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Analytical method and characterization of bioactive fatty acids from biological matrices: property and recent trend

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Premise

The technical report "***Analytical method and characterization of fatty acids from marine foods matrices: property and recent trend***" is part of the project *"Sperimentazione e validazione di nuove tecnologie per il miglioramento della shelflife dei prodotti ittici attraverso l'uso di un film edibile a base pectinica"- PECTINE*- PON Research and Competitiveness 2007-2013.
The aim was to test and validate a mixture based pectinic able to lengthen the shelf life of fish products, through the study and analysis of the mixture in *toto* for the evaluation of its eco-toxicity and the analysis of useful indicators (polyunsatured fatty acids, biogenic amines, proteolysis, pattern of protein, etc.) to estimate the deterioration of the products. The different assays predicted also *analysis of indicators* to evaluate the nutritional parameters, in particular the qualitative and quantitative analysis fatty acids (Masullo et al., 2015 a; Masullo et al., 2015 b).

1 Introduction

1.1 Important relevance about content lipids and fatty acids on health and nutrition

Dietary fats are the most targeted nutrients related to chronic diseases such as obesity, diabetes, cancer, arthritis, asthma, and cardiovascular disease. The public perception of fats, in general, is not favorable. However a wide range of nutrients and non-nutrients can be used as functional ingredients in fats, providing specific health benefits (Huang et al., 2007). The benefits and functional roles of dietary fat in providing calorie density to the diet, as a source of essential fatty acids and as a vehicle for fat-soluble vitamins, nutrients and antioxidants for the human body have been long established. The fatty acids (FAs) composition of food is very important because lipids are one of the three major constituents of food. Their roles in biological tissues are: (1) source of energy, (2) components of biological membranes, (3) precursor for many different molecules and (4) transport vehicle for vitamin A, D, E and K in and out of the cell (Eder et al., 1995). Humans cannot synthesize double bonds at position 6 or lower such as omega-3 (*n*-3) and omega-6 (*n*-6) and so polyunsatured fatty acids PUFAs are essential fatty acids necessary for human health and must be obtained from the diet. The nature is plentiful of the parent compound of the *n*-6 family, linoleic acid (LA) (C18:2, *cis,cis* Δ9,Δ12, *n*-6). It is present in the seeds of most plants being its content high in oils commonly used in cooking, such as corn and sunflower oil. On the other hand α-linolenic acid (ALA) (C18:3, cis,cis,cis Δ9,Δ12,Δ15, n-3), the parent compound of the omega-3 family, is far less common and is found primarily in soya bean, rapeseed and flaxseed oil. Both α-linolenic acid and linoleic acid can be elongated and desaturated to long-chain PUFAs: linoleic acid to arachidonic acid (AA) (C20:4, *cis,cis,cis,cis* Δ5, Δ8,Δ11,Δ14, *n*-6), and α-linolenic acid to eicosapentaenoic acid (EPA (C20:5, *cis,cis,cis,cis,cis*-Δ5,Δ8,Δ11,Δ14,Δ17, *n*-3) and docosahexaenoic acid (DHA) (C22:6, *cis,cis,cis,cis,cis,cis*- Δ4,Δ7,Δ10,Δ13,Δ16,Δ19, *n*-3) (Michelsen et al., 2014). Consumption of certain foods or fatty acid supplements can modulate abnormal fat and eicosanoid metabolism, both of them associated with the above mentioned chronic diseases. Among all the fats, certain fatty acids have the potential to be used as functional ingredients since their intake has been found positively related to health (Berquin et al., 2012). Polyunsatured fatty acids PUFA, along with γ-linolenic acid (GLA) (C18:3, *cis,cis,cis* Δ6,Δ9,Δ12, *n*-6) and stearidonic acid (STA) (C18:4, *cis,cis,cis,cis*- Δ6, Δ9,Δ12,Δ15, *n*-3) have shown biologically beneficial effects. An increase of α-linolenic acid has been associated with a decrease

in blood cholesterol levels, a reduction in the risk of heart attacks and a growth reduction in breast, colon and prostate cancer (Rodriguez at al., 2009). Linoleic acid has increasing its popularity in cosmetics because of its beneficial properties on the skin. The effective properties of LA are anti-inflammatory, acne reduction and moisture retention properties (Chavarro et al., 2012). It has also been found to reduce actively the serum cholesterol (Menendez et al., 2013). On the basis of the inverse relationship between linoleic acid intake and plasma cholesterol concentration and the link between plasma cholesterol concentration and the incidence of coronary heart diseases, advisory agencies in Western countries have long recommended increasing linoleic acid intake to 4–10% of energy intake (Panchaud et al., 2012). The long-chain *n*-3 PUFAs, such as EPA and DHA prevent and treat hypertension, arthritis, inflammatory and autoimmune disorders, as well as cancer (Chapkin et al., 2013). However, the hallmark effect of these two PUFAs is to decrease plasma triglyceride concentration and therefore to reduce the risk of fatal coronary heart diseases mediated by several mechanisms, including diminution of reduction of triglyceride synthesis and chylomicron secretion from intestinal cells and suppression of fatty acid synthesis and triglyceride production in the liver. In addition to all the beneficial properties described above, low levels of DHA result in a reduction of brain serotonin levels and have been associated with promotion of ADHD (Attention disorder with hyperactivity), Alzheimer's disease, depression, among other diseases. There is mounting evidence that DHA supplementation may be effective in combating such diseases (Svensson et al., 2014). Arachidonic acid or eicosatetranoic acid (AA) is one of the essential fatty acids required by most mammals. It is accumulated rapidly in the developing brain during the last trimester of gestation and a deficit in AA results in reduced growth in infants. On the other hand has also been stated that infant formulas supplemented with AA from fungal oil enhanced the growth of prematural infants. Moreover arachidonic acid supplementation has shown beneficial effects in human amnesic patients, improving the cognitive dysfunction due to organic brain damages or aging (Clandinin et al., 2012). It is involved in the growth suppression of human lung tumor A549 cells (Eder et al., 1995).

1.2 Research on the composition of fatty acids in marine products

Many studies affirm that fatty acids, especially ones in fish lipids**,** are very important nutritional elements for human health. Marine food and particularly marine fish are an important part of the Mediterranean diet (Greenk Ministry of Agricolture 2004). Their beneficial effect has been related basically to the high content of n-3 fatty acids (FAs). Moreover lipids are critical components in fish nutrition as sources of energy and PUFAs affect their metabolic activity, growth rates and reproduction. In Mediterranean Sea the main economic and commercial fishing is represented by small pelagics, in particular anchovy (*Engraulis encrasicolus)* or Sardine (*Sardine philcardus)* (Zlatanos et al., 2007; Patti et al., 2014). The fat content and the fatty acids of the fish are not constant. In fact for exogenous and endogenous factors such as diet, size, age, reproductive cycle, temperature, season, and geographical location, is very difficult predict the fatty acid composition during the year. Experimental evidences suggest the most important change in total lipid and fatty acid composition is observed during the period of reproduction or *spawning period* (Pacetti et al, 2013; Tufan et al., 2011).

Is very important to know the lipid composition of fish. This information can be useful for people to formulate their own diet and also for the industry to determinate when the products of higher quality

can be produced. The n3/n6 ratio has been suggested to be an useful indicator for comparing relative nutritional values of fish products with a recommended ratio of 1-1,5 (Osmann at al., 2011).For example fish oil is the main source of n3-fatty acid. It is extracted from non-consumable parts or byproducts of the fish, with these byproduct being defined as unused or recoverable parts subsequent to traditional methods of treatment of fish such skin, viscera, liver, reproductive elements such eggs or milt (Tufan et al., 2011).

Gas chromatography (GC) is an indispensable analytical technique in the application of fatty acids determination.
With these premises the aim of this report is to definite an reproducibility and fast method to analyze the amounts of fatty acids (SFA-MUFA-PUFA) in biological products for example fishes samples by Gas Cromatography. In particularly have been analyzed fish samples *(Engraulis encrasicolus).*

2 Materials and method

Fatty acids analysis in biological matrices is divided into:

1. extraction of lipids;
2. esterification of fatty acids;
3. separation, identification and quantification of the FAMEs by GC.

2.1 Extraction of fat content in fish samples

A broad range of extraction techniques (Soxhlet, percolation, maceration, digestion, steam distillation, etc.) are used currently for this purpose (Rodriguez et al., 2009). In *Bligh and Dyer 1959* method a chloroform/methanol/water mixture is used to separate the lipids from the matrices in samples containing 80% water. It is employed generally to extract lipids quantitatively in marine matrices. For this reason the extraction of fat content in fish samples was conducted thought *Bligh & Dyer (1959)* method with minor modifications:
fish individuals are beheaded, eviscerated and filleted without removing skin (edible muscle) and an aliquot (2-5 g) of edible muscle was homogenized in chloroform-methanol (1:2, v/v) by multivapor TM Buchi b-12 at 25 °C for 2 min using glass tube of 50 ml to obtain a monophasic system (Figura 1). To this ternary system one part of chloroform is added and the mixture blended for 60 sec and then one part of water, blending continued for 60 sec. The homogenate is filtered through Whatman n.1 filter paper, some mechanical force is used to press the liquid out of tissue. To separate in two layers (lower phase chloroform which are soluble non-polar lipid as tryglicerides, diglycerids, monoglycerides, sterols etc. and an upper phase of water/methanol which are soluble polar lipids as free fatty acids, phospholipids, sphingolipids, etc) is used a separation liquid-liquid method with separating funnel. For quantitative extraction, a successive blending of the residue and filter paper with one part of chloroform follows by filtration. This filtrate is added to filtrate obtained. The lipid content is determined by weighing after evaporation of chloroform layer to dryness 50 °C by multivapor TM Buchi b-12 using glass tube of 16 ml system.

Fig 1: Multivapor TM Buchi b-12

2.2 Transesterification reaction

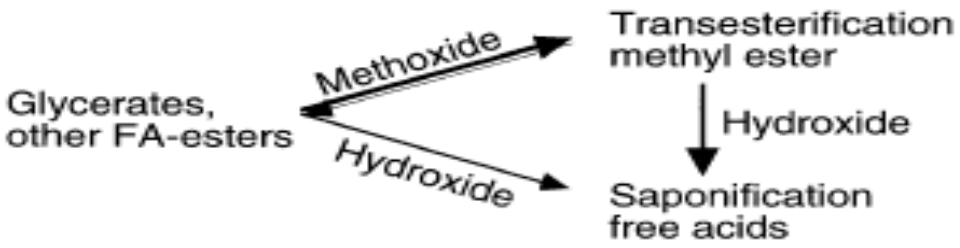
Esterification of lipids can be carried out with several reagents based on acid-catalyzed or base-catalyzed reactions.
The Acid- catalysed transesterification is the most common method used for the transesterification of all types of lipids as tryglicerides, diglycerids, monoglycerides, sterols and free fatty acids. Common reagents used are methanolic, hydrochloric and sulfuric acid, and boron trifluoride in methanol and neither acid-catalysed nor boron-fluoride-catalysed reactions proceed at real temperature; both types of reaction require heating (Eder at al., 1995). In this case it is advisable to protect PUFAs from autoxidation due to heating by adding antioxidants such butylated hydroxytoluene BHT. Formely, the tryglicerides are converted into methyl ester using sodium hydroxide-methanol reagent and then boron trifluoride in methanol reagent step completes the esterification of free acids or re-esterification of all no-esterified fatty acids (AOCS Method Ce-1b-89; Eder et al.,1995). So, samples containing considerable amounts of lipid-bound fatty acids as fish food , the acid step has no benefits and can be omitted (Eder et al., 1995). For this reason the *Base-catalysed transesterification* is the most effective and fast method to analyse the amounts of FAMEs into fish matrix (Eder et al., 1995; Rodriguez et al., 2009). We used it for the analysis of fish samples *(Engraulis encrasicolus)*.

Base-catalysed transesterification:

In contrast to all the acid-catalysed reactions, transesterification with sodium methoxide proceeds at ambient temperature; the risk of decomposition of PUFAs is lower (ambient temperature) and transesterification base at ambient temperature does not require BHT. This is undoubtedly an advantage because in some instances, in the gas chromatographic analysis of FAMEs, it has proved impossible to separate methyl myristate or palmitoleate from BHT and its derivates (Eder at al., 1995). Base-catalysed transesterication has become widely in the most used method to convert all fatty acids into methyl esters, which are in the form of mono-, di-, triglycerides, and sterol esters; however it does not esterify free fatty acids. The methods for base-catalysed transesterication used are determined by the belief that the complete absence of water is a prerequisite. In fact,

steichiometrically some 6% of water in the sample is sufficient for complete reaction. However Eder and collegues (1995) affirmed that fat can be transesterifed in foods even if they consist largely of water. As shown in Fig. 2, reaction methoxide trans-esterifiries triglycerides and other fatty acid esters into methyl esters. In the presence of water, methoxide also forms hydroxide, which may saponify the triglycerides or the newly generated methyl esters. While transesterification is a reversible reaction, saponication is irreversible and, hence, the end point of the reaction if enough time is given to reach it. There is a the equilibrium between methoxide and hydroxide being on the side of the methoxide. It has also been shown that the rate of attack by methoxide is higher than that of hydroxide. This enables the conversion of triglycerides and other esters of fatty acids into methyl esters even in presence of water, provided that the reaction is stopped before the methyl esters are saponifed. The best time to avoid the saponification reaction is 1 min. (Suter at al.,1997). Figura 3 shows key elements of direct transesterification.

Fig 2: Transesterification and saponification (Eder et al., 1995)



The steps *Base-catalysed transesterification* are:

Methyl esters were prepared by transmethylation using 2 M potassium hydroxide (KOH) (Merck, Darmstadt, Germany) in methanol (Sigma-Aldrich, Steinhein, Germany) according to the method described by ISO 5509-2000 with minor modifications:

1. 20-40 mg of extracted oil was dissolved in 1 mL hexane;
2. addition of 0.5 mL of 2 M methanolic KOH;
3. the tube were vortexed by multivapor TM Buchi b-12 at 25 °C for 1 min at ambient temperature (Figura 1);
4. after homogenization, the hexane layer was taken for GC analyses.

Figure 3 Key elements of direct transesterification

- Reaction in the homogenized food
- Fast transesterification: 1 min at ambient temperature
- Conditions providing robust optimum
- Stopping the reaction before relevant saponication occurs
- Internal standards verifying transesterication for each sample

2.3 Chromatographic condition

Optimum qualitative and quantitative GC analysis of complex mixtures presupposes:

1 good resolution, as shown by sharp and symmetric peaks (Figure 5-Figure 6- Figure 7);
2 high repeatability and reproducibility of retention times;
3 high precision and accuracy in quantitation based on peak area measurements, i.e. no discrimination of components through volatility, polarity or concentration.

The analysis of FAMEs was carried out using a Agilent Technologies 7890 B GC System e Agilent Technologies 7000 C GC/MS/MS Triple Quad, equipped with Agilent HP 5ms column (30 m x 0,25 mm i.d. x 0,25 µm film thickness) (Figure 4). Injection temperature was set 250 °C and detector temperature was set 270 °C with splitless system. Septum flow to split vent was 3 ml/min and purge flow to splint vent was 15 ml/min. Oven temperature was programmed at the beginning 40 °C , increased to 250 °C with a 2 °C/min increment (hold time 15 min) and finally reached to 270 °C with a 10 °C/min increment. Peaks were identified by comparison with standards *Supelco™ Component FAME Mix Sigma Aldrich* (Figure 4 and Figure 5) and using the NIST mass spectral database. Mix FAMEs (from C4:0 to C24:1) including saturated, monounsaturated and polyunsaturated FAMEs (Table 1). A further comparison was made from the literature for the values of m/z of saturated, monounsaturated and polyunsaturated respectively to 74, 55, 67 and 79 m/z (AOCS Lipid library).

Figure 4 Agilent Technologies 7890 B GC System e Agilent Technologies 7000 C GC/MS/MS Triple Quad

Figure 5: Chromatogram of standards *Supelco™ Component FAME Mix Sigma Aldrich* by GC/MS

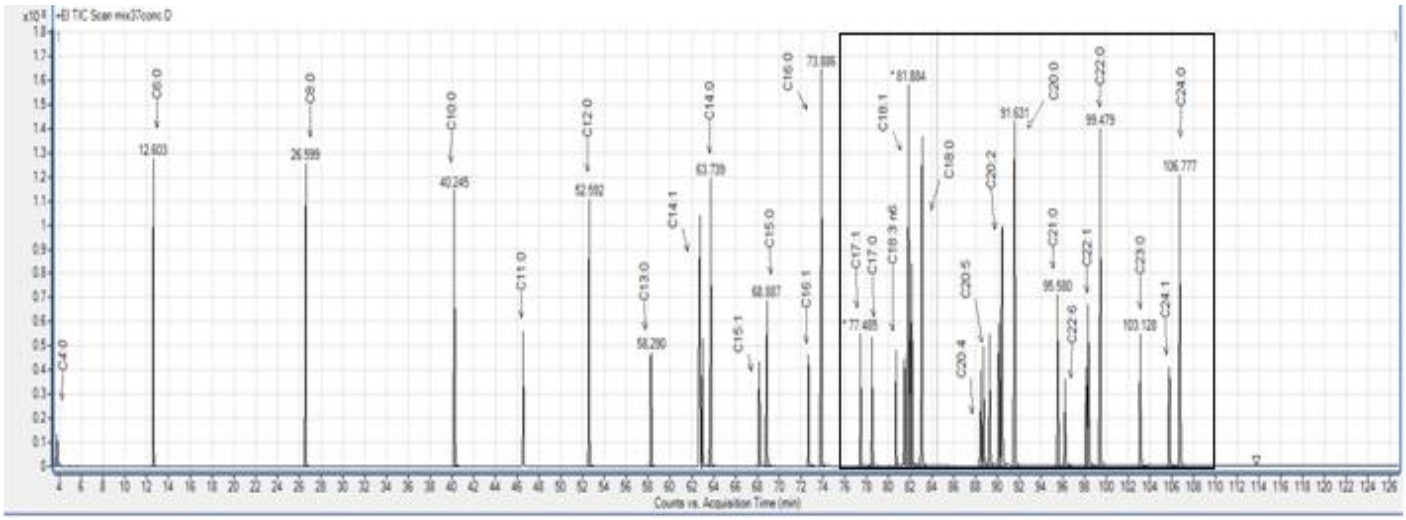
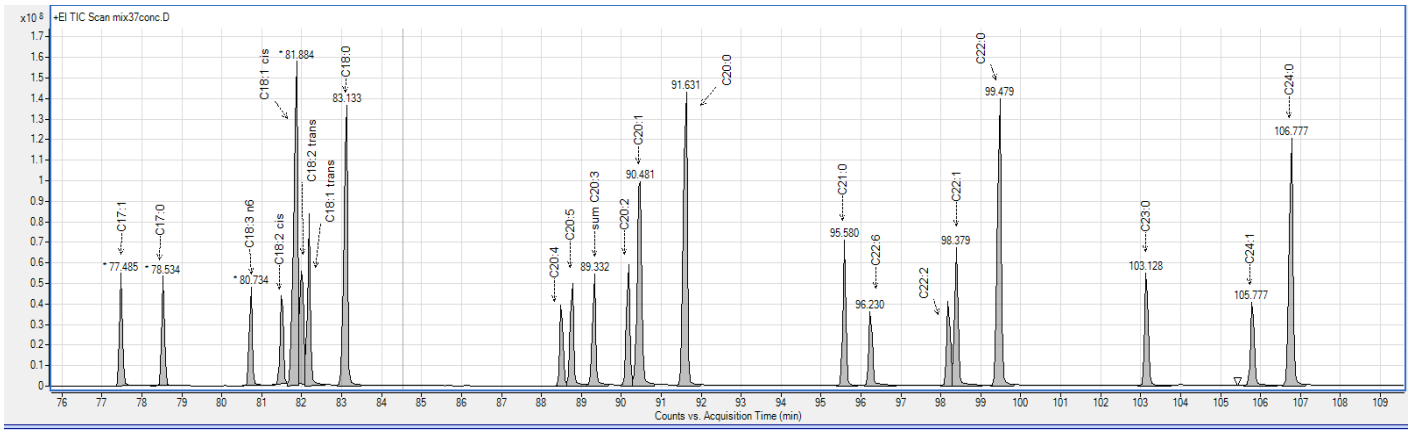


Figure 6: Chromatogram of standards *Supelco™ Component FAME Mix Sigma Aldrich* by GC/MS, zoom



from C17:0 to C 24:0

Figure 5 and Figure 6 show the peaks of SFA, MUFA and PUFA in the standard *Supelco Component FAME MIX Sigma Aldrich.* The names of fatty acids in output order from the chromatographic column are reported in table 1 (the name is reported as Cx:y with X= number of atom Carbon and Y = number of insaturation). Agilent HP 5ms column allows a good resolution of FAMEs, in particular esolve *cis* and *trans* FAMEs, with the *cis* isomer eluting prior to the *trans* isomer (for example 18:1 cis and 18:1 trans). The figure 5 shows better the elution patterns for the C18:1 n9t and C18:1 n9c and C18:2 n6t and C18:2 n6c isomers.

Table 1 Fatty acid methyl ester in *Supelco™ Component FAME Mix Sigma Aldrich* in output order from the chromatographic column Agilent HP 5ms.

Fatty acid methyl ester	Cx:y	Fatty acid methyl ester	Cx:y
1 Butyric acid methyl ester	C4:0	20 Elaidic acid methyl ester	C18:1 t n9
2 Caproic acid methyl ester	C6:0	21 Stearic acid methyl ester	C18:0
3 Caprylic acid methyl ester	C8:0	22 Arachidonic acid methyl ester	C20:4 n6
4 Capric acid methyl ester	C10:0	23 Cis 5,8,11,14,17 Eicosapentaenoic acid methyl ester	C20:5 n3
5 Undecanoic acid methyl ester	C11:0	24 ∑Eicosatrienoic acid methyl ester	C20:3
6 Lauric acid methyl ester	C12:0	25 Cis11, 14 Eicosadienoic acid methyl ester	C20:2
7 Tridecanoic acid methyl ester	C13:0	26 Cis 11-Eicosenoic acid methyl ester	C20:1
8 Myristoleic acid methyl ester	C14:1	27 Arachidic acid methyl ester	C20:0
9 Myristic acid methyl ester	C14:0	28 Heneicosanoic acid methyl ester	C21:0
10 Cis-10 Pentadecenoic acid methyl ester	C15:1	29 Cis 4,7, 10,13,16, 19Docosahexaenoic acid methyl ester	C22:6 n3
11 Pentadecanoic acid methyl ester	C15:0	30 Cis 13,16 Docosadienoic acid methyl ester	C22:2
12 Palmitoleic acid methyl ester	C16:1	31 Erucic acid methyl ester	C22:1 n9
13 Palmitic acid methyl ester	C16:0	32 Behenic acid methyl ester	C22:0
14 Cis-10- Heptadecenoic acid methyl ester	C17:1	33 Tricosanoic acid methyl ester	C23:0
15 Heptadecanoic acid methyl ester	C17:0	34 Nervonic acid methyl ester	C24:1 n9
16 γ-linolenic acid methyl ester	C18:3c n6	35 Lignoceric acid methyl ester	C24:0
17 Linoleic acid methyl ester	C18:2 c n6		
18 Oleic acid methyl ester	C18:1 c n9		
19 Linolelaidic acid methyl ester	C18:2 t n6		

Figure 7 : Chromatogram of sample muscle *Engraulis encrasicolus*

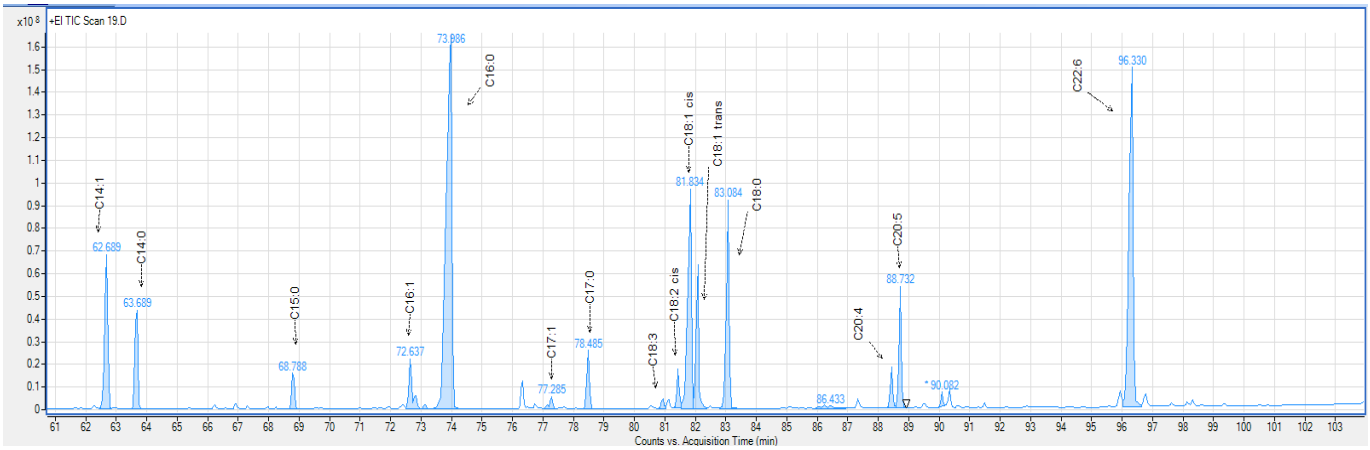


Figure 7 shows a typical Chromatogram of sample muscle *Engraulis encrasicolus.* The analytical method and instruments already described allows a good resolution of FAMEs also in the sample fish, in particular allows the separation among *cis* and *trans* FAMEs for example 18:1 *cis* and 18:1 *trans*. According to literature (Pacetti et al., 2013; Zlatanos et al., 2007), the fatty acids typically found in fishes include saturated, monounsatured and polyaturated, specially omega-3 and omega-6. In particular peak area shows C16:0 and C18:0 the most abundant among SFA while the oleic acid C18:1 *cis* among the MUFA; the large amounts of the omega-3 fatty acids, such as C20:5n3 and C22:6n3 and minor amounts of omega-6 fatty acids are such as 18:3n6, 18:2n6, 20:4n6. Both omega-3 than omega-6 are *cis* isomer.

The analytic procedures herein described enabled the identification and characterization of fatty acids content in different biological matrices for different purposes. It can be used, for example, as valid method to estimate the amounts of polyunsatured fatty acids during degradation processes (according to others parameters such as biogenic amines, protein etc.) or to conduct routine quantitative and qualitative analysis of fatty acids. It has many advantages such as few steps during the extraction phase of lipids, the speed of esterification reaction and the use of small quantities of sample. Moreover GC analysis allows a good resolution of FAMEs in particular esolves *cis* and *trans* FAMEs.

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