Fish Oil Extraction and Analysis of Docosahexaenoic and Eicosatetraenoic Acids by Gas Chromatography Mass Spectrometry

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Abstract
Fish oil is one of the most valuable dietary complements for healthy bodily functions. The effects of this product vary from fast and healthy development of cognitive abilities to prevention of cardiac diseases. One of most important components of fish oil is Omega-3, made from a variety of fatty acids (FA), such as Eicosatetraenoic Acid (EPA) and Docosahexaenoic acid (DHA), on which the present study has focused. As the human body can hardly synthesize these elements on its own, it is crucial to include them as part of a daily diet. However, fish oil capsules on the market are highly priced, and might contain chemical additives. This work's main goal was to propose a valorization method for the waste of *Sardina pilchardus*, of which choice was based on its oily properties, and on the fact that it is the most present and consumed species in Morocco. Fish oil extraction was completely organic, and performed without any additives or chemicals. This oil can be used as animal feed additive and supplement. Additionally, the presence of multiple FA, specially EPA and DHA, was herein proved by using Gas Chromatography-Mass Spectrometry analysis. Obtained quantities of pure oil exceeded 50 mL/kg fish waste.

Keywords*:* fish oil; fish waste; GS-MC; Middle-atlas; Morocco; Omega-3; *Sardina pilchardus*; valorization.

Introduction•

Fish leftovers are one of the main problems in waste management around the world [1]. This category of waste can rapidly become hazardous if not properly treated and taken care of [2]. Growing global awareness on the importance of fish and its by-products as primary elements of daily meals resulted in exponential increase in fish waste generation.

As a countermeasure to this environmental and economic problem, valorizing fish waste into useful elements is key. Fish waste is considered as every unused part or

•The abbreviations list is in page 103.

total of it and its by-products unfit for human consumption [3]. The common link between all mentioned parts is their mineral and fatty acids (FA) components, which can be extracted following precise techniques.

Fish oil, a popular nutritional product, is most valued. Fish oils are excellent dietary sources of important FA, containing especially polyunsaturated fatty acid (PUFA), Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [4]. Long-chain omega-3 and omega-6 PUFA are the two most important FA, due to their various biological benefits, such as oxidative stress mitigation and cardiovascular protection [5]. An appropriate intake of omega-3 FA may prevent the onset of chronic illnesses, such as cardiovascular diseases, and relief from symptoms linked to ulcerative colitis, menstrual and joint pains. Furthermore, adequate intake of omega-3 FA may also aid in optimal cognitive performance [6]. PUFA are highly concentrated in the brain, and help in the early development of cognitive function and visual sharpness [5].

Nonetheless, PUFA are inefficiently produced by the body, and must be obtained from diets. Their known best source is marine food. However, allergies or lack availability of fish in some parts of the world may be drawbacks for this kind of nutrition. Thus, fish oil capsules are a much more convenient and affordable resource, although marketed brands are highly priced, and might contain some industrial additives such as preservatives.

The main aim of this work was to present a method for valorizing fish waste, by extracting pure organic fish oil. Raw materials were acquired from the local fish market of Khenifra, in middle Atlas Morocco. The oil analysis by GC-MS method was carried out to prove the presence of both EPA and DHA in the final product. The produced oil was extracted without any complex technology and with low cost, which justifies the feasibility of this method for fish waste valorization.

Material and methods

The raw material fish waste of common sardines (*Sardina pilchardus*) was acquired from a local market in Khenifra. All fish waste, composed of bones, heads, skins, viscera and flesh, was used in the extraction process, to avoid the cost of segregating each part from the mix.

The extraction process started by mixing 1 kg fish waste with 1 kg distilled water.

The cooking process was generally carried out at a minimum temperature of 75 °C, to ensure that the oils in the mix were liberated and separated from solid waste. Herein, cooking was performed by a pressure cooker, at temperatures varying from 75 to 80 °C, for 15 to 20 min. This temperature helped reduce the quantity of used water, and ensure that the mix was hygienic. After cooking, the mix was strained to separate any solids from the liquid, in a primary straining process to eliminate large chunks of fish waste. Remaining solids still contained high quantities of water and oil, which required the use of a pressing process, with a machine that separated oil from grains, to ensure that the whole liquid was extracted from the strained mix.

After the whole solid products were pressed, dry fish waste, or so-called pressed cakes and liquid mix were obtained. Press-liquor is composed of water, oil and sludge. While every mentioned element can be valorized, since the main goal of this work was to obtain oils, separation process was primarily based on the difference in specific gravities, by settling the press-liquor under the influence of gravity. However, after letting the mix settle for 10 days, oils, water and sludge were not completely separated.

As gravitational separation proved to be inefficient, a centrifugation approach was crucial to separate water, oil and sludge. The mixture was then put in small tubes dedicated for centrifugation, using a syringe, and then centrifuged at 13,4*1000 tr/min, for 30 min. After centrifugation, separation process was complete, clearly showing oils at the tube's top, water in the middle and sludge at the bottom. The final step of the extraction process was to precisely separate oil in each tube.

After extraction and separation, the fish oil sample was prepared following ISO 12966-2 norm for preparing fatty acid methyl esters (FAME), which can be summarized as follows: around 50 mg of the test sample were transferred into a 10 mL ground-neck flask; using a funnel, 2 mL 0.2 mol/L sodium methoxide solution were added to methanol, and brought to regulate boiling; a reflux condenser was connected, shaken and brought to a boil, and the mixture was refluxed until a clear solution was obtained, (which, for most oils, takes about 5 min., but requires up to 20 min., for long-chain or concrete saturated oils); the flask was removed from the heat source; after reflux stopped, the condenser was removed, and two drops of phenolphthalein were added; after sulfuric acid was added to the 1 mol/L methanol solution, so that it became colorless, further 0.2 mL were added; the condenser was connected and brought back to boil for 5 min; the flask was removed from the heat source and cooled under running water; after removing the condenser, 4 mL NaCl were added to the flask, which was shaken; 1 mL isooctane were added to the flask, which was closed and vigorously shaken for 15 sec; the flask was left to stand until the two phases had separated; finally, more NaCl was added, until the aqueous phase had reached the lower end of the flask neck. Fig. 1 shows final result after separation.

Figure 1: Fish oil after preparation of FAME.

Isooctane upper phase is suitable for GC analysis in accordance with ISO 12966-4. GC-MS conditions were according to [7]. For temperature programming, the oven was maintained at 80°C, during one min. The rate was increased from 10 °C per min to 250°C, and then slowed to 8 °C per min, until 280°C was reached, being maintained for 5 min. Split injection was conducted at a split ratio of 10:1, and He was used as carrier gas, at a rate of 0.8 mL/min, with injection volume of 1µl. GS-MS was performed in electron-impact mode, at 200 °C, with precolumn pressure of 70 kPa, injection temperature of 250 °C, interface temperature of 280 °C, electron energy of 70 eV, and solvent delay of 5.5 m.

Results and discussion

The obtained oil was of pure quality and highly condensed, since the extraction method was completely deprived of any chemical additives. The process was simple, and it may easily be applied for mass production, as it has low costs. For human consumption, the whole operation has to follow higher standards, to ensure elimination of any health risks.

The quantity obtained from 1 kg fish waste was almost 50 mL, which exceeded all expectations. Considering waste management costs that would have been spent on bringing fish waste into landfills, oil extraction is a much more beneficial procedure.

After preparing the oil according to ISO 12966-2 [8] norms, results for GC-MS screening reports on each major peak are shown in Figs 2 and 3, with different retention times (RT).

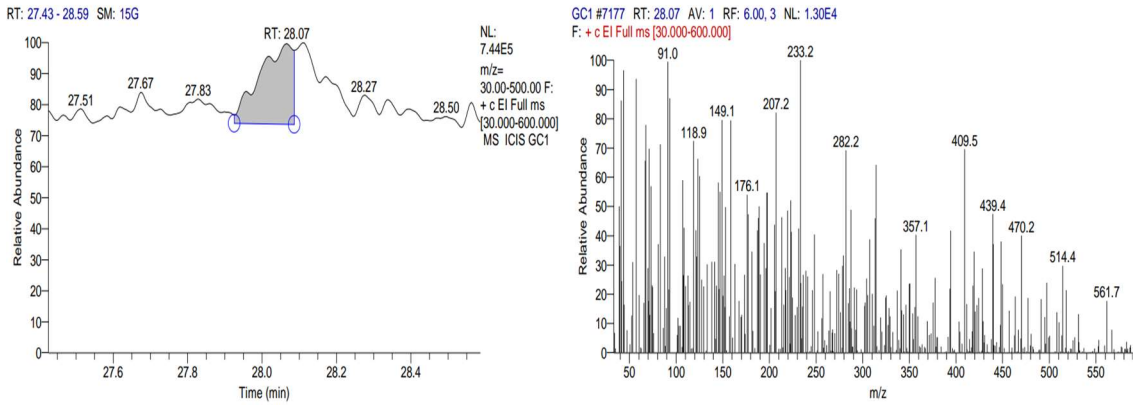

Figure 2: GC-MS screening report of the first peak.

Each of the peaks represents either a FA or one of its variants, with the same components, but in different compositions [9] and order. The analyzed oil is very rich in FA, with short and long chains. The most interesting ones in the scope of this work are EPA and DHA, Omega-3's main and most sought-after components. Unfortunately, with the absence of a database to compare GC-MS spectrum with, it was impossible to accurately quantify of EPA and DHA concentration in the

analyzed fish oil. However, molecular ion peaks correspond to molecular weights from EPA and DHA, in their FAME form derived for GC-MS analysis. EPA/FAME's (C20:5) molecular weight was 302 Da and DHA/FAME (C22:6) was 328 Da (7).

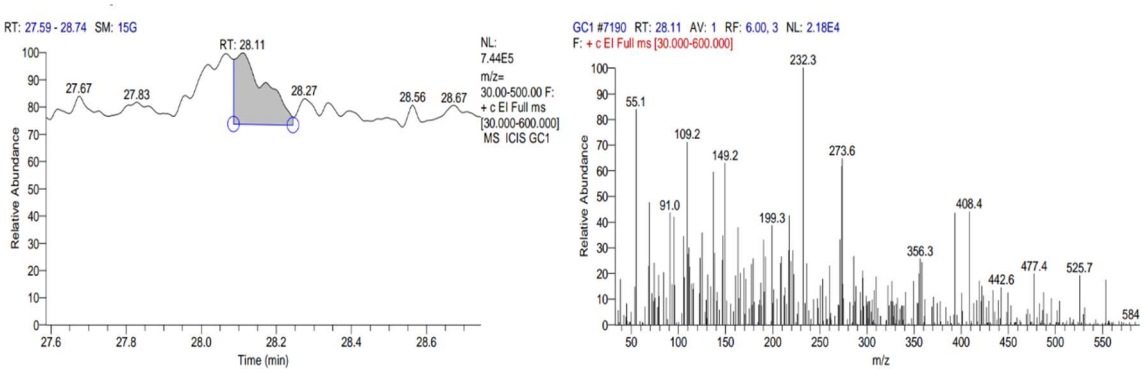

Figure 3: GC-MS screening report of the second peak.

Based on GC-MS data from the first report, it may be reasonable hypothesized that the sample contains EPA and DHA, even though direct molecular ion peaks were not observed (m/z 302 and 328 for EPA and DHA, respectively).

Firstly, RT observed in the report aligned with what was expected for PUFA. EPA typically elutes before DHA, due to its shorter carbon chain (20, compared to 22 from DHA). Peaks observed at RT ~28.07 and ~28.11 min, for EPA and DHA, respectively, were relatively close together, fitting expected elution order. The peak at RT ~28.11 min may correspond to DHA (5), which tends to elute later, due to its larger size and higher unsaturation.

Secondly, although molecular ion peaks were not detected, fragmentation patterns provide strong evidence for the presence of PUFA. The peak at RT 28.07 min shows fragments at m/z 233, 282 and 409, which are consistent with what is found in larger unsaturated molecules. Similarly, the peak at RT 28.11 min shows fragments at m/z 273, 408 and 525, which further suggest a complex molecule, consistent with DHA's known fragmentation pattern.

The absence of molecular ion peaks does not rule out the presence of EPA and DHA. These compounds often fragment heavily in GC-MS analysis [10], and the absence of molecular ions could be due to this extensive fragmentation. Additionally, if the sample was not derived into methyl esters (FAMEs), this could further explain why molecular ions are not detected clearly.

Given that EPA and DHA are typically found in marine and biological sources, RT and fragmentation patterns observed in the spectrum are consistent with these compounds. While the evidence is not definitive without molecular ion peaks, data strongly suggests that EPA and DHA might be present.

To confirm the presence of EPA and DHA, further analysis should be conducted using known EPA and DHA standards under identical GC-MS conditions. Derivation of the sample into FAME would also help improve identification of these FA. Moreover, re-examining m/z 302 and m/z 328 regions could help identify missing molecular ions.

In conclusion, while definitive identification is not possible without the molecular ion peaks, data strongly indicates the possible presence of EPA and DHA in the sample.

Precise determination of EPA and DHA and their respective quantities in the studied sample required a deeper analysis and comparison with fixed models. The main aim of this analysis was to prove that valorization of fish waste into fish oil can indeed create pure high-quality oils with the presence of its most important Omega-3 components.

Conclusions

Depending on the approach, fish waste can either be a huge problem or a valuable asset. Elimination in landfills is no longer a solution, due to the daily increase of waste production around the world. This work presents a method of valorization using a simple process to create valuable fish oil completely organic and rich in FA, especially EPA and DHA. The method can be further perfected to improve the quality of the final product, and make it fit for human consumption without any risks.

The extraction procedure is just a basic approach to prove the feasibility of the process. With more investments and technological advancement, the quality and quantity of the final product will increase exponentially, becoming a sustainable and affordable Omega-3 source.

Authors' contributions

H. Essabiri: main idea and primary author for original manuscript. **M. Hachi**: conception and revision. **R. Damrani**: proof reading and editing. **El H. Abba**: supervision and correction.

Conflict of interest

The authors declare that there are no conflict of interests regarding the publication of this manuscript. In addition, ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely addressed by the authors.

Availability of data and materials

Data that support the findings of this study are available at reasonable request from the corresponding author.

Abbreviations

DHA: Docosahexaenoic acid
EPA: Eicosapentaenoic acid
FA: fatty acids
FAME: fatty acid methyl ester
GC-MS: Gas Chromatography Mass Spectrometry
m/z: mass-to-charge ratio
PUFA: polyunsaturated fatty acids
RT: retention time

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