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APPLICAZIONE DI DIVERSE TECNICHE ANALITICHE STRUMENTALI ALLA VALUTAZIONE SELETTIVA DI COMPONENTI BIOSENSIBILI IN MATRICI DI ORIGINE ANIMALE E VEGETALE

-APPLICATION OF DIFFERENT INSTRUMENTAL TECHNIQUES TO THE SELECTIVE EVALUATION OF BIO-SENSIBLE COMPOUNDS IN FOODSTUFF OF ANIMAL AND VEGETABLE ORIGIN-

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“Litterarum radices amarae sunt,
fructus iucundiores”

Cato

PhD's RESEARCH ACTIVITIES

Publications

Castagnetti GB, Delmonte P, Melia S, **Gori A**, Losi G (2007). The effect of extruded whole linseed flour intake on the variation of CLA (Conjugated Linoleic Acid) content in milk, and OFA (Oxydated Fatty Acid) on cheese obtained - The Reggiana cattle's case. Sci. Tecn. Latt-Cas., 58 (6), 363-382.

Carretero AS, Carrasco-Pancorbo A, Cortacero-Ramíreza S, Cañabate-Díaz B, **Gori A**, Cerretani L, Fernández-Gutiérrez A (2008). Direct analysis of 4-desmethyl sterols and two dihydroxy triterpenes in saponified vegetal oils using LC-MS. Eur. J. Lipid Sci. Technol., 110, 1142–1149

Castagnetti GB, Delmonte P, Melia S, **Gori A**, Losi G (2008). The effect of extruded whole linseed flour intake on the variation of CLA (Conjugated Linoleic Acid) content in milk - The Reggiana cattle's case - L'effetto dell'integrazione della razione con farina di lino estrusa sul contenuto in CLA (Acido Linoleico Coniugato) nel latte - il caso della razza Reggiana. Prog. Nutr., 10, (3), 174-183.

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Workshops

119th AOAC INTERNATIONAL, Orlando, Florida, September 2005.

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13th Workshop on the Developments in the Italian PhD Research on Food Science and Technology, Centro Ricerche Soremartec-Ferrero, Alba (CN), 10-12 September 2008

INDEX

1

SUMMARY

This PhD thesis describes the application of some instrumental analytical techniques suitable to the study of fundamental food products for the human diet, such as: extra virgin olive oil and dairy products. These products, widely spread in the market and with high nutritional values, are increasingly recognized healthy properties although their lipid fraction might contain some unfavorable components to the human health. The research activity has been structured in the following investigations:

- *"Comparison of different techniques for trans fatty acids analysis"*
- *"Fatty acids analysis of outcrop milk cream samples, with particular emphasis on the content of Conjugated Linoleic Acid (CLA) and trans Fatty Acids (TFA), by using 100m high-polarity capillary column"*
- *"Evaluation of the oxidited fatty acids (OFA) content during the Parmigiano-Reggiano cheese seasoning"*
- *"Direct analysis of 4-desmethyl sterols and two dihydroxy triterpenes in saponified vegetal oils (olive oil and others) using liquid chromatography-mass spectrometry"*
- *"Quantitation of long chain poly-unsatured fatty acids (LC-PUFA) in base infant formulas by Gas Chromatography, and evaluation of the blending phases accuracy during their preparation"*
- *"Fatty acids composition of Parmigiano Reggiano cheese samples, with emphasis on trans isomers (TFA)"*

Keywords: dairy fats, vegetable oils, separation techniques, fatty acids, TFA, CLA, OFA.

2

INTRODUCTION

The increasing interest regarding the relation between nutrition and wellness, has led the scientific research to study the importance of nutrients contained in both the raw material and their derived food products. Extra virgin olive oil and dairy products, considered as fundamental food products for the diet of million of people, are source of liposoluble vitamins (A, D, E), anti-oxidant compounds and mineral salt, although are simply deemed by consumers as “food dressing” and high energetic foods (since they are predominantly constituted of fat).

Healthy properties are widely recognized to these food products by the scientific panel. To the extra virgin olive oil, for example, are attributed several beneficial effects: the high content in mono-unsatured fatty acids, with the oleic acid as the principal constituent, shows to have gastro-protective properties, inhibits biliary lithiasis and improves the intestinal transit regulating it, reduces the LDL cholesterol levels (Low Density Lipoprotein, the potentially harmful cholesterol with atherogenic effects) leaving unaltered the HDL levels (High Density Lipoprotein, prevent the oxidized cholesterol sticks to artery walls), it has antioxidant effect on the LDL (1), enforces the immune system reducing the risk of autoimmune diseases/ breast cancer/colon-recto cancer (2). Extra virgin olive oil contains antioxidant compounds with proved anti-inflammatory and anti-carcinogenic actions (3, 4). Moreover, since it does not subjected to the refinetion process (on the contrary of what happens for all the others edible oils), it does not meet to the formation of *trans* fatty acids.

Several investigations about milk fat and, as a consequence, butter and cheese fat, confirmed the high potential in human nutrition of CLA (Conjugated Linoleic Acid), in particular, of rumenic acid (C18:2 cis-9, trans-11), and consequently, of its forerunner vaccenic acid (C18:1 trans-11) that represent the principal isomer of the fatty acids group called TFA (*trans* Fatty Acid).

At the present, CLA have attracted considerable attention because of their attitude to contrast the risk of some lifestyle-related diseases, such as: tumors, atherosclerosis, diabetes, obesity, and hypercholesterolemia. Moreover, they enhance the immune system modulation (5, 6, 7). These properties have been verified in animal models and human cellular lines (8, 9).

Unlike the olive oil, dairy products naturally contain significant levels of fatty acids in *trans* configuration. Several studies have reported the negative effects of these isomers to human health. In 2004, the European Food Safety Authority (EFSA) affirmed that the negative effects of TFA on human health might be worst than those of saturated fatty acids. The origin of TFA is due to the rotation of the molecule around a "double bound", leading the natural configuration *cis*-*cis* to *cis*-*trans*. This configurational overthrow has noteworthy negative effects on human health: TFA, besides increasing LDL levels, make the cell membrane more permeable allowing molecules, even toxics, to filter inside the cell. They can weaken the immune system and worsen the deficiency of essential fatty acids, thwarting the production of the prostaglandins, which regulate the vascular smooth mussels in blood vessels, kidneys functions, and the inflammatory responses (10). Although *trans* fats are naturally present in the ruminant fat as the result of an enzymatic production (formation of specific isomers), they should be

distinguished from the "artificial" *trans* fat industrially produced under catalytic conditions (more random distribution of isomers) such as Partially Hydrogenated Vegetable Oils (PHVO). Since the TFA isomers responsible for the negative effects have not yet been identified, it makes it difficult to asses whether the TFA from PHVO and ruminant fats present similar risk factor (11). Moreover, according to the results of a Danish study, not only the intake of TFA from ruminant fats affect the risk of coronary health disease, but even more, they do not show any correlation (12).

Another concern, relative to the group which CLA belong to, the Poli-unsatured Fatty Acids (PUFA), is their susceptibility to the oxidation process, especially whether they are in the free form (13). Nutritionally, such oxidation can be translated as a negative health effect because of the inclination to increase the LDL levels, meaning a higher risk of pathologies related to the atherosclerosis disease (14).

Therefore, it raise spontaneous the need to elaborate scientific methods of analysis that both permit to value, identify, and eventually improve the fatty acid composition of olive oils and butters (protecting consumers from sophistications and frauds), and to point out the tight correlation between natural/ anthropic factors correlated to the production and the chemical-physical-organoleptic properties, that is "the typicality".

2.1. LIPIDS

Despite the existence of several definition of lipids, due to their complexity and heterogeneity, they are generally reported as a broad group of compounds that are soluble in organic solvents (diethyl ether, hexane, benzene, chloroform or methanol) but only sparingly soluble in water. They are major components of adipose tissue and, together with proteins and carbohydrates, they constitute the principal structural

components of all living cells. The terms *fats* and *oils* refer traditionally to glycerol esters of fatty acids, which make up to 99% of the lipid of plant and animal origin. The two terms are used interchangeably and the choice of terms is usually based on the physical state of the material at ambient temperature and tradition. Generally, fats appear solid at ambient temperatures and oils appear liquid.

Lipids are important components that contribute very significantly to the nutritional and sensory value of almost all kinds of foods, except for most fruits, sweets and beverages. Food lipids are either consumed in the form of "visible" fats, such as butter, lard and shortening or as constituents of basic foods, such as milk, cheese and meat.

The effect on food quality is mainly related to the contents, distribution in the food matrix, chemical composition and reactivity of the lipids, as well as to their physical properties (crystalline structure, melting properties) and changes due to processing and the interactions with other components. Indeed, during the processing, storage and handling of foods, lipids undergo complex chemical changes (i.e.: lipolysis, oxidation) and react with other food constituents, producing several compounds both desirable and deleterious to food quality.

2.2. CLASSIFICATION

Lipid structures can be classified depending on:

- The physical properties at room temperature. For instance, oils appear liquid and fats appear solid;
- The polarity. Neutral lipids include fatty acids, alcohols, glycerides and sterols, while polar lipids, glycerophospholipids and glyceroglycolipids;
- Their essentiality for humans (essential and nonessential fatty acids);

• The structure, which can be respectively simple or complex.

Based on structure, lipids can be classified as derived, simple or complex. The *derived lipids* include fatty acids and alcohols, which are the building blocks for the simple and complex lipids. *Simple lipids*, made up of fatty acids and alcohol components, include acylglycerols, ether acylglycerols, sterols and their esters and wax esters. In general terms, *simple lipids* can be hydrolyzed to two different components, usually an alcohol and an acid. *Complex lipids* include glycerophospholipids (phospholipids), glyceroglycolipids (glycolipids), and sphingolipids. These structures yield three or more different compounds on hydrolysis.

A general classification of lipids based on their structure is proposed in **table 2.1.** even though it should be taken as a guide since other classifications may be more useful. The most abundant class of food lipids is the acylglycerol, which dominate the composition of depot fats in animals and plants. The polar lipids are found almost entirely in the cellular membranes (phospholipids being the main components of the bilayer) with only very small amounts in depot fats. In some plants, glycolipid constitute the major polar lipids in cell membranes. Waxes are found as protective coating on skin, leaves and fruits. Edible fats are traditionally classified in different subgroups illustrated in **table 2.2.**

Table 2.1. – *Classification of lipids*

Major classes	Subclasses	Descriptions
Simple lipids	Acylglycerols	Glycerol + fatty acids
	Waxes	Long-chain alcohol + long-chain fatty acid
Compound lipids	Phosphoacylglycerols (or glycerophospholipids)	Glycerol + fatty acids + phosphate + another group usually containing nitrogen
	Sphingomyelins	Spingosine + fatty acid + phosphate + choline
	Cerebrosides	Spingosine + fatty acid + simple sugar
	Gangliosides	Spingosine + fatty acid + complex carbohydrate moiety (including salicilic acid)
Derived lipids	Lipid materials not simple or compound	Carotenoids, steroids, fat-soluble vitamins

Table 2.2. – *Lipid subgroups*

Lipid subgroups	Decription of the kind of fat	Main fatty acids
Milk fats	Fats from the milk of ruminants (dairy cows)	Palmitic, oleic, stearic and appreciable amounts of short chain fatty acids (C4:0 to C12:0), small amounts of branched, odd-numbered and trans
Lauric acids	Fats from certain species of palm (coconut, babasu)	Lauric acid (40-50%), moderate amounts of C6:0, C8:0 and C10:0, low in unsaturated acids
Vegetable buters	Fats from the seed of various tropical trees: vegetable butters (cocoa butter) used in the manufacture of confections	Saturated fatty acids
Oleic-linoleic acids	Oils of vegetable origin: cottonseed, corn, peanut, sunflower, saflower, olive, palm and sesame oils	Oleic and linoleic acid, less than 20% saturated fatty acids
Linolenic acids	Soybean, rapeseed, flaxsed, wheat germ, hempseed and perilla oils	Substantial amount of linolenic acid
Animal fats	Fats from domestic land animals (lard and tallow), egg lipids	Large amount of C16 and C18 fatty acids, medium amount of unsaturated acids (oleic, linoleic) and small amount of odd-numbered acids

2.3. NOMENCLATURE OF FATTY ACIDS

The term fatty acid (FA) refers to any aliphatic monocarboxylic acid that can be liberated by hydrolysis from naturally occurring fats. Most of FA were originally described under “trivial” or common name and even after adopting the Internation Union of Pure and Applied Chemistry (IUPAC) system for nomenclature, the habit of assigning trivial names to FA acids continues.

In standard IUPAC terminology, the fatty acid is named after the parent hydrocarbon with the same name of carbon atoms. The terminal letter *e* in the name of the parent hydrocarbon is replaced with *oic*. For example, an 18-carbon carboxylic acid is called octadecanoic acid, from octadecane, the 18-carbon aliphatic hydrocarbon.

Unsaturated FA can be named after the parent unsaturated hydrocarbon and replacement of the terminal *anoic* by *enoic* indicates unsaturation and *di*, *tri* and so on represent the number of double bonds (i.e.: hexadecenoic acid for 16:1, octadecatrienoic acid for 18:3).

The simplest way to specify the location of double bonds is to put, before the name of the acid, one number for each unsaturated linkage (Δ configuration) representing the distance from the carboxyl carbon. Oleic acid is, for example, named Δ9-octadecenoic acid or simply 9-octadecenoic, with one double bond between carbons 9 and 10 (carboxyl group is regarded as carbon 1). Nevertheless, unsaturated FA are often distinguished by the location of the first double bond from the methyl end of the molecule, that is, the omega (ω) carbon (shorthand identification). The methyl group is number 1 (the last character in the Greek alphabet is ω, hence the end): linoleic acid (cis-9,12-octadecadienoic acid) is therefore 18:2ω6 (or n-6) acid. **Fig. 2.1.** illustrates the difference between IUPAC Δ and shorthand numbering systems.

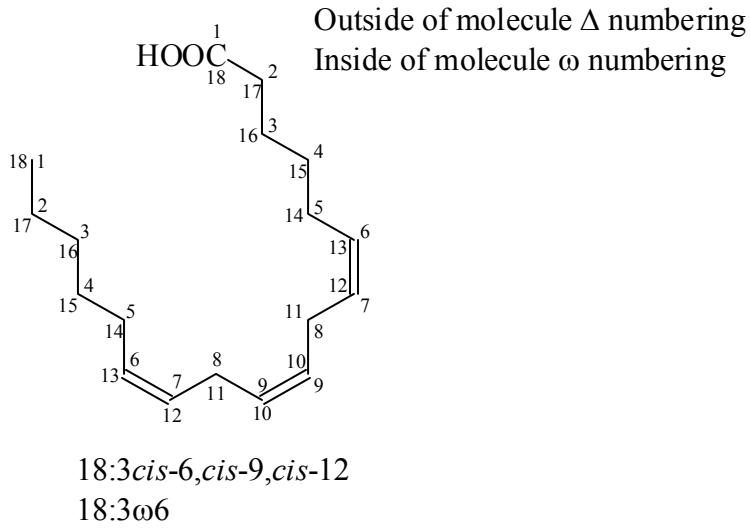

Figure 2.1. – *IUPAC Δ and common ω numbering system*.

The geometric configuration of double bonds is usually designated by the use of terms *cis* (Latin, on this side) and *trans* (Latin, across), indicating whether the alkyl group are on the same or opposite sides of the molecule (**Figure 2.2.**). The prefixes *cis* and *trans* can be abbreviated as *c* and *t* in structural formulas. In shorthand notation, the unsaturated fatty acids are assumed to have *cis* bonding and, if the fatty acid is polyunsaturated, double bonds are in the methylene interrupted positions.

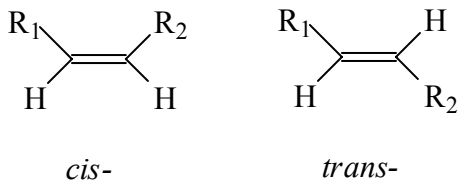

Figure 2.2. – *Example of cis/trans nomenclature.*

In the following page a list of some of the most common FA found in natural fats is reported (**Table 2.3.)**, indicating both systematic and common name for each FA.

Table 2.3. – *Nomenclature of some common fatty acids.*

Abbreviation	Systematic name	Common or trivial name
4:0	Butanoic	Butyric
6:0	Hexanoic	Caproic
8:0	Octanoic	Caprylic
10:0	Decanoic	Capric
12:0	Dodecanoic	Lauric
14:0	Teradecanoic	Myristic
16:0	Hexadecanoic	Palmitic
16:1 n-7	*cis*-9-Hexadecenoic	Palmitoleic
18:0	Octadecanoic	Stearic
18:1 n-9	*cis*-9-Octadecenoic	Oleic
18:1 n-7	*cis*-11-Octadecenoic	Vaccenic
18:2 n-6	*cis*-9,12-Octadecadienoic	Linoleic
18:3 n-3	*cis*-9,12,15-Octadecatrienoic	α-Linolenic
20:0	Eicosanoic	Arachidic
20:4 n-6	*cis*-5,8,11,14-Eicosatetraenoic	Arachidonic
20:5 n-3	*cis*-5,8,11,14,17-Eicosapeantaenic	EPA
22:1 n-9	*cis*-13-Docosenoic	Erucic
22:5 n-3	*cis*-7,10,13,16,19-Docosapentaenoic	DPA
22:6 n-3	*cis*-4,7,10,13,16,19-Docosahexaenoic	DHA

2.4 VEGETABLE OILS AND FATS

Reference for this section: 15, 16

Most fats and oils consist of triacylglycerides which differ in fatty acid composition to a certain extent. Other constituents which make up less than 3% of fats and oils, are the unsaponifiable fraction (phospholipids, tocopherols, sterols, resins, carbohidrates, pesticides, proteins trace metals, and pigments) and a number of acyl lipids (traces of free fatty acids, mono and diacylglycerols). Their composition in fatty acids can differ greatly, and depends for several factors: plant fat is affected by the cultivar and growth environment, such as climate and location of the plant; animal fat depends by the kind and breed of animal and by the feed.

Oils and fats naturally occur in a wide range of sources, each one providing a separate and distinctive material. Hundreds of seeds and fruits bear oil, all animal produce fat, and marine sources also provide oils; however, only a few of these sources are of economic importance. The factors that have always influenced their distribution and eating habit of our ancestors result to be, unequivocally, climate and availability. For instance, in northern European countries, consumers generally obtain their edible fats from animals, whereas people in southern Europe, Asia, and Africa acquire their edible oils from vegetable sources. The food products developed in these different regions use the available fats and oils products, and consequently, the cuisine of central and northern European countries developed around the use of solid fats such as butter and lard, while the diets of inhabitants from warmer climates around the use of liquid oils for their food products. Since the second half of last century, food products from the Mediterranean countries have definitely increased of importance in food science due to their beneficial effects on human health, especially for their principal source of fat: olive oil and dairy fat.

2.4.1. OLIVE OIL

Reference for this section: 15, 16

Between the vegetable oils, olive oil is the one that unequivocally have ever played an important role in the word market of oils. As reported in **figure 2.3** and **2.4**, more than 90% of the world's olive harvest takes place in the Mediterranean region, due to the unique agronomic and climatic factors of this area. Olive oil consumption has been for centuries restricted to the Mediterranean people. For the rest of the word olive oil was an unfamiliar oil, much more expensive than other vegetables (seed) oils. Due to

nutritional and economical factors, this tendency begun to change since the second half of last century, where non-producing countries (U.S.A., Australia, Canada, Brazil, Japan, France) increased olive oil consumption, and nowadays, this trend is still growing.

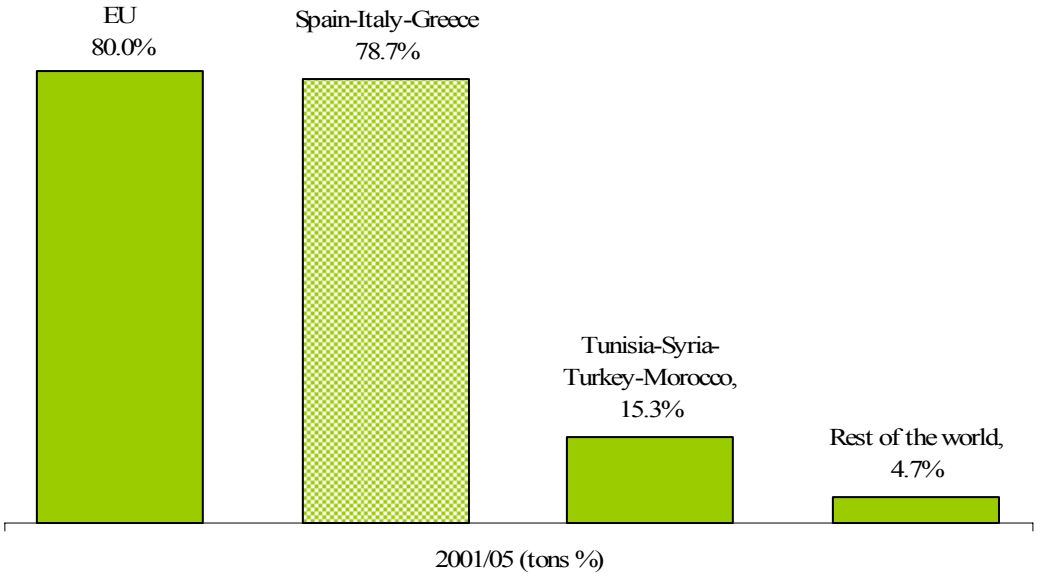

Figure. 2.3. – *The world olive oil production (quantities in tons %)*

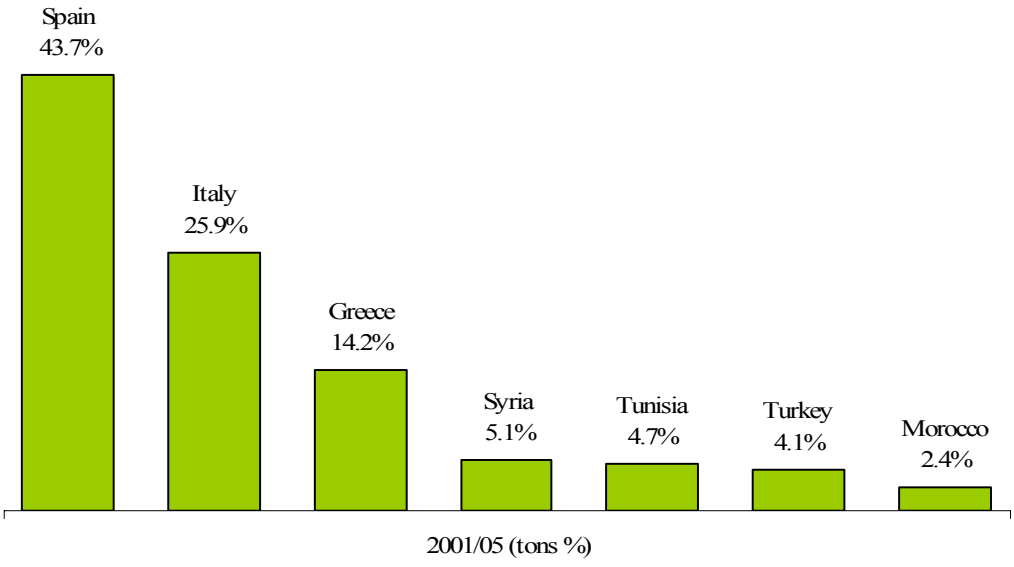

Figure. 2.3. – *The olive oil production in the Mediterranean area (quantities in tons %)*

2.4.1.1. Fatty Acids

Olive oil fatty acid composition may differ from sample to sample, depending on the area of production, the latitude, the climate, the variety, and the fruit maturity.
As reported in **table 2.4**, the main fatty acids are: palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3). Others fatty acids are present in trace amount.

Table. 2.3. – *Olive Oil Fatty Acid Composition* (%)

Oleic	C18:1 n-9	55 to 83
Palmitic	C16:0	7.5 to 20
Linoleic	18:2 n-6	3.5 to 21
Stearic	C18:0	0.5 to 5
Linolenic	18:3 n-3	<0.9
Palmitoleic	16:1 n-7	0.3 to 3.5
Arachidic	C20:0	<0.6
Margaric	C17:0	<0.3
Margaroleic	C17:1 n-8	<0.3
Lignoceric	C24:0	<0.3
Gadoleic	C20:1 n-11	0.1 to 0.4
Behenic	C22:0	<0.2
Myristic	C14:0	<0.1

Greek, Italian, and Spanish olive oils are low in linoleic and palmitic acids and they have a high percentage of oleic acid. Tunisian olive oils are high in linoleic and palmitic acids and lower in oleic acid. On the basis of the analysis of samples from various countries olive oils are classified in two types, one with a low linoleic-palmitic acid and high oleic acid content, and the other with a high linoleic-palmitic acid and low oleic acid content. This effect has been associated with the stage of maturity of the fruit, and an antagonistic relationship between oleic and palmitic, palmitoleic and linoleic acids have been observed.

2.4.1.2. Triacylglycerols, and Partial Glycerides

The triacylglycerols found in significant proportions in olive oil are OOO (40-59%), POO (12-20%), OOL (12.5-20%), POL (5.5-7%) and SOO (3-7%). Smaller amounts of POP, POS, OLnL, LOL, OLnO, PLL, PLnO and LLL are also encountered. Fully saturated moieties have not been reported and the same applies for the tri-unsaturated ones containing linolenic acid. Stearic and palmitic acids are absent from the 2-position of unsaturated species (tri- and tetraunsaturated) or from the molecule when there are more than five double bonds. Trilinolein or ECN 42 triacylglycerol content (as corrected recently), which is used as an authenticity marker by the EU, is the sum of the amounts of LLL, PoPoPo, SLnLn, PoPoL, PPoLn, OLLn, PLLn and PoOLn (positional isomers included).

The presence of partial glycerides in olive oil is due either to incomplete triacylglycerol biosynthesis or hydrolytic reactions. In virgin olive oil, concentration of diacylglycerols (DG) range from 1 to 2.8%. In the diacylglycerol fraction C-34 and C-36 compounds prevail. Monoacylglycerols are present in much smaller quantities (less than 0.25%) whereas 1-species are considerably higher than the respective 2-monoglycerides. Their ratio depends on oil acidity, and the storage conditions affect the distribution of fatty acids. 1.2-Diacylglycerols present in fresh oil tend to isomerize to the more stable 1.3-diacylglycerols. This rearrangement gives information about the age of the oil and storage conditions. The ratio of 1.3-/1.2-DG is considered as a useful ratio to monitor quality.

2.4.1.3. Hydrocarbons

Two hydrocarbons are present in considerable amounts in olive oil, squalene and β-carotene that will be discussed in the pigments section. Squalene, or 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, is the last metabolite preceding sterol ring formation. Its presence is regarded as partially responsible for the beneficial health effects of olive oil and its chemo-preventive action against certain cancers. It is the major constituent of the unsaponifiable matter (referring to the whole quantity of substances present in the oil or fat which after saponification by an alkaline solution extraction by a specific solvent, are not soluble in aqueous alkali and non-volatile under the condition of test) and makes up more than 90% of the hydrocarbon fraction. It ranges from 200 to 7500 mg/kg of oil, even though higher levels up to 12,000 mg/kg have been also reported in literature. Squalene content depends on olive cultivar, oil extraction technology, and it is dramatically reduced during the process of refining. Except for squalene, the hydrocarbon fraction of virgin olive oil is composed of diterpene and triterpene hydrocarbons, isoprenoidal polyolefins, and n-paraffins.

2.4.1.4. Tocopherols

These compounds are present in olive oil in concentrations of about 150 – 250mg/Kg, Normally present are the α, β, γ and δ forms of which α (vitamin E) is the most abundant (90 – 95% of the total tocopherol content). Low amounts of the homologues β-tocopherol (~10 mg/kg), δ-tocopherol (~10 mg/kg) and γ-tocopherol (~20 mg/kg) are usually reported. The level of α-tocopherol may be related to the high levels of chlorophyll pigments and the concomitant requirement for singlet oxygen deactivation,

while it seem to reduce with the fruit ripeness. Tocopherols carry out an anti-oxydant action in oils exposed to “light”(ultaviolet radiation).

2.4.1.5. Pigments

Olive oil color is the result of green and yellow hues due to the presence of chlorophylls and carotenoids. It is influenced by olive cultivar, maturation index production zone, extraction system, and storage conditions. Therefore it is considered as a quality index though no standardized method exists for its measurement. Chlorophylls are encountered as pheophytins. Among the latter pheophytin α (Pheo α) is predominant, while pheophytin β is also present though in minute amounts. The presence of Pheo α is related to processing conditions and enzymatic or enzymatic-like activity. Handling and duration of storage cause further changes in pheophytin α content. It has been reported that the presence of pheophytin degradation products (such as: epimers, pyro-forms and allomers) can be related with the storage condition. These products, on the basis of previously reported findings, were identified as pyropheophytin α, 151-OH lactone pheophytin α and 132-OH-pheophytin α. Under light exposure green pigments degrade causing oil bleaching.

The main carotenoids present in olive oil are lutein and β-carotene

The presence of carotenoids in olive oil is closely related to that of green pigments and is influenced by the same factors. The carotenoid fraction may also include several xanthophylls (violaxanthin, neoxanthin, luteoxanthin, antheraxanthin, mutatoxanthin, and β-cryptoxanthin). The ratio between the two major carotenoids seems to be cultivar dependent.

2.4.1.6. Aliphatic and aromatic alcohols

Aliphatic and aromatic alcohols present in olive oil are found in free and esterified form. The most important are fatty alcohols and diterpene alcohols. Alkanols and alkenols with less than ten carbon atoms in their molecule, which are present in free and esterified form, and some aromatic alcohols (benzyl alcohol and 2-phenylethanol) are constituents of the olive oil volatile fraction. Benzyl esters of hexacosanoic and octacosanoic acid have been also reported in olive oil.

Fatty Alcohols are a class of minor constituents consisting of linear saturated alcohols with more than 16 carbon atoms which are present in the free and esterified form. The main fatty alcohols present in olive oil are docosanol, tetracosanol, hexacosanol, and octacosanol. Others fatty alcohols with odd carbon atoms (tricosanol, pentacosanol, and heptacosanol) may be found in trace amounts. Virgin olive oil total fatty alcohols level is affected by cultivar, crop year, fruit ripeness, and processing, even though is not usually higher than 250 mg/kg. The most abboundand fatty alcohols were found to be tetracosanol and hexacosanol. Esters of fatty alcohols with fatty acids (waxes) are also classified as minor olive oil constituents, and they can be used as a criterion to differentiate various olive oil types (EC Regulation 2568, 1991). The main waxes detected in olive oil are esters of oleic or palmitic acid with 36, 38, 40, 42, 44, and 46 carbon atoms. Virgin olive oils contain waxes at levels lower than 150 mg/kg and their content and composition is also affected by cultivar, crop year, and processing.

Phytol and geranylgeraniol are two acyclic diterpenoids present in the aliphatic alcohol fraction of olive oil in the free and esterified form. Phytol, which probably originates from chlorophyll, has been found in monovarietal virgin olive oils at levels ranging from 25 to 595 mg/kg. Geranylgeraniol is reported to be present in virgin olive oil from

a new olive cultivar (I-77) at levels lower than 50 mg/kg. Its levels are used in the calculation of the alcoholic index, a useful parameter for detecting solvent extracted olive oil in virgin olive oil. Esters identified in the wax fraction of extra virgin olive oil are oleate, eicosanoate, eicosenoate, docosanoate, and tetracosanoate, mainly as phytyl derivatives.

2.4.1.7. Sterols

Sterols are important lipids related to the quality of the oil and broadly used for checking its genuineness. Four classes of sterols occur in olive oil: common sterols (4-desmethylsterols), 4α-methylsterols, triterpene alcohols (4, 4-dimethylsterols), and triterpene dialcohols.

Common Sterols (4α-desmethylsterols) contained in Olive oil are mainly in free and esterified form, although they have also been found as sterylglucosides and lipoproteins. The main components of this sterol fraction are β-sitosterol, Δ5-avenasterol, and campesterol. Other sterols present in smaller quantities or in trace amounts are stigmasterol, cholesterol, brassicasterol, chlerosterol, ergosterol, sitostanol, campestanol, Δ7-avenasterol, Δ7-cholestenol,Δ7-campestenol, Δ7-stigmastenol, Δ5,23-stigmastadienol, Δ5,24-stigmastadienol, Δ7,22-ergostadienol, Δ7,24-ergostadienol, 24-methylene-cholesterol, and 22,23- dihydrobrassicasterol. Total sterol content of virgin olive oils varies mainly between 1000 mg/kg, which is the lower limit set by the European Union Commission (EC Regulation 2568, 1991), and 2000 mg/kg. Lampante olive oils contain higher amounts of total sterols, while refined olive oils contain lower levels because the refining process gives rise to significant losses of sterols, which may be as high as 25%. Total sterol content of solvent extracted olive oils is up to three

times higher than that of virgin olive oils. Studies on olive oil sterol composition show that β-sitosterol makes up 75 to 90% of the total sterol fraction, Δ5-avenasterol usually ranges between 5% and 20%, campesterol and stigmasterol make up 4% and 2% respectively (campesterol levels are always higher than those of stigmasterol). The rest of the sterols occur in minute quantities. The levels of Δ5- and Δ7-avenasterol, Δ7-stigmastenol, stigmasterol, and chlerosterol are used to determine whether virgin, refined, and solvent extracted olive oils. Sterol composition and total sterol content are affected by cultivar, crop year, degree of fruit ripeness, storage time of fruits prior to oil extraction, processing, and also by geographic factors.

4-Methylsterols are intermediates in sterol biosynthesis, and they are present in olive oil in small quantities in free and esterified form. The predominating components are obtusifoliol, gramisterol, cycloeucalenol, and citrostadienol. They are Δ7- or Δ8-sterols except cycloeucalenol which has a 9,19-cyclopropane ring in the steroid skeleton. The levels of total 4α-methylsterols are lower than that of common sterols and triterpene alcohols and vary between 50 and 360 mg/kg.

2.4.1.8. Triterpene acids

Hydroxy pentacyclic triterpene acids are important olive fruit constituents. They are biologically active compounds and are present at trace amounts in olive oil.

The main triterpene acids present in virgin olive are Oleanolic (3β-hydroxyolean-12-en-28-oic acid) and maslinic acid (2α, 3β-dihydroxyolean-12-en-28-oic acid) are the main triterpene acids present in virgin olive. Both compounds and traces of ursolic acid (3β-hydroxyurs-12-en-28-oic acid) are located in the reticular lipid layer of olive skin.

Total triterpene acid content of extra virgin olive oils obtained from fruits of different olive cultivars was found to range between 40 and 185 mg/kg. Recently, the main factor influencing the level of hydroxy pentacyclic triterpene acids in olive oil was studied. Olive oil acidity resulted to be the principal contributor while olive cultivar, olive ripeness, and oil extraction system have less influence on the levels of these acids.

2.4.1.9. Volatile and aroma compounds

Approximately two hundred and eighty compounds have been identified in the volatile fraction of virgin olive oils. They are hydrocarbons (more than 80 compounds), alcohols (45 compounds), aldehydes (44 compounds), ketones (26 compounds), acids (13 compounds), esters (55 compounds), ethers (5 compounds), furan derivatives (5 compounds), thiophene derivatives (5 compounds), pyranones (1 compound), thiols (1 compound), and pyrazines (1 compound). From this large number of compounds, only 67 were found to be present at levels higher than their odor threshold contribute to the flavor of virgin olive oils with sensory defects. The potent odorants of olive oil have been evaluated by applying aroma extract dilution analysis (AEDA) and gas chromatography-olfactometry analysis of headspace.

2.4.1.10. Other minor constituents

Some classes of minor constituents are present only in the crude oil. Filtration reduces the initial levels to a great extent whereas refining process leads to their removal. Olive oil contains a small amount of phospholipids and, even if experimental work for their identification is rather limited, phosphatidylcholine, phospatidylethanolamine, phosphatitylinositol, were reported to be the main constituent of this fraction.

2.4.2. DAIRY PRODUCTS

Reference for this section: 15, 17, 18, 19.

Milk and dairy products are major components of the human diet in Western countries, providing about 30% of dietary proteins and lipids and about 80% of dietary calcium. Current annual production of milk is about 600 x 10^6 tons, of which about 85% are bovine (**Figure 2.4**).

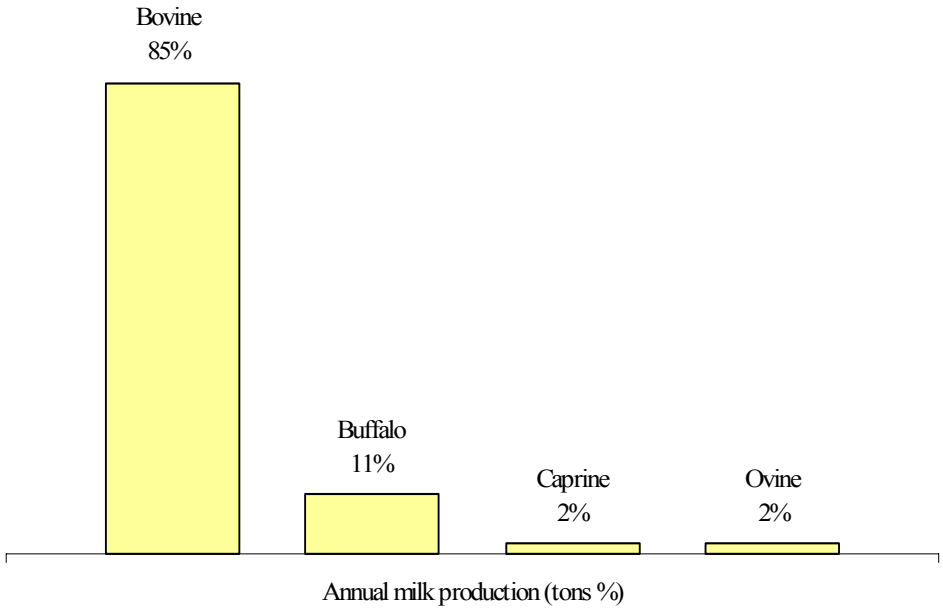

Figure. 2.4. – *Current annual milk production.*

Although some raw milk is still consumed, the vast majority of milk is processed to at least some extent. Liquid (beverage) milk is a major food item in all developed dairying countries, representing about 40% of total milk production, while the remainder is processed into one of several thousand products (**Figure 2.5**). For this reason the dairy industry is probably the most diverse and flexible sector of the food industry. The flexibility of milk as a raw material resides in the chemical and physical-chemical

properties of its constituents, many of which are unique. The principal constituents of milk can be modified by enzymatic, chemical and/or physical methods, permitting the production of new products. The natural function of milk is to supply the neonatal mammal, of which there are about 4500 species, with its complete nutritional and some of its physiological requirements. Because the nutritional requirements are species-specific and change as the neonate matures, the composition of milk shows very large interspecies differences. Inter-species differences in the concentrations of many of the minor constituents are even greater than those of the macro-constituents. Milk from domesticated animals has been used by humans since at least 8000 BC. Although sheep and goats were the first domesticated dairy animals, because they are more easily managed than cattle, the latter, especially certain breeds of Bos taurus, are now the dominant dairy animals.

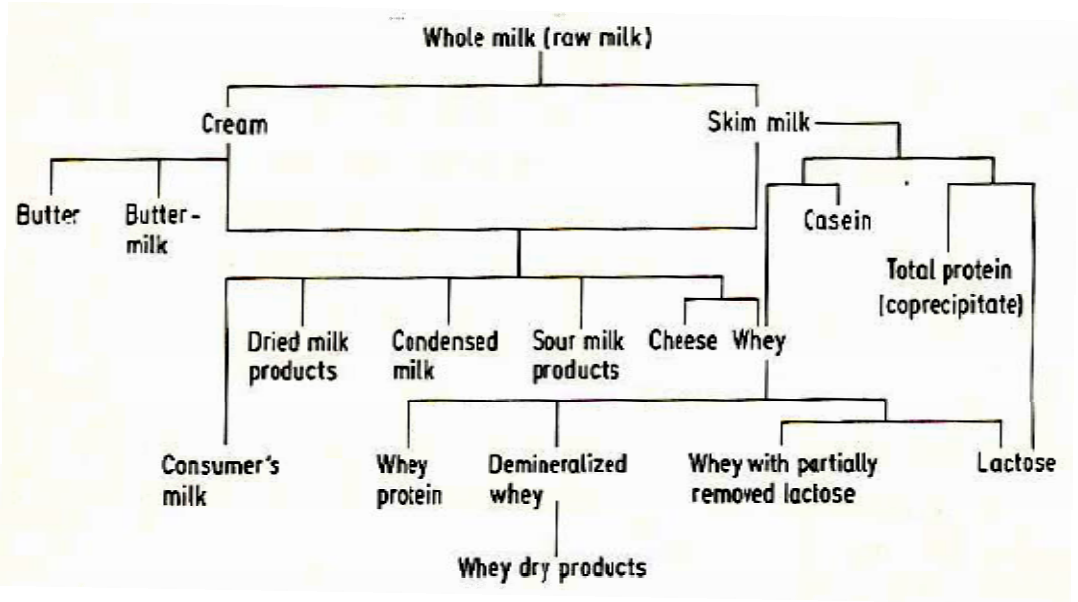

Figure 2.5. – *Schematic presentation of milk processing.*

Therefore, to avoid misunderstanding, this section will concentrate on the properties of bovine milk or simply “milk”, as term used today as synonymous. Milk is a very

flexible raw material from which several thousand types of dairy products are produced around the world in a great diversity of flavors and forms, including thousand varieties of cheese. The total world milk yield is used for the production of the principal dairy products, that are in order: liquid (beverage) milk, cheese, butter, whole milk powder, skimmed milk powder, concentrated milk products, fermented milk products, casein and infant formulae. This flexibility and diversity are a result of the properties, many of them unique, of the constituents of milk, the principal of which are easily isolated from milk, permitting the production of valuable food ingredients. Moreover the processability and functionality of milk and milk products are determined by the properties and concentrations of its principal constituents: proteins, lipids, lactose and salts. The lipids occur as globules, 0.1–20 μm in diameter, surrounded by the milk fat globule membrane (MFGM), which serves as an emulsifier. Their concentration varies with species, breed, individual animal, stage of lactation, mastitis infection, plane of nutrition, interval between milkings, and point during milking when the sample is take.

2.4.2.1. Fatty acids

Ruminant milk fat contains a wider range of fatty acids than any other lipid system, up to 400 fatty acids have been reported in bovine milk fat; the principal fatty acids are the homologous series of saturated fatty acids, C4:0, C18:0 and C18:1. The outstanding features of the fatty acids in milk fat are a high concentration of short and medium chain acids (ruminant milk fats are the only natural lipids that contain butanoic acid) and a low concentration of polyunsaturated fatty acids (PUFA). In ruminants, the fatty acids for the synthesis of milk lipids are obtained from triglycerides in chylomicrons in the blood or synthesized de novo in the mammary gland from acetate or β–hydroxybutyrate

produced by microorganisms in the rumen. The triglycerides in chylomicrons are derived from the animal's feed or synthesized in the liver. Butanoic acid (C4:0) is produced by the reduction of β-hydroxybutyrate which is synthesized from dietary roughage by bacteria in the rumen and therefore varies substantially with the animal's diet. All C6:0– C14:0 and 50% of C16:0 are synthesized in the mammary gland via the malonyl-CoA pathway from acetyl-CoA produced from acetate synthesized in the rumen. Essentially 100% of C18:0, C18:1, C18:2 and C18:3 and 50% of C16:0 are derived from blood lipids (chylomicrons) and represent about the 50% of total fatty acids in ruminant milk fat. Unsaturated fatty acids in the animal's diet are hydrogenated by bacteria in the rumen unless they are protected (encapsulation). Seasonal variation can cause very significant changes in the fatty acid profile of milk fat. A grass-based diet is rich in PUFA (see **Figure 2.6**), and these are subjected to the bio-hydrogenation process by bacteria in the rumen (e.g.: *Butyrivibrio fibrisolvens*) and then converted to mono-unsatured fatty acids in *cis* and *trans* configurations, that they will be found consequently in milk fat and the relative dairy products obtained.

PUFA are considered to be nutritionally desirable and, consequently, there has been interest in increasing their concentration in bovine milk fat, e.g. linoleic acid, that is an essential fatty acid and must be supplied in the diet since it cannot be synthesized by mammals. This can be done by feeding encapsulated PUFA-rich lipids or crushed PUFA-rich oil seeds to the animal.

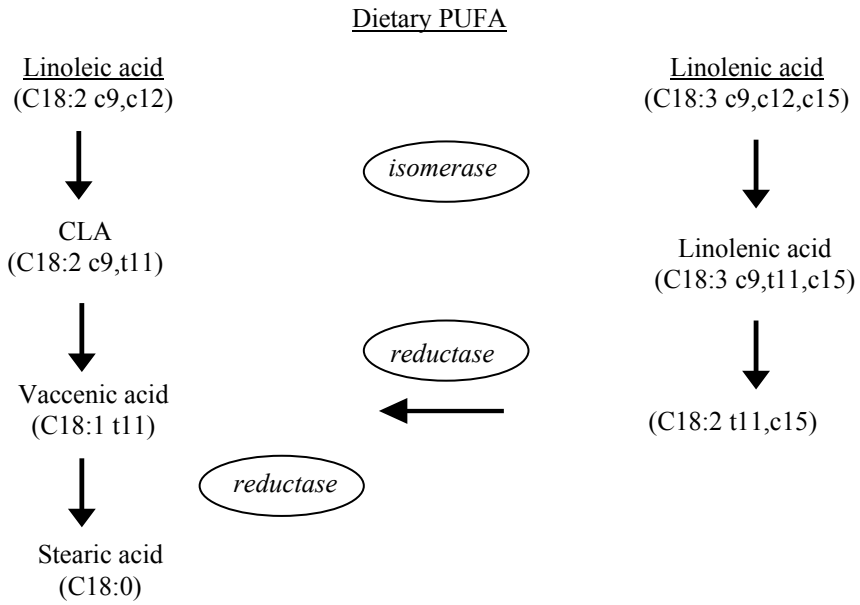

Figure. 2.6. – *Biohydrogenation of Dietary Poliunsatured Fatty acids in the rumen*

Unsaturated fatty acids may occur as *cis* or *trans* isomers (**Figure 2.7**); *trans* isomers, which have higher melting points than the corresponding cis isomers, are considered to be nutritionally undesirable. Bovine milk fat contains a low level (5%) of trans fatty acids in comparison with chemically hydrogenated (hardened) vegetable oils, in which the value may be 50% due to non-stereospecific hydrogenation.

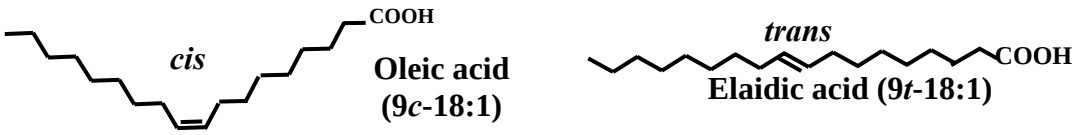

Figure. 2.7. – *Fatty acids in cis and trans configuration.*

Another group of fatty acids that have attracted very considerable attention recently, is the CLA (**Figure 2.8**). It is a mixture of eight positional and geometric isomers of

linoleic acid which have a number of health-promoting properties. Of the eight isomers of CLA, only the C18:2 cis 9, trans 11 isomer is biologically active.

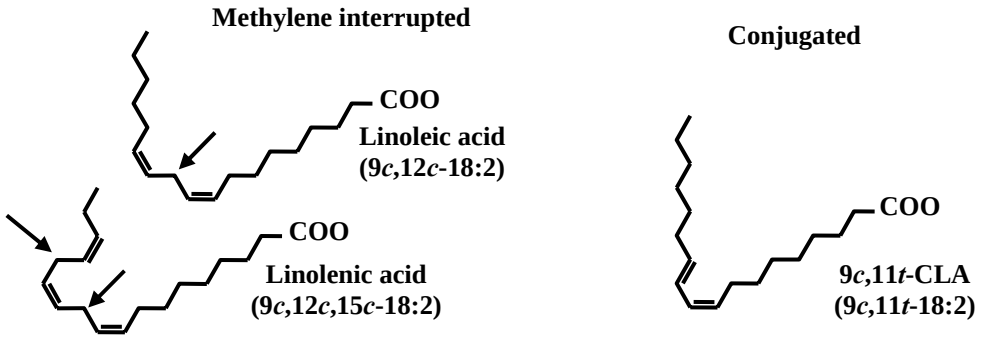

Figure. 2.8. – *Conjugated and non-conjugated fatty acids.*

This compound is effective at very low concentrations, 0.1 g per 100 g diet. Fat-containing foods of ruminant origin, especially milk and dairy products, are the principal sources of dietary CLA which is produced as an intermediate during the biohydrogenation of linoleic acid by the rumen bacterium, *Butyrivibrio fibrisolvens*, and isomerized by delta-9 desaturase in the mammary gland from vaccenic acid (trans-11 C18:1), that is an intermediate of polyunsaturated fatty acid biohydrogenation in the rumen (**Figure 2.9**). Since CLA is formed from linoleic acid, it is not surprising that the CLA content of milk is affected by diet and season, being highest in summer when cows are on fresh pasture rich in PUFA and higher in the fat of milk from cows on mountain than on lowland pasture. The concentration of CLA in milk fat can be increased 5–7 fold by increasing the level of dietary linoleic acid, e.g., by duodenal infusion or by feeding a linoleic acid-rich oil, e.g., sunflower oil.

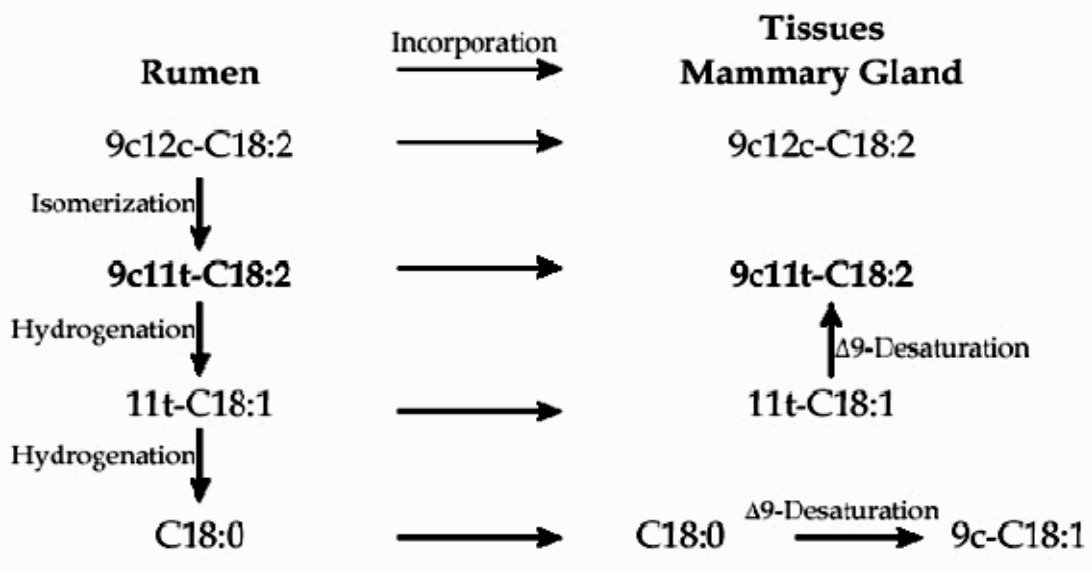

Figure. 2.9. – *Pathways of rumenic acid in rumen and mammary gland*

2.4.2.2. Glycerides

Triacylglycerols, called triglycerides for short, make up the bulk (generally more than 98%) of the lipids and accordingly, largely determine the properties of milk fat (**Table 2.4**). These properties vary with the fatty acid composition. Because the number of different fatty acid residues is great, the number of different triglycerides is much greater. The distribution of fatty acid residues over the position in the triglyceride molecule is far from random: Butanoic and hexanoic acids are esterified almost entirely, and octanoic and decanoic acids predominantly, at the sn-3 position; as the chain length increases up to C16:0 an increasing proportion is esterified at the sn-2 position (more marked for human than for bovine milk fat, especially in the case of palmitic acid); stearic acid (C18:0) is esterified mainly at sn-1; unsaturated fatty acids are esterified mainly at the sn-1 and sn-3 positions, in roughly equal proportions. The position of the fatty acid residues in the triglyceride molecules considerably affects the crystallization behavior of milk fat.

Some of di- and monoglycerides occur in fresh milk fat. Lipolysis increases their quantities. Diglycerides are predominantly apolar and do not differ much from triglycerides in properties. Monoglycerides, present in far smaller quantities, are somewhat polar; they are surface active and thus accumulate at an oil–water interface. Most lipolytic enzymes, including that of milk, especially attack the 1- and the 3-position of the triglyceride molecule. This means that most monoglycerides have a fatty acid residue at the 2-position, and that most of the free fatty
acids formed originate from the other positions, including the short-chain types that are predominantly in the 3-position.

Table. 2.4. – *Percentage of glycerides in milk fat.*

	% in milk fat
Neutral glycerides	**98.7**
Tryglycerides	98.3
Diglycerides	0.3
Monoglycerides	0.003

2.4.2.3. Hydrocarbons

Several hydrocarbons occur in milk in trace amounts. Of these, carotenoids are the most significant. In quantitative terms, carotenes occur at only trace levels in milk but they contribute 10-50% of the vitamin A activity in milk and are responsible for the yellow colour of milk fat. The carotenoid content of milk varies with breed and very markedly with season. The latter reflects differences in the carotenoid content of the diet (since they are totally derived from the diet); fresh pasture, is much richer in carotenoids than hay or silage (due to oxidation on conservation) or cereal-based concentrates. The higher the carotenoid content of the diet, the more yellow will be the colour of milk and milk fat.

2.4.2.4. Tocopherols

Vitamin E is a generic term used to indicate tocopherols and tocotrienol, and α-tocopherol has the greatest activity for humans. Vitamin E is a very effective antioxidant, protects the lipids (particularly polyunsaturated fatty acids) and membranes in the body against damage caused by free radicals. The role of vitamin E is of particular importance in the lungs where exposure of cells to oxygen is greatest. Vitamin E also exerts a protective effect on red and white blood cells. It has been suggested that the body has a system to regenerate active vitamin E (perhaps involving vitamin C) once it has acted as an antioxidant. The concentration of vitamin E in cows' milk is quite low (0.09mg per l00g) and is higher in summer than in winter milks.

2.4.2.5. Sterols

These compounds are found in the unsaponifiable fraction of milk lipids and consist mostly of cholesterol with some Δ7-cholesterol and β-Sitosterol.

They are polycyclic alcohols having a secondary –OH group at position 3, and the presence of this group makes sterols more polar than triglycerides. Moreover, in milk fat globules, cholesterol, both free (90%) and esterified, is one of the constituents of the globule membrane. The occurrence of sterols in milk fat is about 0.3%.

2.4.2.6. Phospholipids

The phospholipids comprise approximately 1 % of the total lipid in bovine milk. While quantitatively minor, the ability of the phospholipids to form stable colloidal suspensions or emulsions in aqueous solution cause them to be important in the

formation and secretion of milk fat. They consist of a glycerol backbone on which one or two fatty acids and a phosphate residue with different organic groups may be linked Their physical properties as bipolar molecules and their relatively high concentration of unsaturated fatty acids also make them an important factor to consider during the storage and processing of milk. They are relatively susceptible to oxidation because of their polyunsaturated fatty acid content. As the total milk lipid increases in a milk product, so does the phospholipid concentration. However, the ratio of phospholipid to total lipid varies greatly. Skim milk contains the smallest concentration of phospholipid but the highest ratio of phospholipid to total lipid. The opposite relationship is seen in cream and butter. Most milk lipid exists as fat globules suspended in the aqueous phase of milk. The size of the milk fat globules varies from 0.1 to 2 p in diameter. The core of the globule is primarily TG, which is surrounded by the milk fat globule membrane (MFGM), as reported in **figure 2.10**. This membrane contains protein, glycoproteins, enzymes, phospholipids, and other polar materials. It is a major source of cholesterol and phospholipid in milk. The major glycerophospholipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. Sphingolipids are a group of phosholipids that consist of an a-polar sphingosine backbone on which a fatty acid is bound to form a ceramide, and it can contain a similar organophosphate group like choline (sphingomyelin) or a mono- or disaccharide (glycosphingolipids). Important dairy sphingolipids are sphingomyelin, glucosylceramide and lactosylceramide.

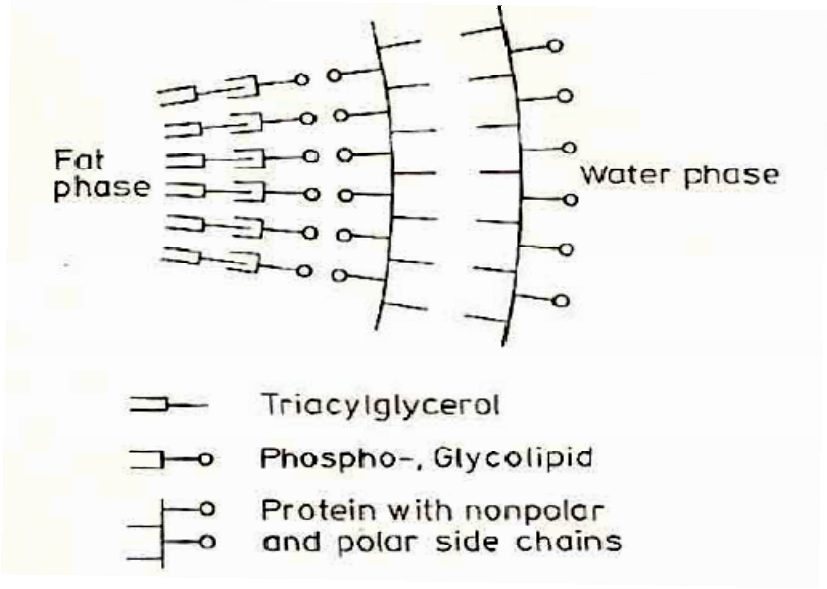

Figure. 2.10. – *Schematic representation of a milk fat globule.*

Sphingolipids are known to exhibit various biological properties and are therefore important in human nutrition, such as, the capability to reduce the cholesterol uptake, and their inhibitory effect on colon

cancer.

Table. 2.5. – Percentage of Phospholipids, Cerebrosides, Gangliosides in milk fat

	Alcohol Residue + Other Costituent	*% in milk fat*
Phospholipids	**Phospho group**	**0.8**
Ph. Choline	Glycerol + Choline	0.27
Ph. Ethanolammine	Glycerol + Ethanolammine	0.26
Ph. Serine	Glycerol + Serine	0.03
Ph. Inositide	Glycerol + Inositol	0.04
Sphingomyelin	Sphingosine + Choline	0.2
Cerebrosides	**Sphingosine + Hexose**	**0.1**
Gangliosides	**Sphingosine + Hexose**	**0.01**

2.4.2.7. Free fatty acids

They occurrence of free fatty acids in milk fat is about 0.1% and lipolysis increases their amount. Especially the shorter acids are somewhat soluble in water. In water, the acids can, of course, dissociate into ions; their pK is about 4.8. In milk plasma, they are thus predominantly in the ionized form (i.e., as soaps), and these are much more soluble in pure water than the pure fatty acids. Fatty acids dissolve well in oil, though only in the nonionized form. Moreover, they tend to associate into dimers, by forming hydrogen bonds. The partition of the acids over the oil and water phases is rather intricate. All in all, the shorter acids (C4:0 and C6:0) are predominantly in the plasma, the longer ones (from C14:0 on) in the fat. The other acids are distributed between both fractions, though more go into the fat with decreasing pH. This is even more complicated because the fatty acids, especially the long-chain ones, are surface active and tend to accumulate in the oil–water interface. The distribution over the phases is of much importance because acids dissolved in the aqueous phase (in the form of soaps) — hence, the shorter acids — are responsible for the soapy-rancid flavor perceived after lipolysis.

2.4.2.8 Lipo-soluble vitamins

The fat-soluble vitamins are retinol (vitamin A), tocopherols (and related compounds, vitamin E), calciferols (vitamin D), and phylloquinone (and related compounds, vitamin K). Since vitamin A and E were previously treated (see Hydrocarbon and Tocopherols), this part will concern on vitamin D and K.

Unlike other vitamins, cholecalciferol (vitamin D,) can be formed from a steroid precursor, 7 dehydrocholesterol, by the skin when exposed to sunlight; with sufficient exposure to the sun, no preformed vitamin D is required from the diet.UV light (280-

320 nm) causes the photoconversion of 7-dehydrocholesterol to pre-vitamin D_3. This pre-vitamin can undergo further photoconversion to tachysterol and lumisterol or can undergo a temperature-dependent isomerization to cholecalciferol. At body temperature, this conversion requires about 28 h to convert 50% of previtamin D_3, to vitamin D_3. Thus, production of vitamin D, in the skin can take a number of days. Preformed vitamin D, is obtained from the diet. This latter, must undergo two hydroxylations to become fully active in liver and kidneys respectively. However, at least 37 metabolites of vitamin D, have been identified, but only 1,25-dihydroxycholecalciferol $1,25(OH)_2D_3$ is the most biologically active metabolite of vitamin D,. The major form of vitamin D in both cows' and human milk is 25-hydroxycholecalciferol $25(OH)_2D_3$. Whole cows' milk contains only about 0.03 μg vitamin D per 100g. The principal physiological role of vitamin D in the body is to maintain plasma calcium by stimulating its absorption from the gastrointestinal tract, its retention by the kidney and by promoting its transfer from bone to the blood. Vitamin D acts in association with other vitamins, hormones and nutrients in the bone mineralization process. In addition, vitamin D has a wider physiological role in other tissues in the body, including the brain and nervous system, muscles and cartilage, pancreas, skin, reproductive organs and immune cells.

The structure of vitamin K is characterized by methylnaphthoquinone rings with a side chain at position 3.Menaquinones are synthesized only by bacteria (which inhabit the human gastrointestinal tract and thus provide some of the vitamin K required by the body).Whole cows' milk contains 0.4-1.8 μg vitamin K per 100g. The physiological role of vitamin K is in blood clotting and is essential for the synthesis of at least four of the proteins (including prothrombin) involved in this process. Vitamin K also plays a

role in the synthesis of a protein (osteocalcin) in bone. Vitamin K deficiency is rare but can result from impaired absorption of fat. Vitamin K levels in the body are also reduced if the intestinal flora is killed (e.g. by antibiotics).

2.4.2.9 The fat in milk products

Because the various lipids are unevenly distributed among the physical fractions of milk (Table 2.8), the fat composition of different milk products varies. The largest differences originate from variations in the amount of material from the fat globule membranes. Examples are given in **table 2.6**. Anhydrous milk fat is prepared from butter by melting it, and by separating and drying the oil layer obtained; its composition is virtually equal to the fat in the core of the milk fat globules.

Table. 2.6. – *Approximate content of lipids in some milk products*

	Composition (% w/w)			
Product	**Total Fat**	**Phospholipids**	**Sterols**	**Free Fatty Acids**
Separated milk	0.06	0.015	0.002	0.002
Milk	4	0.035	0.013	0.008
Cream	10	0.065	0.03	0.017
Cream	20	0.12	0.06	0.032
Cream	40	0.21	0.11	0.06
Buttermilk from 20% cream	0.4	0.07	0.005	0.002[a]
Buttermilk from 40% cream	0.6	0.13	0.011	0.002[a]
Butter (unsalted)	82	0.25	0.21	0.12[a]
Anhydrous milk fat	> 99.8	0.00	0.25	0.15[a]

[a] Higher if the cream has been subject to lipolysis, especially after its separation.

2.5. CHEMICAL ASPECTS

Reference for this section: 20, 21.

2.5.1. LIPOLYSIS

Hydrolysis of ester bonds in lipids may occur by enzyme action or by heat and moisture, resulting in the liberation of free fatty acids from glycerides. Since edible animal fats are not usually refined, prompt rendering is of particular importance. The temperatures commonly used in the rendering process are capable of inactivating the enzymes responsible for hydrolysis.

The release of short-chain fatty acids by hydrolysis is responsible for the development of an undesirable rancid flavour (hydrolytic rancidity) in raw milk. On the other hand, certain typical cheese flavors are produced by deliberate addition of microbial and milk lipases (endogenous). In contrast to animal fats, olive oils may have undergone substantial hydrolysis by the time the fruits are harvested, giving rise to significant amounts of free fatty acids; so, it may start while the fruit is still on the tree. The endogenous lipase does not manifest its activity until the fruit starts turning purple. Bacteria, yeasts and molds (that may grow on the fruit) elaborate their own lipases. If the fruit is stored before processing and especially if the storage results unsatisfactory, then the combined effect of the endogenous and microbial lipases may result in considerable rise of the acidity of the oil to the detriment of its quality.

2.5.2. AUTOXIDATION

Lipid oxidation in food systems is a detrimental process and is one of the major causes of food spoilage. It deteriorates the sensory quality and nutritive value of a product,

poses a health hazard and presents a number of analytical problems. It leads to the development, in edible oils and fat-containing foods, of various off flavours and off odours generally called rancid (oxidative rancidity, which render these foods less acceptable. In addiction, oxidative reaction can decrease the nutritional quality of food, and certain oxidation products are potentially toxic. On the other hand, a limited degree of lipid oxidation is sometimes desirable, as in aged cheeses.

The term autoxidation is referred to the reaction with molecular oxygen via a self-catalytic mechanism, which is the main reaction involved in oxidative deterioration of lipids. Although photochemical reactions have been known for a long time, only recently the role of photosensitized oxidation and its interaction with autoxidation emerged. In food systems lipids can be oxidized both by enzymic and non enzymic mechanisms.

2.6. PROCESSING OF FATS AND OILS

Reference for this section: 20, 21.

2.6.1. PROCESSING OF FAT AND OIL

Apart from some oils obtained by cold pressing, most of the oils obtained using expeller, screw or hydraulic presses, solvent extraction or by melting at elevated temperatures are not suitable for immediate consumption. Depending on the raw material and the oil recovery process, the oil contains polar lipids especially phospholipids, free fatty acids, some odor- and taste-imparting substances, waxes, pigments (chlorophyll, carotenoids and their degradation products), phenolic compounds, trace metal ions, contaminants and autoxidation products.

A refining process may comprise the following steps: vegetable lecithin removal, degumming, free fatty acid removal, bleaching and deodorization. All the undesired compounds and contaminants are removed. In practice, the refining steps used, depend on the quality of the crude oil and its special constituents. Moreover, the absence of oxygen, the avoidance of heavy metal contamination and the maintaining of processing temperatures as low and duration as short as possible, are precautionary misures that must be taken during the refining in order to avoid undesirable autoxidation and polymerization reaction.

2.6.2. HYDROGENATION

Liquid oils are supplied mostly from natural sources. However a great demand exists for fats which are solid or semi-solid at room temperature (such as shortenings and margarine). The process that convert liquid oil into solid fat is called “fat hardening” and consists in the addiction of hydrogen to double bonds in the fatty acid chains. In practice, the oil is first mixed with a suitable catalyst (e.g. nickel), heated to the desired temperature (140°-225°), then exposed, while stirred, to hydrogen at pressure up to 60 psig. The course of the hydrogenation reaction is usually monitored by determining the change in the refractive index, strictly related to the degree of saturation of the oil. When the desired end point is reached, the hydrogenated oil is cooled and the catalyst is removed by filtration. During hydrogenation, not only some of the double bonds are saturated, but some may also be relocated and/or transformed from the usual *cis* to the *trans* configuration. Partial hydrogenation thus may result in the formation of a relatively complex mixture of reaction products, depending on which of the double

bonds are hydrogenated, the type and degree of isomerization, and the relative rates of these various reaction.

2.6.3. INTERESTERIFICATION

It has been mentioned that natural fats do not contain a random distribution of fatty acids among the glyceride molecules. The tendency of certain acids to be more concentrated at specific *sn* position varies from one species to another an is influenced by factors such as environment and location in the plant or animal. The physical characteristic of a fat are greatly affected not only by the nature of constituent fatty acids but also by their distribution in the triacilglycerol molecules. Indeed, unique fatty acid distribution patterns of some natural fats may limit their industrial application. Interesterification is one of the processes that can be applied to improve the consistency of such fats and to improve their usefulness, involving a fatty acids rearrangement so they become distributed randomly among the triacylglycerol molecules of the fat.

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3

COMPARISON OF DIFFERENT TECHNIQUES FOR TRANS-FATTY ACIDS ANALYSIS

3.1. SUMMARY AND KEYWORDS

FDA's final rule on trans fat labeling requires that amounts of trans fat per serving be listed on the nutrition facts panel. AOCS Official Method of Analysis Ce 1h-05 can be used for analysis of trans fatty acids (TFA) in vegetable or non-ruminant animal oils and fats. Difficulties in quantitating low amounts of TFA and partial co-elution of cis- and trans- 18:1 fatty acids suggest the need for fractionating TFA before gas chromatographic (GC) analysis. Silver ion-HPLC (Ag+-HPLC) and silver ion solid phase extraction (Ag+-SPE) have been used for separating the trans-18:1 fatty acid methyl esters (FAME). Representative samples of fats and oils were studied. Trans 11-18:1 fatty acid n-butyl ester (FABE) was included as an internal standard. The TFA content was also measured by direct Ag+-HPLC analysis, using 3 ChromSpher 5 Lipids silver ion loaded columns in series with 0.1 % MeCN in hexane mobile phase at 1.0 mL/min. and 196 nm UV detection. Ag+-HPLC fractionation was carried out using the same mobile phase at 3 mL/min and a ChromSpher 5 Lipids semipreparative column. Fractionation of TFA prior to GC analysis simplified the interpretation of chromatograms, but provides quantitation only of trans-18:1 FAME. Results of the analysis of 10 samples of different oils and fats obtained by the different techniques will be described.

Keywords: trans-fatty acids, gas chromatography, Ag+-HPLC, Ag+-SPE, ATR-FT-IR

3.2. INTRODUCTION

Interest in quantitation trans fat has increased following addition of the trans fat content to the Nutrition Facts panel on food labels. Trans fat is calculated by adding the content of all the fatty acids with at least one trans double bond (TFA), excluding fatty acids with conjugated double bonds as conjugated linoleic acid (CLA). After extracting the lipid fraction from food, TFA are derivatized into fatty acid methyl esters (FAME) and quantitated by gas chromatography (GC) using long polar capillary columns (1). The interpretation of the FAME separation is often particularly complex, because TFA are not completely separated from other fatty acids present in oil and fats. An alternative approach is the application of silver ion high performance liquid chromatography (Ag+-HPLC). The TFA as FAME can either be directly quantitated by Ag+-HPLC or fractionated prior to GC analysis (2, and 3). This technique is currently limited to the quantitation or fractionation of the trans-18:1 fatty acids that generally constitute more than 95% of the trans fat in fats and oils (3). Attenuated total reflection FT-IR (ATR-FT-IR), measuring the specific IR absorption of trans double bonds at 966 cm-1, provides quantitation of total trans fat without giving any information on the content of individual fatty acids (4). An advantage ATR-FT-IR is that it can be applied to the direct quantitation of TFA in neat fats and oils, thus eliminating the derivatization step. In this study, we compared the analysis of 10 different fats and oils using different techniques of analysis.

3.3. MATERIALS AND METHODS

3.3.1. SAMPLES PREPARATION

Ten samples of common fats and oils were obtained from the American Oil Chemist Society. The samples were methylated according to Official Method AOCS Ce 2-66. For Ag+-HPLC analysis and Ag+-HPLC fractionation, 1 mg of t11-18:1 FABE as internal standard was added to c.a. 20-30 mg neat FAME of each sample. t11-18:1 FABE was purchased from Nu Chek Prep, as a special preparation.

3.3.2. ANALYSIS BY GAS CHROMATOGRAPHY

Samples (**Figure 3.1.)** were analyzed with an Agilent 6890N gas chromatograph equipped with an FID detector and a Varian CP-Sil 88 capillary column (Varian, 100 m x 0.25 mm i.d., 0.2 μm thickness). The oven was maintained at 180°C, the detector at 300°C, and the injection port at 250°C. Hydrogen as carrier gas was eluted at 1.0 ml/min and the split ratio was maintained at 1:200.

3.3.3. ANALYSIS BY AG+-HPLC

Analysis were performed with a Waters 2695 separations module equipped with a Waters 2996 PDA detector and a Waters 2420 ELSD detector. Three silver ion HPLC columns (ChromSpher 5 Lipids, 4.6 x 250 mm, 5 μm particle size, Varian) were used in series and maintained at 20°C in a water circulating bath. The mobile phase was 0.15% MeCN in hexane at 1.0 ml/min., and the injection volume was 5 μl. The UV signal was acquired between 190 and 300 nm. Chromatograms for purpose were extracted at 203 nm (**Figure 3.2.)**.

3.3.4. AG+-HPLC FRACTIONATION FOLLOWED BY GC ANALYSIS

Fractionation was achieved using a Waters Delta Prep 4000 preparative HPLC equipped with a Waters 717 Plus autosampler and a Waters 2996 PDA detector.

A semi-preparative ChromSpher 5 Lipids column (10 x 250 mm, 5 μm particle size, Varian) was maintained at room temperature. The mobile phase was 0.1% MeCN in hexane at 3.0 ml/min. Fractions were collected manually. Collection times were adjusted to compensate for the drift in the TFA retention times (**Figure 3.3.**).

3.3.5. ATTENUATED TOTAL REFLECTION FT-IR

Samples as pure fat or oil were analyzed with a Varian (Randolph, MA) FTS 7000e IR spectrometer controlled by Resolution Pro software (**Figure 3.4.**). Fourier transform (FT) IR spectra were collected between 4000 to 600 cm-1 at a resolution of 4 cm-1. 256 scans were acquired for each sample (approximately 4 min), and the signal averaged. A reference background spectrum was measured for air. A PIKE (Madison, WI) heated single reflection diamond ATR cell was used, maintained at 65°C. The height of the negative second derivative of the 966 cm-1 band was measured. The instrument was calibrated in the interval 0-100% TFA using mixtures of neat tri-elaidin (TE) in tri-palmitin (TP). TE and TP were supplied by Nu Check Prep, Inc. (Elysian, MN).

3.4. RESULTS AND DISCUSSION

Table 3.1. shows the comparison of the quantitation of TFA in 10 samples representative of the lipid fraction of foods, fats and oils available in North American

markets. ATR-FT-IR over-estimated trans fat content, compared with GC, in samples with less than 1% trans fat. Direct analysis by Ag+HPLC can quantify only trans-18:1 FAME, and is not suitable for determining the total trans fat content according to current food labeling regulations. The Ag+HPLC fractionation followed by GC is also limited to the quantitation of trans-18:1 FAME and showed higher limits of detection compared to direct GC analysis. At this time, for samples containing 1% or less trans fat, GC appears to be the most suitable technique.

TABLES

Table 3.1 - *Comparison of the Trans fat contentas % of total fat in ten samples determined by four teqniques.*

	IR	GC	Ag^{+}HPLC -GC	Ag^{+}HPLC
hydrogenated lard	1.19	0.91	0.41	0.49
magarine oil	11.69	12.27	9.65	9.21
canola oil	25.14	27.72	22.28	22.29
lard	1.19	1.38	-	0.52
sunflower oil	0.35	0.41	0.49	-
coconut oil	1.02	0.14	N.D.	-
canola oil	25.14	27.74	29.12	18.08
vegetable shortening	41.53	48.90	28.25	31.00
cocoa butter	0.43	0.13	N.D.	0.02
coconut oil	0.94	0.20	0.12	-

FIGURES

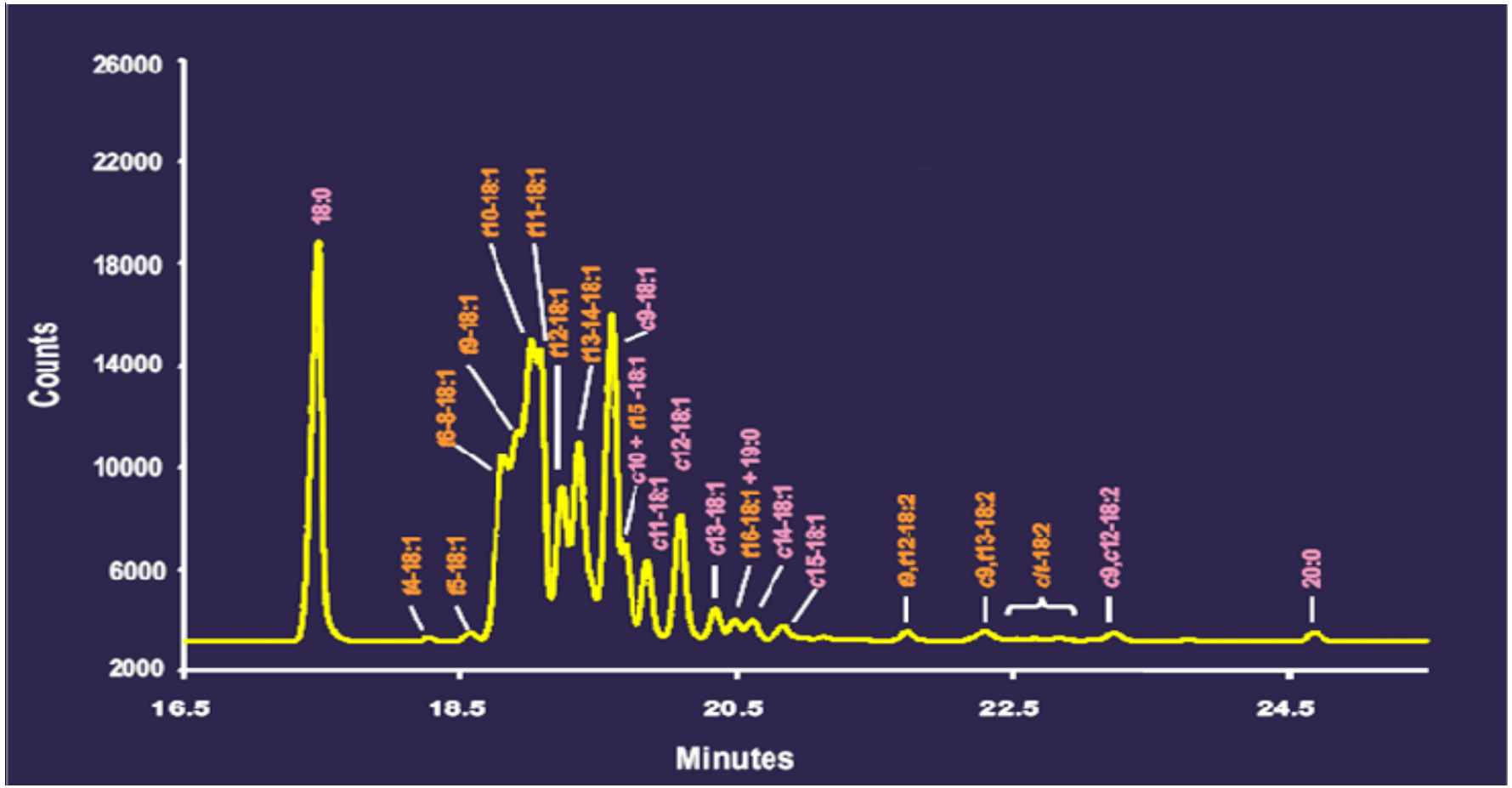

Figure 3.1. - *Partial gas chromatogram of a partially idrogenated oil sample.*

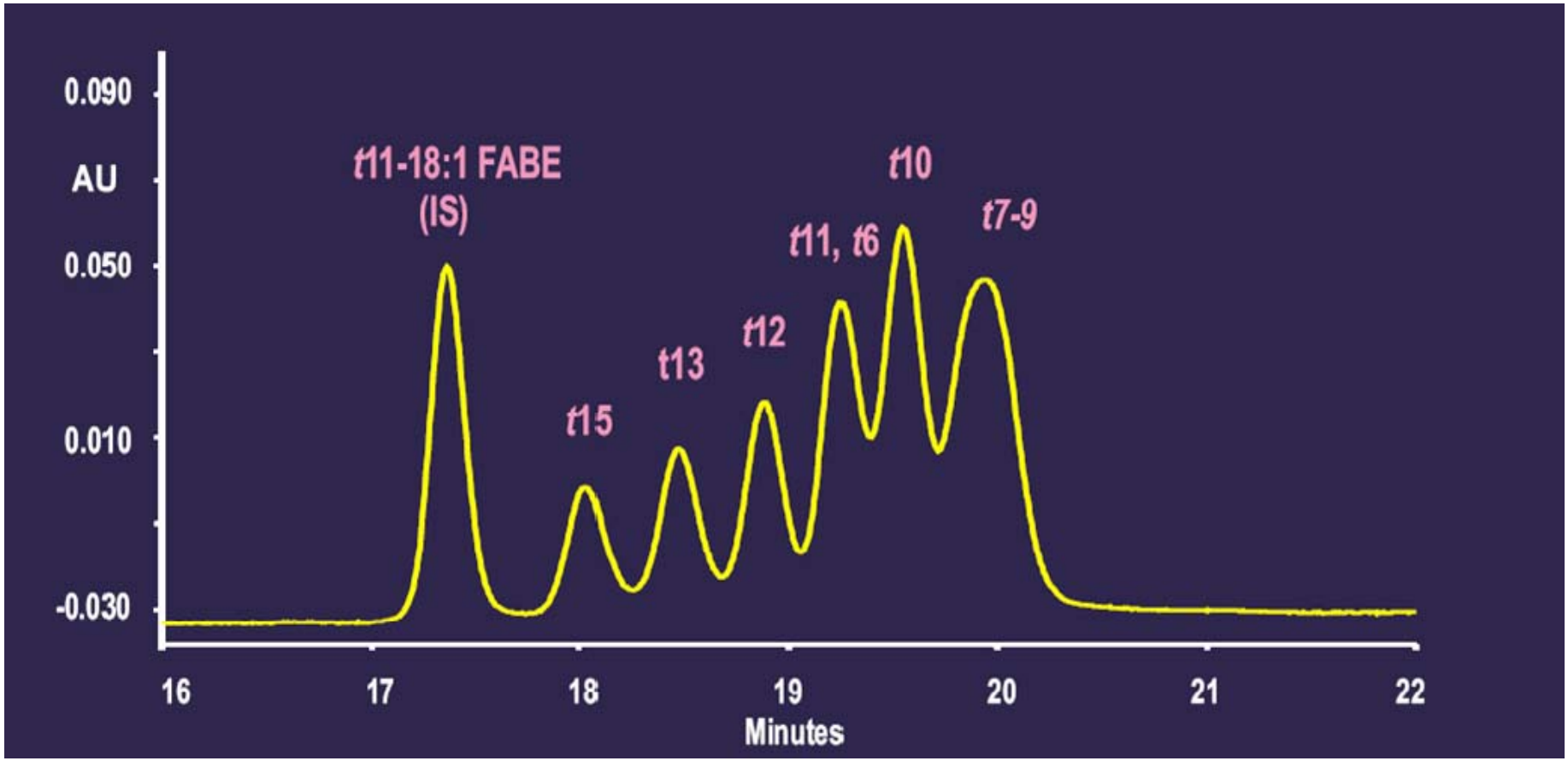

Figure 3.2. – *Ag+-HPLC analysis of a partially hydrogenated oil sample.*

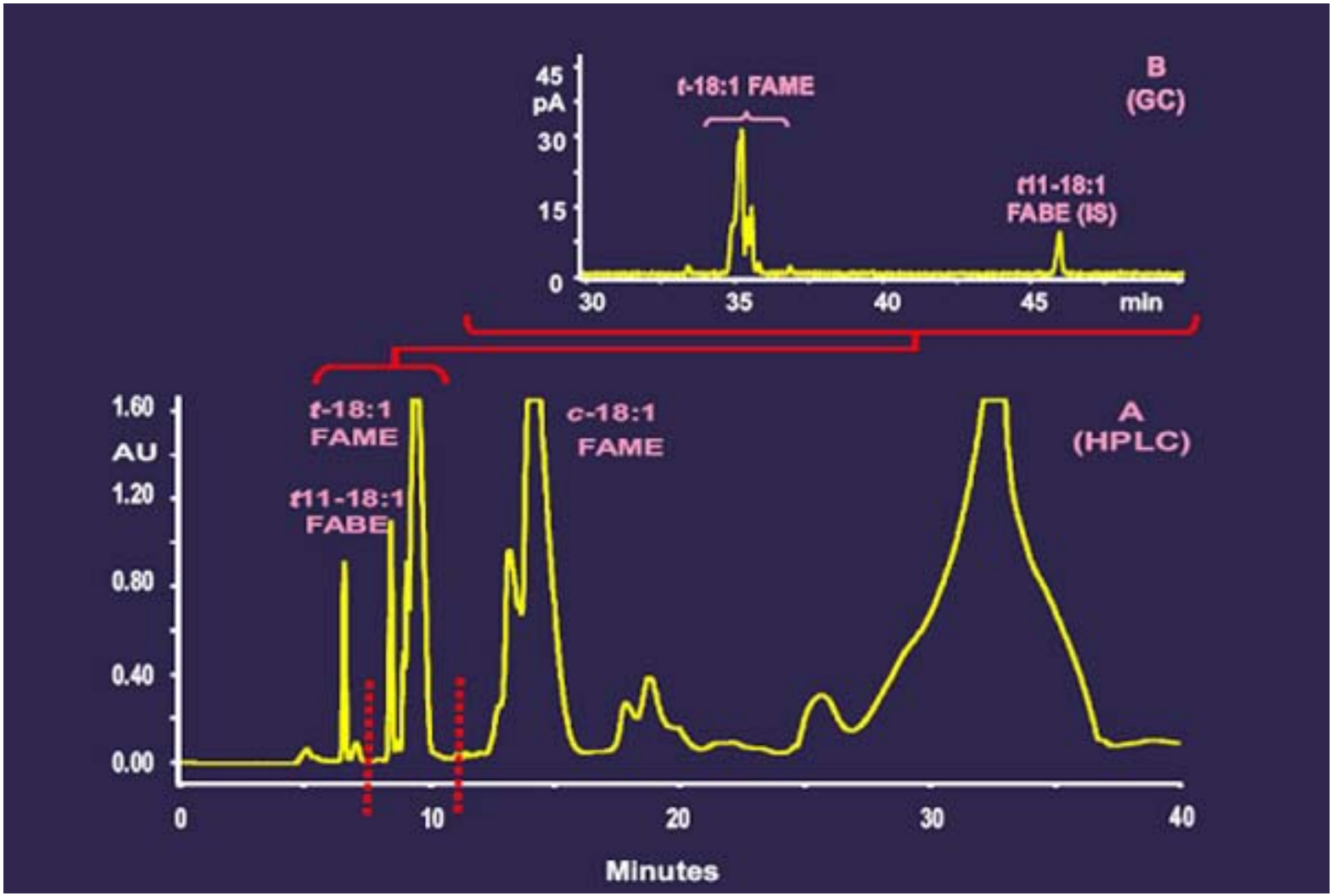

Figure 3.3. - *Ag+-HPLC fractionation of a partially hydrogenated oil sample, followed by GC analysis.*

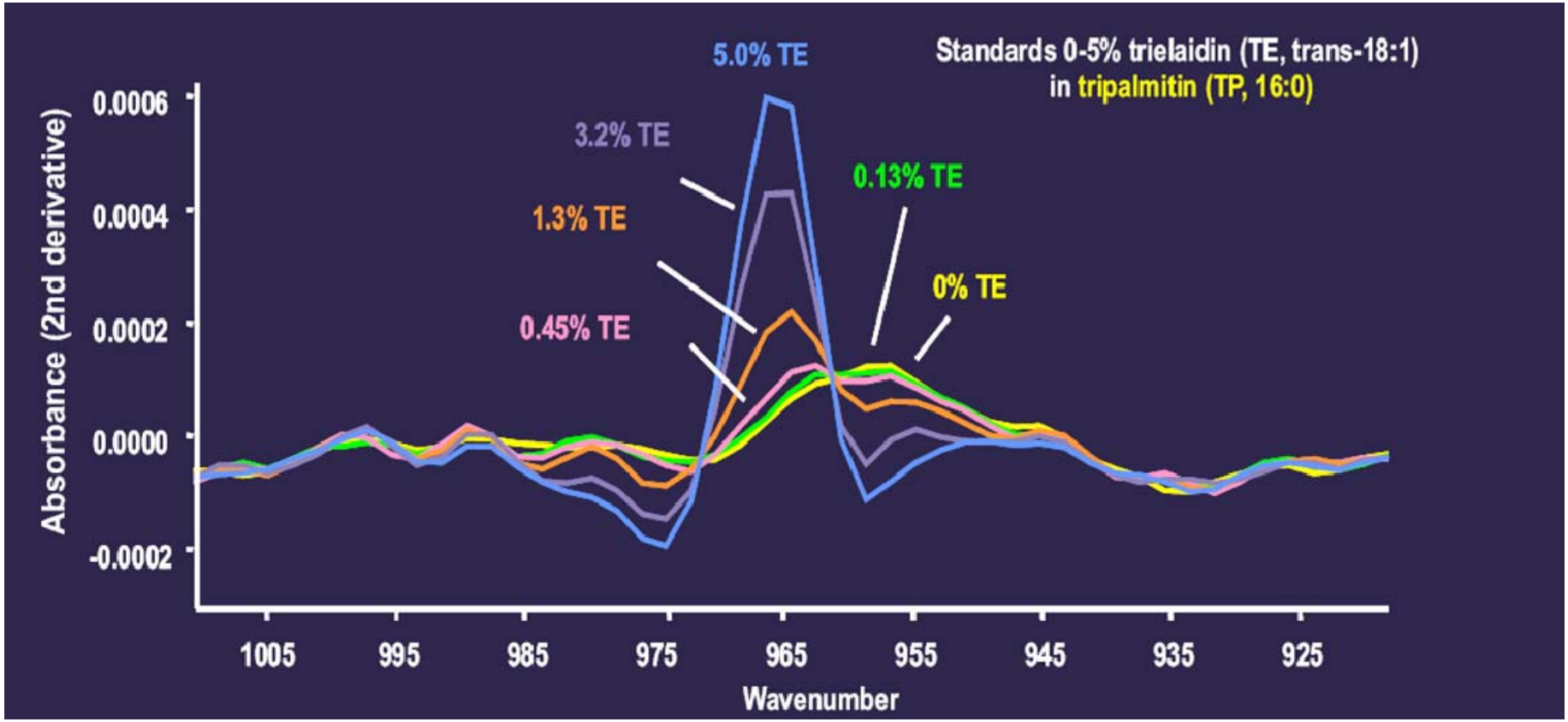

Figure 3.4. - *Attenuated Total Reflection FT-IR spectra of the calibration solution in the interval 1-5% trans fat. The height of the negative second derivatative of the 966 cm⁻¹ band was measure*

3.5. REFERENCES

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4

THE EFFECT OF EXTRUDED WHOLE LINSEED FLOUR INTAKE ON THE VARIATION OF CLA (CONJUGATED LINOLEIC ACID) CONTENT IN MILK, AND OFA (OXYDATED FATTY ACID) ON CHEESE OBTAINED - THE REGGIANA CATTLE'S CASE.

4.1. SUMMARY AND KEYWORDS

The experiment was conducted on Reggiana cattle over an eight months period in 2005. By integrating extruded whole linseed flour (300 g/d) to the basal ration (fresh or preserved forage) which changes according to the season, the possibility to modify both the milk fatty acid composition in general, and the CLA content (Conjugated Linoleic Acids) in particular, has been confirmed. The principal and statistically significant results find that Rumenic Acid (C18:2 c9-t11) and Vaccenic Acid (C18:1 trans-11) have risen up to 45% and 47% respectively on the milk fat content, subsequent to the extruded whole linseed flour administration. Moreover the results confirm the correlation between these two fatty acids in milk. However, saturated and monounsaturated fatty acids (SFA, MUFA) are not suitable to be modified, with the exception of the monounsaturated Oleic Acid that increases during the extruded whole linseed flour integration and decreases when it is suspended. In conclusion, it is possible to increase the CLA content in milk and dairy products and to improve their nutritional values in human nutrition through an appropriate and targeted cattle's ration integration.

Keywords: cow's milk, Conjugated Linoleic Acid (CLA), rumenic acid (C18:2 c9t11), vaccenic acid (C18:1 *trans*-11).

4.2. INTRODUCTION

It is well known how many efforts have been made so far to improve the fatty acids composition in vegetable oils. However, not enough research concerning animal fat and dairy products has been conducted, despite the concern of consumers. Only in the last few years a serious investigation to enhance the fatty acid profile in milk and dairy fat) has been started even in our country, achieving interesting results that lead the research to a prosecution of this route. In fact, an increasing number of studies and researches aimed to the beneficial effects played from some of the Essential Fatty Acids have been risen. One of the most important in this group is represented by Linoleic Acid (C18:2 cis-9, cis 12); such fatty acid has to be introduced through a proper diet cause of human incapacity for an endogenous production. Linoleic Acid is considered the forerunner of all the CLA (Conjugated Linoleic Acids), a generic term used to describe positional and geometric isomers of octadecadienoic fatty acids containing conjugated double bonds (1). The cis-9, trans-11 CLA (rumenic acid) is "the one that unequivocally shows anticarcinogenic activity with animal models" (1-7) as well as positive biological effects against others pathologies (6-17). Furthermore, unlike other isomers that have exogenous origin because formed by Butyrivibrio Fibrisolvens, which is widely presents in ruminant diet, rumenic acid shows to have an endogenous source (in the mammary gland and adipose tissue) from the conversion of vaccenic acid t-11 C, 18:1 as a consequence of Delta-9 desaturase enzyme (3-6, 12, 15, 18-22). CLA content in ruminant milk and dairy products appears to be higher than in beef, and the cis-9, cis 12 configuration represents the main isomer from 80% to 90% (3, 6, 7, 14, 15, 18, 21, 23-26). The natural variation of CLA content depends on several factors including the seasonal trend (1, 7, 27-30) the diet, lactation phase, and the breed (4, 6, 15, 22, 31, 32).

Thus, animal scientists have set up diets and food strategies for ruminants oriented to increase the content of these fatty acids in milk and, thereby, in dairy products (4, 6, 10, 15, 19-2131, 33-36), most likely modifying the food composition (14,15, 18, 22, 25, 26, 28, 31, 35, 37-42). Then The purpose of the present work has been to verify the possibility to modify both the milk fatty acid composition and the CLA content, by changing the diet as the basal ration (fresh or preserved forage), by integrating extruded whole linseed flour, and by comparing results we achieved with analogous experiments.

4.3. MATERIALS AND METHODS

4.3.1. SAMPLES COLLECTION

The present work was conducted on a Reggiana cattle farm, located in the district of Reggio Emilia, in the Parmigiano Reggiano yield area. The normal and usual cows rationing has been modified by integrating extruded whole linseed flour (300 g/die) to both the constant concentrated fodder (1kg/3L milk yield) and to the basal ration (fresh or preserved forage) which changes according to the season, and achieved with the collaboration of the farm personnel. In **table 4.1.** the experimental scheme reports what is explained above

According to the different diets we adopted in the respective experimental periods, from the milk yield obtained and delivered to the cheese factory later, 150 mL of outcrop cream has been collected in a specific container after has being mixed for 15 minutes. Moreover, Parmigiano Reggiano cheese was produced in a dairy factory from the massal milk yield following the production's technical policy. The sampling were carried out following the specific policy FIL-IDF (44), and at 6,12, and 24 month of seasoning

4.3.2. SAMPLES PREPARATION

Milk cream samples were collected and stored at -20°C until analysis. About 0.5 g of cream was placed in a 1000 ml separatory funnel and 50 ml diethyl ether (DE) was added. The solution was mixed one minute, 50 ml of 50:50 petroleum ether (PE) /DE (% by volume) was added, was mixed for one minute, then 50 ml of PE was added before one minute of energetic mixing. Two hundred ml of saturated sodium chloride solution was added and the separator funnel was gently mixed for one minute. The organic phase (upper) was recovered and filtered over anhydrous sodium sulphate. The extraction was repeated, and the extracts were combined. The organic solvent was removed in a stream of nitrogen, and the extract was stored at -20°C under nitrogen. The preparation of the methyl esters was performed following the modified procedure of Cruz-Hernandez *et al*. About 20 mg of dry fat was put in a test tube and 2 ml of hexane was added, followed by 40 µl of methyl acetate and 300 µl of 0.5N sodium methoxyde in methanol (#33080, Supelco Inc., Bellefonte, PA). The tube was purged with nitrogen, mixed, heated 10 minutes at 50C in a silicon oil bath, and then frozen. 180 µl oxalic acid (0.5 g in 15 mL di ethyl ether) was added and thoroughly mixed, then the sodium-oxalate precipitate was separate by centrifugation and the hexane fraction was filtered through anhydrous sodium sulphate.

The cheese samples obtained from cows fed with two sperimental diet (Fresh Forage/Fresh Forage and extruded whole linseed flour), were sampled and stored at -40˚C until analysis. Fat was extracted following the Rose-Gottlieb method modified by Secchiari (45). According to Rovellini and Cortesi (46) fat samples were transesterified with 1.0 M sodium benzyloxyde in benzyl alcohol before the analysis by HPLC-DAD.

4.3.3. GAS CHROMATOGRAPHIC QUANTITATION

FAME were analyzed with an Agilent 6890 gas chromatograph (Hewlett Packard, Wilmington, DE) equipped with a CP Sil 88 fused silica capillary column (100 m x0.25 mm (i.d.), 0.2-μm film thickness; Varian, Inc.), a FID detector, and a split injector. Hydrogen was used as carrier at the constant flow rate of 1.0 min/min. The FID detector was maintained at 300°C with air flow rate of 400 ml/min, hydrogen flow rate of 30 ml/min, and helium (make up gas) flow rate of 30 ml/min. The split injector was maintained at 250°C with the split ratio of 1:100. The temperature program was as follow: 4 minutes at 80°C, ramp 7°C/min to 180°C maintained 30 minutes, ramp 4°C /min to 225°C maintained 20 minutes. The quantitation was based on FID theoretic response factors, from AOCS Ce-1h05 Official Method, and the fatty acids percent composition was calculated considering 100% the sum of the corrected areas.

4.3.4. HPLC ANALYSIS

Cheese samples were analyzed by HPLC-DAD equipped with a Spherisorb ODS-2 column (5 μm, 4,6 mm x 25 cm, 100 Å), 1 ml/min flow rate. Chromatograms were recorded at 255nm.

4.3.5. STATISTYCAL ANALISYS

Calculation and statistics were performed with the Tukey test (version 6.0) to evaluate the different levels of fatty acids in cream samples, according to the diet we adopted.

4.3.6. CHEESE SENSORY ANALISYS

After 24 month of seasoning, a trained panel examinated cheese samples by sensory analisys. The panel was composed of 7 persons, members of TINVAL society, spin-off *Alma Mater Studiorum* University of Bologna. Cheese samples were tested by judges at 16°C following a random order, and served in a parallelepiped (L:50/80 mm; h:15mm; w:15mm) (47). Each judge evaluated either the samples structural parameters and the sense of smell/taste. Mineral water and crackers were used as means of counteraction between each test. The sensory profile was defined applying the Etana model (48), and processing the data with Microsoft Excell 2003, ANOVA, and SPSS for Windows (vers. 13.0, SPSS Inc., Chicago, Illinois, USA).

4.4. RESULTS AND DISCUSSION

Tables 4.1.-4.4. show the results obtained from the samples analysis carried out in duplicate. Sixty seven fatty acid have been separated, identified and quantified; nineteen are saturated, 17 monounsaturated, 19 poliunsaturated and 12 are mono and diiunsaturated trans. From **table 4.2.** rise that 17 of the 19 fatty acids identified and quantified as saturated do not change their content, while the C4 and the C6 significantly decrease their percentage parameters ($P<0,05$) when the linseed flour is introduced. As reported in **table 4.3.** most of the 17 monounsaturated fatty acids do not undergo a change with the linseed flour integration while the only exception is represented by the linoleic acid which increases considerably. **Table 4.4.** shows the variation of the 19 poliunsaturated fatty acids. By administering a linseed flour enriched diet, a percentage gain of rumenic acid (C18:2 c9-t11) and a downward trend of arachidonic acid (C20:4) can be observed, while normalize their self when the linseed

flour integration is suspended. The variation of rumenic acid appears to be statistically significant (P<0,05). Relatively to the unsaturated fatty acids with a trans configuration, as reported in **table 4.5.**, the results point out another relevant matter: an increase of vaccenic acid (C18:1 trans-11) level when preserved forage is administered instead of fresh forage, and even when the extruded whole linseed flour is integrated to the fresh forage ration; the trans vaccenic level normalizes itself when the linseed integration is suspended. The latter observation is also valid for the elaidinic acid (C18:1 trans-9). **Figure 4.1.** shows the full chromatogram with the overall fatty acids separation and identification, while **figure 4.2.** and **4.3.** report the chromatograms with the cis/trans C18:1 fatty acids elution area (i.e. CLA), which is our principal purpose in this investigation. On **figure 4.4.** the course of the content of rumenic and vaccenic acid during the test period can be noticed. Moreover, **figure 4.5.** reports the principal variations of the 4 fatty acid groups observed. Thus, this investigation demonstrates the possibility to increase noticeably the CLA content in milk fat, by integrating extruded whole linseed flour (300 g/die) to the basal ration of the cows. Higher levels of CLA in milk fat have been found by M. Monici et al (14), who carried out an experimental dietary study over 2000 cows to increase the ω3 (EPA and DHA) levels in milk fat. They were able to achieve 1,308 g of CLA/100 g of fat, versus 1,160 g of CLA/100 g of fat obtained in our research. We have to point out 2 investigations of Strocchi *et al.* (28, 29) from 1967 (40 years ago), where 200 butter samples from Emilia has been studied; they firstly separated and identified more than 30 fatty acids, and afterwards they established a positive correlation between the content in trans-monounsaturated (vaccenic acid) and conjugated-diens (i.e. CLA). In these studies, the authors noticed how the suchlike fatty acids composition of butters from the Parmigiano Reggiano yield

area differs from the fatty acid profile of butters collected in others district of Emilia Romagna, even though it does not coincide with the most recent study from Chiavari *et al*. (49). Lastly, Capella *et al*. (50) in 1974 reported the most important acquisition about 71 fatty acids identified with different analytical techniques.

Moreover, a preliminary study on the content of the OFA in Parmigiano Reggiano cheese sample collected at 6, 12, 24 month of seasoning was conducted. In this case cheese samples from two experimental diets were analyzed: 1) Fresh Forage 2) Fresh Forage with extruded whole linseed flour integration .

Since the CLA diary fat enrichment of the cows under study, during the processing was equally distributed between butter and cheese, we analyzed the OFA in the Parmigiano Reggiano cheese during the ripening. The data obtained are reported in figure3. More precisely, the purpose of this investigation was to verify whether the extruded whole linseed flour might be involved in the increase of the OFA content, because it could modify the lipid class in general and, more precisely the MONO- and POLI- unsatured fraction, which is strongly sensible to the oxidation (51). The lipid oxidation products are involved on the development of atherosclerosis process even though not much is known for the human health about their assimilation through the diet (52, 53). The results obtained are reported in **table 4.6.** and **figure 4.6.**

Furthermore, a sensory evaluation of the same Parmigiano Reggiano cheese samples from the two experimental diets (with and without integration) was conducted. The results did not show so relevant differences between samples. Although, between the sensory descriptor considerated in the Etana model (**figure 4.7.),** two of them showed significant differences (P>0,001) relatively to the sour and to the friability. Their values

resulted to be lower in cheese samples from the experimental diet with extruded whole linseed flour integration.

4.5. CONCLUSIONS

Over the last eight years, more than 2,600 scientific articles about CLA in dairy products have been published. The increasing interest in CLA arise from the remarkable health and dietary effects in human nutrition.

In this work, conducted over an 8 months period on a Reggiana cow farm (located in the district of Reggio Emilia in the Parmigiano Reggiano yield area), important considerations can be made by examining the statistical results.

First of all, according to the literature (25, 26, 40) by administering linseed flour enriched diet to dairy cows, the possibility to increase the rumenic acid content in milk has been confirmed. In order to point out the tight correlation between rumenic acid and trans-vaccenic acid when linseed flour is integrated to the cows ration, an increment of 47% and 49% respectively has been seen in milk fat (3, 6, 15, 18-20, 22, 36, 40). This first result is particularly interesting especially for the entity of rumenic acid quantitative variation. On the other hand, the increment in trans-vaccenic acid has to be seen negatively since both USA and EU health care are debating on low total trans fatty acid (TFA) intake, even though we believe that is an important value to be pointed out. TFA are generally present in food products containing hydrogenated fat and, as a consequence, they influence both the low and high density lipoprotein levels (LDL, HDL), increasing the risk coronary heart disease (54-56).The second result, closely related with the first, concern an increment of 36% of the total TFA in milk by

introducing linseed flour in cow's diet, while it undergoes a decrease of 22% when the linseed integration has been suspended.

Lastly, the investigation about the OFA content in the Parmigiano Reggiano cheese samples, shows an interesting result: as it might be thought, instead of being constant or increasing in the amount, the OFA content decreased during the ripening, and it reduced further in cheese samples where cows were fed with extruded whole linseed flour integration. These results can be explained considering that the state of ripening induce to create such a strong reducing system inside the cheese, preventing the formation of the oxidized fatty acids.

TABLES

Table 4.1. – *Experimental scheme*

Period	**Food ration**	**Observations**
25 March - 29 April	*Preserved forage (hay + MCI*)*	4
30 April - 31 May	*Fresh forage + MCI*	4
15 August - 30 September	*Fresh forage + MCI + extruded whole linseed flour (300 g/d)*	7
12 October - 9 November	*Fresh forage + MCI after suspension of extruded whole linseed flour integration*	3

Table 4.2. – *Percentage composition of saturated fatty acids and branched chain by administering different diets.*

Unsatured	**Preserved forage**	**Fresh forage**	**Linseed flour integration**	**Fresh forage**
C4:0	3,55 ^{b} ± 0,10	3,52 ^{b} ± 0,04	3,20 ^{a} ± 0,08	3,26 ^{a} ± 0,05
C5:0	0,02 ^{a} ± 0,01	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,00
C6:0	2,12 ^{b} ± 0,03	2,08 ^{b} ± 0,07	1,90 ^{a} ± 0,02	1,97 ^{a} ± 0,03
C7:0	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,00
C8:0	1,25 ^{b} ± 0,02	1,22 ^{b} ± 0,05	1,11 ^{a} ± 0,03	1,19 ^{b} ± 0,03
C9:0	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,00
C10:0	2,73 ^{b} ± 0,05	2,67 ^{b} ± 0,13	2,38 ^{a} ± 0,07	2,64 ^{b} ± 0,05
C11:0	0,31 ^{b} ± 0,01	0,30 ^{a,b} ± 0,01	0,28 ^{a} ± 0,01	0,31 ^{a,b} ± 0,00
C12:0 +11:1	3,21 ^{b} ± 0,06	3,13 ^{b} ± 0,16	2,79 ^{a} ± 0,09	3,12 ^{b} ± 0,05
C13:0 +12:1	0,17 ^{b} ± 0,01	0,17 ^{b} ± 0,01	0,15 ^{a} ± 0,01	0,17 ^{b} ± 0,00
C14:0	11,15 ^{b} ± 0,08	10,96 ^{b} ± 0,49	10,20 ^{a} ± 0,22	10,94 ^{b} ± 0,11
C15:0	1,26 ^{a} ± 0,02	1,27 ^{a} ± 0,04	1,20 ^{a} ± 0,05	1,26 ^{a} ± 0,03
C16:0	29,07 ^{b} ± 0,66	28,64 ^{b} ± 1,08	25,74 ^{a} ± 0,85	27,76 ^{b} ± 0,07
C17:0	0,76 ^{a,b} ± 0,02	0,79 ^{b} ± 0,02	0,73 ^{a} ± 0,02	0,77 ^{a,b} ± 0,01
C18:0	10,54 ^{a} ± 0,22	11,52 ^{b} ± 0,31	12,56 ^{c} ± 0,33	11,17 ^{a,b} ± 0,19
C19:0	0,03 ^{a} ± 0,05	0,11 ^{a} ± 0,00	0,15 ^{a} ± 0,18	0,08 ^{a} ± 0,03
C20:0	0,20 ^{a,b} ± 0,01	0,21 ^{b,c} ± 0,01	0,22 ^{c} ± 0,01	0,18 ^{a} ± 0,01
C22:0	0,08 ^{a} ± 0,01	0,10 ^{a} ± 0,01	0,09 ^{a} ± 0,01	0,08 ^{a} ± 0,01
C24:0	0,07 ^{a} ± 0,00	0,07 ^{a} ± 0,00	0,07 ^{a} ± 0,01	0,07 ^{a} ± 0,00

Different letters indicate significant differences (Tukey test with $p<0,05$).

Table 4.3. – *Percentage composition of mono-unsaturated fatty acids (cis + trans) by administering different diets.*

Mono-unsatured	Preserved forage	Fresh forage	Linseed flour integration	Fresh forage
C13:1	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C14:1	0,90 ^{a,b} ± 0,03	0,87 ^{a,b} ± 0,03	0,83 ^{a} ± 0,05	0,93 ^{b} ± 0,03
C15:1	0,01 ^{a} ± 0,01	0,01 ^{a} ± 0,01	0,01 ^{a} ± 0,00	0,01 ^{a} ± 0,00
C16:1 *c*9	1,31 ^{b} ± 0,02	1,26 ^{a,b} ± 0,03	1,17 ^{a} ± 0,06	1,32 ^{b} ± 0,04
C17:1 *c*	0,27 ^{a,b} ± 0,01	0,27 ^{a,b} ± 0,01	0,25 ^{a} ± 0,01	0,28 ^{b} ± 0,01
C18:1 *c*9 +*t*15	20,91 ^{a} ± 0,34	21,44 ^{a} ± 0,54	22,62 ^{b} ± 0,50	21,85 ^{a,b} ± 0,57
C18:1 *c*11	0,55 ^{a} ± 0,01	0,56 ^{a} ± 0,03	0,55 ^{a} ± 0,02	0,45 ^{a} ± 0,20
C18:1 *c*12	0,26 ^{a} ± 0,01	0,27 ^{a} ± 0,01	0,31 ^{a} ± 0,02	0,32 ^{a} ± 0,16
C18:1 *c*13	0,07 ^{a} ± 0,01	0,08 ^{a} ± 0,00	0,09 ^{a} ± 0,01	0,05 ^{a} ± 0,04
C18:1 *c*15	0,11 ^{a} ± 0,01	0,12 ^{a} ± 0,00	0,14 ^{a} ± 0,06	0,07 ^{a} ± 0,05
C19:1 *c*7	0,11 ^{b} ± 0,00	0,11 ^{b} ± 0,00	0,06 ^{a} ± 0,12	0,03 ^{a,b} ± 0,06
C20:1 *c*5	0,01 ^{a} ± 0,01	0,01 ^{a} ± 0,01	0,01 ^{a} ± 0,02	0,02 ^{a} ± 0,00
C20:1 *c*8	0,14 ^{a} ± 0,01	0,15 ^{a} ± 0,01	0,12 ^{a} ± 0,05	0,14 ^{a} ± 0,01
C20:1 *c*11	0,05 ^{a} ± 0,01	0,05 ^{a} ± 0,00	0,06 ^{a} ± 0,03	0,05 ^{a} ± 0,00
C22:1	0,01 ^{a} ± 0,00	0,01 ^{a} ± 0,01	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,01
C24:1	0,01 ^{a} ± 0,00	0,02 ^{a} ± 0,01	0,01 ^{a} ± 0,01	0,01 ^{a} ± 0,00

Different letters indicate significant differences (Tukey test with p<0,05).

Table 4.4. – *Percentage composition of poly-unsaturated fatty acids by administering different diets.*

Poly-unsaturated	Preserved forage	Fresh forage	Linseed flour integration	Fresh forage
18:2 *c*9-*c*12	2,15 ^{a} ± 0,18	2,13 ^{a} ± 0,05	2,14 ^{a} ± 0,08	2,19 ^{a} ± 0,02
C18:2 *c*9-*t*11	0,80 ^{a} ± 0,08	0,80 ^{a} ± 0,02	1,16 ^{b} ± 0,07	0,94 ^{a} ± 0,06
C18:2 *t*9-*c*11	0,04 ^{a} ± 0,01	0,05 ^{a} ± 0,03	0,04 ^{a} ± 0,02	0,04 ^{a} ± 0,01
C18:2 *t*11-*c*13	0,01 ^{a} ± 0,01	0,02 ^{a} ± 0,01	0,06 ^{b} ± 0,03	0,03 ^{a} ± 0,00
C18:2 *t*11,*t*13	0,00 a ± 0,01	0,01 a ± 0,02	0,00 a ± 0,01	0,00 a ± 0,00
C18:2 *t*9,*t*11,*t*10,*t*12	0,02 a ± 0,01	0,01 a ± 0,01	0,03 a ± 0,02	0,01 a ± 0,01
C18:2 *c*9-*t*13	0,18 ^{a} ± 0,02	0,19 ^{a} ± 0,01	0,26 ^{b} ± 0,02	0,21 ^{a} ± 0,01
C18:3 *c*-*c*-*c* n-3	0,75 ^{a} ± 0,07	0,72 ^{a} ± 0,05	0,96 ^{b} ± 0,10	0,78 ^{a} ± 0,05
C18:3 *c*-*c*-*c* n-6	0,05 ^{b} ± 0,00	0,04 ^{b} ± 0,00	0,03 ^{a} ± 0,01	0,04 ^{a,b} ± 0,00
C20:2	0,03 ^{a} ± 0,00	0,03 ^{a} ± 0,00	0,03 ^{a} ± 0,01	0,03 ^{a} ± 0,00
C20:3 n-3	0,02 ^{a} ± 0,00	0,02 ^{a} ± 0,00	0,01 ^{a} ± 0,01	0,02 ^{a} ± 0,00
C20:3 n-6	0,11 ^{a} ± 0,01	0,11 ^{a} ± 0,01	0,10 ^{a} ± 0,01	0,11 ^{a} ± 0,01
C20:4	0,15 ^{b} ± 0,00	0,15 ^{b} ± 0,01	0,11 ^{a} ± 0,04	0,14 ^{a,b} ± 0,01
C20:5	0,06 ^{a,b} ± 0,00	0,07 ^{b} ± 0,00	0,05 ^{a} ± 0,02	0,06 ^{a,b} ± 0,00
C22:2	0,03 ^{a,b} ± 0,00	0,04 ^{b} ± 0,01	0,03 ^{a} ± 0,02	0,04 ^{b} ± 0,01
C22:3	0,00 ^{a} ± 0,01	0,00 ^{a} ± 0,00	0,00 ^{a} ± 0,00	0,00 ^{a} ± 0,00
C22:4	0,02 ^{a} ± 0,00	0,03 ^{a} ± 0,00	0,02 ^{a} ± 0,01	0,02 ^{a} ± 0,00
C22:5	0,12 ^{a} ± 0,00	0,12 ^{a} ± 0,00	0,11 ^{a} ± 0,01	0,12 ^{a} ± 0,01
C22:6	0,00 ^{a} ± 0,00	0,01 ^{a} ± 0,01	0,01 ^{a} ± 0,02	0,01 ^{a} ± 0,00

Different letters indicate significant differences (Tukey test with p<0,05).

Table 4.5. – *Percentage composition of mono and di-unsaturated trans fatty acids by administering different diets.*

Unsatured trans	Preserved forage	Fresh forage	Linseed flour integration	Fresh forage
C16:1 *t*	0,08 [a] ± 0,01	0,08 [a] ± 0,00	0,11 [a] ± 0,04	0,10 [a] ± 0,00
C18:1 *t*4	0,02 [a] ± 0,00	0,02 [a] ± 0,00	0,03 [b] ± 0,00	0,02 [a] ± 0,00
C18:1 *t*5	0,02 [a] ± 0,01	0,02 [a] ± 0,00	0,02 [a] ± 0,01	0,01 [a] ± 0,01
C18:1 *t*6-8	0,21 [a] ± 0,01	0,22 [a] ± 0,00	0,28 [b] ± 0,01	0,23 [a] ± 0,02
C18:1 *t*9	0,30 [a] ± 0,01	0,32 [a,b] ± 0,00	0,35 [b] ± 0,03	0,31 [a] ± 0,01
C18:1 *t*10	0,32 [a] ± 0,03	0,34 [a] ± 0,03	0,31 [a] ± 0,03	0,32 [a] ± 0,01
C18:1 *t*11	1,68 [a] ± 0,06	1,80 [a,b] ± 0,03	2,65 [c] ± 0,18	2,06 [b] ± 0,02
C18:1 *t*12	0,26 [a] ± 0,02	0,27 [a] ± 0,00	0,36 [b] ± 0,03	0,26 [a] ± 0,01
C18:1 *t*16	0,30 [a] ± 0,14	0,32 [a,b] ± 0,01	0,51 [b] ± 0,06	0,30 [a,b] ± 0,16
C18:2 *t*9,*t*12	0,00 [a] ± 0,00	0,00 [a] ± 0,00	0,02 [a] ± 0,02	0,00 [a] ± 0,00
C18:1 *t*13+*t*14 (+*c*6-8)	0,62 [a] ± 0,02	0,68 [a] ± 0,01	0,89 [b] ± 0,06	0,66 [a] ± 0,03
C18:2 *c*9-*t*12	0,24 [a] ± 0,10	0,10 [a] ± 0,01	0,19 [a] ± 0,14	0,16 [a] ± 0,13
C18:2 *t*9-*c*12	0,10 [a] ± 0,01	0,12 [a] ± 0,01	0,28 [b] ± 0,13	0,20 [a] ± 0,04

Different letters indicate significant differences (Tukey test with p<0,05).

Table 4.6. – *Trend of oxidized fatty acids (OFA) in 4 moulds of Parmigiano Reggiano cheese at 6,12 and 24 months of ripening in relation to food ration (mg OFA /100 mg of lipids).*

Food ration	Months		
	6	12	24
Diet with fresh forage	4,15 ± 1,15	0,81 ± 0,04	1,39 ± 0,24
Diet with fresh forage	4,44 ± 1,26	0,93 ± 0,01	0,92 ± 0,06
Extruded whole linseed flour integration	3,23 ± 0,92	0,64 ± 0,60	0,49 ± 0,38
Extruded whole linseed flour integration	3,38 ± 0,91	0,75 ± 0,88	0,59 ± 0,22

FIGURES

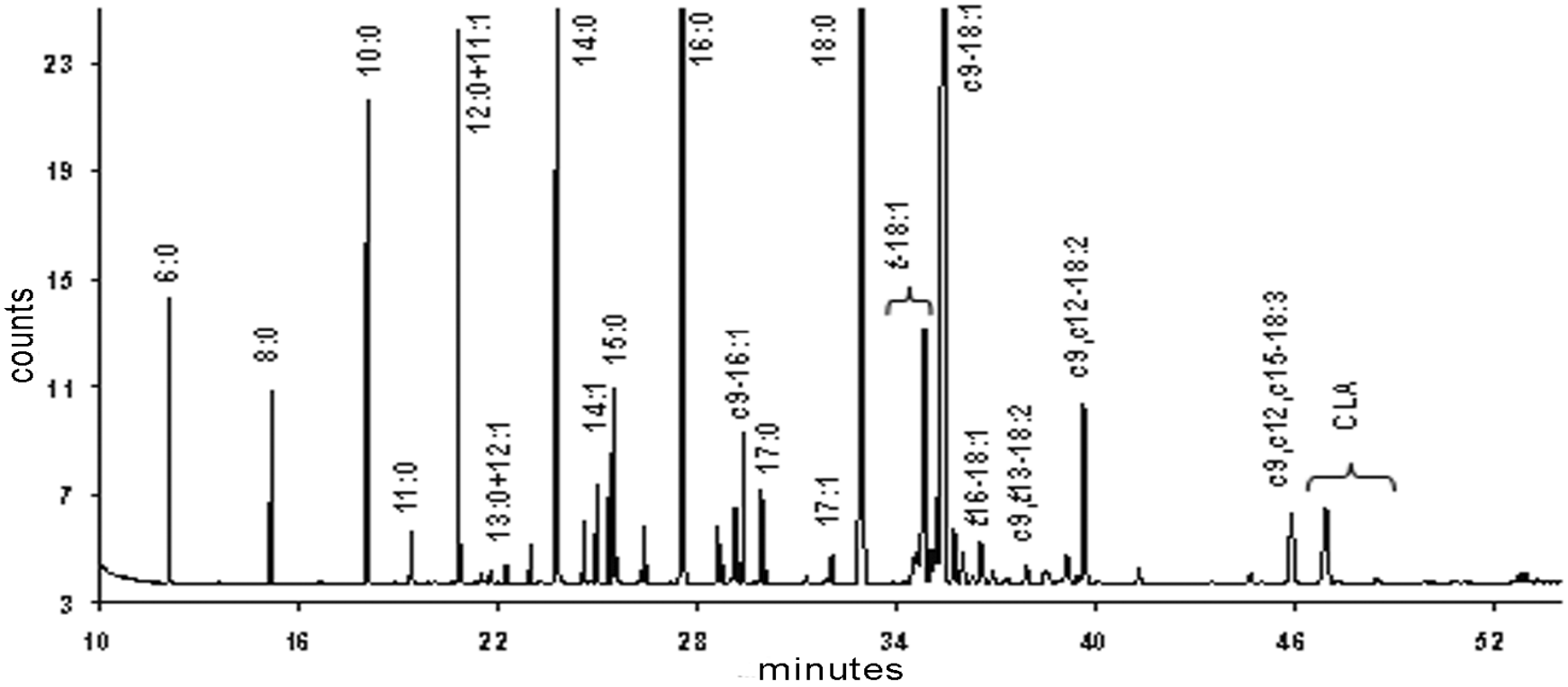

Figure 4.1. - *Chromatogram of fatty acid*

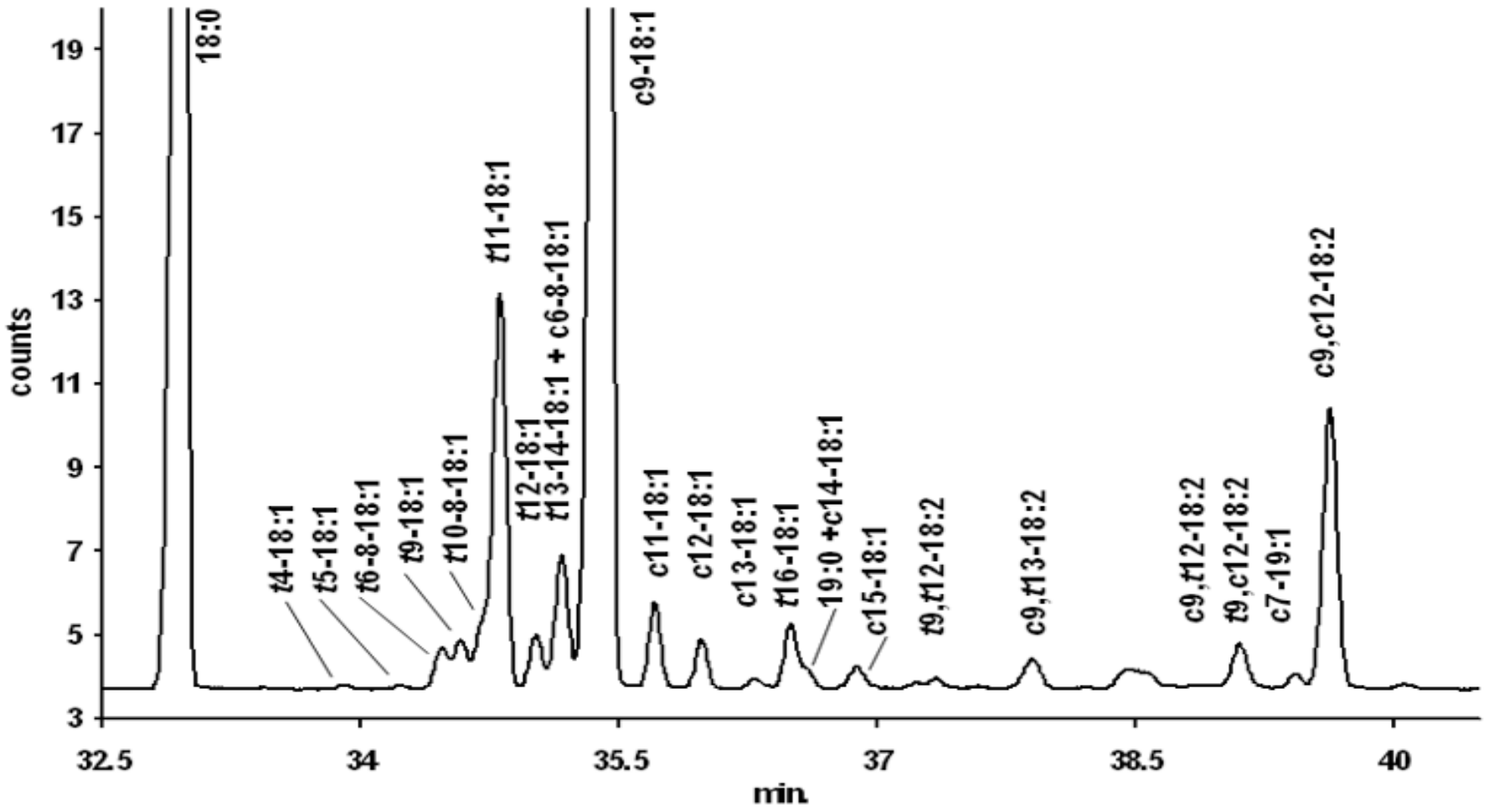

Figure 4.2. – *Partial chromatogram of cis/trans-18:1 acids.*

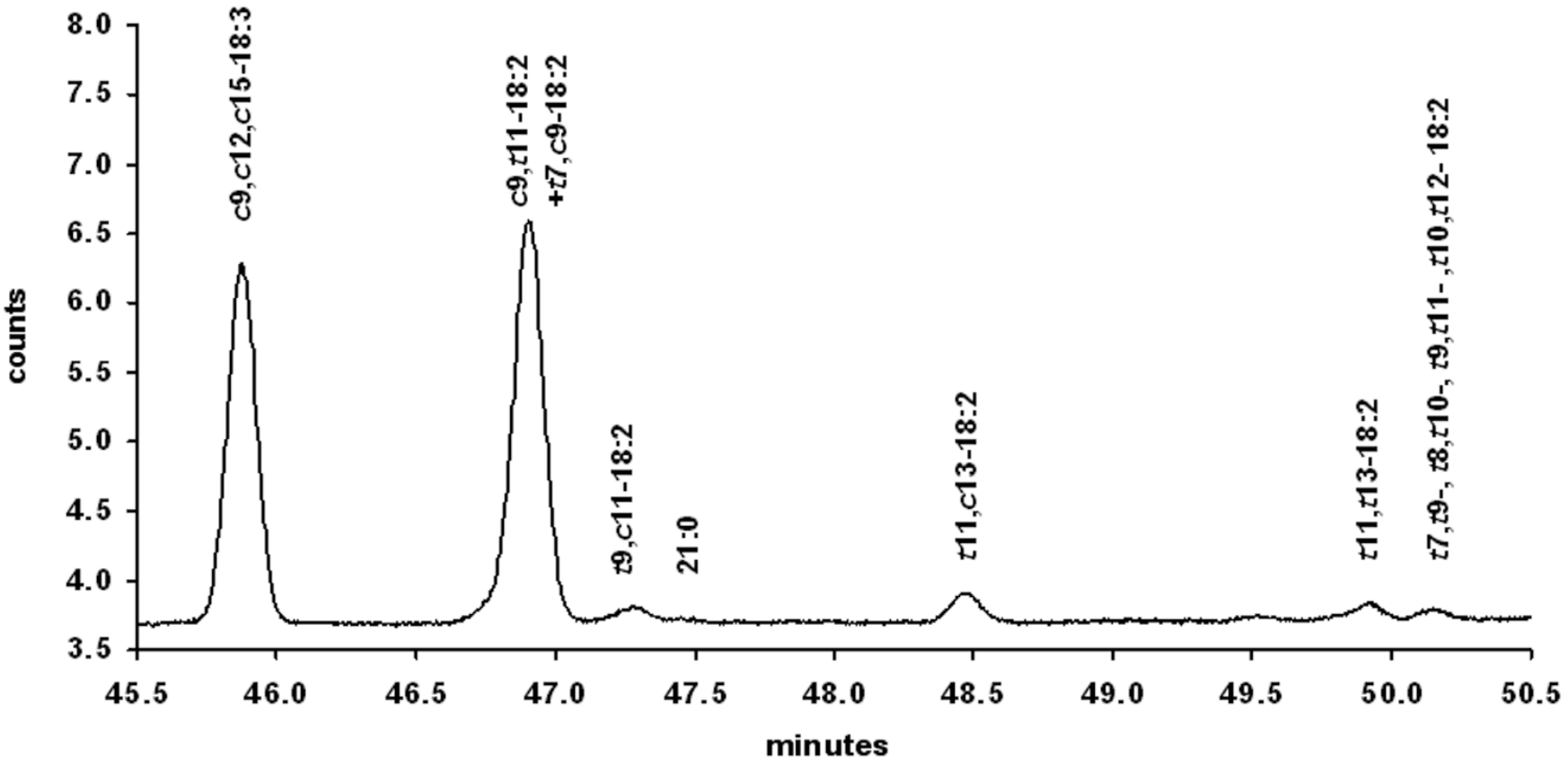

Figure 4.3. - *Partial chromatogram of CLA.*

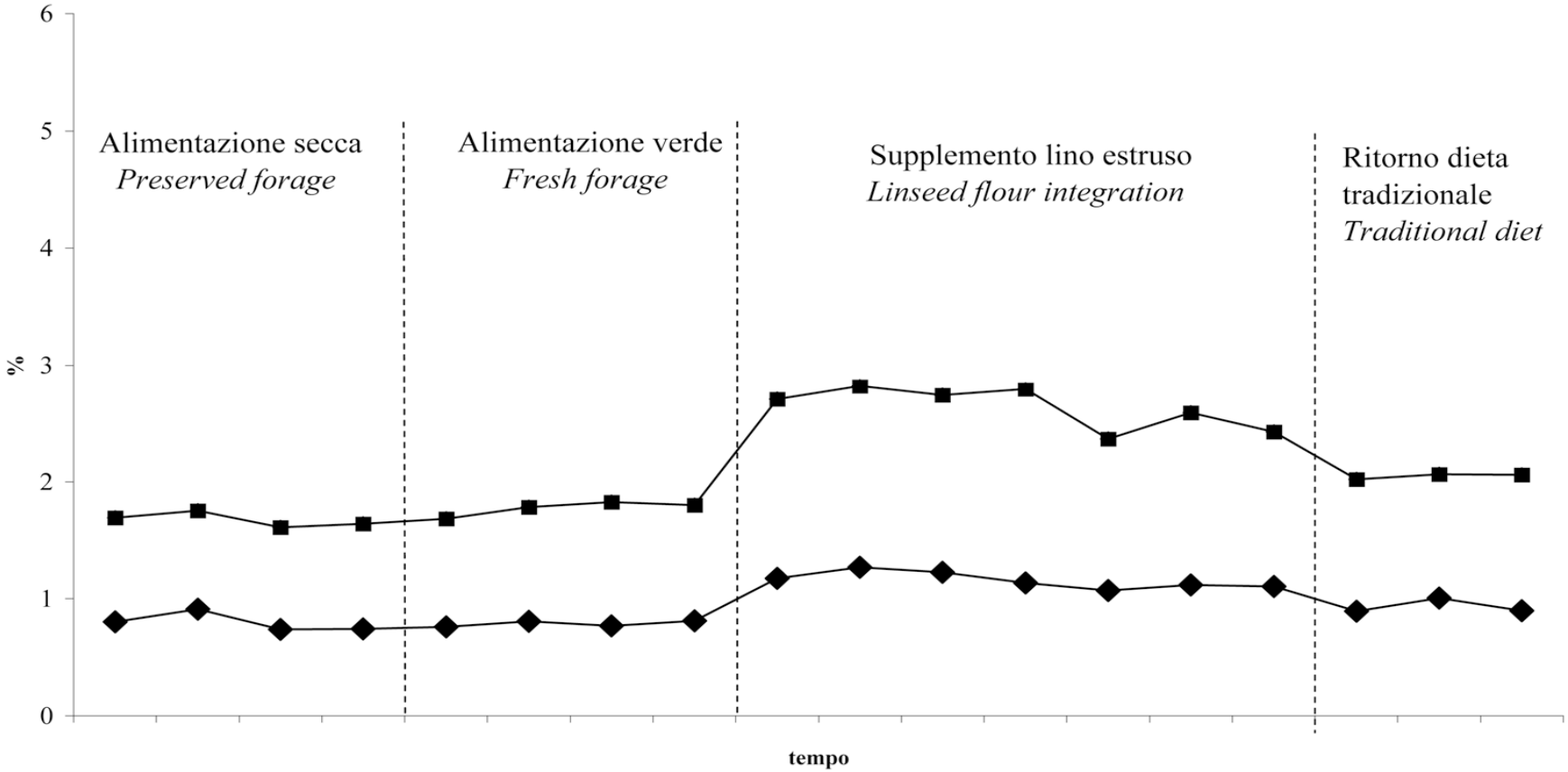

Figure 4.4. - *Effect of linseed flour integration: vaccenic acid and rumenic acid variations by varying cow's diet (■ trans vaccenic acid percentage; ♦ rumenic acid percentage).*

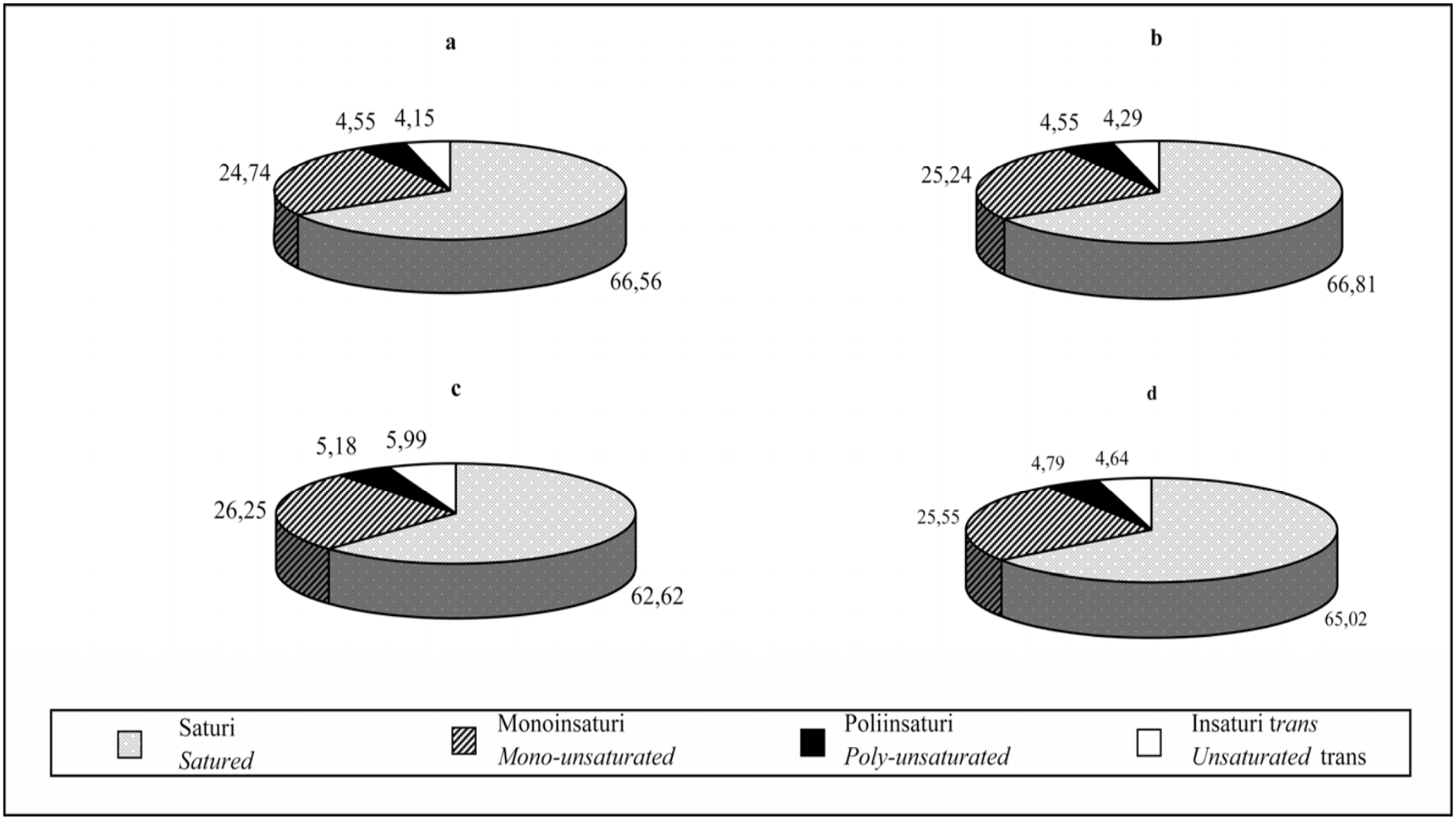

Figure 4.5. - *Effect of linseed flour integration: variation of acidic composition: (a: preserved forage; b): fresh forage; c) enriched extruded whole linseed flour; d) fresh forage, non enriched fresh forage).*

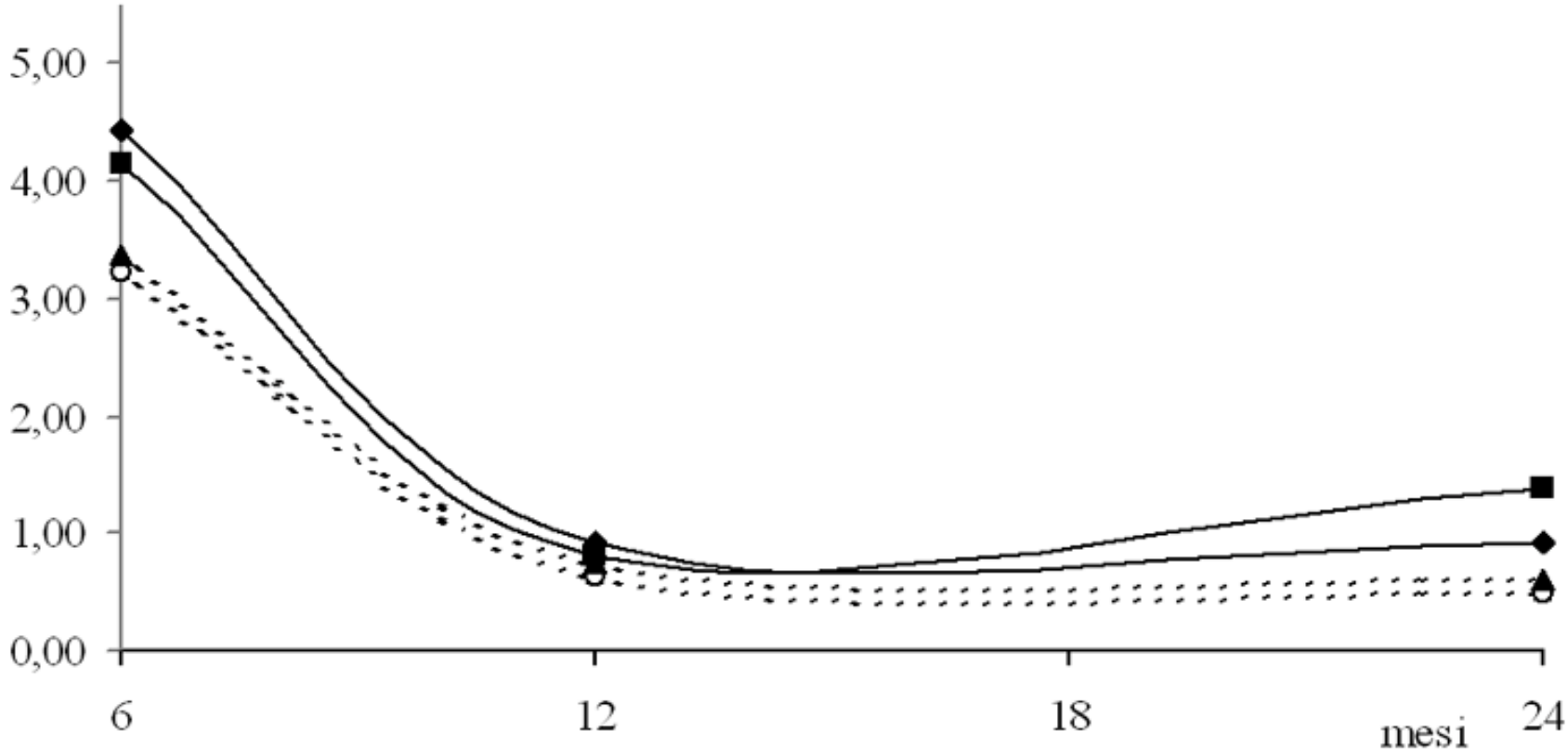

Figure 4.6. - *Intake trend of oxidized fatty acids (mg OFA /100 mg of lipids) in Parmigiano Reggiano cheese samples collected at different ripening times referred to two experimental batches (two samples for batches: ■ - ♦ diet with fresh forage; ○ - ▲ extruded whole linseed flour integration).*

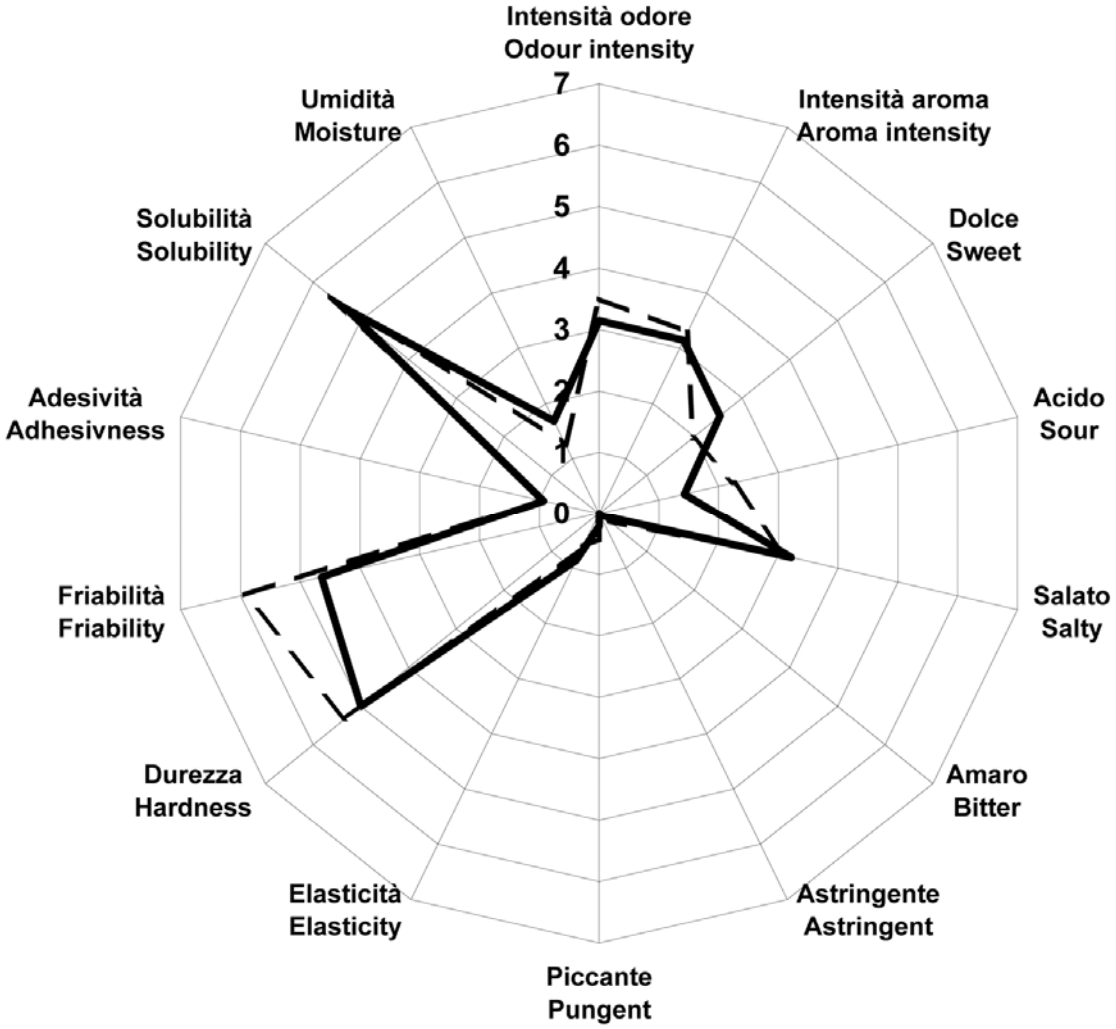

Figure 4.7. - *Etana profiles of two experimental batches (—— extruded whole linseed flour integration; ------ control).*

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5

A SIMPLIFIED METHOD FOR HPLC-MS ANALYSIS OF STEROLS IN VEGETABLE OIL

5.1. SUMMARY AND KEYWORDS

A liquid-chromatographic method using atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) detection in positive mode has been developed. This method was used to separate and identify 15 sterols and 2 dihydroxy triterpenes in saponified oils, enabling the analysis of these compounds directly from saponified samples without recourse to thin-layer chromatography; this fact thus significantly simplifies the process. The analyses were made using a Waters Atlantis 5 mm dC_{18} 15062.1 mm column with a gradient of acetonitrile/water (0.01% acetic acid) at a flow rate of 0.5 ml/min and a column temperature of 30°C. The quantification of several of these compounds in soybean oil, palm oil, seed oil, sunflower oil, olive-pomace oil and virgin olive oil was carried out using their commercial standards, and the results were compared satisfactorily with the official method.

Keywords: High-performance liquid chromatography, Mass spectrometry, Oils, Olive oil, Sterols.

5.2. INTRODUCTION

Sterols are widely occurring natural substances and make up the greatest proportion of the unsaponifiable fraction of lipids (1, 2). Plant fats and oils contain phytosterols as naturally occurring constituents (3). The most important natural sources of plant sterols in the human diet are oils and margarines, although they are also found in a range of seeds, legumes, vegetables and unrefined vegetable oils (4–6). Their composition depends on the plant species (7) and, in oils, it may vary according to agronomic and climatic conditions, the quality of the fruits or seeds, extraction and refining procedures and storage conditions. Phytosterols can be classified on a structural or biosynthetic basis as 4-desmethyl sterols, 4α-monomethyl sterols and 4,4-dimethyl sterols. In addition, the 4-desmethyl sterols may be subdivided into Δ5-sterols, Δ7-sterols and Δ5,7-sterols, depending on the position of the double bonds in the B ring (8). The predominant phytosterol is β-sitosterol; minor components are campesterol, stigmasterol, Δ5-avenasterol, Δ7-avenasterol and brassicasterol. These compounds are biogenetic precursors of numerous metabolites, including plant steroid hormones, and have anti-inflammatory, antibacterial, antifungal, antiulcerogenic and antitumoral activity (9).

The characterization and quantification of phytosterols can be carried out by a wide variety of chromatographic techniques including column chromatography, thin-layer chromatography (TLC), gas chromatography (GC), normal phase high-performance liquid chromatography (HPLC), reverse-phase HPLC, capillary electrochromatography (10) and also online-coupled HPLC-GC. These compounds can be detected with flame ionization detection (FID), UV detection (UV), evaporative light scattering detection, infrared detection, nuclear magnetic resonance detection, and mass spectrometry (MS).

GC using FID or MS (11–21) and HPLC with different detection systems are the more widely used techniques (22–25), although the most accurate identifications have been performed by MS. Recently, some papers on the determination of sterols with HPLC-MS (26–29) have been published; in the article of Cañabate et al. (29), a liquid chromatographic method for the identification and quantification of seven phytosterols in olive oil and the sterols was developed and the compounds were quantified by liquid chromatography with MS detection in positive atmospheric pressure chemical ionization (APCI) mode. This is due to the fact that, in HPLC-MS analysis, baseline separation of peaks is not always necessary because of the high specificity and selectivity of the detection system. Because sterols are highly lipophilic and have few polar functional groups, they are difficult to ionize by conventional electrospray methods (30); APCI is the most widely used ionization technique for sterol analysis. HPLC-MS with APCI has been found to be suitable for sterol analyses in different sample matrices. It has been used to identify sterols in soybean oil (26), to characterize phytosterols in spelt (30), to determine ergosterol levels in bulrush (31) and to measure cholesterol oxides in various foods (32).

The official method (33–35) for the analysis of total sterols from oils involves the saponification of the lipids, extraction of the unsaponifiable matter with diethyl ether and washing the extract with water (liquid-liquid extraction), followed by separation by TLC on silica gel plates, derivatization of the sterols with trimethylsilyl derivatives and subsequent GC analysis. Therefore, sample preparation is laborious, the technique is highly wasteful in terms of reagents, and the separation of sterols by TLC is deceptive. Herein is described a simple HPLC-APCI-MS method to analyze 4-desmethyl sterols and two dihydroxy triterpenes in oil samples after saponification and a simple liquid-

liquid extraction. This proposed method is useful as it permits to identify 17 compounds (4-desmethyl sterols and two dihydroxy triterpenes) and quantify seven of them (vs. their commercial standards) which are relevant in olive oils and other oils that are recognized by the International Olive Oil Council and included in European legislation. The use of MS as detection system provides an accurate identification of the compounds under study.

5.3. MATERIALS AND METHODS

5.3.1. CHEMICALS

Cholesterol came from Riedel-de Haën (Seelze, Germany). β-Sitosterol (90%), erythrodiol (97%) and stigmasterol (95%) were from Fluka (Buchs, Switzerland). Sitostanol (95%), uvaol (95%), Δ5-avenasterol (95%), 2,7-dichlorofluorescein, anhydrous pyridine, hexamethyl disilazone and trimethylchlorosilane were from Sigma-Aldrich (St. Louis, MO, USA). Stocks solutions containing 50 mg/mL of sterols were prepared in HPLC-grade methanol and stored in the dark at 4°C. The concentration of the final stock solution was calculated taking into account the purity of commercial standards. Working standard solutions were prepared from these solutions and diluted with methanol prior to analysis. HPLC-grade solvents (ethanol, methanol, hexane, diethyl ether, 2-propanol, toluene, acetone, hexane, ethyl ether and chloroform) were from Panreac (Barcelona, Spain). Potassium hydroxide and anhydrous sodium sulfate were from Sigma, and Silicagel 60 TLC plates (20x20 cm) from Merck (Barcelona, Spain). Water was deionized with a Milli-Q system (Millipore, Bedford, USA).

5.3.2. INSTRUMENTATION

GC analyses were done with a Fison GC 8000 series (Fison Instrument, France) using for the separation a WCOT-fused silica 30 m x 0.25 mm i.d. coating CP FIL 8CB, DF = 0.25 μm column (J&W Scientific Inc. Agilent, Spain), and a flame ionization detector (Fison Instrument, France). The carrier gas was helium. A 10 μl GC microsyringe with a hardened needle was used.

HPLC analyses were carried out using an HPLC system from Thermo Separation Products (UK) equipped with an autosampler, a degasser and a heated column. Separation was done on an Atlantis dC_{18} 150 column (2.1 mm i.d., 5 mm; Waters) under gradient conditions at an injection volume of 10 μL at 30°C. The mass spectrometer system was a Finnigan AQA (ThermoQuest, USA) with an APCI interface. Data were collected by Xcalibur data system software on a personal computer.

5.3.3 ANALYTICAL METHOD

5.3.3.1. Official Method

To assure that our results are reliable, the samples were analyzed with the official method. The operating conditions of this method are as follows: column temperature, 260 ± 5°C; injector temperature, 280°C; detector temperature, 290°C; linear velocity of the carrier gas: helium 20 cm/s, hydrogen 30 cm/s; splitting ratio of 1 : 75; amount of substance injected, 1 μl of trimethylsilyl (TMSE).

5.3.3.2. Proposed Method

The analyses were made using a Waters Atlantis 5mm dC18 150 x 2.1 mm column with a gradient of acetonitrile/water (0.01% acetic acid) at a flow rate of 0.5 mL/min and a

column temperature of 30°C. The MS conditions were: a nebulizer temperature of 450°C, source temperature of 120°C; corona discharge of 10 μA; accelerating voltage of 25 V; cone gas flow rate of 50 L/h; and a desolvation gas flow rate of 350 L/h.

5.3.4. SAMPLES

Different vegetable oils (soybean, palm, seed, sunflower, olive-pomace and virgin olive oil) were acquired on a local market and used as received.

5.3.5. SAMPLE PREPARATION FOR STEROLS AND DIHYDROXY TRITERPENES

5.3.5.1. Official Method

In the official method (33–35), the isolation of the sterol fractions in olive oils involves:

(1) *Saponification with KOH in ethanol solution:* Usually, 5 g of oil is saponified by refluxing with 50 ml of an ethanolic solution of 2 M KOH for 1 h.

(2) *Extraction of the unsaponifiable fraction with diethyl ether:* 100 ml water is added after cooling to room temperature and, subsequently, after phase separation in a separatory funnel, the aqueous phase is washed three times with diethyl ether.

(3) *Washing with water:* The diethyl ether fractions are collected, washed with water and dried with anhydrous sodium sulfate.

(4) *Separation by TLC on silica gel plates, derivatization of the sterols and subsequent chromatographic analysis:* 100 ml of a 5% solution of the unsaponifiable fraction in chloroform is streaked on a chromatographic plate as thinly and uniformly as possible and then allowed to elute until the solvent front is approximately 1 cm from the upper

edge of the plate. The plate is removed from the developing chamber and the solvent evaporated either in a flow of hot air or by being left for a short while under a hood.

5) *Recovery of the sterols by means of scratching and their extraction with chloroform:* The plate is sprayed lightly and uniformly with the 2,7-dichlorofluorescein solution and the silica gel in the marked area is scraped off with a metal spatula.

6) *Derivatization of sterols:* The silylation reagent is added to the test tube, which is then stopped. It is shaken carefully until the sterols are completely dissolved and then centrifuged for a few minutes. The clear solution is then ready for GC analysis.

7) *Analysis by GC-FID*.

5.3.5.2. Proposed Method

We describe here a simplified way of pre-treating the sample, which is complete after the third step. The diethyl ether fractions are collected, washed with water and dried with anhydrous sodium sulfate. They are then filtered and evaporated to dryness using a rotary evaporator at reduced pressure. The residue is dissolved in methanol. Sample extracts are filtered through a membrane filter (0.45 μm) before being analyzed by HPLC-APCI-MS.

5.4. RESULTS AND DISCUSSION

5.4.1. ISOLATION OF STEROLS AND DIHYDROXY TRITERPENES FROM THE UNSAPONIFIABLE FRACTION USING THE REDUCED METHOD

The sterol fractions in virgin olive oil and other oils were tested by HPLC-APCI-MS with and without using TLC as described by the official method. We succeeded in

reducing the time needed to isolate these compounds in comparison to the official method. The official method was simplified prior to separation by TLC. At this point, the solvent was evaporated and the extract re-dessolved in methanol. **Figure 5.1.** shows the differences found between the two extracts of virgin olive oil with and without the use of TLC. As it can be seen, the simplified extraction method (without the use of TLC) gave a similar profile to that obtained by the official extraction protocol with TLC.

5.4.2. HPLC-MS CONDITIONS

The composition of the mobile phase, the solvent ratio and the flow rate was studied. After preliminary studies, it was decided that the optimum flow rate should be 0.5 ml/min. Different gradients using water (0.01% acetic acid), acetonitrile and methanol, and different isocratic compositions at a constant flow of 0.5 ml/min were assayed to obtain the best resolution of the chromatographic peaks. In mixtures containing methanol, the resolution and intensity of the peaks was worse. After realizing that water (with 0.01% acetic acid) (as phase A) and acetonitrile (as phase B) were the most appropriate mobile phases to achieve our aim, we checked a wide number of gradients, taking into account the resolution among peaks, the intensity in the mass spectrometer and the analysis time as analytical parameters.

In general, we observed that an increase in the percentage of phase A at the beginning of the analysis slowed down the analysis time. Using the following gradient, from 0 to 40 min, from 90% B/10% A to 100% B, we obtained a clear resolution between peaks, so we focused our experimental work upon the study of linear gradients, i.e. 85 to 100%, 90 to 100% and 95 to 100% of phase B, all over a period of 40 min. In this study,

we observed that the resolution between peaks decreased concomitantly with an increase in the initial percentage of acetonitrile. When using an initial percentage of acetonitrile of 85%, the resolution improved, but the peaks were retarded considerably. Therefore, we studied in detail the variation of the initial acetonitrile concentration from 90 to 95% over 60 min in order to obtain clear resolution between peaks. We finally found that with a C18 stationary phase and a gradient between 90% acetonitrile/10% water + 0.01% acetic acid and 92% acetonitrile/8% water + 0.01% acetic acid, from 0 to 60 min at a flow rate of 0.5 ml/min and a column temperature of 30 °C, we achieved the best separation.

Optimum MS conditions were: a nebulizer temperature of 450 °C, a source temperature of 120 °C; corona discharge of 10 μA; accelerating voltage of 25 V; cone gas flow of 50 L/h; and a desolvation gas flow of 350 L/h. HPLC-MS determinations were obtained by operating the mass spectrometer in the positive ion mode. Full-scan mass spectra were acquired within the 300–450 m/z range.

The identification of each compound was carried out using the migration time and the fragmentation, and for the quantification the area of each ion extracted was used.

Figure 5.2. shows a chromatogram of a standard solution of five sterols and two dihydroxy triterpenes under the optimum conditions chosen.

5.4.3. IDENTIFICATION OF STEROLS AND DIHYDROXY TRITERPENES IN DIFFERENT SAMPLES

Samples obtained by using the new simple extraction procedure were used for the quantification of sterols and dihydroxy triterpenes by HPLC-APCI-MS and samples obtained by the official procedure extraction for the quantification by GC-FID. The

presence of erythrodiol, uvaol, cholesterol, Δ5-avenasterol, stigmasterol, β-sitosterol and sitostanol was confirmed by comparing their mass spectra and retention times with the data obtained from the standard compounds. Peak identification was also performed with spiked real samples at different concentration levels. For the identification of the other sterols, the analyses carried out by GC-FID on each of the oil samples in question were took into account. The different oils studied contained specific quantities of some of the sterols, as they were determined previously by GC-FID. Although the migration time of sterol by GC-FID is different than by HPLC-APCI-MS, the elution order is known; such results were helpful to identify them in the samples analyzed by HPLC. In other words, when the official method (GC-FID) is used, the sterols elution order result to be as follow: cholesterol, brassicasterol, 24-methylene cholesterol, campesterol, campestanol, stigmasterol, Δ7-campesterol, Δ5,23-stigmastadienol, chlerosterol, β-sistosterol, sitostanol, Δ5-avenasterol, Δ5,24-stigmastadienol, Δ7-stigmasterol, Δ7-avenasterol. This means all the mentioned compounds can be identified and quantified by GC-FID, using the integrated area and the external standards. Thus, these results and the relative concentrations of the different compounds can be used to identify those analytes in the HPLC profiles.

β-Sitosterol was the principal component in all the oils studied. Cholesterol was more abundant in palm and seed oils, while brassicasterol only appeared in seeds and olive-pomace oils (in minor quantities in the latter); campesterol, campestanol, stigmasterol and sitostanol were predominant in soy oil, and erythrodyol and uvaol were very abundant in olive pomace oil. The percentage of each component was determined in each sample of oil analyzed, in order to assign the corresponding component to each peak in the chromatogram obtained by using HPLC-APCI-MS.

The spectra were characterized by the protonated molecules of the analytes and an abundant signal corresponding to fragment ions due to the loss of a water molecule (see **Table 5.1.**).

5.4.4. ANALYTICAL PARAMETERS

The detection limits ($DL = 3S_0/b$) and quantification limits ($QL = 10S_0/b$) of the method were tested against erythrodiol, uvaol, cholesterol, Δ5-avenasterol, stigmasterol, β-sitosterol and sitostanol using the IUPAC method (36), where S_0 is the standard deviation of the blank using as the blank the signal to- noise (S/N) ratio and b is the slope of the calibration plot.

All the calibration curves showed good linearity: Cholesterol, stigmasterol and sitostanol were linear from QL (0.25) to 20 mg/L, β-sitosterol from QL (0.15) to 200 mg/L, Δ5-avenasterol from QL (0.25) to 50 mg/L, and erythrodiol and uvaol from QL (0.50) to 200 mg/L. Each point of the calibration plot was repeated three times in an independent solution prepared in the same way. The calibration plots indicate good correlation between peak areas and analyte concentrations. The regression coefficients (r^2) were 0.999 for all the compounds quantified.

The repeatability of the method was checked by analyzing the same sample seven times in one day and five times on different days. The intraday relative standard deviation (RSD) of the retention times for uvaol, erythrodiol, stigmasterol and β-sitosterol was around 1% and the RSD of the areas for the same compounds was around 2%. The interday RSD of the retention times and areas was around 1.5 and 4.0%, respectively, for the same compounds.

5.4.5. APPLICATION AND QUANTIFICATION TO REAL SAMPLES

Using the described extraction protocol and the HPLC-APCI-MS method, six different oil samples (soybean oil, palm oil, seed oil, sunflower oil, olive-pomace oil and virgin olive oil) were analyzed. All samples were injected into the HPLC instrument five times (n = 5).

Sample preparations of all oils were made as described in **Section 5.4.**. The analyses were carried out under the optimum conditions described in **Section 5.4.2.,** and quantification was carried out using the extracted ion chromatogram at 369, 395, 397, 399 and 425 m/z. Erythrodiol, uvaol, cholesterol, Δ5-avenasterol, stigmasterol, β-sitosterol and sitostanol were quantified individually in mg/kg (**Table 5.2.**), while erythrodiol and uvaol were quantified together because they eluted at the same time and provided the same m/z in MS.

The chromatograms of six samples of different oils are shown in **figure 5.3.**. The main peaks in the chromatograms can be observed within the retention time range between 20 and 35 min and belong to Δ7-avenasterol, cholesterol, Δ5-avenasterol, Δ7-campesterol, campestanol, clerosterol, stigmasterol, Δ7-stigmastenol, Δ5,24-stigmastadienol and β-sitosterol.

β-Sitosterol was the most abundant sterol in all the oils. 24-Methylene cholesterol was only present in soybean and palm oil. Brassicasterol was only present in seed and olive pomace oil. High quantities of erythrodiol and uvaol were also found in olive pomace oil. This kind of oil is frequently mixed with virgin olive oil and marketed and sold as olive oil.

Regarding this last point, it is possible to say that our method could be capable of detecting fraudulent mixtures of virgin olive oils and olive pomace oils, both

quantitatively and qualitatively, analyzing the data of dihydroxy triterpenes in a few minutes, while the official method of the International Olive Oil Council takes more than 20 min to achieve the same purpose.

5.4.6. COMPARATIVE STUDY

To assure our results are reliable, all the samples were also analyzed with the official method. The operating conditions were as follows: column temperature, 260 ±5°C; injector temperature, 280°C; detector temperature, 290°C; linear velocity of the carrier gas: helium 20 cm/s, hydrogen 30 cm/s; splitting ratio of 1 : 75; amount of substance injected, 1 ml of TMSE solution.

The results are shown in **Table 5.2.** as individual sterol concentrations in mg/kg of fatty material. A comparison of such a data, reveals that the results obtained with the proposed method are in good agreement with those obtained with the official method. Erythrodiol and uvaol can be quantified in all oils except in palm oil with the proposed method, while they can only be quantified in olive pomace and virgin olive oils with the official method. However, sitostanol can be quantified in all the oils analyzed with the official method, but only in soybean, olive pomace and virgin olive oils with the proposed new method.

5.5. CONCLUSIONS

This study describes the use of HPLC-APCI-MS to identify efficiently 17 compounds and to quantify seven of them that are legislated upon by several regulations and

trademarks laid down by the International Olive Oil Council and the European Union. The advantages of the proposed method are:

1) simplicity in the preparation of sample, 2) robustness: good repeatability taking into account retention time and areas, and 3) cheaper process of the samples compared to the official method. The results found with the proposed method for the analytes studied are in good agreement with the obtained data using the official method.

TABLES

Table 5.1. – *Ions observed in the APCI mass spectra of sterols in positive mode and their retention times.*

Analytes	Ion $[M+H-H_2O]^+$	Retention time [min]
Erythrodiol	425	3.72
Uvaol	425	3.72
Δ5,23-Stigmastadienol	395	17.21
24-Methylene cholesterol	381	17.21
Brassicasterol	381	19.90
Δ7-Avenasterol	395	19.90
Cholesterol	369	21.13
Δ5-Avenasterol	395	21.13
Δ7-Campesterol	383	23.87
Clerosterol	395	25.44
Campesterol	383	25.50
Campestanol	385	25.50
Stigmasterol	395	26.95
Δ7-Stigmastenol	397	29.81
Δ5,24-Stigmastadienol	395	29.81
β-Sitosterol	397	31.82
Sitostanol	399	39.60

Table 5.2. – *Concentrations of sterols, expressed as mg/kg of the different vegetable oils quantified by HPLC-APCI-MS and the official GCFID method; (¤Value = \bar X¤ ± SD)*

Analytes	Method	Different oils					
		Soybean	Palm	Seed	Sunflower	Olive-pomace	Virgin olive
Erythrodiol + uvaol	HPLC-APCI-MS	8.5 ± 0.5	nd	16.2 ± 1.1	12.8 ± 0.5	509.9 ± 15.3	18.9 ± 1.4
	GC-FID	nd	nd	nd	nd	518.0 ± 17.2	19.9 ± 1.2
Δ5-Avenasterol	HPLC-APCI-MS	56.0 ± 5.5	8.9 ± 0.7	255.7 ± 15.3	93.1 ± 2.5	41.5 ± 1.8	154.9 ± 9.0
	GC-FID	51.9 ± 3.0	9.3 ± 0.8	225.6 ± 15.3	92.7 ± 1.9	40.8 ± 0.8	156.8 ± 5.0
Cholesterol	HPLC-APCI-MS	7.7 ± 1.5	20.1 ± 2.5	23.1 ± 1.9	3.3 ± 0.4	3.5 ± 0.6	2.2 ± 0.3
	GC-FID	10.0 ± 0.5	21.3 ± 2.0	21.5 ± 2.3	3.1 ± 0.4	3.2 ± 0.2	2.0 ± 0.1
Stigmasterol	HPLC-APCI-MS	575.3 ± 18.0	74.9 ± 3.5	102.4 ± 7.5	14.3 ± 0.7	26.3 ± 1.8	8.5 ± 0.4
	GC-FID	577.3 ± 25.1	76.2 ± 5.5	105.4 ± 6.5	13.8 ± 0.5	27.2 ± 1.7	8.7 ± 0.2
β-Sitosterol	HPLC-APCI-MS	1593.2 ± 80.3	338.6 ± 10.0	2380.3 ± 105.5	1816.6 ± 147.5	2125.6 ± 180.3	985.9 ± 58.3
	GC-FID	1604.2 ± 72.5	340.1 ± 8.5	2230.7 ± 125.2	1735.1 ± 125.2	2272.4 ± 160.5	1003.8 ± 55.2
Sitostanol	HPLC-APCI-MS	50.5 ± 2.5	nd	nd	nd	46.5 ± 3.9	9.0 ± 0.9
	GC-FID	52.9 ± 3.3	2.8 ± 0.2	18.7 ± 0.5	16.5 ± 0.7	48.8 ± 2.6	9.7 ± 0.8

FIGURES

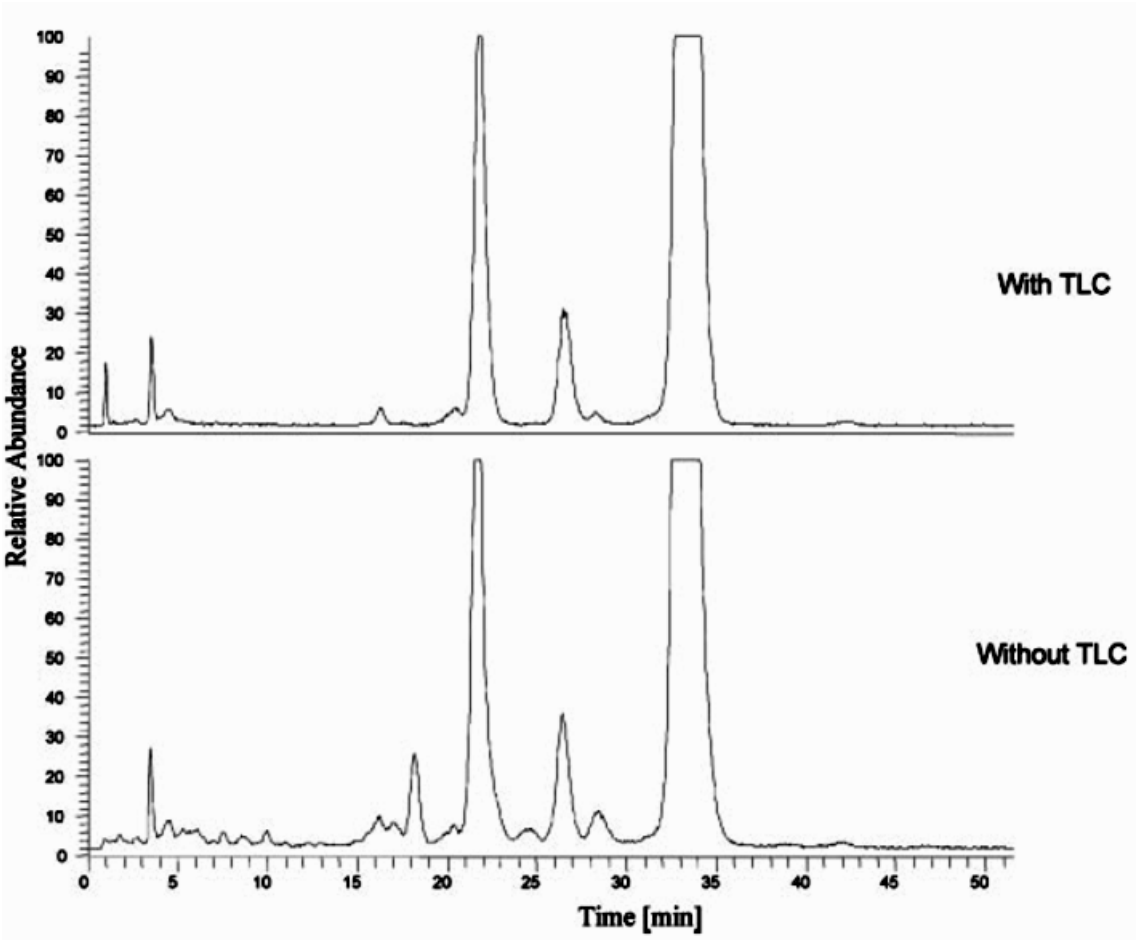

Figure 5.1. - *Chromatogram of two extracts of virgin olive oil using isolation of total sterols both with and without TLC.*

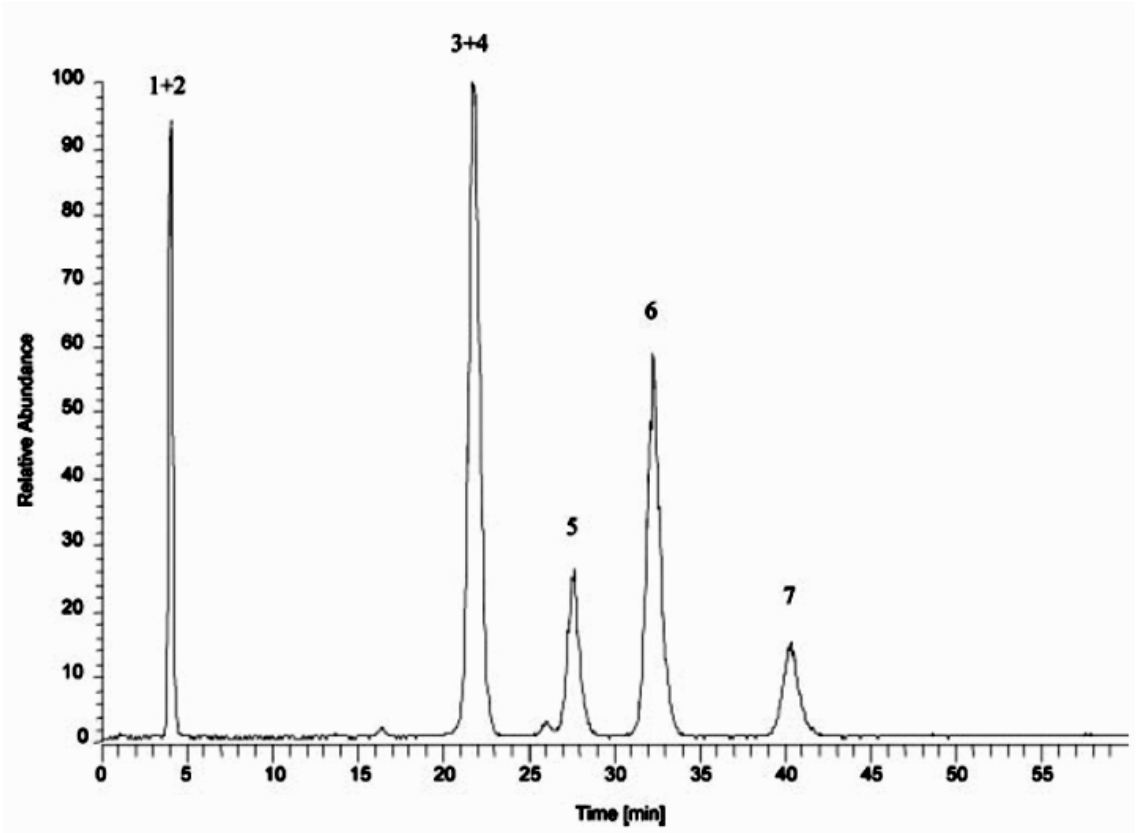

Figure 5.2. – *HPLC-APCI-MS chromatogram of a standard solution of five sterols and two triterpenic alcohols. Gradient from 90% acetonitrile/10% water + 0.01% AcOH to 92% acetonitrile/8% water + 0.01% AcOH from 0 to 60 min. Peaks: (1+2) erythrodiol and uvaol, (3+4) cholesterol and Δ5-avenasterol, (5) stigmasterol, (6) β-sitosterol, (7) sitostanol.*

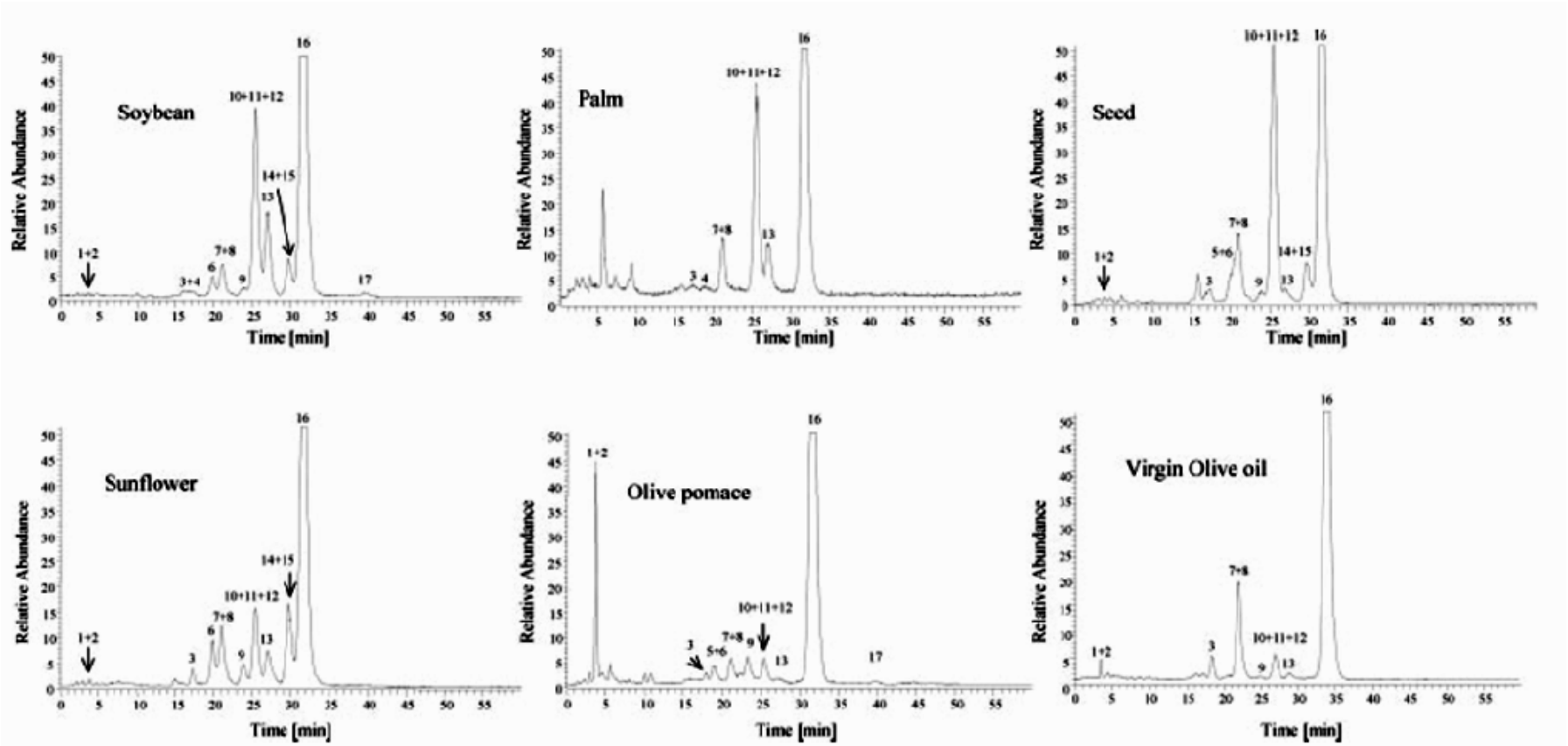

Figure 5.3. - *Chromatograms of six different vegetable oils. (1) Erythrodiol, (2) uvaol, (3) Δ5,23-stigmastadienol, (4) 24-methylene cholesterol, (5) brassicasterol, (6) Δ7-avenasterol, (7) cholesterol, (8) Δ5-avenasterol, (9) Δ7-campesterol, (10) clerosterol, (11) campesterol, (12) campestanol, (13) stigmasterol, (14) Δ7-stigmastenol, (15) Δ5,24-stigmastadienol, (16) β-sitosterol and (17) sitostanol.*

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6

QUANTITATION OF LONG CHAIN POLY-UNSATURED FATTY ACIDS (LC-PUFA) IN BASE INFANT FORMULAE BY GAS CHROMATOGRAPHY, AND EVALUATION OF THE BLENDING PHASES ACCURACY DURING THEIR PREPARATION

6.1. SUMMARY AND KEYWORDS

The purpose of this investigation has been the quantitation of Long Chain Poly-unsatured Fatty Acids (LC-PUFA), such as arachidonic acid (ARA , C20:4 n-6) and docosahexaenoic acid (DHA , C22:6 n3), and the evaluation of their stability during the blending phases for the preparation of base infant formula added with ARA and DHA less than 0,5%. The investigation has been also oriented to set up a method that can permit to reduce the uncertainty values associated with the mesurement of ARA and DHA, to a compatible level with the their certain determination in the samples examinated.

Keywords: Infant Formula, LC-PUFA, ARA, DHA.

6.2. INTRODUCTION

Infant milk formulae are designed to provide infants with the required nutrients for optimal growth and development. They are formulated to mimic breast milk composition in order to obtain an ‘‘ideal’’ substitute (infant formula) for babies. Human breast milk contains a full complement of all polyunsaturated fatty acids (PUFA), including the two essential PUFA, linoleic acid (LA,18:2 n-6) and α-linolenic acid, (ALA, 18:3 n-3) and also a range of LC-PUFA that have been shown to have benefits for both preterm and term infants, such as promoting sensory and neuronal maturation. LC-PUFA are present mainly in highly specialized membranes, e.g., retina and synapses, and in membranes of excitable cells. Moreover, like DHA, ARA is an essential fatty acid for adequate function of the central nervous system (1).

New research has shown that both preterm and term infants can actively convert the essential fatty acids LA and ALA, to LC-PUFA (2, 3, 4). However, the amount of LC-PUFA being produced, particularly of DHA, may not be sufficient to meet the developmental requirement of the infant. An important question that is still challenging investigators is whether formula fed term infants can make enough LCPUFA from the essential fatty acids that are provided in formula or whether they require added LC-PUFA. Research is ongoing and there is no final answer to this question, although there is some evidence that a supply of LC-PUFA to the infant may be beneficial.

Most national and international authorities have based their recommendations for the manufacturing of infant formulae on mature human milk fatty acids composition as the gold standard. The Commission of the European Union issued regulations on infant formula composition, including permission for addition of LCPUFA up to 1 and 2%, respectively, of total fat content as n-3 and n-6 LC-PUFA (5).

Infant formulae are usually marketed as spray-dried powders to be reconstituted with water, or ready-to-feed infant infant formulae. Their manufacture may differ from producers, but it generally consists in a dry blending process. It begins with the receipt of the ingredients, and stored until they are tested for conformance to specifications. Then, dry ingredients are blended in large batches in a ribbon blender or other large scale blending equipment. The ingredients are blended until the are uniformly distributed throughout the batch. Later the product is passed through a sifter, and then transferred to bags for storage, or directly to the packaging line.

Therefore, manufacturers consider of extremely importance to verify the proper distribution and the stability of some ingredients in the infant formula during the blending, since may be present in low numbers and may be non-randomly distributed within the lot. In this particular case, the object of the investigation is a base infant formula added with ARA and DHA less than 0,5% of total fat, with the purpose to set up a method of analysis that permits to reduce the uncertainty values associated with the measurement of these fatty acids, to a compatible level with the certain determination of such LC-PUFA in the samples examined. Such a requirement rises from the limits in the application of the methods of analysis UNI EN ISO 5508 (1998) and UNI EN ISO 5509 (2000), which do not allow to fully verify the effective content of ARA and DHA in the product, because they determine high uncertainty values for the typology of the samples under investigation, and because ARA and DHA are present in low concentration in the formulation.

6.3. MATERIALS AND METHODS

6.3.1. SAMPLES COLLECTION

In order to carry out the investigation, the following samples of base infant formulae were collected. Each sample was stored at -20°C until analysis:

- 10 powder samples (450 g each one) corresponding to the "inter-blend phase", and collected at the end of each blend phase (**Table 6.1.)**.
- 3 powder samples (450 g each one) corresponding to the "intra-blend phase", and collected at the beginning/half/end of 5° blend phase (**Table 6.2.)**.
- 11 powder samples (900 g each one) collected at the end of each blend and then packaged. They correspond to the commercial products (**Table 6.3.)**.
- 2 powder samples containing the raw material employed for the addiction of ARA and DHA.

6.3.2. SAMPLES PREPARATION

Before the extraction, base infant formulae were reconstituted following the specifications reported in the label. In this case, 4,3 g of powder were weighted and dissolved in 30 ml of water, obtaining a concentration of 0,14 mg/ml. Later, the fat was extracted in double following the Rose-Gottlieb official method (6), and then, the preparation of the methyl esters was carried out applying the method of Christopherson and Glass (7): about 20 mg of dry fat was put in a test tube, 50 µl of KOH MeOH 2N and 500 µl of hexane were added respectively, and then the tube was mixed for 30 seconds; 0,5 mg of C13:0 (Sigma-Aldrich, St. Louis, MO, USA) were used for the fatty acids quantitation as internal standard, while the 53 FAME GLC-reference standard mix-463 (NU-CHEK-PREP, Elysian, MN) for the fatty acids identification. After the

phase separation has been acted, 300 µl of surnatant was collected and put in a vial. Analysis by gas chromatography has been followed.

6.3.2. GAS CHROMATOGRAPHIC QUANTITATION

FAME were analyzed with a Perkin Elmer Clarus 500 gas chromatograph equipped with a RTX 2330 (Restek, Bellefonte, PA, USA) fused silica capillary column (30 m x0.25 mm i.d., 0.2-µm film thickness), a FID detector, and a split injector. Helium was used as carrier at the constant flow rate of 0.75 min/min. The FID detector was maintained at 240°C with air flow rate of 400 ml/min, hydrogen flow rate of 40 ml/min, and helium (make up gas) flow rate of 30 ml/min. The split injector was maintained at 240°C with the split ratio of 1:50. The temperature program was as follow: 1 minute at 60°C, ramp 7°C/min to 240°C and maintained 10 minutes.

6.3.3. STATISTYCAL ANALYSIS

Calculation and statistics were performed with Statistica for windows (1998), and Microsoft excel for windows (2003).

6.4. RESULTS AND DISCUSSION

The investigation has been focalized on the deepening of the following steps:

a. Characterization of the raw materials.
b. Set up of the method.
c. Fatty acid analysis of the intra-blend samples.
d. Fatty acid analysis of the inter-blend samples.
e. Fatty acid analysis of the commercial products.

a. The characterization of the raw materials has permitted to optimize the condition for the fatty acids separation by gas chromatography, necessary to their identification and quantification in the complex mixture.

b. A deep verification of the method of analysis has been carried out in order to verify the steps and the manipulations requested, starting from the matrix under study. In this case, the matrix was base infant formula, and the lipidic fraction has been extracted with solvent following the Rose-Gottlieb official method. This method, which can be also used on liquid milk, takes place in basic condition with the purpose to break the milk fat globule membrane. In milk powder the fat is not completely structured such as in non-modified milk, even though it must be stable in the liquid product when reconstituted. However, the extraction must be conducted considering the residual globules with membrane, which are more problematic to break with solvent due to the smaller size and the higher ratio area/volume of the globules. Therefore, the repetibily of the method has been verified by identifying and quantifying the fatty acids in one of the commercial samples for five times, and the results are reported on **table 6.4.**. The quantification has been carried out on the principal fatty acids, besides the fatty acids added to the powder for the formulation. Other minor fatty acids have been grouped under "others". On **figure 6.1.** are reported the chromatograms relative to the samples of raw materials employed for the addiction of ARA and DHA, and a sample from the inter-blend. The verification of the method have permitted to calculate the limit of detection (LOD) and the limit of quantitation (LOQ), that are respectively of 20 and 60 ppm. The error, relatives to the fatty acids under investigation, have been calculated on the commercial samples

according with the equation of William Horwitz (8, 9) that estimate the uncertainty of measure (**Table 6.5.)**. The equation is:

$$\%RSD_R=2^{(1-0.5\log C)}$$

where $\%RSD_R$ is the inter-laboratory Coefficient of Variation, and C is the concentration of analyte in the sample as a decimal fraction.

c. 3 powder samples corresponding to the "intra-blend phase" and collected at the beginning/half/end of 5° blend phase, were analyzed in double by gas chromatography. In **table 6.6.** are reported the mean, the standard deviation, and the variance of the fatty acids identified and quantified in the samples. The fatty acids of interest are underlined in grey, while fatty acids quantified as less than 0,1g/100g are classified as "others" at the bottom of the table. The variance analysis of the fatty acids of interest is reproduced on **figure 6.2.**.

d. **Table 6.7.** shows the results of the analysis by gas chromatography of the inter-blend samples carried out in double. The mean, the standard deviation, and the variance of the fatty acids identified and quantified in the samples have been calculated. The fatty acids of interest are underlined in grey. Moreover, in **figure 6.3.** is reported the variance analysis of the fatty acids under investigation. Fatty acids quantified as less than 0,1g/100g are classified as "others" at the bottom of the table.

e. The fatty acid analysis of the 11 commercial samples carried out in double, is showed in **table 6.8.**. Fatty acids of interest are underlined in grey, while those quantified as less than 0,1g/100g are classified as "others" at the bottom of the table. The results include the mean, the standard deviation, and the variance of the fatty acids identified and quantified in the samples, while in **figure 6.4.** is reported the variance analysis of the fatty acids under investigation.

6.5. CONCLUSIONS

The investigation, oriented to set up a method that can permit to reduce the uncertainty values associated with the measurement of ARA and DHA and to verify the effective content of these fatty acids in base infant formulae, have permitted to achieve the following goals:

First of all, the elaboration of the uncertainty values associated with the measurement of ARA and DHA and the other fatty acids under study (**Table 6.5.**) has been obtained from the analysis by gas chromatography of a commercial sample. The estimation of the reproducibility calculated with the equation of William Horwitz can provide the assessment of the uncertainty value, which can be verified through the comparison with the single measurement of the fatty acids.

Moreover, as reported in **table 6.4.**, the method of analysis shows a good repeatability when considering the mean, the standard deviation and the variance of the fatty acids.

Intra-blend samples, collected at the beginning/half/end of 5° blend phase (**Table 6.6., Figure 6.2.**), present values absolutely satisfactory; the variance and the standard deviation appear low, and the mean values are by far less than the error of the method (**Table 6.5.**).

Same consideration can be made for the samples corresponding to the inter-blend (**Table 6.7., Figure 6.3.**), even though the standard deviation and the variance result to be higher, that is may due to the different lot of production.

On **table 6.8.** and **figure 6.4.** are reported the results about the commercial samples. They show to be more homogeneous than the inter-blend samples, probably because the latter represent an intermediate step in the production; as it can see in **table 6.9.**, the percent standard deviation results to be less than 10% for all the fatty acids considered.

A statistical comparison between intra-blend samples and commercial samples is reported in **table 6.10.**, where their principal parameters are compared, in particular the student's t-test that permits to describe the estimation of the data's similarity. It rise that with a level of test significative of 95%, the differences between the mean values observed are not statistically significatives with $p<0.05$, indicating how the two groups are non-different. **Figure 6.5. (a to i)** graphically shows the comparison of the considered parameters in **table 6.10.**, about the total lipids extracted and mono-, di-, poli-unsatured fatty acids. The same figure report the mean, the standard error, and a 95% confidence interval.

TABLES

Table 6.1. – *Powder samples corresponding to the inter-blend phase.*

Number of blends	Name on the can streaker	Meaning
1	**EB1, BB2**	End of 1° Blend
2	**EB2 BB4**	End of 2° Blend
3	**EB3, BB6**	End of 3° Blend
4	**EB4, BB8**	End of 4° Blend
5	**EB5, BB10**	End of 5° Blend
6	**EB6, BB12**	End of 6° Blend
7	**EB7, BB14**	End of 7° Blend
8	**EB8, BB16**	End of 8° Blend
9	**EB9, BB18**	End of 9° Blend
10	**EB10, BB20**	End of 10° Blend

Table 6.2. – *Powder samples corresponding to the intra-blend phase (5° Blend).*

Number of blends	Name on the can streaker	Meaning
5	**BB5, BB9**	Beginning
5	**MB5, BB9**	Half
5	**EB5, BB10**	**End**

Table 6.3. – *Powder samples corresponding to the commercial products.*

Number of blends	Name on the can streaker	Meaning
2	FP-BB3 / FP-BB4	Obtained from 2° Blend
3	FP-BB6	Obtained from 3° Blend
4	FP-BB8	Obtained from 4° Blend
5	**FP-BB10 / FP-BB9**	Obtained from 5° Blend
6	FP-BB12	Obtained from 6° Blend
7	FP-BB14	Obtained from 7° Blend
8	FP-BB16	Obtained from 8° Blend
9	FP-BB18	Obtained from 9° Blend
10	FP-BB20	Obtained from 10° Blend

Table 6.4. – *Repeatability of the method of analysis.*

(g/100g of powder)	*AG19_2-1*	*AG19_2-2*	*AG19_2-3*	*AG19_2-4*	*AG19_2-5*	*mean*		*SD*	*VAR*
FAT	***22.81***	***25.00***	***24.91***	***25.64***	***22.86***	***24.24***	±	***1.32***	1.73
Satured	***7.93***	***8.43***	***8.26***	***8.72***	***7.76***	***8.22***	±	***0.38***	*0.15*
C4:0	0.27	0.32	0.30	0.32	0.28	0.30	±	0.02	0.00
C6:0	0.20	0.23	0.21	0.23	0.20	0.21	±	0.01	0.00
C8:0	0.14	0.00	0.14	0.15	0.13	0.11	±	0.06	0.00
C10:0	0.30	0.31	0.29	0.31	0.27	0.30	±	0.02	0.00
C11:0	0.01	0.01	0.01	0.01	0.01	0.01	±	0.00	0.00
C12:0	0.36	0.36	0.34	0.36	0.32	0.34	±	0.02	0.00
C14:0	1.07	1.10	1.06	1.12	1.00	1.07	±	0.05	0.00
C15:0	0.10	0.11	0.11	0.11	0.10	0.11	±	0.00	0.00
C16:0	3.86	4.22	4.08	4.29	3.83	4.06	±	0.21	0.04
C17:0	0.07	0.08	0.08	0.07	0.07	0.08	±	0.00	0.00
C18:0	1.30	1.44	1.42	1.49	1.32	1.39	±	0.08	0.01
C20:0	0.09	0.10	0.10	0.10	0.09	0.10	±	0.01	0.00
C22:0	0.08	0.08	0.08	0.09	0.08	0.08	±	0.00	0.00
C23:0	0.01	0.01	0.01	0.01	0.01	0.01	±	0.00	0.00
C24:0	0.06	0.05	0.05	0.06	0.05	0.06	±	0.00	0.00
Mono-unsatured	***9.93***	***11.08***	***11.28***	***11.30***	***10.09***	***10.74***	±	***0.67***	***0.45***
C14:1	0.15	0.15	0.14	0.15	0.14	0.15	±	0.01	0.00
C16:1c	0.24	0.26	0.25	0.27	0.24	0.25	±	0.01	0.00
C17:1	0.05	0.05	0.05	0.04	0.05	0.05	±	0.01	0.00
C18:1 c 9	9.00	10.08	9.86	10.31	9.18	9.68	±	0.57	0.32
C18:1 c 11	0.28	0.30	0.30	0.32	0.28	0.30	±	0.02	0.00
C18:1 c 12	0.02	0.02	0.02	0.03	0.02	0.02	±	0.00	0.00
C18:1 t16-c14	0.05	0.05	0.50	0.03	0.05	0.13	±	0.20	0.04
C20:1 n9	0.10	0.10	0.10	0.11	0.10	0.10	±	0.01	0.00
C22:1	0.03	0.04	0.04	0.04	0.03	0.03	±	0.00	0.00
C24:1	0.02	0.02	0.02	0.02	0.02	0.02	±	0.00	0.00
Poli-unsatured	***4.94***	***5.50***	***5.37***	***5.62***	***5.01***	***5.29***	±	***0.30***	***0.09***
C18:2 9t 12t	0.05	0.06	0.04	0.04	0.04	0.05	±	0.01	0.00
C18:2 C/t	0.03	0.03	0.03	0.03	0.02	0.03	±	0.00	0.00
C18:2 n6	4.18	4.66	4.57	4.78	4.25	4.49	±	0.26	0.07
C18:3 n6	0.01	0.01	0.01	0.01	0.01	0.01	±	0.00	0.00
C18:3 n3	0.45	0.49	0.49	0.51	0.45	0.48	±	0.03	0.00
C20:2	0.01	0.01	0.01	0.01	0.01	0.01	±	0.00	0.00
C20:3 n6	0.02	0.02	0.02	0.02	0.02	0.02	±	0.00	0.00
C20:4 n6 (ARA)	0.10	0.11	0.12	0.12	0.11	0.11	±	0.01	0.00
C20:5 n3 (EPA)	0.02	0.03	0.02	0.03	0.02	0.02	±	0.00	0.00
C22:6 n3 (DHA)	0.08	0.08	0.08	0.08	0.08	0.08	±	0.00	0.00

Table 6.5. – *Error of the method, relatives to the fatty acids under investigation, and estimated on the commercial samples.*

Fatty acids	*Error of the method (g/100g of powder)*
C18:1 c9	4.04
C18:2 n6	1.88
C18:3 n3	0.20
C20:4 n6 (ARA)	0.04
C22:6 n3 (DHA)	0.03

Table 6.6. – *Fatty acids composition (g/100 g of powder) of the 3 intra-blend samples.*

(g/100g of powder)	*BB5 BB9*	*MB5 BB9*	*EB5 BB10*	*mean*		*SD*	*VAR*
FAT	***22.17***	***23.20***	***22.52***	***22.63***	±	***0.52***	***0.27***
Satured	***7.35***	***7.96***	***7.71***	***7.68***	±	***0.30***	***0.09***
C4:0	0.28	0.31	0.29	0.29	±	0.01	0.00
C6:0	0.21	0.23	0.22	0.22	±	0.01	0.00
C8:0	0.13	0.15	0.15	0.14	±	0.01	0.00
C10:0	0.26	0.31	0.29	0.29	±	0.02	0.00
C12:0	0.30	0.33	0.32	0.32	±	0.02	0.00
C14:0	0.94	1.00	0.98	0.97	±	0.04	0.00
C15:0	0.09	0.10	0.10	0.10	±	0.00	0.00
C16:0	3.74	3.85	3.76	3.78	±	0.06	0.00
C18:0	1.12	1.35	1.31	1.26	±	0.12	0.02
Mono-unsatured	***9.82***	***10.07***	***9.79***	***9.89***	±	***0.15***	***0.02***
C14:1	0.13	0.14	0.14	0.13	±	0.00	0.00
C16:1c	0.23	0.24	0.24	0.24	±	0.00	0.00
C18:1 c 9	9.11	9.25	9.00	9.12	±	0.12	0.02
C18:1 c 11	0.14	0.23	0.22	0.20	±	0.05	0.00
C20:1 n9	0.10	0.10	0.10	0.10	±	0.00	0.00
Poli-unsatured	***5.00***	***5.17***	***5.01***	***5.06***	±	***0.10***	***0.01***
C18:2 n6	4.20	4.33	4.21	4.24	±	0.07	0.00
C18:3 n3	0.46	0.47	0.45	0.46	±	0.01	0.00
C20:4 n6 (ARA)	0.11	0.12	0.10	0.11	±	0.01	0.00
C22:6 n3 (DHA)	0.06	0.07	0.06	0.07	±	0.00	0.00
Others*				0.58			

Others* < 0.1 g/100g (C11:0; C17:0; C17:1 c; C18:1 c 12; C18:1 t16-c14; C18:2 c/t; C18:2 9t12t; C18:2 c9, t11 (CLA) C18:3 n6; C18:3t; C20:0; C20:2; C20:3 n6; C20:5 n3 (EPA); C21:0; C22:0; C22:1; C24:1; C23:0; C24:0)

Table 6.7. – *Fatty acids composition (g/100 g of powder) of the 10 inter-blend samples.*

(g/100g of powder)	*EB1 BB2*	*EB2 BB4*	*EB3 BB6*	*EB4 BB8*	*EB5 BB10*	*EB6 BB12*	*EB7 BB14*	*EB8 BB16*	*EB9 BB18*	*EB10 BB20*	*mean*		*SD*	*VAR*
FAT	***23.89***	***24.91***	***21.46***	***23.2***	***22.52***	***23.56***	***25.62***	***27.71***	***24.92***	***24.92***	***22.06***	±	***1.76***	***3.08***
Satured	***7.88***	***8.40***	***7.48***	***8.13***	***7.71***	***8.02***	***8.62***	***9.39***	***8.40***	***8.40***	***7.49***	±	***0.54***	***0.29***
C4:0	0.29	0.31	0.31	0.35	0.29	0.31	0.32	0.31	0.30	0.30	0.28	±	0.02	0.00
C6:0	0.23	0.24	0.23	0.26	0.22	0.23	0.25	0.26	0.24	0.24	0.22	±	0.01	0.00
C8:0	0.15	0.16	0.15	0.17	0.15	0.15	0.16	0.18	0.16	0.16	0.14	±	0.01	0.00
C10:0	0.30	0.32	0.31	0.36	0.29	0.31	0.32	0.35	0.32	0.32	0.29	±	0.02	0.00
C12:0	0.31	0.35	0.34	0.38	0.32	0.33	0.36	0.39	0.35	0.35	0.32	±	0.02	0.00
C14:0	0.97	1.05	0.97	1.07	0.98	1.00	1.08	1.18	1.06	1.06	0.95	±	0.07	0.00
C16:0	3.68	4.05	3.52	3.79	3.76	3.87	4.25	4.60	4.10	4.10	3.61	±	0.31	0.10
C18:0	1.53	1.45	1.22	1.30	1.31	1.37	1.50	1.63	1.45	1.45	1.29	±	0.12	0.01
Mono-unsatured	***10.45***	***10.92***	***9.28***	***10.01***	***9.79***	***10.29***	***11.24***	***12.12***	***10.96***	***10.96***	***9.64***	±	***0.81***	***0.66***
C14:1	0.13	0.14	0.13	0.15	0.14	0.14	0.15	0.16	0.15	0.15	0.13	±	0.01	0.00
C16:1c	0.21	0.25	0.22	0.24	0.24	0.24	0.25	0.29	0.26	0.26	0.22	±	0.02	0.00
C18:1 c 9	9.69	10.06	8.43	9.02	9.00	9.46	10.36	11.16	10.05	10.05	8.84	±	0.79	0.62
C18:1 c 11	0.20	0.23	0.28	0.32	0.22	0.23	0.24	0.25	0.26	0.26	0.23	±	0.03	0.00
Poli-unsatured	***5.56***	***5.58***	***4.70***	***5.05***	***5.01***	***5.25***	***5.76***	***6.20***	***5.57***	***5.57***	***4.93***	±	***0.43***	***0.19***
C18:2 n6	4.72	4.70	3.94	4.24	4.21	4.39	4.84	5.19	4.66	4.66	4.14	±	0.36	0.13
C18:3 n3	0.47	0.50	0.42	0.46	0.45	0.47	0.52	0.56	0.50	0.50	0.44	±	0.04	0.00
C20:4 n6 (ARA)	0.11	0.11	0.10	0.11	0.10	0.12	0.12	0.13	0.12	0.12	0.10	±	0.01	0.00
C22:6 n3 (DHA)	0.05	0.07	0.07	0.07	0.06	0.07	0.07	0.08	0.07	0.07	0.06	±	0.01	0.00
Others*											0.79			

Others* < 0.1 g/100g (C11:0; C15:0; C17:0; C17:1 c; C18:1 c 12; C18:1 t16-c14; C18:2 c/t; C18:2 9t12t; C18:2 c9, t11 (CLA) C18:3 n6; C18:3t; C20:0; C20:1 n9; C20:2; C20:3 n6; C20:5 n3 (EPA); C21:0; C22:0; C22:1; C24:1; C23:0; C24:0)

Table 6.8. – *Fatty acids composition (g/100 g of powder) of the 11 commercial samples.*

(g/100g of powder)	*FP BB3*	*FP BB4*	*FPBB6*	*FP BB8*	*FP BB9*	*FP BB10*	*FP BB12*	*FP BB14*	*FP BB16*	*FP BB18*	*FP BB20*	*mean*		*SD*	*VAR*
FAT	***23.16***	***23.88***	***21.80***	***24.19***	***25.26***	***22.15***	***24.60***	***22.15***	***26.33***	***25.28***	***25.28***	***24.00***	±	***1.52***	***2.31***
Satured	***7.74***	***8.01***	***7.48***	***8.41***	***8.56***	***7.51***	***8.47***	***7.52***	***8.94***	***8.55***	***8.55***	***8.16***	±	***0.52***	***0.27***
C4:0	0.29	0.27	0.30	0.34	0.31	0.28	0.33	0.28	0.32	0.31	0.31	0.31	±	0.02	0.00
C6:0	0.22	0.20	0.23	0.26	0.23	0.21	0.25	0.21	0.25	0.24	0.24	0.23	±	0.02	0.00
C8:0	0.14	0.13	0.15	0.17	0.15	0.14	0.17	0.14	0.16	0.16	0.16	0.15	±	0.01	0.00
C10:0	0.28	0.27	0.30	0.35	0.31	0.28	0.34	0.27	0.33	0.32	0.32	0.31	±	0.03	0.00
C12:0	0.31	0.31	0.33	0.39	0.35	0.31	0.37	0.30	0.37	0.35	0.35	0.34	±	0.03	0.00
C14:0	0.97	1.00	0.95	1.09	1.08	0.94	1.08	0.94	1.12	1.07	1.07	1.03	±	0.07	0.00
C15:0	0.10	0.10	0.09	0.10	0.11	0.09	0.11	0.09	0.11	0.11	0.11	0.10	±	0.01	0.00
C16:0	3.84	4.00	3.56	3.96	4.21	3.68	4.04	3.70	4.37	4.20	4.20	3.98	±	0.26	0.07
C18:0	1.30	1.41	1.26	1.38	1.49	1.31	1.42	1.29	1.55	1.49	1.49	1.40	±	0.10	0.01
Mono-unsatured	***10.21***	***10.52***	***9.50***	***10.49***	***11.07***	***9.72***	***10.71***	***9.72***	***11.53***	***11.10***	***11.10***	***10.52***	±	***0.67***	***0.44***
C14:1	0.13	0.14	0.13	0.15	0.15	0.13	0.15	0.13	0.15	0.15	0.15	0.14	±	0.01	0.00
C16:1c	0.23	0.24	0.22	0.25	0.25	0.23	0.25	0.23	0.27	0.25	0.25	0.24	±	0.01	0.00
C18:1 c 9	9.46	9.75	8.67	9.53	10.20	8.91	9.76	8.98	10.60	10.21	10.21	9.66	±	0.62	0.39
C18:1 c 11	0.18	0.18	0.28	0.32	0.23	0.24	0.30	0.17	0.26	0.26	0.26	0.24	±	0.05	0.00
C20:1 n9	0.10	0.10	0.10	0.11	0.11	0.09	0.11	0.09	0.12	0.11	0.11	0.10	±	0.01	0.00
Poli-unsatured	***5.21***	***5.35***	***4.81***	***5.30***	***5.63***	***4.92***	***5.41***	***4.91***	***5.86***	***5.63***	***5.63***	***5.33***	±	***0.34***	***0.12***
C18:2 n6	4.40	4.51	4.05	4.45	4.73	4.13	4.54	4.15	4.92	4.76	4.76	4.49	±	0.29	0.08
C18:3 n3	0.47	0.48	0.43	0.48	0.51	0.45	0.49	0.45	0.53	0.51	0.51	0.48	±	0.03	0.00
C20:4 n6 (ARA)	0.10	0.11	0.10	0.11	0.12	0.10	0.12	0.09	0.12	0.12	0.12	0.11	±	0.01	0.00
C22:6 n3 (DHA)	0.06	0.06	0.06	0.07	0.07	0.06	0.07	0.05	0.08	0.07	0.07	0.07	±	0.01	0.00
Others*												0.62			

Others* < 0.1 g/100g (C11:0; C17:0; C17:1 c; C18:1 c 12; C18:1 t16-c14; C18:2 c/t; C18:2 9t12t; C18:2 c9, t11 (CLA) C18:3 n6; C18:3t; C20:0; C20:2; C20:3 n6; C20:5 n3 (EPA); C21:0; C22:0; C22:1; C24:1; C23:0; C24:0)

Table 6.9. – *Comparison of the mean values of inter-blend and commercial samples*

mean (g/100g)	*inter-blend*	*Commercial sample*	*SD (%)*
FAT	*22.06*	*24.00*	*8.09*
Satured	*7.49*	*8.16*	*8.15*
C4:0	0.28	0.31	7.85
C6:0	0.22	0.23	5.10
C8:0	0.14	0.15	4.93
C10:0	0.29	0.31	5.06
C12:0	0.32	0.34	6.39
C14:0	0.95	1.03	7.94
C15:0	0.09	0.10	8.63
C16:0	3.61	3.98	9.16
C18:0	1.29	1.40	7.71
Mono-unsatured	*9.64*	*10.52*	*8.33*
C14:1	0.13	0.14	8.12
C16:1c	0.22	0.24	7.63
C18:1 c 9	8.84	9.66	8.48
C18:1 c 11	0.23	0.24	6.89
C20:1 n9	0.09	0.10	8.32
Poli-unsatured	*4.93*	*5.33*	*7.53*
C18:2 n6	4.14	4.49	7.76
C18:3 n3	0.44	0.48	8.19
C20:4 n6 (ARA)	0.10	0.11	4.71
C22:6 n3 (DHA)	0.06	0.07	6.24

Table 6.10. – *statistical comparison between intra blend and commercial sample*

	Intra blend	Commercial samples	t student value	df	p value	N Intrablend samples	N commercial samples	SD Intrablend	SD commercial samples	Variance ratio F	p Variances
FAT	24.27100	24.00727	0.369294	19	0.715992	10	11	1.753178	1.519665	1.330933	0.659612
Satured	8.24300	8.15818	0.366968	19	0.717698	10	11	0.536678	0.521974	1.057136	0.924410
Mono-unsatured	10.60200	10.51545	0.267785	19	0.791750	10	11	0.812866	0.666984	1.485277	0.545361
C18:1 c 9	9.72800	9.66182	0.215151	19	0.831942	10	11	0.785774	0.621302	1.599518	0.474376
Poli-unsatured	5.42500	5.33273	0.544885	19	0.592172	10	11	0.430278	0.344647	1.558647	0.498559
C18:2 n6	4.55500	4.49091	0.450074	19	0.657750	10	11	0.361855	0.289774	1.559370	0.498120
C18:3 n3	0.48500	0.48273	0.147377	19	0.884387	10	11	0.039511	0.031013	1.623083	0.461012
C20:4 n6 (ARA)	0.11400	0.11000	0.883504	19	0.388007	10	11	0.009661	0.010954	1.285714	0.715949
C22:6 n3 (DHA)	0.06800	0.06545	0.723261	19	0.478329	10	11	0.007888	0.008202	1.081169	0.916107

FIGURES

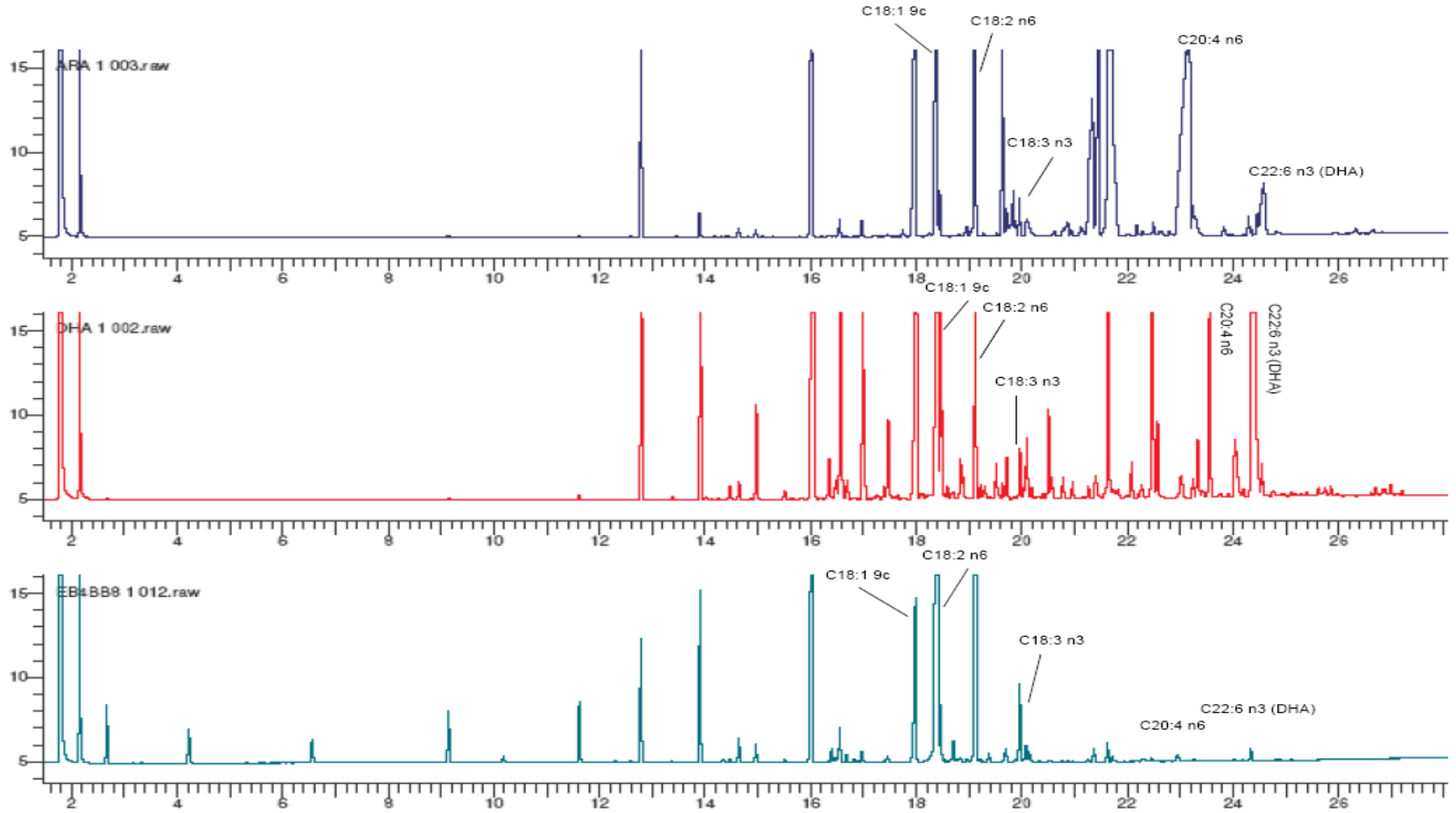

Figure 6.1. - *Chromatograms relative to the samples of raw materials employed for the addiction of ARA and DHA, and a sample from the inter-blend.*

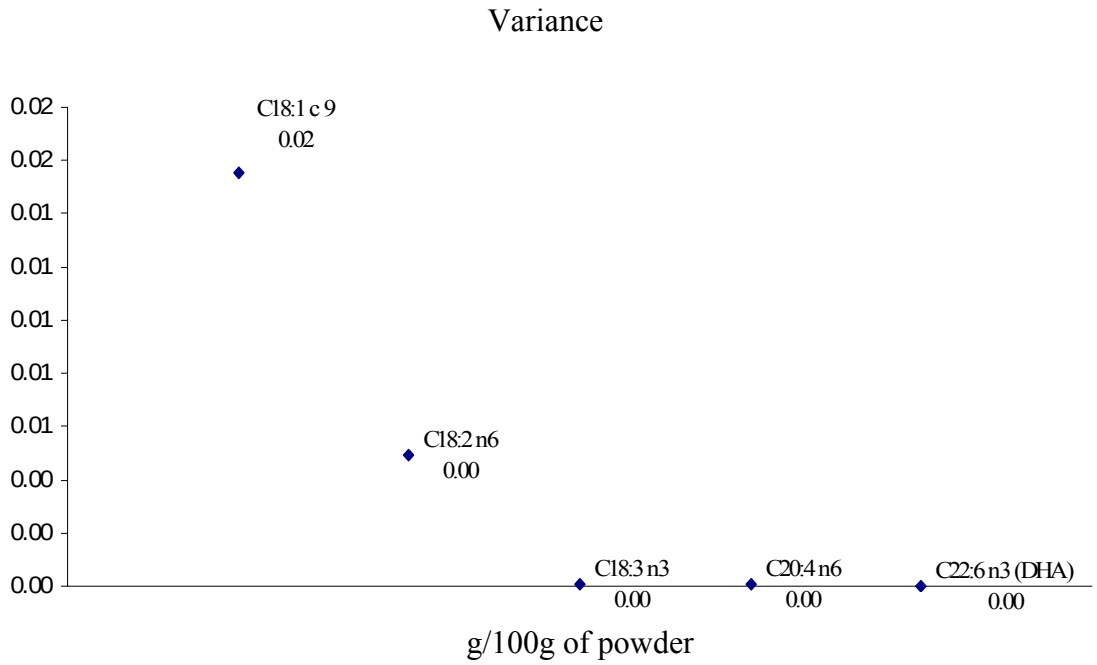

Figure 6.2. - *Variance of the fatty acids under investigation, and relatives to the intra-blend samples.*

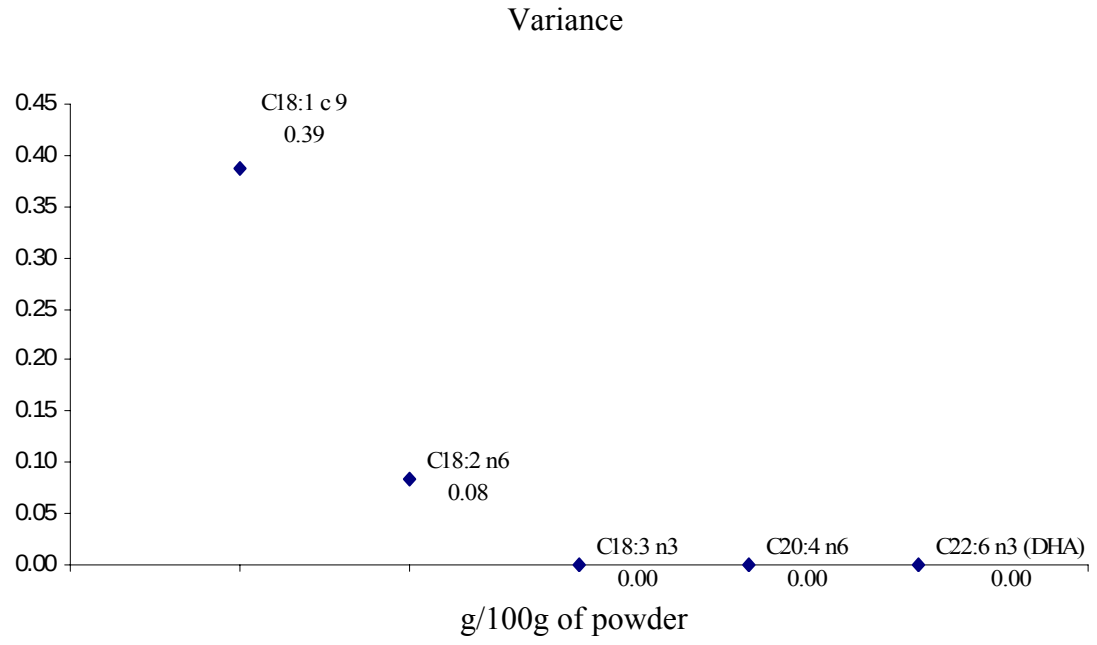

Figure 6.3. - *Variance of the fatty acids under investigation, and relatives to the inter-blend samples.*

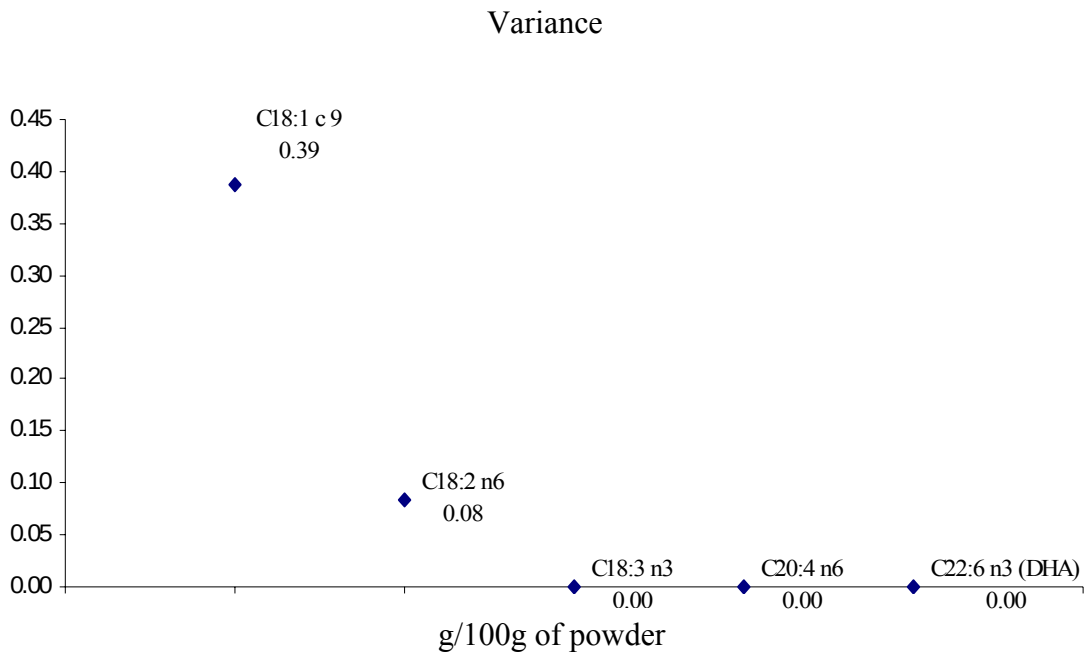

Figure 6.4. - *Variance of the fatty acids under investigation, and relatives to the commercial samples.*

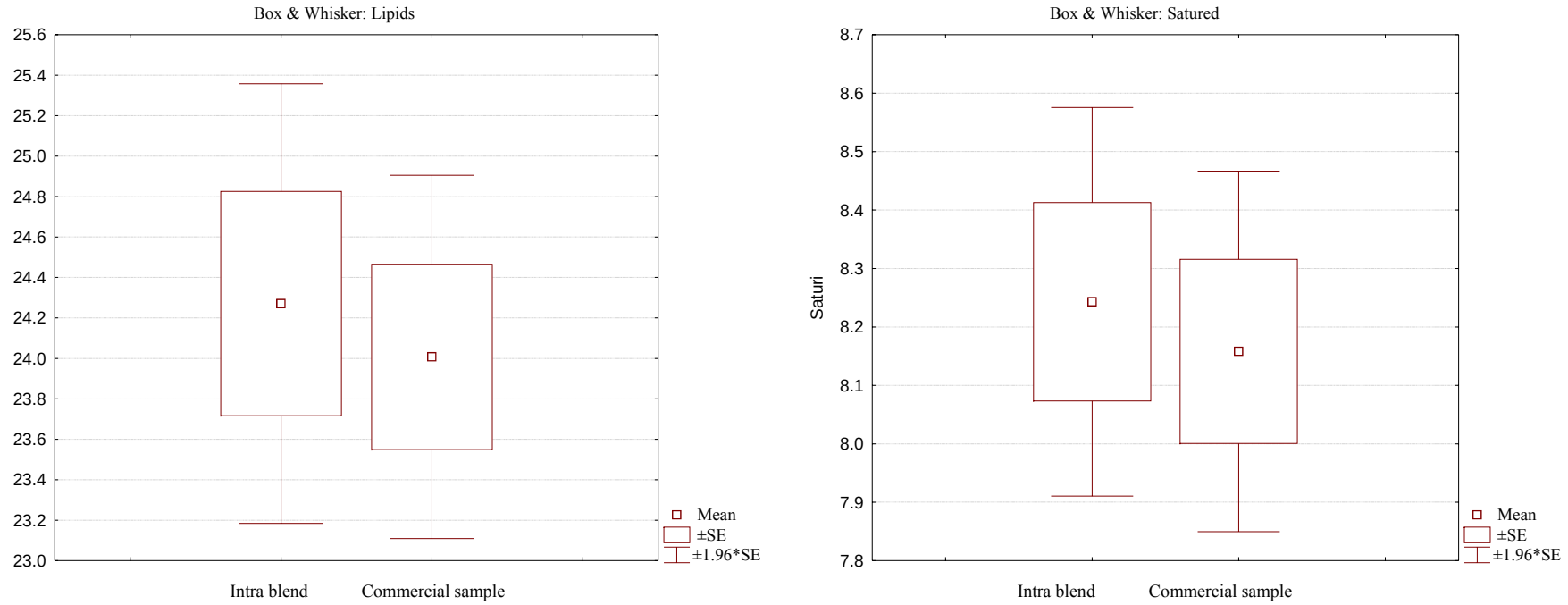

Figure 6.5a,b. – *Lipids and Satured: comparison of the mean, standard error, and a 95% confidence interval.*

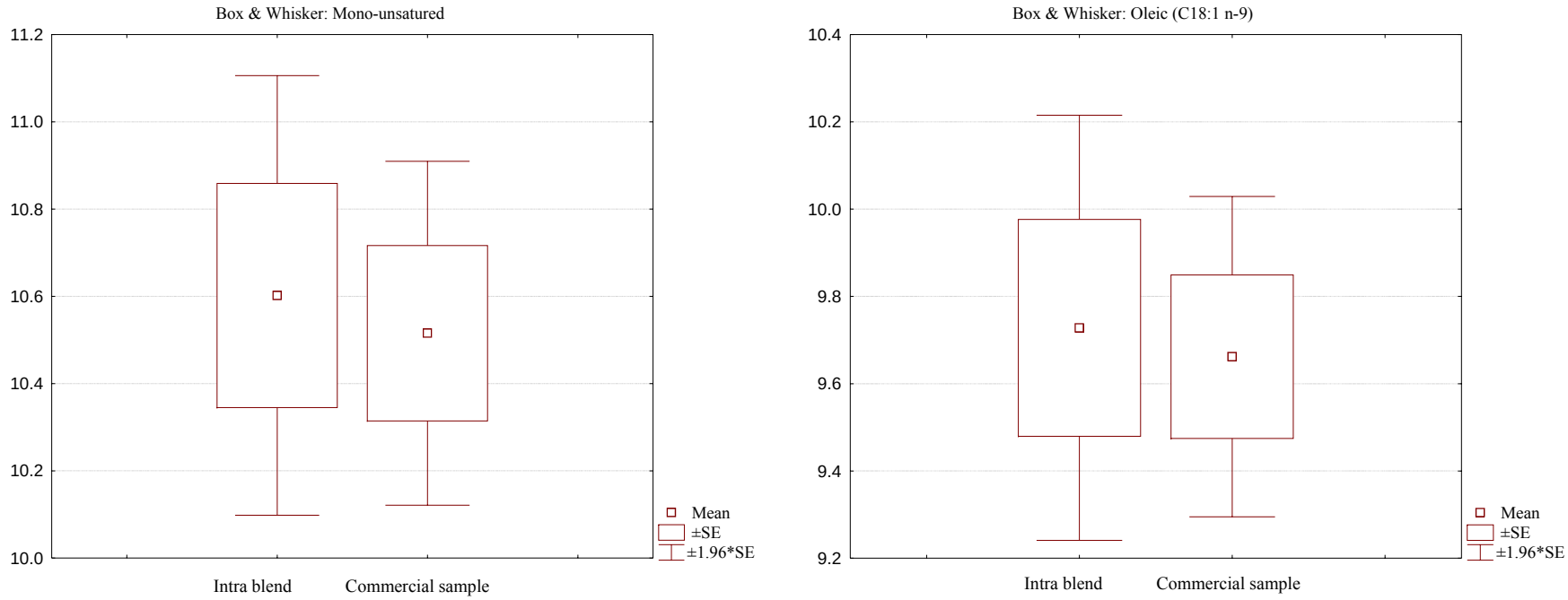

Figure 6.5c,d. – *Mono-unsatured and Oleic acid: comparison of the mean, standard error, and a 95% confidence interval.*

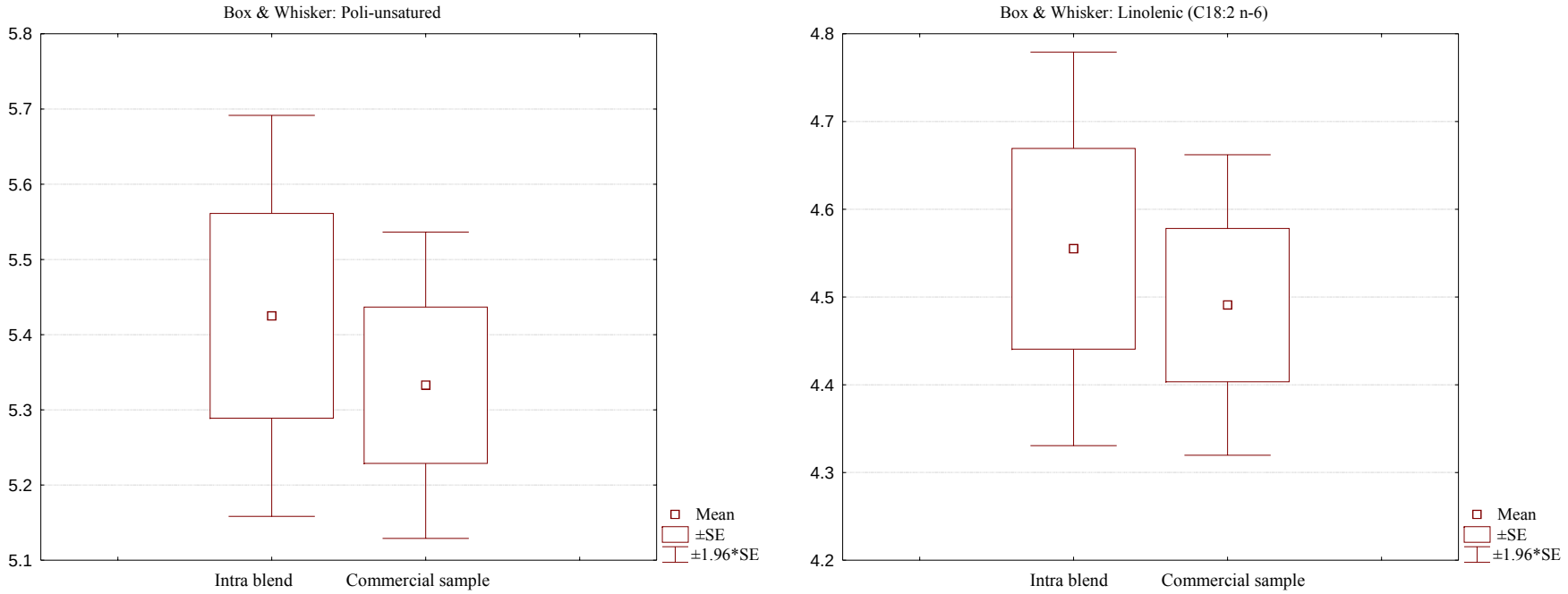

Figure 6.5e,f. – *Poli-unsatured and Linoleic acid: comparison of the mean, standard error, and a 95% confidence interval.*

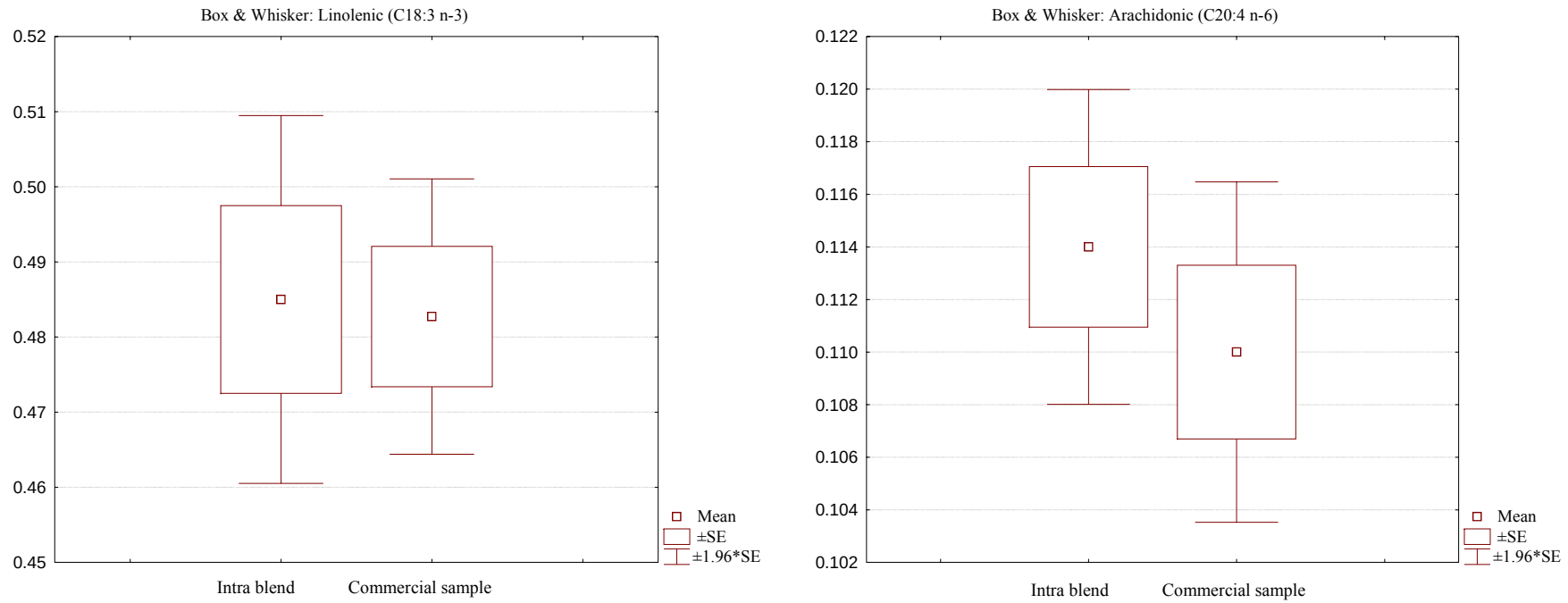

Figure 6.5g,h. – *Linolenic and Arachidonic acid: comparison of the mean, standard error, and a 95% confidence interval.*

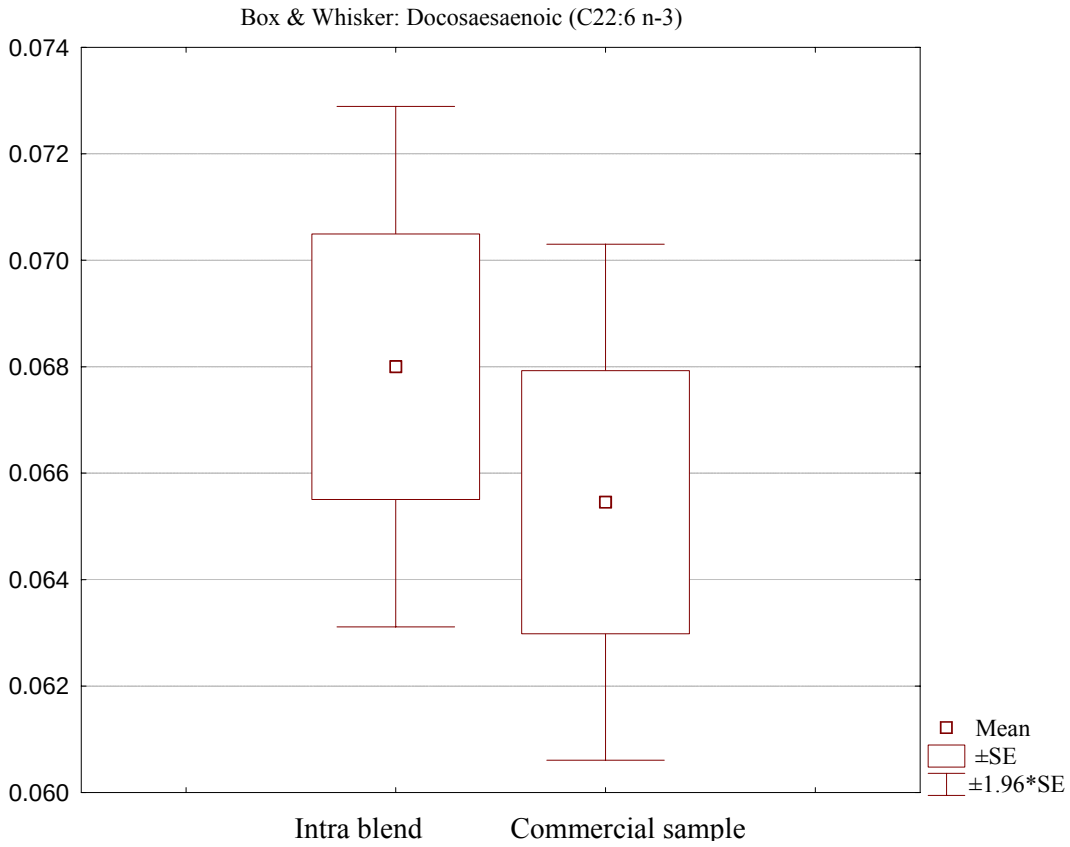

Figure 6.5i. – Docosahexaenoic *acid: comparison of the mean, standard error, and a 95% confidence interval.*

6.6. REFERENCES

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7

FATTY ACIDS COMPOSITION OF PARMIGIANO REGGIANO CHEESE SAMPLES, WITH EMPHASIS ON *TRANS* ISOMERS (TFA).

7.1. SUMMARY AND KEYWORDS

A wide debate in the scientific panel regarding the human consumption of food products containing fatty acids in *trans* configuration (TFA) have been raised, influencing either the nutritional recommendations proposed by national health cares, and food laws for nutritional labeling purpose. Despite the low levels of TFA in foods products from animal origin, and their particular composition in these products, even dairy products have been involved in the diatribe. Therefore, due to the lack of specific information about the TFA content in the Parmigiano Reggiano cheese, in this work the study of the fatty acid profile with particular emphasis to the TFA in Parmigiano Reggiano cheese samples has been proposed.

Keywords: *trans* fatty acids, Parmigiano Reggiano, food labels.

7.2. INTRODUCTION

The current investigation, dedicated to describe the fatty acid profile (with particular interest on *trans* fatty acids) of 13 samples of Parmigiano Reggiano cheese, collected in the 4 districts of the yield area (Bologna, Mantova, Parma, Reggio Emilia), and at different time of seasoning, is related to the increasing concern regarding unfavorable roles on human health of TFA in coronary heart diseases (CHD) and, lately, in prostate cancer risk (1). The discovery of their negative health effects concurrently to the advances in medicine and food science research, sparked a great interest in the scientific panel, inducing many governments to implement legislation to reduce the total TFA content of food products by introducing mandatory labeling of total TFA or restricting the sale of industrially produced fats and oils with more than a certain amount. Unfortunately, the debate, is still today an object of simplifications and mistakes that origin perplexity (2).

The predominant dietary sources of TFA in the western diet are: vegetable-oil-based margarines, shortenings, and cooking oils that have been subjected to the industrial process called hydrogenation; ruminat fats (dairy products and beef), where TFA are naturally produced by the process called biohydrogenation and present in a considerable lower amount. Recently, new studies found an inverse association between ruminant TFA and risk of CHD, implying that ruminant TFA intake might be innocuous or even protective against CHD (3, 4). Moreover, the TFA isomers from ruminant and industrial fat may differ in their relative abundance; the TFA isomers in industrial fats are produced under catalytic conditions that results in a more random distribution of isomer, while the isomers in ruminant fats are enzymatically produced resulting in the formation of specific TFA isomers. Therefore, the new investigations on total TFA negative health

effects should consider the differences between TFA isomers from ruminant and industrial fat, as well as the nutritional information to consumers and the definition of nutritional profiles that determine the food quality in dairy products should be seen under a different point of view.

7.3. MATERIALS AND METHODS

7.3.1 SAMPLES COLLECTION

13 samples of Parmigiano Reggiano cheese were collected in different dairy factory with the collaboration of the farm personnel.

7.3.2. PRELIMINARY ANALYSIS OF THE ORGANIC AND INORGANIC FRACTION

Each sample was analyzed in its organic and inorganic fraction composition by Food Scan (Foss)-NIT (**Table 7.1.**) at the Parmigiano Reggiano cheese Consortium, and then stored at - 40°C until analysis.

7.3.3. SAMPLE PREPARATION

The fat was extracted in double following the method of Blight and Dyer (5), and then, the preparation of the methyl esters was carried out applying the method of Christopherson and Glass (6): about 20 mg of dry fat was put in a test tube, 50 µl of KOH MeOH 2N and 500 µl of hexane were added respectively, and then the tube was mixed for 30 seconds; 0,5 mg of C13:0 (Sigma-Aldrich, St. Louis, MO, USA) were used for the fatty acids quantitation as internal standard. After the phase separation was

acted, 300 µl of surnatant was collected and put in a vial. It followed the analysis by gas chromatography.

7.3.4. GAS CHROMATOGRAPHIC QUANTITATION

FAME were analyzed with a Perkin Elmer Clarus 500 gas chromatograph equipped with a Supelco 2560 (Supelco Inc., Bellefonte, PA) fused silica capillary column (100 m x0.25 mm i.d., 0.2-µm film thickness), a FID detector, and a split injector. Helium was used as carrier at the constant flow rate of 0,75 min/min. The FID detector was maintained at 250°C with air flow rate of 400 ml/min, hydrogen flow rate of 40 ml/min, and helium (make up gas) flow rate of 30 ml/min. The split injector was maintained at 250°C with the split ratio of 1:67. The temperature program was as follow: ramp 1) 3°C/min from 100°C to 180°C and maintained 10 minutes, then ramp 2) 3°C/min from 180°C to 240°C and maintained 30 minutes. For the fatty acids identification was used the 53 FAME GLC-reference standard mix-463 (NU-CHEK-PREP, Elysian, MN).

7.4. RESULTS AND DISCUSSION

The fatty acid profile of the Parmigiano Reggiano cheese samples has been determined (**Table 7.2.**). Likewise the milk fatty acid distribution, the major fatty acids have resulted to be the palmitic acid (30,13 %), oleic acid (19,28 %), stearic acid (10,40 %), and miristic acid (10,39 %). The analysis of TFA content has followed: for definition, *trans* fats, are defined as all the unsaturated fatty acids that contain one or more isolated, non-conjugated, double bonds in a trans geometric configuration. Conjugated fatty acids with a trans double bond, including CLA isomers, are excluded from the definition of *trans* fats. By observing **table 7.3.** and **figure 7.1.** the dominant *trans* isomer in the

samples analyzed result to be the vaccenic acid (C18:1 *t*11), that represent the 74 % of the overall TFA content, while their total content fluctuates between the minimum of 2,5 % and a maximum of 5.4 % of total fatty acids composition, turning out to be in accordance with the literature (7). Several studies have observed variation of TFA among same dairy samples; seasonal variation is probably the most important cause of differing their fatty acid composition (8). Specifically, during the outdoors feeding period the intake of polyunsaturated and monounsaturated fatty acids increases and this leads to an increase in the proportions of oleic acid and trans fatty acids and a corresponding decrease in saturated fatty acids of milk (9). This trend has also been observed in the samples examined (**Figure 7.2.**), even though the cattle diet was unknown; this is difficult to determine in retrospect but since the samples were collected during different times, and seasoned differently, seasonal variation probably accounted for much of the differences found between the 13 samples in the present study.

7.5. CONCLUSIONS

The TFA composition found in the 13 Parmigiano Reggiano cheese samples results to be in accordance with the literature for dairy products and reflects, logically, the TFA content of milk. Nevertheless, it has been necessary to obtain such information from analysis about this unique cheese, in order to support with objective data the food labeling regulation, whereas Parmigiano Reggiano cheese is exported in countries where is mandatory the TFA declaration on the food label.

Moreover, from a nutritional point of view, the data have confirmed the specificity of TFA composition in dairy products, especially, if compared with food products containing hydrogenated fat. The vaccenic acid (C18:1 *t*11), the most abundant isomer,

is also the forerunner of rumenic acid (C18:2 *c*9 *t*11), the principal isomer of the group of CLA, and the presence of conjugated poli-unsatured fatty acids is very significative, since nutritional and healthy properties have been attributed.

TABLES

Table 7.1. – *Organic and inorganic fraction composition of Parmigiano Reggiano cheese samples.*

	Umidity	**Fat**	**Protein**	**Salt**
35	31.26	**31.39**	31.82	1.6
40	30.13	**32.58**	32.87	1.45
41	31.12	**30.61**	32.33	1.69
43	30.04	**33.62**	31.29	1.88
45	31.52	**31.64**	31.91	1.34
47	31.76	**31.29**	31.39	1.4
49	32.35	**31.75**	30.92	1.87
52	31.56	**29.43**	34.1	1.51
53	31.92	**30.99**	32.81	1.51
D5	30.62	**33.03**	31.07	1.43
5	35.02	**28.83**	30.65	1.36
6	35.1	**25.07**	33	1.63
7	31.12	**30.59**	33.77	1.61

Table 7.2. – *The fatty acid profile of the Parmigiano Reggiano cheese samples.*

Sample #	**35**	**40**	**41**	**43**	**45**	**47**	**49**	**52**	**53**	**5**	**6**	**7**	**D5**
Satured (100g of fat)													
c4:0	1.81	2.39	1.34	1.75	1.62	1.47	1.56	1.32	1.15	1.84	1.64	1.56	1.65
c6:0	0.87	1.79	1.17	1.23	1.31	1.25	1.23	1.18	1.06	1.42	1.27	1.19	1.29
c8:0	0.55	1.10	0.93	2.90	0.87	0.85	0.79	0.84	0.73	0.92	0.89	0.84	0.91
c10:0	1.42	2.95	2.47	1.97	2.27	2.27	2.03	2.46	1.94	2.40	2.36	2.27	2.58
c11:0	0.22	0.22	0.28	0.22	0.23	0.26	0.14	0.09	0.19	0.24	0.16	0.20	0.23
c12:0	2.80	4.00	2.74	2.60	2.98	3.07	2.64	3.45	2.58	3.07	3.01	2.94	3.59
c14:0	10.90	13.58	11.72	10.80	11.44	11.64	9.81	11.57	10.06	11.29	10.24	10.41	12.03
c15:0	1.10	1.52	1.40	1.36	1.24	1.44	1.06	1.52	1.17	1.23	1.10	1.09	1.39
c16:0	31.47	39.22	32.64	34.11	32.84	35.60	30.90	32.18	30.47	31.48	26.03	28.41	36.47
c17:0	0.76	0.95	0.80	0.84	0.80	0.91	0.66	0.80	0.77	0.84	0.64	0.67	0.81
c18:0	11.76	12.55	10.38	10.39	12.14	10.75	11.62	9.65	11.35	11.42	13.22	11.48	8.87
c20:0	0.16	0.12	0.17	0.16	0.19	0.16	0.13	0.14	0.16	0.17	0.16	0.13	0.15
c21:0	0.01	0.01	0.04	0.03	0.02	0.03	0.01	0.02	0.03	0.03	0.02	0.02	0.02
c22:0	0.07	0.06	0.09	0.00	0.08	0.08	0.05	0.12	0.07	0.08	0.07	0.08	0.07
c23:0	0.02	0.00	0.05	0.04	0.03	0.03	0.01	0.00	0.01	0.04	0.02	0.02	0.03
c24:0	0.03	0.10	0.07	0.03	0.04	0.05	0.02	0.03	0.04	0.05	0.02	0.03	0.02
Total	***64.01***	***80.57***	***66.36***	***68.53***	***68.11***	***69.85***	***62.67***	***65.37***	***61.78***	***66.52***	***60.85***	***61.34***	***70.10***
g/100g of cheese	***20.09***	***26.24***	***20.31***	***23.03***	***21.54***	***21.85***	***19.89***	***19.23***	***19.14***	***19.17***	***15.25***	***18.76***	***23.15***

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Sample #	**35**	**40**	**41**	**43**	**45**	**47**	**49**	**52**	**53**	**5**	**6**	**7**	**D5**
Mono-ins.cis (100g of fat)													
c14:1	0.80	0.50	1.02	0.84	0.81	0.87	0.83	1.05	0.88	0.84	0.73	3.16	1.00
c16:1c	1.86	1.33	1.86	1.82	1.68	1.93	1.88	2.01	1.98	1.72	1.40	1.62	1.94
c17:1	0.22	0.11	0.24	0.22	0.18	0.23	0.19	0.23	0.15	0.21	0.15	0.18	0.20
c18:1 c 9	23.34	12.07	20.97	19.73	19.51	18.73	23.58	22.29	23.52	21.27	23.71	22.25	18.94
c18:1 c 11	0.72	0.38	0.48	0.53	0.56	0.54	0.67	0.95	0.72	0.58	0.58	0.68	0.64
c18:1 c 12	0.30	0.18	0.24	0.21	1.06	0.20	0.36	0.33	0.36	0.35	0.73	0.52	0.21
c18:1 c 13	0.06	0.01	0.04	0.03	0.01	0.03	0.05	0.06	0.06	0.04	0.07	0.07	0.03
c18:1 c15	0.12	0.10	0.16	0.13	0.11	0.12	0.13	0.10	0.14	0.13	0.18	0.18	0.14
c20:1 n9	0.06	0.00	0.04	0.02	0.04	0.04	0.05	0.02	0.06	0.05	0.02	0.04	0.02
Total	***27.48***	***14.69***	***25.06***	***23.54***	***23.95***	***22.69***	***27.75***	***27.03***	***27.86***	***25.19***	***27.55***	***28.70***	***23.12***
g/100g	***8.62***	***4.78***	***7.67***	***7.91***	***7.57***	***7.10***	***8.81***	***7.95***	***8.63***	***7.26***	***6.90***	***8.77***	***7.63***
Poli-uns. cis (100g of fat)													
c18:2 n6	2.60	1.68	2.08	2.17	2.31	1.97	3.22	2.82	3.59	2.36	4.17	3.60	2.59
c18:3 n6	0.02	0.00	0.01	0.01	0.02	0.01	0.02	0.02	0.03	0.02	0.02	0.02	0.04
c18:3 n3	0.52	0.31	0.75	0.76	0.47	0.54	0.59	0.58	0.58	0.81	0.97	0.97	0.65
c20:2	0.02	0.00	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.01	0.02	0.00
c20:3 n6	0.11	0.06	0.10	0.10	0.10	0.08	0.12	0.15	0.14	0.13	0.15	0.12	0.12
c:20:4 n6	0.14	0.07	0.13	0.12	0.12	0.11	0.15	0.11	0.16	0.16	0.17	0.13	0.16
c22:2	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
c20:5 n3	0.03	0.01	0.06	0.03	0.03	0.04	0.03	0.03	0.04	0.06	0.02	0.04	0.02
c22:6 n3	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Total	***3.44***	***2.12***	***3.15***	***3.20***	***3.06***	***2.77***	***4.18***	***3.73***	***4.56***	***3.56***	***5.51***	***4.91***	***3.61***
g/100g of cheese	***1.07***	***0.69***	***0.96***	***1.07***	***0.96***	***0.86***	***1.32***	***1.09***	***1.41***	***1.02***	***1.38***	***1.50***	***1.19***

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Sample #	35	40	41	43	45	47	49	52	53	5	6	7	D5
CLA (100g of fat)													
c18:2 c9, t11	***0.77***	***0.2***	***0.9***	***0.75***	***0.64***	***0.58***	***0.69***	***0.4***	***0.72***	***0.57***	***0.69***	***0.59***	***0.39***
g/100g	***0.24***	***0.06***	***0.27***	***0.25***	***0.2***	***0.18***	***0.21***	***0.11***	***0.22***	***0.16***	***0.17***	***0.18***	***0.12***
TFA (100g of fat)													
Total	***4.31***	***2.42***	***4.54***	***3.98***	***4.23***	***4.11***	***4.71***	***3.45***	***5.08***	***4.16***	***5.39***	***4.46***	***2.79***
g/100g of cheese	***1.35***	***0.78***	***1.38***	***1.33***	***1.33***	***1.28***	***1.49***	***1.01 g***	***1.57***	***1.19***	***1.35***	***1.36***	***0.92***

Table 7.3. – *TFA compositionof the Parmigiano Reggiano cheese samples.*

		TFA (% FAME)						
AGE	*SAMPLE*	*C13:1*	*C16:1*	*C18:1 t11*	*C18:2*	*C18:3*	*TOTAL TFA*	**g/100g of cheese**
24	***#40***	0.08	0.36	1.74	0.20	0.03	***2.42***	***0.79***
23	***#D5***	0.09	0.30	1.85	0.44	0.11	***2.79***	***0.92***
6	***#5***	0.12	0.37	3.06	0.47	0.14	***4.16***	***1.20***
24	***#47***	0.11	0.40	3.06	0.43	0.11	***4.11***	***1.28***
24	***#45***	0.09	0.39	3.23	0.40	0.12	***4.23***	***1.34***
26	***#43***	0.13	0.40	2.88	0.44	0.13	***3.98***	***1.34***
12	***#6***	0.07	0.28	4.17	0.77	0.10	***5.39***	***1.35***
26	***#52***	0.04	0.28	2.47	0.55	0.11	***3.45***	***1.35***
26	***#35***	0.08	0.36	3.34	0.39	0.13	***4.31***	***1.35***
36	***#7***	0.06	0.29	3.35	0.65	0.11	***4.46***	***1.37***
24	***#41***	0.16	0.45	3.24	0.54	0.15	***4.54***	***1.39***
25	***#49***	0.06	0.33	3.61	0.59	0.12	***4.71***	***1.49***
24	***#53***	0.08	0.41	3.83	0.63	0.13	***5.08***	***1.57***
						MEAN	***4.12***	***1.29***

FIGURES

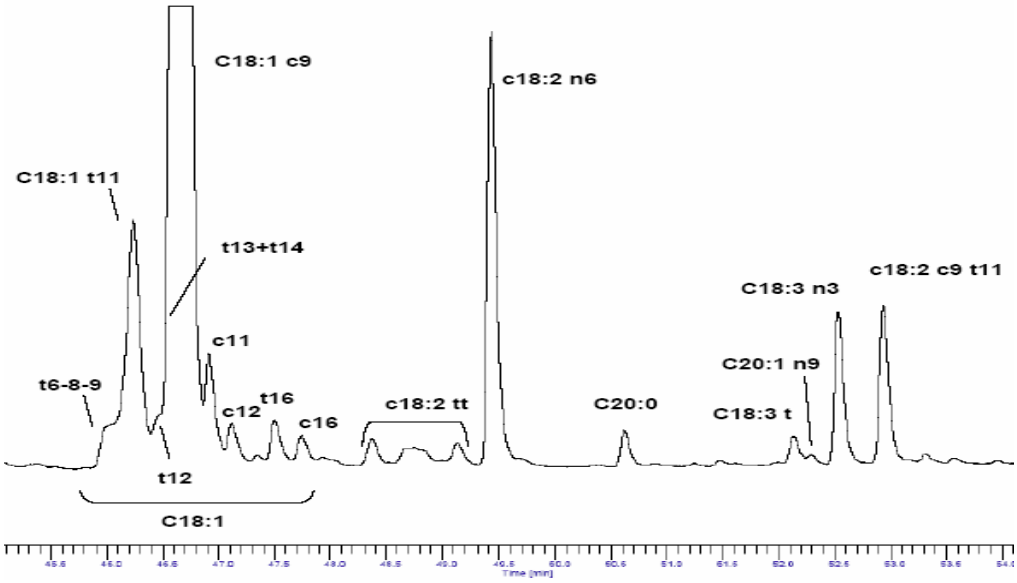

Figure 7.1. – *Partial chromatogram of the TFA elution area.*

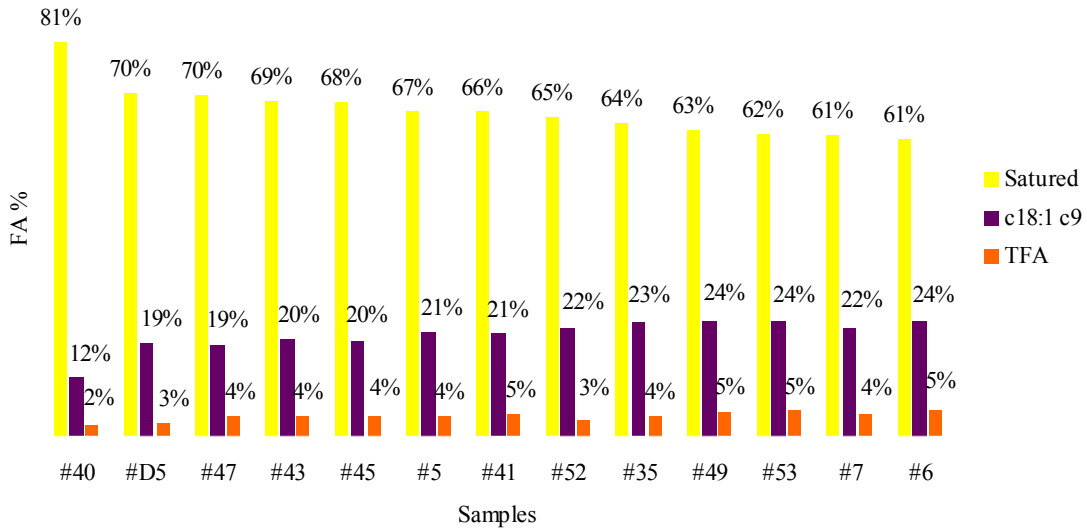

Figure 7.2. – *Percentual variation of satured FA, oleic acid, and TFA in each sample.*

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