Physicochemical Characterization and Nutritional Quality of Fish By-Products: 
In vitro Oils Digestibility and Synthesis of Flavour Esters

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Abstract
Three fish species (Annular sea bream, sardine and golden grey mullet) were examined as the most Tunisian fishes consumed and could be used as a valuable bio-resource. The fillet and the pyloric caeca from these fish have been investigated for their proximate composition, minerals, nutritional quality and oil physicochemical properties. Fish fillets and viscera showed higher macro-mineral concentrations. Moreover, unsaturated fatty acids were found to be predominant over the saturated ones. The lipid health indexes and the predominance of PUFAs acids in all studied fish could meet people’s needs. Interestingly, a higher stability of polyene, peroxide values and carotenoids were observed during the storage for 30 days at -20°C, which allows higher oils stability. In vitro digestibility model showed that fish oils were efficiently hydrolyzed by pancreatic lipase, which suggests the higher assimilation of fish oils by consumers. Furthermore, fish lipases revealed an acceptable potential to produce aromatic esters.

Keywords: Marine fish; Oil characterization and stability; Nutritional quality; Higher digestibility; Flavour esters synthesis

Abbreviations
ω3-PUFA: Omega-3 Poly Unsaturated Fatty Acids; MUFA: Mono Unsaturated Fatty Acid; SFA: Saturated Fatty Acid; PUFAs: Poly Unsaturated Fatty Acids; EPA: Eicosa Pentaenoic Acid; DHA: Docosa Hexaenoic Acid; UFAs: Unsaturated Fatty Acids; FAMEs: Fatty Acid Methyl Esters; GC: Gas Chromatography; AI: Atherogenic Index; TI: Thrombogenic index; TPL: Turkey Pancreatic Lipase; ASDL: Annular Sea Bream Digestive Lipase; SaDL: Sardine Digestive Lipase; GmDL: Grey Mullet Digestive Lipase; SaDLi: Immobilized Sardine Digestive Lipase

Introduction
Marine organisms and particularly fish have the potential of delivering valuable nutritive products. Maximizing the use of the entire fish is of great importance because of environmental regulations and in the desire to obtain more value [1]. Fish processing industries generate large amounts of waste material including viscera, which represent an important part of the animal mass (about 15 to 25% of the original catch depending on the season) [2]. However, this material can be a valuable bio-resource from which waste represents an important commercial loss. Recently, there have been heightened interests in the lipid and fatty acid (FA) composition of fish. Fish oils are the main source of ω-3 fatty acids [3]. The benefits of these important fatty acids are not only associated with the synthesis of eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes [3], but also they are equipped by an anti-inflammatory effect with the aim of minimizing the inflammation and improving clinical outcomes [4].

Many alternative methods of fish viscera processing have been examined, including enzyme extraction [5,6] and preparation of protein hydrolysates, utilisable as feed in aquaculture diets [7] or for microbial growth [8]. However, there are few published studies on the thermal stability and rheological properties of fish oil. The digestion of lipids continues to reach considerable scientific interest, with food emulsions increasingly being seen as a mechanism by which lipid uptake may be controlled. There are considerable developments in understanding the relationship between interfacial quality and lipolysis in human gastrointestinal tract. The digestion of dietary lipids is a complex process that involves different enzyme activities [9]. It takes place mainly in the intestine by pancreatic lipase [9]. There is an increasing interest in using in vitro digestion models, to understand fish and fish oil digestibility and to simulate human digestion; thus, many approaches have been suggested. A recent review by Hur et al. [10] has shown different models which depends on pH, the use of one, two or more gastrointestinal phases and enzyme sources. To better simulate human physiological conditions, we have to aspirate intestinal human model. To our knowledge, no comparisons of fish oils digestion were carried out.

The golden grey mullet (Mugil auratus, Mugilidae), sardine (Sardinella aurita, Actinopterygii) and annular sea bream (Diplodus annularis, Sparidae) are the widely distributed fish species in Tunisian coasts. Among the small pelagic fish, the sardine is one of the most abundant species in Mediterranean Sea, which feeds mainly on planktonic crustaceans, appendicularians and other organisms [11]. In 2012, its catches reached 21,478 tons in Tunisia, respectively (FAO, 2013). Grey mullets, so called Mugilidae, are common in all warm and temperate seas of the world and live in a wide range of habitats [12]. The golden grey mullet (Mugil auratus) is a coastal species that occurs in marine and brackish habitats from the Mediterranean and Black Seas. It feeds on algae and small crustaceans [12]. The Sparidae family has a widespread distribution from the Mediterranean to Black and Adriatic seas [13]. The most Sparidae species found in the Mediterranean Sea was the annular sea bream, Diplodus annularis. This species is a demersal marine fish found in groups over sandy bottoms and sea grass bed habitats, at depths ranging from 0 to 50 m [13]. Despite their wide distribution in the Mediterranean Sea, there is a lack of published data on nutritional quality and composition of these three species.

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species. Physicochemical composition, oil stability and digestibility by pancreatic lipase were assessed for these three species. We evaluated the potential use of fish digestive lipases in the synthesis of industrially important flavour esters such as isoamyl acetate, butyl acetate and butyl laurate.

Materials and Methods

Fishes

Samples of three bony fish species were used in this study; the golden grey mullet (Mugil auratus), sardine (Sardinella aurita) and annular sea bream (Diplodus annularis) were collected from Tunisian coasts (Sidi Mansour, Sfax). Specimens were transported on ice to the laboratory where they were washed. Measurements were recorded for body length and total body wet weight (Table 1) and immediately processed. Totally, nine individuals for each species were used. In each case meat and pyloric caeca were separated manually, blended and stored at -20°C until analyzed for moisture, lipid, mineral composition, crude protein and fatty acid compositions.

Mineral composition

Determination of Na, Mg, Ca, Zn, Fe and Cu levels was carried out by flame atomic absorption spectrometry. An external calibration method was used for quantitative analysis. Portion samples (5 g - 10 g wet weight) were dried at 500°C under a gradual temperature increase. Ash was dissolved in concentrated HCl (12 N) and the solution obtained was evaporated to dryness. Quantification of these elements was performed using a spectrophotometer (Spectr AA-20) with deuterium background correction.

Proximate composition analysis

After preparation of edible parts of fish as described, proximate composition analyses were performed according to AOAC, 1990 (Official methods of analyses of association of analytical chemist) procedures. The moisture was determined by weight difference of the homogenized tissue (1 g - 2 g) before and after 24 h at 105°C. Dried samples were used for determination of crude protein and mineral contents. Crude protein content was calculated by using nitrogen content obtained by Kjeldahl method. A conversion factor of 6.25 was used for calculation of protein content. Crude fat was measured by solvent extract ion method in a Soxhlet system using hexane as solvent.

Fatty acids analysis

Lipid extraction was carried out according to the method described by Folch et al. [14] with some modification. After lipid extraction, samples were dissolved in 0.5 ml of hexane. Then, 0.2 ml of potassium hydroxide in methanol (2 N) was added for the fatty acid methylation process. The mixture was vortexed then centrifuged and the upper layer was removed. Then, 0.1 ml of methanol was added. A solution of concentrated HCl (12 N) was added depending the used lipase. The olive oil emulsion oil and 20 ml of buffer (2 mm Tris-HCl pH 8). Bile salts (NaDC) was added depending the used lipase. The olive oil emulsion was centrifuged at 4000 g for 10 min, the freshly prepared emulsion was used for the

In vitro digestibility

Briefly, the pH of each emulsion was adjusted to 8 in order to simulate pancreatic phase because it corresponds to a mean value of the pH of the duodenum medium [9]. The reaction contains 10 ml of emulsified fish oil and 20 ml of buffer (2 mm Tris-HCl pH 8, 50 mm NaCl). Bile salts (NaDC) was added depending the used lipase. The olive oil emulsion was used as a control. Enzymatic hydrolysis was performed using purified digestive lipases from different origin; i) turkey pancreatic lipase (TPL) [19], ii) sardine digestive lipase (SaDL) [5], iii) annular sea bream digestive lipase (AsDL) [20], and iv) the golden grey mullet lipase [6]. Enzymatic hydrolysis was performed at pH 8 using the pH-stat technique [21].

| Species Subfamily Average length (cm) Average weight (g) Main food type Life styles Number tested (n) |
|------------------|------------------|------------------|------------------|------------------|------------------|
| Annular sea bream (D. annularis) Sparidae 13.84 ± 0.68 18 Benific invertebrates Demersal 8 |
| Sardine S. aurita Actinopterygii 15.26 ± 1.24 22 Zooplantonic, fish larvae, phytoplantonic Pelagic 8 |
| Golden grey mullet (M. auratus) Mugilidae 17.3 ± 1.2 40.62 ± 1.63 Algae, crustaceans, small wormsand molluscs Demersal 8 |

Table 1: The main information on biological characteristics of the examined fish species D. annularis, M. auratus and S. aurita.
Immobilization of lipases

For immobilization, the crude lipase preparations, obtained after sulphate precipitation step for different lipases (AsDL, SaDL and AsDL) [5,6,20] were immobilized by a simple adsorption technique into CaCO₃ support as described by Ghamgui et al. [22]. The mixture was incubated 1 h at 4°C under mild agitation. Afterwards, the lipase-absorbed into CaCO₃ support was washed two times with 10 ml of chilled acetone at-20°C, filtered through a Buchner funnel and dried in vacuum dissecator at room temperature. The yield of the immobilized lipase activity was defined as the ratio of the adsorbed activity recovered at the end of the immobilization period divided by the total soluble lipase activity initially added to 1 g of the support.

Potential of CaCO₃-immobilized lipases in flavor esters synthesis

Flavor esters synthesis was carried out as described by kharrat et al. [23]. These ester synthesis reactions were carried out in screw capped flasks containing 4 ml hexane and 2 g of a substrate mixture (acetic acid to alcohol molar ratio equal to 1) using 10 IU of CaCO₃ - immobilized lipases at 40°C under shaking (400 g). A parallel reaction under the same conditions without addition of the enzyme was prepared and used as a control. Aliquots of 200 µl were withdrawn periodically from the reaction mixture.

The immobilized enzyme was removed by centrifugation at 9000 g for 15 min and the residual acid content was measured by titration with 0.5 N sodium hydroxide using phenolphthalein as an indicator and 2 ml of ethanol as a quenching agent. The conversion yield (%) was based on the amount of the consumed acid.

Statistical analysis

All analysis was carried out in triplicates and results were expressed as mean values ± standard deviation (SD) (n=3). The differences were calculated using one-way analysis of variance (ANOVA), and statistically significant differences were reported at p < 0.05. Data analyses were done with the use of SPSS 10.0 software.

Results and Discussion

Species belonging to three fish families; the golden grey mullet (Mugil auratus, Mugilidae) and the annular sea bream (Diplodus annularis, Sparidae) were sampled opportunistically during October 2015 from the local port (Sfax, Tunisia) on the same day. The main information about biologic characteristics of the three fish species (standard length, weight, etc.) are reported in Table 1. Subsequently, fillet and viscera from these marine species were used for mineral determination, lipid extraction, characterization and oils digestibility by a pancreatic lipase in order to simulate the human gastrointestinal process.

Mineral composition

Marine foods are very rich sources of minerals. The main functions of essential minerals include skeletal structure and they are also important components of hormones and enzymes [24]. It is known that mineral content of marine foods slightly changes from a species to another. Its composition is closely related to seasonal and biological discrepancies (species, age, and sex), geographical area, and environmental conditions (salinity and temperature) [25]. The mineral contents (six mineral elements) of the three edible species pyloric caeca and meat were determined after incineration of the tissues at 500°C. They are expressed in mg/g except for Cu and Fe which are expressed in mg/kg of wet weight. Similar patterns were observed for all elements in the pyloric caeca and in the meat (Table 2). The main elements were Na, Ca and Mg. Among the six minerals investigated, the most abundant was Na followed by Ca. The Na contents in Golden grey mullet, sardine and annular sea bream meat were found to be 358.05, 408.06 and 337.5 mg/g, respectively. In general, Na contents are considerably higher in shellfish than in finfish [24]. The calcium content was higher in the meat of annular sea bream (577.5 mg.100g⁻¹) than in sardine and golden grey mullet (Table 2). These values were higher than these reported for the marine snail [24]. Similar mineral concentrations were also found in the pyloric caeca of the three studied fish. Fe, Zn, and Cu, are known as essential minerals for humans. According to National Research Council Recommended dietary allowances, the Zn deficiency can lead to loss of appetite, skin changes and immunological abnormalities. Fe, Zn, and Cu were present in lower amounts. Their contents were variable among fish fillets (Table 2). Zn concentrations were higher in the pyloric caeca of different species than in meat. Similar values were reported for other marine cephalopods (40-42) and marine snail [24].

Proximate composition

Fillets and pyloric caeca from the golden grey mullet, the sardine and the annular sea bream were used as starting materials for lipid, moisture and lipid determination. Although, the three fish species have similar diets and occupy the same geographical areas, their fillet and pyloric caeca show significant differences (P < 0.05) in moisture, protein and fat contents. The moisture content was found to be 20.99 ± 0.021%, 18.67 ± 0.015% and 25.55 ± 0.022% in sardine, annular sea bream and golden grey mullet meats, respectively (Figure 1A). In contrast to sardine and annular sea bream, the moisture content in pyloric caeca of golden grey mullet was significantly higher than in the meat (Figure 1B). Crude proteins represent the major component of the dry matter in both meat and pyloric caeca of the three fish. Lipid content was higher in meat (mean values range: 8.03% to 26.67%) than in pyloric

| Elements | Meat (P. caeca) | Meat (P. caeca) | Annular sea bream | Annular sea bream |
|----------|----------------|----------------|-------------------|-------------------|
| Na (mg/g) | 408.06 ± 2.25  | 322.75 ± 2.16  | 358.05 ± 2.16     | 186.25 ± 2.86     |
| Ca (mg/g) | 382.22 ± 1.75  | 265.87 ± 1.47  | 300.65 ± 3.17     | 201.60 ± 1.75     |
| Mg (mg/g) | 46.208 ± 1.32  | 63.50 ± 0.98   | 43.416 ± 1.62     | 28.95 ± 1.61      |
| Zn (mg/g) | 0.431 ± 0.09   | 8.906 ± 0.65   | 3.026 ± 0.91      | 3.765 ± 0.73      |
| Cu (mg/kg)| 139 ± 1.65     | 90 ± 1.13      | 100 ± 2.31        | 80 ± 1.55         |
| Fe (mg/kg)| 556 ± 5.28     | 100 ± 1.75     | 467 ± 5.02        | 180 ± 2.13        |

Results were expressed as mean values ± standard deviation (SD) (n=3).

Table 2: Mineral contents (wet basis) in three fishes (M. auratus, S. aurita and D. annularis) tissues.
caeca (mean values range: 1.84% to 2.61%) (Figure 1A). Proximate values obtained for the three edible fish were similar to those reported by Kacem et al. [26].

Oil characterization

The physicochemical properties of the extracted oil from the studied fish are studied. Oil parameters such as iodine index (II), density and carotenoids content are measured and presented in Table 3. The density of the three oils seems to be similar, it range from 0.901 to 0.967.

Similar values were reported for oils extracted from cartilaginous fish such as stingray [27]. Iodine index (II = 105.6 ± 2.23 mg I2/g oil) and carotenoids (9.79 ± 0.23 mg/100g) of sardine oil were higher than those of golden grey mullet and annular sea bream. These values were lower than those reported for the stingray (II =3.50 ± 2.30 mg I2/g oil and 2.72 ± 0.08 mg/100g) [27]. The polyene index (PI) of the sardine oil (PI=1.8) was higher than those measured for the other two fish (Table 3). Polyene indexes of mullet and sea bream oils are similar to that reported for the bogue (Boops boops) [28], but lower than those reported for gilthead sea bream [29].

The fatty acid composition of lipids extracted from fillet and pyloric caeca were presented in Tables 4 and 5. Fourteen fatty acids, from C12:0 to C22:6n-3 were identified and compared among the different species. There was a wide variation and significant differences (p < 0.05) among the fatty acid (FA) profiles of the fish species in terms of total saturated and unsaturated FAs. The amount of fatty acid groups in all fish species, in a decreasing order was as follows: Monounsaturated fatty acids (MUFA)> Saturated fatty acids (SFA)> Polyunsaturated fatty acids (PUFA).

Saturated fatty acids (SFA): The saturated fatty acids represented more than one-third of total fatty acids due to the high value of palmitic acid

![Figure 1](image_url)

**Figure 1**: (A) Proximate composition (%) of the sardine (S. aurita), golden grey mullet (M. auratus) and annular sea bream (D. annularis) fillets. (B) Proximate composition in fish pyloric caeca. (C) Histogram showing the amount of SFA, MUFA, PUFA in fish fillets. (D) ω-3 and ω-6 PUFAs, ω-3/ω-6 and DHA/EPA ratios in fish fillets.

| Species              | Density (g/ml ± SD)  | Carotenoids (mg/100g) ± SD | Polyene index ± SD | Iodine index ± SD |
|----------------------|----------------------|-----------------------------|--------------------|------------------|
| Sardine oil          | 0.922 ± 0.032        | 9.79 ± 0.23                 | 1.8 ± 0.02         | 105.6 ± 2.23     |
| Grey mullet oil      | 0.901 ± 0.062        | 2.23 ± 0.56                 | 0.74 ± 0.01        | 88.2 ± 1.60      |
| Annular sea bream oil| 0.967 ± 0.058        | 7.64 ± 0.69                 | 0.9 ± 0.03         | 95.22 ± 0.71     |

Results were expressed as mean values ± standard deviation (n=3). Iodine index was expressed in mg I2/g oil. Total carotenoid was expressed in mg/100 g oil. Density was expressed in g/ml.

**Table 3**: Physicochemical characteristics of oils from sardine, golden grey mullet and annular sea bream.
in the three fish fillets (Figure 1A). The highest average contents of SFA were those of grey mullet and annular sea bream, (42%) (Figure 1A). However, sardine contained the smallest proportion of palmitic acid (6.11 ± 0.86%) (Table 4). The palmitic acid was also identified as the dominant compound of saturated FA in fish pyloric caeca (Table 5). The golden grey mullet pyloric caeca contained the highest percentage of palmitic acid (47.26%). Comparable results were obtained with other fish species wherein palmitic acid represents about 70% of the total SFAs [30]. Comparable values of stearic acid (C18:0) were measured in the three fish tissues (Figure 2).

Monounsaturated fatty acids (MUFAs): During the past decades, MUFA s have received increasing attention because of their heart-healthy benefits after studies of the olive oil highly consumed by the populations of the Mediterranean region. MUFA constituted nearly half of the total fatty acids of sardine and annular sea bream but less than 40% in golden grey mullet fillets. This fact is due to the abundance of palmitoleic acid (C16:1) and oleic acid (C18:1) (Table 4). Annular sea bream fillet contains the highest percentage of oleic acid. Our results are coherent with those reported by Alasalvar et al. [25] these authors show that oleic acid is the predominant MUFA (60% to 75%) in fish lipids. The pyloric caeca of annular sea bream and sardine contained high amounts of MUFA lipids as compared to the golden grey mullet. The palmitoleic acid was the second most abundant MUFA after the oleic acid in the pyloric caeca with the highest values in sardine and golden grey mullet. For the three fish pyloric caeca, the MUFA s are more abundant than SFAs (Table 5).

Polyunsaturated fatty acid (PUFA): It was indicated that the fatty acid profile of fish fillets reflects the content of the dietary lipid sources [30]. Fish are the main contributors of n-3 PUFA for the human diet. Compared with freshwater fish, marine species present higher levels of PUFAs, especially DHA and EPA. Essential fatty acids, such as linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3), have great physiological importance. These essential fatty acids, not synthesized by humans, either accumulate in adipose tissues or are converted into long-chain unsaturated fatty acids such as arachidonic acid, EPA and DHA. Significant variations in the relative proportion of total PUFAs were found among the three fish fillets. The PUFA portion accounted for 16.6% to 23.38% of total FAs in the three edible fish fillets. The major contributor to n-3 PUFAs in all samples was DHA (C22:6n-3), followed by EPA (C20:5n-3), that accounted together for over 76% of the total n-3 PUFAs in golden grey mullet, sardine and annular sea bream (Table 4). These results are in agreement with previous studies on other marine species [31].

Several studies reported that some ω-6 fatty acids like arachidonic acid are pro-inflammatory [3]. Therefore, the absence of this polyunsaturated fatty acid in the annular sea bream and sardine may be advantageous to consumers for cardiovascular health. The presence of arachidonic acid (3.99%) in the golden grey mullet can be explained by the observation that this pelagic fish feed predominantly on the phytoplankton food, which contains high levels of C20:4n-6 [32]. In addition, the most abundant ω-6 PUFA in the three tested fish fillets was the linoleic acid (C18:2n-6). The percentages of PUFAs found in pyloric caeca of the three edible species were important. The highest levels of PUFAs were observed for sardine samples (16.48%) with an abundance of DHA and C22:6n-3 (Table 5). Therefore, digestive tissues like pyloric caeca seem to be a good source of omega-3 fatty acids recognized for their health benefits. These findings were in line with those measured in the viscera of other Mediterranean species like saupe (Sarpa salpa) and cuttlefish (Sepia officinalis) [26]. We can conclude that differences in lipid contents of the three studied fish were due to species nature, the tissue, the diet, the season and the geographical origin [33].

Nutritional quality

Lipids play an important docket in the nutritional quality of fish fillets. With increasing consumer awareness of the risks associated with high fat intake, food manufacturers should monitor the physical and chemical properties of lipids. A good knowledge of the nutritional properties of fish species with a low commercial price represents the first step towards a valorization of fish by-products and divulgation to the market and to consumers of their quality. The desirable fatty acids (DFA), ω-3/ω-6, DHA/EPA, PUFA/SFA and hypercholesterolaemic/hypocholesterolaemic (h/H) ratios are extensively used to evaluate the nutritional quality of fish lipids [34]. To evaluate the nutritional quality of the annular sea bream, sardine and golden grey mullet, the different indexes mentioned above were measured on oils extracted from the three studied fish (Table 6). Interestingly, these fish oils show a higher level of unsaturated fatty acids which represent about 75% of total lipids. This fact can increase the desirable fatty acids (DFA) in the fish fillets (Table 6).

The ω3/ω6 fatty acid ratio: It’s well established that an increase in the human dietary ω3/ω6 fatty acid ratio helps to prevent coronary heart disease by reducing plasma lipids and to reduce cancer risk [3]. The ratio of ω3/ω6 PUFAs varied significantly among fish species, ranging between 1.71 for golden grey mullet, 3.84 for annular sea bream and 1.6 for sardine. These results were similar to those reported by other studies [31]. Interestingly, the ratios of ω3/ω6 found in this study were lower than the value limit (4.0) allowed by UK Department

| Pyloric caeca | Fish fillets | FFA (%) |
|--------------|-------------|--------|
| G. grey mullet | 4.12 ± 0.02 | 5.23 ± 0.012 | 4.32 ± 0.03 |
| Sardine      | 2.24 ± 0.24 | 1.94 ± 0.03  | 0.99 ± 0.06 |
| A. sea bream | 16.22 ± 0.44 | 6.11 ± 0.22  | 13.39 ± 0.66 |
| C14:0        | 5.16 ± 0.54 | 6.19 ± 0.09  | 5.07 ± 0.18 |
| C18:0        | 4.54 ± 0.55 | 2.98 ± 0.14  | 2.79 ± 0.65 |
| C18:1        | 37.97 ± 1.32 | 24.04 ± 0.57 | 28.73 ± 1.72 |
| C18:2        | 22.34 ± 1.67 | 32.25 ± 1.2  | 19.89 ± 0.01 |
| C18:3        | 1.70 ± 0.03  | 4.75 ± 0.23  | 6.16 ± 0.03 |
| Σ SFA        | 16.32 ± 1.02 | 21.53 ± 0.98 | 30.13 ± 0.04 |
| Σ MUFA       | 38.66 ± 2.69 | 58.53 ± 1.25 | 64.66 ± 0.05 |
| C18:2 ω-6    | 4.63 ± 0.25 | 6.68 ± 0.64  | 3.43 ± 0.01 |
| C18:3 ω-3    | 2.68 ± 0.45  | 1.52 ± 0.05  | 1.04 ± 0.03 |
| C20:4 ω-6 (ARA) | 3.99 ± 0.05 | 0         | 0         |
| C20:5 ω-3 (EPA) | 5.33 ± 0.32  | 1.52 ± 0.04  | 2.89 ± 0.25 |
| C22:6 ω-3 (DHA) | 5.04 ± 0.01  | 6.23 ± 0.71  | 9.24 ± 0.65 |
| Σ PUFA       | 23.38 ± 1.20 | 17.43 ± 1.7  | 16.6 ± 0.85 |
| Σ PUFAs/SFA  | 0.615 ± 0.09 | 0.72 ± 0.02  | 0.58 ± 0.01 |
| ω3 /ω6       | 14.75 ± 0.26 | 10.75 ± 0.02 | 13.07 ± 0.12 |
| ω3/ω6        | 0.82 ± 0.21  | 6.68 ± 0.26  | 3.43 ± 0.54 |
| ω3/ω6        | 1.71 ± 0.04  | 1.6 ± 0.09   | 3.84 ± 0.21 |
| DHA/EPA      | 1.19 ± 0.01  | 2.08 ± 0.21  | 3.22 ± 0.42 |

Table 4: Acid composition of total lipids extracted from the fillet s of the golden grey mullet (M. auratus), sardine (S. aurita) and annular sea bream (D. annularis), expressed as a percentage (% w/w) of total fatty acid content.

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of Health, indicating that the three Mediterranean fish species bring a good lipid composition for human health and should be recommended for dietary inclusion to reduce cardiovascular diseases.

The DHA/EPA ratio: The percentage of DHA in three edible fishes always exceeded that of EPA. The DHA/EPA ratios in the analyzed fish oils were 1.19% in golden grey mullet, 2.08 for sardine and 3.22% in annular sea bream. For golden grey mullet, Özogul et al. [30] reported an opposite trend with an EPA content exceeding DHA, showing a DHA/EPA ratio of 0.33.

The PUFA/SFA ratio: Several studies reported an inverse proportional relation between PUFA/SFA ratio and cardiovascular risks, suggesting the replacement of SFA with PUFA in the feed diet would decrease cardiovascular diseases [3]. In our study the PUFA/SFA ratio was found to be around 0.6 in all studied species. Özogul et al. [30] reported similar results for saupe and grey mullet with PUFA/SFA ratios of 0.6 and 0.51, respectively.

The hypocholesterolemic/hypercholesterolemic (h/H) ratio: The hypocholesterolemic index (∑(C 14:0 + C 16:0)) varied significantly between the examined fish species, showing the highest value for the golden grey mullet (18.46%) followed by the annular sea bream (14.38%) (Table 6). Whereas, sardine oil shows the lowest level which, did not exceed 7.05%. This is due especially to the high concentration of palmitic acid in the mullet and in the sea bream fillets. The hypocholesterolemic/hypercholesterolemic (h/H) ratio, which indicates the fatty acids effects on cholesterol metabolism, is used as an indicator of the cholesterol fractional value of the lipid source [35]. Thus, higher values for the h/H ratio are desirable. The h/H ratio was found to have significant differences between the three edible fish (Table 6). The sardine displays the higher h/H index (5.52), followed by the annular sea bream (3.25). Nevertheless, the golden grey mullet shows the lowest h/H ratio. These h/H index values were higher than those reported for lamb meat [35].

Atherogenic (AI) and the Thrombogenic (TI) indexes: Fatty acids can also reduce the potential peril of atherosclerosis and coronary thrombosis, due to their low density lipoprotein cholesterol concentration. For such reason, Ulbrich and Southgate [18] have introduced the atherogenic (AI) and the thrombogenic (TI) indexes. It was established that low values of AI and TI are recommended for a healthy diet [34]. The recommended level should not exceed 0.4 – 0.5, to be considered beneficial for humans. Saturated fatty acids such as myristic (C14:0) and palmitic (C16:0) fatty acids are known to be the most

![Figure 2](image-url)
The oxidative stability of fish oils was tested by measuring the peroxide values (PV). The PVs were found to be 1.2, 1.6 and 2.6 meq O\textsubscript{2}/kg for the freshly extracted oils from annular sea bream, sardine and golden grey mullet, respectively (Table 3). A storage experiment was designed, where the stabilities of the three fish oils were compared (Figure 2A). Oils stability during storage

| FFA (%) | G. grey mullet | Sardine | A. sea bream |
|---------|----------------|---------|--------------|
| C12:0   | 7.65 ± 0.04    | 7.45 ± 0.02 | 6.56 ± 0.01 |
| C14:0   | 2.80 ± 0.02    | 1.35 ± 0.01 | 5.16 ± 0.12 |
| C16:0   | 24.41 ± 0.56   | 7.54 ± 0.12 | 19.98 ± 1.02 |
| C17:0   | 2.75 ± 0.01    | 5.12 ± 0.21 | 4.99 ± 0.02 |
| C18:0   | 5.14 ± 0.25    | 6.23 ± 0.02 | 4.45 ± 0.01 |
| C20:0   | 4.51 ± 0.61    | 2.33 ± 0.01 | 2.81 ± 0.01 |
| ΣSFA    | 47.26 ± 1.24   | 30.02 ± 1.02 | 43.95 ± 1.02 |
| ΣMUFA   | 41.85 ± 0.68   | 53.50 ± 0.56 | 45.77 ± 1.56 |
| ΣPUFA   | 10.90 ± 0.65   | 16.48 ± 0.65 | 10.28 ± 0.65 |
| SFA     | 0.23 ± 0.05    | 0.55 ± 0.01 | 0.23 ± 0.01 |
| MUFA    | 1.44 ± 0.01    | 0.91 ± 0.01 | 0.76 ± 0.01 |
| PUFA    | 2.61 ± 0.01    | 5.98 ± 0.02 | 2.55 ± 0.02 |
| Σω6 FA  | 2.10 ± 0.05    | 0.80 ± 0.01 | 0.56 ± 0.01 |
| Σω3 FA  | 2.79 ± 0.07    | 3.12 ± 0.12 | 1.67 ± 0.02 |
| Σω6ω3   | 1.95 ± 0.12    | 5.67 ± 0.32 | 4.74 ± 0.01 |
| Σω3ω6   | 10.90 ± 0.65   | 16.48 ± 0.65 | 10.28 ± 0.65 |
| Σω3 FA  | 0.23 ± 0.05    | 0.55 ± 0.01 | 0.23 ± 0.01 |
| Σω6 FA  | 4.71 ± 0.25    | 6.78 ± 0.13 | 3.11 ± 0.12 |
| Σω3ω6   | 1.31 ± 0.02    | 1.43 ± 0.03 | 2.3 ± 0.21 |
| DHA/EPA | 0.7 ± 0.01     | 1.81 ± 0.01 | 2.85 ± 0.14 |

Table 5: Acid composition (% of total fatty acids) of total lipids extracted from the pyloric caeca of golden grey mullet (Mugil auratus), sardine and annular sea bream (Diplodus annularis) (mean value ± SD).

atherogenic, while the oleic acid (C\textsubscript{18:1}) is considered to be thrombogenic [34]. To bring further information about the nutritional quality of the three studied fishes, the AI and TI values were measured (Table 6). Both indexes differed significantly among the three fish oils. The TI values varied from 0.13 for sardine oil to 0.34 for golden grey mullet oil (Table 6). Consequently, the golden grey mullet displays an important decrease of the PV and this was also observed at 0°C. Therefore, for a better nutritional quality, it’s recommended to store fish at -20°C. Several authors have used also the polyene index to further study the oxidation degree in fish oil [38]. They suggest that this index relates the EPA and DHA amounts as compared to palmitic acid (C\textsubscript{16:0}). During the storage (at 0°C or 20°C) for one month, no significant fluctuation was found for this index (data not shown). These findings agree with the peroxide values which show a high stability at-20°C after 30 days of storage and strengthen the hypothesis that EPA and DHA didn’t changes during the storage at-20°C.

Total carotenoids

The fish is not able to synthesize carotenones, so they need to get them from the feed diet [39]. Therefore, fish incorporate carotenones through the algae, phytoplankton and small crustaceans which are the fundamental parts of their feeding. Due to their liposoluble nature, carotenones are extracted with the oil [39]. Therefore, we have determined the carotenone concentrations in the oils extracted from the three fishes (Table 3). Carotenone contents of oils extracted from fish muscles varied significantly (<0.05) among the studied species with values ranging from 2.23 ± 0.56 mg/100g for golden grey mullet oil, to 9.79 ± 0.23 mg/100g for sardine oil (Table 3). The carotene content in the muscle extracted oil of three fish was lower than that extracted from the liver of D. brevis (16 mg/100g oil), but superior to that of oil from liver of G. marmorata (0.2 mg/100g oil) [40]. These variations in the amount of carotenones were dependent on growth phase when fish are harvested, the feeds and the season [40]. It is likely that consumption of microalgae ensures high carotenones intake for sardine. During storage, oxygen is depleted in the oil and carotenones act thus as antioxidants and protect the oil from oxidation as well as from the in vivo peroxidation [39]. During the storage for 30 days at 0°C and -20°C, total carotenones contents were measured (Figures 2B and 2C). At 0°C, the carotenones concentrations decreased significantly after 20 days for all the three species. It was found to be about 9.79 ± 0.23, 2.23 ± 0.56 and 7.64 ± 0.69 mg/100g for sardine, golden grey mullet and annular sea bream oils (Figure 2B). During the first 20 days of storage, the carotenones concentrations of different oils are stable, and then a slower decrease was observed. Apparently the loss of carotenones is due to oxidation of oil followed by the appearance of primary oxidation mechanisms. For 30 days of storage at -20°C, the carotenones contents for the three edible fish seem not to be affected and are maintained higher than those of the samples stored at 0°C (Figure 2C). A decrease of the PV and the carotenones contents was observed with the storage time of the different oils. Therefore, it can be recommended to store fish species products at -20°C in order to gain longer storage life.

In vitro digestibility of fish oils

To further evaluate the nutritional quality of fish fillets, we tried to simulate the human intestinal phase of hydrolysis of triglycerides.
compared the rates of hydrolysis of fish oil and olive oil emulsions by a pancreatic lipase. Our results show that the rate fish oil emulsion hydrolysis was greater when adding the BSA to the reaction medium (Data not shown). This is to be explained by the fact that the physicochemical properties of the oil/water interface could affect also the penetration of the lipase to the interface and the hydrolysis rate, as previously suggested [41]. In vitro digestion of fish oil emulsions was carried out using purified turkey pancreatic lipase (TPL) [19] and fish digestive lipases purified from sardine (SaDL), golden grey mullet (GmDL) and annular sea bream (AsDL) [5,6,20]. We tested the ability of these lipases to hydrolyze various oils. Lipase solution containing 30 UI was used in the test systems. The released fatty acids by different lipases were measured at pH 8 during the time course of hydrolysis by titration of released fatty acids with 0.1M NaOH using the pH-stat (Figure 3).

All oil emulsions were efficiently hydrolyzed by lipases within 15 min (Figure 3A). The pancreatic lipase could process similarly fish oils and olive oil. The TPL presents linear kinetics, when hydrolyzing all fish oil emulsions; it was more active on golden grey mullet oil than on annular sea bream and sardine oils. These results can be explained by the fact that the golden grey mullet oil has lower unsaturated FA concentration, whereas, both sardine and annular sea bream oils were wealthy in oleic (C18:1) and other unsaturated fatty acids (Table 4), which might reduce the interface accessibility for the enzyme, as it was described previously for the Human pancreatic lipase [42].

The golden grey mullet lipase (GmDL) seems to be active on all emulsions with the same rates (Figure 3B). Like TPL, the GmDL has a linear kinetics towards all fish oils and was more active on the golden grey mullet oil (Figure 3B). This is in coherence with our recent studies, showing that GmDL has a wide spectrum of substrate specificity and hydrolyses efficiency short chain, and long-chain triacylglycerols at comparable rates [6].

The kinetic of fish oils hydrolysis by annular sea bream (AsDL) and the sardine (SaDL) lipases are shown in Figure 3C. Unlike TPL and GmDL, AsDL and SaDL) show non-linear hydrolysis kinetics. This is probably due to an enzyme inactivation with time due to an inappropriate substrate interface for the enzymes. The grey mullet oil was always the
Many short chain aliphatic esters are capable to produce pleasant fruity flavors [6]. These kind of esters are generally produced either traditionally by extraction from natural sources, by fermentation or by chemical synthesis. The use of biotechnology could be an alternative in the production of flavor esters [7]. They are widely used as additives in food, cosmetic and pharmaceutical industries [8]. Flavor esters have a great interest in several industrial applications. They are widely used as additives in food, cosmetic and pharmaceutical industries [7]. These kind of esters are generally produced either traditionally by extraction from natural sources, by fermentation or by chemical synthesis. The use of biotechnology could be an alternative in the production of flavor esters [7]. They are widely used as additives in food, cosmetic and pharmaceutical industries [8].

The immobilization of lipases onto solid support contributes to increasing their thermo-stability and to extend their biotechnological potential. For this reason, different fish lipases were successfully immobilized onto CaCO₃ to be used for synthesis reactions.

**Potential of immobilized lipases in flavor esters synthesis**

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Flavor esters have a great interest in several industrial applications. They are widely used as additives in food, cosmetic and pharmaceutical industries [7]. These kind of esters are generally produced either traditionally by extraction from natural sources, by fermentation or by chemical synthesis. The use of biotechnology could be an alternative in the production of flavor esters [7]. They are widely used as additives in food, cosmetic and pharmaceutical industries [8].

**Table 6: Nutritional quality indexes measured in the fillet of three fishes.**

| Species               | AI      | TI     | DFA     | HH     |
|-----------------------|---------|--------|---------|--------|
| Sardine oil           | 0.25 ± 0.042 | 0.13 ± 0.002 | 77.56 ± 1.21 | ± 0.032 |
| Grey mullet oil       | 0.47 ± 0.012 | 0.34 ± 0.024 | 74.62 ± 1.65 | ± 0.062 |
| Annular sea bream oil | 0.30 ± 0.042 | 0.246 ± 0.09 | 76.33 ± 1.24 | ± 0.058 |

Results were expressed as mean ± standard deviation (n=3). AI = (C12:0 + 4*C14:0 + C16:0)/(MUFAs + 3*PUFAs); TI = [(C14:0) + C16:0 + 18:0] / (0.5*MUFAs + ω-6 PUFAs) + 3*MUFAs / (ω-3/ω-6)); DFA = (UFA + C18:0); HH = (C18:1n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:6n-3) / (C14:0 + C16:0).

**Figure 4: Time course of isoamyl acetate, butyl acetate and butyl laurate synthesis using CaCO₃ immobilized SaDL.** Reaction conditions were: 10 U of immobilized SaDL, acetic acid/ alcohol molar ratio of 1.4 ml of hexane and incubation at 40°C.

**Conversion yield (%) vs. Time (min) for ISO, BUT and BUTL.**

The best substrate for the studied lipases likely due to its lower unsaturated fatty acids content. Interestingly, the AsDL and the SaDL hydrolize sardine and annular sea bream oils at similar rates whereas, their activities on olive oil are lower (Figure 3C). This might be explained by the fact that olive oil was mostly composed by unsaturated fatty acids (oleic acid).

**Conclusion**

This study is a step towards the characterization and classification of some economically important Mediterranean (Tunisia coasts) fish species. Three edible fish (Annular sea bream, sardine and golden grey mullet) are characterized by high contents of minerals and beneficial fatty acids such as EPA and DHA. Their fillets displayed ω-3/ω-6 ratios that allow them to be suitable for coronary heart disease prevention. The study indicated that there is a large variation in the fatty acid compositions and oil content among studied species. The lipid stability, measured by evaluating PUFA damage and oxidative indicators was higher during a storage time of 30 days at-20°C, which is a recommended storage temperature in order to maintain the product quality. In vitro hydrolysis showed that fish oils, rich in PUFAs, were efficiently hydrolyzed by a pancreatic lipase. This suggests their high digestibility in the intestinal tract. The fish lipases show a great potential to synthesize aromatic esters designed for food industry.

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