracts.*

| Extract | Extract Number | Extraction Yield (%) |
|---|---|---|
| UAE, hexane | 1 | 75.1 |
| UAE, 2-butanol | 2 | 74.9 |
| UAE, Hexane: isopropanol (2:3) | 3 | 84.6 |
| MAE, hexane | 4 | 30.3 |
| MAE, 2-butanol | 5 | 29.5 |
| MAE, Hexane: isopropanol (2:3) | 6 | 44.4 |

In order to qualitatively and quantitatively evaluate the PUFA content of the different extracts, GC analysis was performed. In the obtained chromatograms five important peaks were detected, which were identified as follows: 1. C14:0 (myristic acid), 2. C16:0 (palmitic acid), 3. C18:1 (oleic acid), 4. C18:0 (stearic acid), 5. C22:6 (DHA). The profile and exact content of fatty acids in each extract are shown in Table 2 and Table 3.

***Table 2.*** *PUFA profile in C. cohnii UAE extracts. The numbers of the extracts correspond to Table 1.*

| **Extract Number** | | **1** | **2** | **3** |
|---|---|---|---|---|
| **PUFA** | **Retention Time, RT (min)** | **Concentration (μg/ml)** | **Concentration (μg/ml)** | **Concentration (μg/ml)** |
| **C14:0** | 13.7 | 180.3 | 281.4 | 332.7 |
| **C16:0** | 17.7 | 224.3 | 452.9 | 442.7 |
| **C18:1** | 21.1 | 42.4 | - | 123.9 |
| **C18:0** | 21.7 | 38.5 | - | 86.9 |
| **C22:6** | 28.7 | 121.5 | 337.9 | 370.5 |
| **Total** | - | **607.0** | **1072.2** | **1356.7** |

***Table 3.*** *PUFA profile in C. cohnii MAE extracts. The numbers of the extracts correspond to Table 1.*

| **Extract Number** | | **4** | **5** | **6** |
|---|---|---|---|---|
| **PUFA** | **Retention Time, RT (min)** | **Concentration (μg/ml)** | **Concentration (μg/ml)** | **Concentration (μg/ml)** |
| **C14:0** | 13.7 | 200.1 | 239.6 | 145.1 |
| **C16:0** | 17.7 | 256.7 | 316.7 | 189.8 |
| **C18:1** | 21.1 | - | 62.4 | 46.7 |
| **C18:0** | 21.7 | - | 121.9 | 36.2 |
| **C22:6** | 28.7 | 151.4 | 293.4 | 210.1 |
| **Total** | - | **608.2** | **1034** | **627.9** |

The relative abundance of each PUFA in each extract is presented in Table 4.

***Table 4.*** *Relative abundance of PUFAs in C. cohnii extracts.*

| **PUFA Extract Number** | **C14:0** | **C16:0** | **C18:1** | **C18:0** | **C22:6** |
|---|---|---|---|---|---|
| **1** | 29.7% | 37.0% | 7.0% | 6.3% | 20.0% |
| **2** | 26.2% | 42.2% | | | 31.6% |
| **3** | 24.5% | 32.6% | 9.1% | 6.4% | 27.4% |
| **4** | 32.9% | 42.2% | | | 24.9% |
| **5** | 23.2% | 30.6% | 6.0% | 11.8% | 28.4% |
| **6** | 23.1% | 30.2% | 7.4% | 5.8% | 33.5% |

In the UAE extract of 2-butanol and MAE extract of hexane, a complete separation of C18:1 and C18:0 was not detected.

Comparing the UAE with the MAE, the former method not only reached higher yields, but also achieved better recovery of DHA (C22:6) and PUFA (Tables 1 & 2). The improved performance of UAE is the result of cavitation forces spread by ultrasounds, where bubbles explode and produce

targeted pressure. This phenomenon causes the rupture of microalgae cells and favours the release of intracellular content in the solvent [14].

On the other hand, MAE achieved lower yields. In general, microwaves are emitted as waves, which can penetrate the tissues and interact with polar molecules, such as water, to create heat. In MAE, the dielectric constant of the selected solvent plays a very important role in the extraction efficiency, as the absorbance of microwave energy is an important characteristic. The dielectric constant describes the polarity of a molecule in the electric field. Typically, the higher the dielectric constant of a solvent, the higher the degree of microwave absorption, as the solvent molecules absorb the microwave energy and are polarized. Thus, this method is somewhat limited to polar compounds, as non-polar molecules, such as fatty acids, cannot be heated by microwaves [15].

Furthermore, comparing the different solvents used in the extractions, butanol extracts (2 and 5) and the extracts of hexane: isopropanol (3 and 6) allowed the highest oil extraction in both cases (UAE and MAE) and demonstrated the highest PUFA content. On the contrary, hexane extracts did not recover remarkable quantities of fatty acids. Compared to hexane, butanol and isopropanol are smaller molecules, which may easily penetrate the *C. cohnii* cell walls.

The UAE using hexane: isopropanol (2:3) as solvent was proven to be the best combination and therefore this system was selected to be further examined, changing the extraction parameters, extraction time, solid to solvent ratio and ultrasound power.

The extraction yields in correlation to the solvent to biomass ratio (Figure 1a), extraction time (Figure1b) and ultrasound power (Figure 1c) are presented in the following diagrams, in order to examine the effect of these three parameters.

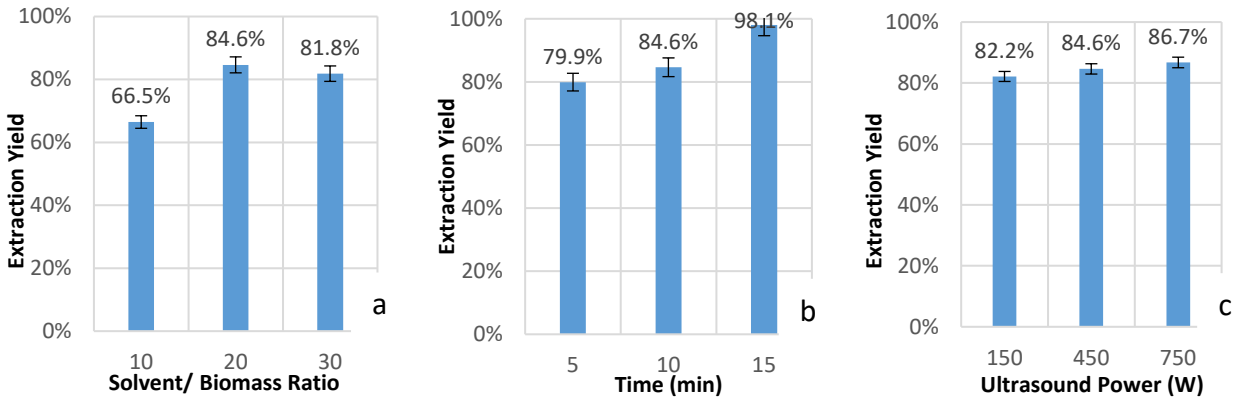


***Figure 1.*** *Extraction yields (%) of C. cohnii biomass after UAE using hexane: isopropanol (2:3) as solvent for: a. solvent/biomass ratio 10, 20 and 30, b. time 5, 10 and 15 min, and c. ultrasound power 150, 450 and 750 W.*

The results demonstrated that the extraction power did not affect significantly the extraction yield, ranging from 82.2 to 86.7% (Figure 1c), while the extraction time (Figure 1b) and the solvent/biomass ratio (Figure 1a) were more crucial parameters, more influencing the extracts behaviour. Specifically, when the solvent to biomass ratio was small (10), the solvent could not penetrate efficiently the *C. cohnii* biomass and the minimum yield was obtained. The most effective solvent/biomass ratio was 20. Moreover, during longer extractions (15 min) the solvent and *C. cohnii* biomass had more time to interact, achieving higher diffusion of the targeted compounds.

## CONCLUSIONS

In conclusion, *C. cohnii* represents a valuable source of ω-3 PUFA, which can be recovered through the gentle and environmentally benign methods of UAE and MAE using green solvents. Between the two techniques, the former demonstrated higher PUFA content, especially when hexane:

isopropanol (2:3) was used as solvent. Finally, the most appropriate UAE conditions were 15 min, 750 W and 20 solvent/biomass ratio.

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