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Valorisation of fish-cannery by-products targeting the recovery of ω3 lipids

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ABSTRACT

Over the past years, ω3 fatty acids, namely EPA and DHA, have been recognized as presenting multiple health benefits. Several studies consider fish oil as the most important source of EPA and DHA.

Nowadays, canned fish industry plays a very important role in Portuguese economy. However, expansion of this business brought some environmental concerns due to the high amount of by-products generated. Nevertheless, this problem can be substantially reduced by the recovery of some of the by-product components, diminishing its contamination load and simultaneously obtaining value-added products.

This study was born from the growing interest in obtaining new sources of lipids rich in ω3 fatty acids, combined with environmental concerns related to the production of wastes from the fish canning industries, rich in these compounds. It thus intends to evaluate lipid extraction methods in liquid by-products from the fish canning industry, aiming to obtain fractions rich in ω3 fatty acids. Additionally, in a biorefining concept, the protein content of the remaining aqueous fractions was also quantified.

KEYWORDS: Cannery industry; fish by-products; lipid extraction; ω3 fatty acids; high hydrostatic pressure

RESUMO

Nos últimos anos, têm sido reconhecidos aos ácidos gordos ómega-3, nomeadamente EPA e DHA, múltiplos benefícios para a saúde. Além disso, vários estudos consideram o óleo de peixe como a fonte mais importante de EPA e DHA.

Hoje em dia, a indústria conserveira desempenha um papel muito importante na economia Portuguesa. Contudo, a expansão deste negócio originou algumas preocupações ambientais devido à quantidade de subprodutos gerados. No entanto, este problema pode ser substancialmente reduzido através da recuperação de alguns componentes presentes nos subprodutos, diminuindo assim a carga de contaminação dos mesmos e obtendo simultaneamente produtos de valor acrescentado.

Este trabalho teve origem no crescente interesse em obter novas fontes de lípidos ricos em ácidos gordos ω3, aliado a preocupações ambientais relacionadas com a produção de resíduos procedentes da indústria conserveira, ricos nestes compostos. O presente trabalho teve como principal objetivo o estudo de diversos métodos de extração de lípidos dos subprodutos líquidos originados na indústria conserveira, com o objetivo de obter frações ricas em ácidos gordos ómega-3. Adicionalmente, e numa perspetiva de biorefinaria, o teor proteico das frações aquosas remanescentes foi também quantificado.

PALAVRAS-CHAVE: Indústria conserveira; subprodutos de peixe; extração de lípidos; ácidos gordos ω3; alta pressão

TABLE OF CONTENTS

ACKOWLEDGEMENTS ii

ABSTRACT iii

RESUMO iv

INDEX OF TABLES vii

INDEX OF FIGURES viii

ACRONYMS x

CHAPTER 1 – INTRODUCTION 1

1.1. Portugal and the fish market 1

1.2. Fish canning industry 3

1.3. By-products generated: environmental impact and potential economic value 5

1.3.1. Environmental impact 5

1.3.2. Potential economic value 6

1.4. Extraction methodologies 8

1.4.1. Chemical Methods 9

1.4.2. Physical methods 11

1.4.3. Physico-chemical methods 11

1.4.4. Biological methods 12

CHAPTER 2 – EXPERIMENTAL 13

2.1 Samples 13

2.2 Extraction processes 13

2.3.1 Physico-Chemical methods 13

2.2.1.1. Temperature and solvents 13

2.2.1.2. High Hydrostatic Pressure (HHP) and solvents 13

2.3.2 Biological methods 15

2.3 Analytical assays 15

2.3.1 Biochemical composition 15

2.3.1.1. Total lipid content 15

2.3.1.2. Protein content 16

2.3.1.3. Fatty acid profile 16

2.4. Statistical analysis 17

CHAPTER 3 – RESULTS AND DISCUSSION 18

3.1 Extractions with temperature and solvents 18

3.2 High Hydrostatic pressure (HHP) and solvents 19

3.2.1 1st set of experiments 19

3.2.1.1. Total lipid content 19

3.2.1.2. Protein content 21

3.2.1.3. Fatty acid profile of lipid content 22

3.2.2 2nd set of experiments 25

3.2.2.1. Total Lipid content 25

3.2.2.2. Protein content 26

3.2.2.3. Fatty acid profile of lipid content 27

3.3 Biological methods 30

3.2.1 DH, total Lipid and protein content and fatty acid profile 30

CHAPTER 4 - CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK 34

4.1 Conclusions 34

4.2 Suggestions for future work 35

4.2.1 Alternative solvents 35

4.2.2 Qualitative evaluation of lipid and protein fractions 35

REFERENCES 36

INDEX OF TABLES

Table 1- Amount of catched fish by groups of species in Portugal (DGRM) 1
Table 2 - Fish by-products, possible value-added products and their applications (adapted from Ferraro et al., 2013)... 7
Table 3 - Experimental conditions used in HHP experiments. 14
Table 4 - Fatty acid profile in g per Kg of lipid extracted in mackerel wastewater in the 1st set of experiments...24
Table 5 - Fatty acid profile in g per Kg of lipid extracted in mackerel wastewater in the 2nd set experiments...28
Table 6- Fatty acid profile in g per Kg of lipid extracted in mackerel wastewater in the 2nd set experiments (continuation). 29
Table 7 - Fatty acid profile in g per Kg of lipid extracted in mackerel wastewater in hydrolysis. 33

INDEX OF FIGURES

Fig. 1 - Variation of the captured fish volumes in the last 3 years (DGRM). 2

Fig. 2 - Canning steps for sardine, sardine-type fish and mackerel, according to the Mediterranean tradition, along with the residues generated (Ferraro et al., 2013). 4

Fig. 3- Components of sardine and mackerel and their average proportion (Penven et al., 2013). 6

Fig. 4 – Chemical structure of EPA and DHA molecules (Kralovec et al., 2012). 8

Fig. 5 – Extraction process categories and their processes. 9

Fig. 6 - Lipid amount in g per Kg of mackerel wastewater, for different temperatures (50°, 70° and 90°C) and different centrifugation velocities (4000, 8000 and 12000 rpm). 18

Fig. 7 Lipid amount in g per Kg of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 MPa of pressure for 10 min. 20

Fig. 8 - Lipid amount in g per Kg of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 and 500MPa of pressure for 10 min. 20

Fig. 9- Protein amount in g per Kg of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 MPa of pressure for 10 min. 21

Fig. 10 - Protein amount in g per Kg of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 and 500 MPa of pressure for 10 min. 21

Fig. 11- ω3 amount in g per Kg of lipid of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 MPa of pressure for 10 min. 22

Fig. 12 - ω3 amount in g per Kg of lipid extracted in mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 and 500 MPa of pressure for 10 min. 22

Fig. 13 – Lipid amount in g per Kg of mackerel wastewater for 2nd set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 150, 300 and 450 MPa of pressure for 10 min or 20 min. 25

Fig. 14 - Protein amount in g per Kg of mackerel wastewater for 2nd set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 150, 300 and 450 MPa of pressure for 10 min or 20 min. 26

Fig. 15 - ω3 amount in g per Kg of lipid extracted in mackerel wastewater for 2nd set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 150, 300 and 450 MPa of pressure for 10 min or 20 min. 27
Fig. 16 -, Lipid amount in g per Kg of mackerel wastewater for the various hydrolysis degree (0,1, 0,6 and 1,1%) at different reaction time (3h and 6h. 30
Fig. 17, Protein amount in g per Kg of mackerel wastewater for the various hydrolysis degree (0,1; 0,6 and 1,1%) at different reaction time (3h and 6h. 31
Fig. 18 – ω3 amount in g per Kg of lipid extracted in mackerel wastewater for the various hydrolysis degree (0,1; 0,6 and 1,1%) at different reaction time (3h and 6h..........................31

ACRONYMS

DH - Degree of Hydrolysis

DHA - Docosahexaenoic Acid

DME - Liquid Dimethyl Ether

EPA - Eicosapentaenoic Acid

FFAs – Free Fatty Acid

HHP - High Hydrostatic Pressure

ILs - Ionic Liquids

MUFAs - Monounsaturated Fatty Acids

PI – Polarity Index

PUFAs – Polyunsaturated Fatty Acids

SFAs - Saturated Fatty Acid

TAGs - Triacylglycerols

$\omega 3$ – Omega-3 Fatty Acids

CHAPTER 1 – INTRODUCTION

1.1. Portugal and the fish market

Favoured by its geographical position and coastline with 830 kilometres, Portugal is a country with a very rich shore in what concerns to flora and fauna (Pereira, 2004). The relative abundance of species is provided by the location in a transition zone to warmer ecosystems. Although this aspect contributes to a high biodiversity, in the case of some species such as fish, there are a few constraints caused by physical and biological conditions that influence their quantities (DGRM, 2013).

In the last few years, the global amount of captured fish in Portuguese seashore was around 100 000 ton/year, despite its expected decrease (DATAPESCAS, 2014). Such diminution is a consequence of the over-exploitation of marine resources - that caused a considerable reduction of raw material - as also the obstacles in fishing industry such as the increase of fuel price and the high production costs. According to Direção-Geral de Recursos Naturais, Segurança e Serviços Marítimos (DGRM), the amount of captured fish in 2014 was 14,2% lower when compared to 2013. Besides all the reasons previously refereed, another cause in this particularly case was the application of *Portaria 188-A/2014*, that forbid the sardine fishing in Portuguese continental shelf once the maximum limit of catch was reached (INE, 2015; Penven *et al*, 2013). The global amount of fish catch by groups of species in Portugal, in the past 3 years is represented in Table 1.

Table 1- *Amount of catched fish by groups of species in Portugal (DGRM)*

Groups of species (tons)	2012	2013	2014
Mackerel	27 968.3	29 892.0	21 535.8
Sardine	20 639.9	20 115.2	16 034.7
Horse mackerel	11 673.2	12 442.2	13 012.0
Octopus	5 964.6	9 648.8	6 658.4
Hake	2 018.6	2 120.8	1 971.3
Jack mackerel	3 152.2	2 036.0	1 784.2
Black scabbardfish	1 994.0	1 569.3	1 617.3
Pout	1 703.6	1 264.5	1 476.6
Clams	980.7	932.0	1 329.5
Blue whiting	1 767.3	1 710.3	1 182.0
Cuttlefish	1 019.8	1 135.5	1 096.7
Cockle	823.2	670.4	1 023.0
Conger	956.2	780.1	912.8

Anchovies	656.2	324.5	811.5
Skates	844.3	831.7	801.4

In order to better visualize the variations occurred during this period of time, the volumes of captured fish for the two most important species in volume (sardine and mackerel) are depicted in Figure 1.

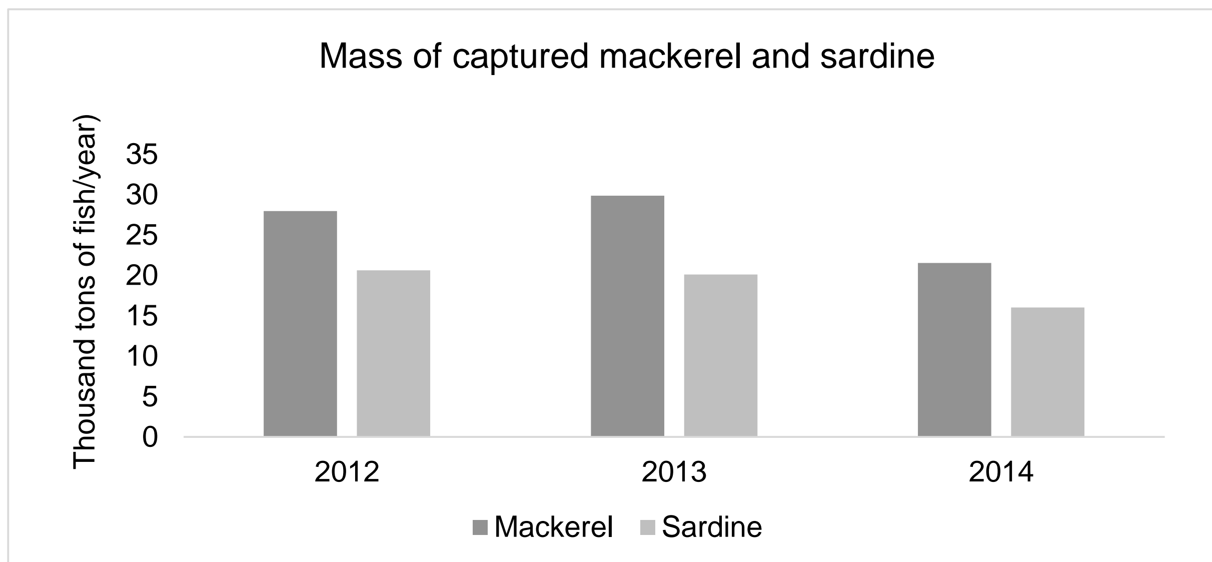


Fig. 1 *- Variation of the captured fish volumes in the last 3 years (DGRM).*

The strong inbound with the sea, linked to political and social factors and the necessity of food preservation triggered the establishment and increase of fish canning industry in Portugal. In fact, this country was the main global producer of fish canning industry before I World War, and canning industry had moments of strong activity and growth, as well as several moments of crisis (Serra, 2007). In recent times, an important economic development was verified: between 2010 and 2012, fish canning exportations increased 32,7%, representing 186,6 millions of € in 2012 (Nunes, 2013).

One of the major concerns of this industry is related with the amount of wastes generated, since they may reach 75% of the initial raw material (Ferraro et al., 2013). Indeed, the expansion of industrial fish processing, namely fish canning, has the adverse effect of increasing the amount of residues produced, which may represent around 14,4% of the global fish residues. These compounds can generate a serious environmental problem, due to their very rich nutritional content. Therefore, the scientific community is doing an effort to improve existing technologies and develop new ones for removing these nutrients in the several residues produced, so that they can be released into the environment with less potential hazard

(Ferraro et al., 2013). On the other hand, the recovery of these nutritional compounds may become a relevant source of revenue to the companies, allowing the creation of new jobs as well as an improvement of the environment (Blanco *et al*, 2007).

The framework of this study is to explore procedures to recover lipids, with special emphasis on polyunsaturated ω3 fatty acids, from liquid by-products arising from cannery industries. This goal may simultaneously reduce the organic level of effluents generated, and provide functional ingredients (polyunsaturated ω3 fatty acids) to incorporate in other food products.

1.2. Fish canning industry

Statistical monitoring data reveals that ca. 21% of all fish taken from the sea is not tapped for human consumption (Blanco *et al.*, 2007). Among this fraction one can consider discards, wastage on board and wastage ashore. Discards represent all the lost fish, most of which is returned to the sea, mainly due to the presence of by-catch fish. Other important fraction is wasted aboard, as residues generated during processing within the fishing boats. The last non-used portion is the one exploited in this study and is related with the wastage produced ashore, again during processing, specifically during canning processing (Blanco *et al.*, 2007).

The term "waste" is not to be understood as excluding substances with potential economic re-utilization. In fact, the concept does not presume that the holder disposing of a substance intends to exclude all economic reutilization of the substance or object by others (Directives 75/442/EEC and 78/319/EEC1). Food supply chain waste (i.e., the organic material produced for human consumption that is discarded, lost or degraded), has been identified as particularly interesting due to the volumes produced and the range of chemical compounds with known properties and markets it contains. The food industry generates considerable amounts of waste under the form of off-specification products, processing by-products, residues and wastewater.

The search for solutions to recover by-products and decrease the potential hazard of wasted material generated during canning processing is of paramount importance. However, in order to reach these goals, the identification and characterization of by-products and wastes generated during industrial processing is mandatory.

Generally, there are two distinct canning methods for fish: the raw pack method (also known as the Traditional Mediterranean method), and another one in which a hot smoking step is incorporated, rather than pre-cooking in the can (the Norwegian method) (López, 1999). The traditional Portuguese canning process and the corresponding leftovers are described in Figure 2.

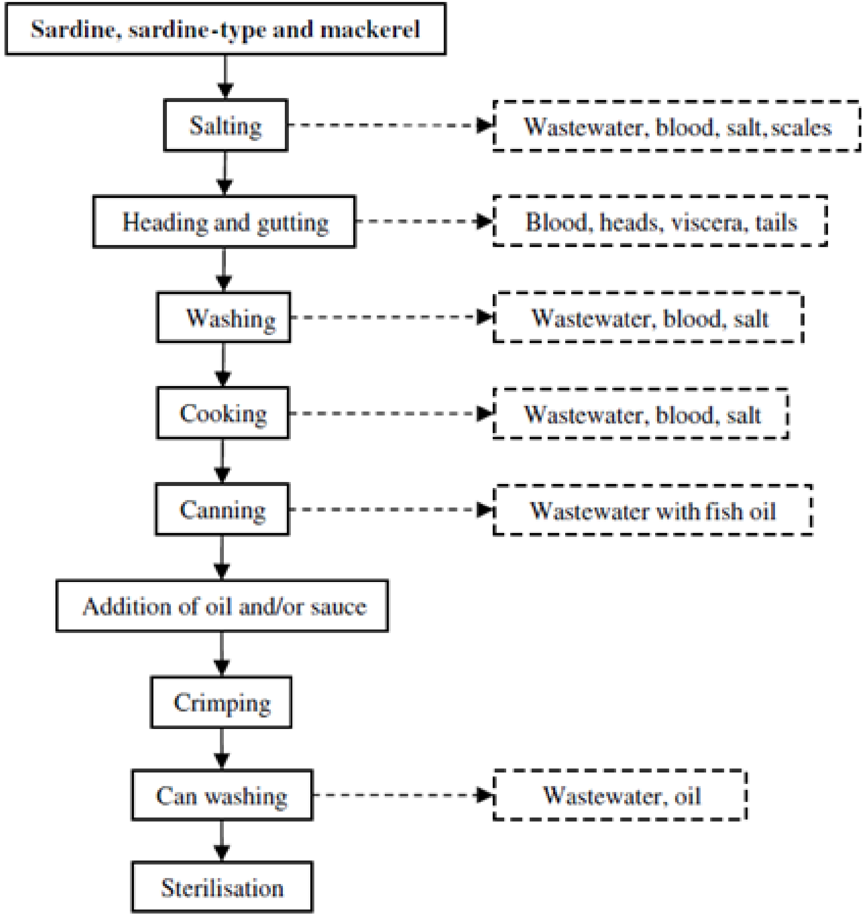


Fig. 2 *- Canning steps for sardine, sardine-type fish and mackerel, according to the Mediterranean tradition, along with the residues generated (Ferraro et al., 2013).*

As observed in Figure 2, the initial step of traditional canning process is the fish brining, whose main purpose is to provide flavour to the product. The length of this procedure depends on the size and fat content of pelagic species, but in sardine it usually takes fifteen to twenty minutes to obtain a final salt content between 1-2%. Furthermore, the presence of salt increases the osmotic pressure inside the cells, thus causing dehydration, and since the fat material is not in contact with the air, the rancidity is substantially decreased. Around 10% of global canning process wastewaters arise from this stage, containing mainly blood, salt and scales (Ferraro *et al.*, 2013; López, 1999).

Heading and gutting is the next stage, which can be either mechanical or handmade. However, the heterogeneous size of sardine and mackerel in different batches render the

manual manufacture more common. The resulting residues are blood, heads, viscera and tails (Ferraro *et al.*, 2013). The subsequent step consists in washing, heading and eviscerating fish, to avoid the presence of non-desired solids. This process represents about 35% of all wastewaters of the cannery industry; blood and salt are also scrapped (Ferraro *et al.*, 2013; López, 1999).

The subsequent stages are cooking and canning, either in this sequence or in a reverse one. In the former, the prepared fish is placed on perforated trays that facilitate the spillage of oil and water, followed by a thermal treatment with steam at approximately 100°C and atmospheric pressure. The objectives of this thermal cooking are: (i) to eliminate part of the water in the meat, so that it is not liberated inside the can during the sterilization; (ii) eliminate part of the oils/grease, which may provide strong flavours to the final product; (iii) coagulate fish proteins, facilitating the later removal of the skin, spine and provide certain characteristics to the product such as colour, texture and flavour (López, 1999). The amount of liquid effluents generated in this part of the process is around 15% of the global liquid residues produced. Once fish is cooked, it is packaged in cans. Depending on the final product, oil and/or sauce may also be added and the can is ready to be sealed in crimping machines (Ferraro *et al.*, 2013; López, 1999).

Finally, cans are washed and consecutively sterilized. The can washing ensures that all remain residues from the process are removed; this is the step that represent the major fraction of liquid effluents, 40%. Like cooking, sterilization submits the canned fish to a thermal treatment with the appropriate duration to destroy or inactivate microorganisms (Ferraro *et al.*, 2013; López, 1999).

1.3. By-products generated: environmental impact and potential economic value

1.3.1. Environmental impact

In what concerns the canning process already described, it is possible to emphasize some factors that have large influence on the environmental impact of this type of activity. The first one is the use of water: this natural resource is used in brining, in distinct washing steps and even in cases where steam generation is required. Other core resource is electric power, intended to operating equipment, lighting, refrigeration, etc. It should also be taken into account the energy expended in the production of steam. Thus, besides fish, water and electricity represent the most used inputs on the entire production process (López, 1999). Detergents used in washing steps, which are dumped in wastewaters and may cause water pollution can't be ignored too. Nor can fail to mention the presence of strong odours mainly present when the cooking of raw material is performed (López, 1999).

Lastly and extremely important are the different generated by-products or wastes with organic components, which can be separated into two groups, concerning their physical state: solids or liquids. Solid ones (Figure 3) can represent between 40-70% of the animal, and are usually sold to produce fishmeal (Ferraro *et al.*, 2013; Penven *et al.*, 2013), whereas liquid by-products are usually discarded.

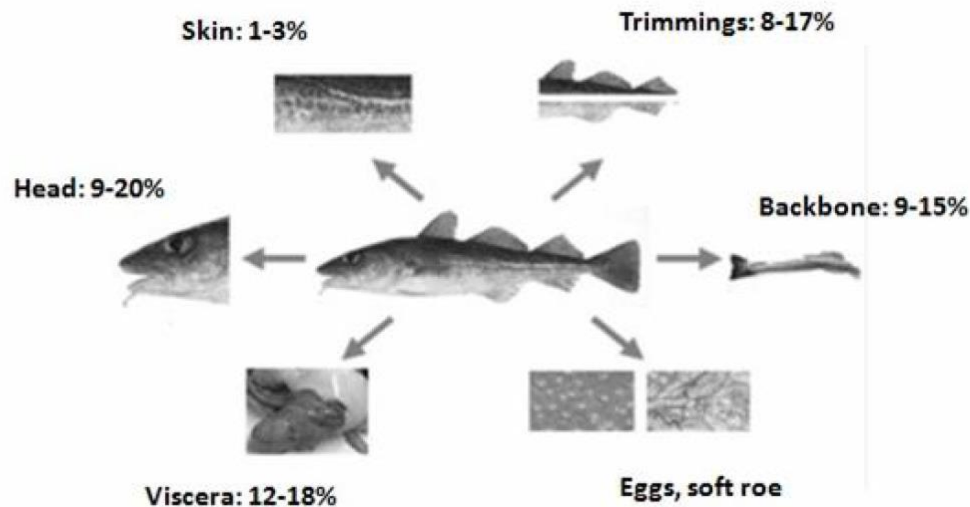


Fig. 3- *Components of sardine and mackerel and their average proportion (Penven et al., 2013).*

1.3.2. Potential economic value

Although fishmeal represents the largest destiny of by-products, such solution is not economically interesting because they are sold at very low prices; alternatively, the recovery of bioactive compounds from by-products can generate a payback 80 times higher or more, depending on their purity (Ferraro *et al.*, 2013). However, the isolation of high-value by-products is a process that requires high implementation and maintenance costs, thus increasing the payback period of the investment. As a result, very few fish origin value-added by-products were able to reach the market and be sold in large quantities until now (Olsen *et al.*, 2014). Possible explanations rely on overestimation of market possibilities, too small amounts of high quality by-products available on a regular basis, and very high costs of isolating specific components often present (Olsen *et al.*, 2014). Furthermore, the utilization of by-products for human consumption as food ingredients requires the implementation of quality systems and is regulated by specific legislation (Secretariat of the Pacific Community, 2014). However, some of these limitations may be surpassed, e.g. by focusing on the recovery of bioactive compounds with already existent (and high-valued) market, such as ω3 fatty acids.

Depending on the type of by-product, it is possible to obtain different value-added compounds with distinct characteristics and applications (Table 2).

Table 2 *- Fish by-products, possible value-added products and their applications (adapted from Ferraro et al., 2013).*

By-products	Value-added compounds	Applications
Skin, scales and bones	Collagen, collagen hydrolysates, gelatine	Cosmetic, pharmaceutical and food industries
Scales and bones	Hydroxyapatite	Fertilisers and biomaterial industry
Viscera	Proteolytic enzymes	Food and feed industries
Heads, tails, viscera, Skin, bones, fins, scales and frames	Total proteins, minerals, lipids (ω−3 fatty acids)	Food industry
Flesh residues and cooking wastewater	Total proteins, lipids (ω−3 fatty acids)	Food industry
Salting wastewater	Free amino acids and bioactive peptides	Food and pharmaceutical industry

Among the various possible value-added compounds extracted, ω3 fatty acids are one of the most known, due to their effects in human health. Omega-3 fatty acids belong to a broad group of polyunsaturated fatty acids (PUFA), which are fatty acids with long chain carbonic molecules. The letter "ω" represents the position of the first double bond counting from the last methyl group, and the number "3" is the amount of carbon atoms associated to the previously mentioned position. Docosahexaenoic (DHA) and eicosapentaenoic acids (EPA) are the most important and researched ω3, presenting 22 and 20 C-atoms with 6 and 5 cis-double bonds, respectively (Gupta *et al* 2012; Montañés *et al*, 2012).

Several studies demonstrated that the intake of ω3 fatty acids prevents cardiovascular diseases, some types of cancer and inflammatory autoimmune diseases, including type 2 diabetes; they have also an important effect on brain function and retina, since they play a key role in cell structure; besides that a positive impact on attention deficit/hyperactivity disorder (ADHD), dyslexia, skin disorders and asthma were notice. On the other hand, a poor feeding in these fatty acids accelerates the aging process and increases the likelihood to develop degenerative and cardiovascular diseases (Bermúdez-Aguirre & Barbosa-Cánovas, 2010; Montañés *et al*, 2012).

EPA and DHA molecules are depicted in Figure 4; due to the presence of bisallylic methylene groups and all double bonds being in the cis-configuration, they have a preponderant easiness to modify their structure (Kralovec *et al*, 2012).

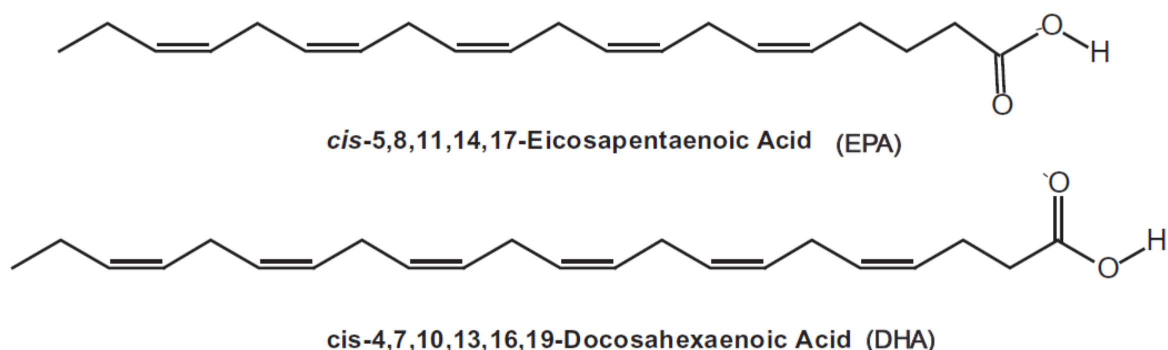


Fig. 4 *– Chemical structure of EPA and DHA molecules* (Kralovec et al., 2012).

These bioactive compounds are mainly found in fish oil, reaching ca. 30% of the total fatty acid content, although this value is dependent on water temperature, salinity of the water, fish species, fatty acid composition of the available diet, catch season, gonad maturation stage and fish age and sex. According to some authors, the spawning period represent an important decrease of the total lipid and fatty acid composition of fish, once in this stage all the lipids stored will be send to gonads in order to ensure maturation (Pacetti, 2013). In the case of sardine, EPA and DHA content can reach around 1,2g in 100g of wet sardine, and for mackerel the value is equivalent (Usydus & Szlinder-Richert, 2012; Bouderoua *et al*, 2011). The daily recommended doses for normal adults are between 0,25 and 0,5g of EPA plus DHA, depending on their health condition (EFSA, 2012).

1.4. Extraction methodologies

When extracting lipids or fats, the selected method is extremely important to achieve a complete or close to complete extraction. Otherwise, the extraction may be incomplete, or the extract may contain a large amount of impurities. Extraction techniques may be roughly divided into 4 groups: chemical, physical, physico-chemical and biological processes.

Chemical methods are processes that allow the extraction of lipids using one or more organic solvents. Since they normally have relatively simple procedures and are well studied, they are the most commonly used. In contrast, they have a very significant environmental impact, due to the large amounts of solvents used and subsequently discarded (Adeoti & Hawboldt, 2014).

Physical methods use mechanical operations and changes in temperature and/or pressure. One disadvantage is that the high temperatures that may be required can damage the extracting material and represent an increase in energy consumption. Nevertheless, these methods are usually employed to obtain fishmeal and fish oil (Adeoti & Hawboldt, 2014).

In the case of physico-chemical methods, as the name indicates, characteristics of chemical and physical methods are allied. These procedures link the utilization of organic solvents with temperature and pressure; in recent years they have also being associated with alternative technologies (e.g. microwaves, supercritical extraction, etc.). The use of these methods intends to conjugate the high yields offered by organic solvents with physical methods, thus allowing the reduction in organic solvents (Adeoti & Hawboldt, 2014).

Finally, biological processes are widely used in the concentration of proteins, also providing the obtainment of high amounts of oil due to increasing lipid release. These processes are essentially characterized by the use of enzymatic or bacterial activity. The absence of organic solvents, requirements of low temperature and low energy consumption make them less hazardous to the environment (Adeoti & Hawboldt, 2014). However, for lipid extraction, although the use of enzymes may help to release bound lipids, thus enhancing extraction yields, an additional step with organic solvent extraction is mandatory, in order to recover the lipid fraction. Figure 5 summarizes the distinct categories of extraction methods.

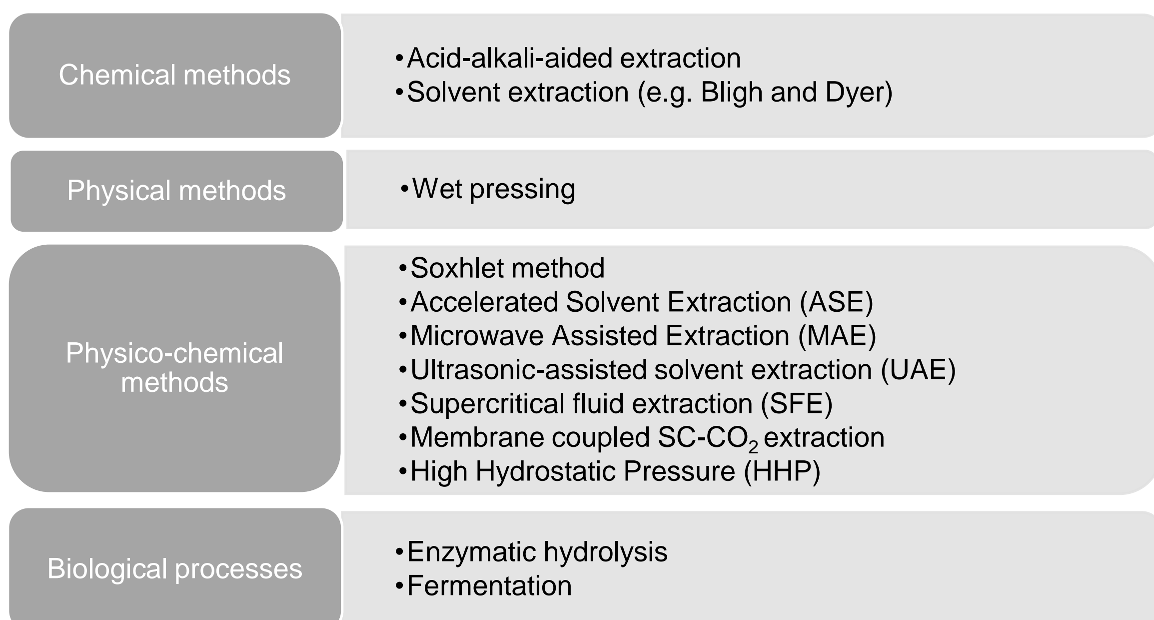


Fig. 5 *– Extraction process categories and their processes.*

1.4.1. Chemical Methods

The most general techniques to extract lipids from fish matrices are solvent extraction and acid-alkali-aided extraction.

Solvent extraction is a widely used technique for the extraction of lipids, and the most commonly used solvents are hexane, benzene, cyclohexane, acetone, methanol and chloroform. Oils, including fish oils, are generally soluble in organic solvents, which break the cell walls or split the bonds between lipids and tissue matrix, allowing their extraction. In order to choose the most appropriate solvent for each type of compound, some factors must be taken into account: it is recommended to know the solubility of the compounds to be extracted, work with solvents with low boiling point and take into consideration the solvent price, toxicity, availability and reusability. Furthermore, for an effective extraction, it is desirable that the solvent penetrates the lipid completely; therefore, solvent polarity must coincide with the polarity of the target compounds (Adeoti & Hawboldt, 2014). As lipids are relatively non-polar molecules, they can be extracted from a sample by using relatively non-polar solvents. With a non-polar solvent, only non-polar molecules in the sample dissolve, while polar ones do not. Problems arise however, in cases where lipids are bound in animal or plant cell membranes. Animal and plant cell membranes are made up of molecules that have both polar and non-polar regions, such as triglycerides (molecule with polar glyceride heads and non-polar fatty acid tails) and phospholipids (similar to triglycerides, but a phosphate group replaces the fatty acid tail). These molecules group together with their polar heads sticking outwards and non-polar tails inwards, making it difficult for non-polar solvents to interact with the non-polar tails and extract them. As these molecules are composed from a part non-polar and a part polar, extracting solvents must present both of these characteristics; this is the main reason why a mixture of solvents is usually employed.

Bligh and Dyer procedure (Bligh & Dyer, 1959) is one of the methods with higher performance in the extraction of lipids from fish tissues, which uses a mixture of chloroform and methanol in water. This procedure is based on Folch procedure (Folch *et al.*, 1957), combining the use of polar and non-polar solvents, but with different volumes, in order to extract the maximum amount of lipids as possible. Some of the mixtures used in this process had the best throughput obtained up to now. However, for large-scale, this method generates extensive amounts of solvents discarded, making the recycling high priced and raising various security issues. The possible contamination of food with these organic solvents is another concern (Adeoti & Hawboldt, 2014). In order to use less environmentally hazardous solvents, alternative methods using isopropanol and hexane (Hara & Radin, 1978) have been developed.

The acid-alkali-aided extraction is a procedure that employs an alkali or acid digestion of the sample. The major disadvantage of this method is the simultaneous extraction of other non-lipid compounds. Very important too is the high destructive power of acid hydrolysis that may degrade some fatty acids, hindering their extraction (Adeoti & Hawboldt, 2014). Nevertheless, extraction yields are usually high.

1.4.2. Physical methods

The most common physical method in fish industry is wet pressing, which enables recovery of a high volume of fish oil (between a mass fraction of 1,4% and 40,1% (Adeoti & Hawboldt, 2014). Briefly, after cooking, which promotes protein coagulation (thus contributing for the release of oil and water), solid and liquid phases are separated by pressing or centrifugation and draining (Adeoti & Hawboldt, 2014).

1.4.3. Physico-chemical methods

Almost all physico-chemical methods emerged as modifications from existing procedures. The most known is probably Soxhlet method, which is based on a solid-liquid extraction with a refluxing solvent at high temperatures. Although the solid does not get in direct contact with the solvent, the reflux system allows that, after condense, the solvent percolate in the solid sample, solubilizing the substances to extract. As in the abovementioned solvent extraction methods, this approach can also provide considerable efficiencies, depending on the properties of the solvent and the type of compound to be extracted. On the other side, this is a time-consuming and not automated process, which hinders its implementation in the industry, and produces large quantities of organic wastes (Adeoti & Hawboldt, 2014).

The Accelerated Solvent Extraction (ASE) is a modification of Soxhlet method. As many of the solvents used have high boiling points, this method proposed that the reflux system operates using only an increase of pressure and not an increase of temperature. Although it solves some of the limitations of the Soxhlet method, such as accelerating the process and increasing reproducibility, also in this case large amounts of waste solvents are generated. While it may be used in the extraction of lipids, it was not possible to recover all the lipids; besides, recovered quantities were not always significant, making it necessary to further study the method (Adeoti & Hawboldt, 2014).

Likewise other technologies, Microwave assisted extraction (MAE) may extract specific compounds of the samples with solvents and heat, in this case using microwave energy. This type of heating allows greater selectivity, thus minimizing energy losses, as it acts directly in polar solvents or material. It can be used to lipid extraction with low temperatures, simultaneous extractions with different samples, good efficiency, reproducibility and mechanization (Adeoti & Hawboldt, 2014).

Ultrasonic-assisted solvent extraction operates at the same way as the MAE, the interaction between the solvent and the sample is also at lower temperatures, but in this case through particle vibration, permitting the rupture of the cell. The largest difference between the two methods is related to the amount of water present in the oil, once when microwaves are used the reduce both polar and/or water content of fat cells (Adeoti & Hawboldt, 2014).

Supercritical Fluid Extraction (SFE) with carbon dioxide is another alternative. The major advantage of this method is the abolishment of organic solvents, minimizing toxicity and accelerating extraction and separation processes. Carbon dioxide is the preferred solvent due to its availability and low cost, low toxicity and non-flammable properties; since this solvent is in the gas state at room temperature and pressure, it is easily separated from the solute. For this procedure the pressure, temperature, CO_2 flow rate and extraction time are crucial. It was already been used to extract sardine oil but, as the moisture acts as a barrier against CO_2 diffusion into the sample and diffusion of lipids out of the cell, freeze drying of the sample is required prior SFE. Additionally, the equipment necessary for this procedure is very expensive and complex (Adeoti & Hawboldt, 2014).

Membrane coupled SFE add a nanofiltration system to the conventional system. This process allows the direct extraction of triglycerides from fish oil, separating them in short-chained triglycerides as permeate, and long chained triglycerides as retentate (containing EPA and DHA). Despite the large investment required for this process, the final product has extremely high purity (Adeoti & Hawboldt, 2014).

Finally, high hydrostatic pressure (HHP), conventionally used for pasteurization processes, can also be employed for extraction of bioactive compounds. Under HHP, the differential pressure between the interior and the exterior of cell membranes is extremely large, thus leading to rapid permeation, due to cell deformation and cell wall damage, and faster equilibrium concentration between the cell interior and the exterior, while increased solubility can also occur for several compounds. This procedure can be performed with low temperatures, allowing its use in thermo-sensitive compounds such as fatty acids (Santos *et* al, 2013).

1.4.4. Biological methods

Regarding biological processes, and depending on the origin of enzymes (internal or exogenous), the process can be labelled as autolysis or hydrolysis. To obtain silage, the most common is the autolysis of fish muscle, which uses enzymes from fish viscera (Adeoti & Hawboldt, 2014). Some proteolytic enzymes such as pepsin, trypsin, chymotrypsin, gastricins and elastase, when obtained from fish industry wastes, might be used in autolysis. The key benefits are high catalytic efficiency, even in low temperatures, lower thermostability and cold stability (Blanco *et al.*, 2007). Considering that the procedure avoids the recourse to drastic conditions as chemicals and heat, lipid release is increased when compared with other methods (Dumay *et al.*, 2009). Otherwise, exogenous lipases like *Thermomycetes lanuginosus* can also represent a good option, particularly, due to the capacity to remove shorter chain fatty acids while retaining EPA and DHA on the glycerol backbone (Kralovec *et al.*, 2012).

CHAPTER 2 – EXPERIMENTAL

2.1 Samples

Raw material used in this study was mackerel (*Scomber japonicus)* cooking wastewater, provided by La Gondola company (Matosinhos, Portugal). Mackerel was catched in Portugal seashore by purse seine fishery from August to September 2014. During canning processing, the fish was cooked, in order to reduce the amount of water present within the flesh; the corresponding condensate was collected in metal trays, filtered with cotton cheesecloth (in order to remove solid particles in suspension), recovered in plastic bottles and stored at -20°C until use.

2.2 Extraction processes

2.3.1 Physico-Chemical methods

2.2.1.1. Temperature and solvents

Lipids were extracted according to the procedure described by Hara and Radin (1978) with modifications (Alonso *et al*, 2003), using a mixture of isopropanol and hexane (4:3) at 50°C, 70°C and 90°C, under continuous mixing at 1500 rpm in a heating magnetic stirrer (AREC.X, Velp Scientifica), for 30 minutes. Phase separation was achieved by centrifugation at different velocities (4000 rpm, 8000 rpm and 12000 rpm), in a centrifuge (Heraus Megafuge 16R, Thermo Scientific). The upper organic phase was collected and the solvent was evaporated under low pressure (Buchi Rotavapor R-200, with Vacuum Controller V-850 and Heating Bath B-490), for quantification of total lipids by gravimetry.

2.2.1.2. High Hydrostatic Pressure (HHP) and solvents

Assays were conducted in a Hydrostatic press (FPG7100, Stanstead Fluid Power, Stanstead, United Kingdom), at Aveiro University, equipped with a pressure vessel of 100 mm inner diameter and 250 mm height, and surrounded by an external jacket to control the temperature. Pressure, time, nature of solvents and their proportion (thus, generating mixtures with different polarity index) were studied in two different experiments, as described in Table 3.

Table 3 - *Experimental conditions used in HHP experiments.*

1st set of experiments

Assay	Pressure (MPa)	Time (min)	Sample (ml)	Isopropanol+ Hexane (ml)	Hexane (ml)	Water (ml)	Ethanol 70% (ml)	PI
A	300	10	80	80+60	-	-	-	5,2
B	300	10	40	40+30	-	-	-	6,5
C	300	10	80	-	70	-	-	5,5
D	300	10	80	-	35	-	-	7,1
E	500	10	80	80+60	-	-	-	5,2
F	500	10	80	-	35	-	-	7,1

Different letters in assay identification mean experiments with different pressure, time, or solvents

2nd set of experiments

Assay	Pressure (MPa)	Time (min)	Sample (ml)	Isopropanol+ Hexane (ml)	Hexane (ml)	Water (ml)	Ethanol 70% (ml)	PI
A1	150	10	16	-	-	28	-	10,2
A2	150	10	16	-	-	-	28	7,97
A3	150	10	16	16+12	-	-	-	5,15
B1	300	10	16	-	-	28	-	10,2
B2	300	10	16	-	-	-	28	7,97
B3	300	10	16	16+12	-	-	-	5,15
C1	450	10	16	-	-	28	-	10,2
C2	450	10	16	-	-	-	28	7,97
C3	450	10	16	16+12	-	-	-	5,15
D1	300	20	16	-	-	28	-	10,2
D2	300	20	16	-	-	-	28	7,97
D3	300	20	16	16+12	-	-	-	5,15

Different letters in assay identification mean different conditions of pressure or time; different numbers following the same letter mean experiments with equivalent conditions of pressure and time, but different solvents

In the second set of experiments, tests with water and ethanol originated final mixtures with only one phase, due to the low amount of lipid fraction, thus preventing the recovery of an organic phase. Therefore, it was necessary to add an extra extraction step, in which 24 mL of the previously treated sample were mixed with 9 mL of hexane, placed 1 min in the vortex and separated by centrifugation. After centrifugation, the upper organic layer was recovered, dried and weighted.

2.3.2 Biological methods

Enzymatic hydrolysis was carried out under controlled conditions (pH 8 and 50°C), with different amounts of Alcalase® 2.4L (Sigma-Aldrich): 0.1%, 0.6% and 1.1% (v/v) in 20 mL of wastewater. After 3 or 6 hours of hydrolysis, enzymes were inactivated by heating at 95ºC for 15 minutes. The resulting aqueous solution was subsequently submitted to an extraction with isopropanol and hexane at 1500 rpm for 30 min, in order to allow lipids to migrate and be collected within the organic phase. Finally, determination of total lipids and fatty acid profile occurred in the organic phase, whereas the aqueous phase was also collected for protein quantification.

The hydrolysis degree (DH) was calculated according to the following equation:

$$\%DH = \frac{B \times M_b}{M_p \times \alpha \times h_{tot}}$$

where B is the volume of sodium hydroxide consumed during hydrolysis (mL), M_b is the molar concentration of sodium hydroxide, M_p is the mass of protein (g) in the raw material (determined according to Lowry method), α is the dissociation factor for α-NH2 groups (corresponding to a value of 0.88 at 50ºC), and h_{tot} is the total number of peptide bounds in the protein (corresponding to a value of 8,6 eq. g kg-1protein in fish samples) (Dumay *et al.*, 2009).

2.3 Analytical assays

2.3.1 Biochemical composition

Total lipids were determined gravimetrically by the method of Hara and Radin (1978) with modifications (Alonso *et al*, 2003), and total proteins were assayed by the Lowry method (Lowry *et al.*, 1951). Detailed protocol for both methods is provided below.

2.3.1.1. Total lipid content

Lipid extraction was performed by mixing 4 mL of sample with 4 mL of isopropanol, and 3 mL of hexane. After homogenization in a vortex, when adequate, a physical extraction step was performed. To allow phase separation, the mixture was centrifuged for 5 min at 8000 rpm and 4 °C. The supernatant was filtered to a glass tube and evaporated at 30-40 °C under vacuum. The results were obtained as g of lipid per kg of mackerel wastewater.

2.3.1.2. Protein content

A sample amount of 1 mL was inserted into a tube, subsequently 5 mL of reagent I was added; after 10 min of wait in the dark, 1 mL of reagent II was added. After vortexing, the solution rested for 30 minutes. The optical density was read at 750 nm and the amounts of protein were determined by comparison of the measured absorbance values with those obtained from a calibration curve, using bovine serum albumin as standard. The results were obtained as g of protein per kg of mackerel wastewater.

Reagent I: prepared by mixture of 48 mL of sodium carbonate solution (5%, w/v), 1 mL of Na,K-tartrate solution (2%, w/v) and 1 mL of copper sulphate solution (1%, w/v).

Reagent II: prepared by dilution of Folin-Ciocalteau reagent with water, in a 1:2 proportion

2.3.1.3. Fatty acid profile

Fatty acid methyl esters were obtained by transesterification of triplicate lipid samples, according to the acidic method described by Lepage and Roy (1984), with the modifications introduced by Cohen *et al.* (1988), using heptadecanoic acid as internal standard and acetyl chloride as catalyst.

Hence, the composition of lipid fraction was determined adding 1 mg of internal standard and the sample to a teflon-capped glass tube. Thereupon 2 mL of a freshly prepared mixture of acetyl chloride and methanol (5:100, v/v) were added. The tubes were heated at 90-100ºC for 1 hour, then cooled to 30-40ºC, 1 mL of hexane (with 0.01% BHT) was added and the solution was mixed in the vortex for a few seconds. Afterwards, 1 mL of pure water was added, it was gently mixed and the phase separation was allowed. Finally the upper phase was removed and collected in a GC vial. After GC analysis the results were expressed as g of fatty acid per Kg of lipid of mackerel wastewater.

The analysis of those esters was carried out with a Shimadzu GC-2010 gas chromatograph with AOC-20i Auto Injector, equipped with a flame ionization detector and a polar, 60 m long capillary column of fused silica (CP-Sil 88, Agillent). The injector and detector temperatures were 250 and 270°C, respectively, and the column temperature was placed at 100ºC for 5 min and subsequently increased until 215°C at a rate of 1°C min^{-1}. Pure standards (Sigma) were used for fatty acid identification, which was based on comparison of peak retention times of samples and standards. Peak areas were quantified and calculations were performed according to the AOCS Official Method Ce 1b-89 (AOCS, 1994).

2.4. Statistical analysis

Analysis of variance and Tukey (HSD) or Games-Howell post hoc tests were employed to statistically analyse the results, using IBM® SPSS® 22 Statistics software for Windows (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $p<0.05$.

CHAPTER 3 – RESULTS AND DISCUSSION

This chapter includes the results for all assays, with different parameters, for lipid extraction with temperature and solvents, for lipid extraction with HHP and solvents, both 1st and 2nd set of experiments, and finally lipid extraction for hydrolysis experiments. For each experiment total lipid amount, total protein amount, sum value of ω3 fatty acid amount and fatty acids profile of total lipids extracted were analysed, except for the first assays, where only total lipid amount was analysed. As previously stated, the main goal of this study was to ascertain the effects of different extraction methods on the total amount of lipids obtained from liquid by-products, and their fatty acid profile, with special emphasis on the amount of ω3 fatty acids. The amount of protein was also determined in order to evaluate the potential recovery of the aqueous phase, in a biorefinery concept.

3.1 Extractions with temperature and solvents

These tests were intended to perceive the roles of temperature during lipid extraction and centrifugation speed during solvent separation phase. For this purpose, tests with a mixture of isopropanol:hexane at different temperatures (50°, 70° and 90°C) and different centrifugation velocities (4000, 8000 and 12000 rpm) were performed.

By the observation of figure 6 it can be seen that for a speed of 4000 rpm the variation of the amount of lipid is quite low, which leads to believe that for this speed the temperature has no great influence. Also for 8000 rpm centrifugation velocity, a tendency behaviour was noted, for that velocity the rise of sample temperature result in an increase amount of lipid extracted.

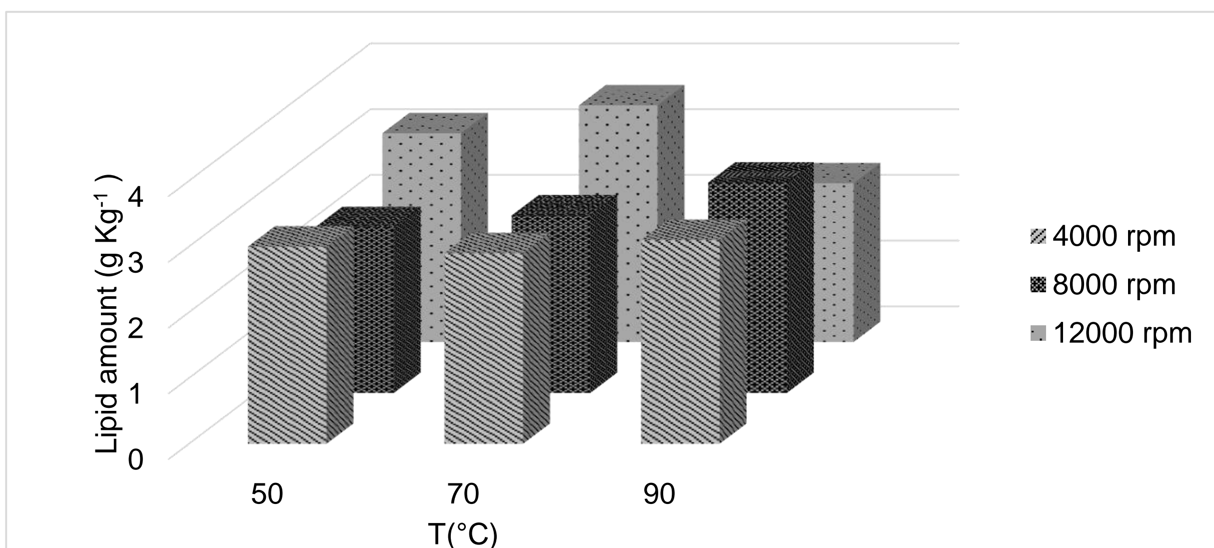


Fig. 6 *- Lipid amount in g per Kg of mackerel wastewater, for different temperatures (50°, 70° and 90°C) and different centrifugation velocities (4000, 8000 and 12000 rpm).*

Higher values of extracted lipids were obtained for temperatures of 50-70ºC and centrifugation speed of 12 000 rpm, which may indicate that temperatures higher than 70ºC during extraction process may hinder lipid extraction, whereas a higher centrifugation speed seems to facilitate phase separations. The higher amount of lipid obtained by the solvent extraction from mackerel wastewater was 3,6 g Kg^{-1}, at 70°C and 12 000 rpm. Most literature concerning lipid valorisation from marine by-products concern solid by-products, and comparative results to analyse were very difficult to obtain. A study from Civit, Parin and Lupin (1982) of the effect of pH and temperature on the recovery of protein and oil from fishery bloodwater waste, refers that temperatures above 75-80ºC do not improve the recovery, which was also found in these experiments. Another study from Garcia-Sanda, Omil and Lema (2003) on the recovery of wastes in tuna cooking effluents, reports amounts of 2 g/L in total lipids.

3.2 High Hydrostatic pressure (HHP) and solvents

3.2.1 1st set of experiments

As mentioned in material and methods for 1st set of experiments, the samples were submitted to HHP. Thus, the influence of pressure, the type of solvent and their volume were studied in pursuance to obtain the total lipids amount, fatty acid profile and protein amount recovered.

3.2.1.1. Total lipid content

Since different solvents with different volumes were used, the polarity index (PI) was used to enable a simplification of data. PI values were calculated as weighted values of the individual PI for each solvent and for water (the main component of samples). Therefore, those assays in which the amount of sample (i.e., water) is high will have an increased PI, because the water PI is higher than for other solvents used.

Results suggest that at the same pressure (300 MPa) the higher value of total lipid amount (1,6 mg of lipid per mL of sample) occurs for lower PI (5,2) as described in figure 7. For the remaining PI values, there are apparently no significant differences of lipid amount. These results are not unexpected, since lipids are compounds with low polarity and are readily soluble in organic solvents with low polarity. Thus, matching polarity of the targeted compounds increase the relative strength of interactions between the solvent and lipid molecules, thus enhancing the extraction process (Adeoti & Hawboldt, 2014).

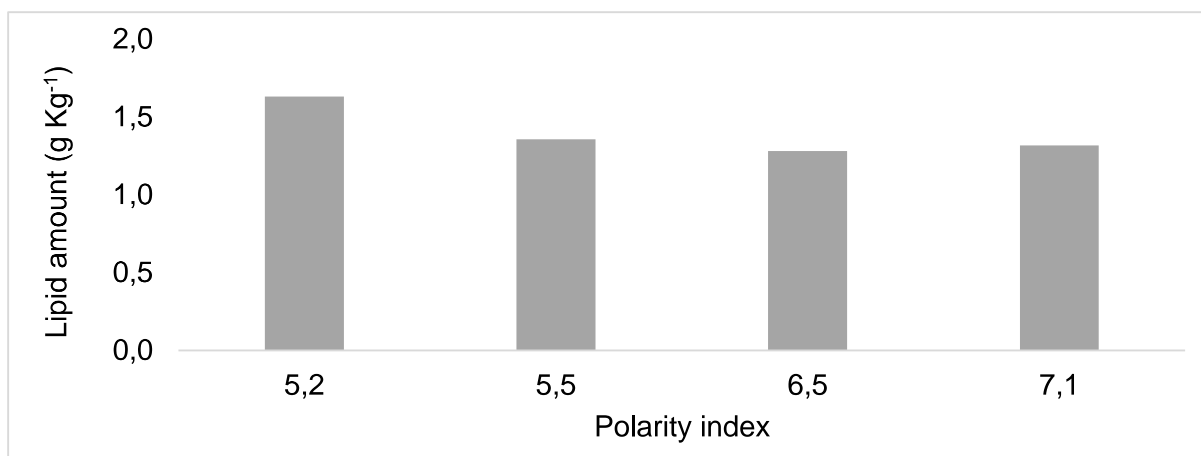


Fig. 7 *Lipid amount in g per Kg of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 MPa of pressure for 10 min.*

On the other hand, when comparing results with different PI and different pressures (figure 8), it is observed that the best result (27,6 g Kg^{-1}) was achieved with the highest values of both parameters (500 MPa and polarity index of 7,1). These results are not consistent with what has been said previously about the influence of polarity in the amount of lipid. However, the explanation may rely in an experimental error occurred during the preparation of samples. Due to the low solubility of fatty matter in water at room temperature, the wastewater samples could have been not completely homogenized, i.e., it is possible that the samples in assays A-E did not had the same initial amount of lipids, which would lead to differences in the final amounts of lipids quantified for each assay.

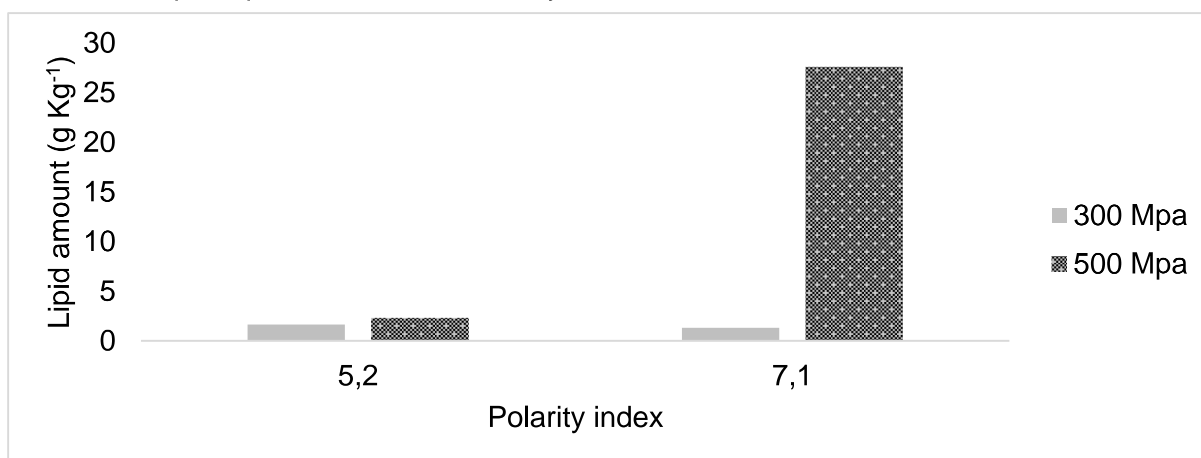


Fig. 8 *- Lipid amount in g per Kg of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 and 500MPa of pressure for 10 min.*

3.2.1.2. Protein content

Whereupon protein amounts, under a constant pressure (300 MPa), figure 9 shows that for different PI, data do not have a clear trend. In any case, contrary to the quantity of total lipids for this pressure, the largest amount of protein in mackerel wastewater was 58,1 g Kg^{-1} and was obtained for the higher PI (7,1).

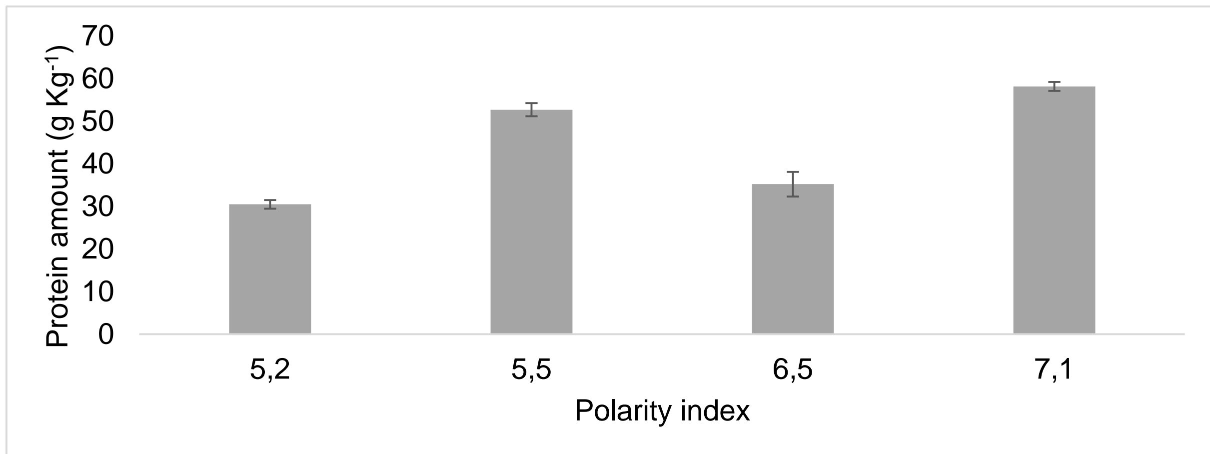


Fig. 9- *Protein amount in g per Kg of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 MPa of pressure for 10 min.*

Comparing the results obtained when using different pressures, the best results of protein concentration was 58,1 g Kg^{-1} and was verified for lower pressure (300 MPa) and higher polarity index (7,1), as showed in figure 10. Again, it is difficult to compare our results with those from literature, because the matrix is not the same. The amount of protein extracted from solid mackerel wastes was 184 g Kg^{-1} (García-Moreno *et al.*, 2013), but solid wastes are always more concentrated in both proteins and lipids than liquid wastes.

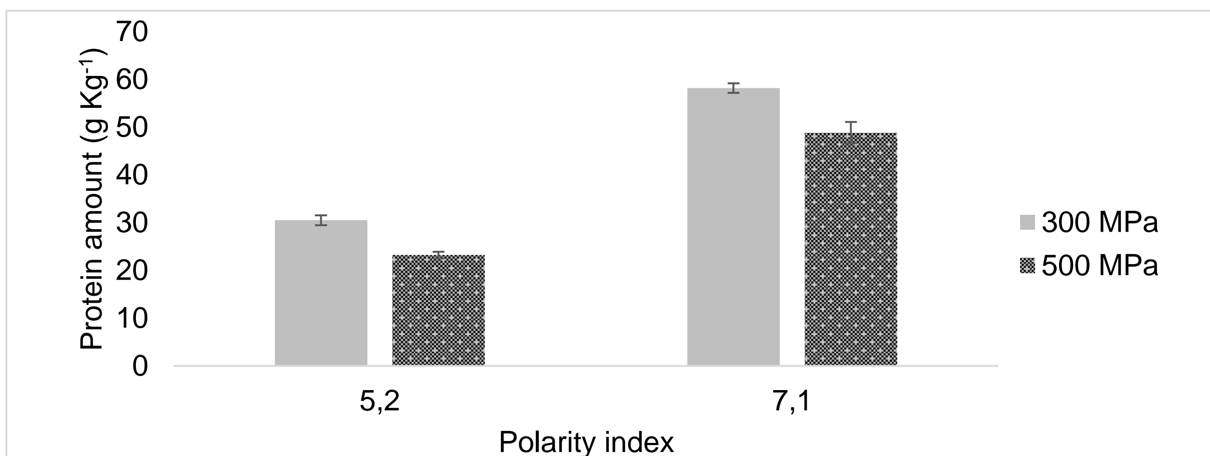


Fig. 10 *- Protein amount in g per Kg of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 and 500 MPa of pressure for 10 min*

3.2.1.3. Fatty acid profile of lipid content

Another important result of this experiment is the sum of the amount of ω3 fatty acids present in total lipids. Alike total protein compounds, figure 11 shows that under a 300 MPa constant pressure, data do not have a clear trend and the greatest amount of ω3 existent in the lipid extracted was 221.8 g Kg^{-1}, obtained for the higher PI (7,1).

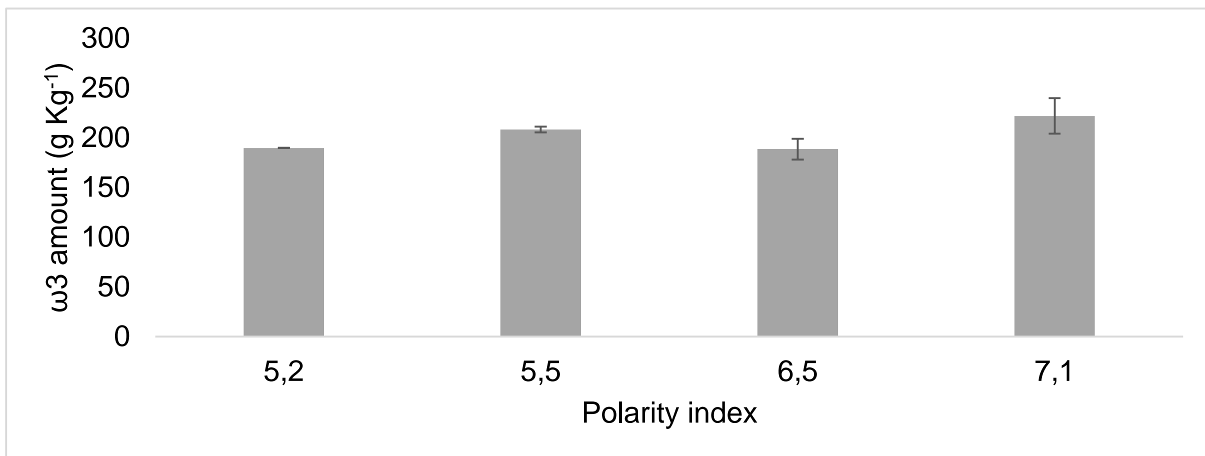
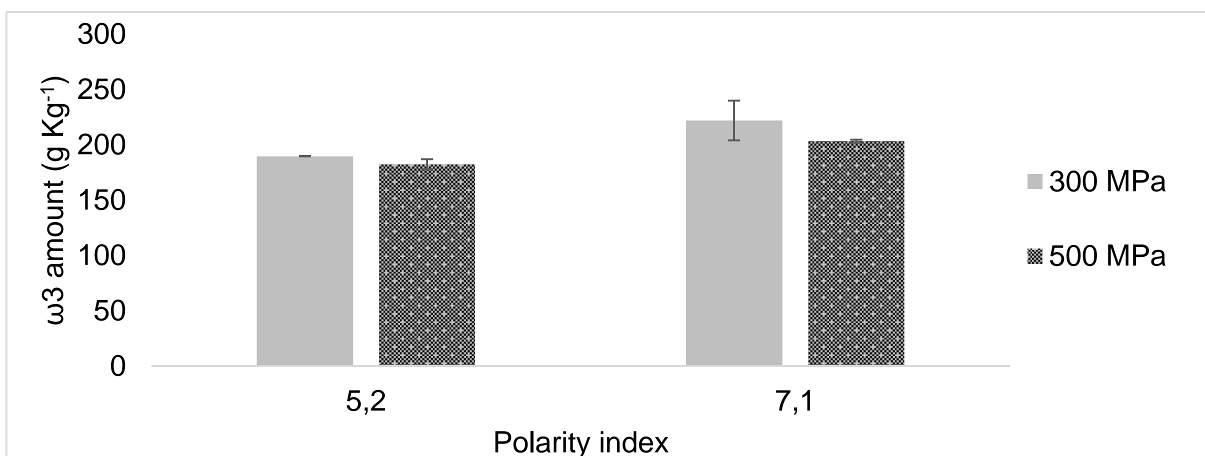


***Fig. 11**- ω3 amount in g per Kg of lipid of mackerel wastewater for 1st set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 300 MPa of pressure for 10 min.*

To finalize the analysis of ω3 amount remains to say that the increase in pressure does not seem to significantly affect results the assays is favoured by the increase of pressure and once again the major quantity of ω3 was obtain at 300 MPa and PI (7,1), this situation is present in figure 12.



were realised with different polarity index's and submitted to the HHP treatment under 300 and 500 MPa of pressure for 10 min.

The qualitative fatty acid profile of lipid fish wastewater extracts for this survey is described in table 4. In general, the amounts of each fatty acid for the various surveys are not very distinct.

In general, major fatty acids identified, were the SFAs palmitic-acid (C16:0), MUFA oleic acid (C18:1 ω9 cis) and PUFAs EPA (C20:5 ω3) and DHA (C22:6 ω3). Moreover, MUFAs amounts are superior to SFAs amounts. These results are in line with previous studies on fatty acid profile of lipids extracted from mackerel (García-Moreno *et al*, 2013).

With concern to ω3 fatty acids, a statistical analysis was performed in order to compare ω3 fatty acid values in each assay and show the statistically significant differences between each other. Results indicate that the amount of alpha-linolenic acid (C18:3 ω3) is different for all the assays, i.e., both pressure and solvents exert influence on the amounts extracted. For EPA, docosapentaenoic acid (C22:5 ω3) and DHA, the amounts extracted on assays A and C are statistically equivalent, whereas the remaining values are different for all the assays.

It should be noted that in this test the sample volume, and consequently the used solvents are substantially higher than the volumes used in the 2nd set of experiments and hydrolysis tests.

Table 4 *- Fatty acid profile in g per Kg of lipid extracted in mackerel wastewater in the 1st set of experiments.*

	A	B	C	D	E	F
SFA						
c14:0	29,5 ± 0,01	26,0 ± 1,76	29,1 ± 0,25	30,6 ± 1,92	27,6 ± 0,79	30,0 ± 0,70
c16:0	112,7 ± 2,70	107,9 ± 5,67	112,7 ± 1,12	119,4 ± 8,61	107,6 ± 2,82	117,7 ± 1,99
c18:0	27,9 ± 1,83	28,2 ± 1,47	28,6 ± 0,28	30,3 ± 2,23	27,8 ± 0,88	29,4 ± 0,48
c24:0	0,4 ± 0,00	0,3 ± 0,02	0,3 ± 0,11	0,4 ± 0,09	0,3 ± 0,01	0,4 ± 0,06
MUFA						
c16:1 ω7	27,4 ± 1,48	25,4 ± 0,85	26,8 ± 0,17	28,6 ± 2,08	24,9 ± 0,71	27,0 ± 0,61
c18:1 ω9 cis	69,2 ± 3,79	67,8 ± 3,83	72,3 ± 0,70	76,7 ± 5,56	67,3 ± 1,82	69,8 ± 1,20
c18:1 ω7 cis	16,7 ± 1,27	16,9 ± 1,00	17,8 v 0,25	18,9 ± 1,29	16,8 ± 0,41	17,7 ±0,20
PUFA						
c18:2 ω6 cis	8,9 ± 0,08	8,4 ± 0,44	9,0 ± 0,14	9,5 ± 0,58	8,2 ± 0,14	8,8 ± 0,24
c18:3 ω3	7,7 ± 0,01[a]	7,2 ± 0,36[b]	7,9 ± 0,10[c]	8,5 ± 0,55[d]	7,2 ± 0,19[e]	7,7 ± 0,11[f]
c20:3 ω6	0,4 ± 0,01	0,4 ± 0,03	0,5 ± 0,01	0,5 ± 0,04	0,4 ± 0,03	0,5 ± 0,04
c20:4 ω6	9,4 ± 0,15	11,7 ± 0,76	12,9 ± 0,12	13,5 ± 1,18	11,8 ± 0,39	12,5 ± 0,19
c20:5 ω3	58,5 ± 0,12[a]	57,2 ± 3,09[b]	63,0 ± ,79[a]	67,2 ± 5,21[c]	55,6 ± 1,45[d]	61,4 ± 1,30[e]
c22:5 ω3	11,2 ± 0,14[a]	11,4 ± 0,67[b]	12,6 ± 0,24[a]	13,3 ± 1,15[c]	11,1 ± 0,37[d]	11,8 ± 0,29[e]
c22:6 ω3	112,2 ± 0,22[a]	112,6 ± 6,27[b]	124,7 ± 1,77[a]	132,8 ± 10,94[c]	108,5 ± 2,50[d]	119,8 ± 2,39[e]

Values are means ± SD

*Values with different letters are significantly different.

3.2.2 2nd set of experiments

As mentioned in the previous chapter, the 2nd set experiments are similar to the ones performed in the 1st set, with slight modifications: (i) solvents used were water, 70% ethanol, and isopropanol:hexane; solvent volumes used; (iii) pressure at which the samples were submitted; and (iv) the time of treatment.

3.2.2.1. Total Lipid content

As appended in the 1st set, in general, assays with lower PI solvents promote a higher amount of extracted lipids; it was also observed that assays with a lower PI show very high standard deviation (figure 13). Again and for the same reason abovementioned, these results are in agreement with the literature. The best result of wastewater lipid content (9.7 g Kg^{-1}) was obtained for the lower PI (5.15), under a pressure of 300 MPa for 10 min.

The influence of time is not clearly observed since in the case of PI (7,97) the extension of HHP treatment benefits the results, for the other two PI assays it seems to make no difference.

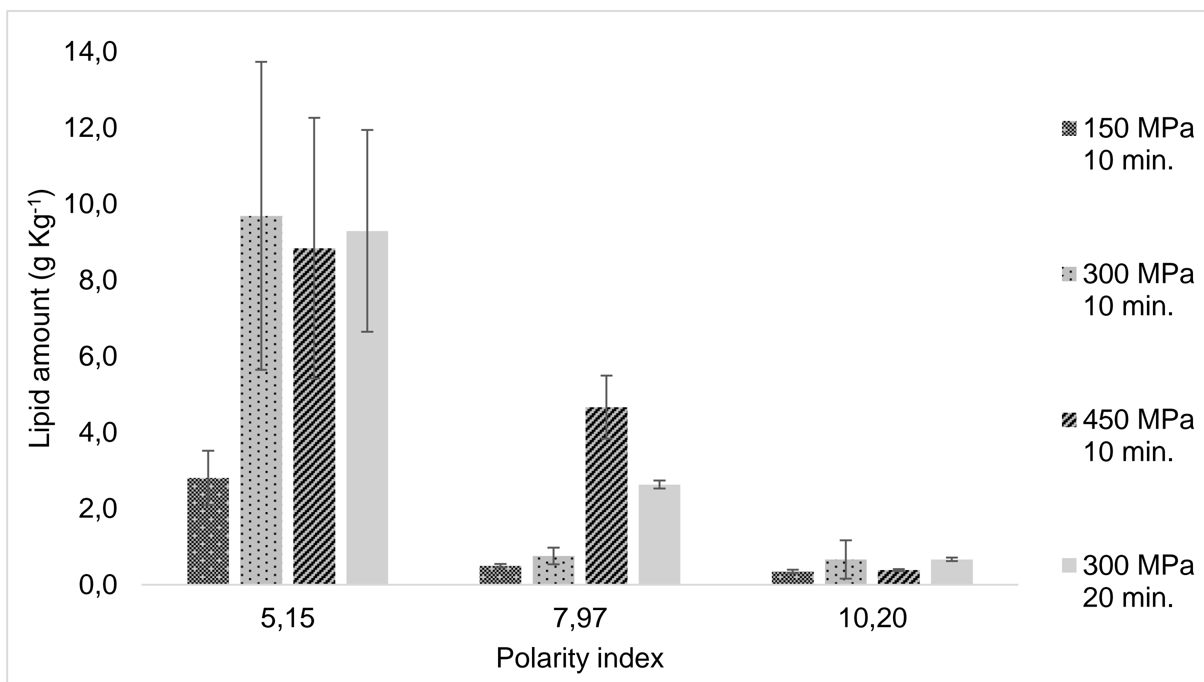


***Fig. 13** – Lipid amount in g per Kg of mackerel wastewater for 2nd set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 150, 300 and 450 MPa of pressure for 10 min or 20 min.*

3.2.2.2. Protein content

The amount of proteins in fish wastewater is maximal (17,6 g Kg^{-1}) for the assay conducted at higher pressure (450 MPa) during the minimum time (10 min) and with a lower degree of polarity (5,15). As the amount of extracted lipids, also the amount of protein increases with decreasing of PI (figure 14). Although it is not possible to establish a comparison with results of other authors, these results make sense, because a strong interaction between the solvents and lipids allows a better lipid extraction yield, thus avoiding the presence of proteins in the organic phase.

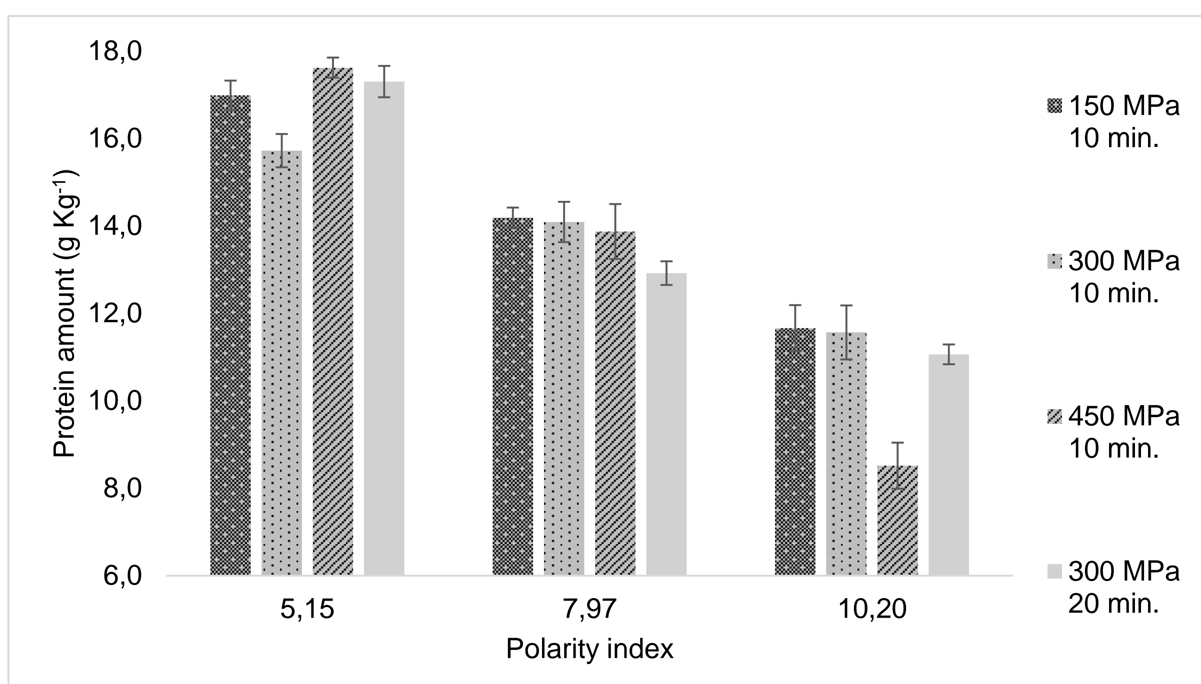


Fig. 14 *- Protein amount in g per Kg of mackerel wastewater for 2nd set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 150, 300 and 450 MPa of pressure for 10 min or 20 min.*

With respect to pressure, only in case of PI (7,97), the amount of protein is higher at lower pressures and lower time. In remain cases it is not possible to establish considerable conclusions.

Previous research concerning the effect of HHP treatment on lipid and protein content is scarce for marine products, so was not possible compare the obtained results with literature results obtain by this method.

3.2.2.3. Fatty acid profile of lipid content

Also for the ω3 the maximum amount (319,2 g Kg^{-1}) was reached for the test with the lowest PI (5,15), this time under the lowest pressure (150 MPa) and lower time (10 min) as shown in figure 15.

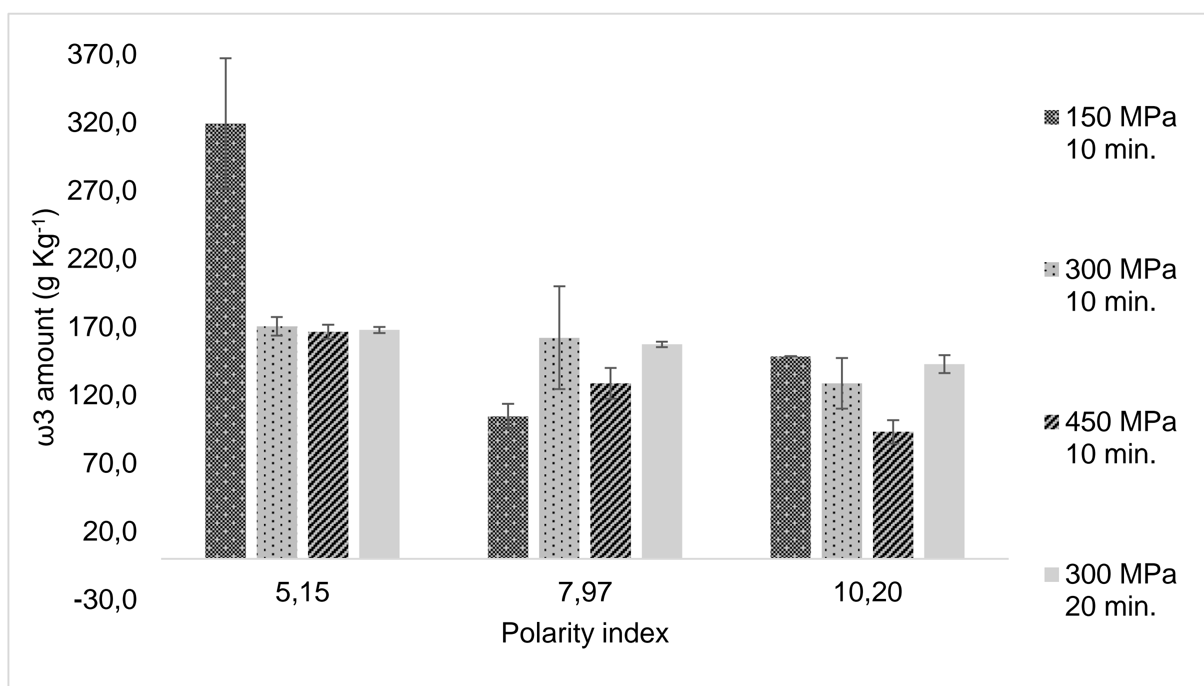


Fig. 15 *- ω3 amount in g per Kg of lipid extracted in mackerel wastewater for 2nd set of experiments, the samples were realised with different polarity index's and submitted to the HHP treatment under 150, 300 and 450 MPa of pressure for 10 min or 20 min.*

The tables 5 and 6 contain the fatty acid profile data for different tests, it can be seen that the amount of each compound does not significantly vary from test to test.

In general, the major fatty acids identified, common for all the assays and in agreement with previous results, were the SFAs palmitic-acid (C16:0), MUFA oleic acid (C18:1 ω9 cis) and PUFAs EPA (C20:5 ω3) and DHA.

The best results for EPA and DHA were 99,8 g Kg^{-1} and 192,8 g Kg^{-1}, respectively; this results were obtained for the test conducted under 150 MPa during 10 min with the mixture isopropanol:hexane.

As in the 1st set of experiments, a statistical analysis was performed in order to compare ω3 fatty acid values in each assay and show the statistically significant differences between each other. Results indicate that the amounts of alpha-linolenic acid (C18:3 ω3), EPA, docosapentaenoic acid (C22:5 ω3) and DHA are different for all the assays, i.e., both pressure, time and solvent exert influence on the amounts extracted.

Table 5 - Fatty acid profile in g per Kg of lipid extracted in mackerel wastewater in the 2nd set experiments.

	A1	A2	A3	B1	B2	B3
SFA						
C14:0	27,6 ± 0,89	19,1 ± 2,04	54,8 ± 9,05	28,3 ± 2,32	28,0 ± 9,39	26,2 ± 0,93
C16:0	111,6 ± 4,01	79,9 ± 7,88	217,5 ± 37,06	110,9 ± 6,28	112,9 ± 33,79	98,5 ± 2,42
C18:0	30,7 ± 0,99	24,3 ± 1,43	57,3 ± 10,57	29,0 ± 0,23	30,9 ± 6,85	24,3 ± 1,24
C24:0	0,3 ± 0,16	0,2 ± 0,00	0,8 ± 0,12	0,3 ± 0,01	0,3 ± 0,09	0,3 ± 0,00
MUFA						
C16:1 ω7	24,8 ± 0,71	16,9 ± 1,80	48,8 ± 7,96	25,4 ± 2,35	25,4 ± 8,27	24,7 ± 0,83
C18:1 ω9 cis	62,9 ± 1,27	43,4 ± 5,46	124,9 ± 20,51	64,7 ± 5,68	65,3 ± 22,71	59,9 ± 2,89
C18:1 ω7 cis	16,1 ± 0,40	11,2 ± 1,45	32,0 ± 5,67	16,3 ± 1,24	16,6 ± 5,49	14,5 ± 0,84
PUFA						
C18:2 ω6 cis	7,6 ± 0,10	5,5 ± 0,98	15,0 ± 2,23	6,7 ± 0,72	8,1 ± 2,62	7,4 ± 0,30
C18:3 ω3	6,3 ± 0,06[a]	4,5 ± 0,73[b]	12,6 ± 2,13[c]	5,4 ± 0,79[d]	6,5 ± 2,24[e]	6,6 ± 0,26[f]
C20:3 ω6	0,6 ± 0,03	0,3 ± 0,00	0,8 ± 0,21	0,4 ± 0,01	0,5 ± 0,05	0,4 ± 0,02
C20:4 ω6	17,7 ± 0,69	0,4 ± 0,06	16,5 ± 4,35	17,3 ± 0,93	11,5 ± 1,85	4,8 ± 0,58
C20:5 ω3	46,5 ± 0,07[a]	33,3 ± 3,57[b]	99,8 ± 16,18[c]	40,3 ± 5,75[d]	52,8 ± 14,95[e]	52,9 ± 2,12[f]
C22:5 ω3	9,1 ± 0,71[a]	5,9 ± 0,61[b]	14,0 ± 3,17[c]	7,6 ± 1,03[d]	9,3 ± 3,09[e]	9,5 ± 0,33[f]
C22:6 ω3	86,7 ± 0,51[a]	60,9 ± 7,95[b]	192,8 ± 32,88[c]	75,5 ± 11,06[d]	93,6 ± 33,08[e]	101,5 ± 4,08[f]

Values are means ± SD

*Values with different letters are significantly different

Table 6- *Fatty acid profile in g per Kg of lipid extracted in mackerel wastewater in the 2nd set experiments (continuation).*

	C1	C2	C3	D1	D2	D3
SFA						
C14:0	24,4 ± 4,44	20,5 ± 1,54	26,1 ± 0,25	33,4 ± 0,63	25,5 ± 0,07	26,0 ± 0,34
C16:0	100,5 ± 16,75	78,3 ± 5,22	99,3 ± 0,94	131,8 ± 1,37	98,7 ± 0,24	99,4 ± 1,05
C18:0	28,2 ± 4,50	19,0 ± 1,10	24,2 ± 0,15	34,3 ± 0,26	24,3 ± 0,07	24,3 ± 0,43
C24:0	0,4 ± 0,04	0,1 ± 0,11	0,02 ± 0,01	0,4 ± 0,00	0,2 ± 0,00	0,05 ± 0,02
MUFA						
C16:1 ω7	20,7 ± 3,73	20,2 ± 1,69	24,6 ± 0,36	29,6 ± 0,44	24,6 ± 0,15	24,8 ± 0,20
C18:1 ω9 cis	55,6 ± 9,17	55,5 ± 10,03	59,5 ± 1,30	76,1 ± 1,09	59,4 ± 0,02	59,6 ± 0,68
C18:1 ω7 cis	14,1 ± 2,96	4,2 ± 4,93	14,8 ± 0,20	19,1 ± 0,32	14,5 ± 0,03	14,4 ± 0,33
PUFA						
C18:2 ω6 cis	6,0 ± 0,84	5,9 ± 0,47	7,4 ± 0,16	8,6 ± 0,03	7,4 ± 0,05	7,4 ± 0,06
C18:3 ω3	4,3 ± 0,89[g]	5,2 ± 0,45[h]	6,5 ± 0,14[i]	6,7 ± 0,17[j]	6,3 ± 0,06[k]	6,6 ± 0,07[l]
C20:3 ω6	0,3 ± 0,00	0,3 ± 0,02	0,3 ± 0,01	0,4 ± 0,06	0,4 ± 0,01	0,4 ± 0,01
C20:4 ω6	14,9 ± 2,13	5,0 ± 2,71	9,6 ± 0,96	18,9 ± 0,26	8,6 ± 0,01	10,3 ± 0,02
C20:5 ω3	29,6 ± 3,21[g]	40,5 ± 3,74[h]	51,6 ± 1,50[i]	45,2 ± 2,20[j]	49,2 ± 0,53[k]	52,3 ± 0,70[l]
C22:5 ω3	6,3 ± 0,40[g]	7,3 ± 0,55[h]	9,5 ± 0,62[i]	8,4 ± 0,36[j]	8,9 ± 0,09[k]	9,5 ± 0,13[l]
C22:6 ω3	53,1 ± 4,87[g]	75,8 ± 6,54[h]	99,0 ± 2,89[i]	82,6 ± 3,77[j]	92,9 ± 1,26[k]	99,6 ± 1,28[l]

Values are means ± SD

*Values with different letters are significantly different.

3.3 Biological methods

As previously mentioned, the biological method used in this work was the enzymatic hydrolysis. Alcalase enzyme was used in different concentrations (0,1%; 0,6% and 1,1% of enzyme in wastewater volume) and different incubation times (3h and 6h).

3.2.1 DH, total Lipid and protein content and fatty acid profile

The hydrolysis degree reached during incubation with Alcalase ranged between 77,8 and 79,6%, and there were no significant differences among the different incubation times or amounts of enzyme used. The highest amount of lipids was obtained with 0,1% of enzyme at incubation for 3 h, and these figures decrease with increasing enzyme concentration when incubating for 3 h, and keep constant for all the enzyme amounts during incubation at 6 h (figure 16).

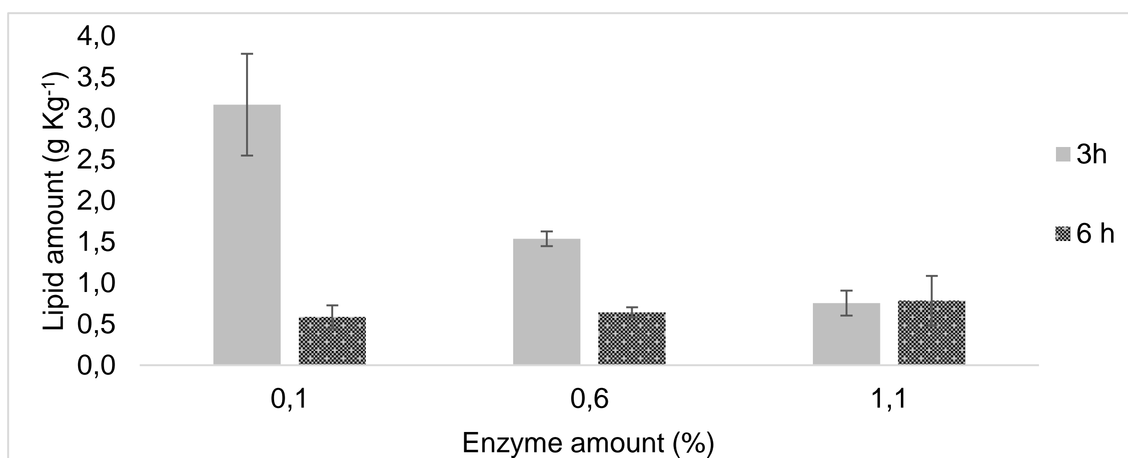


Fig. 16 -, *Lipid amount in g per Kg of mackerel wastewater for the various hydrolysis degree (0,1, 0,6 and 1,1%) at different reaction time (3h and 6h.*

Due to a lapse, protein quantification test was not performed for 0,1 % of enzyme and an incubation time of 3 h. Despite that, as can be seen further on, the results are in agreement with the literature. Protein amounts are higher for incubation at 3 h, although equivalent for all the enzyme concentrations tested, whereas the same equivalence is observed in incubations at 6 h (Figure 17). These results are not surprising, since the amount of protein in the wastewater raw material was not very high (ca. 18-24 g L^{-1}) and thus, the lowest amount of enzyme and the smallest period of time were enough to promote an almost complete hydrolysis of protein and concomitant release of bound lipids (Dumay *et al.*, 2009). The observed decrease in protein contents at 6 h may reflect an excessive bond cleavage, leading to an increased presence of amino acids. It must be kept in mind that the protein determination was achieved through Lowry test, which

uses copper ions that interact with a compound that contains two or more peptide bonds, thus resulting in the formation of a violet/purple-coloured product. Therefore, the decrease in peptides and/or proteins due to hydrolysis may lead to a measured decrease in Lowry results and thus explain the decrease in total protein content with increasing hydrolysis incubation time. The observed decrease in total lipid content with increasing amounts of enzyme and incubation time may be due to lipid oxidation occurred during hydrolysis, since this reaction was performed at 50ºC.

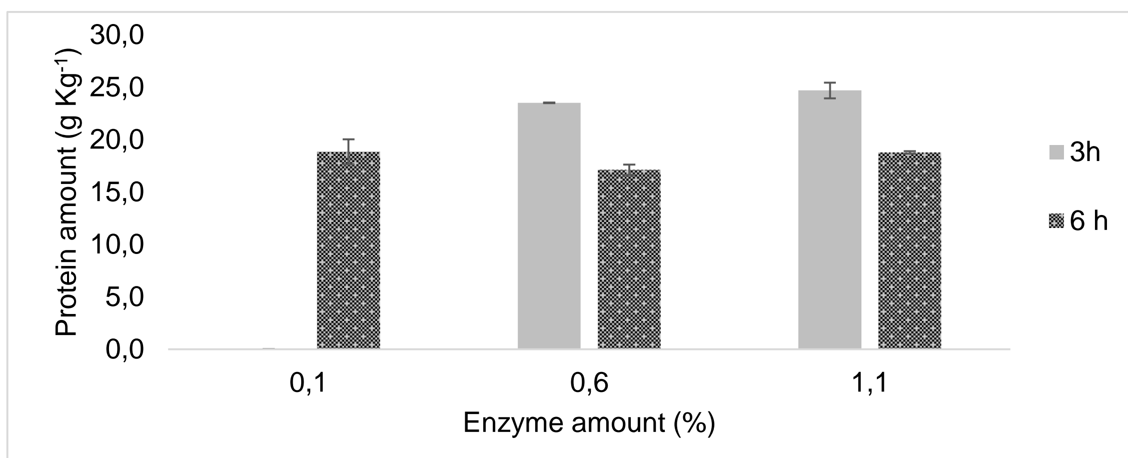


***Fig. 17**, Protein amount in g per Kg of mackerel wastewater for the various hydrolysis degree (0,1; 0,6 and 1,1%) at different reaction time (3h and 6h.*

The best result for the amount of ω3 (170,0 g Kg^{-1}) was obtained for the hydrolysis carried out for 3h with 0,1% Alcalase (figure 18).

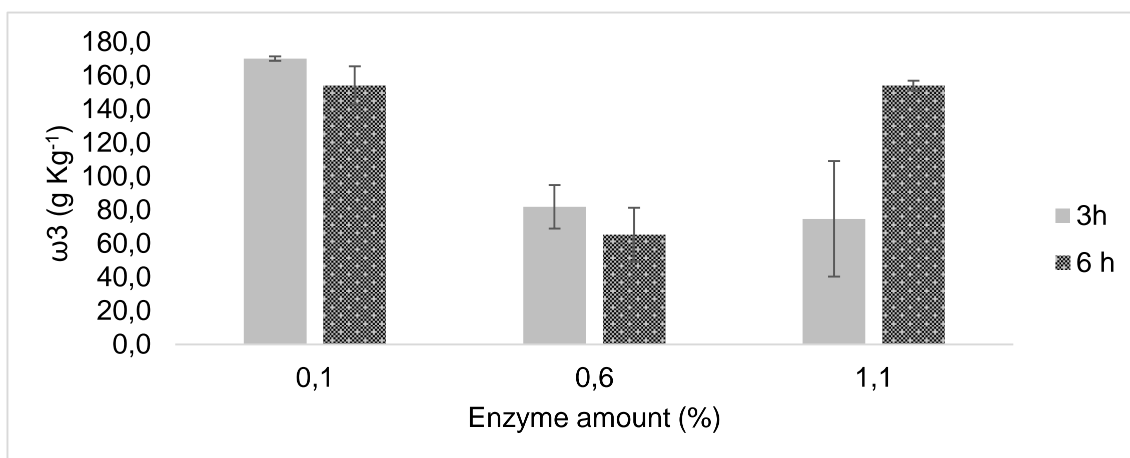


***Fig. 18** – ω3 amount in g per Kg of lipid extracted in mackerel wastewater for the various hydrolysis degree (0,1; 0,6 and 1,1%) at different reaction time (3h and 6h.*

The fatty acid profile data for the various hydrolysis conditions studied. In general, major fatty acids identified, as was in previous trials, were the SFAs palmitic-acid (C16:0), MUFA oleic acid (C18:1 ω9 cis) and PUFAs EPA (C20:5 ω3) and DHA (C22:6 ω3).

The best results for EPA (53,0 g Kg^{-1}) and DHA (100,7 g Kg^{-1}) contents were obtained for the hydrolysis performed with 0,1 % of Alcalase and 3 h of reaction (table 7).

In the survey, a statistical analysis was also performed in order to compare ω3 fatty acid values in each assay and show the statistically significant differences between each other.

In conclusion, for HHP treatment of 1st set, apparently, when decreasing the volume of isopropanol:hexane, the amount of lipid extracted also decreased; for 2nd set, the amount of total lipid recovered increased when PI decreased. Lastly for hydrolysis surveys, the best amounts of total lipid were obtain for trials executed in the shortest time. Effectively, the best result was obtained for the assay of the 1st set at 500 MPa for 10 min, using hexane as a solvent. However, the analysis of 2nd set of results demonstrated that a pressure enhance did not reflected an increase of lipid extracted amount. On the other hand, the 1st set of experiments concern preliminary tests, thus the amount of solvents and sample were not measured with high accuracy, so it is probable that some flaws have occurred. Additionally, the higher pressures are certainly associated with higher energy output, so, although the amount of extracted lipid is greater for higher pressures, the return might not be advantageous. The above facts lead to the conclusion that the best result was obtained for experiments performed at 300 MPa for 10 min, with the mixture of isopropanol and hexane.

About protein amount the analysis of the results for 1st set of experiments allow to conclude that the amount of protein is favoured for trials with lower pressure (300 MPa). In the case of 2nd set of experiments the amount of protein increase with the diminution of PI. For hydrolysis, as happen with total lipid amount, also for the protein amount lower times allow best results The best result was obtained for the test realised with HHP treatment and carried out under 300 MPa for 10 min and with a PI 7,1.

In 1st set of experiments the ω3 fatty acids were favoured by the increase of the pressure, while for the remaining treatments a logical trend do not exist. The best result was obtained for the HHP treatment under 150 MPa for 10 min, with PI 5,15. But as the amount of fatty acids depends on the amount of lipid extracted, by calculating the amount of fatty acid per amount of sample, the best result was also obtained in the test performed at 300 MPa for 10 min with isopropanol and hexane.

Table 7 *- Fatty acid profile in g per Kg of lipid extracted in mackerel wastewater in hydrolysis.*

	0,1% 3h	**0,6% 3h**	**1,1% 3h**	**0,1% 6h**	**0,6% 6h**	**1,1% 6h**
SFA						
C14:0	28,0 ± 0,16	14,4 ± 0,49	13,4 ± 6,42	30,9 ± 0,89	13,8 ± 3,51	30,7 ± 0,98
C16:0	114,0 ± 1,13	62,1 ± 0,35	57,7 ± 27,22	128,8 ± 0,45	62,4 ± 21,02	126,0 ± 2,11
C18:0	32,0 ± 0,83	20,8 ± 4,15	18,6 ± 9,21	35,5 ± 0,30	21,2 ± 9,92	34,4 ± 0,60
C24:0	0,3 ± 0,02	0,2 ± 0,01	0,2 ± 0,06	0,4 ± 0,05	0,3 ± 0,15	0,4 ± 0,01
MUFA						
C16:1 ω7	25,2 ± 0,35	13,1 ± 0,35	11,4 ± 5,19	27,0 ± 0,41	12,3 ± 3,26	27,2 ± 0,79
C18:1 ω9 cis	67,0 ± 1,58	33,0 ± 1,86	29,6 ± 12,65	70,1 ± 1,34	31,0 ± 7,88	70,1 ± 1,72
C18:1 ω7 cis	17,9 ± 0,37	8,6 ± 0,24	7,7 ± 3,55	17,9 ± 0,40	7,9 ± 2,07	17,8 ± 0,34
PUFA						
C18:2 ω6 cis	8,6 ± 0,78	4,1 ± 0,21	3,7 ± 1,64	8,1 ± 0,09	3,7 ± 0,99	8,4 ± 0,03
C18:3 ω3	6,7 ± 0,08[a]	3,4 ± 0,43[b]	3,2 ± 1,56[c]	6,4 ± 0,16[d]	2,8 ± 0,66[e]	6,6 ± 0,01[f]
C20:3 ω6	0,4 ± 0,06	0,2 ± 0,06	0,2 ± 0,02	0,5 ± 0,07	0,2 ± 0,06	0,4 ± 0,00
C20:4 ω6	16,2 ± 0,03	2,3 ± 0,33	2,3 ± 1,24,	12,2 ± 2,53	7,9 ± 2,22	11,2 ± 0,78
C20:5 ω3	53,0 ± 0,61[a]	25,8 ± 3,50[b]	23,8 ± 10,93[c]	46,3 ± 0,45[a]	20,6 ± 5,03[d]	48,1 ± 0,75[e]
C22:5 ω3	9,7 ± 0,20[a]	4,4 ± 1,36[b]	4,3 ± 2,03[c]	8,3 ± 1,75[d]	4,2 ± 1,35[e]	9,1 ± 0,29[f]
C22:6 ω3	100,7 ± 0,48[a]	48,2 ± 7,69[b]	43,4 ± 19,83[c]	87,1 ± 4,12[d]	37,7 ± 8,98[e]	90,3 ± 1,79[f]

Values are means ± SD

*Values with different letters are significantly different

CHAPTER 4 - CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

4.1 Conclusions

The society is realizing the necessity to use renewable feedstocks for the production of chemicals, materials and fuels. This pressure led companies to study the environmental impact of their supply chains beyond their own operations, and thus introducing specifications along the entire supply chain for new and existing products. The reutilization of wastes from food industry has the advantages of occurring in large volumes and may be subjected to traceability, a key parameter for consumer acceptance on new products from reused wastes.

With this work it is intended to meet this trend, seeking viable solutions for the recovery of by-products of the canning industry, turning them into value-added products.

An industrial approach is also interesting, so La Gondola company bakes about 120 m^3 per month of fish wastewater. Using the HHP pre-treating with 300 MPa of pressure for 10 min and a mixture with isopropanol and hexane as solvent it would be possible to get is about 240 Kg of ω3 per month.

A bibliographic research on the processing of fish wastewaters revealed their characterization and cleaning processes in order to be reused (Cristóvão *et al.*, 2015; Muthukumaran *et al.*, 2013), whereas another study pointed out the development of a bioprocess for conversion of wastes of fish processing into single-cell oil and single-cell protein through microalgal cultivation, with parallel water reuse, under the scope of a biorefinery (Queiroz *et al.*, 2013). As previously mentioned, the literature concerning valorisation of liquid wastes from fish processing is extremely scarce, and thus, the present study brings about some necessary insights into the subject.

The extraction assays performed during the experimental plan were always executed with isopropanol and hexane. Although these solvents are less effective than chloroform and methanol for lipid extraction, they are classified as food grade, and this was considered a critical issue so that the process could be later implemented at industrial scale.

4.2 Suggestions for future work

4.2.1 Alternative solvents

For future work we propose the study of other solvents used in the extraction process, both organic solvents and ionic liquids. For organic solvents the performance of hexane:ether mixture, methanol, liquid dimethyl ether (DME) and *d*-limonene, can be studied. *d*-Limonene is an agricultural by-product from the citrus industry. According to Virot (2008), the major handicap of using d-limonene instead of n-hexane is the higher energy consumption related to solvent recovery by evaporation due to the higher boiling point (175°C) when compared to n-hexane (69 °C). Yields of d-limonene's olive extracts were almost equivalent to those obtained using n-hexane. DME can be used to extract neutral and polar lipids from wet or dry samples and it also have a good performance in lipophilic compounds extraction (Cho *et al*, 2012; Virot *et al*, 2008).

The use of ionic liquids (ILs) has been to arouse the attention of the scientific community in recent times, since they are been studied regarding the application as an alternative to solvents to extract value-added compounds from biomass. The main advantages presented by this type of liquid are the isolation of target compounds coupled to the recovery and reuse of ILs (Passos *et al*, 2014).

4.2.2 Qualitative evaluation of lipid and protein fractions

Besides the evaluation of the amounts of both lipids and proteins it would also be interesting to study some qualitative parameters of these compounds, such as the antioxidant activity of lipid fraction and the antihypertensive activity of the protein fraction.

It is also suggested a detailed study of the possible oxidation of the samples as well as the antimicrobial activity of the lipid fraction, as some fatty acids are reported to have antimicrobial activity. Finally, a life-cycle analysis study, in order to weight the economic and environmental aspects of the proposed solutions would also be adequate.

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