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Sample preparation techniques coupled to advanced chromatographic methods for marine organisms investigation☆

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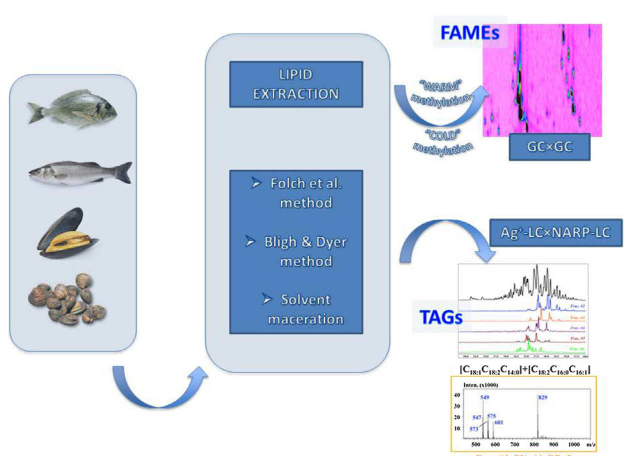
HIGHLIGHTS

- Folch's, Bligh & Dyer's and maceration for isolation of lipids from marine species.
- For derivatization of free fatty acids into methyl esters, two approaches were tested.
- Triacylglycerols analyzed by off-line Ag^+-LC/NARP-LC.

GRAPHICAL ABSTRACT



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ABSTRACT

Objective: of this work was to develop suitable extraction methodologies for the isolation of lipids from fish, mussels and clams from the Mediterranean sea, and their successive analysis by means of advanced chromatographic instrumentation. More specifically, three different sample preparation methodologies were adopted: Folch's, Bligh & Dyer's and maceration. The lipidic extracts, after application of two different methylation procedures, were subjected to monodimensional and comprehensive two-dimensional GC analyses, in order to compare the fingerprints of samples derived from different extraction and transesterification methodologies. Triacylglycerols (TAGs) were analyzed by an off-line combination of silver-ion liquid chromatography with non-aqueous reversed phase liquid chromatography. In both LC and GC analyses, mass spectrometric detectors were used, which greatly supported the identification procedure. In particular, with respect to HPLC, mass spectrometry with atmospheric pressure chemical ionization in positive mode was applied.

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1. Introduction

Fish fat includes different classes of lipids: triglycerides, phospholipids, sphingomyelins, waxes and sterols. Pioneer epidemiological studies on the low incidence of heart disease in Eskimos [1] ended up with the scientific evidence of beneficial effects of fish lipids on human health. Fish fat is characterized by the presence of polyunsaturated fatty acids (PUFAs), the most important being docosahexaenoic acid (C22:6n3, DHA) and eicosapentaenoic acid (C20:5n3, EPA). EPA and DHA are today universally recognized as nutraceuticals, for their numerous positive properties on the cardiovascular and nervous systems [2]. Beyond EPA and DHA, several other fatty acids have been reported in fish (i.e., oleic, palmitic, eicosenoic and cetoleic acids). However, fat composition is deeply affected by various factors: animal species, habitat, availability of food, and season [3].

Fat composition of mussels and clams is dominated by the presence of triacylglycerols (TAGs) and phospholipids [4]. Compared to fish, the fraction of saturated fatty acids (SFAs) is quantitatively higher, with consistent amounts of palmitic, myristic and oleic acids.

A survey of literature highlights big discrepancies in the percentages reported for the different classes of fatty acids, namely, saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids [5–8]. On average, ranges reported for these three groups are 25–50% SFAs, 15–30% MUFAs, and 20–60% PUFAs.

As for fish, this highly variable composition of the fat is dependent on: animal species, geographical site, season, and animal's organs.

Objective of this work was to develop suitable extraction methodologies for the isolation of lipids from fish, mussels and clams, and their successive analysis by means of advanced chromatographic instrumentation.

It is unquestionable that the most successful methodologies utilized in the last decades for total lipid extraction from animal tissues are those initially proposed by Folch et al. [9] in 1957, later modified by Bligh and Dyer [10] in 1959. Folch and co-workers emphasized the importance of the proportions of extractants (chloroform:methanol:water 8:4:3 by volume) and washing of crude extracts toward the exhaustive extraction of lipids from tissues. Originally, this protocol was developed over a variety of biological tissues, basically human brain, since Folch was an eminent neuroscientist. The method proposed by Folch and co-workers became so popular that, by far, around 560 papers make use of it [11]. In an attempt to reduce the amount of solvents employed for the extraction, the Bligh & Dyer methodology earned success as well, in particular for the extraction of lipids from fish tissues, which contain much lower fat and much more water. Numerous adaptations of both the Folch and Bligh & Dyer methods have been presented during the years, depending upon the matrix under investigation. In the shade of these two predominant methodologies, other extraction techniques have been applied to marine species, namely, supercritical fluid extraction [12–14], microwave-assisted extraction [15–17], accelerated solvent extraction [18], and Soxhlet [18,19]. In this study, the lipidic extracts were characterized by means of advanced chromatographic techniques. More specifically, triacylglycerols (TAGs) were determined by means of multidimensional HPLC techniques. Due to the great number of possible fatty acids combinations on the glycerol backbone, the determination of TAG profile represents a very challenging task. An effective solution has been demonstrated to be the use of both silver ion liquid chromatography (Ag^+-LC), which provides separation mainly on the basis of the unsaturation degree (number of double bonds, DBN); and non-aqueous reversed phase liquid chromatography (NARP), which provides separation on the basis of hydrophobicity, according to increasing partition number (PN). Partition number is defined as PN = CN − 2 DB, where CN is the total number of carbon atoms of the three acyl chains and DB is the total number of double bonds.

Various works have previously reported successful separations of complex lipidic samples by exploiting a combination of argentation chromatography with NARP-HPLC [20–23]. On the other hand, the lipidic extracts, upon methylation, were subjected to GC analysis, in order to compare the fingerprints of samples derived from different extraction and transesterification methodologies. In both LC and GC analyses, mass spectrometric detectors were used, which greatly supported the identification procedure. In particular, with respect to HPLC, mass spectrometry with atmospheric pressure chemical ionization ($APCI^+$-MS) is considered the most convenient and effective detector for the separation of regioisomers of TAGs [24].

2. Experimental

2.1. Samples and chemicals

Samples of clams (*Venerupis aurea* var. *laeta*) and of mussels (*Mytilus galloprovincialis*) were kindly provided by mollusc farms, located in the neighborhood of Messina (Sicily, Italy) on the salted lakes of Ganzirri and Faro. Fishes under investigation were sea bass (*Dicentrarchus labrax*) and gilthead bream (*Sparus aurata*), either purchased at the local fish market or collected from the wild, in the Strait of Messina (Mediterranean Sea). Methanol, chloroform and sodium methoxylate were supplied by Sigma–Aldrich (Milan, Italy). Hexane was purchased from PanReac AplliChem (Barcelona, Spain). Isopropyl alcohol (IPA), butyronitrile (BCN) and acetonitrile (ACN) (all LC–MS grade) were purchased from Sigma–Aldrich (Milano, Italy). Potassium hydroxide was from Fluka (Buchs, Switzerland). 1-Palmitin-2-olein-3-stearin (POS), triolein (OOO), trilinolein (LLL), trilinolenin (LnLnLn), triarachidin (ArArAr), trieicosapentaenoin (EpEpEp), and tridocosahexaenoin (DhDhDh) were purchased from Larodan (Solna, Sweden).

2.2. Sample preparation

Extraction of lipids from fish and molluscs took place by means of three different extraction procedures.

2.2.1. Bligh & Dyer method [10]

Ten grams of animal's flesh were ground in a mortar with 10 mL of chloroform ($CHCl_3$) and 20 mL of methanol (MeOH). The ground mixture obtained was added again with 10 mL of $CHCl_3$, plus 10 mL of distilled water, and stirred. Successively, the mixture was filtered through paper and the obtained solution was allowed to stand. Finally, the solution was centrifuged for 15 min at 3000 rpm. The bottom layer was then collected and transferred into a rotating evaporator, model P/N Hei-VAP Precision ML/G3 (Heidolph Instruments GmbH & Co., Schwabach, Germany). For an exhaustive extraction, the upper layer was subjected again to all the steps above described.

2.2.2. Folch method [9]

An aliquot of 10 g of animal's flesh was ground in a mortar with 67 mL (20:1 solvent/tissue parts) of a solution 2:1 $CHCl_3$/MeOH. The mixture was then placed in an ice bath and stirred for 30 min. Successively, the suspension was transferred into a separating funnel, and agitated for a couple of minutes. The upper layer was then centrifuged for 15 min at 3000 rpm. Finally, the bottom layer was evaporated to dryness and the upper layer subjected again to the extraction process for other two times.