ALMA MATER STUDIORUM – UNIVERSITÀ DEGLI STUDI DI BOLOGNA

FACOLTÀ DI AGRARIA

DOTTORATO DI RICERCA IN SCIENZE DEGLI ALIMENTI

Tesi per il conseguimento del titolo di Dottore di Ricerca

Triennio Accademico 2004/2007 – XIX Ciclo di Dottorato

Settore scientifico-disciplinare: AGR/15

APPLICATION OF CHROMATOGRAPHIC AND SPECTROSCOPIC TECHNIQUES IN THE EVALUATION OF THE LIPID FRACTION OF ANIMAL PRODUCTS

Tutor:
Dott.ssa Tullia Gallina Toschi

Tesi presentata da:
Dott. Federico Ferioli

Coordinatore:
Prof. Claudio Cavani

INDEX

1. AIM AND DESCRIPTION OF THE PhD PROJECT

Lipolysis and oxidation of lipids in foods are the major biochemical and chemical processes that cause food quality deterioration, leading to the characteristic, unpalatable odour and flavour called rancidity. In addition to unpalatability, rancidity may give rise to toxic levels of certain compounds like aldehydes, hydroperoxides, epoxides and cholesterol oxidation products.

In this PhD study chromatographic and spectroscopic techniques were employed to determine the degree of rancidity in different animal products and its relationship with technological parameters like feeding fat sources, packaging, processing and storage conditions.

To achieve this goal capillary gas chromatography (CGC) was employed not only to determine the fatty acids profile but also, after solid phase extraction, the amount of free fatty acids (FFA), diglycerides (DG), sterols (cholesterol and phytosterols) and cholesterol oxidation products (COPs). To determine hydroperoxides, primary products of oxidation and quantify secondary products UV/VIS absorbance spectroscopy was applied.

Most of the foods analysed in this study were meat products. In actual fact, lipid oxidation is a major deterioration reaction in meat and meat products and results in adverse changes in the colour, flavour and texture of meat. The development of rancidity has long recognized as a serious problem during meat handling, storage and processing. On a dairy product, a vegetal cream, a study of lipid fraction and development of rancidity during storage was carried out to evaluate its shelf-life and some nutritional features life saturated/unsaturated fatty acids ratio and phytosterols

content. Then, according to the interest that has been growing around functional food in the last years, a new electrophoretic method was optimized and compared with HPLC to check the quality of a beehive product like royal jelly.

This manuscript reports the main results obtained in the five activities briefly summarized as follows:

1) comparison between HPLC and a new electrophoretic method in the evaluation of authenticity of royal jelly;
2) study of the lipid fraction of a vegetal cream under different storage conditions;
3) study of lipid oxidation in minced beef during storage under a modified atmosphere packaging, before and after cooking;
4) evaluation of the influence of dietary fat and processing on the lipid fraction of chicken patties;
5) study of the lipid fraction of typical Italian and Spanish pork dry sausages and cured hams.

Keywords: animal foods, high performance chromatographic techniques, lipid fraction, lipolysis, oxidative rancidity, UV/VIS spectroscopy.

2. INTRODUCTION

2.1. LIPIDS AND FOOD QUALITY AND TECHNOLOGY

References for this Section: Nawar, 1996, Kołakowska and Sikorski, 2003; EUFIC, 2006.

Even no exact definition of *lipids* exists, 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.

2.1.1. The content of lipids in foods

Fats in foods have either animal or vegetable origin. All plants and animals eaten by humans contain lipids, which are essential components for a healthy body, providing a source of energy and carrying vital nutrients. Only a few food products are devoid of lipids; for example, sugar, honey and clarified juices while various vegetables and most fruits belonging to the food commodities are very low in fats (0.3%).

The main sources of animal fat in Europe are meat and meat products, eggs and dairy products like butter, cheese, milk and cream. Fat can also be found in plant seeds (i.e.: rapeseed, sunflower, maize), fruits (i.e.: olive, avocado) and nuts (i.e.: peanuts, almonds). In this case oil is obtained by crushing the seeds, fruits or nuts, heating them and removing the oil through extraction processes. The oil is then refined to remove undesirable taste, smell, colour or impurities. Some oils like virgin olive oil are pressed straight from the seed and obtained without any further refining.

The lipid content in the muscle tissue of lean beef, fish, white poultry and shellfish is about 2%, about 3.7% in cow's milk, from 2 to 4% in grains, about 30% in fatty pork, about 32% in an egg yolk, and up to 35% in fillets of fatty fish. Oil-bearing nuts and seeds contain from 20% fat in soybeans to 65% fat in walnuts.

The factors that affect the lipid content in food raw materials include the species, genotype and variety of the plant or animal, as well as the part of the plant or organ of the carcass; for example, there is up to 70% oil in cod liver and only 1/100 of this amount in the fillet. The temperature and other conditions of vegetation or breeding are

also important, as well as the maturity of the plant at harvest, feeding and age, sex, maturity of the slaughter animal and stage of development f the gonads in fish.

In processed foods, the fat content depends on the raw material specificity and the required sensory properties of the products. The fat content in bread is 0.5 to 1.5%, in chocolate 22 to 36% and in confectionery products from 3.4% for a wafer to 36% for a praline; margarine is 80% or from 39 to 41%, depending on the type of a product; butter from 81 to 85%; other dairy products from 0.5 to 30% and meat products from 13 to 50%. In **Table 2.1.** are reported some fat containing foods both of animal and vegetable origin.

Table 2.1. – Food sources rich in the various types of fatty acids

Type of fat	Sources
Saturated	Butter, cheese, meat, meat products (sausages, hamburgers), full-fat milk and yoghurt, pies, pastries, lard, dripping, hard margarines and baking fats, coconut and palm oil.
Monounsaturated	Olives, rapeseed, nuts (pistachio, almonds, hazelnuts, macadamia, cashew, pecan), peanuts, avocados, and their oils.
Polyunsaturated	Omega-3 polyunsaturated: Salmon, mackerel, herring, trout (particularly rich in the long chain omega-3 fatty acids EPA or eicosapentaenoic acid and DHA or docosahexaenoic acid). Walnuts, rapeseed, soybean flax seed, and their oils (particularly rich in alpha linolenic acid).
	Omega-6 polyunsaturated: Sunflower seeds, wheat germ, sesame, walnuts, soybean, corn and their oils.Certain margarines (read the label).
Trans fatty acids	Some frying and baking fats (e.g. hydrogenated vegetable oils) used in biscuits, cakes and pastries, dairy products, fatty meat from beef and sheep.

2.1.2. Role of lipids in food technology

The characteristics of fats and oils play a very important role in the manufacture and cooking of foods and in the texture and appearance of the final product.

- **Aeration:** products such as cakes or mousses need air incorporated into the mixture in order to give a well-risen texture. This is usually achieved by trapping bubbles of air in a fat/sugar mixture to form a stable foam.

- **Shortening:** a crumbly texture found in some pastry and biscuits is achieved by fat coating the flour particles to prevent them from absorbing water.
- **Flakiness:** fat helps separate the layers of gluten and starch formed in the dough when making flaky or puff pastry or biscuits. The fat melts during cooking, leaving minute air pockets and the liquid present produces steam which evaporates and causes the layers to rise.
- **Moisture retention:** fat helps retain a product's moisture content and therefore increase its shelf life.
- **Glaze:** fats give a glossy appearance for example when added to hot vegetables and also add shine to sauces.
- **Plasticity:** solid fats do not melt immediately but soften over a range of temperatures. Fats can be processed to rearrange the fatty acids and alter their melting point. This technology has been used to produce spreads and cheeses that will spread straight from the fridge.
- **Heat transfer:** in deep frying the food is completely surrounded by the frying fat which acts as a very efficient heat-transfer medium.

2.1.3. Effects of lipids on the sensory attributes of foods

Palatability is a major determinant of food choice and fat contributes to the palatability of foods by its texture and flavour and affecting the mouth-feel. Some examples may be the richness of whole milk as opposed to the blank taste of skim milk or the smoothness of high-quality ice cream. All fats and oils act as carriers for fat-soluble flavour compounds.

Lipid primarily affect colour, rheological properties and flavour of foods, depending on their content, chemical composition, chemical, enzymatic and physical changes that take place during storage and food manufacturing operations.

2.1.3.1. Colour

Lipids are involved in food colour formation by carrying different coloured substances and by participating as substrates in reactions leading to the generation of colored compounds. The surface pigmentation of marine animals is largely due to different carotenoproteins, which may be yellow, orange, red, purple, blue or green, depending on the structure of the complexes – the kind of carotenoid, predominantly astaxanthin, cantaxanthin and β-carotene, as well as the properties of the proteinaceous component. Dissociation of the protein moiety from the complex in bright light rings about fading of the colours. Carotenoid pigments are also responsible for the colour of the flesh oil of redfish (*Sebastes marinus*). Vegetable oils also contain different carotenoids, generally in concentrations below 0.1%. In palm oil, the carotenoid pigments (about 0.3%) are responsible for the orange colour.

2.1.3.2. Texture

The rheological properties are affected by fat in meat and meat products, in fishery products, in dairy commodities and in pastry, cakes and mayonnaise. The desirable texture of culinary meat is due to proper marbling of the muscles with thin fat layers whereas that of comminuted sausages is conditioned by an adequate fat content in the formulation. Baltic sprats caught in the summer are unsuitable as raw material for the canned product known as smoked Baltic sprats in oil because, at a fat content of less

than 6%, the texture of the fish is too hard. High-quality hot smoked mackerel can be assured only by using raw material containing about 30% fat. The cream for producing whipped cream without any whipping agents should contain about 30% fat. The desirable sensory sensation caused by melting of chocolate in one's mouth is due to the narrow range of melting temperature (28 to 36°C) of the lipids in cocoa butter.

2.1.3.3. Aroma and flavour

Lipid degradation products in low concentration contribute to the mild, rather pleasant, plant-like, melon-like, seaweedy aroma of very fresh fish. Due to reactions catalyzed by endogenous lipoxygenases, hydroxyperoxide lyases, Z,E-enal isomerases and alcohol dehydrogenases, the PUFA of fish lipids are degraded to aldehydes, ketones and alcohols with 6, 8, and 9 carbon atoms, respectively. The gradual loss in the intensity of the fresh fish aroma is caused, in part, by microbial conversion of the carbonyl compounds into alcohols, which have higher aroma threshold values. During the storage of frozen fish, an off-flavour develops due to the oxidation of lipids. The desirable flavour in many cheeses is created, in part, by lipid oxidation products, such as ketones and aldehydes. In lipids consisting of short-chain FA, both oxidation and lipolysis influence off-flavour.

2.2. NOMENCLATURE OF FATTY ACIDS

References for this Section: Nawar, 1996, O'Keefe, 2002; Nichols and Sanderson, 2003.

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.

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 (**Fig. 2.1.**). 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.

In **Table 2.2.** a list of some of the most common FA found in natural fats is given, reporting both systematic and common name for each FA while **Fig. 2.2.** illustrated the difference between IUPAC Δ and shorthand numbering systems.

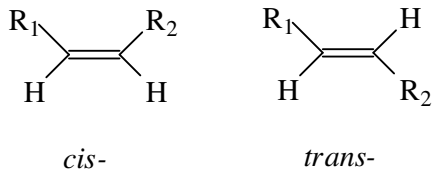


Fig. 2.1. – Example of *cis/trans* nomenclature.

Table 2.2. – 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

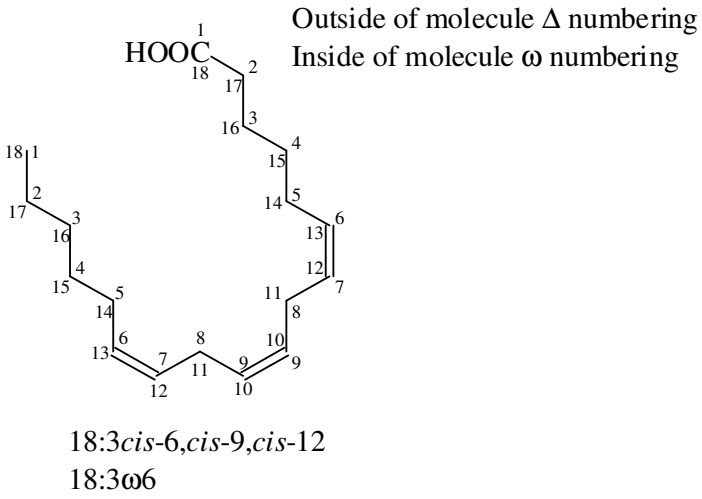


Fig. 2.2. – IUPAC Δ and common ω numbering system.

2.3. CLASSIFICATION OF LIPIDS

References for this Section: Nawar, 1996.

Classification of lipid structures is possible based on physical properties at room temperature (oils are liquid and fats are solid), their polarity (polar and neutral lipids), their essentiality for humans (essential and nonessential fatty acids) or their structure (simple or complex). Neutral lipids include fatty acids, alcohols, glycerides and sterols, while polar lipids include glycerophospholipids and glyceroglycolipids.

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*, composed 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 2.3.** even 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 2.4.**

Table 2.3. – 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 2.4. – 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

2.4. CHEMICAL ASPECTS OF LIPIDS: OXIDATION AND LIPOLYSIS

References for this Section: Hamilton, 1989; Nawar, 1996; Shahidi and Wanasundara, 2002; Kołakowska, A. 2003; Wąsowicz. E. 2003.

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. Lipid oxidation is affected by numerous internal and external factors such as FA composition, content and activity of pro- and antioxidants, irradiation, temperature, oxygen pressure, surface area in contact with oxygen and water activity (a_w). The complex process of food lipid oxidative changes is interpreted in terms of an oxidation mechanism derived from model studies, predominantly involving a single FA. Lipid oxidation in foods is assumed to proceed along a free radical route (autoxidation), photoxidation route, and/or lipoxygenase route. The oxidation mechanism is basically explained by invoking free-radical reactions, while the photoxidation and lipoxygenase routes differ from it at the initiation stage only.

It is generally agreed that "autoxidation", the reaction with molecular oxygen via a self-catalytic mechanism, is the main reaction involved in the 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.

2.4.1. Autoxidation or free radical oxidation

The classical oxidation route depends on the production of free radicals R· from lipid molecules RH by their interaction with oxygen in the presence of a catalyst and involved three stages: initiation, propagation and termination.

Initiation: $RH + O_2 \xrightarrow{catalyst} R\cdot + \cdot OOH$

$RH \xrightarrow{catalyst} R\cdot + \cdot H$

Propagation: $R\cdot + O_2 \rightarrow RO_2\cdot$

$RO_2\cdot + RH \rightarrow RO_2H + R\cdot$

Termination: $R\cdot + R\cdot \rightarrow R\text{–}R$

$RO_2\cdot + R\cdot \rightarrow RO_2H$

The initiation can occur by the action of external energy sources such as heat, light or high energy radiation or by chemical initiation involving metal ions or metalloproteins such as haem. The mechanism of the initiation step is still not completely understood. The free radical R· produced in the initiation step can then react to form a lipid peroxy radical ROO· which can further react to give the hydroperoxide ROOH. The second reaction of the propagation step also provides a further free radical R·, making it a self-perpetuating chain process. In this way a small amount of catalyst such as copper ions, can initiate the reaction, which then produces many hydroperoxide molecules, which finally break down to cause rancidity. The self-propagating chain can be stopped by termination reactions, where two radical compounds combine to give products which do

not feed the chain reactions.

Qualitative and quantitative analyses of the isomeric hydroperoxides from oleate and linoleate have been conducted. When this mechanism is applied to the autoxidation of methyl oleate (**Fig. 2.3.**), hydrogen abstraction on C-8 and C-11 forms two allylic radicals, each of which can be represented by two canonical forms. These forms help to explain why not only the 8-hydroperoxides but also the 10-hydroperoxides is obtained from one allylic radical and the 9- and 11- hydroperoxides from the other allylic radical. The double bond position is scrambled since there are other hydroperoxides present in addition to the Δ9 hydroperoxide and the configuration may be changed from *cis* to *trans*.

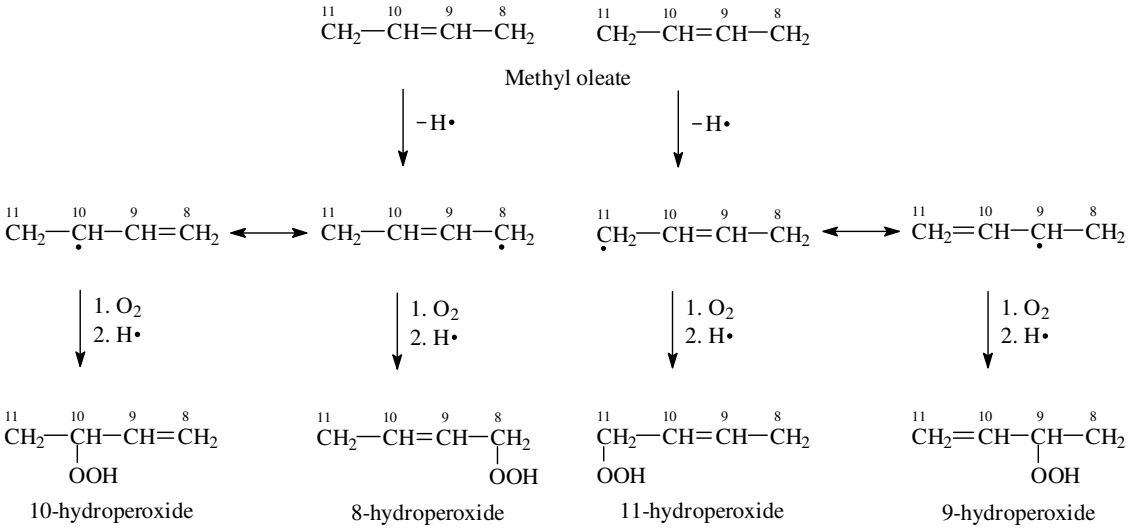


Fig. 2.3 – Autoxidation of methyl oleate.

The autoxidation of methyl linoleate starts with the abstraction of a hydrogen at the doubly reactive methylene at C-11 (**Fig. 2.4.**). Hydrogen abstraction at this position produces a pentadienyl radical intermediate, which upon reaction with molecular oxygen produces an equal mixture of conjugated 9- and 13-diene hydroperoxides. Evidence reported in

literature indicates that the 9- and 13-*cis*, *trans*-hydroperoxides undergo interconversion, along with some geometric isomerization, forming *trans*,*trans*-isomers. Thus, each hydroperoxides (9- and 13-) is found in both the *cis*,*trans* and the *trans*,*trans* forms.

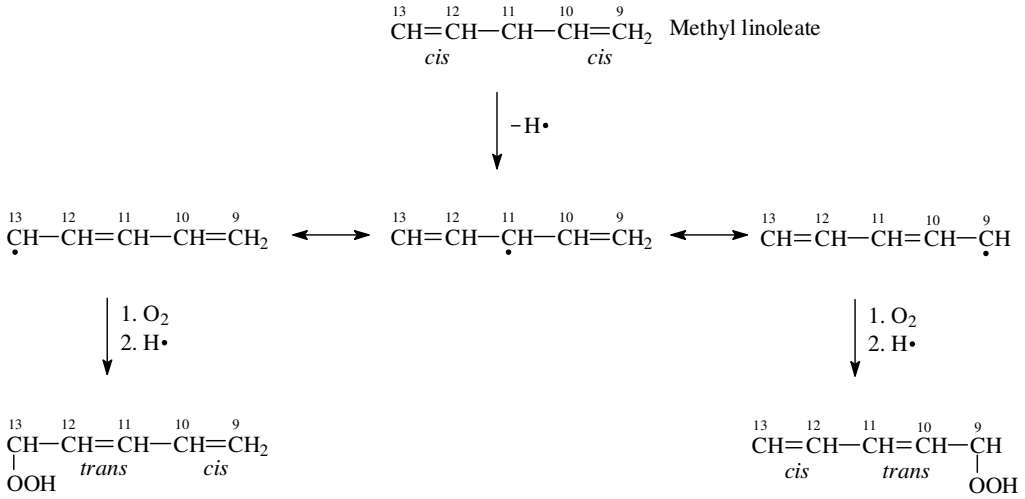


Fig. 2.4. – Autoxidation of methyl linoleate.

Induction period

When autoxidation of fat is followed experimentally, for example by measuring the amount of oxygen absorbed or the peroxide value (PV), it is found that the oxidation proceeds though two distinct phases. During the first phase , the oxidation goes slowly and a uniform rate. After a certain point the reaction enters a second phase, which has a rapidly accelerating rate of oxidation and the eventual rate is many times greater than that observed in the initial phase. The initial phase is called induction period and it is found that the autoxidation rate increases with increasing number of double bonds in fatty acids. Actually methyl linoleate react more quickly than methyl oleate and has a shorter induction period

2.4.2. Photo-oxidation

Photo-oxidation is an alternative route to the free radical mechanism, because it is found that different hydroperoxides are formed when light and certain photosensitiser molecules are present. Photo-oxidation involves the formation of hydroperoxides in a direct reaction of singlet oxygen to unsaturated lipids, without radical formation. The singlet oxygen $^1O_2$ emerges during a reaction of sensitisers (chlorophyll, haemoglobin, myoglobin, erythrosine, riboflavin and heavy metal ions) with atmospheric oxygen. Photosensitization can also occur *in vivo*. Singlet oxygen react about 1500 times faster with methyl linoleate than does triplet oxygen and, as formerly stated, it reacts directly with double bonds by addition at either end of the double bond, producing an allylic hydroperoxide in which the double bond has been shifted in the *trans* configuration (**Fig. 2.5.**). With this kind of oxidation, no induction period is known. Two mechanisms have been proposed for photo-oxidation.

Type 1

$$\text{Sensitiser} + X + h\nu \rightarrow [\text{Intermediate I}]$$

$$[\text{Intermediate I}] + {}^3O_2 \rightarrow \text{Products} + {}^1\text{Sensitiser}$$

$${}^1\text{Sens} + h\nu \rightarrow {}^1\text{Sens}^* \rightarrow {}^3\text{Sens}^*$$

$${}^3\text{Sens}^* + X\ (\text{acceptor}) \rightarrow [\text{Intermediate I}]$$

$$[\text{Intermediate I}] + {}^3O_2 \rightarrow XO_2 + {}^1\text{Sens}$$

Type 2

$$\text{Sensitiser} + O_2 + h\nu \rightarrow [\text{Intermediate II}]$$

$$[\text{Intermediate II}] + X \rightarrow \text{Products} + \text{Sensitiser}$$

$$\text{Sensitiser} + h\nu \rightarrow {}^1\text{Sensitiser}$$

$${}^1\text{Sensitiser} \rightarrow {}^3\text{Sensitiser}$$

$${}^3\text{Sensitiser} + {}^3O_2 \rightarrow \text{Sensitiser} + {}^1O_2$$

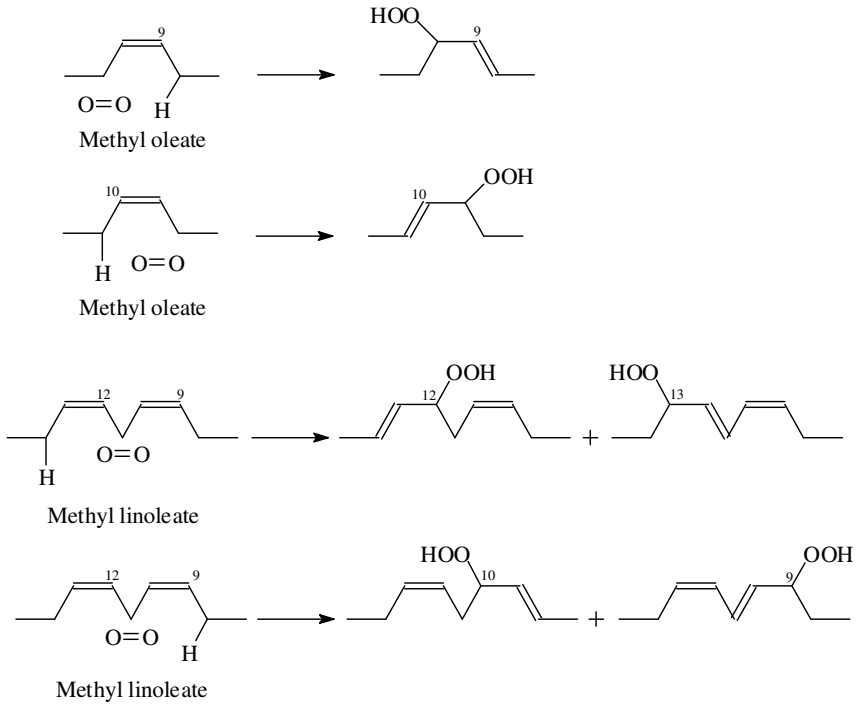


Fig. 2.5. – Photo-oxidation route of hydroperoxide formation.

2.4.3. Lipoxygenase (LOX) route

The enzyme LOX is believed to be widely distributed throughout the plant and animal kingdoms. LOX-catalyzed oxidation differs from the free radical reaction by the formation

of hydroperoxides at a certain position of the chain. Although the basic stoichiometry of LOX is the same as for autoxidation, LOX, in common with most of the enzymes, is very specific about the substrate and how the substrate is oxydized. Linoleic acid is oxidized at positions 9 and 13 by LOX isolated from most natural sources. LOX prefers free fatty acids as substrates and the regiospecificity and sterospecificity of the reaction are illustrated in **Fig. 2.6.**

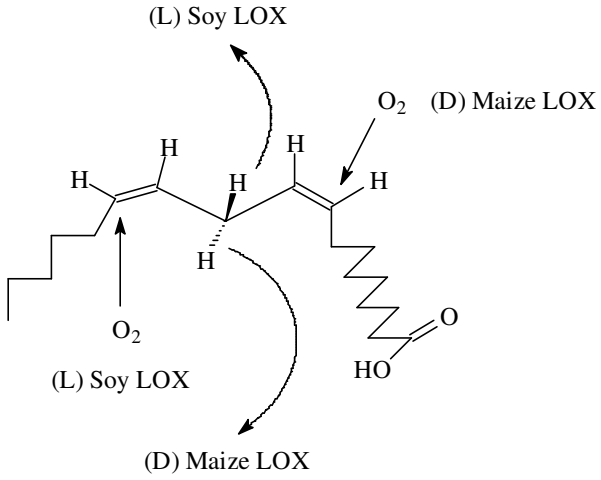


Fig. 2.6. – Steroespecific oxygenation of linoleic acid by lipoxygenase.

2.4.4. Secondary oxidation products

Lipid hydroperoxides are very unstable compounds and break down in several steps, yielding a wide variety of decomposition products. Each hydroperoxide produces a set of initial breakdown products that are typical of the specific hydroperoxide and depend on the position of the peroxide group in the parental molecule. Peroxides first decompose to an alkoxy free radical which break down, mainly by cleavage on either side of the carbon atom bearing the oxygen atom (**Fig. 2.7.**).

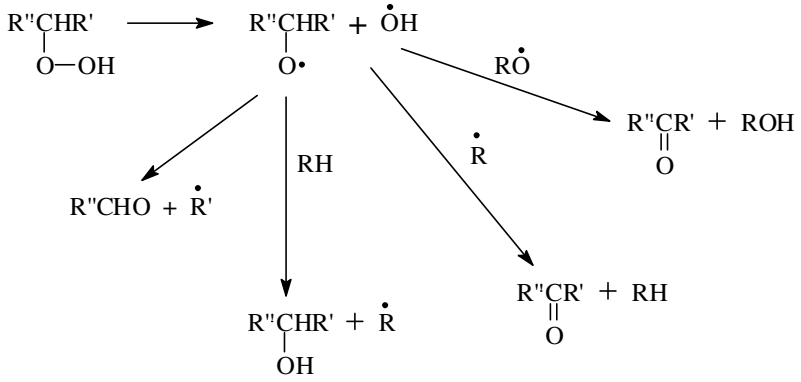


Fig. 2.7.

2.4.4.1. Aldehydes

The mechanism for the cleavage of the alkoxy free radical depends in the cleavage on either side of the carbon atom containing the oxygen atom. The two odd electrons produced on neighbouring atoms can then form the carbonyl double bond. The example illustrated in **Fig. 2.8.** shows the cleavage of 11-hydroperoxyoleic acid methyl ester.

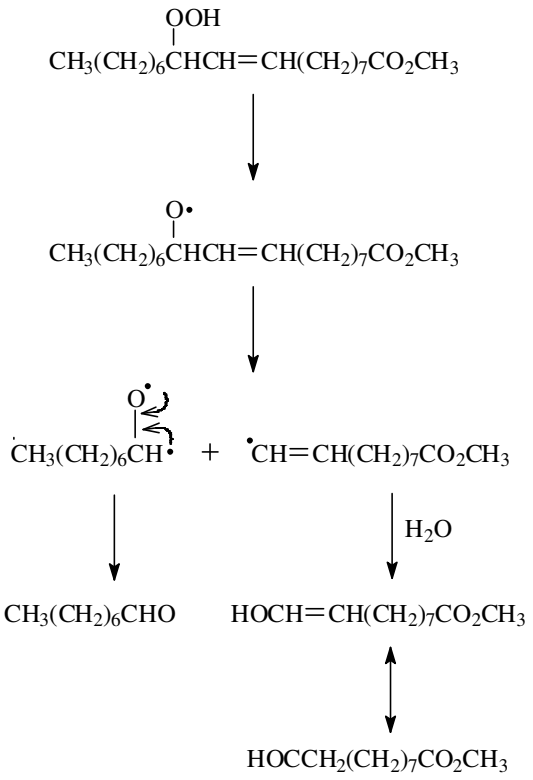


Fig. 2.8.

Thus aldehyde groups can be produced directly and indirectly via an enol, which is simply the tautomeric form of an aldehyde (in this case giving octanal and methyl 10-oxodecanoate respectively). Clearly with the range of hydroperoxides available there are a great many aldehydes which can be produced.

Aldehydes give rise to flavours which are described as ranging from sweet, pungent to oxidized milk. The saturated aldehydes are said to contribute power, warmth, resonance, depth, roundness and freshness to the flavour, whilst the 2-enals and 2,4-dienals are said to contribute sweet, fruity or fatty and oily characters to the flavour. The saturated aldehydes are described as C_2 (fresh pungent), C_3 (fresh, milky), through C_6 (fresh green), C_8 (fresh, citrus), to C_{11} (fatty).

2.4.4.2. Alcohols

The alcohols can be formed by a mechanism which is similar to that for aldehydes. Te alkoxy free radical cleaves t ogive an aldehyde and a hydrocarbon free radical which can pick up an OH· radical to give the alcohol or alternatively pick up an H· radical to form an hydrocarbon as shown in **Fig. 2.9.**

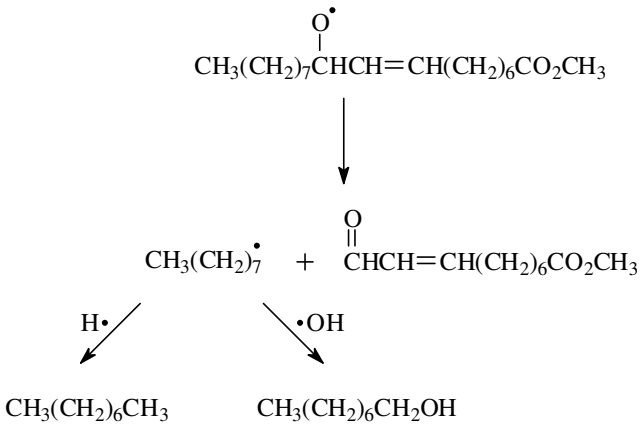


Fig. 2.9.

The alcohols are believed to contribute to the flavour in he same manner as the aldeydes, but in a milder way, ranging from the C_3 saturated alcohol which is described as solventy, nondescript to C_6, decribed as grassy, green, to C_9, described as fatty, green.

2.4.4.3. Hydrocarbons

It is possible to postulate mechanisms similar to those for aldeydes and alcohols to account for the hydrocarbons. In addition, if we postulate that the H·radical is acquired from R'H, we form a new radical R'· with the result that further chain reactions can occur (**Fig. 2.10.**).

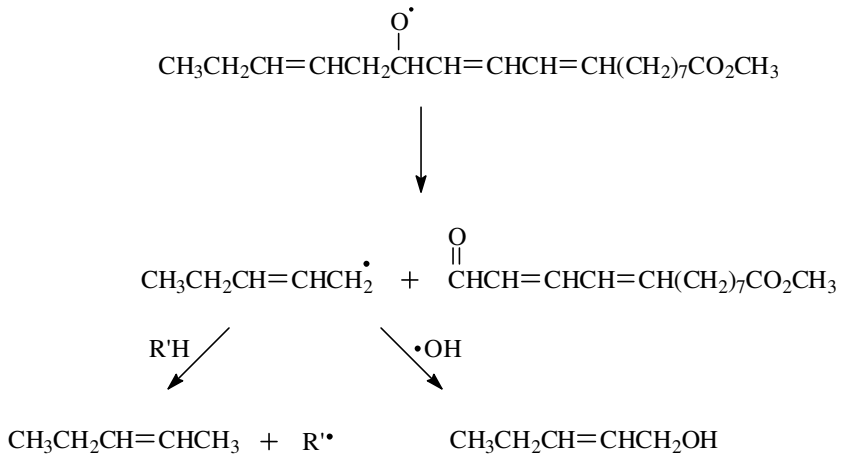


Fig. 2.10.

When these general cleavage methods are applied to methyl linolenate hydroperoxides, a variety of products are obtained, some of which are shown in **Fig. 2.11.**

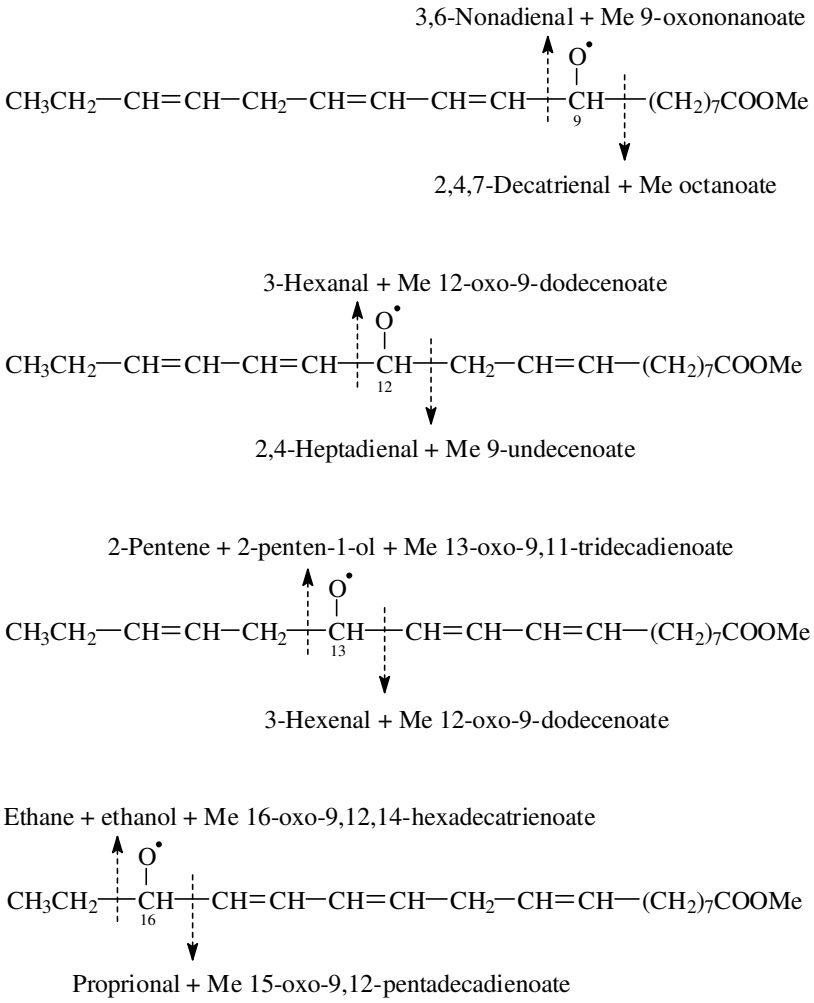


Fig. 2.11. –Decomposition of methyl linolenate hydroperoxides

2.4.5. Hydrolitic rancidity (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 that are virtually absent in fat of living animal tissue. They can however form by enzyme action after the animal is killed.

The release of short-chain FA by hydrolysis is responsible for the development of an undesirable rancid flavour in raw milk. On the other hand, certain typical cheese flavours are produced by deliberate addition of microbial and milk lipases. A controlled and

selective lipolysis is also used in the manufacture of other foods such as yogurt and bread. In contrast to animal fats, oils in mature oil seeds may have undergone substantial hydrolysis by the time they are harvested, giving rise to significant amounts of free fatty acids. Neutralization with alkali is thus required for most vegetable oils after they are extracted. Lipolysis is a major reaction occurring during deep-fat frying due to the large amounts of water introduced from the food and the relatively high temperatures used.

From a chemical standpoint, methyl ketones, the lactones and the esters may be formed primarily by hydrolytic reactions. Thus the glyceride molecule, under the action of heat and moisture, may break down to keto acids, which lose carbon dioxide readily (**Fig. 2.12**.). The release of hydroxyl fatty acids can provide the precursor for γ- or δ-lactones. (**Fig. 2.13.**)

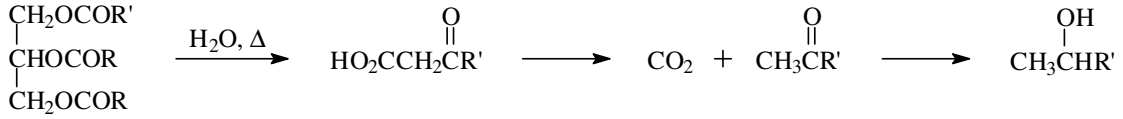


Fig. 2.12.

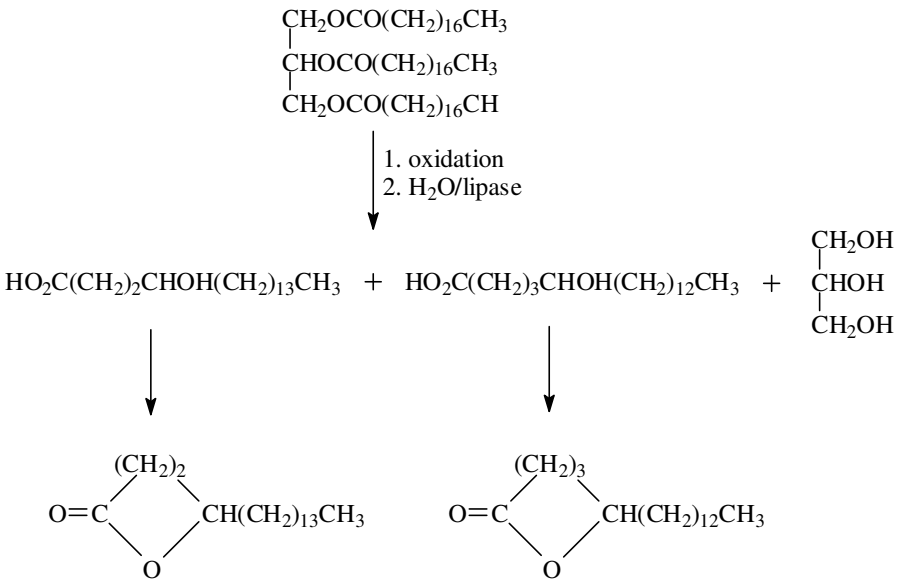


Fig. 2.13.

As formerly stated, hydrolytic reactions provide free fatty acids which can undergo more rapidly autoxidation. Methyl ketones contribute a piercing sweet fruitiness, ranging from C_3, pungent, sweet, through C_7 blue cheesy, to C_{11} fatty , sweet. The aliphatic acids contribute to the flavour by being sour, fruity, cheesy or animal-like. Their contribution ranges from C_2 vinegary, C_3 sour, Swiss cheesy, C_4 sweaty cheesy, C_8 goat cheesy, C_9 paraffinic, to C_{14}-C_{18} with very little odour.

2.4.6. Antioxidants

Antioxidants (AH) are substances that, added in low concentration, can delay onset, or slow rate, of oxidation of autoxidizable materials. Literally hundreds of compounds, both natural and synthesized, have been reported to posses antioxidant properties. Their use in foods, however, is limited by certain requirements not least of which is adequate proof of safety.

The main lipid-soluble antioxidants currently used in food are monohydric or polyhydric phenols with various ring substitutions (**Fig. 2.14**).

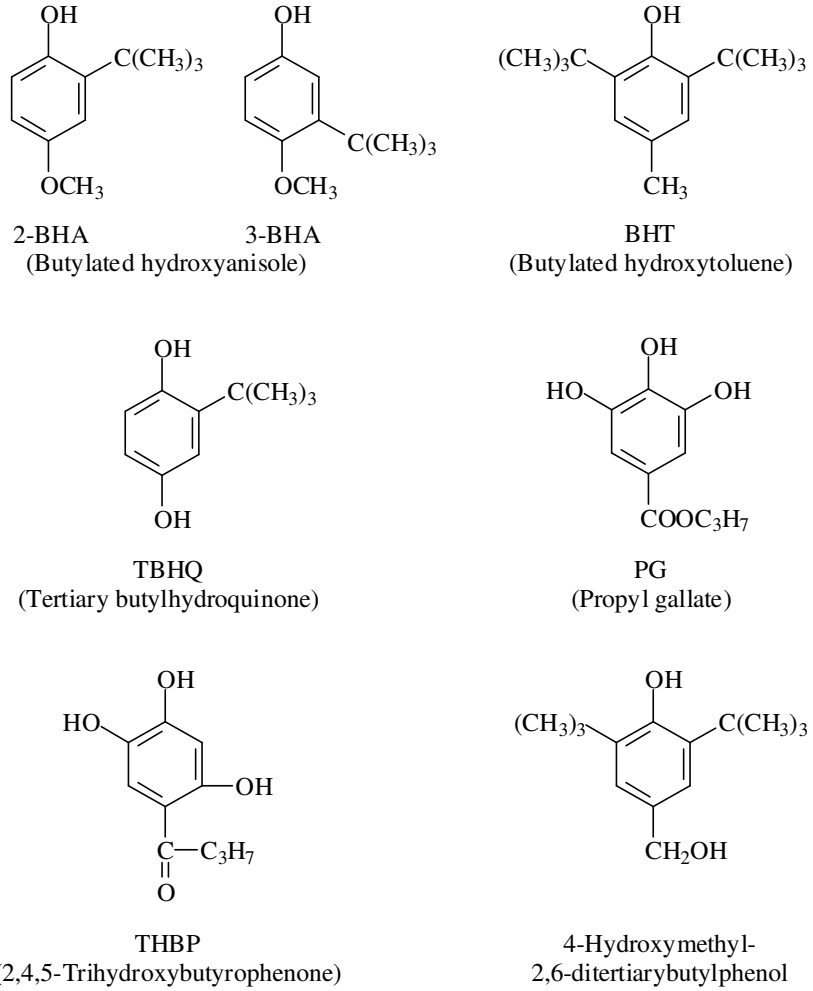


Fig. 2.14. – Major antioxidants used in foods.

Anti-oxidants can interfere with either chain propagation or initiation as follows:

$$ROO\cdot + AH \rightarrow ROOH + A\cdot$$

$$A\cdot + ROO\cdot \rightarrow \text{non radical products}$$

$$A\cdot + A\cdot \rightarrow \text{non radical product}$$

Anti-oxidants commonly used in food lose their efficiency at high temperatures because the hydroperoxides formed as above break down.

There are also preventive anti-oxidants, which act by reducing the rate of chain initiation. Metal inactivators, which coordinate with metal ions capable of catalysing chain initiation, include citric, phosphoric and ascorbic acids. Some preventive anti-oxidants can absorb radiations without forming radicals. Carbon black, phenyl salicylate and hydroxybenzophenone are examples of UV deactivators.

Synergism is the effect obtained when two of these stabilisers are used together. The mixing of the two has a much better effect that either of the stabilisers alone. If a chain-breaking and a preventive anti-oxidant are mixed, both initiation and propagation are suppressed.

2.4.7. Cholesterol and its oxidation products

Cholesterol, with a C 27 carbon skeleton, is a sterol characteristic for higher animals. It is a steroid that is present in all animal tissues as a major structural component of cellular membranes. It is the precursor of bile acids, provitamin B, and the steroid hormones. Cholesterol can be present in the free form or esterified at the hydroxyl group with fatty acids of various chain length and saturation. Cholesterol also occurs in plants, usually in very small quantities, and marine algae. The relationship between dietary cholesterol and total serum cholesterol has been extensively investigated, along with the suggestion that dietary cholesterol contributes a risk factor in the development of coronary heart disease. A lower intake of high-cholesterol foods has been suggested as an effective method for

lowering serum cholesterol levels. The content of cholesterol in some foods is illustrated in **Table 2.5.**

Table 2.5. – Cholesterol content in selected food products

Product	**Cholesterol (mg/100g)**
Skim milk	1.8
Whole milk	13.6
Curd cheese	5-37
Process and hard cheese	51-99
Cream and sweet cream	35-106
Butter	183-248
Pork	72-100
Lard	92
Beef	65-82
Tallow	109
Chicken, whole	75
Turkey, light meat	60
Liver	300-360
Raw whole egg	450
Raw egg yolk	1260
Tuna	38
Cod	73
Lobster	95
Shrimp	152

The expression “cholesterol oxidation products” (COP) or “oxysterols” refers to a group of sterols similar in structure to cholesterol but containing an additional hydroxyl, ketone or epoxide group on the sterol nucleus or a hydroxyl group on the side chain of the molecule. In **Table 2.6.** are presented the names of most prominent COP formed in foods, plasma and tissues.

Table 2.6. – Nomeclature of some cholesterol oxidation products (COP)

Systematic name	Common name	Abbreviated name
Cholest-5-en-3β,7α-diol	7α-Hydroxycholesterol	7α-HC
Cholest-5-en-3β,7β-diol	7β- Hydroxycholesterol	7β-HC
5-Cholestane-3β,5α,6β-triol	Cholestanetriol	CT
5,6α-Epoxy-5β-cholestan-3β-ol	Cholesterol-α-epoxide	α-CE
5,6β-Epoxy-5β-cholestan-3β-ol	Cholesterol-β-eppxide	β-CE
Cholest-5-en-3β-ol-7-one	7-Ketocholesterol	7-kC
Cholest-5-en-3β,20α-diol	20-Hydroxycholesterol	20-HC
Cholest-5-en-3β,25-diol	25-Hydroxycholesterol	25-HC

Oxidation of cholesterol is of major concern because certain oxidation products have been reported to produce cytotoxic, angiotoxic and carcinogenic effects. Cholesterol autoxidation is a well-established free radical process that involves the same chemistry that occurs for the oxidation of unsaturated lipids.

Cholesterol contains one double bond at the carbon-5 position; therefore, the weakest points in its structure are at the carbon-7 and carbon-4 positions. However, due to the possible influence of the hydroxyl group at carbon-3 and the tertiary carbon atom at carbon-5, the carbon-4 position is rarely attacked by molecular oxygen and therefore the abstraction of an allylic hydrogen predominantly occurs at carbon-7 and gives rise to a series of A and B ring oxidation products. In the chain reaction, usually initiated by free radicals, epimeric hydroperoxides of cholesterol and cholesterol epoxides are formed. The presence of tertiary atoms at C-20 and C-25 in side chain adds to the center's sensitivity to oxidation, forming oxysterols (usually called side-chain oxysterols). In general, the epimeric 7α- and 7β-hydroperoxides are recognized as the initial products, with the 7β-hydroperoxides being more abundant that the α-isomers. Decomposition of the hydroperoxides gives rise to the isomeric 7α- and 7β-hydroxycholestyerols, cholesterol α-

and β-epoxides and 7-ketocholesterol, with the latter being a major product. Cholesterol oxidation pathways are shown in **Fig. 2.15.**

Cholesterol oxidation products have been identified in several processed foods including dried eggs, meat and dairy products, fried foods and heated fats.

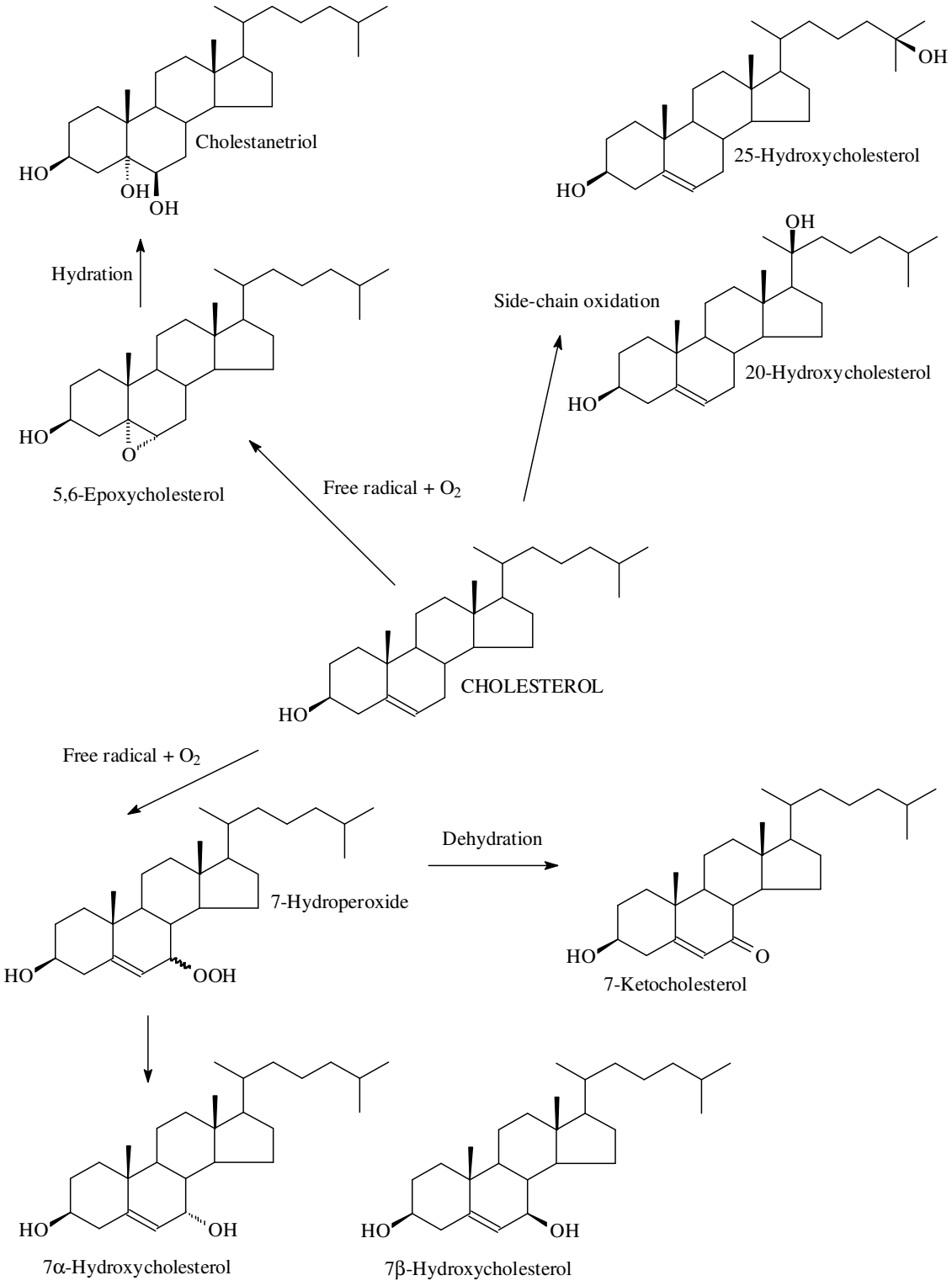


Fig. 2.15. – Cholesterol oxidation pathways.

2.4.8. Methods for measuring lipid oxidation

Lipid oxidation is an exceedingly complex process involving numerous reactions that cause a variety of chemical and physical changes. Although these reactions appear to follow recognized stepwise pathways, they often occur simultaneously and competitively. A single test cannot measure all oxidative events at once, nor can it be equally useful at all stages of the oxidative process, and for all fats, all foods and all conditions of processing. For many purposes, a combination of tests is needed.

2.4.8.1. Peroxide value (PV)

Peroxides are the main initial products of autoxidation. The classical method for quantitation of hydroperoxides is the determination of peroxide value (PV). The hydroperoxide content, generally referred to as PV, is determined by a iodometric method. This is based on the reduction of the hydroperoxide group (ROOH) with iodide ion (I^-). The amount of iodine (I_2) liberated is proportional to the concentration of peroxide present. Released I_2 is assessed by titration against a standardized solution of sodium thiosulfate ($Na_2S_2O_3$) using a starch indicator.

Chemical reactions involved in PV determination are given below:

$$ROOH + 2H^+ + 2KI \rightarrow I_2 + ROH + H_2O + 2K^+$$

$$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaI$$

Potential drawbacks of this method are absorption of iodine at unsaturation sites of fatty acids and liberation of iodine from potassium iodide by oxygen present in the solution to be

titrated. Results may also be affected by the structure and reactivity of peroxides as well as reaction temperature and time. The iodometric method for determination of PV is applicable to all normal fats and oils, but it is highly empirical and any variation in procedure may affect the results. This method also fails to adequately measure low PV because of difficulties encountered in determination of the titration end point. Colorimetric methods are based on the oxidation of Fe^{2+} to Fe^{3+} and determination of Fe^{3+} as ferric thiocyanate.

In studies on the oxidation of biological tissues and fluids, measurement of fatty acid hydroperoxides is more common than measurement of their decomposition products. FA hydroperoxides can be analyzed by high performance liquid chromatography (HPLC) or their corresponding hydroperoxy acid reduction products may be determined by gas chromatography-mass spectrometry (GC-MS). Fluorescence methods have also been developed to determine hydroperoxides by allowing them to react with substances such as luminol and dichlorofluorescein, which form fluorescent products. Although determination of peroxide value is common, its usefulness is generally limited to the initial stages of lipid oxidation. During the course of oxidation, peroxide values reach a peak and then decline.

2.4.8.2. Thiobarbituric acid test (TBA)

Measurement of secondary oxidation products as indices of lipid oxidation is more appropriate since secondary products of oxidation are generally odour-active, whereas primary oxidation products are colourless and flavourless. Secondary oxidation products include aldehydes, ketones, hydrocarbons and alcohols, among others.

TBA test is one of the oldest and most frequently used tests for assessing lipid oxidation in foods and other biological systems. The extent of lipid oxidation is reported as the TBA value and is expressed as milligrams of malonaldehyde (MA) equivalents per kilogram sample or as micromoles MA equivalents per gram sample. MA is a relatively minor product of oxidation of polyunsaturated fatty acids that reacts with the TBA reagent to produce a pink complex with an absorption maximum at 530–532 nm The adduct is formed by condensation of two molecules of TBA with one molecule of MA (**Fig. 2.16.**). Other products of lipid oxidation, such as 2-alkenals and 2,4-alkadienals, also react with the TBA reagent.

There are several procedures for the determination of TBA values. The TBA test may be performed directly on the sample, its extracts or distillate. In case of the distillation method, volatile substances are distilled off with steam. Then the distillate is allowed to react with the TBA reagent in an aqueous medium. The advantage of the distillation method is the absence of interfering substances. In the extraction method, TBA-reactive substances (TBARSs) are extracted from food material into an aqueous medium (i.e., aqueous trichloroacetic acid) prior to colour development with the TBA reagent.

In general, TBA-reactive material is produced in significant amounts from fatty acids containing three or more double bonds. Various compounds, other than those found in oxidized lipids, have been found to react with TBA to yield the characteristic red pigment. Sucrose and some compounds in wood smoke have been reported to give a red colour upon reaction with TBA and act like interfering compound. On the other hand, abnormally low TBA values can result if some of the malonaldehyde reacts with proteins in an oxidizing system. Moreover, flavour scores for different system cannot be consistently estimated

from TBA values because the amount of TBA products from a given amount of oxidation varies from product to product. The TBA test is often useful for comparing samples of a single material at different stages of oxidation.

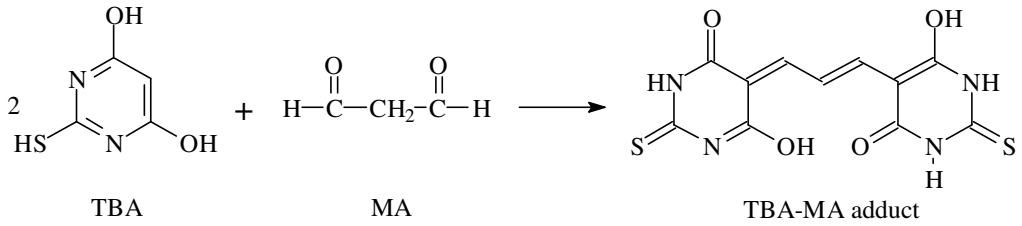


Fig. 2.16. – Reaction of 2-thiobarbituric acid (TBA) and malonaldehyde (MA).

2.4.8.3. Anisidine value

p-Anisidine value (*p*-AnV) is defined as 100 times the optical density measured at 350 nm in a 1.0-cm cell of a solution containing 1.0 g of oil in 100 mL of a mixture of solvent and reagent, according to the IUPAC method. This method determines the amount of aldehyde (principally 2-alkenals and 2,4-alkadienals) in animal fats and vegetable oils. Aldehydes in an oil react with the *p*-anisidine reagent under acidic conditions. The reaction of *p*-anisidine with aldehydes affords yellowish products, as shown in **Fig. 2.17.**

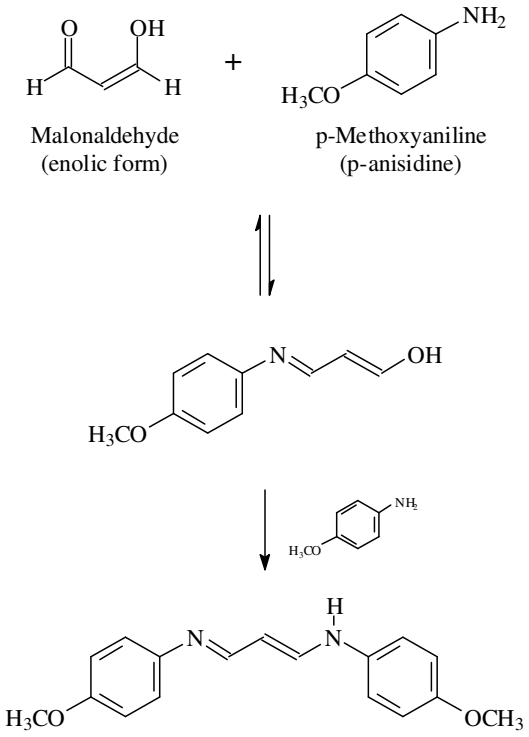


Fig. 2.17. – Possible reaction between *p*-anisidine reagent and maloaldehyde.

2.4.8.4. Total and volatile carbonyl compounds

Methods for determining total carbonyl compounds are usually based on measurement of hydrazones that arise from reaction of aldehydes or ketones (oxidation products) with 2,4-dinitrophenylhydrazine. However, under the experimental condition used for these tests, carbonyl compounds may be generated by decomposition of unstable intermediates, such as hydroperoxides, thus detracting from accuracy of the results. Attempts to minimize such interference have involved reduction of hydroperoxides to noncarbonyl compounds prior to determination of carbonyls or conducting the reaction at a low temperature.

Because the carbonyl compounds in oxidized fats are of relatively high molecular weight, they can be separated by a variety of techniques from lower molecular weight volatile

carbonyl compounds. The lower molecular weight volatile carbonyl compounds are of interest because of their influence on flavour. The volatile carbonyl are usually recovered by distillation at atmospheric or reduced pressure and then determined by the reaction of the distillate with appropriate reagents or by chromatographic methods. Quantitative measurement of hexanal by headspace analysis is a common technique.

2.5. REFERENCES

EUFIC – The European Food Information Council 2006. Fats. In: EUFIC – The Basics 06/2006. Available at the web page: <http://www.eufic.org/article/en/page/BARCHIVE/expid/basics-fats>.

Hamilton, R. J. 1989. The Chemistry of Rancidity in Foods. In: Rancidity in Foods – Second Edition (edited by J. C. Allen and R. J. Hamilton). Pp.: 1-22. London and New York: Elsevier Applied Science.

Kołakowska, A. 2003. Lipid Oxidation in Food Systems. In: Chemical and Functional Properties of Food Lipids (edited by Z. E. Sikorski and A. Kołakowska). Pp.: 133-166. Boca Raton, London, New York, Washington, D.C.: CRC Press.

Kołakowska, A. Sikorski, Z. E. 2003. The Role of Lipids in Food Quality. In: Chemical and Functional Properties of Food Lipids (edited by Z. E. Sikorski and A. Kołakowska). Pp.: 1-8. Boca Raton, London, New York, Washington, D.C.: CRC Press.

Nawar, W. W. 1996. Lipids. In: Food Chemistry – Third Edition (edited by O. R. Fennema). Pp.: 225-319. New York, Basel, Hong Kong: Marcel Dekker.

Nichols, D. S., Sanderson, K. 2003. The Nomenclature, Structure, and Properties of Food Lipids. In: Chemical and Functional Properties of Food Lipids (edited by Z. E. Sikorski and A. Kołakowska). Pp. 29-59. Boca Raton, London, New York, Washington, D.C.: CRC Press.

O'Keefe, S. F. 2002. Nomenclature and Classification of Lipids. In Food Lipids – Chemistry, Nutrition, and Biotechnology – Second Edition, Revised and Expanded (edited by C. C. Akoh, D. B. Min). Pp.: 1-40. New York, Basel: Marcel Dekker.

Shahidi, F., Wanasundara, U. N. 2002. Methods for Measuring Oxidative Rancidity in Fats and Oils. In Food Lipids – Chemistry, Nutrition, and Biotechnology – Second Edition, Revised and Expanded (edited by C. C. Akoh, D. B. Min). Pp.: 465-487. New York, Basel: Marcel Dekker.

Wąsowicz. E. 2003. Cholesterol and Phytosterols. In: Chemical and Functional Properties of Food Lipids (edited by Z. E. Sikorski and A. Kołakowska). Pp. 93-107. Boca Raton, London, New York, Washington, D.C.: CRC Press.

3. DETERMINATION OF (*E*)-10-HYDROXY-2-DECENOIC ACID CONTENT IN PURE ROYAL JELLY: A COMPARISON BETWEEN A NEW CZE METHOD AND HPLC

3.1 SUMMARY AND KEYWORDS

A new CZE method was developed and compared with HPLC for the determination of (*E*)-10-hydroxy-2-decenoic acid (10-HDA) in royal jelly (RJ) samples of different geographical origin. The results obtained with the CZE method were highly correlated with those of HPLC ($p < 0.01$). Under optimized conditions, CZE employed minimal amounts of 50 mM tetraborate buffer as BGE, without the addition of organic solvents, EOF or pH modifiers. The CZE method showed a wide linear response range (0.006-0.808 mg 10-HDA/ml), a good sensitivity (LOD and LOQ were 0.002 and 0.004 mg/ml, respectively) and a satisfactory instrumental repeatability with respect to migration time and peak area (RSD% less than 1.0 and 2.0% on migration time for intra and interday assay respectively and less than 2.0 and for 4.0% on peak area for intra and interday assay respectively). The 10-HDA content in RJ ranged from 0.8 to 3.2 g/100 g of RJ and a significant difference ($p < 0.05$) was found between the Italian and extra-European average values: 2.5 and 1.5 g/100 g of RJ respectively, according to the CZE data. The possibility of application of CZE for routine analyses on RJ and RJ based products to verify their authenticity was here

highlighted.

Keywords: CZE, HPLC, (*E*)-10-hydroxy-2-decenoic acid, royal jelly.

3.2. INTRODUCTION

Royal jelly (RJ) is a yellowish and creamy secretion from hypopharyngeal and mandibular glands of young worker bees (*Apis mellifera* L.) to feed all larvae for the first three days of their life and the queen bee for both her larval life and adulthood. RJ is always fed directly to the queen and larvae as it is secreted and not stored (Piana, 1996). Actually, the significant differences in morphology, development period, life span and behaviour between the queen and worker bees are related to the feeding during the larval stage. Thus RJ is reported as the major cause of this cast differentiation. Although the physiological effects of RJ in humans are not still completely understood, several healthy properties and benefits have been reported (Piana, 1996). To date, RJ is seen as an attractive natural product that undergoes a minimal processing and as a functional food too. RJ is currently consumed not only pure as a dietary supplement but also as ingredient in some foods and preparations like honey, yogurt, jam, fruit juices and medicine-like formulations for its stimulatory effects and cosmetics (Piana, 1996). RJ and especially its protein fraction also showed a high antioxidant activity and a scavenging ability against free radicals that may account for its use in health foods and medicines (Nagai et al., 2001; Nagai and Inoue, 2004). Owing to the growing scientific and economical interest towards this beehive

product, reliable and fast analytical methods to check the quality and authenticity of RJ and RJ based products are required.

A unique and chemically interesting feature in RJ is its lipid fraction which represents 6.2 – 13.2% of dry matter and consists to 80 – 90% (by dry weight) of uncommon short chain (8 to 10 carbon atoms) hydroxy and dicarboxylic free fatty acids (Lercker et al., 1981, 1992-93. These functionalized fatty acids are responsible for most of the biological properties of RJ (Schmidt and Buchmann, 1992). The principal compound is (*E*)-10-hydroxy-2-decenoic acid (10-HDA) which accounts for more than 50% of the free fatty acids and about 1 – 6% of the product (Lercker et al., 1981, 1992-93; Bloodworth et al., 1995; Jia et al., 1995; Genç and Aslan, 1999; Antinelli et al., 2003; Koshio et al., 2003). No other beehive or natural product contains 10-HDA (Barker et al., 1959), thus this fatty acid appears to be specific and may represent a proper marker to access the authenticity of RJ and of those products that claim to contain RJ.

Gas chromatography enables the simultaneous determination of 10-HDA and all the main fatty acids Lercker et al., 1981; Caboni et al., 1994) but it is time-consuming, requiring the extraction of lipid fraction and the successive fatty acids derivatization (Caboni et al., 2004). CE and HPLC are more suitable for the only 10-HDA determination because they do not need any lipid extraction or derivatization processes. Hydroalcoholic or water/acetonitrile/THF mixtures have been used as dissolving agents and mobile phases (Bloodworth et al., 1995; Genç and Aslan, 1999; Antinelli et al., 2003; Koshio et al., 2003) for the 10-HDA determination by HPLC. In HPLC, the 10-HDA elution is obtained in a few minutes but it requires the consumption of large amounts of organic solvents. CE has also been used in the 10-HDA quantification (Jia et al., 1995), adding to BGE an EOF and

an organic modifier to improve efficiency and resolution. In fact CE is a promising analytical technique that in the last years has been extensively reviewed (Monnig and Kennedy, 1994; Cancalon, 1995; Issaq, 1997, 1999; Corradini and Cavazza, 1998), especially for its advantages with respect to gas chromatography and HPLC. The main benefits of CE are a good separation efficiency, ranging from 10^5 to 10^6 theoretical plates, small sample and mobile phase volumes needed and the possibility of working with water mobile phases. This latter feature enables a reduction in laboratory costs with regards to solvents purchase and their environment friendly disposal.

In this study CZE was chosen as the CE operative mode to simplify preparative conditions and because it has been the most employed one for the electrophoretic separation and quantification of small organic acids (Klampfl and Buchberger, 1997; Soga and Ross, 1999; Roselló et al., 2002). In CZE capillary is filled only with a proper BGE and the separation of analytes is obtained by the differential migration of charged solutes in an electric field. The main goal of this work was to develop a new, straightforward and fast CZE method to be daily used for the determination of 10-HDA. As a second purpose, a comparison, which has not been reported to date, between CZE and HPLC was carried out in the 10-HDA quantification in RJ samples with different geographical origin. The two methods were compared in terms of analytical results, analysis time, efficiency, LOD, LOQ and solvent consumption.

3.3. MATERIALS AND METHODS

3.3.1. Chemicals and solvents

10-HDA (assay 98%) was purchased from Larodan AB (Malmö, Sweden). HPLC-grade methanol and water, sodium hydroxide (NaOH) in pellets (assay ≥99%) used for preparing 1 M and 0.1 M NaOH and 85% *orto*-phosphoric acid (H_3PO_4) were from Merck (Darmstadt, Germany). HPCE-grade water and sodium tetraborate decahydrate ($B_4Na_2O_7 \cdot 10H_2O$, assay ≥99.5%) were from Fluka (Buchs, Switzerland). Deionized water was obtained from an Elix 10 water purification system from Millipore (Bedford, MA, USA).

3.3.2. RJ samples

Eight RJ samples were purchased from Italian local beekeepers and were named as IT1, IT2, IT3, IT4, IT5, IT6, IT7 and IT8 while seven commercial samples were from different extra-European countries and were named as E1, E2, E3, E4 (the latter two both from Australia), E5 (from China) and E6 (from South America). The Italian samples were from the Emilia-Romagna region, in particular they were produced in an area, including the provinces of Bologna and Ferrara and Romagna district, situated in the North-East of Italy. The geographical origin of the samples E1 and E2 was unknown. All pure RJ samples came in the form of cream. They were kept, in dark pots or repaired from light, at 4 °C just after harvesting except E3 that was stored at room temperature for three months and then at 4 °C like all the other samples. E3 and E4 came from the same RJ stock. All the Italian samples

were obtained through the traditional way except IT3 which was obtained using an organic method. The production ways adopted for the extra-European samples were unknown. All the samples were harvested in 2003. To validate the accuracy and feasibility of the CZE method E1 sample was employed.

3.3.3. Electrophoretic and chromatographic conditions

The CZE analyses were performed with a CE instrument P/ACE 5500 from Beckman (Fullerton, CA, USA) equipped with a single wavelength UV/VIS detector. The processing and data acquisition were accomplished through a software from Beckman (Beckman P/ACE Station - Capillary Electrophoresis Software, version 1.21). The capillary cartridge contained a polyimide coated fused silica tube (375 μm o.d., 50 μm i.d.) supplied from Beckman. The total capillary length was 47 cm whereas the effective length was 40 cm. The running buffer was 50 mM sodium tetraborate (pH = 9.42) prepared dissolving a proper amount of the salt in HPLC-grade water, filtering the obtained solution through a cellulose acetate 0.45 μm syringe filter from Orange Scientific (Braine-l'Alleud, Belgium) and then sonicating for 10 min. 0.1 and 1.0 M NaOH solutions used for washing steps were prepared in HPLC-grade water. At the beginning of the work, the capillary was conditioned, at 30 °C, by flushing with 1 M NaOH for 5 min, 0.1 M NaOH for 5 min, HPCE-grade water for 5 min and the running buffer for 5 min. Each injection was performed hydrodinamically at the anodic end: the sample was loaded onto the capillary for 5 s at low pressure mode (0.5 psi, 1 psi = 6894.76 Pa) whereas all the conditioning and washing steps were performed at high pressure mode (20 psi). The electrophoretic runs

were carried out at 27 kV for 5 min at 35 °C, for a resulting current ranging from 110 to 120 μA. The capillary was rinsed once at the beginning of the day with the running buffer for 5 min and between each run with 0.1 M NaOH for 2 min, HPCE-grade water for 2 min and running buffer for 2 min. At the end of each electrophoretic run the capillary was rinsed for 2 min with and stored in HPCE-grade water to prevent BGE crystallization. The running buffer was changed after the first daily run and then every two runs. The overall run time was 13 min. The detection was performed at 214 nm whereas other instrumental parameters like rise time, ramp time and data rate were respectively set at 0.2 s, 0.17 s and 10 Hz. 10-HDA was identified using a standard solution and comparing the migration times while the quantification of the same compound in the different samples was accomplished by means of a calibration curve.

The HPLC analyses were performed on an apparatus from Jasco (Tokyo, Japan), equipped with a binary pump (model PU-1580), an autosampler (model AS-2055 Plus) and a diode array UV/VIS detector (model MD-1510, quartz flow cell, 10 mm optical path). The data processing was performed with a software from Jasco (Jasco-Borwin, version 1.50). A column Luna 5 μm C_{18} (2), 250 × 4.6 mm i.d., 5 μm particle size from Phenomenex (Torrance, CA, USA) was used at room temperature. The mobile phase was water/methanol 35/65 (v/v), adjusted at pH 2.50 with *orto*-phosphoric acid, filtered through a nylon 0.20 μm filter disk from Albet (Barcelona, Spain) and degassed in an ultrasonic bath for 10 min. Methanol and water were of HPLC-grade. An isocratic elution was carried out at a flow rate of 1.0 ml/min; the run time was 9 min and the injection volume was 20 μl. Each chromatogram was recorded at 210 nm whereas the absorption spectra between 195 and

400 nm. The 10-HDA identification and quantification were accomplished in the same way described for the CZE analyses.

3.3.4. Recovery evaluation

About 1 g of honey purchased from a local supermarket was weighed in a 100 ml glass bottle, added with 50 ml of deionized water and 1 ml of a water standard solution of 10-HDA at a concentration of 0.808 mg/ml. The mixture was sonicated until complete dissolution, diluted twice in a 10 ml volumetric flask and finally centrifuged at 3000 rpm for 10 min. This procedure was repeated five times (n = 5) and 10-HDA recovery was assessed using the CZE optimized method. The recovery was calculated as a percentage, comparing the amount of 10-HDA determined by CZE and the known amount added at the beginning of the test.

3.3.5. Sample preparation before CZE and HPLC analyses

About 400 mg of each RJ sample were accurately weighed in a 100 ml glass bottle, added with 50 ml of deionized water, sonicated in an ultrasonic bath at room temperature until a complete dissolution of RJ (30-60 min with occasional shaking) and finally diluted five times in a 25 ml volumetric flask. The CZE and HPLC analyses were carried out on the supernatant fraction after centrifugation at 3000 rpm for 10 min. The procedure above described was repeated in triplicate (n = 3) on each RJ sample.

3.3.6. Calibration curves, sensitivity and efficiency evaluation

Two stock solutions of 10-HDA were prepared dissolving 41.2 and 25.6 mg of the standard

compound in 30-40 ml of deionized water in a 50 ml volumetric flask, sonicating until a complete dissolution and making the volume to the mark, for a final concentration of 0.808 and 0.502 mg/ml respectively. Each standard solution was then used for the preparation of less diluted solutions: from 0.006 to 0.101 mg/ml for CZE and from 0.001 to 0.125 mg/ml for HPLC. The solutions were injected three times and twice respectively for CZE and HPLC. LOD and LOQ were estimated evaluating the noise of the electropherograms and HPLC chromatograms. LOD and LOQ were respectively set at S/N = 3 and S/N = 7 where S/N was the signal-to-noise ratio. Efficiency was evaluated in terms of and expressed as the number of theoretical plates (N) and calculated by the following formula: $N = 5.54 \times (MT / w_{½})$ where MT is 10-HDA migration time and $w_{½}$ is the peak width at half-height.

3.3.7. Determination of the moisture content

The moisture content was determined by the Karl Fischer (KF) titration method. The KF titrations were carried out on a Micro TT2050 titrator from Crison (Barcelona, Spain), applying the two component technique with Hydranal Composite 5 as titrating solution and Hydranal Composolver E as working medium, both purchased from Riedel-de Haën (Seelze, Germany). The correction factor was determined titrating 10 µl of deionized water. About 30 mg of each sample were exactly weighted and directly introduced into the titration cell.

3.3.8. Statistics

The results were expressed as the mean of the values obtained for each sample (n = 3), SD

was chosen as spreading index while RSD% was used to express the precision of each determination. The data underwent one-way analysis of variance (ANOVA) using the software Statistica 6.0 from StatSoft (Tulsa, OK, USA). Unless specified, Tukey's honest significant differences and Pearson's linear correlations were evaluated at a $p < 0.05$ level.

3.4. RESULTS AND DISCUSSION

3.4.1. CZE method optimization

The CZE method optimization was performed using the sample E1. The washing steps described in Section 2.3 were the same adopted elsewhere (Bonoli et al., 2004). A tetraborate buffer was chosen as BGE because of it enabled 10-HDA elution in a few minutes, without employing any further additives as organic, EOF or pH modifiers. The buffer pH was not changed. BGE was tested at different concentrations, from 10 to 100 mM. Although the highest efficiency ($2.5 – 3.0 \times 10^5$ theoretical plates) and the lowest 10-HDA migration time (2.0 – 2.5 min) were achieved employing 10 and 20 mM tetraborate, the best compromise in terms of efficiency ($\approx 1.5 \times 10^5$ theoretical plates), baseline stability, migration time and current intensity was obtained with 50 mM tetraborate as shown in **Fig. 3.1.** Other instrumental parameters investigated were temperature and voltage applied during running. A raise in both of them (from 20 to 40 °C and from 15 to 30 kV) caused a decrease in the 10-HDA migration time and did not significantly affect separation efficiency ($\approx 1.5 \times 10^5$ theoretical plates). Nevertheless, a worsening in baseline was observed at the same time. Thus 35 °C and 27 kV were respectively chosen as

operative voltage and temperature. Different injection times were also assayed. From 1 to 7 s, as expected, an increase in the 10-HDA area was observed even a low progressive peak broadening took place and efficiency fell under 1.0×10^5 theoretical plates ($\approx 0.8 \times 10^5$). The best compromise between sensitivity and efficiency was found at 5 s. Under optimized conditions 10-HDA eluted in 3.9 min (**Fig. 3.2.**) and current was comprised between 110-120 μA. No current problem occurred during analyses.

3.4.2. Effect of the sample treatment and the type of solvent on the 10-HDA recovery

Different tests were performed to check which were the pre-injection sample treatment and the type of solvent more suitable for RJ. Two RJ aliquots (about 400 mg each) from the same RJ sample (E1) were dissolved using respectively 50 ml of deionized water (test A1) and 50 ml of water/methanol 1/1 (v/v) (test A2). After a complete dissolution in an ultrasonic bath and a five-time dilution in a 25 ml volumetric flask, from each mixture ten aliquots (about 2-3 ml each) were taken: five ones were centrifuged before CZE analysis at 3000 rpm for 10 min, while the other ones were filtered through 0.45 μm cellulose acetate syringe filter. To prove the only effect of solvent, ten aliquots of the same RJ sample, about 400 mg each, were weighed: five aliquots were dissolved in water (test B1) while the other ones in water/methanol 1/1 (v/v) (test B2). After dilution, centrifugation was performed (3000 rpm × 10 min). In **Table 3.1.** are shown the results from this investigation.

When RJ was dissolved in water, filtration caused a significant loss (about 40%) in the 10-HDA content: 0.9 vs. 1.5 g of 10-HDA/100 g of RJ using filtration and centrifugation respectively. On the contrary, no significant difference was found when a hydroalcoholic

mixture was employed, although with this mixture a significantly lower 10-HDA content was achieved. To verify the latter outcome, different aliquots of the same RJ sample were weighed and successively dissolved in water and water/methanol 1/1 (v/v). Water led to a 10-HDA content significantly higher than that obtained using the hydroalcoholic mixture, confirming the former results. Tests B1 and B2 also enabled a repeatability evaluation of the whole analytical method. Repeatability was better employing water in place of water/methanol: 4.0 vs. 9.0 in terms of RSD% respectively. The RJ solutions in water were clearer than those in water/methanol, depending on the bigger dissolving power of water. Hydroalcoholic mixture did not allow a complete dissolution of 10-HDA that could be still bound to the protein fraction. This amount of 10-HDA which was not dissolved did not interact with syringe filter surface and was not kept during filtration but lost in centrifugation as a white precipitate. Thus no significant difference was found in 10-HDA content after filtration or centrifugation when water/methanol was employed as solvent. Water and centrifugation proved to be definitely the most suitable dissolving agent and clean-up procedure for this kind of product.

3.4.3. Recovery and repeatability study

The recovery trials were carried out spiking a commercial honey sample with a 10-HDA standard solution. The average percentage recovery (n = 5) was 90.5 ± 3.8 (RSD% = 4.2). The result was satisfactory considering the low 10-HDA concentration in the spiked honey aliquots dissolved in water (≈ 0.008 mg/ml). The absence of 10-HDA in honey was verified through CZE before performing recovery assays. The instrumental repeatability was accessed for CZE and HPLC employing a 10-HDA water solution (≈ 0.1 mg/ml) and a RJ

sample (E1) dissolved in water (≈ 0.03 mg 10-HDA/ml). The two solutions were consecutively injected ten times on the same day (intraday precision, n = 10) and on three consecutive days (interday precision, n = 30); the RSD% of the 10-HDA peak area and migration time were determined. The results are shown in **Table 3.2.**

As expected, both for CZE and HPLC, the intraday precision was generally higher than the interday precision. HPLC showed on the whole a better intra and interday precision, probably depending on the goodness of the external autosampler system connected to the HPLC apparatus. Nevertheless, the CZE method showed a good repeatability that never exceeded 2.0% (intraday) and 4.0% (interday).

3.4.4. CZE and HPLC performances

The HPLC determinations were accomplished according to Bloodworth et al. (1995) with a slight modification regarding the mobile phase composition: the ratio water/methanol (v/v) was changed from 55/45 to 35/65. Under the conditions formerly described, 10-HDA eluted in about 6 min as shown in **Fig. 3.3.**

The optimized CZE method showed a separation efficiency about 10 times higher than HPLC: $\approx 1.0 \times 10^5$ theoretical plates for CZE vs. $\approx 1.0 \times 10^4$ for HPLC. This was due first to the plain profile of EOF generated in CZE and also to the small amount of sample that had to be loaded onto the capillary (less than 50 nl in CZE vs. 20 µl in HPLC).

The main parameters of the two calibration curves employed in CZE and HPLC are illustrated in **Table 3.3.** In HPLC, LOD and LOQ were an order of magnitude lower than those found in CZE, owing to the smaller optical path of the UV/VIS detector in CZE (50

μm, detection "on-column") with respect to that of the system fitted on HPLC (10 mm). Considering that in HPLC the volume of sample injected was nearly a thousand times higher than the one loaded in CZE, LOD and LOQ performed by CZE were highly satisfactory. Both of the two calibration curves which were built to quantify 10-HDA showed a high correlation between peak area and analyte concentration. The calibration range employed to quantify 10-HDA in CZE was from 0.006 to 0.101 mg/ml even a high correlation was found for a wider range: 0.006 – 0.808 mg/ml (r^2 = 0.999).

In comparison to the previous CZE method (Jia et al., 1995), the one optimized here offered similar performances in terms of sensitivity (0.002 vs. 0.0005 mg of 10-HDA/ml regarding LOD, even if in Jia et al. (1995) LOD was set at S/N = 2) and analysis time (13 min vs. 15 min.), without employing any kind of additives. CZE offered a minimal solvent consumption: for 100 analyses, CZE needed approximately 50 ml of tetraborate buffer while in HPLC about 1000 ml of hydroalcoholic mobile phase were consumed.

3.4.5. Analyses of pure RJ by CZE and HPLC

Table3.4. shows the 10-HDA content in the various RJ samples. The statistical analysis accessed a significant correlation between the mean values obtained in CZE and HPLC (r^2 = 0.971, $p < 0.01$) and no significance difference between the couples of data for each sample, confirming the goodness of the CZE method here-proposed. The CZE and HPLC data showed a good repeatability: RSD% values never exceeded 6% for CZE and 5% for HPLC. Only for the sample E5 RSD% was higher than 15%, since the presence of white granules did not enable a homogeneous sampling.

A significant difference was found between the 10-HDA content in the Italian and the extra-European RJs (**Table 3.4.**): in the Italian RJ the average amount of 10-HDA was about 60% higher than in the extra-European one. Seven out of the eight Italian samples showed a 10-HDA content higher than 2.0 g/100 g of RJ whereas amongst the extra-European RJs, no-one showed an amount higher than 2.0 g/100 g of RJ. In agreement with formerly reported results (Antinelli et al., 2003), 10-HDA proved not to be a suitable criterion to evaluate RJ freshness. Indeed, sample E3 presented a higher 10-HDA content than E4 despite E3 was stored for three months at room temperature. Even though RJ has a limited shelf-life, 10-HDA degradation was low (from 8 to 17 %) during a long term storage (one year) at 4 °C (Antinelli et al., 2003). It was remarkable that the 10-HDA content found in the sample IT7, the only Italian RJ produced according to an innovative technique, was the lowest in all the samples analyzed. The result obtained for IT7 suggested that a deeper investigation between the 10-HDA content and the production technique adopted should be helpful in spreading light on the differences between Italian and extra-European RJs. Moreover, other reasons that may account for these findings are handling during storage (Antinelli et al., 2003) (repeated homogenization, short variations of temperature), harsh heat treatments or the addition to RJ of other bee products like honey. To date, no study has dealt with the stability of 10-HDA in RJ and the effect of processing on this parameter.

3.4.6. Moisture content

To verify whether differences previously discussed with respect to the 10-HDA content depended on the water amount, the moisture content was evaluated in RJ (data not showed)

employing the Karl Fischer (KF) titration method. KF method has previously been adopted for RJ (Garcia-Amoedo and Alemeida-Muradian, 2002) and honey (Isengard et al., 2001). No significant correlation was accessed between the water and 10-HDA content and no significant difference was found between the mean values for Italian (average value: 53.9 ± 4.2 g of water/100 g of RJ) and extra-European RJ (average value: 50.0 ± 5.2 g of water/100 g of RJ). The data confirmed that moisture did not account for the differences found in the 10-HDA content between the Italian and extra-European samples. On the contrary, unexpected high values were found in those samples like IT3 (54.6 g of water/100 g of RJ) and IT5 (57.5 g of water/100 g of RJ) that proved to be the richest in 10-HDA.

3.5. CONCLUDING REMARKS

A fast and reliable CZE method for the determination of 10-HDA in RJ was developed in this study and compared with HPLC. The two methods were applied in the quantification of 10-HDA in pure RJ samples of different geographical origin. This study demonstrated that CZE gives comparable performance to HPLC in terms of analytical results, efficiency, sensitivity and analysis time without employing any EOF, pH or organic modifiers. A high instrumental repeatability, a lower solvent consumption and the use of aqueous solution as BGE make CZE an effective alternative to HPLC for accessing the quality of RJ and RJ based preparations in routine analyses. This study represented a first attempt to compare and characterize Italian and extra-European RJs according to their 10-HDA content. A higher amount of 10-HDA was found in the Italian RJ than in the extra-European one. The

different moisture content did not account for this finding. Actually further investigations should be carried out to evaluate how environmental and primarily technological factors may affect the 10-HDA content in RJ.

3.6. REFERENCES

Antinelli, J. F., Zeggane, S., Davico, R., Rognone, C., Faucon, J. P., Lizzani L. 2003. Evaluation of (E)-10-hydroxydec-2-enoic acid as a freshness parameter for royal jelly. Food Chemistry, 80, 85-89.

Barker, S. A., Foster, A. B., Lamb, C. D., Hodgson, N. 1959. Identification of 10-hydroxy-Δ^2-decenoic acid in royal jelly. Nature, 183, 996-997.

Bloodworth, B. C., Harn, C. S., Hock, C. T., Boon, Y. O. 1995. Liquid chromatographic determination of trans-10-hydroxy-2-decenoic acid content of commercial products containing royal jelly. Journal of the AOAC International, 78, 1019-1023.

Bonoli, M., Marconi, E.,Caboni, M. F. 2004. Free and bound phenolic compounds in barley (*Hordeum vulgare* L.) flours: Evaluation of the extraction capability of different solvent mixtures and pressurized liquid methods by micellar electrokinetic chromatography and spectrophotometry. Journal of Chromatography A, 1057, 1-12.

Caboni, M. F., Sabatini, A. G., Lercker, G. 2004. La gelatina reale: origine, proprietà e composizione. APOidea-Italy, 1, 72-79.

Caboni, M. F., Solazzo, P., Lercker G. 1994. Proposta di un metodo per la valutazione quali-quantitativa della gelatina reale nei prodotti alimentari. Bollettino dei Chimici Igienisti-Italy. Chim. Igien.-Italy, 45, 195-202.

Cancalon, P. F. 1995. Capillary electrophotesis: a new tool in food analysis. Journal of the AOAC International, 78, 12-15.

Corradini, C., Cavazza, A. 1998. Application of capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) in food analysis. Italian Journal of Food Science, 10, 299-316.

Garcia-Amoedo, L. H., Almeida-Muradian, L. B. 2002. Comparação de metodologias para a determinação de umidade em geléia real. Química Nova, 25, 676-679.

Genç, M., Aslan, A. 1999. Determination of trans-10-hydroxy-2-decenoic acid content in pure royal jelly and royal jelly products by column liquid chromatography. Journal of Chromatography A, 839, 265-268.

Isengard, H. D., Schultheiß, D., Radović, B., Anklam E. 2001. Alternatives to official analytical methods used for the water determination in honey. Food Control, 12, 459-466.

Issaq, H. J. 1997. Capillary electrophoresis of natural products. Electrophoresis, 18, 2438-2452.

Issaq, H. J. 1999. Capillary electrophoresis of natural products-II. Electrophoresis, 20, 3190-3202.

Jia, L., Zhang, H. X., Kou, X. L., Hu, Z. D. 1995. Separation and determination of 10-hydroxy-2-decenoic acid in royal jelly by capillary electrophoresis. Chromatographia, 41, 605-609.

Klampfl, C. W., Buchberger, W. 1997. Determination of low-molecular-mass organic acids by capillary zone electrophoresis. Trends in Analytical Chemistry, 16, 221-229.

Koshio, S., Almeida-Muradian, L. B. 2003. Aplicação da clae para determinação do ácido

10-hidróxi-2-decenóico (10-HDA) em geléia real pura e aficionada a mel brasileiro. Química Nova, 26, 670-673.

Lercker, G., Caboni, M. F., Vecchi, M. A., Sabatini, A. G., Nanetti, A. 1992-93. Caratterizzazione dei principali costituenti della gelatina reale. Apicoltura-Italy, 8, 27-37.

Lercker, G., Capella, P., Conte, L. S., Ruini F. 1981. Components of royal jelly: I. Identification of the organic acids. Lipids 16, 912-919.

Monnig, C. A., Kennedy, R. T. 1994. Capillary electrophoresis. Analytical Chemistry, 66, 280R-314R.

Nagai, T., Inoue, R. 2004. Preparation and the functional properties of water extract and alkaline extract of royal jelly. Food Chemistry, 84, 181-186.

Nagai, T., Sakai, M., Inoue, R., Inoue, H., Suzuki N. 2001. Antioxidative activities of some commercially honeys, royal jelly, and propolis. Food Chemistry, 75, 237-240.

Piana, L. 1996. Royal Jelly. In: Krell, R., Value-added Products from Beekeeping, FAO Agricultural Services Bulletin 124, Rome.

Roselló, S., Galiana-Balaguer, L., Herrero-Martínez, J. M., Maquieira, A., Nuez, F. 2002. Simultaneous quantification of the main organic acids and carbohydrates involved in tomato flavour using capillary zone electrophoresis. Journal of the Science of Food and Agriculture, 82, 1101-1106.

Schmidt, J. O., Buchmann, S. L. 1992. Other Products of the Hive. In: Graham, J.M., The Hive and the Honey Bee (edited by J. M. Graham). Pp.: 927-988. Hamilton, IL: Dadant & Sons.

Soga, T., Ross, G. A. 1999. Simultaneous determination of inorganic anions, organic acids,

amino acids and carbohydrates by capillary electrophoresis. Journal of Chromatography A, 837, 231-239.

3.7. TABLES

Table 3.1. – Results from the optimization of pre-injection sample treatment steps

Test[1)]	Solvent	Sample treatment[2)]	10-HDA content[3)] Mean ± SD (n = 5)	RSD%
A1	H_2O	C	1.5 ± 0.0^{a}	2.7
	H_2O	F	0.9 ± 0.0^{c}	4.5
A2	H_2O/MeOH	C	1.2 ± 0.1^{b}	4.3
	H_2O/MeOH	F	1.2 ± 0.1^{b}	6.1
B1	H_2O	C	1.5 ± 0.1^{y}	4.0
B2	H_2O/MeOH	C	1.4 ± 0.1^{z}	9.0

[1)]In tests A1-A2 and B1-B2 were respectively evaluated the effect of the sample clean-up treatment and the type of solvent on the 10-HDA content; each test is described in Section 3.2

[2)]C: centrifugation; F: filtration through cellulose syringe filter

[3)]g/100 g of RJ determined by CZE (sample E1)

[a-c, y-z)]Different superscript letters on the mean values indicate significant differences ($p < 0.05$)

Table 3.2. – Instrumental precision of CZE and HPLC. The values reported are RSD% of the analytical parameters evaluated

Analytical technique	10-HDA standard solution (≈ 0.1 mg/ml)				RJ sample (≈ 0.03 mg/ml)[1)]			
	Area[2)]		MT[3)]		Area		MT	
	Intra[4)]	Inter[5)]	Intra	Inter	Intra	Inter	Intra	Inter
CZE	0.6	3.7	0.4	1.9	1.6	1.5	0.5	1.4
HPLC	1.0	0.7	0.2	1.4	0.2	1.0	0.3	1.2

[1)]10-HDA concentration of the solution analyzed

[2)]10-HDA peak area

[3)]10-HDA migration time

[4)]Intraday assay (n = 10)

[5)]Interday assay (n = 30)

Table 3.3. – CZE and HPLC calibration curves parameters

Analytical technique	10-HDA concentration (mg/ml)	Regression equation[1)]	r^2	S_m[2)]	S_b[3)]	LOD (mg/ml)[4)]	LOQ (mg/ml)[5)]
CZE	0.006 – 0.101	$A = 293079.2c - 63.7$	0.999	2299.09	119.8	0.002	0.004
HPLC	0.001 – 0.125	$A = 68748119.1c + 89996.9$	0.999	705554.7	36131.7	0.0001	0.0002

[1)] A is 10-HDA peak area and c is 10-HDA concentration expressed in mg/ml.

[2)] S_m: standard deviation of the slope

[3)] S_b: standard deviation of the intercept

[4)] LOD = 3 × S/N where S/N is the signal-to-noise ratio

[5)] LOQ = 7 × S/N

Table 3.4. – 10-HDA content, determined by CZE and HPLC, in Italian and extra-European RJ samples. The values are expressed as g of 10-HDA/100 g of RJ (n = 3).

	CZE		HPLC	
Samples	Mean ± SD	RSD%	Mean ± SD	RSD%
IT1	2.5 ± 0.1^{c}	3.8	2.6 ± 0.1^{e}	2.3
IT2	$2.9 \pm 0.1^{a,b}$	4.6	$2.8 \pm 0.0^{c,d}$	1.5
IT3	3.1 ± 0.2^{a}	5.4	$3.0 \pm 0.0^{a,b}$	0.8
IT4	3.2 ± 0.1^{a}	2.5	3.2 ± 0.0^{a}	0.2
IT5	3.0 ± 0.1^{a}	3.6	$2.9 \pm 0.1^{b,c}$	2.1
IT6	$2.6 \pm 0.1^{b,c}$	2.5	$2.6 \pm 0.0^{d,e}$	1.5
IT7	0.8 ± 0.0^{g}	4.3	0.8 ± 0.0^{k}	4.2
IT8	2.3 ± 0.0^{c}	2.1	2.3 ± 0.1^{f}	3.6
E1	$1.5 \pm 0.0^{e,f}$	2.0	$1.5 \pm 0.0^{h,i}$	0.6
E2	$1.7 \pm 0.0^{d,e}$	1.1	1.7 ± 0.0^{h}	1.6
E3	1.9 ± 0.0^{d}	1.5	1.9 ± 0.0^{g}	0.3
E4	$1.5 \pm 0.1^{e,f}$	3.7	1.4 ± 0.0^{i}	1.6
E5	$1.7 \pm 0.3^{d,e}$	19.8	1.2 ± 0.2^{j}	16.9
E6	1.2 ± 0.0^{f}	2.3	1.2 ± 0.0^{j}	3.9
Italian[1)]	2.5 ± 0.7^{y}	28.9	2.5 ± 0.7^{y}	28.9
Extra-European[2)]	1.6 ± 0.3^{z}	16.3	1.5 ± 0.3^{z}	19.2

[1)]n = 24

[2)]n = 18

[a-k, y-z)]Different superscript letters on the mean values within the same row indicate significant differences ($p < 0.05$)

3.8. LEGENDS TO FIGURES

Figure 3.1. – Effect of tetraborate buffer concentration on the 10-HDA separation in CZE. Buffer concentrations: 10 mM (trace A), 20 mM (trace B), 50 mM (trace C) and 100 mM (trace D). Other instrumental conditions (not optimized): fused silica capillary 47 cm × 50 μm i.d.; applied voltage: 30 kV; capillary temperature: 30 °C; hydrodynamic injection: 0.5 psi for 3 s; detection accomplished at 214 nm. Beside each trace the relative current intensity is reported. The asterisk-marked peak is 10-HDA whereas other peaks were not identified.

Figure 3.2. – Typical CZE electropherogram under optimized conditions of a RJ sample (E1) dissolved in water. Separation conditions: fused silica capillary 47 cm × 50 μm i.d.; applied voltage: 27 kV; capillary temperature: 35 °C; BGE: 50 mM tetraborate buffer (pH 9.42); hydrodynamic injection: 0.5 psi for 5 s; detection accomplished at 214 nm. Other peaks different from 10-HDA were not identified.

Figure 3.3. – Typical HPLC chromatogram of a RJ sample (E1) dissolved in water. Separation conditions: column Luna 5 μm C_{18} (2), 250 × 4.6 mm i.d., 5 μm particle size; mobile phase: water/methanol 35/65 (v/v), pH 2.50 with 85% *orto*-phosphoric acid; flow rate: 1.0 ml/min; injection volume: 20 μl; detection performed at 210 nm. Other peaks different from 10-HDA were not identified.

3.9. FIGURES

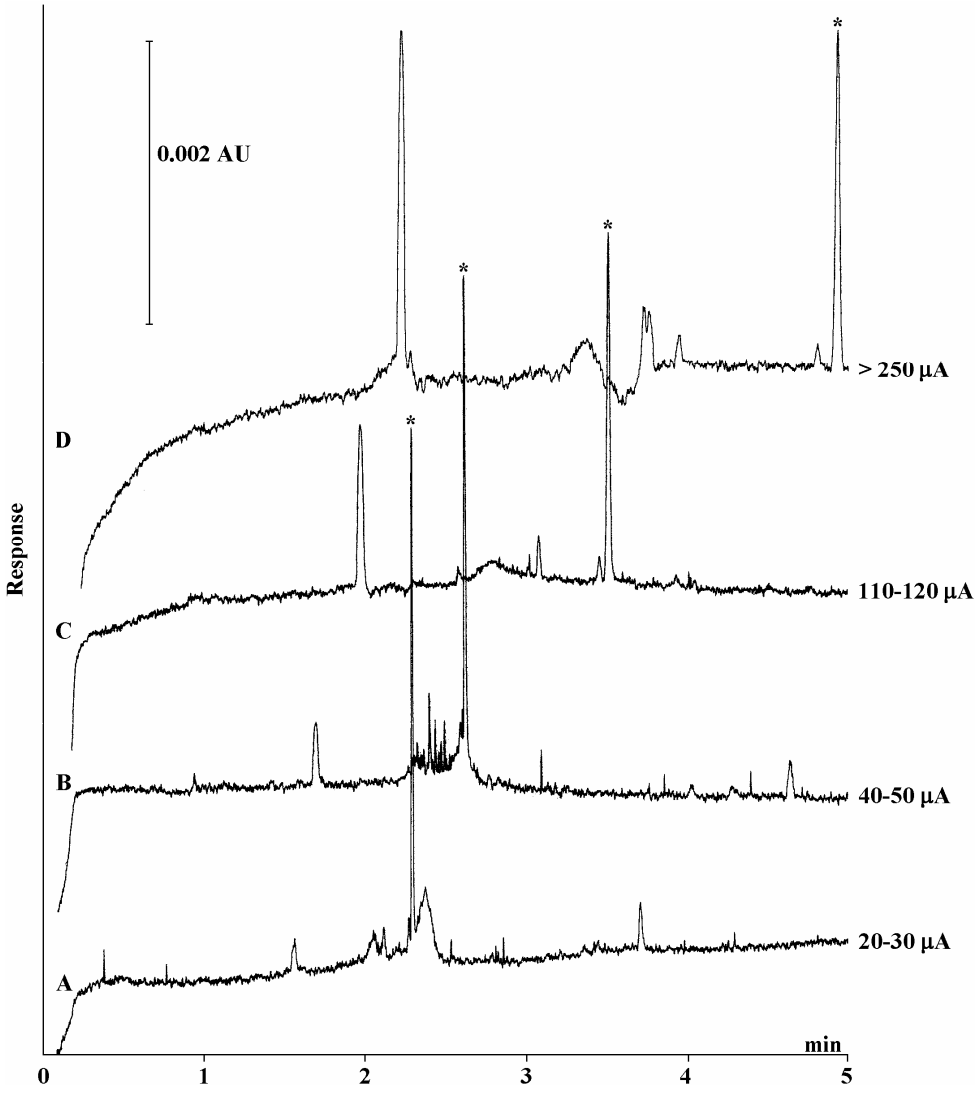


Fig. 3.1.

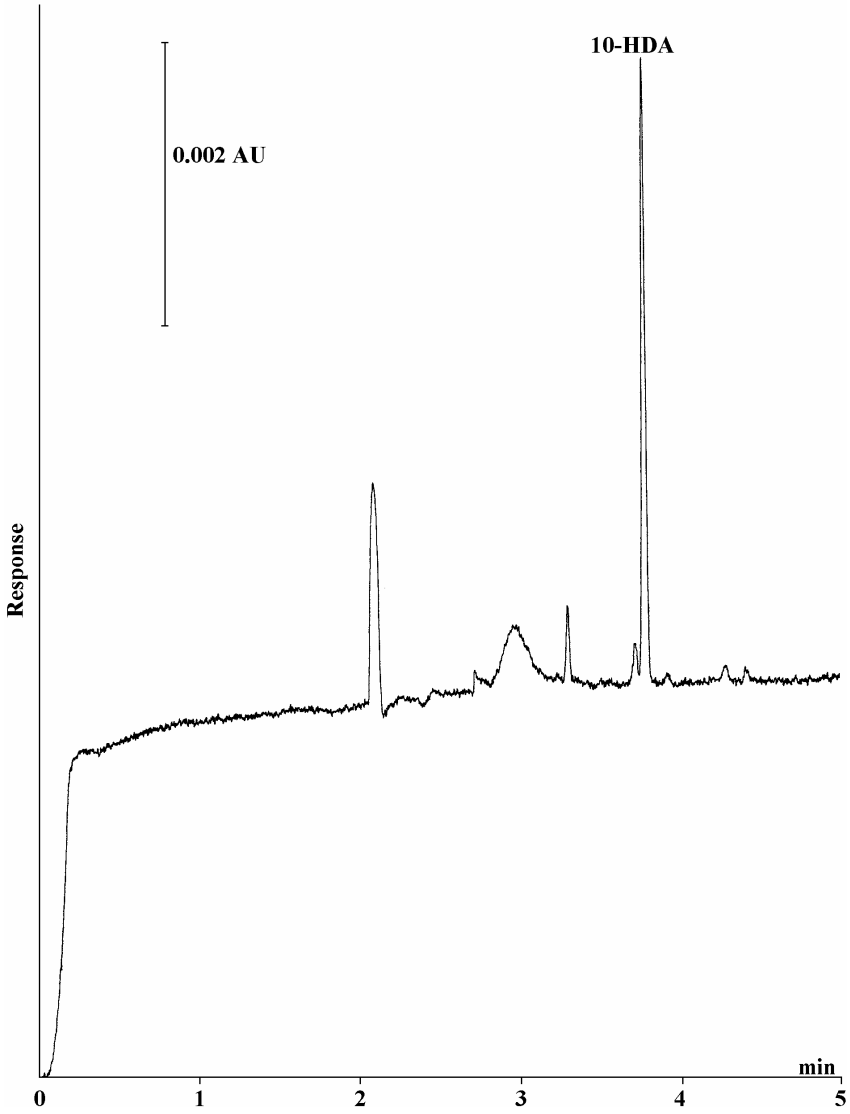


Fig. 3.2.

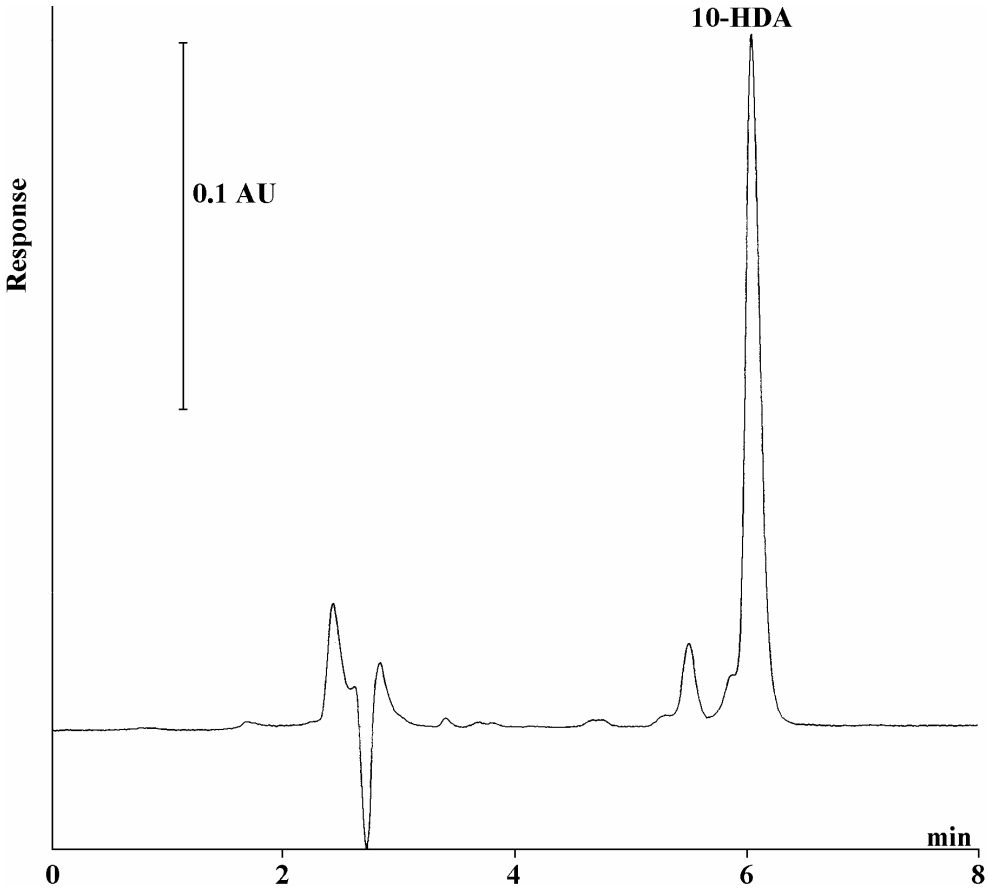


Fig. 3.3.

4. EFFECT OF DIFFERENT STORAGE CONDITIONS ON THE LIPID FRACTION OF A VEGETABLE CREAM

4.1. SUMMARY AND KEYWORDS

The study of the shelf-life of a vegetable cream was carried out. Total and free fatty acids, diglycerides, peroxides and a first sensory evaluation were determined on fresh cream and on samples stored for six months at different temperatures. The product showed a higher unsaturated fatty acids amount (≈ 50% of fatty acids) with respect to milk fat and a low cholesterol level (< 0.01%). Phytosterols content (≈ 14 mg/100 g of cream) was not high enough to contribute to decrease cholesterolemia. Lipid oxidation kept low during storage (peroxides: 2.0-3.0 meq O_2/kg of fat) but a small increase was observed under normal retail conditions after six months (about 6.0 meq O_2/kg of fat). Free fatty acids never exceed 0.3% of fat. The preservation at 4 and 15 °C led to an improvement as regards sensory profile and a delay in lipolysis development in comparison to other storage conditions.

Keywords: cholesterol, lipolysis, oxidative rancidity, phytosterols, shelf-life, storage conditions, unsaturated fatty acids, vegetable cream.

4.2. INTRODUCTION

Cream is a fat-in-water emulsion and a dairy product which is employed as dressing or ingredient in the preparation of several dishes or foods. It is usually composed of the higher-butterfat layer skimmed from the top of milk before homogenization. While in un-homogenized milk, over time, the lighter fat rises to the top, in the industrial production of cream the process of separation is accelerated by using centrifuges called separators that work at a speed of 6500-7000 rpm. Cream represents the raw material for butter production and it appears like a yellowish liquid featuring a tasty odour, a slight flavour and a varying firmness. In Italy cream is sold in several grades depending on its total butterfat content: light or coffee cream (fat content ranging from 10 to 20%), kitchen cream (fat content more than 20%) and whipping cream (fat content more than 30%), with the latter generally employed in the preparation of cakes. After milk skimming, every kind of cream, despite of its fat content, has to undergo a heating treatment. While whipping cream is pasteurized (processing temperatures: 60 and 85 °C for 0.5-3.0 min) in order not to decrease or destroy its whippeabilty, kitchen cream, as the one here-analyzed, undergoes before packaging a UHT processing (140-150 °C for a few seconds) to sterilize the product, improving its firmness after cooling and ensuring it a long shelf-life.

The kitchen cream object of this study and recently put on the market by an Italian dairy company, may be seen and is proposed as an healthy alternative with respect to cream of animal origin sold in stores. Indeed, it was produced replacing, after skimming, the animal fat from milk with a refined vegetable oil and subsequently adding high biological-valued

milk proteins, flavours and other additives. The new cream is presented as a product containing a minimal amount of cholesterol, that is reported on label to be less than 0.01% and with a better unsaturated-to-saturated fatty acids ratio. Moreover the employment of a vegetable fat should enrich the product with some biologically active major and minor compounds like essential fatty acids (linoleic and α-linolenic acids) and phytosterols, also known as plant sterols.

Actually, these nutritional features meet the recent dietary guidelines that suggest, prior to moderately reduce daily fat intake to 20-35% of total calories, to improve first the quality of the consumed fat. Amongst these suggestions, the consumption of less than 300 mg/die of cholesterol, a saturated fatty acids daily intake comprised between 7-10% of total calories, a n-6-to-n-3 fatty acids ratio not higher than 5 and keeping *trans* fatty acids consumption as low as possible are worth to be cited (INRAN – MIPAF 2003; HHS/USDA, 2005).

The vegetable cream is declared to have a six-month shelf-life and, under normal retail conditions, is stored at room temperature. The first and main aim of this work was to study the quality of lipid fraction in samples stored, during a six-month storage period, under different temperatures and under normal retail conditions. Oxidation and lipolysis, the two known biochemical pathways that lead to lipid rancidity, were evaluated through the determination of peroxide value (primary oxidation products), free fatty acids and diglycerides (lipolysis products). Indeed, lipid oxidation is one of the major causes of food spoilage, leading to the development in edible oils and fat-containing foods of off-flavours (Robards *et al.*, 1988; Nawar, 1996) that shorten the shelf-life of these foodstuffs. Free and polyunsaturated fatty acids are especially susceptible to lipid oxidation, the latter ones

owing to the double bonds in their hydrocarbon chains (Nawar, 1996; Min and Boff, 2002; Kołakowska, 2003).

Then, to shed a first light over the nutritional feature of this cream, the total fatty acids profile and the total sterols (cholesterol and phytosterols) content of the different samples were determined while, throughout preservation, pH, colorimetric determinations and consumer tests were also performed to evaluate the effect of different storage temperatures on cream sensory attributes. So far, no studies have carried out regarding the composition of lipid fraction and the development of rancidity in this kind of dairy product.

4.3. MATERIALS AND METHODS

4.3.1. Samples

The vegetable cream object of this study was prepared according to the technological process shown in **Fig. 4.1.** All the samples were supplied by an Italian dairy company and were made of commercial vegetable cream packaged in white polylaminate brik (100 g of product per brik). They underwent different storage conditions during a six-month period, kept in the dark at 4, 15, 30 °C and under normal retail conditions (room temperature: 10-25 °C) and analyzed after production (sample V_0) and after three (samples V_4-3, V_{15}-3, V_{30}-3 and V_{VAR}-3) and six months (expiring date, samples V_4-6, V_{15}-6, V_{30}-6 and V_{VAR}-6) since production. In **Fig. 4.2.** the plan of storage, sampling and the label each sample was identified with are reported. After sampling, the samples were stored at -45 °C and, before analyses, thawed at 4 °C overnight and then at room temperature for about 30 min. Unless

specified, every analytical determination was performed in triplicate (n = 3) for each kind of sample.

4.3.2. Reagents, solvents and standards

Ammonium thiocyanate (NH_4SCN, ≥97.5%), barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$, 99%) and ferrous sulfate eptahydrate ($FeSO_4 \cdot 7H_2O$, ≥99%) were from Carlo Erba Reagenti (Rodano, Italy). Heptadecanoic acid (C17:0, >98%) and trimethylchlorosilane (TMCS) were from Fluka (Buchs, Switzerland). Acetic acid, chloroform, diethyl ether, *n*-hexane, hydrochloric acid (HCl, 37%) used for preparing 0.4 M and 10 M HCl solutions employed in peroxides determination, potassium hydroxide in pellets (KOH, ≥85%) used for preparing 2 M KOH in methanol and sodium hydroxide in pellets (NaOH, ≥99%) used for preparing 1 M NaOH in methanol were from Merck (Darmstadt, Germany). Heneicosanoic acid methyl ester (C21:0), cholesterol (min. 99%), dihydrocholesterol (98.7%), dioleoylglycerol (1,2+1,3-diolein, approx. 99%), 1,3-diolein (approx. 99%), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, ≥98%), campesterol, β-sitosterol, stigmasterol, hexamethyldisilazane (HMDS), pyridine (Pyr) and sodium sulfate anhydrous (Na_2SO_4, ≥99%) were from Sigma (St. Louis, MO, USA). *i*-Propanol was from Riedel-de Haën (Seelze, Germany). Squalane (≥95%) was from Roth (Karlsruhe, Germany).

Diazomethane was prepared as an ethereal solution according to Fieser and Fieser (1967). Two standard mixtures of fatty acid methyl esters, GLC 463 and FAME 189-19 were purchased from Nu-Check (Elysian, MN, USA) and Sigma respectively. Deionized water

was obtained from an Elix 10 water purification system from Millipore (Bedford, MA, USA). All solvents, unless otherwise stated, were of analytical grade.

4.3.3. Consumer test

A consumer test was carried out on fresh, mid-term and expiring samples involving each time 6-7 non-professional tasters in order to evaluate common consumer's preference about the product at different stage of storage. The tasters expressed their preference as regards the following sensory attributes: colour, odour, flavour and palatability. Each organoleptic properties was evaluated by the tester drawing a mark comprised between 0 and 10 on a row.

4.3.4. pH measurements

pH measurements were performed with a pH-meter Knick Digital from Knick (Berlin, Germany). The glass probe was kept in the sample until a stable pH value was read.

4.3.5. Colour measurements

The reflectance colour measurements were conducted with a Minolta reflectance CR300 colorimeter from Konika Minolta (Tokyo, Japan). The CIELab 1976 colorimetric system was applied (CIE, 1978) and the three chromaticity dimensions L^* (lightness), a^* (redness) and b^* (yellowness) were recorded; from these data, colour difference, saturation and hue were evaluated. The first property was calculated with respect to the sample V_0 and by the

formula: $[(\Delta L)^2-(\Delta a^*)^2-(\Delta b^*)^2]^{1/2}$. The second property was obtained by the equation $(a^{*2}+b^{*2})^{1/2}$ while the third one was the angle whose inverse tangent was the ratio b^*/a^*.

4.3.6. Lipid extraction

Lipid extraction was carried out according to Bligh and Dyer (1959). About 3.5 g of cream were accurately weighed in a screw-cap bottle, added with 15 ml of methanol, 12 ml of water and 8 ml of chloroform and homogenized for 1.5-2.0 min. Successively, 8 ml of chloroform were added and a second homogenization was carried out for 1.5-2.0 min. The content of the bottle was transferred in a second screw-cap bottle while the residual matter was rinsed with 5 ml of methanol and 5 ml of chloroform. The mixture was centrifuged at 1800 rpm for 10 min and the supernatant hydroalcoholic phase was then discarded. To let a better separation between the organic matter and the residual methanol/water, the mixture was kept at 4 °C for 1 h and then filtered through sodium sulfate anhydrous. The fat recovered was dried under reduced pressure using a vacuum evaporator and stored -45 °C in *n*-hexane/*i*-propanol 4/1 (v/v) until further analyses. Cream fat content had been previously determined by the staff of the dairy company that supplied the samples employing a butyrometric method.

4.3.7. Gas chromatographic determination of total fatty acids

A cold transmethylation was performed on fat according to Christopherson and Glass, 1969, in order to convert fatty acids to the corresponding methyl esters (FAME). About 15 mg of fat were exactly weighed in a 10 ml Teflon lined screw-cap vial, dissolved in 1 ml of

a standard solution of the internal standard (1.1 mg of heneicosanoic acid methyl ester dissolved in *n*-hexane) and added with 1 ml of 2N KOH in methanol. The mixture was then vigorously shaken for 2 min, added with 1 ml of *n*-hexane and further shaken for 2 min. After the separation of the two phases, 1 ml of the supernatant transmethylated fraction was diluted with 9 ml of *n*-hexane.

Then 1 µl of the organic solution was injected and analyzed by capillary gas chromatography (CGC) employing a fused silica capillary column CP-Select CB for FAME (50 m × 0.25 mm i.d., 0.25 µm f.t.) from Varian (Palo Alto, CA, USA) which were fitted on a AUTO/HRGC/MS MFC 500 gas chromatograph from Carlo Erba Instruments (Rodano, Italy). The injector and detector temperatures were respectively set at 220 and 230 °C. Helium was used as carrier gas at the flow of 1.2 ml/min. The oven temperature was held at 140 °C for 1 min, increased from 140 °C to 220 °C at 3.5 °C/min and finally held at 220 °C for 2 min. The split ratio was set at 1:14. The peaks identification was accomplished comparing the peaks retention times with those of the FAMEs of two standard mixtures. Gas chromatographic data were filed and processed by the software Turbochrom (version 6.2.0.0.0.:B27) from Perkin Elmer (Shelton, CT, USA).

4.3.8. Gas chromatographic determination of sterols

Sterols were quantified by CGC after a cold saponification that was carried out on fat according to Sander *et al.* (1989). About 150-200 mg of fat were accurately weighed in a 10 ml Teflon lined screw-cap vial, added with 0.05 mg of dihydrocholesterol (internal standard) dissolved in *n*-hexane/*i*-propanol 4/1 (v/v) and dried under nitrogen. Then the fat

sample was added with 10 ml of 1M NaOH in methanol, vigorously shaken, covered with aluminium foil and left under stirring for 18 h. Subsequently, the mixture was transferred to a separatory funnel and the residue rinsed with 15 ml of diethyl ether and 15 ml of water. The lower aqueous layer was extracted a first time and then twice more with diethyl ether (2 × 10 ml). All ethereal extracts were pooled, washed with 0.5 M KOH (2 × 10 ml) and water (2 × 10 ml, plus NaCl before the first washing step), dried for about 1.5 h over sodium sulphate anhydrous, filtered on the same salt and finally evaporated under reduced pressure. Prior to CGC, the unsaponifiable matter was methylated with diazomethane (Fieser and Fieser, 1967) and silylated to convert the residual free fatty acids and sterols to the corresponding methyl esters and trimethylsilyl (TMS) derivatives respectively. The silylation procedure was carried out according to Sweeley *et al.* (1963), employing 200 µl of a mixture Pyr/HMDS/TMCS 5/2/1 (v/v/v) and keeping the sample at 70-80 °C for 45 min. After removing the silylating agent under nitrogen, 200 µl of *n*-hexane were added and then the mixture was briefly shaken and centrifuged at 3000 rpm for 5 min.

One µl of the derivatized unsaponifiable matter was analysed by CGC in the same instrument previously described and equipped with a fused silica capillary column (SE-52, 30 m × 0.25 mm i.d., 0.10-0.15 µm f.t.) coated with 5%-diphenyl-95%-dimethyl-polysiloxane from Mega (Milano, Italy). The injector and detector temperatures were set at 330 °C. Helium was used as carrier gas at the flow of 1.2 ml/min. The oven temperature was increased from 270 to 325 °C at 3.0 °C/min and held at 325 °C for 10 min. The split ratio was set at a 1:14 ratio. The sterols were identified comparing the peaks retention times

with those of a standard mixture containing cholesterol, dihydrocholesterol, β-sitosterol, campesterol and stigmasterol.

4.3.9. Spectrophotometric determination of peroxide value (PV)

PV was determined according to Shantha and Decker (1994). This method is based on the spectrophotometric determination of ferric ions (Fe^{3+}) formed by the oxidation of ferrous ions (Fe^{2+}) by hydroperoxides in the presence of ammonium thiocyanate. Thiocyanate ions (SCN^-) react with ferric ions to give a red-colored chromogen that can be determined spectrophotometrically. About 70 mg of fat were exactly weighed and dissolved in a 10 ml volumetric flask with chloroform/methanol 2/1 (v/v), taking care to make the volume up to the mark with the same solvent mixture. The sample was then added with aqueous solutions of ammonium thiocyanate and iron(II) chloride (50 µl each) and left in the dark for 5 min. Then the absorbance of the solution was read at 500 nm with a double beam UV/Vis spectrophotometer UV-1601 from Shimadzu (Kyoto, Japan). To quantify PV, an iron(III) calibration curve was built from iron(III) chloride and with a calibration range from 0.1 to 5.0 µg/ml ($r^2 = 0.984$). PV was expressed as meq of O_2/kg of fat.

4.3.10. Purification and gas chromatographic determination of free fatty acids (FFA)

FFA were purified from the less polar lipid compounds according to Parisi (2001). About 15 mg of fat were exactly weighed in a conical vial, dissolved in 200 µl of chloroform/*i*-propanol 2/1 (v/v) and loaded onto an ammino-propilic bonded phase cartridge for solid phase extraction (SPE) from Isolute, Mid-Glamorgan, UK (NH_2-SPE cartridges, 500 mg

stationary phase, 3 ml reservoir volume). Before fat loading, each cartridge had been placed in a SPE elution apparatus from Isolute, loaded with a small amount of sodium sulfate anhydrous and conditioned with 3 ml of *n*-hexane. The cartridge was eluted with 6 ml of chloroform/*i*-propanol 2/1 (v/v) and then with 10 ml of a 2% (v/v) solution of acetic acid in diethyl ether to remove and collect FFA. The latter fraction was evaporated under reduced pressure and methylated with diazomethane according to Fieser and Fieser (1967). Subsequently, the polar fraction containing FFA was diluted with 100 µl of *n*-hexane, added with a proper amount of the internal standard (5.3 µg of heptadecanoic acid methyl ester dissolved in *n*-hexane) and analyzed in CGC.

The instrumental conditions applied were the same of these used in the FAME analysis. Only the temperature program was slightly changed: the oven temperature was held at 130 °C for 2 min, increased from 140 to 220 °C at 4.0 °C/min and finally held at 220 °C for 4 min.

4.3.11. Purification and gas chromatographic determination of diglycerides (DG)

Diglycerides were isolated and concentrated as reported by Bortolomeazzi *et al.* (1990). About 100 mg of fat were accurately weighed in a conical vial, dissolved in 700 ml of *n*-hexane and loaded onto a silica cartridge for SPE (STRATA cartridges, 500 mg stationary phase, 3 ml reservoir volume) from Phenomenex (Torrance, CA, USA). Before fat loading, each cartridge underwent the same treatment formerly reported for FFA analysis. The cartridge was eluted with 3 ml of *n*-hexane, 3 ml of *n*-hexane/diethyl ether 8/2 (v/v), 4 ml of *n*-hexane/diethyl ether 1/1 (v/v) and 3 ml of methanol. The two last fractions, containing

DG, were pooled, dried under reduced pressure, added with 0.5 mg of squalane dissolved in *n*-hexane (internal standard) and methylated with diazomethane (Fieser and Fieser, 1967) to convert the FFA extracted to the corresponding methyl esters. Prior to CGC analysis, the polar fraction was silylated according to Sweeley *et al.* (1963) in the same way described for the sterols determination. After removing the silylating agent under nitrogen, 1 ml of *n*-hexane was added. The mixture was briely shaken and centrifuged at 3000 rpm for 5 min. One µl of the organic phase was analyzed by CGC, employing the same instrument previously employed in CGC analyses which was equipped with a fused silica capillary column (Rtx 65TG, 30 m × 0.25 mm i.d., 0.10 µm f.t.) coated with 65%-diphenyl-35%-dimethyl-polysiloxane from Restek (Bellefonte, PA, USA). The injector and detector temperatures were set at 350 °C. Helium was used as carrier gas at the flow of 1.2 ml/min. The oven temperature was programmed from 180 to 350 °C at 3.0 °C/min and finally held at 350 °C for 20 min. The split ratio was set at 1:25. To identify 1,2 and 1,3-diglycerides, a thin layer chromatographic (TLC) separation was performed, according to Frega *et al.* (1993), on 12 mg of fat from sample V_{VAR}-6. The diglycerides present in the separated fractions were identified taking into account the differences with respect to the hydrocarbon chain length and the degree of unsaturation as described in the afore-mentioned work.

4.3.12. Statistics

The results of the analytical determinations were reported as the mean values obtained for each sample (n = 3). Standard deviation (SD) was chosen as spreading index. The data underwent one-way analysis of variance (ANOVA) using the software Statistica 6.0 from

StatSoft (Tulsa, OK, USA). Unless specified, Tukey's honest significant differences (HSD) test and Pearson's linear correlations were evaluated at a $p < 0.05$ level.

4.4. RESULTS AND DISCUSSION

4.4.1. Sensory, colorimetric and pH evaluations

The statistical analysis applied to the sensory scores (data not shown) did not access significant differences between the cream samples as regards the four organoleptic properties here evaluated (colour, odour, flavour and palatability). From one side it was likely owing to the low expertise of the tasters who were not professional panelists. From the other side this result may be seen as a first proof of a good shelf-life of this product. Considering the trend of the average values obtained for each sample regarding the different properties, temperature seemed to affect on the whole the commercial acceptability of the product. At 30 °C the lowest colour, flavour and palatability scores, after three and six months of storage, were recorded while odour seemed to be less influenced by temperature. On the contrary, the highest scores were usually recorded at 4 °C. Moreover at 4 and 15 °C and under retail conditions, the scores obtained after three months were often higher than those recorded for V_0. A sort of ripeness and improvement of product organoleptic profile seemed to take place during the mid-term storage while in the second half a slight worsening in sensory profile occurred. A test involving skilled panelists should be definitely carried out to verify these hypotheses.

In Table 1 are shown the results from the colour measurements. Despite consumer can not directly access the colour of the product at the moment of purchase, his/her judgement on the overall quality of the cream is deeply influence by its appearance. The fresh product appeared bright white even if during storage, like other diary products, a slow worsening of its appearance and the comparison of a yellowish film on its surface took place. Consumers often associate this process to the development of rancidity, especially in spoiled foods. Thus, the attention was focused on the colorimetric parameters b^* (yellowness index), hue, saturation and the colour difference with respect to V_0.

Yellownees index (b^*) did not change significantly during storage except for the samples stored at 30 °C in which a significant increase happened probably due to a more intense development of Maillard reaction at this temperature. About hue or colour shade, it may be defined as the property of colours by which they can be perceived as ranging from red through yellow, green, and blue. Hue is the main colorimetric attribute perceived and identified with the common names given to the different colours. When the cream was stored at 4 °C no variation in hue was found with respect V_0 whereas at the other storage condition significant differences were found increasing the temperature and storage time. Nevertheless, at 30 °C just after 3 months of storage hue was significantly different from all the other samples. A similar trend was accessed for b^* and also accessed as regards saturation that represents a measure of how the colour is different from grey and is usually expressed as depth, vividness or purity of colour. No significant change was observed with respect V_0 in samples stored at 4, 15 °C and under retail conditions and the tendency to a small decrease in saturation was observed while in sample store at 30 °C saturation increased and was significantly higher than V_0 after six months. It likely depended to the

corresponding increase in the yellowness index. Finally the colour differences here found between each sample and V_0 were all comprised in the range 0.5-1.5 that denotes only a small difference not easily noticeable. The highest difference were observed at 30 °C. In particular in V_{30}-6 the difference found was close to the range of values 2-3 representing a significant colour difference that can be observable.

pH values were reported in Table 3. At 4 and 30 °C were respectively recorded the lowest and the highest pH increase compared with V_0, confirming the dependence of pH on temperature and also the trend formerly observed as regards sensory and colorimetric evaluations and. On the whole the cream showed a good buffer capacity and the fall in pH since production did never exceed 0.35 pH units. The storage at 30 °C may have catalyzed, as confirmed by free fatty acids analysis, lipolysis and also a faster development in the Maillard reaction, another process that lead to a decrease in pH.

4.4.2. Total fatty acids composition

Table 2 shows the total fatty acid composition of the cream samples while in **Fig. 4.3.** is shown a typical gas chromatographic trace of FAME. Palmitic and oleic acids were the most abundant fatty acids and represented more than 80% of FAME. The fatty acids composition of the product was similar to that of palm oil. Actually, according to literature data (O'Brien, 2004), the primary fatty acids in this edible oil are as follows: palmitic (from 41.8 to 46.8%), oleic (from 37.3 to 40.8%), linoleic (from 9.1 to 11.0%) and stearic (4.5 to 5.1%) acids, followed by small amount of lauric (≈ 0.2 %), myristic (≈ 1.1%), linolenic (≈ 0.4%) and arachidic (≈ 0.4%) acids.

The major differences found with respect to milk fat were the absence of short-chain fatty acids (C4:0 – C10:0, ≈ 10% of total fatty acids in milk) and higher amounts of palmitic, oleic and linoleic acids which in milk fat represent typically 26.9, 28.5 and 3.2% of fatty acids respectively (O'Brien, 2006). The higher oleic and linoleic content led to a more balanced unsaturated-to-saturated fatty acids ratio (1.0 vs. ≈ 0.5 in milk fat according to the data formerly cited) that meets dietary recommendations to increase unsaturated fats intake. No *trans* fatty acids were detected: the limit of detection (LOD) was set at S/N = 3 (S/N: signal-to-noise ratio) and under experimental condition it was 0.3 g/100 g of fat. As most of vegetable oil, in which their content does not exceed 1% of fatty acids, even the one employed in the production of cream was poor in n-3 fatty acids for an imbalanced n-6/n-3 ratio. Recently, the importance to keep n-6/n-3 ratio comprised between 4 and 5 as an healthy factor was underlined (Simopoulos, 1999, 2002).

A significant result was that no decrease was observed as regards the relative amount of polyunsaturated fatty acids, even after six month of storage. Polyunsaturated fatty acids, owing to the their chemical properties, may faster generate hydroperoxides and then secondary oxidation products that unfavourably affect the organoleptic quality of foods. In the past, changes in fatty acids composition were presented as an indirect measure to evaluate lipid oxidation, especially in muscle foods (Melton, 1983). Nevertheless, in the same review, contradictory results obtained employing fatty acids as a mean to evaluate oxidation were reported, suggesting the needs of more consistent and reliable analytical determinations. Nowadays, spectrophotometric and gas chromatographic determinations of oxidation products are preferred to evaluate oxidation in foodstuffs.

4.4.3. Total sterols content

The total sterols content of the samples is reported in Table 3. This determination was performed only on the fresh cream and at the end of the storage period, assuming that cholesterol and phytosterols amount could not change significantly just after three months since production. Phytosterols, also called plant sterols, are minor components of all vegetable oils and the concern that arose around these compounds was due to the possibility of lowering LDL cholesterol level, a well-known modifiable risk factor for cardiovascular diseases, through the consumption of products enrich with phytosterols (Law, 2000; Ros, 2000; de Jong *et al.*, 2003).

No significant difference was found regarding the cholesterol and phytosterols content between the fresh sample and the ones analyzed after six months. As declared on label, the cholesterol content was on average 16 mg/100 g of fat (18% of total sterols identified), corresponding to 3 mg/100 g of sample. Considering that in bovine milk, cholesterol ranges between 0.2-0.4% of fat (Żegarska, 2003), in a similar kitchen cream (fat content: 23.5%), produced without replacing animal fat , the cholesterol level should be comprised in the range 0.05-0.09%, that is about twenty times higher than in the vegetable cream.

About phytosterols, in this study were identified and determined the three most widespread in higher plants (Moreau *et al.*, 2002): campesterol, stigmasterol and β-sitosterol. The more abundant sterol was β -sitosterol (48% of identified sterols), followed by campesterol (23%) and stigmasterol (10%). Even in this case, according to literature compositional data (Phillips *et al.*, 2002) reporting the amount of sterols in different vegetable oils, the phytosterols profile of the cream looked like that of palm oil. With respect to these data, the relative high percentage of cholesterol (1.5 mg/100 g of fat for palm oil according to

Phillips, 2002) depended on the residual amount of membrane-bound cholesterol after skimming.

Dietary advices regarding treatment of high blood cholesterol suggests a phytosterols intake of about 2-3 g per day to have a significant lowering in serum cholesterol level (Ros, 2000; National Institute of Health – National Heart, Lung and Blood Institute, 2001). Actually, the vegetal cream here-analysed did not give an important contribution as regards this specific healthy effect.

4.4.4. Effect of storage temperature on lipid oxidation: peroxide value

PV was adopted to evaluate the development of oxidation during storage and the freshness of lipid matrix since it is a useful indicator of the early stages of rancidity occurring under mild condition and it is a measure the primary lipid oxidation products (Shantha and Decker, 1994). As shown in Table 3, PV was low in V_0, owing to a good quality of the vegetable fat and, on the other side, to the possible contribution that UHT heat-treatment gave in keeping PV low before packaging, leading to a partial breakdown of peroxides already present in the vegetable oil. After three months, no significant increase in PV took place under different conditions with respect to V_0 and PV never exceeded 3.0 meq O_2/kg of fat. On the contrary, even no statistical difference was noticed since the great variability associated to V_{VAR}-6, a noticeable decrease in PV took place at 30 °C and under retail condition. While low temperature like 4 and 15 °C kept PV similar to that of fresh cream, higher temperature causes a small PV fall which, however, was not compensated by the rise of new oxidation products because of the oxidative stability of the product. After six months, PV still remained extremely low and even at 4 and 15°C a decrease took place

while at 30 °C the value obtained was quite similar to that of V_{30}-INT. Nevertheless, a significant increase in PV occurred after this share of time in the sample store under retail conditions. Actually, V_{VAR}-6 showed a PV which was at least twice higher than that found on average in all the other samples. It should be pointed out that despite the higher PV value found in sample V_{VAR}-6, in any sample was observed a PV that exceeded 5.0 meq O_2/kg of fat, which represents the Italian threshold for a refined vegetable oil to be employed in food preparations.

The trend found for PV was likely to reflect the different rate of lipid oxidation depending on storage condition. For the samples stored at controlled temperature, oxidation kept, during the whole storage period, in the initiation phase, in which the increase in peroxides along time was as slow as the absorbance of O_2. The slight PV decrease that took place depended on the transitory nature of peroxides and their decomposition to non-peroxide compounds. Under retail condition, the so-called propagation stage in lipid oxidation took off after three months. In this phase the absorbance of O_2 became higher and the formation of peroxides faster.

Despite the long term storage, the cream showed a good overall stability and different reasons may be taken into account to explain that. In complex foods like milk and dairy products, lipid oxidation is strongly dependent on the interplay between anti and pro-oxidant factors (Lindmark-Månsson and Åkesson, 2000). If natural anti-oxidants (vitamins, enzymic systems) are heat-sensitive and lost during processing, however heat-treatment may lead to a recovery and even an increase in the anti-oxidant efficiency of food. A reducing environment was partially a consequence of proteins unfolding that came from heating and caused the exposure of thiol groups (Tong *et al.*, 2000). Then the anti-oxidant

effect of Maillard reaction products (MRP) have been demonstrated in several carbohydrate-protein model systems (Hayase *et al.*, 1989; Pischetsrieder *et al.*, 1998; Monti *et al.*, 1999; Jayathilakan and Sharma, 2006) and foods like coffee, milk, pasta and tomato (Nicoli *et al.*, 1997; Anese *et al.*, 1999; Calligaris *et al.*, 2004). In a recent study an increase in the oxidative stability of milk was obtained employing strong and brief heat treatments like UHT in place of milder conditions (80 or 90 °C for several hours) that may contribute to enhance milk resistance only after a long time. Furthermore, the cream lipid fraction has a high saturated and monounsaturated fatty acids amount (≈ 90% of FAME) and these lipids are more stable towards oxidation and less than 1% of FAME was represented by long-chain polyunsaturated fatty acids with three or more double bonds.

Moreover, the higher PV and the great variability associated with V_{VAR}-3 and V_{VAR}-6 was a consequence of local overheating process in the bulk of cream and the changes in temperature that both occurred during preservation and led to a heterogeneous distribution of heat in each brik containing cream. The anti-oxidant capability of this product represent a worth feature both from a commercial and nutritional standpoint. First, the onset of lipid oxidation and the appearance of off-flavours and odours of rancidity was significantly delayed, assuring a long shelf-life. Then a stable product such as this cream may be used as a functional vehicle for bio-active compounds like phytosterols, β-carotene or fat-soluble vitamins that are susceptible to oxidative spoilage. It may be also interesting to verify the oxidative stability of a similar cream produced employing vegetable oil with a higher content in n-3 long chain fatty acids to assure a better n-6/n-3 fatty acids ratio in the final product.

4.4.5. Effect of storage temperature on lipolysis: free fatty acids (FFA) and diglycerides (DG)

FFA and DG are the product of lipolysis, the process that involves hydrothermal or enzymic hydrolysis of triglycerides. In Table 4 the amounts of the different FFA and the unsaturated-to-saturated FFA ratio are shown. As expected, in V_0 the FFA content was extremely low, owing to the neutralization process that the vegetable oil underwent during refining to keep FFA in the range 0.01-0.1% of total fat. After three months, at 4 °C no increase occurred whereas only small rises took place at 15 and 30 °C and under retail conditions. On the whole, the development of lipolysis proved to be not noticeable in this period, regardless of the condition of storage. In V_4-6 and V_{15}-6 the total FFA amounts were about twice higher with respect those of the corresponding mid-term samples and V_0. At 30 °C and under retail conditions, after six months the biggest increase occurred and the amount of FFA was about four times higher with respect to the fresh sample. A low temperature like 4 °C, seemed to better preserve the product from the development of lipolysis. Indeed, a progressive increase in FFA with respect to V_0 occurred, after six months, from 4 to 15 °C and from 15 to 30 °C. Regarding sample V_{VAR}-6, it showed the highest FFA content in all samples analysed even it was stored in a range of temperature comprised between 10 and 25 °C: in V_{VAR}-6 the total FFA amount was about four time higher than in V_0. For this sample the catalytic and overheating effect of light has to be taken into account. Actually, both temperature and light are two of the most important factors affecting and catalyzing lipolysis in food (Robards, 1988).

The total FFA amount remained low, especially after three months of storage and never exceeded 0.3% of fat. One reason accounting for this outcome was the UHT processing that

cream underwent before packaging. The high temperatures reached during this technological step caused a complete deactivation of natural milk lipases. Furthermore, the product was packaged under proper hygienic conditions that kept under control microbial pollution and thus the formation of FFA by enzymic action. In **Fig. 4.4.** two gas chromatographic traces of the methyl ester of the FFA of samples V_0 and V_{VAR}-6 are shown.

As regards the compositional data of FFA, the relative amount of different fatty acids were different from those found in FAME analysis. Amongst FFA, a high increase in the relative content of stearic acid and a corresponding decrease in the percentage of palmitic, oleic and linoleic acids were observed. In V_0 and in the samples stored for three months, the percentages of the four main fatty acids were: palmitic (38%), oleic (25-29%), stearic (19-23%) and linoleic (6-7%), thus the relative amount of stearic acid was about five times higher than that found in total FAME. The stearic acid ratio increased more in the last three months of storage: it ranged from 28% (V_4-6) to 42% (V_{VAR}-6) and in V_{VAR}-6 it became the more abundant FFA. The relative amounts in the expiring samples were: stearic (28-42%), palmitic (30-36%), oleic (17-27%) and linoleic (2-10%). The higher contribution that saturated acids, especially stearic acid, gave to the total FFA amount was proved by a unsaturated-to-saturated fatty acids ratio that was comprised between 0.3 to 0.6. This finding was unexpected considering the distribution of fatty acids in the triglycerides molecules of plant fats. In general, seed oils containing common fatty acids show a preferential placement of unsaturated fatty acids at the *sn*-2 position of triglycerides (Nawar, 1996). For instance, in palm oil more than 60% of the triglycerides have a structure like SUS or SUU where S and U mean respectively saturated or unsaturated fatty acid

(Kallio *et al.*, 2001). The position *sn*-2 in the triglyceride molecule is more susceptible to chemical hydrolysis, that is 1,3-DG are usually reported as derivatives from lipolysis of triglycerides (Frega *et al.*, 1993). According to this a rise in the unsaturated-to-saturated FFA ratio should have taken place whereas the opposite trend was observed in real samples.

The chemical process of interesterification that vegetable oil often undergo during the refining phase may partly explain this result. Interesterification is industrially employed to improve the consistency of fats and their usefulness (Nawar, 1996), considering that the physical characteristics of lipid matter are greatly influenced by the distribution of fatty acids in the triglycerides. This process may lead to enhanced technological properties of fats (i.e.: solid content index, plasticity, consistency, performing on baking) without generation, as it takes place in hydrogenation, of health adverse by-products like *trans* fatty acids. Interesterification causes a fatty acid redistribution within and among triglycerides molecules, which leads to substantial changes in lipid functionality. (Rousseau and Marangoni, 2002). In the case of vegetable cream, a transesterification process carried out on the vegetable fat before its employment in the production of cream may have caused an increase of saturated fatty acids occurrence, especially stearic acid, at *sn*-2 position in triglycerides molecules. In a study regarding the physical properties of a mixure soybean oil/tallow (Lo and Handel, 1983), after a thirty-minute random interesterification, a significant change in fatty acids distribution at *sn*-2 position happened. Before processing, palmitic and stearic occurrence at *sn*-2 ranged from 5 to 10% while, after reaction completion, both of them were present at a percentage comprised between 30-35%. On the

contrary only a small decrease in unsaturated fatty acids (oleic, linoleic and α-linolenic), the more abundant at *sn*-2 position, occurred: from 35-45% to less than 35%.

DG represent another parameter to evaluate lipolysis degree in foodstuffs. In particular DG had proved to be useful in evaluating lipolysis in cured or ready-to-eat meat products owing to the degradation that free fatty acids may undergo in long-term storage or under harsh processing (Rodriguez-Estrada, 1997; Bonoli *et al.*, 2007, in press). The DG26 (C12-C14 diglycerides), DG28 (C12-C16 diglycerides), DG30 (C14-C16 and C12-C18 diglycerides), DG32 (C16-C16 and C14-C18 diglycerides), DG34 (C16-C16 diglycerides) and DG36 (C18-C18 diglycerides) series were identified and, employing the analytical method formerly described, it was possible to separate and access the amount of each dyglicerides according to the length of its fatty acids, the disposition of the two fatty acids on the glycerine backbone (1,2 and 1,3-DG, the latter had higher retention times in comparison to 1,2-DG) and the degree of fatty acids unsaturation (unsaturated DG a had a higher retention times than the corresponding saturated ones). In Table 4, the 1,2, 1,3, total DG amounts and 1,2/1,3-DG ratio are shown. The primary DG determined were 1,2-PP (0.1-0.3 g/100 g of fat), 1,3-PP (0.8-1.0 g/100 g of fat), 1,2-PO (0.4-0.6 g/100 g of fat), 1,3-PO (1.1-1.4 g/100 g of fat), 1,2-OO (0.2 g/100 g of fat) and 1,3-OO (0.4-0.5 g/100 g of fat), that accounted for ≈ 75% of total DG. No significant difference was found amongst different samples as regards 1,2-1,3-PO, 1,2-1,3-OO, 1,2-DG, 1,3-DG and total DG. The sample V_0 showed a significant lower amount in 1,2-PP (0.4 vs. 0.1 g/100 g of fat) and an high amount in 1,3-PP (1.1 vs. 1.3 g/100 g of fat) with respect V_{VAR}-6 even these differences were likely due to experimental uncertanity. In **Fig. 4.5.** two gas chromatographic traces of the TMS derivatives of DG of the samples V_0 and V_{VAR}-6 are shown.

In vegetable oil 1,2 isomers derive from an incomplete biosynthesis of triglycerides while 1,3 isomers are mainly derived from triglycerides hydrolysis (Frega *et al.*, 1993). Indeed, the amount of 1,3-DG may be used as a parameter to evaluate the degree of free acidity of the fat before refining, considering that FFA and monoglycerides are eliminated during refining steps while DG are only partially removed and represent an index for the original acidity of the oil. Moreover, the trend of the 1,2-isomers-to-1,3-isomers ratio may be employed to follow the development of lipolysis during the storage of the cream. In V_0 the 1,3 amount ranged from 3.2 to 3.4 g/100 g of fat and thus, considering that from the hydrolysis of one molecule of triglyceride leads to one molecule of DG, containing two fatty acids and to one molecule of FFA, the FFA/DG ratio should be approximately 0.5. Thus, the vegetable oil before refining, should have had a degree of free acidy comprised between 1.6-1.7% of fat. The ratio 1,2/1,3-DG proved not to be a suitable parameter to follow the development of lipolysis during the cream preservation because it did not reflect the same trend observed for free fatty acids. To an increase in free fatty acids content did not correspond a decrease in 1,3-DG or 1,2-DG/1,3-DG ratio. A significant difference was found only between V_0 and V_{VAR}-6 because probably the development of lipolysis that occurred in this sample was intense enough to cause a significant increase in 1,3-DG and a decrease in 1,2-DG/1,3-DG ratio. In the other samples lipolysis did not caused a significant modification in the 1,2-DG/1,3-DG ratio. No correlation was found between total free fatty acids and DG and only a weak correlation ($p < 0.05$, $r^2 = 0.193$) was found between FFA and 1,3-DG.

4.5. CONCLUDING REMARKS

A study of the shelf-life of a vegetable cream was carried out though a qualitative and quantitative investigation of its lipid fraction. Fatty acids profile, total sterols, lipolysis (FFA, DG) and lipid oxidation (PV) were determined on fresh cream and on samples stored for six month under different temperature conditions.

As regards the amount of the different types of fatty acids, from compositional literature data palm oil seemed to be the vegetable fat employed to replace milk fat. Moreover a more balanced unsaturated-to-saturated fatty acids ratio was observed: 1.0 vs. ≈ 2 in milk fat. As declared on label the cholesterol content was less than 0.01% but, depending on the kind of vegetable oil used, the phytosterols amount assured by this cream was too low to give a significant contribute to reach the suggested daily intake. No *trans* fatty acids were detected.

The product proved to be stable to lipid oxidation regardless of the storage temperature and this was likely due to different factors, like the development of a reducing environment caused by heat treatment that the cream underwent, the anti-oxidative properties of the Maillard reaction products and a low amount of long chain polyunsaturated fatty acids. The exposure to light seemed to have a more detrimental effect in enhancing the development of oxidation with respect a controlled temperature. Even lipid hydrolysis remained low and FFA never exceeded 0.3% of fat. At 4 °C the smallest increase in FFA on the whole storage period was observed while at 30 °C and under retail condition a more pronounced rise occurred. DG content did not enable to follow the trend of lipolysis in a refined oil that

underwent neutralization. From colorimetric and sensory determination, the storage at 4 °C kept the colour and organoleptic profile similar to the fresh product while at 30 °C higher changes and a worsening took place even after three months. It was remarkable that the cream proved to be stable to lipid oxidation and lypolisis during a long term storage and especially when preserved at mild temperatures.

Thus the possibility to employ this food as a vehicle for health compound like phytosterols, long-chain polyunsaturared fatty acids and vitamins should be investigated. Besides it could be interesting from a technological and nutritional standpoint, to verify if the oxidative stability of the cream would be kept employing a different type of vegetable fat.

4.6. REFERENCES

Anese, M., Nicoli, M. C., Massini, R., Lerici, C. R. 1999. Effects of drying processing on the Maillard reaction in pasta. Food Research International, 32, 193-199.

Bligh, E. G., Dyer W. J. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37, 911-917.

Bonoli, M., Caboni, M. F., Rodriguez-Estrada, M. T., Lercker, G. 2007. Effect of feeling fat sources on the quality and composition of lipids of precooked ready-to-eat fried chicken patties. Food Chemistry, 101, 1327-1337.

Bonoli, M., Caboni, M. F., Rodriguez-Estrada, M. T., Lercker, G. Effect of processing technology on the quality and composition of lipids of precooked chicken patties. International Journal of Food Science and Technology, in press.

Bortolomeazzi, R., Frega, N., Lercker, G. 1990. Rapid determination of free and esterified sterols using prepacked silica columns. Italian Journal of Food Science, 2, 265-268.

Calligaris, S., Manzocco, L., Anese, M., Nicoli, M. C. 2004. Effect of heat-treatment on the antioxidant activity and pro-oxidant activity of milk. International Dairy Journal, 14, 421-427.

Christopherson, S. W., Glass, R. L. 1969. Preparation of milk fat methyl esters by alcoholysis in an essentially nonalcoholic solution. Journal of Dairy Science, 52, 1289-1290.

CIE 1978. Recommendations on uniform color spaces, color differences, equations. Psychometric color terms. Supplement No.2 to CIE Publications n.15. Commission Internationale de l'Eclairage, Colorimetry. Paris, France.

de Jong, A., Plat, J., Mensink, R. P. 2003. Metabolic effects of plant sterols and stanols (Review). Journal of Nutritional Biochemistry, 14, 362-369.

Fieser, L. F., Fieser, M. 1967. Reagents for organic chemistry. Pp 191-192. New York: Wiley.

Frega, N., Bocci, F., Lercker, G. 1993. High-resolution gas-chromatographic determination of diacylglycerols in common vegetable oils. Journal of the American Oil Chemists' Society, 70, 175-177.

Hayase, F., Hirashima, S., Okamoto, G., Kato, H. 1989. Scavenging of active oxygens by melanoidins. Agricultural and Biological Chemistry, 53, 3383-3385.

HHS/USDA – U.S. Department of Health and Human Services and U.S. Department of Agriculture 2005. Fats. In: Dietary Guidelines for Americans 2005 (6^{th} edition). Pp. 29-34. Washington, DC, USA: Government Printing Office.

INRAN – MIPAF 2003. Grassi: scegli la qualità e limita la quantità. In: Linee guida per una sana alimentazione italiana, revisione 2003. Pp. 28-33. Roma, Italy: Istituto Nazionale di Ricerca per gli Alimenti e la nutrizione (INRAN), Ministero delle Politiche agricole e Forestali (MIPAF).

Jayathilakan, K., Sharma, G. K. 2006. Role of sugar-amino interaction products (MRPs) as antioxidants in a methyl linoleate model system. Food Chemistry, 95, 620-626.

Kallio, H., Yli-Jokipii, K., Kurvinen J. P., Sjövall, O., Tahvonen, R. 2001. Regioisomerism of triacylglycerols in lard, tallow, yolk, chicken skin, palm oil, palm olein, palm stearin, and a transesterified blend of palm stearin and coconut oil analyzed by tandem mass spectrometry. Journal of Agricultural and Food Chemistry, 49, 3363-3369.

Kołakowska, A. 2003 Lipid Oxidation in Food Systems. In: Chemical and Functional Properties of Food Lipids (edited by Z. E. Sikorski and A. Kołakowska). Pp. 133-166. Boca Raton, London, New York, Washington, D.C.: CRC Press.

Law, M. 2000. Plant sterol and stanol margarines and health. British Medical Journal, 320, 861-864.

Lindmark-Månsson, H., Åkesson, B. 2000. Antioxidative factors in milk. British Journal of Nutrition, 84(suppl. 1), S103-S110.

Lo, Y. C., Handel A. P. 1983. Physical and chemical properties of randomly interesterified blends of soybean oil and tallow for use as margarine oils. Journal of American Oil Chemists' Society, 60, 815-818.

Melton, S. L. 1983. Methodology for following lipid oxidation in muscle foods. Food Technology, 37(7), 105-111, 116.

Min, D. B., Boff, J.M. 2002. Lipid Oxidation of Edible Oil. In: Food Lipids: Chemistry, Nutrition, and Biotechnology, Second Edition, Revised and Explained (edited by C. C. Akoh and D. B. Min). Pp. 335-363. New York, Basel: Marcel Dekker.

Monti, S. M., Ritieni, A., Graziani, G., Randazzo, G., Mannina, L., Segre, A. L., Fogliano, V. 1999. LC/MS analysis and antioxidative efficiency of Maillard reaction products from a lactose-lysine model system. Journal of Agricultural and Food Chemistry, 47, 1506-1513.

Moreau, R. A., Whitaker, B. D., Hicks, K. B. 2002. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Progress in Lipid Research, 41, 457-500.

National Institute of Health – National Heart, Lung, and Blood Institute 2001. Third Report of the National Cholesterol Education Program (NCEP), Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) – Executive Summary. Pp 10-12. NIH Publication No. 01-3670.

Nawar, W. W. 1996. Lipids. In: Food Chemistry, Third Edition (edited by O. R. Fennema). Pp. 225-319. New York, Basel, Hong Kong: Marcel Dekker.

Nicoli, M. C., Anese, M., Parpinel, M. T., Franceschi, S., Lerici, C. R. 1997. Loss and/or formation of antioxidants during food processing and storage. Cancer Letters, 114, 71-74.

O'Brien, R. D. 2004. Raw Materials. In: Fats and Oils – Formulating and Processing for Applications (Second edition). Pp. 1-54. Boca Raton, New York, London, Washington, D.C.: CRC Press.

Parisi, E. 2001. Valutazione dello stato di ossidazione di prosciutti crudi e di insaccati non tradizionali. B.Sc. Thesis on Agricultural Sciences and Technologies. Università degli Studi di Bologna, Italy.

Phillips, K. M., Ruggio, D. M., Toivo, J. I., Swank, M. A., Simpkins, A.H. 2002. Free and esterified sterol composition of edible oils and fats. Journal of Food Composition and Analysis, 15, 123-142.

Pischetsrieder, M., Rinaldi, F., Gross, U., Severin, T. 1998. Assessment of the antioxidative and prooxidative activities of two aminoreductones formed during the Maillard reaction: effects on the oxidation of β-catotene, N^{α}-acetylhistidine, and cis-alkenes. Journal of Agricultural and Food Chemistry, 46, 2945-2950.

Robards, K., Kerr, A. F., Patsalides, E. 1988. Rancidity and its measurement in edible oils and snack foods – A review. Analyst, 113, 213-224.

Rodriguez-Estrada, M. T. 1997. Modificazioni nella frazione lipidica del salame durante la stagionatura e dopo il trattamento con radiazione ultravioletta. PhD Thesis on Food Biotechnology. Università degli Studi di Firenze, Italy.

Ros, E. 2000. Intestinal absorption of triglyceride and cholesterol. Dietary and pharmacological inhibition to reduce cardiovascular risk. Atherosclerosis, 151, 357-379.

Sander, B. D., Addis, P. B., Park, S. W., Smith, D. E. 1989. Quantification of cholesterol oxidation products in a variety of foods. Journal of Food Protection, 52, 109-114.

Rousseau, D., Marangoni, A. G. 2002. Chemical Interesterification of Food Lipids: Theory and Practise. In: Food Lipids: Chemistry, Nutrition and Biotechnology, Second Edition (edited by C. C. Akoh and D. B. Min). Pp. 301-333. New York, Basel: Marcel Dekker.

Shantha, N. C., Decker, E. A. 1994. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. Journal of AOAC International, 77, 421-424.

Simopoulos, A. P. 1999. Essential fatty acids in health and chronic disease. American Journal of Clinical Nutrition, 70 (suppl.), 560S-569S.

Simopoulos, A. P. 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomedicine and Pharmacotherapy, 56, 365-379.

Sweeley, C. C., Bentley, R., Makita, M., Wells, W. W. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. Journal of the American Chemical Society, 85, 2497-2507.

Tong, L. M., Sasaki, S., McClements, D. J., Decker, E. A. 2000. Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. Journal of Agricultural and Food Chemistry, 48, 1473-1478.

Żegarska, Z. A. 2003 Milk Lipids. In: Chemical and Functional Properties of Food Lipids (edited by Z. E. Sikorski and A. Kołakowska). Pp. 265-277. Boca Raton, London, New York, Washington, D.C.: CRC Press.

4.7. TABLES

Table 4.1. – Colour characteristics of cream samples. All the results are expressed as mean ± SD (n = 3)

Sample	L^*	a^*	b^*	Hue	Saturation	Colour difference
V_0	$90.3 \pm 0.1^{c,d}$	-2.3 ± 0.0^{e}	9.2 ± 0.1^{b}	-1.3 ± 0.0^{a}	$9.4 \pm 0.1^{b,c}$	-
V_4-3	91.1 ± 0.3^{a}	$-2.1 \pm 0.1^{d,e}$	8.6 ± 0.4^{b}	$-1.3 \pm 0.0^{a,b}$	8.8 ± 0.4^{c}	$1.1 \pm 0.5^{b,c}$
V_{15}-3	91.2 ± 0.1^{a}	$-1.9 \pm 0.1^{c,d}$	8.4 ± 0.1^{b}	$-1.4 \pm 0.0^{b,c}$	8.6 ± 0.1^{c}	$1.2 \pm 0.2^{a,b,c}$
V_{30}-3	$89.8 \pm 0.2^{d,e}$	-1.2 ± 0.0^{a}	10.1 ± 0.3^{a}	-1.4 ± 0.0^{f}	$10.2 \pm 0.3^{a,b}$	$1.5 \pm 0.2^{a,b}$
V_{VAR}-3	$91.0 \pm 0.1^{a,b}$	$-1.7 \pm 0.0^{b,c}$	8.6 ± 0.1^{b}	$-1.4 \pm 0.0^{c,d}$	8.8 ± 0.1^{c}	$1.1. \pm 0.2^{b,c}$
V_4-6	91.2 ± 0.1^{a}	-2.0 ± 0.1^{d}	8.5 ± 0.1^{b}	$-1.3 \pm 0.0^{a,b}$	8.7 ± 0.1^{c}	$1.2 \pm 0.1^{b,c}$
V_{15}-6	$90.5 \pm 0.1^{b,c}$	$-1.7 + 0.1^{b,c}$	9.2 ± 0.4^{b}	$-1.4 \pm 0.0^{d,e}$	9.4 ± 0.4^{c}	0.7 ± 0.2^{c}
V_{30}-6	89.6 ± 0.0^{e}	-1.2 ± 0.0^{a}	10.5 ± 0.0^{a}	-1.5 ± 0.0^{f}	10.6 ± 0.0^{a}	1.9 ± 0.0^{a}
V_{VAR}-6	$90.6 \pm 0.4^{b,c}$	-1.5 ± 0.0^{b}	9.0 ± 0.5^{b}	-1.4 ± 0.0^{e}	9.1 ± 0.5^{c}	$0.9 \pm 0.1^{b,c}$

L^*: lightness; a^*: redness; b^*: yellowness; saturation, hue and colour difference were calculated according to the formulas reported in section Materials and methods.

Different superscript letters within the same row denote significant differences according to Tuckey's test ($p < 0.05$).

Table 4.2. – Total fatty acids profile of the cream samples. The results are expressed as % of the total identified FAME (mean ± SD, n = 3)

Sample	C12:0 lauric	C14:0 myristic	C16:0 palmitic	C 18:0 steraric	C18:1 n-9 oleic	C18:1 n-7 vaccenic	C18:2 n-6 linoleic	C18:3 n-3 α-linolenic
V_0	0.9 ± 0.1	1.2 ± 0.0	43.2 ± 0.2	4.8 ± 0.0^{a,b}	39.1 ± 0.2	0.7 ± 0.1	9.5 ± 0.1	0.6 ± 0.0
V_4-3	0.9 ± 0.0	1.2 ± 0.0	43.0 ± 0.1	4.9 ± 0.1^{a,b}	39.2 ± 0.1	0.7 ± 0.0	9.5 ± 0.1	0.6 ± 0.0
V_{15}-3	0.9 ± 0.0	1.2 ± 0.0	42.8 ± 0.7	4.8 ± 0.0^{b}	39.3 ± 0.5	0.7 ± 0.1	9.6 ± 0.2	0.7 ± 0.0
V_{30}-3	0.9 ± 0.0	1.1 ± 0.0	42.6 ± 0.4	4.8 ± 0.0^{a,b}	39.3 ± 0.2	0.7 ± 0.1	9.8 ± 0.5	0.6 ± 0.1
V_{VAR}-3	0.9 ± 0.0	1.1 ± 0.1	42.8 ± 0.2	4.9 ± 0.0^{a,b}	39.3 ± 0.3	0.8 ± 0.1	9.6 ± 0.1	0.6 ± 0.0
V_4-6	0.9 ± 0.0	1.2 ± 0.0	43.1 ± 0.3	4.9 ± 0.0^{a,b}	39.2 ± 0.2	0.7 ± 0.1	9.5 ± 0.1	0.6 ± 0.0
V_{15}-6	0.9 ± 0.1	1.2 ± 0.1	42.7 ± 0.1	4.9 ± 0.0^{a}	39.4 ± 0.1	0.7 ± 0.0	9.5 ± 0.1	0.6 ± 0.0
V_{30}-6	0.9 ± 0.0	1.2 ± 0.0	42.7 ± 0.5	4.9 ± 0.1^{a,b}	39.6 ± 0.5	0.7 ± 0.0	9.5 ± 0.1	0.6 ± 0.1
V_{VAR}-6	0.9 ± 0.0	1.2 ± 0.0	43.0 ± 0.2	4.9 ± 0.0^{a,b}	39.3 ± 0.1	0.7 ± 0.1	9.4 ± 0.2	0.6 ± 0.0

Cream fat content: 19% (w/w).

C18:3 n-3 (α-linolenic acid) co-eluted with C20:0 (arachidic acid) under the experimental condition.

Different superscript letters within the same row denote significant differences according to Tuckey's test ($p < 0.05$).

Table 4.3. – Total sterols content (mg/100 g sample), PV (meq O_2/kg fat), 1,2, 1,3 and total DG amounts (g/100 g fat), 1,2-DG-to-1,3-DG ratio and pH of different cream samples.

All results are expressed as mean ± SD (n = 3)

Sample	Total cholesterol (mg/100g sample)	Phytosterols (mg/100g sample)	PV (meq O_2/kg fat)	1,2-DG (g/100g fat)	1,3-DG (g/100g fat)	Total DG (g/100g fat)	1,2-DG/1,3-DG	pH
V_0	2.8 ± 0.4	13.7 ± 1.8	3.0 ± 0.0^{a,b}	1.5 ± 0.2	3.3 ± 0.1	4.8 ± 0.3	0.4 ± 0.1^{a}	6.67 ± 0.00^{a}
V_4-3	NE	NE	2.9 ± 0.3^{a,b}	1.3 ± 0.2	3.6 ± 0.2	5.0 ± 0.3	0.4 ± 0.0^{a,b}	6.62 ± 0.00^{b}
V_{15}-3	NE	NE	2.6 ± 0.5^{b}	1.4 ± 0.1	3.7 ± 0.1	5.1 ± 0.1	0.4 ± 0.0^{a,b}	6.60 ± 0.01^{c}
V_{30}-3	NE	NE	2.4 ± 0.0^{b}	1.4 ± 0.1	3.9 ± 0.3	5.3 ± 0.4	0.4 ± 0.0^{a,b}	6.38 ± 0.01^{f}
V_{VAR}-3	NE	NE	2.3 ± 0.2^{b}	1.4 ± 0.2	3.6 ± 0.2	5.0 ± 0.4	0.4 ± 0.0^{a,b}	6.53 ± 0.00^{d}
V_4-6	3.1 ± 0.6	12.3 ± 2.2	2.0 ± 0.2^{b}	1.1 ± 0.2	3.2 ± 0.4	4.3 ± 0.6	0.3 ± 0.0^{a,b}	6.66 ± 0.00^{a}
V_{15}-6	3.0 ± 0.3	14.6 ± 1.7	2.2 ± 0.1^{b}	1.2 ± 0.3	3.3 ± 0.5	4.5 ± 0.8	0.3 ± 0.0^{a,b}	6.54 ± 0.01^{d}
V_{30}-6	3.0 ± 0.3	14.2 ± 1.0	2.3 ± 0.3^{b}	1.3 ± 0.2	3.4 ± 0.3	4.7 ± 0.6	0.3 ± 0.0^{a,b}	6.33 ± 0.00^{g}
V_{VAR}-6	3.1 ± 0.2	15.5 ± 1.3	6.6 ± 3.9^{b}	1.0 ± 0.2	3.3 ± 0.2	4.3 ± 0.4	0.3 ± 0.0^{b}	6.49 ± 0.01^{e}

Cream fat content: 19% (w/w).

NE: not evaluated.

Different superscript letters within the same row denote significant differences amongst the cream samples according to Tuckey's test ($p < 0.05$).

Table 4.4. – FFA composition, saturated, unsaturated and total FFA amounts, unsaturated-to-saturated FFA ratio (U/S) of the different cream samples. Except U/S ratio, all results are expressed as mg/100 g of fat (mean ± SD, n = 3)

Sample	C12:0 lauric	C14:0 myristic	C16:0 Palmitic	C 18:0 steraric	C18:1 n-9 oleic	C18:1 n-7 vaccenic	C18:2 n-6 linoleic	C18:3 n-3 α-linolenic	Saturared FFA	Unsaturated FFA	U/S	Total FFA
V_0	0.9 ± 0.2	4.6 ± 0.9^{c}	25.0 ± 2.8^{c}	14.6 ± 3.2^{d}	16.2 ± 1.9	ND	3.8 ± 0.6	ND	44.8 ± 7.0^{c}	19.9 ± 2.6	0.4 ± 0.0	64.8 ± 9.4^{d}
V_4-3	1.0 ± 0.2	4.7 ± 0.1^{c}	24.0 ± 0.4^{c}	12.8 ± 0.6^{d}	17.5 ± 0.7	ND	4.2 ± 0.2	< 1.0	42.4 ± 1.0^{c}	21.7 ± 0.9	0.5 ± 0.0	64.3 ± 1.2^{d}
V_{15}-3	0.8 ± 0.0	5.1 ± 0.3^{c}	29.0 ± 1.7^{c}	16.8 ± 0.5^{d}	20.4 ± 1.3	ND	4.8 ± 0.3	ND	51.3 ± 2.3^{c}	25.2 ± 1.6	0.5 ± 0.0	76.5 ± 3.8^{d}
V_{30}-3	0.6 ± 0.2	4.9 ± 0.9^{c}	31.6 ± 5.9^{c}	16.2 ± 3.3^{d}	24.5 ± 4.8	ND	5.9 ± 1.1	< 1.0	53.2 ± 10.2^{c}	30.4 ± 5.8	0.6 ± 0.0	83.9 ± 15.6^{c,d}
V_{VAR}-3	1.0 ± 0.1	5.7 ± 0.5^{c}	30.9 ± 1.4^{c}	16.5 ± 0.5^{d}	22.1 ± 1.6	ND	5.6 ± 0.6	ND	54.1 ± 1.6^{c}	27.7 ± 2.2	0.5 ± 0.0	81.8 ± 3.8^{d}
V_4-6	1.0 ± 0.2	7.0 ± 0.4^{b,c}	39.6 ± 5.5^{b,c}	33.8 ± 10.5^{c,d}	33.7 ± 18.5	< 1.0	6.1 ± 1.4	1.3 ± 0.5	81.4 ± 16.1^{b,c}	40.2 ± 20.8	0.5 ± 0.1	123.0 ± 37.1^{c,d}
V_{15}-6	2.5 ± 2.0	8.5 ± 2.1^{a,b}	50.5 ± 13.3^{b}	53.3 ± 15.2^{c}	26.7 ± 6.9	ND	15.5 ± 12.8	2.4 ± 1.3	114.7 ± 32.5^{b}	42.2 ± 19.7	0.4 ± 0.1	159.3 ± 53.5^{b,c}
V_{30}-6	1.9 ± 0.3	10.9 ± 0.7^{a}	81.9 ± 5.3^{a}	78.9 ± 4.8^{b}	40.4 ± 3.8	< 1.0	11.6 ± 0.4	2.7 ± 0.3	173.7 ± 9.7^{a}	52.4 ± 4.1	0.3 ± 0.0	228.8 ± 12.4^{a,b}
V_{VAR}-6	1.4 ± 0.3	10.7 ± 1.1^{a}	80.5 ± 5.6^{a}	112.6 ± 15.2^{a}	50.5 ± 47.8	1.7 ± 1.6	6.3 ± 0.9	2.9 ± 0.3	205.2 ± 18.7^{a}	57.9 ± 50.0	0.3 ± 0.3	265.9 ± 40.4^{a}

Cream fat content: 19% (w/w).

C18:3 n-3 (α-linolenic acid) co-eluted with C20:0 (arachidic acid) under the experimental condition.

U/S: unsaturated-to-saturated free fatty acids ratio.

ND: not detectable; the limit of detection (LOD) was set at S/N = 3 (S/N: signal-to-noise ratio) and corresponded to 0.9 mg/100 g of fat.

Different superscript letters within the same row denote significant differences amongst the cream samples according to Tuckey's test ($p < 0.05$).

4.8. LEGENDS TO FIGURES

Figure 4.1. – Flow-chart of vegetable cream production.

Figure 4.2. – Diagram of storage plan, sampling and samples' names. Samples V_{VAR}-3 and V_{VAR}-6 were stored under normal retail condition and exposed to light while all the other samples were stored in the dark. A six-month storage period corresponded to the product shelf-life reported on label.

Figure 4.3. –. Gas chromatographic trace of FAME obtained, after a trans-methylation on fat, from sample V_0. Peaks identification: 1, C12:0; 2, C14:0; 3, C16:0; 4, C18:0; 5, C18:1; 6, C18:1 n-7; 7, C18:2; 8, C18:3 + C20:0. IS (internal standard): C21:0. For the analytical conditions, see section Materials and methods.

Figure 4.4. – Gas chromatographic traces of the methyl esters of FFA purified after SPE from samples V_0 (left trace) and V_{VAR}-6 (right trace). Peaks identification: 1, C12:0; 2, C14:0; 3, C16:0; 4, C18:0; 5, C18:1; 6, C18:1 n-7; 7, C18:2; 8, C18:3 + C20:0. IS (internal standard): C17:0. Other peaks were not identified. For the analytical conditions, see section Materials and methods.

Figure 4.5. – Gas chromatographic traces of TMS derivatives of DG collected after SPE from samples V_0 (left trace) and V_{VAR}-6 (right trace). The unidentified peaks appearing

beside internal standard (IS) and free cholesterol were likely methyl esters of FFA and TMS derivatives of phytosterols respectively. Peaks identification: Ch, free cholesterol; D26, C12-C14 diglycerides; D28, C12-C16 diglycerides; D30, C12-C18 and C14-C16 diglycerides; D32, C14-C18 and C16-C16 diglycerides; D34, C16-C18 diglycerides; D36, C18-C18 diglycerides. IS: squalane. For the analytical conditions, see section Materials and methods.

4.9. FIGURES

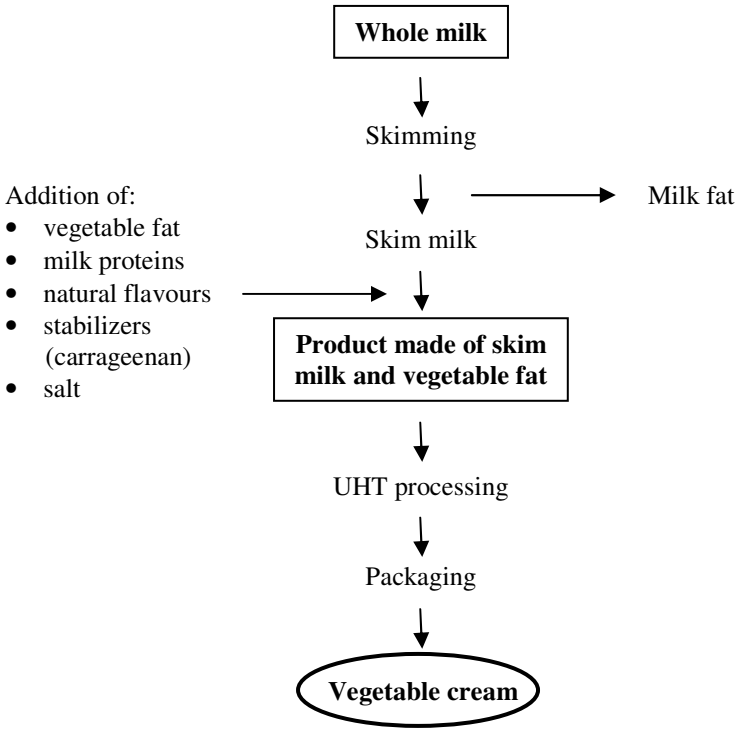


Fig. 4.1.

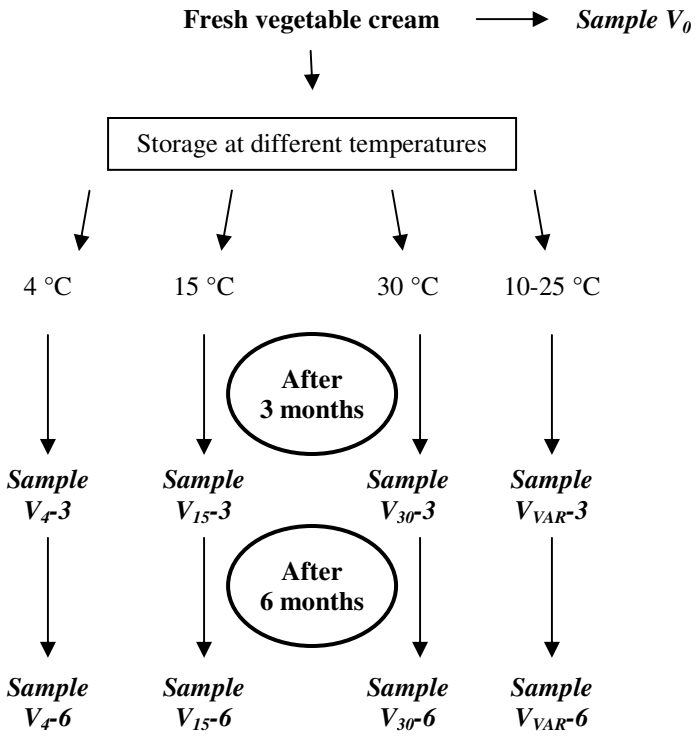

Fresh vegetable cream
Sample V_0
Storage at different temperatures
4 °C
15 °C
30 °C
10-25 °C
After 3 months
Sample V_4-3
Sample V_{15}-3
Sample V_{30}-3
Sample V_{VAR}-3
After 6 months
Sample V_4-6
Sample V_{15}-6
Sample V_{30}-6
Sample V_{VAR}-6

Fig. 4.2.

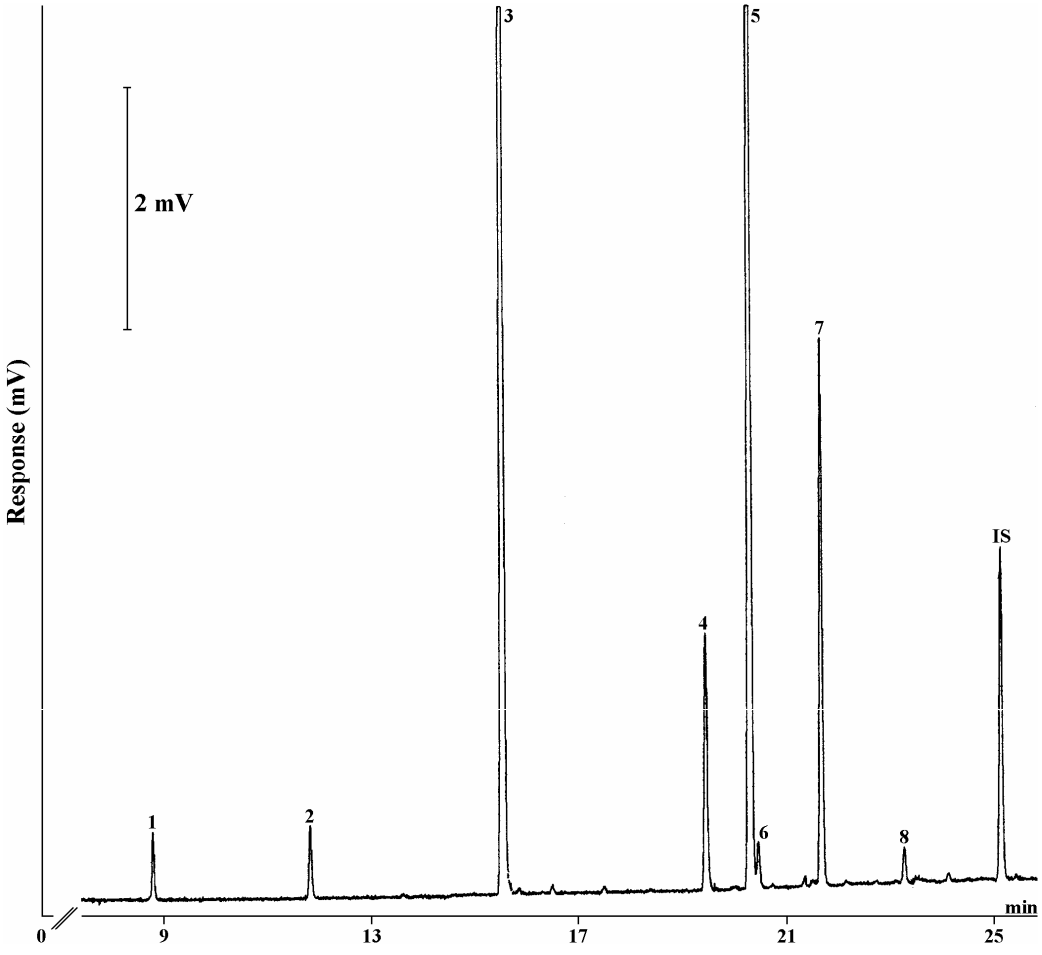


Fig. 4.3.

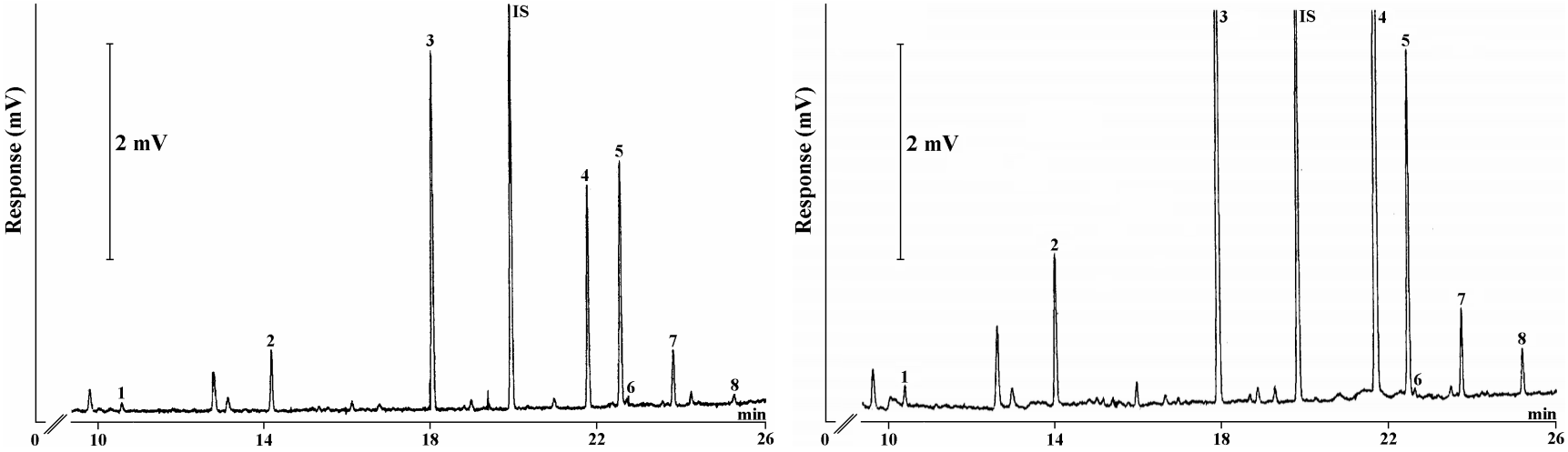


Fig. 4.4.

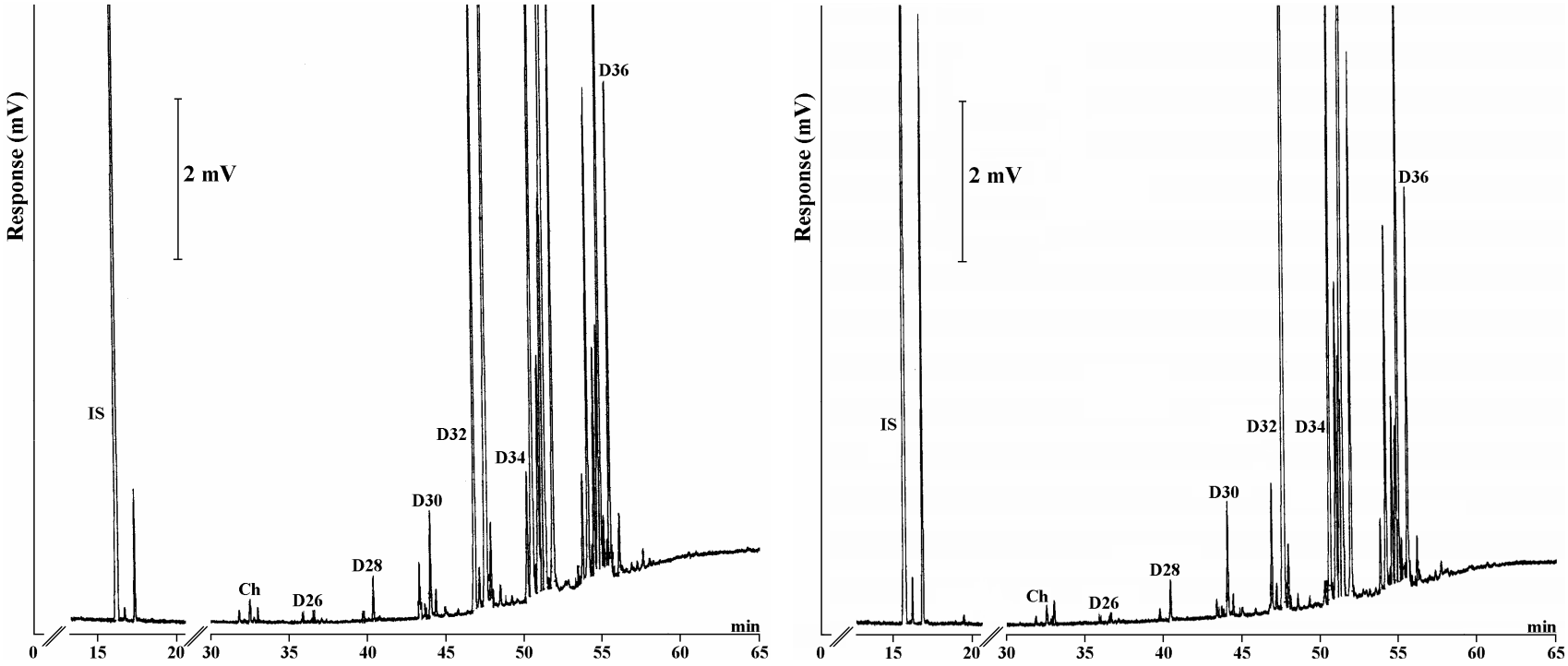

Response (mV)
2 mV
IS
Ch
D26
D28
D30
D32
D34
D36
min
0
15
20
30
35
40
45
50
55
60
65

Fig. 4.5.

5. EVALUATION OF LIPID AND CHOLESTEROL OXIDATION IN RAW AND COOKED MINCED BEEF STORED UNDER AEROBIC AND OXYGEN-ENRICHED ATMOSPHERES

5.1. SUMMARY AND KEYWORDS

Oxygen enriched modified atmosphere packaging (MAP) represents an important mean to stabilize meat colour but may also lead to an increase in lipid rancidity and thus influence the acceptability of the product. In this work the effect on lipid and cholesterol oxidation in ground beef meat stored under aerobic atmosphere and MAP (O_2/CO_2, 80/20) was studied. Total fatty acids, peroxide value (PV), thiobarbituric acid reactive substances (TBARS) and cholesterol oxidation products (COPs) were determined before and after pan frying at different moment of refrigerated storage. While packaging and cooking did not significantly affect fatty acids profile, a rise in lipid peroxidation and COPs formation occurred under MAP. After 8 days at 3-4 °C, COPs increased from 0.7 to 2.0 μg/g in raw meat under MAP but did not change (≈ 1.0 μg/g) under aerobic packaging. Cooking caused an increase in COPs formation after eight days of storage (from 2.0 to 4.0 μg/g and from 0.9 to 3.3 μg/g under MAP and aerobic packaging respectively) but had no significant effect on fresh meat. 7α-Hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol were the more abundant COPs identified.

Keywords: cholesterol oxidation products, lipid oxidation, minced beef, modified atmosphere packaging, pan frying.

5.2. INTRODUCTION

Fresh meat is a highly perishable food matrix but its packaging has only recently been a matter of major concern to meat traders, health officials and scientists. Moreover, packaging may be seen not only as the materials immediately surrounding meat or meat products but is the synthesis of product, processing, labour and machines, for addressing specific functional and/or marketing requirements (Bell, 2001). The primary aim of packaging is to contain, protect and preserve the shelf-life of meat and its products. Packaging materials can be designed to form physical barriers that are capable of inhibiting or reducing the permeation of light or air into muscle foods (Kerry et al., 2002). Modified atmosphere packaging (MAP) requires the use of films with low gas permeabilities: after packaging air is evacuated from the package and replaced injecting a single gas or a mixture of gases followed by clip or heat-seal closure of the bag (Seideman and Durland, 1984; von Elbe and Schwartz, 1996). Some gases are used to control the microbial growth while others to prevent colour deterioration (Seideman and Durland, 1984). As regards the latter issue, the trend to self-service merchandising of fresh meat requires a high standard of colour presentation since consumers' judgement about meat freshness is related to the bright-red colour of oxymyoglobin (Bell, 2001). Indeed, the colour of meat depends mainly on the chemistry of myoglobin, the heme pigment that in properly bled muscle is responsible for more than 90% of the pigmentation (von Elbe and Schwartz, 1996). While reduced myoglobin (Mb) is the

predominant muscle pigment in fresh cut meat and in vacuum packaged meat, the binding of molecular oxygen yields oxymyoglobin (MbO_2) and the colour of meat changes to the customary bright-red. With time, the loss of oxygen and the oxidation of hemic iron from Fe^{2+} (ferrous ion) to Fe^{3+} (ferric ion) lead to the undesirable brown metmyoglobin (MMb) (Fox, 1966; von Elbe and Schwartz, 1996; Miller, 1998a). A high partial pressure of oxygen favours oxygenation, forming MbO_2 and at the same time the formation of MMb is retarded. Thus, in order to enhance oxymyoglobin formation, saturation levels of oxygen in the packaging environment are useful (von Elbe and Schwartz, 1996). MAP employing oxygen-enriched atmospheres represents an important approach to stabilize meat colour and resolve or delay the problem of discoloration in red meat. For red meats, high-oxygen MAP systems typically utilize atmospheres containing from 20% to 30% carbon dioxide, from 60% to 80% oxygen and up to 20% nitrogen as filler. In this way the high oxygen concentration enhances the bright-red colour and the elevated carbon dioxide concentration inhibits the growth of aerobic spoilage microorganisms, ensuring a longer shelf-life (5-10 days) with respect aerobically packaging (Bell, 2001). Nevertheless, the use of oxygen-enriched MAP may result, as a main drawback, in other chemical and biochemical alterations, especially the development of oxidative rancidity that can influence the overall acceptability of meat products. Meat rancidity has been extensively reviewed (Kanner, 1994; Gray et al., 1996; Morrisey et al., 1998) since, like in other foods containing varying amounts of fat, it is one of the major biochemical causes of food spoilage, affecting meat quality both from a sensory and a nutritional standpoint (Nawar, 1996). The rise of unpleasant off-flavours and off-odours is linked to lipid peroxidation even during processing and storage of foods, another detrimental consequence of lipid oxidation is the generation of

toxic compounds such as like cholesterol oxidation products (COPs). The occurrence and determination of COPs in foods have arisen a great concern amongst nutritionist as well as food scientists for their biologically adverse effects, especially regarding the onset of degenerative disease like atherosclerosis and cancer (Paninangvait et al., 1995; Schroepfer, 2000; Garcia-Cruset et al., 2002; Osada, 2002).

Some papers have recently dealt with the effect of packaging conditions on the development of lipid peroxidation and the formation of COPs in beef and other kind of meat during storage, comparing aerobic and vacuum packaging (Galvin et al., 2000; Ahn et al., 2001; Du et al., 2001; Nam et al., 2001) but not taking into account MAP. Only a few reports illustrated the effects of MAP on lipid and cholesterol oxidation during storage, in particular in pork and chicken meat (Cayuela et al., 2004; Conchillo et al., 2005). The aim of this work is a preliminary investigation on the relationship between the type of packaging (modified and aerobic atmosphere) on the oxidative stability of ground beef meat, before and after cooking, at different periods of storage. The analytical parameters that were evaluated to qualify the lipid fraction were peroxides, thiobarbituric acid reactive substances, COPs and total fatty acids.

5.3. MATERIALS AND METHODS

5.3.1. Reagents and chemicals

Barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$), boron trifluoride in methanol ($BF_3 \cdot MeOH$), chloroform, dichloromethane, ferrous sulfate eptahydrate ($FeSO_4 \cdot 7H_2O$), *n*-hexane, 37% hydrochloric acid, hydrogen peroxide (Perhydrol®), iron powder, methanol and dry methanol, 85% *orto*-phosphoric acid, potassium hydroxide in pellets, *i*-propanol,

sodium hydroxide in pellets, sodium sulphate anhydrous, tetraethoxypropane (TEP) and trichloroacetic acid were purchased from Merck (Darmstadt, Germany). Ammonium thiocyanate (NH_4SCN), butylhydroxytoluene (BHT), 19-hydroxicholesterol (19-OH) and sodium chloride were purchased from Sigma (St. Louis, MO, USA). Ethanol was purchased from Solveco Chemicals AB (Rosersberg, Sweden). All solvents and chemicals, unless specified, were of analytical grade. Water solutions were prepared employing deionized water.

5.3.2. Samples

The samples were commercial trays of minced beef meat (about 400 g per tray) purchased from local supermarkets located in Uppsala, Sweden. A first batch was made of trays in which meat was stored under a modified atmosphere packaging (MAP) with a high oxygen content (O_2/CO_2, 80/20) and overwrapped using a low permeable oxygen film. The second batch was made of trays in which meat was packaged aerobically and sealed using a permeable oxygen film. All samples were kept in the dark at a temperature ranging from 3 to 4 °C and analyzed at different moments: after 1, 8 (expiring date as reported on label) and 15 days since packaging as regards the samples stored under MAP and after 1 (expiring date) and 8 days since packaging regarding the samples stored under normal atmosphere. Hamburgers (100 g each, thickness ≈ 0.5 cm) were prepared from samples of raw meat and underwent a cooking treatment in order to study the effect of packaging both on raw and cooked meat. The cooking technique here adopted was pan frying without any additional fat or vegetable oil. The heating device temperature was comprised between 150-160 °C; each hamburger was fried for 15 min (7.5 min per side) until a core temperature of 71-80 °C was reached. The cooked

samples were directly prepared from the raw ones and not stored in order to follow the widespread consumers' practise to store raw meat at low temperatures and cook it just before consumption. The plan of storage and sampling and samples' name are illustrated in **Fig. 5.1.**

5.3.3. Lipid extraction

Lipid extraction was performed according to Hara and Radin (1978) with some modifications. About 25 and 15 g of raw and cooked meat respectively were accurately weighed in a ≈ 200 ml screw-cap plastic bottle suitable for centrifugation, added with 140 ml of *n*-hexane/*i*-propanol 3/2 (v/v) and homogenized (5 × 30 s at maximum speed) using an Ultra-Turrax T 25 Basic homogenizer fitted with a S 25 N - 18 G dispersing tool, both of them from IKA-Werke (Staufen, Germany). The dispersing tool was washed with 10 ml of *n*-hexane/*i*-propanol 3/2 (v/v). Subsequently, the homogenate was added with 60 and 70 ml, for raw and cooked sample respectively, of a 6.67% (w/v) solution of sodium sulphate anhydrous, gently shaken for about 30 s and centrifuged at 4000 rpm for 3 min at a temperature comprised between 7 and 13 °C to let a better separation between the organic and water phases. Then the supernatant organic layer was siphoned away and collected in a 250 ml previously weighed bottom round flask while the mixture left after siphoning was added with 80 ml of *n*-hexane and underwent a second extraction following the procedure formerly described. The upper phase obtained after the second centrifugation was again siphoned and the two organic extracts were pooled and evaporated under reduced pressure. The fat matter was dried under a nitrogen flow for 45-60 min, weighed and stored at -20 °C until further analyses.

5.3.4. Spectrophotometric determination of peroxide value (PV)

PV was determined according to the IDF standard method (1991). This method is based on the spectrophotometric determination of ferric ions (Fe^{3+}) formed by the oxidation of ferrous ions (Fe^{2+}) by hydroperoxides in the presence of ammonium thiocyanate. Thiocyanate ions (SCN^-) react with ferric ions to give a red-colored chromogen that can be determined spectrophotometrically. About 50 or 100 mg of fat were exactly weighed into glass tubes and dissolved in a proper volume (9.85 or 9.8 ml respectively) of chloroform/methanol 7/3 (v/v) to reach a final volume of 9.9 ml. The glass tubes were wrapped with aluminium foil in order to prevent oxidation caused by light. Then 50 µl of aqueous solutions of 30% (w/v) ammonium thiocyanate and ferrous chloride were added to each tube (50 µl each). After an incubation time of 5 min and within 10 min, the absorbance of the solution was read at 500 nm against a blank (9.9 ml chloroform/methanol 7/3 (v/v)) with a double beam UV/Vis spectrophotometer. To quantify PV, an iron(III) calibration curve was built from iron powder and with a calibration range from 0.1 to 5.0 µg/ml. The absorbance of standard solutions was plotted against iron(III) amount (expressed in µg) and each solution was prepared in duplicate. A new calibration curve was built every week. PV was expressed as meq of O_2/kg of fat.

5.3.5. Spectrophotometric determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to the method described by Miller (1998b). This method is based on the capability of malondialdehyde (MDA), one of the major second

oxidation products, to react with thiobarbituric acid to form a pink-coloured chromogen that absorbs at 450, 530 or 538 and then can be determined spectrophotometrically. About 5 g of meat were exactly weighed in a 150 ml glass beaker and added with 1 ml of 0.02% (w/v) BHT in 99.5% ethanol and 45.5 ml of a 10% (w/v) solution of trichloroacetic acid in 0.2 M *orto*-phosphoric acid. The mixture was then homogenized (5 × 30 sec) with the same device employed in lipid extraction and filtered through a Whatman n.1 filter paper (Brentford, England). Two 5 ml aliquots of the filtrate were transferred to10 ml glass tubes, respectively added with 5 ml of 0.02 M thiobarbituric acid and 5 ml of deionized water (sample blank), briefly shaken and kept in the dark overnight for 15-20 h. The absorbances of the two solutions were then read at 530 nm against a blank (deionized water). Sample blank absorbance was subtracted from the sample reading. To quantify TBARS, an MDA calibration curve was built from a 25 μM TEP mother solution and with a calibration range from 0.625 to 6.25 μM. The absorbance of each standard MDA solution was plotted against MDA amount (nmol); each standard solution was prepared in duplicate. A new calibration curve was built every week. TBARS were expressed as mg of MDA/kg of sample.

5.3.6. Cold saponification of fat and COPs enrichment by solid phase extraction (SPE)

A cold saponification was performed on fat with an excess of an ethanolic alkali solution according to Dutta and Åppelqvist (1997). About 200 mg of fat were accurately weighed, dissolved in 3 ml of dichloromethane in a round bottom screw-cap glass tube (≈ 50 ml capacity), added with 2 μg of 19-hydroxycholesterol (19-OH) used as internal standard (solution in dichloromethane), 10 ml of 1 M KOH in 95.5% ethanol, briefly

shaken and left overnight (18 h) in the dark at room temperature. After the addition of 7 ml of dichloromethane and 10 ml of water, the tube was vigorously shaken and centrifuged at 3000 rpm for 3 min. The upper water layer was discarded and the organic phase was added with 5 ml of 0.5 M KOH. The tube was gently shaken, centrifuged at the same condition above described and the water phase was again discarded. Then, the organic phase was washed with 10 ml of water, taking care to gently shake, centrifuge and remove the upper phase. This procedure was repeated until a clear organic solution was obtained. The solvent was removed under nitrogen flow. The unsaponfiable matter was dissolved in 1 ml of *n*-hexane/diethyl ether 3/1 (v/v) and stored at -20 °C until SPE. Enrichment and purification of COPs from other unsaponifiable compounds was performed employing Silica cartridges for SPE from Isolute, Mid-Glamorgan, UK (SI cartridges, 6 ml reservoir, 500 mg sorbent mass). Before sample loading, each cartridge had been placed in a SPE elution apparatus and conditioned with 3 ml of *n*-hexane. To partly remove the apolar materials and unoxidized sterols, after sample loading, the cartridge was eluted with 3 ml of *n*-hexane/diethyl ether 3/1 (v/v) and 3 ml of *n*-hexane/diethyl ether 3/2 (v/v), taking care to let the solvent eluting drop wise and not to let the cartridge dry. COPs were collected eluting the cartridge with 4 ml of acetone/methanol 3/2 (v/v). The COPs containing fraction was dried under nitrogen and added with 1 ml of *n*-hexane/diethyl ether 3/1 (v/v). Subsequently, the entire SPE procedure was repeated to better purify COPs from unoxidized sterols and the last fraction was stored at -20 °C until derivatization.

5.3.7. Preparation of trimethylsilyl (TMS) derivatives of COPs

Prior to gas chromatographic analyses, COPs were converted to the corresponding TMS-ether. Briefly, the COPs fraction from SPE was dried under nitrogen and added with 100 µl of a silylating mixture Tri-Sil® Reagent (pyridine/hexamethyldisilazane/trimethylchlorosilane 10/2/1 (v/v/v)) purchased from Pierce (Rockford, IL, USA). The tube was sonicated for 1 min, kept at 60 °C for 45 min. Thereafter the silylating agent was removed under nitrogen and the tube was added with 100 µl of *n*-hexane, sonicated for 1 min and centrifuged at 2500 rpm for 3 min.

5.3.8. Capillary gas chromatographic determination (CGC) of cholesterol oxidation products (COPs)

The TMS-ethers derivativesof COPs were analyzed by CGC using Varian Star 3400 CX instrument (Varian, Palo Alto, CA, USA) coupled with a flame ionization detector (FID). A combination of two fused- silica capillary columns, DB5-MS and DB17- MS from J&W Scientific (Folsom, CA, USA) and having same dimensions (10m × 0.18 mm i.d., film thickness 0.18 µm) and jointed together by a universal press-fit connector from NTK Kemi (Uppsala, Sweden) was used. The capillary column, DB17-MS was connected to the injector side while the capillary column DB5-MS was connected to the detector side. Helium was used as carrier gas at an inlet pressure of 18 psi and nitrogen was employed as a make-up gas at a flow rate of 30 ml /min. About 0.4 µl of the derivatized sample was injected in splitless mode (split delay time: 1 min). The initial oven temperature was held at 60 °C 1 min, raised at a rate of 50 °C/min to 260 °C, held at 260 °C for 5 min and then raised at 1 °C/min to 280 °C and finally kept at 280 °C for 10 min. The injector and the detector temperatures were set at 260 °C and 310 °C

respectively. The peak areas were computed by Star Chromatography Software version 4.01 from Varian and quantification was done relative to 19-OH.

5.3.9. Identification of COPs by gas chromatography/mass spectrometry (GC/MS)

The GC-MS analyses of TMS derivatives of COPs were performed on a GC 8000 Top Series gas chromatograph (CE Instruments, Milan, Italy) coupled to a Voyager mass spectrometer with MassLab data system version 1.4V (Finnigan, Manchester, UK). The samples were injected in splitless mode with a split delay time of 1 min.. The carrier gas helium was set at an inlet pressure of 20 psi. The COPs were determined on the same column system and conditions as used for GC analysis described above. Injector temperature was set at 260 °C. The mass spectra were recorded at electron energy of 70 eV, and the ion source temperature was 200 °C. The spectra were scanned in the range of m/z 50-600. COPs were identified by comparing the mass spectra with standard samples of COPs

5.3.10. Preparation of fatty acids methylesters (FAME)

Total fatty acids were determined according to Savage et al. (1997). About 10-15 mg of fat were exactly weighed in a 10 ml screw cap glass tube, dissolved in 0.5 ml of *n*-hexane, added with 2 ml of 0.01 NaOH in dry methanol and kept at 60 °C in a water bath for 10 min. Then 3 ml of borum trifluoride in methanol were added to catalyze the methylation of free fatty acids. The tube was kept for further 10 min at 60 °C, cooled under running water for about 3 min and finally added with 2 ml of 20% (w/v) NaCl and 1 ml of *n*-hexane. The tube was vigorously shaken and centrifuged at 2500 rpm for

3 min to allow the two layers to separate. About 1 ml of the upper organic layer was transferred to a smaller glass tube and kept at -20 °C until CGC analysis.

5.3.11. CGC determination of FAME

CGC analysis of FAME was performed on a CP 9001 gas chromatograph from Chrompack (Middelburg, The Netherlands) equipped with an autosampler, a split/splitless injector and a FID. Each sample was injected (1 µl) at a split injection mode and the split ratio was 1/30. The separation of the different FAME was achieved on a fused silica capillary column BGX70 from SGE (Austin, TX, USA) fitted on the gas chromatograph (column dimensions: 50 m × 0.22 mm i.d., 0.25 µm film thickness). The oven temperature was held at 155 °C for 5 min, increased from 155 to 225 °C at 2 °C/min and finally held at 225 °C for 4 min. Injector and FID temperatures were respectively set at 230 and 250 °C. Helium was used as carrier gas at a pressure of 150 kPa while nitrogen was employed as make up gas at a flow rate of 30 ml/min. The peaks identification was accomplished comparing the retention times with those of a standard mixture FO 7 purchased from Larodan AB (Malmö, Sweden). Gas chromatographic data were filed and processed by the software Maestro Chromatography Data System version 2.4 from Chrompack.

5.3.12. Statistics

Each analytical determination was performed in triplicate and the results were reported as the mean of the values obtained (n = 3). Standard deviation (SD) was chosen as spreading index. The data underwent one-way analysis of variance (ANOVA) using the

software Statistica 6.0 from StatSoft (Tulsa, OK, USA). Unless specified, Tukey's honest significant difference (HSD) test was evaluated at a $p < 0.05$ level.

5.4. RESULTS AND DISCUSSION

5.4.1. Fatty acids content

In **Table 5.1.** the content of the main fatty acids and the percentage of polyunsaturated fatty acids (PUFA) of each sample is shown. The content of the different fatty acids was in the range of published reports (Rossell, 1992; Lawrie, 1998). Some differences were found as regards the content of polyenoic acids, since the raw samples stored under MAP showed a lower oleic acid content and a higher linoleic and heicosatrienoic percentages with respect to A_1-R and A_8-R. This difference may have been a consequence of the higher amount of depot fat present in A_1-R and A_8-R in which the fat content (w/w) was 12.1 and 11.0% respectively while in all the samples stored under MAP it ranged from 6.4 to 6.5%. In fact it is known that lipids in muscle contain greater contents of phospholipids than adipose fat and then the degree of unsaturation is higher in lean muscle than in adipose triglycerides (Foegeding et al., 1996). PUFA associated with phospholipids are critical to the development of off-flavour in muscle. Cooking did not significantly modify the fatty acid content even in recent works opposite findings have been reported. Rodriguez-Estrada et al. (1997) verified that different cooking methods (roasting, microwave, boiling and pan frying) caused a significant increase in PUFA (3.5-3.6%) with respect to raw beef hamburgers (3.2%). Nevertheless, Echarte et al. (2003) recently reported that microwave and pan frying in olive oil affected fatty acid profile in beef patties, bringing to a decrease in PUFA amount. In this study, the

choice not to employ frying fat in cooking was the main reason accounting for the negligible changes brought by processing on fatty acid amounts. About the effect of packaging on the polyunsaturated lipids, during the first 8 days of storage no remarkable decrease occurred regardless the different atmosphere employed to preserve the samples. A first significant change took place in meat stored under MAP but after 15 days, a week after the expiring date of the product. The decrease that took place in MAP_{15}-R as regards the amounts of linoleic (C18:2), α-linolenic (C18:3 n-3) and heicosatrienoic (C20:3 n-3) acids with respect to MAP_1-R was 14, 26 and 36% respectively for an overall fall in polyunsaturated fatty acids corresponding to 20%. A similar trend was also observed comparing the fatty acid data of sample MAP_1-C and MAP_{15}-C: the decrease in linoleic, α-linolenic, heicosatrienoic and total PUFA fatty acids that occurred was 13, 20, 32 and 17% respectively. As later discussed, sample MAP_{15}-R proved to be highly oxidized in comparison to other raw samples stored under MAP and aerobically and this explained the decrease in PUFA that was observed. However, it should be pointed out that the qualitative and quantitative determination of total fatty acids did not allow to follow the different trend of lipid oxidation in its early stages and the effect of packaging on the development of rancidity. No significant correlation ($p < 0.05$) was fount between polyunsaturated fatty acid amount and the others parameters employed to evaluate lipid oxidation like PV, TBARS and COPs. This was in agreement with a review about the methods for following lipid oxidation in meat in which changes in polyunsaturated fatty acids composition was proposed as an indirect measure of the meat susceptibility to lipid oxidation (Melton, 1983). Actually, in the same paper the opposite results reported in different previous works were

resumed and the need of more reliable methods to follow rancidity especially in lamb and beef meat was highlighted as well.

5.4.2. Lipid oxidation: peroxide value (PV) and thiobarbituric acid reactive substances (TBARS)

To carry out an evaluation of the effect of storage conditions and cooking technique on the development of lipid peroxidation the parameters PV and TBARS were determined. PV represents the first oxidation products and reflect the quality of the raw materials while TBARS were the products formed from peroxides breakdown. The results are shown in **Table 5.2.** Both kind of fresh samples (MAP_1-R and A_1-R), purchased one day after packaging, showed a similar and good oxidative status: PV were ≈ 1.0 meq O_2/kg of fat for both samples whereas TBARS did not exceed 1.0 mg MDA/kg of meat. According to literature data PV appears as an extremely changeable parameter in unprocessed raw beef hamburgers, ranging from 0.04 (Larkeson et al., 2000) to 8.0 meq O_2/kg of fat (Rodriguez-Estrada et al., 1997) and this depends on the several factors can affect the initial oxidative status of meat like post-mortem storage conditions and processing, the presence of catalysts such as transition metals and antioxidant (tocopherols), carcass temperature, level of myoglobine and oxygen, a different surface-to-volume ratio and the action of light (Gray et al., 1996; Rodriguez-Estrada et al., 1997; Erickson, 2002). Besides the analytical results regarding PV are also affected by the specific procedure used and test temperature (Nawar, 1996) or the possibility for hydroperoxides to react with protein (Melton, 1983).

About raw meat stored under MAP, after eight days of storage only a small increase in PV took place: lipid oxidation was still in the so-called initiation phase in which

peroxide formation and O_2 absorption were extremely slow. Nevertheless in the second week of storage lipid peroxidation took off and a ten-fold rise in PV occurred. In raw meat stored aerobically, it was remarkable that from A_1-R to A_8-R no increase in PV occurred but a small decrease was recorded: the rate of decomposition of peroxides to secondary oxidation products in the first week of storage was faster that their formation, causing a trend in PV that was opposite to that regarding secondary oxidation products. From PV determination, rancidity seemed to be at an earlier stage in raw meat samples stored aerobically in comparison with those stored under an oxygen-enriched MAP. Cooking is a known processing treatment that modifies lipid oxidation (Erickson, 2002). In this study the effect of cooking on peroxide formation appeared to be influenced by the concentration of peroxides in the raw material confirming the results reported elsewhere (Rodriguez-Estrada et al., 1997) in which to an advanced degree of oxidation in raw meat corresponded a decrease in PV after cooking, regardless the technique employed. In the samples A_1-C and A_8-C, the effect of cooking was to duplicate and triplicate respectively the peroxide content in cooked meat with respect the corresponding raw materials: as longer the storage time of raw meat as bigger the PV rise after pan frying. When lipid oxidation was still at a beginning stage, cooking catalyzed the formation of peroxides and heat proved to be a pro-oxidant factor.

In meat under MAP, pan frying led to a rise in PV as regards samples MAP_1-C and MAP_8-C in which peroxides amount was about five and three times higher with respect to MAP_1-R and MAP_8-R respectively. Nevertheless in this case the older the raw meat the lower the rise in PV. Moreover the application of cooking on MAP_{15}-R produced a significant decreased in PV in the corresponding cooked samples MAP_{15}-C, in agreement with Rodriguez-Estrada et al. (1997) as formerly stated. When PV

concentration in meat was too high, the pro-oxidant effect of heating on the formation of peroxides was overcome by a counter effect that catalyzed peroxides breakdown. Despite peroxide enable a first study of the oxidative status of raw meat, PV was not a proper parameter to evaluate the extent of lipid peroxidation in cooking samples, owing to the transient nature of such compound especially in food systems that undergo harsh heat treatments.

TBARS test is one of the most widely method to measure secondary lipid oxidation products in foods (Nawar, 1996; Kołakowska, 2003). The changes in TBARS values are illustrated in **Table 5.2.** and as discussed as follows they indicated that the kind of packaging of meat was more important than cooking treatment on the development of lipid rancidity and that samples stored under aerobic atmosphere showed a higher oxidative stability. TBARS amounts detected in fresh samples A_1-R and MAP_1-R were in agreement with published data. According to Houben et al. (2000, 2002) the levels of TBARS in fresh minced beef meat were comprised in the range 0.7-0.8 mg MDA/kg of meat for samples stored under aerobic atmosphere and 0.9-1.1 mg MDA/kg of meat for meat stored under oxygen-enriched MAP. Nevertheless other investigators have reported higher TBARS values ranging from 1.8 to 10.4 mg MDA/kg of meat (Britt et al., 1998; Formanek et al., 1998; Nam et al., 2001) in fresh beef meat. Indeed TBARS test, due to the possibility of malonaldehyde to react with protein and the interfering action of other compounds, is generally employed in comparing samples of the same material at different stages of oxidation (Nawar, 1996) rather than determining the exact amount of secondary oxidation products. After eight days under MAP, TBARS increased four times with respect MAP_1-R while in the same share of time under aerobic packaging the amount of TBARS found in A_8-R was less than twice the one

determined in A_1-R, confirming that packaging affected the rate of oxidation in raw meat and that an oxygen-enriched environment was effective in weakening the anti-oxidant capacity of meat. A great rise in TBARS, as well as noticed for PV, took place in the second week of storage in which their amount reached a level about fifteen times higher than that in MAP_1-R. The observed effect of packing on TBARS formation rate was similar to the finding of other oxidation studies on beef meat. Houben et al. (2000) reported that TBARS increase from 0.8 to 3.1 mg MDA/kg of meat and from 1.1 to 5.7 mg/kg of meat in minced beef meat respectively stored aerobically and under a MAP with high oxygen level ($O_2/CO_2/N_2$, 65/25/10) during a week's storage. Formanek et al. (1998) verified that TBARS rose from 2.3 to 3.0 mg MDA/kg of meat and from 2.3 to 5.6 MDA/kg in minced beef under aerobic packaging and MAP respectively (O_2/CO_2, 80/20). In other investigations the effect of packaging was not noticeable: Houben et al. (2002) found TBARS values lower in ground beef meat stored for seven days under MAP ($O_2/CO_2/N_2$, 75/20/5) than in meat wrapped in an oxygen-permeable foil: from 0.7 to 3.5 mg MDA/kg of meat in aerobic conditions and from 0.9 to 1.7 mg MDA/kg under MAP. Similarly Gatellier et al. (2001) did not accessed a significant effect of packaging (aerobic vs. MAP O2/CO2 80/20) on lipid oxidation in different cattle muscles stored for 13 days at 3 °C.

In this experimentation, the effect of cooking on meat was to cause a slight decrease in TBARS amount with respect the corresponding raw samples. Actually TBARS are low molecular weigh and relatively polar compounds with respect to fatty hydroperoxides and a reason accounting for the decrease in TBARS occurred after cooking was a loss of these compounds in the water lost during pan frying. Moreover, the longer the preservation time for raw meat, the higher the decreasing effect of cooking on TBARS

amount. In samples MAP_1-C, MAP_8-C and MAP_{15}-C the cooking losses were respectively 39.7, 42.7 and 45.3% while the decrease in TBARS found with respect MAP_1-R, MAP_8-R and MAP_{15}-R were 9.6, 20.4 and 28.1%: to an increase in weigh loss corresponded an increase in TBARS loss after cooking. In meat stored under aerobic atmosphere the weigh loss after 8 days was quite lower with respect other samples (32.1%) and in fact TBARS amount was nearly the same between A_8-R and A_8-C.

5.4.3. Lipid oxidation: cholesterol oxidation products (COPs)

Cholesterol oxidation is an established free radical process that follows the same oxidative pattern involving in the oxidation of unsaturated lipids and that can be induced by reactive oxygen species and peroxy radicals of oxidized neighbouring fatty acids (Paniangvait et al., 1995; Lercker et al., 2002; Wąsowicz, 2003).

COPs identified in meat samples that were object of this study were: 7α and 7β-hydroxycholesterol (7α-HC and 7β-HC), cholesterol-α-epoxide (α-CE), cholesterol-β-epoxide (β-CE), 7-ketocholesterol (7-kC), cholestanetriol (CT) and 25-hydroxycholesterol (25-HC). The two latter compounds were present only in trace (less than 1 μg/kg of fat) while some not identified by-products co-eluted in some samples with α-CE and β-CE and did not enable an overall proper quantification of the two epoxides. Thus in **Tables 5.3.** and **5.4.** are reported the amounts of the three major COPs determined: 7α-OH, 7β-OH and 7-K and the sum of them (≈ 80% of total COPs), respectively expressed as μg/g of fat and μg/g of meat to take into account the weigh loss that occurred during pan frying. The amounts of total COPs found at the beginning of preservation in MAP_1-R and A_1-R (≈ 1 μg/g of meat) were in agreement with results recently reported elsewhere. Pie et al. (1991) and Nam et al. (2001) reported 3.4 and 3.3

μg/kg respectively as the amount of COPs determined in raw beef meat, Hwang and Maerker (1993) and Echarte et al. (2003) detected 0.5 and 0.2 μg/g of meat in beef patties respectively while Larkeson et al. (2000) found 0.9 μg of COPs/g of meat in raw commercial beef hamburgers. The kind of packaging atmosphere had a dramatic effect on cholesterol oxidation in raw beef meat: while meat aerobically stored was stable to cholesterol oxidation during storage and no significant increase in COPs amount was recorded after 1 week, in the same period COPs increased about three times under MAP, confirming the pro-oxidant effect of this packaging. Nam et al. (2001) verified that in beef meat aerobically packaged only a small rise in COPs content occurred at 4 °C after 1 week (from 3.3 to 3.8 to μg/g of meat) even no literature data described the effect of a high-oxygen MAP cholesterol oxidation in beef meat. Nevertheless, Cayuela et al. (2004) illustrated that a packaging atmosphere with a high-oxygen content (O_2/CO_2, 70/30) caused a significant rise in the rate of cholesterol oxidation in pork loin chops stored under different conditions: the total COPs increase was 18.2 and 86.4% under aerobic (6 days at 4 °C) and oxygen-enriched packaging (13 days at 4 °C) respectivelly.

About the effect of pan frying on COPs formation, as a general trend, the longer the storage time, the higher the increase in COPs content after frying with respect the corresponding raw samples. In the thermally treated samples A_1-C and MAP_1-C the amounts of COPS were similar to those of the corresponding untreated samples and no significant increase after cooking took place. After 8 and 15 days since packaging, the COPs amounts in MAP_8-C and MAP_{15}-C were 33 and 71% respectively higher than in MAP_8-R and MAP_{15}-R, expressing COPs as μg/g of fat. Because of the water loss and the consequent rise in fat percentage in meat, these differences were higher calculating

COPs as μg/g of meat: 102 and 180% after 8 and 15 days since packaging under MAP respectively. In aerobically packaged stored meat, the effect of cooking on cholesterol oxidation was even higher and in sample A_8-C a four-fold rise in COPs level was observed.

Different papers focusing on the effect of cooking on the formation of COPS, reported a negligible rise in COPs after different heat treatments as here-observed. De Vore (1988) and Larkeson et al. (2000) did not notice, after microwave and pan frying respectively, a significant and immediate increase in COPs in hand-made and commercial beef hamburgers. Similarly, after different thermal processing (stove, hot plate, microwave plus roasting) the absence of COPs in raw samples were kept as well in cooked beef hamburgers (Vicente and Torres, 2007). On the contrary, Rodriguez-Estrada et al. (1997) observed that the amount of 7-kC in an oxidized raw beef meat (3.5 μg of kC/g of meat) was lower after different cooking treatments and ranging from 1.7 to 2.5 μg/g of meat. Different reasons may be taken into account and have been proposed to explain the low effect that cooking proved to have on cholesterol oxidation in raw fresh meat. The rate of 7-kC breakdown can be higher than its formation during cooking (Rodriguez-Estrada et al., 1997), as observed in this study as regards PV in meat stored under MAP after 15 days. Moreover the interaction between COPs and other compounds can make cholesterol oxides undetectable (Rodriguez-Estrada et al., 1997). In some works (Kim and Nawar, 1993; Chien et al., 1998) 120 °C was reported as the minimal temperature required to start COPs formation during thermal processing. Thus the low temperature (71-80°C) reached at the core of the products cannot cause a remarkable conversion of cholesterol to COPs. Finally, a small amount of COPs can be loss along with the fat released from during cooking (Vicente and Torres, 2007).

Nevertheless the effect of cooking on cholesterol oxidation has still to be completely clarified since in other researches an increase in total COPs has been observed in beef meat. In a work by Pie el al. (1991) the concentration of oxysterols in minced beef meat was increased by cooking in an electric skillet from 3.4 to 5.1 μg/g of meat (after 3 min) and to 5.9 (after 10 min) while Echarte et al. (2003) found that COPs rose from 0.2 to 0.4 μg/g of meat after frying in olive oil.

No literature data were available on the effect of thermal processing on meat after aging at 4°C that was here studied. The finding formerly reported proved that even pan frying had a negligible catalyzing action on the development of cholesterol oxides, this pro-oxidant effect became more significant as longer the aging period of meat. This finding may depend on the decrease in the natural anti-oxidant capacity of raw meat during preservation: lipid peroxidation, as proved from PV and TBARS analyses, was more intense as older was the raw meat analyzed. Thus, the progressive development of lipid rancidity caused a weakening in the resistance of raw meat against lipid but also cholesterol oxidation.

5.5. CONCLUDING REMARKS

The present study represents a first attempt to evaluate the effect of packaging under an oxygen-enriched modified atmosphere on the development of lipid and cholesterol oxidation in raw and cooked beef meat. The results showed that oxidation in raw meat was more intense during storage in beef ground meat stored under MAP than in samples aerobically preserved. Pan frying had a negligible effect on cholesterol oxidation on fresh meat, regardless the type of packaging but as older the meat became as higher the

increase in cholesterol oxide amounts in cooked samples with respect to the corresponding raw ones. The effect of different cooking techniques on oxidative degree in beef meat represents an aspect that should be deeper investigated and clarified. Even MAP should ensure a longer shelf-life to meat, its high oxygen content did not protect the product from a faster development of rancidity and cholesterol oxidation.

5.6. REFERENCES

Ahn, D. U., Nam, K. C., Du, M., Jo, C. 2001. Effect of irradiation and packaging conditions after cooking on the formation of cholesterol and lipid oxidation products in meats during storage. Meat Science 57, 413-418.

Bell, R. G. 2001. Meat Packaging: Protection, Preservation, and Presentation. In: Meat Science and Applications (edited by Y. H. Hui, Wai-Kit Nip, R. W. Rogers, O. A. Young). Pp.: 463-490. New York, Basel: Marcel Dekker.

Britt C., Gomaa E. A., Gray J. I., Booren A. M. 1998. Influence of cherry tissue on lipid oxidation and heterocyclic aromatic amine formation in ground beef patties. Journal of Agricultural and Food Chemistry 46, 4891-4897.

Cayuela, J. M., Gil, M. D., Bañón S., Garrido, M. D. 2004. Effect of vacuum and modified atmosphere packaging on the quality of pork loin. European Food Research and Technology 219, 316-320.

Chien J. T., Wang, H. C., Chen B. H. 1998. Kinetic model of the cholesterol oxidation during heating. Journal of Agricultural and Food Chemistry 46, 2572-2577.

Conchillo, A., Ansorena, D., Astiasarán, I. 2005. Use of microwave in chicken breast and application of different storage conditions: consequences on oxidation. European Food Research and Technology 221, 592-596.

De Vore, V. R. 1988. TBA values and 7-ketocholesterol in refrigerated raw and cooked ground beef. Journal of Food Science 53, 1058-1061.

Du, M., Nam, K. C., Ahn, D. U. 2001. Cholesterol and lipid oxidation products in cooked meat as affected by raw-meat packaging and irradiation and by cooked-meat packaging and storage time. Journal of Food Science 66, 1396-1401.

Dutta, P. C., Appelqvist, L. Å., 1997. Studies on phytosterol oxides. I: Effect of storage on the content in potato chips prepared in different vegetable oil. Journal of the American Oil Chemists' Society 74, 647-657.

Echarte, M., Ansorena, D., Astiasarán, I. 2003. Consequences of microwave heating and frying on the lipid fraction of chicken and beef patties. Journal of Agricultural and Food Chemistry 51, 5941-5945.

Erickson, M. C. 2002. Lipid Oxidation of Muscle Foods. In: Food Lipids – Chemistry, Nutrition, and Biotechnology – Second Edition, Revised and Expanded (edited by C. C. Akoh, D. B. Min). Pp.: 365-411. New York, Basel: Marcel Dekker.

Foegeding, E. A., Lanier, T. C., Hultin, H. O. 1996. Characteristics of Edible Muscle Tissues. In: Food Chemistry, Third Edition (edited by O. R. Fennema). Pp.: 879-942.

Formanek, Z., Kerry, J. P., Buckley, D. J., Morrissey P. A., Farkas, J. 1998. Effects of dietary vitamin E supplementation and packaging on the quality of minced beef. Meat Science 50, 203-210.

Fox, J. B. 1966. The chemistry of meat pigments. Journal of Agricultural and Food Chemistry 14, 207-210.

Galvin, K., Lynch, A. M., Kerry, J. P., Morrissey, P. A., Buckley D. J. 2000. Effect of dietary vitamin E supplementation on cholesterol oxidation in vacuum packaged cooked beef steaks. Meat Science 55, 7-11.

Garcia-Cruset, S., Carpenter, K. L. H., Codony, R., Guardiola, F. 2002. Cholesterol Oxidation Products and Atherosclerosis. In: Cholesterol and Phytosterol Oxidation Products – Analysis, Occurrence, and Biological Effects (edited by F. Guardiola, P. C. Dutta, R. Codony, G. P. Savage). Pp.: 241-277. Champaign, IL: AOCS Press.

Gatellier, P., Hamelin, C., Durand, Y., Renerre, M. 2001. Effect of a dietary vitamin E supplementation on colour stability and lipid oxidation of air- and modified atmosphere-packaged beef. Meat Science 59, 133-140.

Gray J. I., Gomaa E. A., Buckley D. J. 1996. Oxidative quality and shelf life of meats. Meat Science 43, S111-S123.

Hara, A., Radin, N. S., 1978. Lipid extraction of tissues with a low toxicity solvent. Analytical Biochemistry 90, 420-426.

Houben J. H., van DijK A., Eikelenboom, G., Hoving-Bolink A. H. 2000. Effect of dietary vitamin E supplementation, fat level and packaging on colour stability and lipid oxidation in minced beef. Meat Science 55, 331-336.

Houben, J. H., van Dijk, A., Eikelenboom, G. 2002. Dietary vitamin E supplementation, an ascorbic acid preparation, and packaging effects on colour stability and lipid oxidation in mince made from previously frozen lean beef. European Food Research and Technology 214, 186-191.

Hwang, K. T., Maerker, G. 1993. Quantitation of cholesterol oxidation products in unirradiated and irradiated meats. Journal of the American Oil Chemists' Society 70, 371-375.

International Dairy Federation, 1991. International IDF Standard 74A. Brussels, Belgium.

Kanner J. 1994. Oxidative processes in meat and meat products: quality implications. Meat Science, 36, 169-189.

Kerry, J .P., Gilroy, D. A., O'Brien, N. M. 2002. Formation and Content of Cholesterol Oxidation Products in Meat and Meat Products. In: Cholesterol and Phytosterol Oxidation Products – Analysis, Occurrence, and Biological Effects (edited by F. Guardiola, P. C. Dutta, R. Codony, G. P. Savage). Pp.: 162-185. Champaign, IL: AOCS Press.

Kim, S. K., Nawar W. W. 1993. Parameters influencing cholesterol oxidation. Lipids 28, 917-922.

Kołakowska, A. (2003) Lipid Oxidation in Food Systems. In: Chemical and Functional Properties of Food Lipids (edited by Z. E. Sikorski and A. Kołakowska). Pp. 133-166. Boca Raton, London, New York, Washington, D.C.: CRC Press.

Larkeson, B., Dutta, P. C., Hansson, I. 2000. Effects of frying and storage on cholesterol oxidation in minced meat products. Journal of American Oil Chemists' Society 77, 675-680.

Lawrie, R. A. 1998. Chemical and Biochemical Composition of Muscle. In: Lawrie's Meat Science – Sixth Edition. Pp.: 58-95. Cambridge: Woodhead Publishing Limited.

Lercker, G., Rodriguez-Estrada, M. T. 2002. Cholesterol Oxidation Mechanisms. In: Cholesterol Oxidation Products: Other Biological Effects. In: Cholesterol and Phytosterol Oxidation Products – Analysis, Occurrence, and Biological Effects (edited by F. Guardiola, P. C. Dutta, R. Codony, G. P. Savage). Pp.: 1-25. Champaign, IL: AOCS Press.

Melton, S. L. 1983. Methodology for following lipid oxidation in muscle foods. Food Technology 37(7), 105-111, 116.

Miller, D.D., 1998a. Meat Pigments. In: Food Chemistry – A Laboratory Manual. Pp. 101-105. New York, Chichester, Weinhiem, Brisbane, Singapore, Toronto: Wiley-Interscience, John Wiley & Sons.

Miller, D.D., 1998b. Lipid Peroxidation. In: Food Chemistry – A Laboratory Manual. Pp. 57-67. New York, Chichester, Weinhiem, Brisbane, Singapore, Toronto: Wiley-Interscience, John Wiley & Sons.

Morrissey P. A., Sheehy P. J. A., Galvin K., Kerry J. P., Buckley D. J. 1998. Lipid stability in meat and meat products. Meat Science 49, S73-S86.

Nam, K. C., Du, M., Jo, C., Ahn, D. U. 2001. Cholesterol oxidation products in irradiated raw meat with different packaging and storage time. Meat Science 58, 431-435.

Nawar, W. W. 1996. Lipids. In: Food Chemistry, Third Edition (edited by O. R. Fennema). Pp.: 225-319. New York, Basel, Hong Kong: Marcel Dekker.

Osada, K. 2002. Formation and Content of Cholesterol Oxidation Products in Meat and Meat Products. In: Cholesterol Oxidation Products: Other Biological Effects. In: Cholesterol and Phytosterol Oxidation Products – Analysis, Occurrence, and

Biological Effects (edited by F. Guardiola, P. C. Dutta, R. Codony, G. P. Savage). Pp.: 278-318. Champaign, IL: AOCS Press.

Paniangvait P., King, A. J., Jones, A. D., German, B. G. 1995. Cholesterol oxides in foods of animal origin. Journal of Food Science 60, 1159-1174.

Pie, J. E., Spahis, K., Seillan, C. 1991. Cholesterol oxidation in meat products during cooking and frozen storage. Journal of Agricultural and Food Chemistry 39, 250-254.

Rodriguez-Estrada, M .T., Penazzi, G., Caboni, M. F., Bertacco, G., Lercker, G. 1997. Effect of different cooking methods on some lipid and protein components of hamburgers. Meat Science 45, 365-375.

Rossell, J. B. 1992. Chemistry of Lipids. In: The Chemistry of Muscle-based Foods (edited by D. E. Johnston, M. K. Knight, D. A. Ledward). Pp.: 193-202. Cambridge: The Royal Society of Chemistry.

Savage, G. P., McNeil, D. L., Dutta, P. C., 1997. Lipid composition and oxidative stability of oils in hazelnuts (*Corylus avellana* L.) grown in New Zealand. Journal of the American Oil Chemists' Society 74, 755-759.

Schroepfer, G. J. Jr 2000. Oxysterols: modulators of cholesterol metabolism and other processes. Physiological Reviews 80, 361-554.

Seideman, S. C., Durland, P. R. 1984. The utilization of modified gas atmosphere packaging for fresh meat: a review. Journal of Food Quality 6, 239-252.

Vicente, S. J. V., Torres, E. A. F. S. 2007. Formation of four cholesterol oxidation products and loss of free lipids, cholesterol and water in beef hamburgers as a function of thermal processing. Food Control 18, 63-68.

von Elbe, J. H., Schwartz, S. J. 1996. Colorants. In: Food Chemistry, Third Edition (edited by O. R. Fennema). Pp.: 651-722. New York, Basel, Hong Kong: Marcel Dekker.

Wąsowicz. E. 2003. Cholesterol and Phytosterols. In: Chemical and Functional Properties of Food Lipids (edited by Z. E. Sikorski and A. Kołakowska). Pp. 93-107. Boca Raton, London, New York, Washington, D.C.: CRC Press.

5.7. TABLES

Table 5.1.– Total fatty acids composition of the different beef meat samples. All the results are expressed as mean ± SD (n = 3).

Fatty acid (% FAME)	MAP						Aerobic packaging			
	Day 1		Day 8		Day 15		Day 1		Day 8	
	MAP_1-R	MAP_1-C	MAP_8-R	MAP_8-C	MAP_{15}-R	MAP_{15}-C	A_1-R	A_1-C	A_8-R	A_8-C
C14:0 (myristic)	2.5 ± 0.1	2.5 ± 0.1	2.6 ± 0.2	2.5 ± 0.1	2.6 ± 0.1	2.5 ± 0.0	3.1 ± 0.1	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0
C16:0 (palmitic)	29.9 ± 0.8	31.0 ± 0.9	29.8 ± 1.2	30.0 ± 1.0	30.7 ± 0.3	30.8 ± 0.6	27.6 ± 0.3^{2a}	27.7 ± 0.1^{2a}	28.9 ± 0.1^{1a}	29.0 ± 0.1^{1a}
C16:1 (palmitoleic)	3.5 ± 0.1	3.4 ± 0.1	3.8 ± 0.2	3.5 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	4.0 ± 0.1^{1b}	4.6 ± 0.0^{1a}	4.1 ± 0.0^{1a}	4.2 ± 0.0^{2a}
C 17:0 (margaric)	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0^{1a}	1.0 ± 0.0^{2b}	1.0 ± 0.0^{1a}	1.0 ± 0.0^{1b}
C18:0 (stearic)	19.7 ± 0.7	20.4 ± 0.7	19.0 ± 1.3	19.9 ± 1.0	19.8 ± 0.3	20.2 ± 0.4	16.6 ± 0.2^{2a}	15.6 ± 0.0^{2b}	17.5 ± 0.0^{1a}	17.5 ± 0.1^{1a}
C18:1 (oleic)	37.0 ± 1.2	35.3 ± 1.1	37.5 ± 1.7	36.5 ± 1.5	36.7 ± 0.5	36.2 ± 0.8	42.4 ± 0.3^{1a}	42.7 ± 0.0^{1a}	40.2 ± 0.1^{2a}	40.0 ± 0.1^{2a}
C18:1 n-7 (vaccenic)	1.5 ± 0.1	1.5 ± 0.0	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0^{2b}	1.7 ± 0.0^{1a}	1.5 ± 0.0^{1b}	1.6 ± 0.0^{2a}
C18:2 (linoleic)	3.0 ± 0.1^{1a}	2.9 ± 0.1^{1a}	2.9 ± 0.2^{1a}	3.0 ± 0.1^{1a}	2.5 ± 0.0^{2a}	2.6 ± 0.1^{2a}	2.2 ± 0.1^{1b}	2.3 ± 0.0^{1a}	2.1 ± 0.0^{1a}	2.2 ± 0.0^{2a}
C18:3 n-3 (α-linolenic)	0.6 ± 0.0^{1a}	0.6 ± 0.0^{1a}	0.6 ± 0.0^{1a}	0.6 ± 0.0^{1a}	0.5 ± 0.0^{2a}	0.5 ± 0.0^{2a}	0.6 ± 0.0^{1a}	0.6 ± 0.0^{1b}	0.6 ± 0.0^{2a}	0.6 ± 0.0^{1a}
C20:3 (heicosatrienoic)	0.6 ± 0.0^{1a}	0.6 ± 0.0^{1a}	0.6 ± 0.0^{1a}	0.6 ± 0.0^{1a}	0.4 ± 0.0^{2a}	0.4 ± 0.0^{2a}	0.3 ± 0.0^{2a}	0.3 ± 0.0^{2a}	0.3 ± 0.0^{1b}	0.4 ± 0.0^{1a}
PUFA	4.3 ± 0.1^{1a}	4.3 ± 0.2^{1a}	4.2 ± 0.3^{1a}	4.3 ± 0.2^{1a}	3.4 ± 0.1^{2a}	3.5 ± 0.1^{2a}	3.2 ± 0.1^{1a}	3.2 ± 0.0^{1a}	3.1 ± 0.0^{2a}	3.2 ± 0.0^{1a}

PUFA: polyunsaturated fatty acids.

Other fatty acids identified (< 1.0% of total identified FAME) were: C12:0, C 20:0, C 20:1, C 20:2, C20:4, C24:0 and C22:6.

Different superscript numbers denote significant differences ($p < 0.05$) amongst raw or cooked samples stored under the same conditions (i.e. MAP_1-R, MAP_8-R and MAP_{15}-R) whereas different superscript letters denote significant differences between raw and cooked samples at the same storage period (i.e.: MAP_1-R and MAP_1-C).

Table 5.2. – PV and TBARS amounts in beef meat samples under modified and aerobic atmosphere, before and after cooking. All the results are expressed as mean ± SD (n = 3).

Oxidative parameter	MAP						Aerobic packaging			
	Day 1		Day 8		Day 15		Day 1		Day 8	
	MAP_1-R	MAP_1-C	MAP_8-R	MAP_8-C	MAP_{15}-R	MAP_{15}-C	A_1-R	A_1-C	A_8-R	A_8-C
PV (meq O_2/kg of fat)	1.2 ± 0.0^{2b}	5.7 ± 0.5^{1a}	1.7 ± 0.0^{2a}	3.1 ± 0.2^{2a}	12.1 ± 1.4^{1a}	4.8 ± 0.0^{1b}	1.0 ± 0.0^{1b}	1.9 ± 0.0^{1a}	0.4 ± 0.0^{2b}	1.1 ± 0.1^{2a}
TBARS (mg MDA/kg of meat)	0.6 ± 0.0^{3a}	0.5 ± 0.0^{3a}	2.4 ± 0.1^{2a}	1.9 ± 0.1^{2a}	8.6 ± 0.8^{1a}	6.2 ± 0.3^{1b}	0.7 ± 0.1^{2a}	0.4 ± 0.1^{2a}	1.2 ± 0.1^{1a}	1.3 ± 0.2^{1a}

Different superscript numbers denote significant differences ($p < 0.05$) amongst raw or cooked samples stored under the same conditions (i.e. MAP_1-R, MAP_8-R and MAP_{15}-R) whereas different superscript letters denote significant differences between raw and cooked samples at the same storage period (i.e.: MAP_1-R and MAP_1-C).

Table 5.3. – Cholesterol oxides content, expressed as μg of COPs/g of fat, in beef meat samples under modified and aerobic atmosphere, before and after cooking. All the results are expressed as mean ± SD (n = 3).

COPs (μg/kg of fat)	MAP						Aerobic packaging			
	Day 1		Day 8		Day 15		Day 1		Day 8	
	MAP_1-R	MAP_1-C	MAP_8-R	MAP_8-C	MAP_{15}-R	MAP_{15}-C	A_1-R	A_1-C	A_8-R	A_8-C
7α-HC	2.0 ± 0.5^{3a}	3.2 ± 0.1^{2a}	10.3 ± 3.2^{2a}	9.6 ± 1.3^{2a}	26.1 ± 4.8^{1a}	30.7 ± 1.4^{1a}	3.4 ± 0.1^{1a}	6.1 ± 1.3^{2a}	3.4 ± 0.1^{1b}	17.1 ± 1.6^{1a}
7β-HC	3.0 ± 0.7^{3a}	3.9 ± 0.2^{3a}	9.1 ± 0.7^{2a}	12.6 ± 0.5^{2a}	15.6 ± 3.4^{1b}	27.3 ± 0.6^{1a}	2.8 ± 0.6^{1a}	2.2 ± 0.2^{2a}	3.2 ± 0.3^{1b}	8.1 ± 0.8^{1a}
7-kC	5.4 ± 1.2^{2a}	5.0 ± 0.7^{3a}	11.3 ± 3.5^{2b}	18.6 ± 1.7^{2a}	18.7 ± 4.2^{1b}	45.2 ± 0.7^{1a}	1.6 ± 0.2^{1a}	1.5 ± 0.1^{2a}	2.0 ± 0.3^{1b}	4.8 ± 0.3^{1a}
Total COPs	10.4 ± 2.0^{3a}	12.1 ± 0.9^{3a}	30.7 ± 0.3^{2a}	40.9 ± 2.9^{2a}	60.5 ± 12.4^{1b}	103.2 ± 1.4^{1a}	7.9 ± 0.8^{1a}	9.8 ± 1.6^{2a}	8.6 ± 0.1^{1b}	30.0 ± 2.0^{1a}

7α-HC: 7α-hydroxycholesterol; 7β-HC: 7β-hydroxycholesterol; 7-kC: 7-ketocholesterol; Total COPs: sum of the amounts of 7α-HC, 7β-HC and 7-kC.

Different superscript numbers denote significant differences ($p < 0.05$) amongst raw or cooked samples stored under the same conditions (i.e. MAP_1-R, MAP_8-R and MAP_{15}-R) whereas different superscript letters denote significant differences between raw and cooked samples at the same storage period (i.e.: MAP_1-R and MAP_1-C).

Table 4 – Cholesterol oxides content, expressed as μg of COPs/g of meat, in beef meat samples under modified and aerobic atmosphere, before and after cooking. All the results are expressed as mean ± SD (n = 3).

COPs (μg/kg of meat)	MAP						Aerobic packaging			
	Day 1		Day 8		Day 15		Day 1		Day 8	
	MAP_1-R	MAP_1-C	MAP_8-R	MAP_8-C	MAP_{15}-R	MAP_{15}-C	A_1-R	A_1-C	A_8-R	A_8-C
7α-HC	0.1 ± 0.0^{3a}	0.3 ± 0.0^{3a}	0.7 ± 0.2^{2a}	0.9 ± 0.1^{2a}	1.7 ± 0.3^{1b}	3.2 ± 0.2^{1a}	0.4 ± 0.0^{1b}	0.8 ± 0.2^{2a}	0.4 ± 0.0^{1b}	1.9 ± 0.2^{1a}
7β-HC	0.2 ± 0.0^{3a}	0.4 ± 0.0^{3a}	0.6 ± 0.0^{2b}	1.2 ± 0.0^{2a}	1.0 ± 0.2^{1b}	2.9 ± 0.1^{1a}	0.3 ± 0.1^{1a}	0.3 ± 0.0^{2a}	0.4 ± 0.0^{1b}	0.9 ± 0.1^{1a}
7-kC	0.3 ± 0.1^{2a}	0.5 ± 0.1^{3a}	0.7 ± 0.2^{2b}	1.8 ± 0.2^{2a}	1.2 ± 0.3^{1b}	4.8 ± 0.1^{1a}	0.2 ± 0.0^{1a}	0.2 ± 0.0^{2a}	0.2 ± 0.0^{1b}	0.5 ± 0.0^{1a}
Total COPs	0.7 ± 0.1^{3a}	1.1 ± 0.1^{3a}	2.0 ± 0.0^{2b}	4.0 ± 0.3^{2a}	3.9 ± 0.8^{1b}	10.9 ± 0.1^{1a}	1.0 ± 0.1^{1a}	1.4 ± 0.2^{2a}	0.9 ± 0.0^{1b}	3.3 ± 0.2^{1a}

Different superscript numbers denote significant differences ($p < 0.05$) amongst raw or cooked samples stored under the same conditions (i.e. MAP_1-R, MAP_8-R and MAP_{15}-R) whereas different superscript letters denote significant differences between raw and cooked samples at the same storage period (i.e.: MAP_1-R and MAP_1-C).

5.8. LEGENDS TO FIGURES

Figure 5.1. – Flow chart diagram of storage plan and conditions, sampling and samples' names. Each sample was stored in the dark at 3-4 °C. One-day and 8-day storage periods corresponded to the product expiring date reported on label for meat stored aerobically and under MAP respectively.

5.9. FIGURES

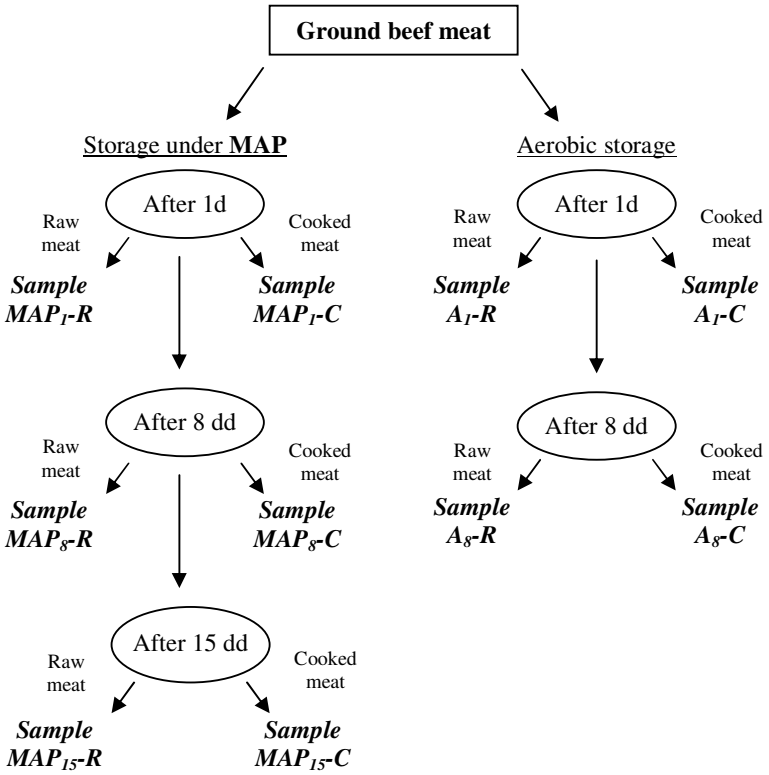


Fig. 5.1.

6. EFFECT OF FEEDING FAT AND PROCESSING TECHNOLOGY ON THE COMPOSITION AND OXIDATIVE QUALITY OF THE LIPID FRACTION OF PRE-COOKED CHICKEN PATTIES

6.1. SUMMARY AND KEYWORS

The study of the effect of feeding fat sources and production technology on the lipid fraction of raw chicken meat and final pre-cooked patties was carried out. The two technologies employed differed each other by the order of application of the cooking steps: flash-frying and cooking in steam-convection oven. Total fatty acids, free fatty acids (FFA), diglycerides (DG), peroxides (PV), thiobarbituric acids reactive substances (TBARS) and fatty acids profile in phospholipids (PS) were determined. A vegetable fat integration increased polyunsaturated acids in raw meats (≈ 43% vs. ≈ 25% of total fatty acids in meat from vegetable and animal fat-based diet respectively) even the oil absorption that occurred during cooking masked these differences and led to a similar lipid composition in cutlets. Oxidation was low in meat (PV < 1.5 meq O_2/kg of fat, TBARS < 0.2 mg/kg) and not affected by dietary fats. A good oxidative status guaranteed a low oxidation development during processing (PV < 3.0 meq O_2/kg of fat and TBARS ≤ 1.0 mg/kg fat in patties). DG were a more available parameter than FFA to check lipolysis extent. Processing slight affected lipolytic degree and in products obtained from traditional technology a lower DG amounts was recorded with respect patties from innovative technology where flash-frying was postpone to oven cooking

(0.6-0.8 g vs. 0.8-1.1 g DG/100g fat in patties from traditional and innovative technology respectively). An unexpected high percentage of monoenoic acid was found in PS of cutlets (≈ 50% vs. ≈ 30-35% in raw meat).

Keywords: feeding fat sources, lipid oxidation, lipolysis, pre-cooked chicken patties, processing.

6.2. INTRODUCTION

The popularity of chicken meat amongst consumers has been increasing in the past few years owing to some healthy perceived features. A relative low fat content (about 5% vs. 4-8% for beef and 9-11% for pork meat as reported by Foegeding et al.(1996)) and an high percentage of polyunsaturated fatty acids, varying from 18.9 (breast) to 15.5% (thigh) with respect to 4.9-6.6% and 5.3-9.2% in Longissimus dorsi and Semimembranosus muscles in beef and pork respectively (Erickson, 2002) give a significant contribution to this positive appeal. Indeed recent dietary recommendations advised not only to slight decrease fat daily intake to less than 30% of total calories but also to increase the ratio of polyunsaturated fatty acids in human diet (INRAN – MIPAF 2003; HHS/USDA, 2005). Beside fresh poultry, pre-cooked preparation based on chicken meat, belonging to the group of so-called convenient or ready-to-eat food, are becoming frequent home meal options and a wide range of innovative ingredients (coatings, new flavour, fat-replacing alternatives, use of antioxidants) for use in the area of poultry foods are now available (Pszczola, 2002). Breaded pre-fried and cooked chicken patties are an example of this kind of food. Even the low fat content, the high

phospholipids content (from 21 to 48% of total lipids in chicken white and red muscle respectively according to Foegeding et al.(1996)) and unsaturation degree make chicken meat an animal substrate and a food ingredient readily susceptible to lipid oxidation. Actually it is well known the correlation between the degree of unsaturation and the oxidizability of lipids and also that the oxidative stability of meat is main influence by the level of membrane and polar lipids like free fatty acids and phospholipids, rather than triglycerides (Nawar, 1996; Morrissey et al., 1998; Erickson, 2002). In particular the proximity of phospholipids to catalytic sites of oxidation (enzymes, heme-compounds) explains the importance of membrane lipids in tissue oxidation (Erickson, 2002). Besides these reasons concerning the composition of chicken fat, in the preparation of ready-to-eat chicken patties are involved several industrial processing treatments that may promote the development of oxidation and also significant changes in the fat composition. In particular, technological steps like mechanical deboning, skinning, grinding and cooking alter cellular compartimentalization, disrupting cellular integrity, facilitating the interactions of pro-oxidatns with polienoic acids, diluting anti-oxidants compounds and increasing the exposure of tissues to oxygen (Gray et al., 1996; Erickson, 2002). Breading and deep fat frying are two common processes employed in chicken patties preparation with the latter one consisting in immersing the breaded products in hot cooking oil for a certain share of time until a partial or complete cook is reached, leading to a significant oil absorption and water loss. The quality and type of the absorbed oil may influence the evolution of oxidation, the development of certain flavours and odours (Erickson, 2002), affects also the discolouration during storage (Ramírez, 2004) and dramatically modifies fatty acids compostion (Bonoli et al., 2007, in press).

Since the fat unsaturation index may affect the oxidative stability in meat products from one side whereas from the other manipulation of membrane lipids fatty acid composition may be accomplished altering feeding fat sources in non-ruminants (fish, poultry, pork) diet (Decker and Xu, 1998), different studies have recently focused on the effect of different type of dietary fats on the composition and quality of chicken lipids (Lopez-Bote et al., 1998a,b; O'Neill et al., 1998; Skřivan et al., 2000; Grau et al., 2001; Bou et al., 2001, 2004). Diets supplemented with α-tocopherol were effective in preventing lipid and cholesterol oxidation in chicken meat while total fatty acids composition was strongly affected by the kind of feeding fat employed: the higher the unsaturation degree in dietary fat the higher the polyunsaturated fatty acids content in meat. Other studies verified that common cooking techniques like grilling, roasting, frying and microwaving caused different increases in lipid oxidation and changes in fatty acids profile with respect to raw chicken meat (Echarte et al., 2003; Conchillo et al., 2004, 2005a,b; Eder et al., 2005). Nevertheless only two papers deal with the study of the composition and quality of the lipid fraction in industrially made pre-cooked chicken patties (Bonoli et al., 2007, in press), focusing respectively on the effect of feeding fat and processing technology on raw meat and final product. The aim of this work was to verified the results achieved in the afore-mentioned papers. In the productions of ready-to-eat chicken cutlets here analyzed were employed raw materials (breasts and thighs) coming from two groups of broilers fed respectively an animal fat and a vegetable fat based diet. Moreover the final patties were obtained with two different production technologies: a traditional processing in which formed and breaded cutlets were flash-fried and then cooked in a stem-convection oven while in the innovative process the two cooking steps were inverted in order to increase crunchiness

and weigh yield. Fat content, fatty acids profile, lipolytic and oxidative parameters like free fatty acids (FFA), diglycerides (DG), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were determined in raw meat, meat mixture before and after ingredients addiction and final products obtained both with traditional <and innovative tecxhnology.

6.3. MATERIALS AND METHODS

6.3.1. Samples

Two groups of male Ross 508 broilers were reared under commercial conditions in separeted housed and fed ad libitum two isoenergetic diets specific for growing broilers but differing each other for the kind of feeding fat employed. Indeed one group of animals was fed a diet containing animal fat (cattle tallow and pork fat) while the other one was fed a diet containing vegetable fat (olive, soybean and sunflower oil). At the age of 54 days, the broilers were slaughtered and processed 24 hours post mortem. Breasts and thighs were separated from carcasses, deboned and used, without skin, as raw materials for the preparation of final products.

The pre-cooked chicken patties were prepared in two separated pilot plants, according to two different production technologies. In the traditional technology (TT), the meat mixture (breasts and thighs plus water and additives) was formed, coated with a starch-protein batter, breaded, flash-fried at 180°C for 40 sec in an oil bath and finally cooked in a humid steam-convection oven (2 min, core temperature = 70°C). In the innovative technology (IT), the formed patties were covered with flour and breadcrumbs (pre-dust),

cooked in a humid-steam oven (2 min, core temperature = 70°C), coated with batter, breaded and finally flash-fried (180°C for 40 sec).

The samples analyzed were ground thighs from animal and vegetable diet (T-A and T-V), ground breasts from animal and vegetable diet (B-A and B-V), meat mixtures composed by 63.5% breast and 36.5% thighs (M-A and M-V), meat mixture plus water and additives (water and additives: 27.5%, samples name: I-A and I-V), final patties from traditional technology (FP-A-TT and FP-V-TT) and final patties from innovative technology (FP-A,IT and FP-V-IT).

Fig. 6.1. summarizes the production of the different kind of patties and the plan of sampling. The samples were stored at -40°C until analyses.

6.3.2. Reagents, solvents and standards

Trimethylchlorosilane (TMCS, min 98.0%) was from Fluka (Buchs, Switzerland). Ammonium thiocyanate (NH_4SCN, min 97.5%), barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$, min99%), ethylenediamine-tetraacetic acid (EDTA) disodium salt (100%±1%), iron(II) sulphate eptahydrate ($FeSO_4 \cdot 7H_2O$, min 99.0%), sodium sulphate anhydrous (min 99.0%) and trichloacetic acid (min 99%) were from Carlo Erba Reagenti (Rodano, Italy). Acetic acid, chloroform, 2,7-dichlorofluorescein, diethyl ether, di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, 99.0-102.0%), ethanol, *n*-hexane, methanol, potassium hydroxide (KOH) in pellets (min. 85%) used for preparing 2 M KOH in methanol, *i*-propanol and pyridine (pyr) were purchased from Merck (Darmstadt, Germany). Squalane was from Roth (> 95%, Karlsruhe, Germany). L-ascorbic acid, hexamethyldisilizane (HMDS), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, min 98%), methyl tridecanoate (min 98%), potassium

chloride (min 99.0%), potassium phosphate monobasic (KH_2PO_4, min 99.0%), 1,1,3,3-tetramethoxypropane (TMP) and tridecanoic acid (min 98%) were from Sigma (St. Louis, MO, USA). Deionized water was obtained from an Elix 10 water purification system from Millipore (Bedford, MA, USA). All reagents and solvents, unless elsewhere specified, were of analytical grade.

6.3.3. Lipid extraction and determination of fat content

Lipid extraction was performed according to Folch et al. (1957) with some modifications as reported by Boselli et al. (2001). About 40 g of sample (raw meat, final patties) were accurately weighed in a 1000 ml glass bottle, added with 500 ml of chloroform/methanol 1/1 (v/v) and homogenized for 3 min. Then the mixture was kept at 60 °c for 20 min, added with 250 ml of chloroform and homogenized again for 1 min. The content of the bottle was filtered under vacuum through Albet 400 paper from Albet (Barcelona, Spain), mixed with 250 ml of 1 M KCl for about 30 sec and left overnight at 4 °C in order to improve the separation between the organic matter and the residual methanol/water supernatant phase. The lower organic phase was collected through the aid of a separatory funnel, dried for about 1.5-2 hours on and filtered over sodium sulphate anhydrous. Fat content was determined gravimetrically removing the organic solvent using a vacuum evaporator and drying the fat under nitrogen flow for about 45 min. The fat recovered was stored -45 °C in *n*-hexane/*i*-propanol 4/1 (v/v) until further analyses. A single fat extraction was also performed, as above described, on 40 g of each kind of chicken feed (integrated with animal or vegetable fat) employed in feeding the two groups of broilers in order to determine its fatty acids compostion.

6.3.4. Gas chromatographic determination of total fatty acids

In order to convert the fatty acids bound to triglycerides molecules to the corresponding methyl esters (FAME), a cold transmethylation was performed on fat according to the method described in the Official Journal of the European Communities (2002). About 50 mg of fat were exactly weighed in a conical vial, methylated twice with an ethereal solution of diazomethane (about 100-150 µl for each methylation, Fieser and Fieser 1967) and dried under nitrogen flow. Then the sample was dissolved in 1 ml of *n*-hexane, added with 2.1 mg of internal standard (tridecanoic acid methyl ester dissolved in *n*-hexane) and 100 µl of 2N KOH in methanol. The mixture was vigorously shaken for 30 sec and centrifuged at 1500 rpm for 3 min 2 min. About 1 ml of the supernatant organic phase was diluted with 2 ml of *n*-hexane and 1 µl of the solution was analyzed by capillary gas chromatography (CGC) employing a fused silica capillary column Rtx-2330 (30 m × 0.25 mm i.d., 0.2 µm f.t.) from Restek (Bellefonte, PA, USA). The column was fitted on a Clarus 500 gas chromatograph from Perkin Elmer (Shelton, CT, USA). The injector and detector (flame ionization detector) temperatures were set at 240 °C. Helium was used as carrier gas at the flow of 1.25 ml/min. The oven temperature was held at 120 °C for 1 min, increased from 120 °C to 240 °C at 4.0 °C/min and finally held at 240 °C for 10 min. The split ratio was set at 1:40. The peaks identification was accomplished comparing the peaks retention times with those of the FAMEs of two standard mixtures: GLC 463 and FAME 189-19 respectively purchased from Nu-Check (Elysian, MN, USA) and Sigma (St. Louis, MO, USA). Gas chromatographic data were filed and processed by the software TotalChrom Navigator (version 6.2.1) from Perkin Elmer.

6.3.5. Spectrophotometric determination of peroxide value (PV)

PV was determined according to Shantha and Decker (1994). This method is based on the spectrophotometric determination of ferric ions (Fe^{3+}) formed by the oxidation of ferrous ions (Fe^{2+}) by hydroperoxides in the presence of ammonium thiocyanate. Thiocyanate ions (SCN^-) react with ferric ions to give a red-colored chromogen that can be determined spectrophotometrically. An amount of fat comprised between 50 and 75 mg was exactly weighed and dissolved in a 10 ml volumetric flask with chloroform/methanol 2/1 (v/v), taking care to make the volume up to the mark with the same solvent mixture. The sample was then added with aqueous solutions of ammonium thiocyanate and iron(II) chloride (50 µl each) and left in the dark for 5 min. Then the absorbance of the solution was read at 500 nm with a single beam UV-VIS spectrophotometer UV mini 1240 from Shimadzu (Kyoto, Japan). To quantify PV, an iron(III) calibration curve was built from iron(III) chloride with a calibration range from 0.1 to 5.0 µg/ml (r^2 = 0.998). PV was expressed as meq of O_2/kg of fat.

6.3.6. Spectrophotometric determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined following the method described by Tarladgis et al. (1960) with some modifications. About 2 g of sample (raw meat and final patties) were exactly weighed in a 25 ml glass bottle and added with 8 ml of a phosphate buffer (pH = 7.00 ± 0.02) and 2 ml of 30% (w/v) trichloroacetic acid. The phosphate buffer was prepared adding 65.8 ml of 0.5 M KH_2PO_4 and 111 ml of 0.5 M $Na_2HPO_4 \cdot H_2O$ (water solutions) in a 500 ml volumetric flask, correcting pH employing either the acid or the basic solution and making the volume up to the mark with water. To delay oxidation and

prevent the pro-oxidative effect of metals, proper amounts of EDTA and ascorbic acid were added to the buffer to reach a final concentration of 0.1% (w/v) for both of them. The mixture was then homogenized for 30 sec and filtered through Albet 400 paper from Albet. The filtrate was collected in a 10 ml Teflon lined screw cap vial. Two ml of the filtrate were pipetted in a second 10 ml Teflon lined screw cap vial, added with 2 ml of 0.02 M thiobarbituric acid and kept at 90 °C for 20 min in a water bath covered with aluminum foil. After cooling at 4 °C for 20 min and at room temperature for about 10 min, the absorbance of the solution was read at 530 nm with the same spectrophotometer employed in PV determination. To quantify TBARS, a malondialdehyde (MDA) calibration curve was built from a 20.4 nM TMP solution in the buffer afore-described, with a calibration range from 0.3 to 8.1 nM (r^2 = 0.995). TBARS were expressed as mg MDA/kg of meat or product.

6.3.7. Purification and gas chromatographic determination of free fatty acids (FFA)

FFA were purified from the less polar lipid compounds according to Parisi (2001). About 20 mg of fat were exactly weighed in a conical vial, added with 0.11 mg of tridecanoic acid (internal standard, solution in *n*-hexane), dried under nitrogen flow, dissolved in about 300 µl of chloroform/*i*-propanol 2/1 (v/v) and finally loaded onto an ammino-propilic bonded phase cartridge (STRATA NH2 cartridges, 500 mg stationary phase, 3 ml reservoir volume) for solid phase extraction (SPE) from Phenomenex (Torrance, CA, USA). Before fat loading, each cartridge had been placed in a SPE elution apparatus from Isolute (Mid-Glamorgan, UK), loaded with a small amount of sodium sulfate anhydrous and conditioned with 3 ml of *n*-hexane. The cartridge was

eluted with 6 ml of chloroform/*i*-propanol 2/1 (v/v) and then with 10 ml of a 2% (v/v) solution of acetic acid in diethyl ether to remove and collect FFA. The latter fraction was evaporated under reduced pressure and methylated three times with an ethereal solution of diazomethane (about 100-150 µl for each methylation) according to Fieser and Fieser (1967). After methylation, 200 µl of *n*-hexane were added and the organic solution containing FFA was analyzed in CGC under the same instrumental conditions adopted for FAME analysis.

6.3.8. Purification and gas chromatographic determination of diglycerides (DG)

Diglycerides were isolated and concentrated as reported by Bortolomeazzi *et al.* (1990). About 100 mg of fat were accurately weighed in a 10 ml Teflon lined screw cap vial, dissolved in 1 ml of *n*-hexane and loaded onto a silica cartridge for SPE (STRATA SI-1 cartridges, 500 mg stationary phase, 3 ml reservoir volume) from Phenomenex (Torrance, CA, USA). Before fat loading, each cartridge had been placed in a SPE elution apparatus from Isolute, loaded with a small amount of sodium sulfate anhydrous and conditioned with 3 ml of *n*-hexane. The cartridge was then eluted with 3 ml of *n*-hexane, 3 ml of *n*-hexane/diethyl ether 8/2 (v/v), 4 ml of *n*-hexane/diethyl ether 1/1 (v/v) and 3 ml of methanol. The two last fractions, containing DG, were pooled, dried under reduced pressure, added with 0.1 mg of squalane (internal standard, solution in *n*-hexane), dried again under nitrogen flow and, prior to CGC analysis, silylated according to Sweeley *et al.* (1963) in order to convert DG to the corresponding trimethylsilyl (TMS) derivatives. The silylation procedure was carried out as follows: 200 µl of a mixture Pyr/HMDS/TMCS 5/2/1 (v/v/v) freshly prepared were added, then the sample was briefly shaken and kept at 40 °C for 20 min. After removing the silylating agent

under nitrogen, 200 µl of *n*-hexane were added; the mixture was shaken on vortex and centrifuged at 3000 rpm for 3 min.

One µl of the organic phase was injected in an AUTO/HRGC/MS MFC 500 gas chromatograph (Carlo Erba Instruments, Rodano, Italy) which was equipped with a fused silica capillary column (Rtx-65TG, 30 m × 0.25 mm i.d., 0.10 µm f.t.) coated with 65%-diphenyl-35%-dimethyl-polysiloxane from Restek (Bellefonte, PA, USA). The injector and detector temperatures were set at 350 °C. Helium was used as carrier gas at the flow of 1.2 ml/min. The oven temperature was programmed from 160 to 350 °C at 3.0 °C/min and finally held at 350 °C for 10 min. The split ratio was set at 1:40.

6.3.9. Isolation of phospholipids (PS) by thin layer chromatography and gas chromatographic determination of PS fatty acids composition

About 20 mg of fat were exactly weighed in a conical vial, dissolved in about 100-150 µl of chloroform and then loaded on a 20 × 20 cm Silica gel 60 TLC plate from Merck in order to isolate PS from other lipid compounds. The mobile phase employed was100 ml of a mixture *n*-hexane/diethyl ether 3/2 (v/v) which was poured in the elution chamber at least 30 min before inserting TLC plate. The plate was then drawn from the chamber when the solvent front was 10 cm above the solvent origin and subsequently dried open air for about 10 min. The PS band was visualized under UV light (254 nm) sprying the TLC plate with a 0.02% (w/v) ethanolic solution of 2,7-dichlorofluorescein sodium salt and then scraped off and collected in a second conical vial. PS were extracted three times with chloroform and all organic extracts were pooled, dried under nitrogen flow, added with 1 ml of *n*-hexane and finally transmethylated with 100 µl of 2 M KOH in methanol, according to the procedure previously described for the

determination of FAME. After centrifugation at 1500 rpm for 3 min to separate the organic layer from the residual silica gel, 1 µl of the transmethylated solution was analyzed by CGC under the same instrumental conditions adopted for total and free fatty acids.

6.3.10. Statistics

Each analytical determination was performed in triplicate and the results were reported as the mean of the values obtained (n = 3). Standard deviation (SD) was chosen as spreading index. The data underwent one-way analysis of variance (ANOVA) using the software Statistica 6.0 from StatSoft (Tulsa, OK, USA). Unless specified, Tukey's honest significant difference (HSD) test and Pearson's correlations were evaluated at a $p < 0.05$ level.

6.4. RESULTS AND DISCUSSION

6.4.1. Effect of feeding fat and processing on lipid content

In **Table 6.1.** is shown the fat content of raw chicken meat, meat mixtures and final products obtained both from traditional and innovative technology. As regards the effects of dietary fat on raw meat, while the fat content in "A" and "V" breasts was very similar regardless the kind of diet, in ground thighs, meat mixtures and meat mixtures added with ingredients coming from "A" diet, the fat content was higher than in the "V" samples, even only between I-A and I-V was found a statistically significant difference. The high uncertainty and consequent RSD% associated with M-A and T-V were likely due to difficulties met in obtaining an homogenous sampling from chicken meat and

also during homogenization steps in fat extraction. “V” thighs, meat mixtures and meat mixtures plus ingredients had a fat content which was 15.2, 14.8 and 20.0% respectively lower than the corresponding samples coming from chicken fed an animal-fat based diet. In this study it was also remarkable that the effect of feeding fat was less relevant in lean muscles like breasts, with a fat content less than 2%.

These results were partly in agreement with the findings reported in the study of Bonoli et al. (2007). In that case no analysis was carried out on separated ground thighs and breasts but lipid content was determined directly on meat mixtures before and after ingredients addiction. The difference between “animal” and “vegetable” mixtures was much higher than that found in this study: 6.0 and 3.2% was the fat content in “A” and “V” ground mixtures respectively while the dilution effect of ingredients and additives led to a similar fat content: 2.9 and 2.8% respectively in “A” and “V” mixtures-plus-ingredients respectively. In two previous studies (Sanz et al., 1999, 2000) that dealt with the effect of feeding fat type on broilers performances, it was found that dietary fat saturation affected fat accumulation in chickens and that the inclusion of saturated fats of animal origin (cattle tallow, lard) in broiler diets produced higher fat accumulation than unsaturated fats of vegetable origin like sunflower oil. To account these results, a different metabolic use of the energy absorbed by dietary saturated and unsaturared fat in broilers was suggested. While energy from unsaturated fats can be used for metabolic purposes, energy from saturated fats is less available and accumulates as body lipids (Sanz et al., 2000).

About final pre-cooked patties, the oil absorption that raw meat underwent in both kind of production technologies during the flash frying step led to a dramatic rise in lipid percentage that exceeded 10% regardless processing and diet and in accordance with

previously reported results (Bonoli et al., 2007, in press). In another work (Echarte et al. 2003) lipid percentage in chicken patties fried in olive oil (180 °C for 3 min till a core temperature of 85-90 °C) was 11.5% and significantly higher than that of raw patties (10%). In the same trend were the results reported by Conchillo et al. (2005b) that found an increase in the fat content of commercial chicken breasts after grilling in sunflower oil (180 °C for 1.5 min, core temperature: 85-90 °C) from 1.3 to 3.0%.

The differences previously met in raw materials disappeared after cooking and, opposite to what previously observed, in cutlets from traditional technology a higher fat content was found in samples from "V" diet. In samples from the innovative technology the fat content was generally lower than in samples produced according to traditional processing even a significant difference was found between FP-V-TT and FP-V-IT but not between FP-A-TT and FP-A-IT. In the new technology, anticipating cooking in steam oven caused amongst ground meat pieces the formation of a heat-induced gel network, involving myofibrillar proteins, that reduced oil absorption that occurred in the flash-frying. To reduce the amount of absorbed fat, increasing at the same time product yield was indeed one of the main objective of the innovative technological route.

6.4.2. Effect of feeding fat and processing on total fatty acids composition

Tables 6.2. and 6.3. illustrate the percentage of the main fatty acids and of the different classes of acids, according to the degree of unsaturation, in raw meat and final cutlets. The data confirmed that the type of feeding fat greatly affected the fatty acids composition in raw meat. In "A" breasts and thighs and, as a consequence, in meat mixture, oleic acid (about 35%) was the most abundant one, followed by palmitic, linoleic, stearic and palmitoleic acids, with a higher oleic and palmitoleic acids content

in thighs while in chicken breasts about 3% of total FAME was represented by eicosatrienoic and arachidonic acids that led to a higher polyunsaturated fatty acids (PUFA) amount with respect T-A. In raw meat from broilers fed a vegetable fat-based diet, linoleic acid was the most abundant and present in a much higher amount in comparison to the corresponding samples coming from "animal" diet: about 35% in B-V and T-V vs. ≈ 20% in B-A and T-A for a PUFA percentage which was almost double in "vegetable" samples. The great rise that took place in T-V and B-V, led to a unsaturated-to-saturated (U/S) fatty acids ratio significantly higher with respect the corresponding T-A and B-A. Indeed, while in raw meat from an animal fat–based diet, monounsaturated fatty acids (MUFA) gave the main contribution to total fatty acids for the high oleic acid content, followed by saturated (SFA, ≈ 30%) and PUFA (≈ 25%), in T-V, P-V and M-V the trend observed was completely different and PUFA were the most abundant classes (more than 40% of total identified FAME), followed by MUFA (30-35%) and SFA (about 25%). These profiles reflected the fatty acids profile of the two kinds of chicken feeds employed and summarized as follows: 32.6, 35.6 and 31.9 were the SFA, MUFA and PUFA percentages respectively in feed integrated with animal fat whereas 15.0 (SFA), 22.9 (MUFA) and 61.8 (PUFA) were the FAME composition of feed containing vegetable oil. In a past study, O'Neill et al. (1998) verified the influence of feeding fat composition on the quality of lipid fraction of white and dark chicken meat. The dietary supplementation with tallow and olive oil led to two different fatty acids profiles in raw meats and the main result was an increase in the percentage of oleic acid that shifted from 37.9 and 39.4% in breasts and thighs respectively from broiler fed a tallow supplemented diet to 54.7 and 57.7% respectively in breasts and thighs from broilers fed an olive oil supplemented diet.

As suggested in **Table 6.2.**, the fatty acid composition of meat mixtures I-A and I-V was very similar to that of the corresponding meat mixture M-A and M-V before the ingredient addiction, owing to the negligible contribution that additives brought to the lipid fraction of mixtures. In **Fig. 6.2.** are reported two FAME gas chromatographic traces of sample M-A and M-V.

These results were similar to those reported by Bonoli et al. (2007) and confirmed that to an increase in the degree of unsaturation in feeding fat sources corresponded a rise in the PUFA percentage in chicken meat. Bonoli et al. verified that SFA, MUFA and PUFA percentages were 33.7, 47.2 and 19.1 in "A" meat mixture (before ingredients addiction) whereas 30.6, 39.1 and 30.3 in "V" mixture. With respect the results here displayed, the polienoic fraction was lower and even in "V" mixture monounsaturated fatty acids were the main class. Probably it depended on a different composition of the feeding fat added to animal diet which was not determined in that study.

As a consequence of oil absorption during flash-frying, cooking step dramatically affected and changed fatty acids composition with respect raw meat. The incorporation of significant amounts of oil from the frying bath caused a high increase in MUFA percentage that was about 60% of total FAME in all kind of cutlets regardless feeding fat type and processing technology. U/S ratio rose (≈ 8 in all cutlets) and was about four times higher than the values observed in raw meat. Other noticeable effects of oil absorption were an increase in α-linolenic acidc amount (≈ 7%) and a fall in saturated fats that never exceeded 12%. Although in cutlets produced from meat coming from broilers fed "vegetable" diet PUFA were significantly higher than in FP-A-TT and FP-A-IT (30% and 28% respectively in "V" and "A" patties) a fall in the differences previously noted in ground meat before processing took place. Flash-frying masked

these differences and after the final productive stage from a qualitative standpoint the fat compositions of the four types of patties were almost undistinguishable, in agreement to what reported in the studies of Bonoli et al. (2007, in press). Processing overcame the effects of dietary fats in such a complex technological sequence. In **Fig. 6.3.** are illustrated two gas chromatograms of total FAME of chicken patties from "animal" and "vegetable" diet both obtained with traditional technology and displaying a similar FAME composition. In a simpler processing carried out on raw chicken breasts (grilling at 180 °C in sunflower oil at 180 °C for 3 min, core temperature 85-90 °C), similar finding were obtained by Conchillo et al. 2004, with a significant increase in unsaturation degree in cooked meat at the expences of saturated and monounsaturated fatty acids. To a decrease in oleic, palmitic and stearic acids from 34.6, 24.9 and 10.2% respectively in raw meat to 32.7, 18.4 and 7.2% in grilled breasts, a important rise in linoleic acid corresponded, from 18.8 to 33.8%. Bou et al. (2001), studying the influence of different dietary fat sources (beef tallow, sunflower oil, oxidized sunflower oil, linseed oil) and anti-oxidants supplementation on different chemical and sensory parameters of dark chicken meat, found that higher the level of PUFA in dietary feed, higher the percentage of PUFA in chicken meat. PUFA content in beef tallow was 23.5% for a corresponding 17.9% in meat while to a 65.2% PUFA content in linseed oil coresonded a 47.0% in meat.

6.4.3. Effect of feeding fat and processing on lipid oxidation: peroxide value (PV) and thiobarbituric acid reactive substances (TBARS)

The development of lipid rancidity in raw meat and final products was studied evaluating both peroxides (PV) and thiobarbituric acid reactive substances (TBARS), as

long as these determinations enable to quantify the primary and secondary oxidation products respectively (Nawar, 1996; Antolovich et al., 2002). In **Table 6.1.** are shown PV and TBARS for each kind of sample. PV determination was employed to evaluate the freshness of raw meat employed in the preparation of patties. Indeed PV are the main initial products of autoxidation and represents a useful measure to follow the development of rancidity at its early stage (Nawar, 1996; Shahidi and Wanasundara, 2002). The PV in raw materials was extremely low both in "A" and "V" samples and never exceeded 1.5 meq O_2/kg of fat. The kind of feeding fat did not seem to affect this parameter. Even though, as previously stated, skinning, grinding and mixing meat were considered steps that may increase oxidation, no significant increase in PV occurred even after additives addiction, although salting has reported to have a pro-oxidant effect (Erickson, 2002). In comparison to the results obtained by Bonoli et al. (2007) that found a PV in meat mixture (chicken breasts plus thighs) ranging from 21.5 ("A" sample) to 26.4 ("V" sample) meq O_2/kg of fat, the oxidative status of the raw meat here analyzed seemed to be better and oxidation only at an early stage. Parameters like storage conditions of meat before grinding, the freshness of meat and of the feeding fat source itself may have influenced this finding and their relationship with rancidity development in fresh meat should be deeper investigated in order to improve the quality of raw materials. As expected heat transfer associated with cooking steps brought to an increase in PV although their value remained low and never bigger than 3.0 meq O_2/kg of fat. In the cutlets here studied PV was about four times lower than in chicken patties analyzed by Bonoli et al. (2007, in press) in which PV ranged from 10.8 to 12.0 meq O_2/kg of fat. PV is not always a proper parameter to evaluate lipid oxidation in products that underwent harsh heat treatment due to labile chemical nature of such compounds.

Moreover, during flash-frying a migration of peroxides from meat to cooking oil bulk may have occurred. Nevertheless, considering that in this study and in the ones previously cited meat mixture underwent similar cooking treatment, it seemed that a good oxidative status in raw materials may affect the final PV amount in finished products. Moreover, it should be also verified the level of rancidity in the oil employed in flash frying that may represent another factor affecting PV in chicken cutlets.

About TBARS, it must be noticed that they followed during cutlets production, the same trend of PV and a significant correlation ($r^2 = 0.878$, $p < 0.05$) was found between these two parameters. In raw meat TBARS never exceeded 0.2 mg MDA/kg of meat. With respect the values recorded in I-A and I-V, just before cooking processes, in the final products a four to five-fold rise took place but in different cutlets TBARS remained still low. The type of feeding fat and processing technology did not affected TBARS too.

Low PV and TBARS were found in fresh chicken meat (breasts) according to Conchillo et al. (2005b) where raw samples did not show detectable amount of peroxides and also after pan frying in sunflower oil PV did not exceed 5 meq O_2/kg of fat. In the same raw and grilled samples TBARS were comprised in the range 0.1-0.3 mg/kg of meat: to a good oxidative status in fresh meat corresponded a low development of rancidity in meat after cooking in a vegetable medium even at high temperatures. The same authors (Conchillo et al. 2005a) found no peroxides and low TBARS (0.1 mg/kg of meat) always in fresh chicken breasts. Although the cooking technique adopted in that work (microwaving) was quite different from the complex processing here adopted, microwaved meat showed low PV (2.5 meq O_2/kg of fat) and TBARS (0.2 mg/kg of meat). Low TBARS were also reported by Lopez-Bote et al. (1998) who found 0.2 and

0.1 mg malonaldehyde/kg of meat in fresh white and dark meat respectively. After cooking in a waterbath (70 °C) for 30 min TBARS reached values similar to those obtained in this study: 1.0 mg/kg for both white and dark chicken meat respectively.

PV and TBARS are usually taken together to better evaluate on the whole the extent of lipid rancidity in foods, considering that TBARS arise from peroxides breakdown in polyunsaturated system containing fatty acids with more than three double bonds (Nawar, 1996) and that the primary oxidation products (hydroperoxides) of fats and oils are transitory intermediates that decompose into various secondary products (Shahidi and Wanasundara, 2002). Moreover secondary oxidation products are the volatile compounds responsible of the comparison of off-odours in meat and other fat containing products. On the basis of what previously noted, TBARS are expected to be present in significant amounts in the advanced stages of oxidation when the concentration of PV is high and their decomposition to secondary products proceed at a higher speed with respect the early stage of oxidation. In the production of chicken patties here analyzed both PV and TBARS were low and slowly increase from raw materials to final products owing to the pro-oxidative effect of heating and to the fact that cooking favoured the decomposition of peroxides to secondary products. The positive correlation found between PV and TBARS proved that oxidation was still in its fist stage in raw materials and final products too otherwise a decrease in PV should have been observed as in the works of Bonoli et al. (2007, in press). In that case PV along cutlets production reflected a sinusoidal behaviour typical of primary oxidation products and fall from more than 20 meq O_2/kg of fat in ground meat to nearly 10 meq O_2/kg of fat in final products. Comparing the oxidative data here reported to those of the afore-mentioned studies, it seemed that the extent of oxidation and thus the overall quality of

the lipid fraction of chicken patties was deeply influenced by the extent of oxidation in raw materials. High PV values in the final patties may represent a negative feature from an organoleptic standpoint, especially during the conservation of the products when from the breakdown of PV originate unpleasant flavours and odours.

6.4.4. Effect of feeding fat and processing on lipolysis: free fatty acids (FFA) and diglycerides (DG)

In **Table 6.1.** are shown the amount of total FFA and DG in different samples. Lipolysis represents the second biochemical route to the development of rancidity and involves triglycerides hydrothermal or enzymic hydrolysis to FFA (Robards et al., 1988). FFA are more susceptible to peroxidation process than fatty acids esterified to glycerol (Nawar, 1996), furthermore lipolysis is a major reaction during frying, where both water introduced from the food, high temperature and heating are present (Robards, 1988; Nawar, 1996). In raw materials (breast, thighs and meat mixure before ingredients addiction) no significant difference was found between “A” and “V” samples. This findings did not confirm what found by Bonoli et al. (2007) that verified a higher activity of animal lipases towards fat from animal sources. In actual fact, in that work FFA contents in “A” and “V” animal mixtures were respectively 0.05 and 0.02 g/100g of meat. In this study, even no statistical difference was notices, it seemed that lipolysis was a bit more intense in meat from vegetable fat-based diet. After ingredients and thus water addiction before cooking lipolysis rose significantly with respect raw meat in I-V but not in I-A. Despite the high temperatures reached during flash-frying and cooking in a steam humid oven, in final patties FFA fell under 1.0 g/100g of fat, following the same trend reported elsewhere (Bonoli et al., 2007, in press) and probably owing to the

migration of FFA in frying oil bath. A decrease in FFA after pan frying with respect raw meat was also observed by Rodriguez-Estrada et al. (1997) in a study about the effect of cooking treatments on the lipid fraction of beef hamburgers. In that case FFA ranged from 715.0 mg/100g of lipid in raw hamburgers to 673.5 mg/100g of lipids in meat after pan frying. The lost of FFA in frying medium (not specified) or their interaction with other molecules during treatment was there assumed.

About the effect of processing, higher amount of FFA were found in samples coming from the innovative technology but no significant difference were found. In Bonoli et al. (in press) a strong difference was found: 0.3 and 0.1 g/100g of fat were the total FFA amount in final patties respectively obtained with traditional and innovative technology respectively ("animal diet"). In **Fig. 6.4.** are shown gas chromatographic traces of patties both from animal fat based diet but obtained with different technologies.

DG determination confirmed the hypothesis about the effect of technology. In raw meat and meat mixtures, as observed in FFA, diet did not affect the degree of lipolysis. Moreover no significant increased in DG amount occurred before cooking. In final patties the effect of heat on lipolysis development was clear, indeed DG increased with respect raw meat about tree times, expressing DG as g/100g of fat while the rise was higher expressing this parameter as g/100g of meat as long as the high fat content in final products. Thus in products that underwent a complex technological treatment, DG appeared as a more reliable parameter to evaluate lipolysis at each productive step. Different reasons may account for the small increase in DG that occurred in the innovative process. In traditional technology lipases deactivation that occurred at the high temperatures reach during deep frying took place at an earlier stage with respect the innovative technology in which this flash-frying is postponed to oven cooking. On

the contrary in the innovative route enzymic lipolysis mat have given a significant contribution in the share of time in between the two cooking treatments: oven cooking and then flash frying. Moreover, in traditional technology flash frying brought to a rapid elimination and a decrease in the availability of water present on the surface of the product while in the innovative process water availability was likely to be higher in the first cooking step that on the contrary cause a moisturization of the product. In **Fig. 6.5.** are shown two gas chromatographic traces which display TMS derivatives collected from samples FP-A-TT (upper trace) and FP-A-TI (lover trace).

About the composition of FFA (data not showed) the relative amount of different fatty acids seemed to be affected by the extent of lipolysis. M-A displayed similar percentages of the three fatty acids classes: 37.9, 29.3 and 32.3% were respectively the relative amounts in FFA fraction of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Thus the percentage of SFA was higher than in total fatty acids and a decrease in MUFA ratio was also observed. Before cooking lipolysis was determined both by enzymes (enzymic lipolysis) and heat and moisture action (chemical hydrolysis) and probably this synergism led to an homogenous distribution of FFA in the three main classes. In M-V polyunsaturated fatty acid were the most abundant amongst FFA but even in this case a rise in SFA percentage with respect to total FAME was recorded. The water addiction that took place together with other ingredients and additives caused a significant change in FFA composition in addition to a rise in their total amount of FFA. The samples I-A and I-V showed the highest relative amount of polyunsaturated free fatty acids. In these sample the large amount of water added made probably chemical lipolysis the most important process involved in and leading to the liberation of FFA from triglycerides molecules. It has

been known that he position *sn*-2 in the triglycerides is more susceptible to chemical hydrolysis, that is 1,3-DG are usually reported as derivatives from lipolysis of triglycerides (Frega et al., 1993). Both in animal and, to a higher degree, in vegetable fat *sn*-2 position in triglycerides and phospholipids is mainly occupied by mono or polyunsaturated fatty acids (Nawar, 1996) and it follows that higher the intensity of chemical lipolysis higher the degree of unsaturation in FFA. In final patties different profiles were notice according to the kind of technology adopted. In the innovative route the FFA composition was similar to that of meat mixture: in this kind of processing a lower oil absorption was supposed to take place and this may have contribute to lesser modify FFA profile.

6.4.5. Effect of feeding fat on fatty acids composition of phospholipids

In **Table 6.4.** is reported the fatty acids composition of phospholipids (PS) in different samples. In lean muscle PS content ranges from 0.5 to 1.0% with a higher amount of polyunsaturated fatty acids with respect neutral lipids like triglycerides (Foegeding et al., 1996). According to the same report, in white and red chicken muscle, phospholipids represent 48 and 21% of total lipids respectively, besides the oxidation of polienoic acids in membrane PS gives an important contribution to the development of rancidity. As stated by Decker and Xu (1998), the manipulation of dietary fat affected the composition of membrane lipids. In thighs, with respect total fatty acids profile, in the PS fraction a higher amount of saturated fatty acids was found: 35.5 and 35.7% in phospholipids of samples T-A and T-V respectively vs. 30.3 and 24.6% in total fatty acids in the same samples. About PUFA, in PS of chicken thighs a decrease in PUFA took place with respect total lipids: 19.3 and 28.6% were the percentage of polienoic

acids in PS of samples T-A and T-V respectively. As regards chicken breasts the percentage of saturated and polyunsaturated fatty acids in PS and total lipid was similar. The higher similarity between the total and PS fatty acids profile in breasts was probably due to the higher contribution that this lipid classes gives in white muscle with respect dark muscle.

In T-A and B-A the most abundant PS fatty acid was oleic, followed by palmitic, linoleic and stearic while, as a proof of the effect of feeding fat on PS fatty acids composition, in T-V and B-V the relative abundance was oleic ≈ linoleic, palmitic and stearic acids. An important contribution to polienoic fraction was given by eicosatrienoic and arachidonic acid that in total FAME never exceeded 2-3%. It was remarkable the effect of cooking treatments that dramatically altered fatty acids profile, masking even in PS fraction the previously discussed differences. Indeed an important increase in monoenoic acids occurred although to a lesser extend than in total fatty acids. This was an unexpected finding: oil absorbed from frying bath was mainly constituted by neutral lipids and gave a little contribution in terms of PS to cooked patties, considering the high PS content in raw meat (Foegeding et al., 1996). The high temperature reaches in flash frying and cooking may have favoured some chemical interaction between PS and triglycerides of the bulk of vegetable oil that modified the fatty acids composition in PS. A deeper investigation has to be carried out to clarified this point.

6.5. CONCLUDING REMARKS

In this study the effect of two feeding fat sources and two processing technologies on the composition and quality of the lipid fraction of raw ground chicken meat and pre-cooked ready-to-eat patties was performed. Two groups of broilers were respectively fed an animal fat (cattle tallow and pork fat) based diet and a vegetable fat (olive, soybean and sunflower oil) based diet. The correspondent cutlets were prepared according with two technologies: a traditional one in which flash frying in a vegetable oil bath was followed by cooking in a steam-convection oven and an innovative one, in which the two steps are inverted. Dietary fat proved to be an effective mean to affect the composition of total fatty acids in raw meat. To the higher degree of unsaturation in vegetable feed corresponded a significant increase in the amount of polyunsaturated fatty acids in breasts and thighs. Nevertheless the complexity of processing and the oil absorption from frying bath made these differences disappear in the final products and the total fatty acids profile of different patties was similar regardless the kind of technology applied and lipid dietary integration. Even the higher degree of unsaturation in "vegetable" raw meat, oxidation was not influenced by fatty acids composition of raw materials and low values as regards PV and TBARS were recorded in meat before and after processing. Although the harsh heat treatments here applied are have been reported to have a pro-oxidant effect, the overall quality of patties was acceptable from an oxidative standpoint. About lipolysis extent, DG were a more suitable parameter to evaluate the development of this biochemical change, considering the losses in FFA that took place in the frying medium. DG increased from raw meat to final patties owing to the relative high amount of water in meat just before cooking and the high temperatures

and heat transfer involved in the final steps. Lipolysis proved to be a bit more intense in the products coming from the innovative technology. This increase may be related to the anticipated deactivation of meat lipases that occurred at the high temperature reached in flash-frying in the traditional technology. In the innovative processing route, flash-frying was postponed, moreover the moisturizing effect in stem-convection oven cooking may have dramatically increased water availability, especially bon the surface of the cutlets. If in the traditional technology a fall in the superficial water availability may be also depended on the immediate oil absorption at the beginning of cooking. PS fatty acids profile displayed a similar trend to that observed for total fatty acids, that is a significant rise in the monoenoic fraction after processing. Frying fat seemed to have altered the composition of PS fatty acids in a way that needs further investigation.

6.6. REFERENCES

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., Robards, K. 2002. Methods for testing antioxidant activity. Analyst, 127, 183-198.

Bonoli, M., Caboni, M. F., Rodriguez-Estrada, M. T., Lercker, G. 2007. Effect of feeding fat sources on the quality and composition of lipids of precooked ready-to-eat fried chicken patties. Food Chemistry, 101, 1327-1337.

Bonoli, M., Caboni, M. F., Rodriguez-Estrada, M. T., Lercker, G. Effect of processing echnology on the quality and composition of lipids of precooked chicken patties. International Journal of Food Science and Technology, in press.

Bortolomeazzi, R., Frega, N. and Lercker, G. (1990). Rapid determination of free and esterified sterols using prepacked silica columns. Italian Journal of Food Science, 2, 265-268.

Boselli, E., Velazco, V., Caboni, M. F., Lercker, G. 2001. Pressurized liquid extraction of lipids for the determination of oxysterols in egg-containing food. Journal of Chromatography A. 917, 239.244.

Bou, R., Guardiola, F., Grau, A., Grimpa, S., Manich, A., Barroeta, A., Codony, R. 2001. Influence of dietary fat source, α-tocopherol, and ascorbic acid supplementation on sensory quality of dark chicken meat. Poultry Science, 80, 800-807.

Bou, R., Guardiola, F., Tres, A., Barroeta, A. C., Codony, R. 2004. Effect of dietary fish, α-tocopheryl acetate, and zinc supplementation on the composition and consumer acceptability of chicken meat. Poultry Science, 83, 282-292.

Conchillo, A., Ansorena, D., Astiasarán, I. 2004. The effect of cooking and storage on the fatty acid profile of chicken breast. European Journal of lipid Science and Technology, 106, 301-306.

Conchillo, A., Ansorena, D., Astiasarán, I. 2005a. Use of microwave in chicken breast and application of different storage conditions: consequences on oxidation. European Food Research and Technology 221, 592-596.

Conchillo, A., Ansorena, D., Astiasarán, I. 2005b. Intensity of lipid oxidation and formation of cholesterol oxidation products during frozen storage of raw and cooked chicken. Journal of the Science of Food and Agriculture, 85, 141-146.

Decker, E. A., Xu, Z. 1998. Minimizing rancidity in muscle foods. Food Technology, 52(10), 54-59.

Echarte, M., Ansorena, D., Astiasarán, I. 2003. Consequences of microwave heating and frying on the lipid fraction of chicken and beef patties. Journal of Agricultural and Food Chemistry 51, 5941-5945.

Eder, K., Grünthal, G., Kluge, H., Hirche, F., Spilke, J., Brandsch, C. 2005. Concentrations of cholesterol oxidation products in raw, heat-processed and frozen-storage meat of broiler chickens fed diets differing in the type of fat and vitamin E concentrations. British Journal of Nutrition, 93, 633-643.

Erickson, M. C. 2002. Lipid Oxidation of Muscle Foods. In: Food Lipids – Chemistry, Nutrition, and Biotechnology – Second Edition, Revised and Expanded (edited by C. C. Akoh, D. B. Min). Pp.: 365-411. New York, Basel: Marcel Dekker.

Fieser, L. F. and Fieser, M. (1967). Reagents for organic chemistry. Pp 191-192. New York: Wiley.

Foegeding, E. A., Lanier, T. C., Hultin, H. O. 1996. Characteristics of Edible Muscle Tissues. In: Food Chemistry, Third Edition (edited by O. R. Fennema). Pp.: 879-942.

Folch, J., Lees, M. and Sloane Stanley G. H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, **226**, 497-509.

Frega, N., Bocci, F., Lercker, G. 1993. High-resolution gas-chromatographic determination of diacylglycerols in common vegetable oils. Journal of the American Oil Chemists' Society, 70, 175-177.

Grau, A., Codony, R., Grimpa, S., Baucells, M. D., Guardiola, F. 2001. Cholesterol oxidation in frozen dark chicken meat: influence of dietary fat source, and α-tocopherol and ascorbic acid supplementation. Meat Science, 57, 197-208.

Gray J. I., Gomaa E. A., Buckley D. J. 1996. Oxidative quality and shelf life of meats. Meat Science 43, S111-S123.

HHS/USDA – U.S. Department of Health and Human Services and U.S. Department of Agriculture 2005. Fats. In: Dietary Guidelines for Americans 2005 (6th edition). Pp. 29-34. Washington, DC, USA: Government Printing Office.

INRAN – MIPAF 2003. Grassi: scegli la qualità e limita la quantità. In: Linee guida per una sana alimentazione italiana, revisione 2003. Pp. 28-33. Roma, Italy: Istituto Nazionale di Ricerca per gli Alimenti e la nutrizione (INRAN), Ministero delle Politiche agricole e Forestali (MIPAF).

Lopez-Bote, C. J., Gray, J. I., Gomaa, E. A., Flegal, C. J. 1998. Effect of dietary oat administration on lipid stability in broiler meat. British Poultry Science, 39, 57-61.

Lopez-Bote, C. J., Gray, J. I., Gomaa, E. A., Flegal, C. J. 1998. Effect of dietary administration of oil extracts from rosemary and sage on lipid oxidation in broiler meat. British Poultry Science, 39, 235-240.

Morrissey P. A., Sheehy P. J. A., Galvin K., Kerry J. P., Buckley D. J. 1998. Lipid stability in meat and meat products. Meat Science 49, S73-S86.

Nawar, W. W. (1996). Lipids. In: Food Chemistry, Third Edition (edited by O. R. Fennema). Pp. 225-319. New York, Basel, Honk Kong: Marcel Dekker.

Official Journal of the European Communities 2002. Commission Regulation (EC) No 796/2002 of 6 May 2002 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis and the additional notes in the Annex to Council Regulation (EEC) No 2658/87 on the tariff and statistical nomenclature and on the Common Customs Tariff. L 128/8, Volume 45, Attachment X.B., 15 May 2002.

O'Neill, L. M., Galvin, K., Morrissey, P. A., Buckley, D. J. 1998. Comparison of effects of dietary olive oil, tallow and vitamin E on the quality of broiler meat and meat products. British Poultry Science, 39, 365-371.

Parisi, E. 2001. Valutazione dello stato di ossidazione di prosciutti crudi e di insaccati non tradizionali. B.Sc. Thesis on Agricultural Sciences and Technologies. Università degli Studi di Bologna, Italy.

Pszczola, D. E. 2002. Beefing up innovations for meat and poultry ingredients. Food Technology, 56(3), 54-66,79.

Ramírez, M. R., Morcuende, D., Estévez, M., Cava, R. 2004. Effects of the type of frying with culinary fat and refrigerated storage on lipid oxidation and colour of fried pork loin chops. Food Chemistry, 88, 85-94.

Robards, K., Kerr, A. F., Patsalides, E. 1988. Rancidity and its measurement in edible oils and snack foods. Analyst, 113, 213-224.

Rodriguez-Estrada, M .T., Penazzi, G., Caboni, M. F., Bertacco, G., Lercker, G. 1997. Effect of different cooking methods on some lipid and protein components of hamburgers. Meat Science 45, 365-375.

Sanz, M., Flores, A., Perez de Ayala, P., Lopez-Bote, C. J. 1999. Higher lipid accumulation in broilers fed on saturated fats than in those fed on unsaturated fats. British Poultry Science, 40, 95-101.

Sanz, M., Flores, A., Lopez-Bote, C. J. 1999. The metabolic use of energy from dietary fat in broilers affected by fatty acid saturation. British Poultry Science, 41, 61-68.

Shahidi, F., Udaya N. Wanasundara, U. N. 2002. Methods for measuring oxidative rancidity in fats and oils. In: Food Lipids – Chemistry, Nutrition, and

Biotechnology – Second Edition, Revised and Expanded (edited by C. C. Akoh, D. B. Min). Pp: 465-487. New York, Basel: Marcel Dekker.

Shantha, N. C., Decker, E. A. 1994. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. Journal of AOAC International, 77, 421-424.

Skřivan, M., Skřivanová, V., Marounek, M., Tůmová, Wolf, J. 2000. Influence of dietary fat source and copper supplementation on broiler performance, fatty acid profile of meat and depot fat, and on cholesterol content in meat. British Poultry Science, 41, 608-614.

Sweeley, C. C., Bentley, R., Makita, M., Wells, W. W. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. Journal of the American Chemical Society, **85**, 2497-2507.

Tarladgis, B. G., Watts, B. M., Younathan, M. T. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. Journal of the American Oil Chemists' Society, 37, 44-48.

6.7. TABLES

Table 6.1. – Fat content, PV, TBARS, total FFA and DG content of groun raw meat (chicken brests and thighs), meat mixuture (before and after ingredients addiction) and final pre-cooked patties. All the results are expressed as mean ± SD (n = 3).

Sample	Fat content (%, w/w)	PV (meq O_2/kg fat)	TBARS (mg MDA/kg meat)	Total FFA g/100 g fat	Total FFA g/100 g meat	DG g/100 g fat	DG g/100 g meat
T-A	6.4 ± 0.1^{2a}	0.9 ± 0.6^{2a}	0.1 ± 0.0^{2a}	$1.1 \pm 0.4^{1,2a}$	$0.07 \pm 0.02^{1,2a}$	0.1 ± 0.0^{4b}	0.01 ± 0.00^{3b}
T-V	5.5 ± 0.6^{3a}	1.4 ± 0.3^{2a}	0.0 ± 0.0^{3b}	$1.7 \pm 0.1^{2,3a}$	0.09 ± 0.01^{1a}	0.2 ± 0.0^{3a}	0.01 ± 0.00^{2a}
B-A	1.6 ± 0.1^{4a}	1.4 ± 0.5^{2a}	0.1 ± 0.0^{2a}	2.0 ± 0.9^{1a}	0.03 ± 0.02^{2a}	0.4 ± 0.2^{3a}	0.01 ± 0.00^{3a}
B-V	1.5 ± 0.2^{5a}	1.1 ± 0.2^{2a}	0.0 ± 0.0^{3a}	2.2 ± 0.5^{2a}	0.03 ± 0.01^{2a}	$0.6 \pm 0.1^{1,2a}$	0.01 ± 0.00^{2a}
M-A	4.1 ± 0.5^{3a}	0.7 ± 0.1^{2a}	0.1 ± 0.0^{2a}	$1.3 \pm 0.1^{1,2b}$	$0.05 \pm 0.01^{1,2a}$	$0.2 \pm 0.0^{3,4b}$	0.01 ± 0.00^{3b}
M-V	3.6 ± 0.3^{4a}	1.0 ± 0.1^{2a}	0.1 ± 0.0^{3a}	$2.4 \pm 0.6^{1,2a}$	0.09 ± 0.03^{1a}	$0.3 \pm 0.0^{2,3a}$	0.01 ± 0.00^{2a}
I-A	3.1 ± 0.1^{3a}	0.7 ± 0.2^{2b}	0.2 ± 0.1^{2a}	2.2 ± 0.3^{1b}	$0.07 \pm 0.01^{1,2a}$	$0.2 \pm 0.1^{3,4b}$	0.01 ± 0.00^{3a}
I-V	2.6 ± 0.1^{5b}	1.1 ± 0.2^{2a}	0.2 ± 0.0^{2a}	3.4 ± 0.6^{1a}	0.09 ± 0.02^{1a}	$0.4 \pm 0.1^{2,3a}$	0.01 ± 0.00^{2a}
FP-A-TT	11.2 ± 0.9^{1a}	2.6 ± 0.4^{1a}	0.9 ± 0.1^{1a}	0.5 ± 0.0^{2a}	$0.06 \pm 0.01^{1,2a}$	0.8 ± 0.1^{2a}	0.09 ± 0.00^{2a}
FP-V-TT	12.3 ± 0.3^{1a}	2.7 ± 0.2^{1a}	0.8 ± 0.1^{1a}	0.6 ± 0.1^{3a}	$0.07 \pm 0.02^{1,2a}$	$0.6 \pm 0.2^{1,2a}$	0.07 ± 0.03^{1a}
FP-A-IT	10.8 ± 0.2^{1a}	3.0 ± 0.4^{1a}	1.0 ± 0.1^{1a}	0.8 ± 0.2^{2a}	0.09 ± 0.02^{1a}	1.1 ± 0.0^{1a}	0.12 ± 0.00^{1a}
FP-V-IT	10.6 ± 0.4^{2a}	2.6 ± 0.2^{1a}	0.8 ± 0.0^{1a}	0.9 ± 0.1^{3a}	0.10 ± 0.01^{1a}	0.9 ± 0.2^{1a}	0.09 ± 0.02^{1a}

Different superscript numbers denote significant differences ($p < 0.05$) between samples of the same kind (for example: T-A and T-V or FP-A-TT and FP-V-TT) differing each other for the broilers feeding fat integration whereas different superscript letters denote significant differences amongst different samples coming from broilers fed the same diet (A or V samples).

Table 6.2. – Total fatty acids composition of the different samples. All the results are expressed as mean ± SD (n = 3).

Sample	C14:0 Myristic	C16:0 palmitic	C16:1 palmitoleic	C 18:0 steraric	C18:1 n-9 oleic	C18:1 n-7 vaccenic	C18:2 n-6 linoleic	C18:3 n-3 α-linolenic	C20:3 n-3 + C20:4 eicosatrienoic + arachidonic
T-A	1.0 ± 0.0^{1a}	22.2 ± 0.4^{1a}	5.1 ± 0.0^{1a}	6.6 ± 0.3^{2a}	36.9 ± 0.5^{2a}	2.7 ± 0.1^{3a}	20.4 ± 0.4^{3b}	1.9 ± 0.0^{2b}	0.8 ± 0.1^{2b}
T-V	0.4 ± 0.0^{1b}	17.8 ± 0.1^{1b}	2.3 ± 0.0^{1b}	6.1 ± 0.2^{2a}	28.6 ± 0.2^{2b}	2.1 ± 0.0^{3b}	36.2 ± 0.4^{1a}	3.1 ± 0.0^{2a}	1.5 ± 0.1^{3a}
B-A	0.9 ± 0.0^{2a}	22.0 ± 0.1^{1a}	3.8 ± 0.2^{3a}	8.2 ± 0.3^{1a}	32.8 ± 0.7^{4a}	3.0 ± 0.1^{2a}	$20.9 \pm 0.0^{2,3b}$	1.7 ± 0.1^{3b}	2.7 ± 0.4^{1b}
B-V	0.3 ± 0.0^{2b}	18.1 ± 0.4^{1b}	1.6 ± 0.1^{3b}	7.5 ± 0.4^{1a}	26.4 ± 0.5^{4b}	2.3 ± 0.1^{2b}	33.6 ± 1.0^{2a}	2.6 ± 0.1^{3a}	3.7 ± 0.5^{1a}
M-A	1.0 ± 0.0^{1a}	22.1 ± 0.0^{1a}	4.8 ± 0.0^{2a}	6.8 ± 0.1^{2a}	35.6 ± 0.4^{3a}	$2.8 \pm 0.1^{2,3a}$	21.2 ± 0.2^{2b}	1.9 ± 0.0^{2b}	1.1 ± 0.1^{2b}
M-V	$0.4 \pm 0.0^{1,2b}$	$17.6 \pm 0,3^{1b}$	2.0 ± 0.1^{2b}	6.2 ± 0.2^{2b}	27.7 ± 0.1^{3b}	$2.2 \pm 0.1^{2,3b}$	36.7 ± 0.6^{1a}	3.0 ± 0.1^{2a}	$1.9 \pm 0.1^{2,3a}$
I-A	1.0 ± 0.0^{1a}	22.0 ± 0.3^{1a}	4.7 ± 0.2^{2a}	6.8 ± 0.1^{2a}	34.7 ± 0.2^{3a}	2.7 ± 0.0^{3a}	21.9 ± 0.5^{1b}	2.0 ± 0.0^{2b}	1.3 ± 0.0^{2b}
I-V	$0.4 \pm 0.0^{1,2b}$	17.9 ± 0.1^{1b}	2.0 ± 0.0^{2b}	6.4 ± 0.1^{2b}	27.0 ± 0.1^{4b}	2.1 ± 0.0^{3b}	36.5 ± 0.1^{1a}	3.0 ± 0.0^{2a}	2.2 ± 0.1^{2a}
FP-A-TT	0.2 ± 0.0^{3a}	7.5 ± 0.2^{2a}	0.8 ± 0.0^{4a}	2.2 ± 0.1^{3a}	55.1 ± 0.2^{1a}	3.7 ± 0.1^{1a}	$21.5 \pm 0.1^{1,2b}$	6.6 ± 0.0^{1a}	0.2 ± 0.0^{3a}
FP-V-TT	0.1 ± 0.0^{3b}	7.1 ± 0.1^{2a}	0.5 ± 0.0^{4b}	2.3 ± 0.0^{3a}	53.9 ± 0.1^{1b}	3.6 ± 0.0^{1a}	22.9 ± 0.0^{3a}	6.8 ± 0.0^{1b}	0.2 ± 0.0^{4b}
FP-A-IT	0.2 ± 0.0^{3a}	7.8 ± 0.1^{2a}	0.8 ± 0.0^{4a}	2.3 ± 0.0^{3a}	54.6 ± 0.1^{1a}	3.5 ± 0.1^{1a}	$21.5 \pm 0.1^{1,2b}$	6.6 ± 0.0^{1b}	0.2 ± 0.0^{3a}
FP-V-IT	0.1 ± 0.0^{3b}	6.8 ± 0.3^{2b}	0.4 ± 0.0^{4b}	2.1 ± 0.2^{3a}	54.1 ± 0.1^{1b}	3.7 ± 0.1^{1a}	23.3 ± 0.4^{3a}	6.8 ± 0.0^{1a}	0.2 ± 0.0^{4a}

In the table are reported the main fatty acids, expressed as percentage of total FAME identified and representing more than 97% of total FAME.

Different superscript numbers denote significant differences ($p < 0.05$) between samples of the same kind (for example: T-A and T-V or FP-A-TT and FP-V-TT) differing each other for the broilers feeding fat integration whereas different superscript letters denote significant differences amongst different samples coming from broilers fed the same diet (A or V samples).

Table 6.3. – Relative amounts, expressed as percentage of total FAME identified, of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in different samples. All the results are expressed as mean ± SD (n = 3).

Sample	SFA	MUFA	PUFA	U/S
T-A	30.3 ± 0.7^{2a}	45.7 ± 0.5^{2a}	23.8 ± 0.2^{5b}	2.3 ± 0.1^{3b}
T-V	24.6 ± 0.3^{2b}	33.3 ± 0.2^{2b}	41.8 ± 0.3^{2a}	3.0 ± 0.1^{3a}
B-A	31.6 ± 0.1^{1a}	40.4 ± 0.8^{4a}	27.4 ± 0.6^{2b}	2.1 ± 0.0^{3b}
B-V	26.4 ± 0.7^{1b}	30.6 ± 0.5^{5b}	$42.2 \pm 0.7^{1,2a}$	2.8 ± 0.1^{3a}
M-A	30.4 ± 0.2^{2a}	44.2 ± 0.4^{3a}	25.1 ± 0.2^{4b}	2.3 ± 0.0^{3b}
M-V	24.5 ± 0.5^{2b}	32.3 ± 0.1^{3b}	42.9 ± 0.4^{1a}	3.1 ± 0.1^{3a}
I-A	30.3 ± 0.2^{2a}	43.1 ± 0.4^{3a}	26.3 ± 0.6^{3b}	2.3 ± 0.0^{3b}
I-V	24.9 ± 0.0^{2b}	31.4 ± 0.1^{4b}	43.2 ± 0.2^{1a}	3.0 ± 0.0^{3a}
FP-A-TT	10.9 ± 0.3^{3a}	60.6 ± 0.2^{1a}	28.4 ± 0.1^{1b}	8.2 ± 0.3^{1a}
FP-V-TT	10.7 ± 0.1^{3a}	59.1 ± 0.1^{1b}	30.1 ± 0.0^{3a}	8.3 ± 0.1^{2a}
FP-A-IT	11.3 ± 0.1^{3a}	60.1 ± 0.1^{1a}	28.5 ± 0.1^{1b}	7.8 ± 0.1^{2b}
FP-V-IT	10.1 ± 0.5^{3b}	59.3 ± 0.2^{1b}	30.5 ± 0.4^{3a}	8.9 ± 0.5^{1a}

Different superscript numbers denote significant differences ($p < 0.05$) between samples of the same kind (for example: T-A and T-V or FP-A-TT and FP-V-TT) differing each other for the broilers feeding fat integration whereas different superscript letters denote significant differences amongst different samples coming from broilers fed the same diet (A or V samples).

Table 6.4. – Fatty acid composition of phospholipid fraction of different samples. The amount of each fatty acid is gives as the percentage of all FAME identified. All the results are expressed as mean ± SD (n = 3).

Sample	C16:0 palmitic	C 18:0 steraric	C18:1 n-9 oleic	C18:1 n-7 vaccenic	C18:2 n-6 linoleic	C18:3 n-3 α-linolenic	C20:3 n-3 + C20:4 eicosatrienoic + arachidonic	SFA	MUFA	PUFA
T-A	22.5 ± 4.2^{1a}	9.3 ± 1.3^{2b}	40.1 ± 3.5^{1a}	$3.0 \pm 0.6^{2,3a}$	14.6 ± 2.9^{2b}	1.4 ± 0.3^{2}	$3.8 \pm 1.5^{2,3a}$	35.5 ± 8.7^{1a}	$45.2 \pm 4.4^{1,2a}$	19.3 ± 4.8^{2b}
T-V	$21.2 \pm 0.4^{1,2a}$	13.8 ± 1.0^{1a}	27.6 ± 4.9^{2b}	2.3 ± 0.1^{2a}	$26.3 \pm 1.7^{1,2a}$	< 1.0	7.1 ± 1.6^{1a}	$35.7 \pm 0.8^{1,2a}$	29.4 ± 3.5^{2b}	$34.5 \pm 2.9^{2,3a}$
B-A	$21.0 \pm 1.6^{1,2a}$	9.7 ± 1.0^{2a}	31.9 ± 3.7^{2a}	3.9 ± 0.1^{1a}	18.4 ± 1.8^{2b}	0.8 ± 0.2^{2a}	$5.9 \pm 1.1^{1,2a}$	$33.5 \pm 2.0^{1,2a}$	$36.9 \pm 3.6^{2,3a}$	28.6 ± 3.7^{1b}
B-V	$16.7 \pm 1.9^{2,3b}$	$9.7 \pm 2.4^{1,2,3a}$	26.3 ± 4.9^{2a}	3.2 ± 0.0^{1b}	$28.2 \pm 4.8^{1,2a}$	1.1 ± 0.4^{2a}	8.4 ± 2.4^{1a}	$27.0 \pm 4.4^{2,3a}$	29.9 ± 4.8^{2a}	$41.3 \pm 2.5^{1,2a}$
M-A	$21.9 \pm 0.4^{1,2a}$	12.6 ± 0.6^{1a}	26.6 ± 3.0^{2a}	$3.3 \pm 0.2^{1,2a}$	18.6 ± 0.9^{2a}	0.6 ± 0.2^{2a}	7.3 ± 0.9^{1a}	37.4 ± 1.5^{1a}	31.4 ± 3.0^{3a}	29.9 ± 2.6^{1a}
M-V	24.3 ± 3.8^{1a}	$12.0 \pm 2.0^{1,2a}$	28.9 ± 4.2^{2a}	2.5 ± 0.2^{2b}	19.9 ± 4.0^{2a}	0.8 ± 0.3^{2a}	$5.6 \pm 2.3^{1,2a}$	37.9 ± 5.1^{1a}	32.7 ± 4.0^{2a}	28.4 ± 4.3^{3a}
I-A	22.7 ± 1.6^{1a}	$7.6 \pm 1.3^{2,3a}$	26.7 ± 2.5^{2a}	2.3 ± 0.4^{3a}	28.6 ± 0.8^{1a}	1.4 ± 0.2^{2a}	$3.6 \pm 1.7^{2,3a}$	$34.3 \pm 3.1^{1,2a}$	30.2 ± 2.2^{3a}	35.1 ± 3.2^{1b}
I-V	$18.9 \pm 1.5^{2,3b}$	$9.0 \pm 2.6^{2,3,4a}$	22.9 ± 4.8^{2a}	2.4 ± 0.2^{2a}	33.9 ± 4.5^{1a}	1.3 ± 0.3^{2a}	$6.2 \pm 3.3^{1,2a}$	$28.6 \pm 3.6^{2,3a}$	25.9 ± 4.6^{2a}	44.3 ± 2.8^{1a}
FP-A-TT	$15.7 \pm 2.8^{2,3a}$	$5.8 \pm 1.1^{3,4a}$	44.3 ± 2.4^{1a}	$3.2 \pm 0.1^{1,2,3a}$	23.8 ± 2.2^{1a}	4.5 ± 0.7^{1a}	0.6 ± 0.2^{3a}	$22.6 \pm 4.8^{2,3a}$	50.0 ± 3.3^{1a}	28.7 ± 2.5^{1a}
FP-V-TT	14.8 ± 0.4^{3a}	$5.4 \pm 0.4^{3,4a}$	43.3 ± 2.1^{1a}	3.2 ± 0.1^{1a}	$26.6 \pm 2.5^{1,2a}$	4.7 ± 0.2^{1a}	0.7 ± 0.6^{2a}	20.7 ± 0.9^{3a}	48.9 ± 3.7^{1a}	32.0 ± 2.8^{3a}
FP-A-IT	13.3 ± 1.1^{3a}	4.7 ± 0.4^{4a}	44.8 ± 3.0^{1a}	$3.3 \pm 0.2^{1,2a}$	26.6 ± 1.3^{1a}	4.8 ± 0.2^{1a}	< 0.1	19.2 ± 1.8^{3a}	51.6 ± 3.7^{1a}	31.7 ± 1.4^{1a}
FP-V-IT	14.0 ± 1.4^{3a}	4.7 ± 0.3^{4a}	43.9 ± 0.6^{1a}	3.2 ± 0.1^{1a}	$25.9 \pm 1.0^{1,2a}$	4.9 ± 0.2^{1a}	0.4 ± 0.0^{2}	20.9 ± 1.9^{3a}	49.8 ± 1.7^{1a}	31.1 ± 1.2^{3a}

Different superscript numbers denote significant differences ($p < 0.05$) between samples of the same kind (for example: T-A and T-V or FP-A-TT and FP-V-TT) differing each other for the broilers feeding fat integration whereas different superscript letters denote significant differences amongst different samples coming from broilers fed the same diet (A or V samples).

6.8. LEGENDS TO FIGURES

Figure 6.1. – Flow chart diagram of chicken cutlet production from raw ground meat to final product.

Figure 6.2. – Gas chromatographic trace of FAME obtained, after a trans-methylation on fat, from samples M-A (upper trace) and M-V (lower trace). Peaks identification: IS (internal standars): C13:0; 1, C14:0; 2, C16:0; 3, C16:1; 4, C18:0; 5, C18:1; 6, C18:1 n-7; 7, C18:2; 8, C18:3; 9, C20:3 n-3 + C20:4. For the analytical conditions, see section Materials and methods.

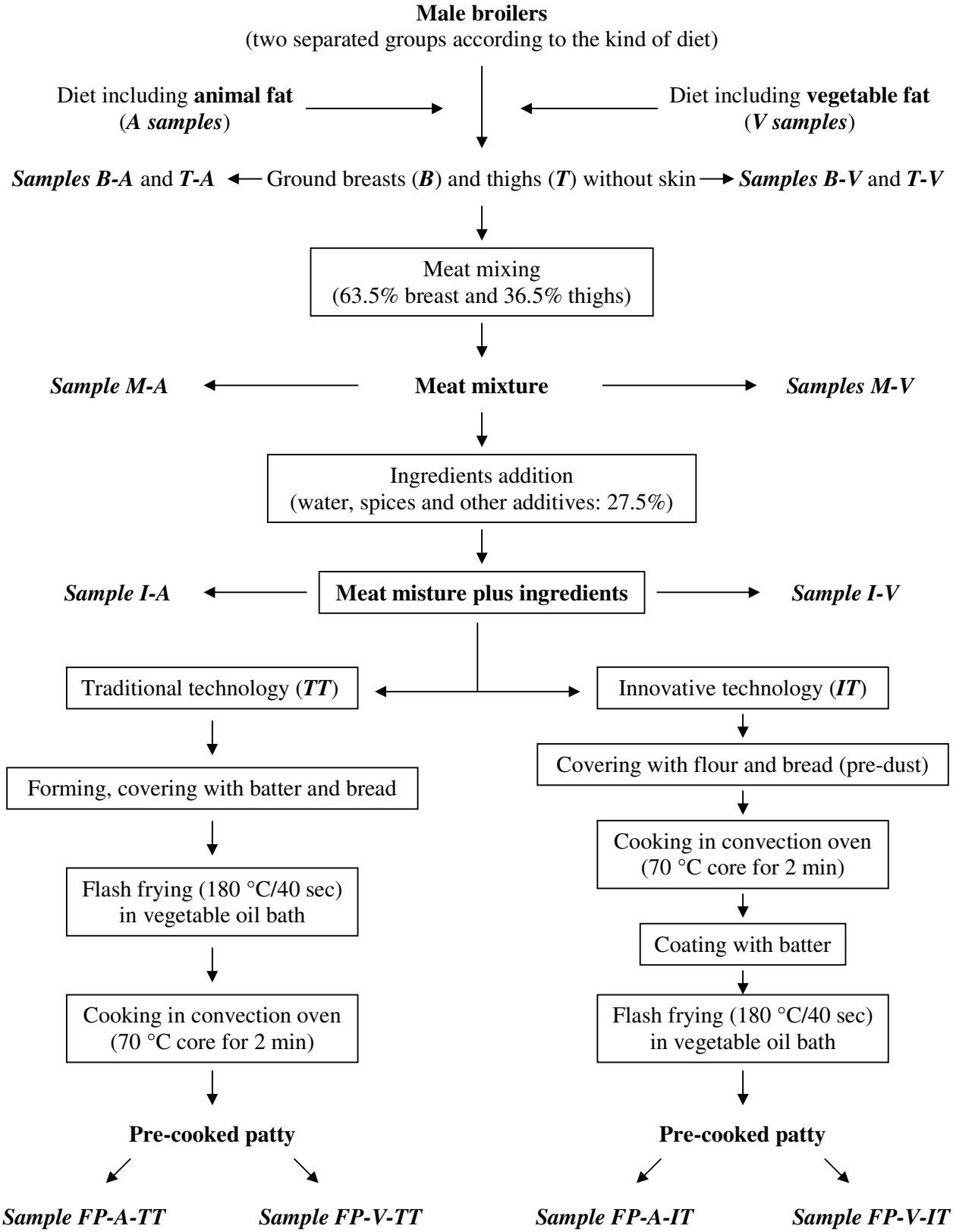
Figure 6.3. – Gas chromatographic trace of FAME obtained, after a trans-methylation on fat, from samples FP-A-TT (upper trace) and FP-V-TT (lower trace). Peaks identification: IS (internal standars): C13:0; 1, C14:0; 2, C16:0; 3, C16:1; 4, C18:0; 5, C18:1; 6, C18:1 n-7; 7, C18:2; 8, C20:0; 9, C18:3 n-3; 10, C20:1, 11, C20:3 n-3 + C20:4. For the analytical conditions, see section Materials and methods.

Figure 6.4. – Gas chromatographic trace of FFA, after SPE performed on fat and methylation with diazomethane, from sample FP-A-TT (upper trace) and FP-A-IT (lower trace). Gas chromatographic trace of FAME obtained, after a trans-methylation on fat, from samples FP-A-TT (upper trace) and FP-V-TT (lower trace). Peaks identification: IS (internal standars): C13:0; 1, C14:0; 2, C16:0; 3, C16:1; 4, C17:0; 5, C18:0; 6, C18:1; 7; C18:1 n-7; 8, C18:2; 9, C18:3 n-3; 10, C20:1, 11, C20:3 n-3 + C20:4. For the analytical conditions, see section Materials and methods.

Figure 6.5. – Gas chromatographic traces of TMS derivatives of DG collected after SPE from samples FP-A-TT (upper trace) and FP-A-IT (lower trace). Peaks identification: IS (internal standard), squalane; Ch, free cholesterol; D34, C16-C18 diglycerides; D36, C18-C18 diglycerides. Peaks beside internal standard and free cholesterol were respectively methyl esters of FFA and free phytosterols respectively. For the analytical conditions, see section Materials and methods.

6.9. FIGURES

Fig. 6.1.



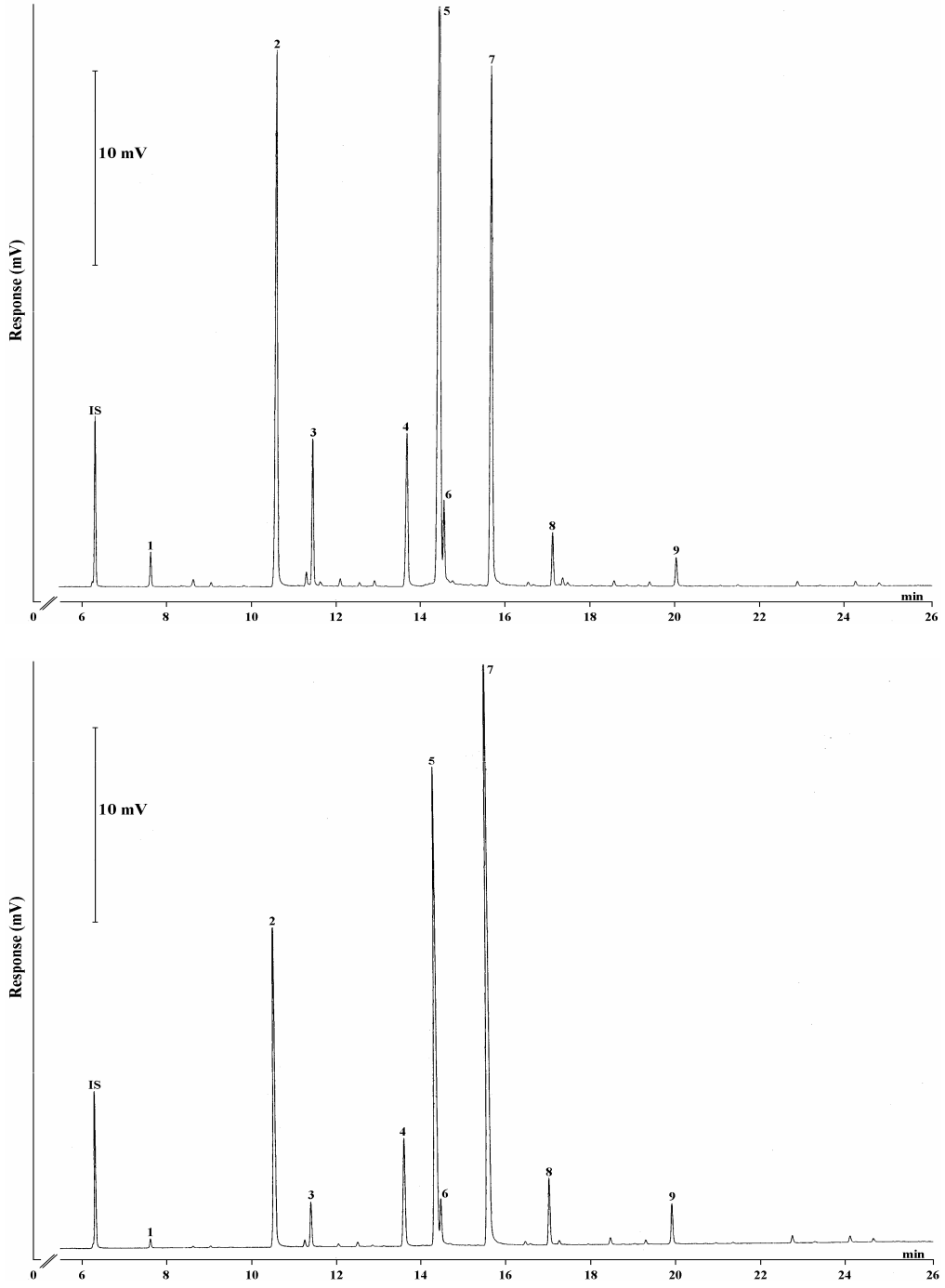

10 mV
Response (mV)
IS
1
2
3
4
5
6
7
8
9
min
0
6
8
10
12
14
16
18
20
22
24
26
10 mV
Response (mV)
IS
1
2
3
4
5
6
7
8
9
min
0
6
8
10
12
14
16
18
20
22
24
26

Fig. 6.2.

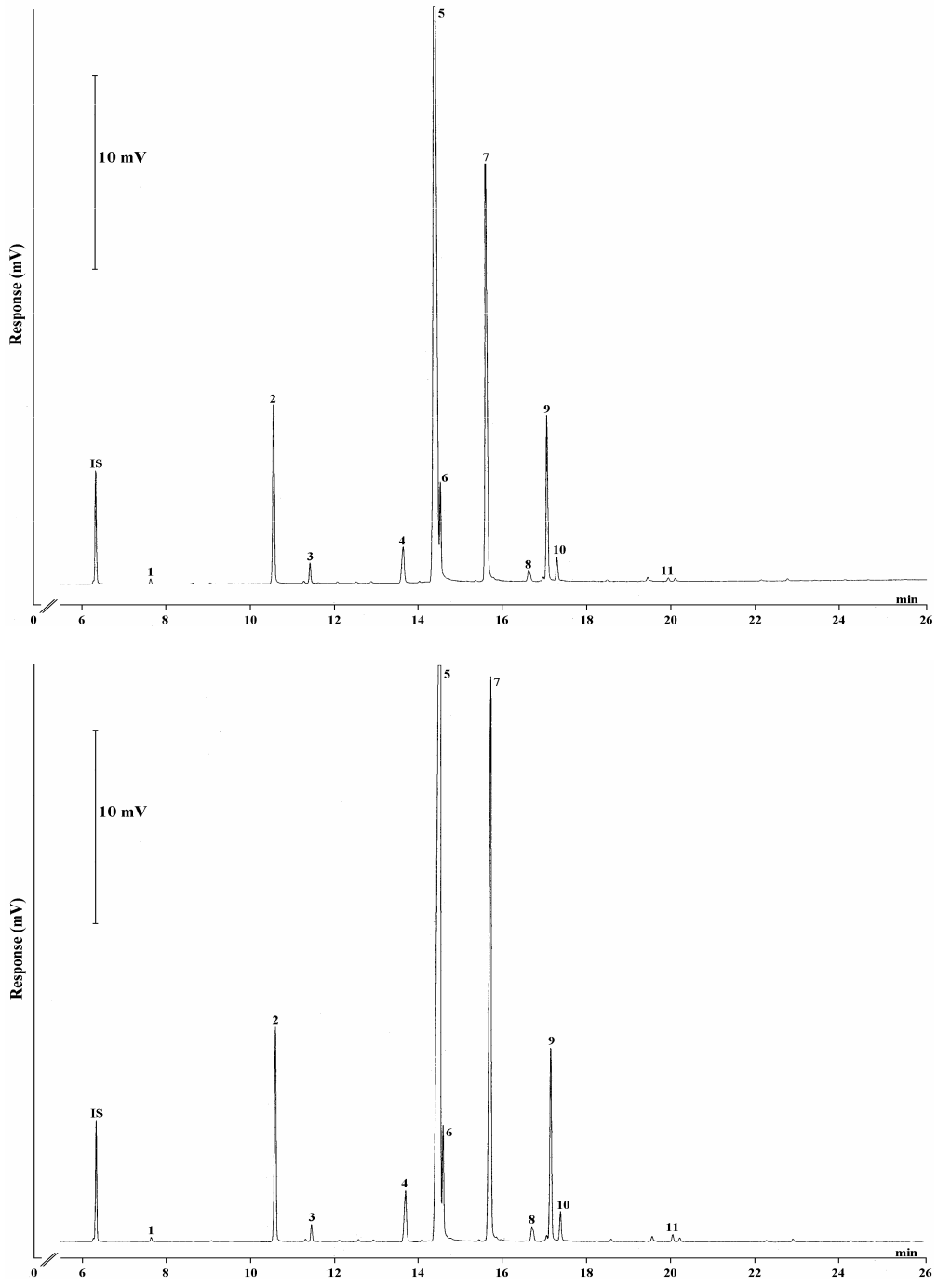

10 mV
Response (mV)
IS
1
2
3
4
5
6
7
8
9
10
11
min
0
6
8
10
12
14
16
18
20
22
24
26

Fig. 6.3.

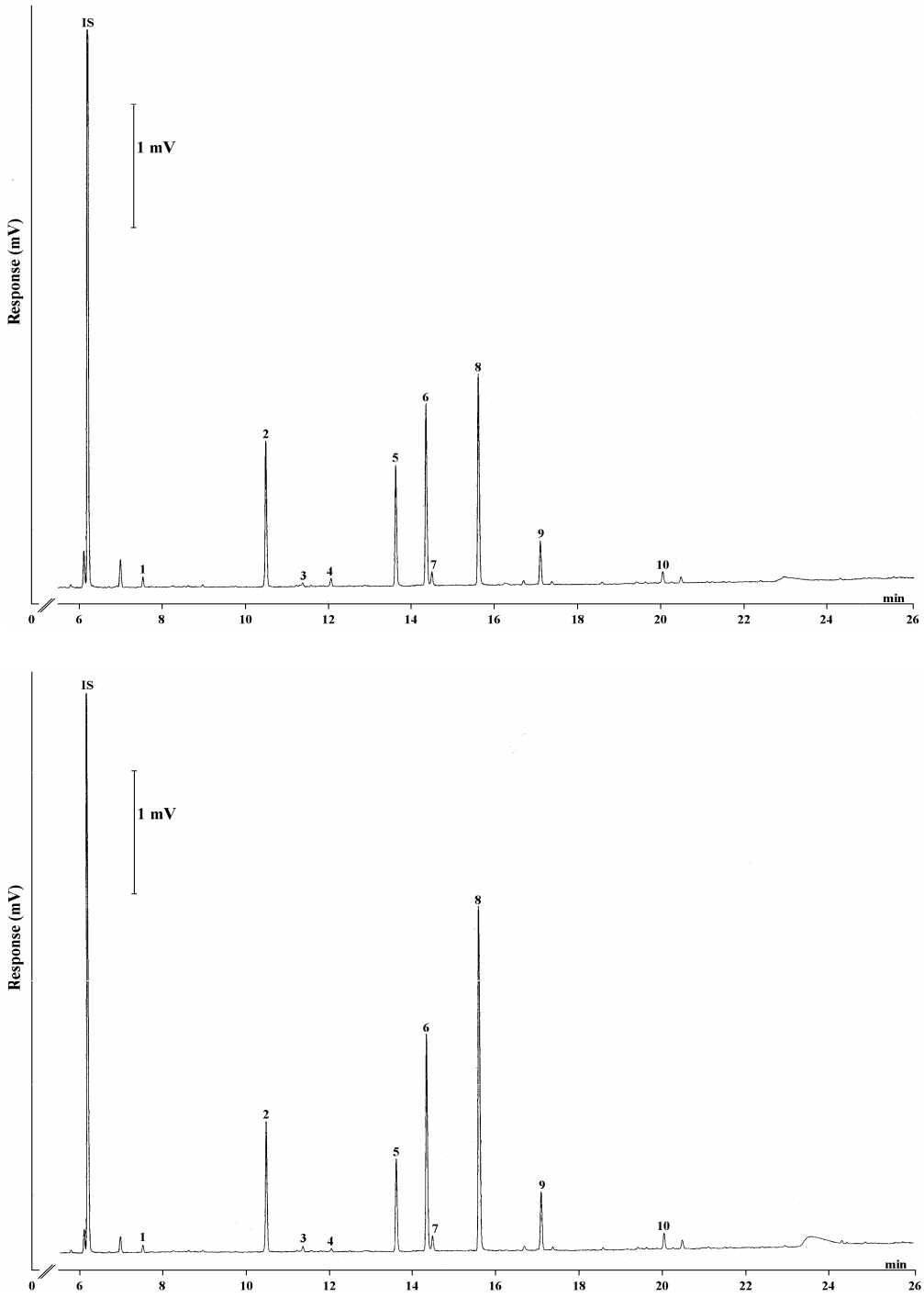

IS
1 mV
Response (mV)
1
2
3
4
5
6
7
8
9
10
min
0
6
8
10
12
14
16
18
20
22
24
26

Fig. 6.4.

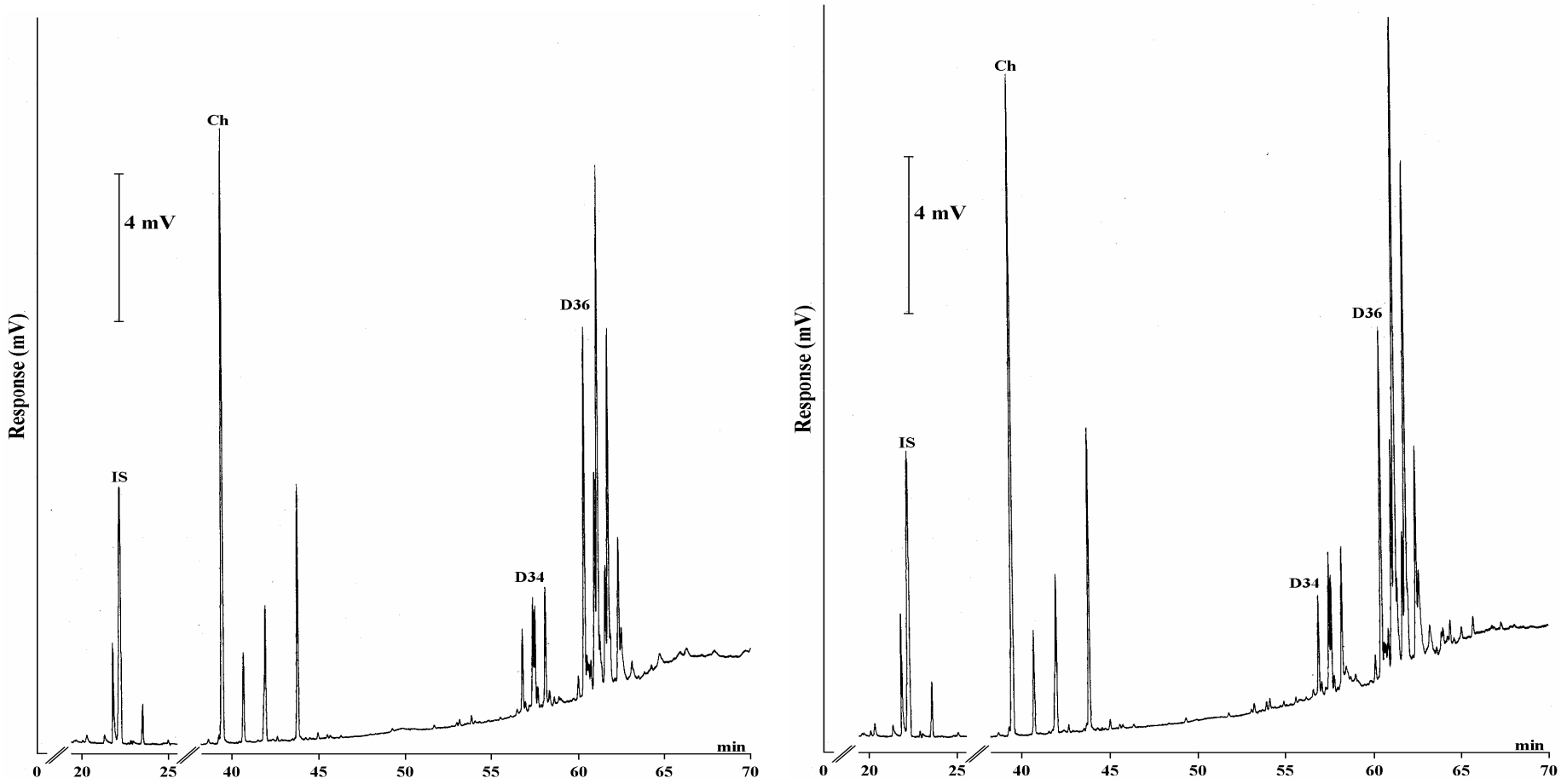


Fig. 6.5.

7. PRELIMINARY STUDY OF THE QUALITY OF THE LIPID FRACTION OF ITALIAN AND SPANISH DRY SAUSAGES AND CURED HAMS STORED UNDER DIFFERENT RETAIL CONDITIONS

7.1. SUMMARY AND KEYWORDS

A preliminary study on the quality of the lipid fraction of Italian and Spanish typical dry sausages and cured hams was carried out. Fat and moisture content, total fatty acids, free fatty acids (FFA), diglycerides (DG), peroxides (PV) and thiobarbituric acid reactive substances (TBARS) were determined. Spanish sausages (Chorizos) proved to be fattier (55 vs. 35%, w/w) than the Italian ones (Salsiccia and Soppressata), owing to the employment of larger amounts of pork fat. In case of hams, the same trend was observed: ≈ 30% was the fat content in Spanish Jamón Serrano while ≈ 20% in Italian Parma and Norcia. The ripening period did not affect these differences. Sausages displayed similar fatty acids profiles regardless their geographical origin while amongst hams differences as regard polyunsaturated fatty acids (PUFA) where more pronounced probably depending on dietary feeding fat sources. The oxidative status of all products stored under vacuum was good: PV never exceeded 5.0 meq O_2/kg fat except one Spanish ham while TBARS were lower than 1.1 mg MDA/kg meat. In Italian ham stored "over the counter", the exposure to air dramatically affected the development of lipid peroxidation and led to high PV: ≈ 55.0 meq O_2/kg fat. Lipolysis affected mainly phospholipids than neutral lipids and brought to a rise in PUFA relative amount in FFA

fraction. Each ham showed a different FFA amount, thus these data combined with the study of headspace compounds may represent a first way to characterize local pork products.

Keywords: cured hams, lipid fraction, lipolysis, oxidation, dry sausages.

7.2. INTRODUCTION

Dry sausages and cured hams are traditional pork products characterized by a high sensorial quality and mainly manufactured in Southern Europe countries like Italy and Spain, according to a basic and simple principle stabilization after a drop in water content and activity and flavour development throughout ripening. To the idea of traditional meat product are often associated those products that were developed ovrr the centuries to preserve meat during harsh climate and along travels (Chizzolini et al., 1998). The production of cured hams has been established for a long time as a process of preservation through salting and subsequent drying (Arboix, 2004). The product quality at the end of ripening is influenced by the raw materials and by technological process and although there are several production technologies for cured hams, the basic aim of all of them is to provide a product which can be kept at room temperature without any risk of deterioration and with the proper sensory qualities (Arboix, 2004). Dry and semi-dry sausages are traditional products too and in most cases have been fermented before and/or during drying, offering a food with special aroma and excellent safety record (Incze, 2004). Cured hams are produced using whole joints or parts of pig

leg, according to a four-step processing: salting, resting period, drying and maturation (Cappelli and Vannucchi, 2005). In Mediterranean countries the dry-salting process is used and a higher level of osmotic dehydration is obtained (Arboix, 2004). After a resting period that lasts about a month and drying under air exposure for some day, maturation or cellar phase begins. In the drying, phase dehydration keeps on and the process of proteolysis and lipolysis that dramatically affect the development of aroma continues. Ripening in cellar ranges from 10-18 months under controlled conditions as regards light intensity and relative humidity which is comprised between 60-80%. Dry-sausages production follow a similar scheme where the first phases are represented by comminution and blending of lean meat and fat, additives and ingredients (milk powder, sugars, spices, nitrite/nitrate and microbial starters if employed) addition and mixture packaging in animal or plastic casings. During fermentation, ripening and drying significant changes take place affecting sausage texture, chemical land physical properties, microbial flora and leading to a product with high sensory qualities and high level of safety.

In both kind of pork product the main biochemical changes involved in the generation of aroma are proteolysis and lipolysis (Toldrà, 1998). Free fatty acids released during the processing of pork products from triglicerides and mainly phospholipids accumulate and give rise to oxidized compound that can act contribute to aroma giving rise to a great number of volatile compound like aldehydes, alcohols and hydrocarbons as described by Hamilton (1989). Nevertheless lipolysis rancidity represent one of the two biochemical pathway leading to rancidity together with lipid oxidation that involves oxygen attack to unsaturated fatty acids (Robards et al., 1988; Nawar, 1996). Depending to its extent lipolysis may contribute to develop a desirable flavour in meat products but

can also cause off-flavours (Incze, 2004). Different papers dealt with the study of the lipid fraction, lipolytic and oxidative processes in pork meat products both from Italy (Ghiretti et al., 1997; Novelli et al., 1998; Vestergaard and Parolari, 1999;Pastorelli et al., 2003; Zanardi et al., 2004) and Spain (Hierro et al., 1997; Cava et al., 1999; Martín et al., 1999; Aguirrezábal et al., 2000; Petrón et al., 2004). The main aim of this study was to carry out a preliminary investigation on the quality of the lipid fraction of typical Italian and Spanish hams and sausages in order to verify their oxidative status under commercial storage conditions, the effect of packaging on the development of rancidity and also to check some differences related to the geographical origin of the products.

7.3. MATERIALS AND METHODS

7.3.1. Samples and sample preparation before analytical determination

The samples object of this experimentation were two dry Spanish sausages "Chorizo Sarta Extra" coming from different local producers, two typical Italian dry sausages "Salsiccia di Calabria piccante" and "Soppressata di Calabria Piccante" both from the Calabria region, two slices (about 1 kg each) of Spanish ham "Jamón Serrano" with and without the addition of paprika and two 1.5-cm thick cuts of Italian typical hams "Parma" and "Norcia". All Spanish samples were supplied directly by the producers and stored under vacuum packaging in the dark and at room temperature till analyses. Italian sausages were purchased from local retail shops where they were stored under vacuum but at light exposure at room temperature while Italian hams slices were bought from local retailers and taken from hams store under light and air exposure at room temperature. Both of Italian sausages and "Parma" ham had the DOP (PDO, Protected

Designation of Origin) European label whereas "Norcia" ham had the IGP (PGI, Protected Geographical Indication) European label. The samples where names as follows: CHOR1 and CHOR2 ("Chorizo Sarta Extra"), JAM-P and JAM ("Jamón Serrano" ham with or without paprika respectively), SAL ("Salsiccia di Calabria"), SOP ("Soppressata di Calabria"), PAR ("Parma" ham) and NOR ("Norcia" ham). After unwrapping, all the samples were cut in 1-2 cm cube-shaped pieces and ground in a meat grinder (2 mm hole diameter). Before grinding, from each ham sample adipose far on the border was cut away and only an approximately 0.5 cm thick layer was left. The ground meat was pooled in aluminium trays, further homogenized with the help of a spoon, collected in glass bottles and, after sampling for lipid extraction, stored under vacuum at -45 °C.

7.3.2. Reagents, solvents and standards

Trimethylchlorosilane (TMCS, min 98.0%) was from Fluka (Buchs, Switzerland). Ammonium thiocyanate (NH_4SCN, min 97.5%), barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$, min99%), ethylenediamine-tetraacetic acid (EDTA) disodium salt (100%±1%), iron(II) sulphate eptahydrate ($FeSO_4 \cdot 7H_2O$, min 99.0%), sodium sulphate anhydrous (min 99.0%) and trichloacetic acid (min 99%) were from Carlo Erba Reagenti (Rodano, Italy). Acetic acid, chloroform, diethyl ether, di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, 99.0-102.0%), ethanol, *n*-hexane, methanol, potassium hydroxide (KOH) in pellets (min. 85%) used for preparing 2 M KOH in methanol, *i*-propanol and pyridine (pyr) were purchased from Merck (Darmstadt, Germany). Squalane was from Roth (> 95%, Karlsruhe, Germany). L-ascorbic acid, hexamethyldisilizane (HMDS), iron(III) chloride hexahydrate

($FeCl_3 \cdot 6H_2O$, min 98%), methyl tridecanoate (min 98%), potassium chloride (min 99.0%), potassium phosphate monobasic (KH_2PO_4, min 99.0%), 1,1,3,3-tetramethoxypropane (TMP) and tridecanoic acid (min 98%) were from Sigma (St. Louis, MO, USA). Deionized water was obtained from an Elix 10 water purification system from Millipore (Bedford, MA, USA). All reagents and solvents, unless elsewhere specified, were of analytical grade.

7.3.3. Determination of moisture content

The determination of the moisture content was performed following the AOAC method (1990). About 10 g of each ground sausage or ham, previously thawed at 4 °C overnight and left at room temperature for about 30 min, were exactly weighed in a small aluminium tray, kept at 105 °C for at least 16 hours, cooled in a desiccator for about 1 hour and than weighed again. The moisture content was calculated from the difference between the tray weight before and after the drying heat treatment.

7.3.4. Fat extraction and determination of lipid content

Lipid extraction was performed according to Folch et al. (1957) with some modifications reported in a work by Boselli et al. (2001). About 15 g of sample (ground sausages or ham) were accurately weighed in a 500 ml glass bottle, added with 200 ml of chloroform/methanol 1/1 (v/v) and homogenized for 3 min. Then the mixture was kept at 60 °c for 20 min, added with 100 ml of chloroform and homogenized again for 1 min. The content of the bottle was filtered under vacuum through Albet 400 paper from Albet (Barcelona, Spain), mixed with 100 ml of 1 M KCl for about 30 sec and left overnight at 4 °C in order to improve the separation between the organic matter and the

residual methanol/water supernatant phase. The lower organic phase was collected in a 1000 ml round flask through the aid of a separatory funnel, dry for about 1.5-2 hours on and filtered over sodium sulphate anhydrous. Fat content was determined gravimetrically removing the organic solvent using a vacuum evaporator. The fat recovered was stored -45 °C in *n*-hexane/*i*-propanol 3/2 (v/v) until further analyses.

7.3.5. Gas chromatographic determination of total fatty acids

Fatty acids bound to triglycerides molecules were converted to the corresponding methyl esters (FAME) through a transmethylation procedure which was carried out according to the method reported in the the Official Journal of the European Communities (2002). About 50 mg of fat were exactly weighed in a conical vial, methylated twice with an ethereal solution of diazomethane (about 100-150 µl for each methylation, Fieser and Fieser 1967) and dry under nitrogen flow. Then the sample was dissolved in 1 ml of *n*-hexane, vigorously shaken on vortex stirrer to completely dissolve fat matter, added with 100 µl of 2N KOH in methanol and shaken again for 30 sec. Subsequently 2.1 mg of tridecanoic acid methyl ester, used as internal standard (solution in *n*-hexane). After a brief shake, the mixture was centrifuged at 1500 rpm for 3 min and 1 ml of the supernatant hexanic phase was diluted in a second vial adding 2 ml of *n*-hexane. One µl of the solution so obtained was analyzed by capillary gas chromatography (CGC) employing a fused silica capillary column Chrompack CP Select CB for FAME (50 m × 0.25 mm i.d., 0.25 µm f.t.) from Varian (Palo Alto, CA, USA). The column was fitted on a Clarus 500 gas chromatograph from Perkin Elmer (Shelton, CT, USA) equipped with a flame ionization detector and a split/splitless injection system. The injector and detector temperatures were set at 240 °C. Helium was

used as carrier gas at the flow of 1.25 ml/min. The oven temperature was held at 100 °C for 1 min, increased from 100 °C to 240 °C at 4.0 °C/min and finally held at 240 °C for 10 min. The split ratio was set at 1:40. The peaks identification was accomplished comparing the peaks retention times with those of the FAMEs of two standard mixtures: GLC 463 and FAME 189-19 respectively purchased from Nu-Check (Elysian, MN, USA) and Sigma (St. Louis, MO, USA). Gas chromatographic data were filed and processed by the software TotalChrom Navigator (version 6.2.1) from Perkin Elmer.

7.3.6. Spectrophotometric determination of peroxide value (PV)

PV was determined according to Shantha and Decker (1994). This method is based on the spectrophotometric determination of ferric ions (Fe^{3+}) formed by the oxidation of ferrous ions (Fe^{2+}) by hydroperoxides in the presence of ammonium thiocyanate. Thiocyanate ions (SCN^-) react with ferric ions to give a red-colored chromogen that can be determined spectrophotometrically. About 50 mg of fat were exactly weighed and dissolved in a 10 ml volumetric flask with chloroform/methanol 2/1 (v/v), taking care to make the volume up to the mark with the same solvent mixture. The sample was then added with aqueous solutions of 30% (w/v) ammonium thiocyanate and iron(II) chloride (50 µl each) and left in the dark for 5 min. Then the absorbance of the solution was read at 500 nm with a single beam UV-VIS spectrophotometer UV mini 1240 from Shimadzu (Kyoto, Japan). To quantify PV, an iron(III) calibration curve was built from iron(III) chloride and with a calibration range from 0.1 to 5.0 µg/ml (r^2 = 0.995). PV was expressed as meq of O_2/kg of fat.

7.3.7. Spectrophotometric determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined following the method described by Tarladgis et al. (1960) with some modifications. About 2 g of sample (ground meat) were accurately weighed in a 25 ml glass bottle and added with 8 ml of a phosphate buffer (pH = 7.00 ± 0.02) and 2 ml of 30% (w/v) trichloroacetic acid. The phosphate buffer was prepared adding 65.8 ml of 0.5 M KH_2PO_4 and 111 ml of 0.5 M $Na_2HPO_4 \cdot H_2O$ (water solutions) in a 500 ml volumetric flask, correcting pH employing either the acid or the basic solution and making the volume up to the mark with water. To delay oxidation and prevent the pro-oxidative effect of metals, proper amounts of EDTA and ascorbic acid were added to the buffer to reach a final concentration of 0.1% (w/v) for both of them.

The mixture was then homogenized for 30 sec and filtered through Albet 400 paper from Albet. The filtrate was collected in a 10 ml Teflon lined screw cap vial. Five ml of the filtrate were pipetted in a second 10 ml Teflon lined screw cap vial, added with 5 ml of 0.02 M thiobarbituric acid and kept at 90 °C for 20 min in a water bath covered with aluminum foil. After cooling at 4 °C for 20 min and at room temperature for about 10 min, the absorbance of the solution was read at 530 nm with the same spectrophotometer employed in PV determination. To quantify TBARS, a malondialdehyde (MDA) calibration curve was built from a 20.2 nM TMP solution in the buffer afore-described, with a calibration range from 0.3 to 8.1 nM (r^2 = 0.995). TBARS were expressed as mg MDA/kg of meat.

7.3.8. Purification and gas chromatographic determination of free fatty acids (FFA)

FFA were purified from the less polar lipid compounds according to Parisi (2001). About 20-25 mg of fat were exactly weighed in a conical vial, added with 0.11 mg of tridecanoic acid (internal standard, solution in *n*-hexane), dry under nitrogen flow, dissolved in about 200 µl of chloroform/*i*-propanol 2/1 (v/v) and finally loaded onto an ammino-propilic bonded phase cartridge (STRATA NH2 cartridges, 500 mg stationary phase, 3 ml reservoir volume) for solid phase extraction (SPE) from Phenomenex (Torrance, CA, USA). Before fat loading, each cartridge had been placed in a SPE elution apparatus from Isolute (Mid-Glamorgan, UK), loaded with a small amount of sodium sulfate anhydrous and conditioned with 3 ml of *n*-hexane. The cartridge was eluted with 6 ml of chloroform/*i*-propanol 2/1 (v/v) and then with 10 ml of a 2% (v/v) solution of acetic acid in diethyl ether to remove and collect FFA. The latter fraction was evaporated under reduced pressure and methylated three times with an ethereal solution of diazomethane (about 100-150 µl for each methylation) according to Fieser and Fieser (1967). After methylation, 200 µl of *n*-hexane were added and the organic solution containing FFA was analyzed in CGC under the same instrumental conditions adopted for FAME analysis.

7.3.9. Purification and gas chromatographic determination of diglycerides (DG)

Diglycerides were isolated and concentrated as reported by Bortolomeazzi *et al.* (1990). About 100 mg of fat were accurately weighed in a 10 ml Teflon lined screw cap vial, dissolved in about 350-400 µl of *n*-hexane and loaded onto a silica cartridge for SPE (STRATA SI-1 cartridges, 500 mg stationary phase, 3 ml reservoir volume) from

Phenomenex (Torrance, CA, USA). Before fat loading, each cartridge had been placed in a SPE elution apparatus from Isolute, loaded with a small amount of sodium sulfate anhydrous and conditioned with 3 ml of *n*-hexane. The cartridge was then eluted with 3 ml of *n*-hexane, 3 ml of *n*-hexane/diethyl ether 8/2 (v/v), 4 ml of *n*-hexane/diethyl ether 1/1 (v/v) and 3 ml of methanol. The two last fractions, containing DG, were pooled, dry under reduced pressure, added with 0.1 mg of squalane (internal standard, solution in *n*-hexane), dry again under nitrogen flow and, prior to CGC analysis, silylated according to Sweeley *et al.* (1963) in order to convert DG to the corresponding trimethylsilyl (TMS) derivatives. The silylation procedure was carried out as follows: 200 µl of a mixture Pyr/HMDS/TMCS 5/2/1 (v/v/v) freshly prepared were added, then the sample was briefly shaken and kept at 40 °C for 20 min. After removing the silylating agent under nitrogen, 200 µl of *n*-hexane were added; the mixture was shaken on vortex and centrifuged at 3000 rpm for 3 min.

One µl of the organic phase was injected in an AUTO/HRGC/MS MFC 500 gas chromatograph (Carlo Erba Instruments, Rodano, Italy) which was equipped with a fused silica capillary column (Rtx-65TG, 30 m × 0.25 mm i.d., 0.10 µm f.t.) coated with 65%-diphenyl-35%-dimethyl-polysiloxane from Restek (Bellefonte, PA, USA), a flame ionization detector and a split/splitless injection system. The injector and detector temperatures were set at 350 °C. Helium was used as carrier gas at the flow of 1.2 ml/min. The oven temperature was programmed from 160 to 350 °C at 3.0 °C/min and finally held at 350 °C for 10 min. The split ratio was set at 1:40.

7.3.10. Statistics

Each analytical determination was performed in triplicate and the results were reported as the mean of the values obtained (n = 3). Standard deviation (SD) was chosen as spreading index. The data underwent one-way analysis of variance (ANOVA) using the software Statistica 6.0 from StatSoft (Tulsa, OK, USA). Unless specified, Tukey's honest significant difference (HSD) test and Pearson's correlations were evaluated at a $p < 0.05$ level.

7.4. RESULTS AND DISCUSSION

7.4.1. Moisture and fat content of sausages and hams

Table 7.1. shows moisture and fat content of Italian and Spanish sausages and hams. Although for SAL and SOP higher ripening periods (at least 30 and 45 days respectively) in comparison to Chorizos (from 21 to 24 days) were reported in the corresponding production protocols, in Spanish dry sausages moisture was comprised in the range 20-25% whereas in Italian product exceeded 30% and SOP showed the highest content in all sausages here analyzed. The same trend was observed also for hams. The maturation process for Italian hams lasts at least 12 months while this share of time is shorter for Spanish hams: about 9 months. According to these data, ripening time did not seem to affect significantly water loss in these cured meat products but other parameters like the relative humidity and environmental temperature in the drying room may have mainly influenced moisture. On the basis of production protocols it was not easy to draw some hypotheses about these differences. Indeed Italian pork products

are still produced according to an handmade way and the description of ripening conditions and procedures was not detailed.

In sausages the fat content was determined by the fat matter-to-lean matter ratio. The two samples of Chorizo proved to be significantly fattier than SAL and SOP and this high fat content was in accordance to the data reported in a recent Spanish report (Anonymous, 2004) where the fat percentage (in dry matter) in different type of Chorizo varied from 51.5 to 64.2%. It was likely to depend on the different composition of the raw material employed in the preparation of the sausages. In actual fact nearly 30% of raw meat used for Chorizo is represented by pork fat (bacon) as reported by Aguirrezábal et al. (2000) while in SAL and SOP fat portion in raw meat mixture was in the range 4-15% and 6-20% (pork lard) respectively. In spite of a lower moisture content, this finding did not explain the higher amount of fat found in Spanish sausages: the differences amongst Chorizos, SAL and SOP were almost identical when fat was expressed in dry matter. According to **Table 7.1.**, moisture was a minor parameter influencing the total fat content for hams too. In these products the quality of raw materials and then the amount of intramuscular fat appeared the main parameter that affected lipid percentage. In particular Spanish swine were fed a traditional diet with respect Italian dietary regime in which cereals can be employed. Nevertheless several other factors like breed, muscle location, animal age and breeding system should be taken into account (Cavani and Bianchi, 2004).

7.4.2. Total fatty acids composition of sausages and hams

In **Table 7.2.** is shown the composition of total fatty acids of each sample whereas in Fig. 1 are displayed two gas chromatographic traces of total fatty acid methyl ester

(FAME) of Chorizo and Parma ham. From a quantitative standpoint, both in Italian and Spanish sausages oleic acids was the most abundant compound in the lipid fraction, representing from 39.1-39.0% of total FAME, followed by palmitic (24.0-24.9%), stearic (12.8-13.2%) and linoleic (11.3-13.5%) acids. Thus monounsaturated fatty acids (MUFA) were about 50% of FAME and together with polyunsaturated fatty acids (PUFA) accounted for almost 60% of fat. This finding was not in agreement with the common consumers' perception they have about sausages, wrongly felt as highly saturated products. Moreover, amongst saturated fat, stearic acid was about one third of total saturated fatty acids (SFA) and in a past research about the dietary effect of this compound it was proved that it was rapidly converted to oleic acid and appeared to be as effective as oleic acid in lowering plasma cholesterol levels when either replaces palmitic acid in the diet (Bonanome and Grundy, 1988).. The four sausages here analyzed presented a similar acidic pattern with only small differences with respect to polyunsaturated fraction. No significant differences were found in saturated and monounsaturated fractions whereas SAL showed a slightly lower amount in PUFA with respect other samples.

In hams, the fatty acids composition was similar to that formerly reported for sausages: oleic (40.8-42.7%), palmitic (23.6-24.2%), stearic (11.3-13.6%) and linoleic (9.1-11.7%) acids. The relative amount of PUFA in each ham was significantly different from that of other hams, with JAM and NOR showing the highest and lowest percentage respectively. In a recent study (Warnants et al., 1999) it was pointed out that the quality of diet affected fatty acids composition in depot and intramuscular pork. In that work the polyunsaturated/saturated fatty acids ratio in backfat was increased from 0.34 in pig fed a diet containing tallow to 0.55 in pigs fed 15% full-fat soybeans for 6 weeks.

Besides while saturated and monoenoic dietary fats did not caused significant changes in the animal fat composition, PUFA are rapidly metabolized in pork tissues (Romans et al., 1995; Fontanillas et al., 1998). Recently Pastorelli et al. (2003) verified that different dietary fat sources (tallow, corn oil and rapeseed oil) had a limited effect on the fatty acid composition of intramuscular fat of dry-cured hams. In that study a treatment effect was noticed for α-linolenic acid content, higher in Parma hams coming from pigs fed a supplemented rapeseed oil diet and for monoenoic acids, higher in products coming from pigs fed a rapeseed and a tallow supplement diet. Thus feeding fat sources may be seen as one of the major parameter the affected the different amount of each lipid class in the hams here studied even no information was known about the specific diet animals were fed. No significant correlation ($p < 0.05$) was here found between the relative amount of PUFA and fat content in sausages and hams.

7.4.3. Evaluation of lipid oxidation in sausages and hams: determination of peroxides (PV) and thiobarbituric acids reactive substances (TBARS) content

The extent of lipid oxidation was evaluated determining the content of PV and TBARS which represents the first and a measure of the amount of secondary oxidation products respectively. In sausages PV were low and never exceed 5.0 meq O_2/kg of fat. To explain this trend the study of Aguirrezábal et al. (2000) may be taken into account. In that work it was pointed out that the addition of paprika (*Capsicum annuum* L.) and garlic together or paprika alone to meat mixtures employed in Chorizo preparation kept PV at low level, showing an anti-oxidant activity and delaying the pro-oxidant effect of salt. In that experimentation significant differences were found between fat and meat-fat batches with and without paprika at the end of ripening: while salt enhanced

peroxidation in the absence of paprika up to 30 meq O_2/kg meat, paprika kept PV always less 30 meq O_2/kg meat. Garlic did not show a noticeable effect on PV. In actual fact, garlic and paprika are used in the preparation of Chorizo and added to meat mixture (fat plus ground lean cuts) in significant amount: 1 and 3% respectively.

The anti-oxidant properties of some spices (rosemary and sage) has been related to their chemical composition and the presence of phenolic compounds acting as radical-scavenger (Madsen and Bertelsen, 1995). Paprika, also known as red pepper, showed one of the lowest reduction potential in comparison to other spices (Palic et al., 1993) and the presence and the quantification of several anti-oxidant compounds like β-carotene, α-tocopherol and ascorbic acid was verified by Daood et al. (1996) confirming what previously stated. Radical scavenger compounds were also found in garlic and the anti-oxidant effect was proportional to their concentration (Yang et al., 1993).

In a recent study on the same kind of sausages, paprika showed a protective effect on the lipid fraction delaying the onset of rancidity for its high carotenoids and capsaicin concentration and ensured a better coloration which remained more stable with time long-lasting colouring for its high carotenoids and capsaicin concentration (Revilla and Vivar Quintana, 2005).

Although Salsiccia and Soppressata (SAL and SOP samples) are produced according to two different protocols and employing different meat cuts, red pepper (sweet or hot) may be used as additive in place of black pepper to convey a special flavour to the products. Nevertheless, in case of Italian sausages, their high oxidative stability did not depend only on the use of spices considering the lower amounts employed with respect Chorizo. Zanardi et al. (2004) verified that the use of spices in low amounts (less than 0.1%) as preservatives in meat formulations led to an increase in the extent of oxidation

with respect the use of other additives like ascorbic acid, nitrites and nitrates. PV values here obtained were similar to those found recently in other two Italian pork products (Novelli et al., 1998). During a six-month frozen storage, PV ranged from 1.7 to 4.2 and from 1.5 to 2.3 meq O_2/kg of fat in Salame and Mortadella respectively.

In this study vacuum packaging proved to be an effective mean to keep PV low regardless exposure to light.

About hams, Spanish hams showed PV value similar to those found by Cava et al. (1999) in a study arried out on Iberian hams where PV was comprised between 1.8 and 9.0 meq O_2/kg of fat (*B. femoris* muscle) at the end of ripening, depending on the kind of diet and breeding system.. Nevertheless in the same work higher PV values (11.1-30.3 meq O_2/kg of fat were found in *Semimembranosus* muscle, owing to a different accessibility for oxygen to the lipid fraction.

The anti-oxidant effect of paprika the lower PV that JAM-P displayed with respect JAM, although the addition of nitrites, nitrates and ascorbic acids was likely to be the main factor affecting lipid oxidation in this case. Besides Cava et al. (1999) observed that at the end of ripeness the extent of oxidation was lower than during the first stage of ham preparation (salting, post-salting and drying), depending on the controlled conditions applied in this technological phase in terms of temperature and relative humidity. A remarkable high PV was found in Italian hams

PV was extremely high in Italian hams with respect Spanish products and the results reported in a recent study carried out on lipid oxidation in Parma hams by Vestergaard and Parolari (1999) where PV ranged from 6.6 to 8.1 me O_2/kg in the second year of ageing. Actually both PAR and NOR were stored in the normal retail “over-the-counter” conditions that means under unwrapped under oxygen exposure and at room

temperature. Moreover in the production protocol of Italian hams it not allowed the addition of natural anti-oxidant or nitrites/nitrates that may have a counteracting effect with oxygen and light.

TBARS analysis was performed to have a complementary information about the oxidative status of pork products, owing to the transient nature of peroxides (Nawar, 1996). For fresh meat, a threshold value for TBARS is 0.5 mg MDA/kg of meat according to Lanari et al. (1995). Nevertheless TBARS determination may be affected by the experimental condition adopted (Antolovich et al., 2002) thus the value formerly cvited should be better considered as a reference. In sausages TBARS ranged from 0.4 to 1.1 mg MDA/kg of meat and were in agreement to PV. Sausages were stored in proper conditions and the extent of oxidation was still in the so called induction period in which oxygen absorbance was slow as the formation of peroxides, owing to the delaying effect of spices. Low TBARS were also recorded both for Spanish and Italians hams, although in PAR and NOR PV was more than five times higher than in JAM-P and JAM. Although oxidation in PAR and NOR was already in the second phase called propagation and characterized by a faster oxygen absorption, a significant peroxide breakdown to the corresponding secondary oxidation products did not take place as indicated by the low TBRAS. This finding was important from an organoleptic standpoint considering that it is from peroxides degradation that are generate those volatile compounds responsible for those “off flavours” and “off-odours” called “rancidity” (Nawar, 1996).

7.4.4. Evaluation of lipolysis in sausages and hams: determination of free fatty acids (FFA) and diglycerides (DG) content

Proteolysis and lipolysis are the two main biochemical processes involved in flavour development in meat and meat preparations, like dry sausages and cured hams, leading to the formation of volatile compounds responsible for aroma (Toldrá, 1998). In particular FFA coming from enzymic or chemical pathways are more susceptible to lipid oxidation with respect fatty acids bound to triglycerides or phospholipids (Nawar, 1996) and give an important contribution in the rise of rancidity. In **Table 7.1.** are reported the total FFA and DG and the FFA-to-DG ratio.

In the two Chorizos and SOP, FFA were about 5 g/100 g of fat while a double FFA amount was found in SAL. The higher extent of lipolysis in SAL was confirmed by DG value. This finding was in agreement with the results reported by in agreement with the data reported by Demeyer et al. (1974) where FFA represented in dry sausages at the end of ripening about 5% of total fat. The more abundant amongst FFA was oleic (1.9-5.3 g/100 g of fat) whereas linoleic acid (1.3-2.2 g/100 g of fat) became the second most important compound in FFA fraction, followed by palmitic (0.8-1.9 g/100 g of fat) and stearic (0.4-0.8 g/100 g of fat) acids. In comparison to total FAME, a significant increase (about 90%) in unsaturation took place in FFA as illustrated in **Table 7.4.** where PUFA were in the range 20-30% of total FFA. To this rise, a decrease in the relative amount of SFA corresponded while the percentage of MUFA was quite similar to that displayed in **Table 7.3.** Actually this trend was about the same observed by Demeyer et al. (1974) where in sausages linoleic acid was the faster to be liberated in FFA fraction by lipases and the rate of lipolysis decreased in the order: linoleic > oleic > stearic and palmitic. A recent studied (Hierro et al., 1997) confirmed these results and

that kind of hierarchy as regards FFA release: linoleic > oleic ≈ palmitoleic > myristic > stearic ≈ palmitic. The results of this and the afore-mentioned studies pointed highlighted the specificity of lipolysis for position *sn*-3 of triglycerides molecules which is manly occupied by octadecenoic acids in pork fat (Nichols and Sanderson, 2003).

An important contribution to lipolysis was also given by the action of lipases towards fatty acids bound to phospholipids since in all sausages DG amount was less than FFA for a similar FFA/DG ratio. At a first glance this value may appear unexpected considering that from the lipolysis of one molecule of triglyceride derives one molecule of fatty acid whose weigh is about half the DG one. This depended on the fact that most of FFA came from lipolysis of PS that were particularly rich in PUFA. On the basis of data reported in **Table 7.1.** it seemed that about 75% of total FFA came from phospholipids while only 25% from triglycerides. In other works it was proved that polar lipids contributed to FFA more than neutral fat (Alasnier and Gandemer, 1998; Martin et al., 1998).

Zanardi et al. (2004) studied lipolytic and oxidative processes in different Mediterranean and Northern type sausages, finding that in cured meat product 60-80% of total lipolysis was brought about by endogenous enzymes. The studies of Zanardi et al. (2004) and Hierro et al. (1997) confirmed that lipolysis in dry fermented meat products was mainly due to the endogenous enzymic pattern and was independent of processing conditions. The quality of raw meat employed and the ratio between fatty and lean meat cuts appeared as the main factor influencing the degree of lipolysis, considering that lipases and phospholipases activity was the driving force.

Hams are meat preparation that need a much longer curing period in comparison with sausages. In this case it was better to take into account DG together with FFA because

in a paper by Toldrá (1998) it was stated that lipolysis was intense especially in the first five months of ripening of dry-cured but after ten months some FFA started to decrease owing to a biochemical spoilage.

The highest FFA amounts were found in Spanish hams while significantly lower values were observed in Italain ones, especially in NOR which displayed less than half FFA than JAM-P and JAM. In Spanish hams and in PAR oleic acid was the most abundant free fatty acids (4.3-7.7 g/100 g fat), follwed by palmitic (2.1-3.8 g/100 g fat), linoleic (2.0-3.1 g/100 g of fat) and stearic (0.9-1.7 g/100 g of fat) while in NOR linoleic acids became the second most important FFA followed by palmitic and stearic acids. The same trend recorded sausages was noticed in hams, with a rise in the relative amount of PUFA with respect to total FAME. As proved by a lower U/S ratio and by the data shown in Tables 4 and 5, a higher percentage of SFA and a low relative amount in PUFA was recorded in hams in comparison with sausages. In the sausages FFA fraction the quantitative order was MUFA > PUFA > SFA while in case of hams it was more simlar to that of total FAME.

Spanish hams and PAR showed similar values for DG while FFA/DG ratio in hams was about twice higher than in sausages. Hams were leaner than sausages and had a lower amount of depot fat but were richer with respect to intramuscular fat and thus membrane phospholipids. On the other hand, in sausages a significant ratio of ingredient was represented by pork adipose fat. NOR showed the lowed FFA and DG values.

In Spanish hams, the high FFA amounts were probably a consequence of the shorter ripening time (about 9 months) applied that delayed the onset of enzymic and oxidative spoilage that could affects FFA in the later months of maturation. In PAR, a product which was cured for more than 12 months, the decomposition of FFA was likely to be

already started. Nevertheless the extent of lipolysis in PAR seemed to be similar to that of JAM-P and JAM on the basis of DG amount almost identical for the three hams. In NOR the ripening period was as long as Parma's even lipolysis development was at a lower stage as proved by DG amount. Some reasons accounting for this outcome may be the employment of larger amount of salt, an anti-microbial agent or a lower lipases activity. The hydrolysis of lipid fraction in hams has bee attributed the presence of muscle enzymes that remain active throughout the curing process (Motilva et al., 1992, 1993). Recently it was pointed out that FFA during ham processing come basically from phospholipids since a decrease in the quantity of fatty acids of phospholipids during ripening occurred (Martín et al., 1999).

7.5. CONCLUDING REMARKS

This study represented a first attempt to characterize the lipid fraction of typical Italian and Spanish dry sausages and cured hams. As a general trend, Spanish products proved to be fattier than the corresponding Italian ones. In case of sausages it depended on a higher amount of animal fat employed in the preparation of meat mixture before the beginning of ripening and thus on a different quantitative ratio between fat and lean parts. Different factors may have affected the results found in hams like: breed, breeding conditions and slaughtering age.

Sausages displayed similar fatty acids profiles regardless their geographic origin while in hams a bit higher variability was noticed as regards PUFA relative amounts. These differences were probably due to dietary regime and feeding fat sources that may influence the relative amount of fatty acids classes in pigs, considering the higher speed

PUFA are metabolyzed in comparison to SFA and MUFA and the fact PUFA are affected by diet to a higher extent.

About oxidation, a noticeable finding was the high PV values (about 55 meq O_2/kg of fat) displayed by Italian hams stored "over-the-counter" and under oxygen exposure. The kind of packaging greatly affected the development of peroxidation that was signifcanlty delayed in pork products stored vacuum packaged. Even different PV values, TBARS remained low although the coreelation between this parameter and organoleptic scores should be verified. A deeper investigation should be carried out between the effect of the traditional retail condition of meat products on their oxidative stability and verify the goodness of alternative packaging or storage condition that may ensure a better oxidative status.

Lipolysis affected mainly the polar lipids (phospholipids) and in all products led to an increase in the polyunsaturated fraction amongst FFA. To better evaluate the development of lipolytic process DG were a useful parameter to be used for this aim together with FFA. Significant differences were found as regards FFA amounts in pork product here analyzed, especially in hams. Thus the simultaneous determination of FFA and volatile compound, whose precursor are indeed fatty acids release by hydrolytic and enzymic processes from triglycerides and phospholipids, may represent a way to characterizes hams and sausages on the basis of their geographical origin.

7.6. REFERENCES

Arnau Arboix, J. 2004. Dry-Cured Ham. In: Encyclopedia of Meat Science (edited by W. K. Jensen, C. Devine and M. Dikeman). Pp. 557-562. Amsterdam, Boston, Heidelberg, London, New York, Oxford, Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo: Elsevier Academic Press.

Aguirrezábal M. M., Mateo J., Domínguez M.C., Zumalacárregui J.M. 2000. The effect of paprika, garlic and salt on rancidity in dry sausages. Meat Science, 54, 77-81.

Anonymous, 2004. Análisis comparativo – Chorizo de Pamplona Extra en vela – Grasa, sal y cholesterol en exceso. Consumer-Spain, March 2004, 26-29.

Antolovich M., Prenzler P. D., Patsalides E., McDonald S, Robards K. 2002. Methods for testing antioxidant activity. Analyst, 127, 183-198.

AOAC – Association of Official Analytical Chemists 1990. Official methods of analysis, 15th Edition, Washington, DC, 147-152.

Bonanome A., Grundy S. M. 1988. Effect of dietary stearic acid on plasma cholesterol and lipoprotein levels. New England Journal of Medicine, 318, 1244-1248.

Bortolomeazzi, R., Frega, N., Lercker, G. 1990. Rapid determination of free and esterified sterols using prepacked silica columns. Italian Journal of Food Science, 2, 265-268.

Boselli, E., Velazco, V., Caboni, M. F., Lercker, G. 2001. Pressurized liquid extraction of lipids for the determination of oxysterols in egg-containing food. Journal of Chromatography A. 917, 239.244.

Cappelli P., Vannucchi V. 2005. Carne e Salumi. In: Chimica degli Alimenti. Pp.: 483-508. Bologna, Italy: Ed. Zanichelli.

Cava R., Ruiz J., Ventanas J., Antequera T. 1999. Oxidative and lipolytic changes during ripening of Iberian hams as affected by feeding regime: extensive feeding and alpha-tocopheryl acetate supplementation. Meat Science, 52, 165-172.

Cavani, C., Bianchi, M. 2004. Carne. In: Chimica degli Alimenti (edited by P. Cabras, A. Martelli). Pp.: 355-379. Padova, Italy: Piccin.

Chizolini, R., Novelli, E., Zanardi, E. 1998. Oxidation in traditional Mediterranean meat products. Meat Science, 49 (suppl. 1), S87-S99

Daood H. G., Vinkler M., Márkus F., Hebshi E. A., Biacs P. A. 1996. Antioxidant vitamin content of spice red pepper (paprika) as affected by technological and varietal factors. Food Chemistry, 55, 365-372.

Demeyer, D., Hoozee, J., Mesdom, H. 1974. Specificity of lipolysis during dry sausage ripening. Journal of Food Science, 39, 293-296.

Fieser, L. F. and Fieser, M. 1967. Reagents for organic chemistry. Pp 191-192. New York: Wiley.

Folch, J., Lees, M., Sloane Stanley G. H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226, 497-509.

Fonatanillas R., Barroeta A., Baucells M. D., Guardiola F. 1998. Backfat acid evolution in swine fed diets high in either *cis*-monounsaturated, *trans* or (*n*-3) fats. Journal of Animal Science, 76, 1045-1055.

Ghiretti, G. P., Zanardi, E., Novelli, E., Campanili, G., Dazzi, G., Madarena, G., Chizzolini, R. 1997. Comparative evaluation of some antioxidants in salame Milano and mortadella production. Meat Science, 47, 167-176.

Hamilton, R. J. 1989. The Chemistry of Rancidity in Food. In: Rancidity in Foods – Second Edition (edited by J. C. Allen, R. J. Hamilton). Pp.: 1-22. London, New York: Elsevier Applied Sceince.

Hierro E., de la Hoz L., Ordóñez J.A. 1997. Contribution of microbial and meat endogenous enzymes to the lipolysis of dry fermented sausages. Journal of Agricultural and Food Chemistry, 45, 2989-2995.

Incze, K. 2004. Typres of Sausages – Dry and Semy-Dry In: Encyclopedia of Meat Science (edited by W. K. Jensen, C. Devine and M. Dikeman). Pp. 1207-1216. Amsterdam, Boston, Heidelberg, London, New York, Oxford, Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo: Elsevier Academic Press.

Lanari M. C., Schaefer D. M., Cassens R. G., Scheller K. K. 1995. Atmosphere and blooming time affect color and lipid stability of frozen beef from steers supplemented with vitamin E. Meat Science, 40, 33-44..

Madsen, H. L., Bertelsen, G. 1995. Spices as antioxidants.Trends in Food Science and Technology, 6, 271-277.

Martín L., Córdoba J. J., Ventanas J., Antequera T. 1999. Changes in intramuscular lipids during ripening of Iberian dry-cured ham. Meat Science, 51, 129-134..

Motilva M. J. Toldrá F., Nieto P., Flores J. 1993. Muscle lipolysis phenomena in the processing of dry-cured ham. Food Chemistry, 48, 121-125.

Motilva, M. J., Toldrá F., Flores, J. 1992. Assay of lipase and esterase activities in fresh pork meat and dry-cured ham. Zeitschrift für Lebensmittle Untersuchung und Forschung, 195, 446-450.

Nawar, W. W. 1996. Lipids. In: Food Chemistry, Third Edition (edited by O. R. Fennema). Pp.: 225-319. New York, Basel, Hong Kong: Marcel Dekker.

Nichols, D. S., Sanderson, K. 2003. The Nomenclature, Structure, and Properties of Food Lipids. In: Chemical and Functional Properties of Food Lipids (edited by Z. E. Sikorski and A. Kołakowska). Pp. 133-166. Boca Raton, London, New York, Washington, D.C.: CRC Press.

Novelli, E., Zanardi, E., Ghiretti, G. P., Campanini, G., Dazzi, G., Madarena, G., Chizzolini, R. 1998. Lipid and cholesterol oxidation in frozen stored pork, salame Milano and mortadella. Meat Science, 48, 29-40.

Official Journal of the European Communities 2002. Commission Regulation (EC) No 796/2002 of 6 May 2002 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis and the additional notes in the Annex to Council Regulation (EEC) No 2658/87 on the tariff and statistical nomenclature and on the Common Customs Tariff. L 128/8, Volume 45, Attachment X.B., 15 May 2002.

Palic, A., Krizanek, D., Dikanovic-Lucan, Z. 1993. The antioxidant properties of spices in dry fermented sausages. Fleischwirtschaft, 73, 670-672.

Parisi, E. 2001. Valutazione dello stato di ossidazione di prosciutti crudi e di insaccati non tradizionali. B.Sc. Thesis on Agricultural Sciences and Technologies. Università degli Studi di Bologna, Italy.

Pastorelli G., Magni S., Rossi R., Paglierini E., Baldini P., Dirinck P., Van Opstaele F., Corino C. 2003. Influence of dietary fat, on fatty acid composition and sensory properties of dry-cured Parma ham. Meat Science, 65, 571-580.

Petrón, M. J., García-Regueiro J. A., Martín L., Muriel, E., Antequera, T. 2003. Identification and quantification of cholesterol and cholesterol oxidation products in

different types of Iberian hams. Journal of Agricultural and Food Chemistry, 51, 5786-5791.

Revilla I., Vivar Quintana A. M. 2005. The effect of different paprika types on the ripening process and quality of dry sausages. International Journal of Food Science and Technology, 40, 411-417.

Robards, K., Kerr, A. F., Patsalides, E. 1988. Rancidity and its measurement in edible oils and snack foods. A review. Analyst, 113, 213-224.

Romans J. R., Johnson R. C., Wulf D. M., Libal G. W., Costello W.J. 1995. Effects of ground flaxseed in swine diet on pig performance and on physical and sensory characteristics and omega-3 fatty acid content of pork: I. Dietary level of flaxseed, Journal of Animal Science, 73, 1982-1986.

Shantha, N. C., Decker, E. A. 1994. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. Journal of AOAC International, 77, 421-424.

Sweeley, C. C., Bentley, R., Makita, M., Wells, W. W. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. Journal of the American Chemical Society, 85, 2497-2507.

Tarladgis, B. G., Watts, B. M., Younathan, M. T. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. Journal of the American Oil Chemists' Society, 37, 44-48.

Toldrá, F. 1998. Proteolysis and lipolysis in flavour development of dry-cured meat products. Meat Science, 49 (suppl. 1) S101-S110.

Vestergaard C. S., Parolari G. 1999. Lipid and cholesterol oxidation products in dry-cured ham, Meat Science, 52, 397-401.

Warnants N., Van Oeckel M. J., Boucqué C. V., 1999. Incorporation of dietary polyunsaturated fatty acids into pork fatty tissues. Journal of Animal Science, 77, 2478-2490.

Yang G. C., Yasaei P. M., Page S. W. 1993. Garlic as anti-oxidants and free radical scavengers. Journal of Food and Drug Analysis, 1, 357-364 (1993).

Zanardi E., Ghidini S., Battaglia A., Chizzolini R., Lipolysis and lipid oxidation in fermented sausages depending on different processing conditions and different antioxidants, Meat Science, 66, 415-423 (2004).

7.7. TABLES

Table 7.1. – Moisture, fat content, PV, TBARS, total FFA content, DG content and FFA-to-DG ratio of Italian and Spanish sausages and hams. All the results are expressed as mean ± SD (n = 3).

Sample	Moisture (%, w/w)	Fat content (%, w/w)	(%, w/w dry matter)	PV meq O_2/kg fat	TBARS mg MDA/kg meat	FFA g/100 g fat	g/100 g meat	DG g/100 g fat	g/100 g meat	DG/FFA
CHOR 1	21.4 ± 0.1^{D}	53.9 ± 2.3^{A}	68.6 ± 3.0^{A}	4.0 ± 0.2^{A}	1.1 ± 0.2^{A}	5.6 ± 1.0^{B}	3.0 ± 0.6^{B}	2.1 ± 0.2^{B}	1.1 ± 0.1^{B}	2.7 ± 0.7^{A}
CHOR 2	25.2 ± 0.3^{C}	55.9 ± 3.8^{A}	74.8 ± 5.1^{A}	4.2 ± 0.2^{A}	0.5 ± 0.1^{B}	5.4 ± 0.4^{B}	3.0 ± 0.3^{B}	1.9 ± 0.3^{B}	1.1 ± 0.1^{B}	2.9 ± 0.4^{A}
SAL	32.2 ± 0.0^{B}	38.8 ± 1.7^{B}	57.2 ± 2.6^{B}	3.1 ± 0.3^{B}	0.5 ± 0.1^{B}	11.9 ± 0.6^{B}	4.6 ± 0.2^{A}	3.9 ± 0.3^{A}	1.5 ± 0.1^{A}	3.1 ± 0.4^{A}
SOP	38.4 ± 0.0^{A}	31.5 ± 1.2^{C}	51.1 ± 2.0^{B}	3.8 ± 0.1^{A}	0.4 ± 0.1^{B}	5.4 ± 0.2^{A}	1.7 ± 0.1^{C}	1.7 ± 0.1^{B}	0.5 ± 0.1^{C}	3.2 ± 0.4^{A}
JAM-P	34.0 ± 1.2^{c}	32.9 ± 2.8^{a}	49.8 ± 4.2^{a}	2.4 ± 0.3^{b}	$0.4 \pm 0.0^{b,c}$	19.0 ± 2.2^{a}	6.2 ± 0.3^{a}	2.8 ± 0.3^{a}	0.9 ± 0.2^{a}	7.0 ± 1.5^{a}
JAM	37.7 ± 1.8^{d}	28.2 ± 1.2^{b}	$45.3 \pm 2.0^{a,b}$	8.6 ± 0.7^{b}	$0.5 \pm 0.2^{a,b}$	15.4 ± 1.3^{b}	4.3 ± 0.6^{b}	2.8 ± 0.7^{a}	0.8 ± 0.2^{a}	5.9 ± 2.3^{a}
PARMA	48.3 ± 0.1^{a}	21.4 ± 0.5^{c}	$41.3 \pm 0.9^{b,c}$	55.1 ± 7.7^{a}	0.3 ± 0.0^{c}	11.4 ± 0.4^{c}	2.4 ± 0.1^{c}	2.8 ± 0.1^{a}	0.6 ± 0.0^{a}	4.0 ± 0.3^{a}
NORCIA	45.3 ± 0.2^{b}	20.7 ± 0.5^{c}	37.8 ± 1.0^{c}	53.0 ± 7.8^{a}	0.7 ± 0.0^{a}	8.3 ± 1.0^{c}	1.7 ± 0.2^{c}	1.3 ± 0.1^{b}	0.3 ± 0.0^{b}	6.5 ± 1.1^{a}

Different superscript letters denote significant differences ($p < 0.05$) between samples of the same kind (capital letters refer to sausages).

Table 7.2. – Total fatty acids composition of the different samples. The relative amount of each fatty acid is expressed as percentage of total fatty acid methyl esters (FAME) identified. All the results are expressed as mean ± SD (n = 3).

Sample	C14:0 myristic	C16:0 palmitic	C16:1 palmitoleic	C 18:0 steraric	C 18:1*t* *trans* isomers	C18:1 n-9 oleic	C18:1 n-7 vaccenic	C18:2 n-6 linoleic	C18:3 n-3 α-linolenic	C20:1 eicosenoic	C20:2 eicosadienoic	C22:0 + C20:3 n-3 + C20:4 behenic + eicosatrienoic + arachidonic
CHOR 1	1.3 ± 0.0^{A}	24.0 ± 0.3^{A}	2.2 ± 0.0^{B}	12.8 ± 0.1^{A}	0.3 ± 0.0^{A}	39.9 ± 0.2^{A}	2.9 ± 0.0^{A}	12.6 ± 0.1^{A}	0.8 ± 0.0^{A}	0.8 ± 0.0^{A}	0.5 ± 0.0^{B}	0.5 ± 0.0^{B}
CHOR 2	1.4 ± 0.0^{A}	24.2 ± 0.1^{A}	2.1 ± 0.0^{C}	12.9 ± 0.0^{A}	0.2 ± 0.0^{C}	39.1 ± 0.1^{A}	2.8 ± 0.0^{B}	13.5 ± 0.0^{A}	0.7 ± 0.0^{A}	0.9 ± 0.0^{A}	0.6 ± 0.0^{A}	0.5 ± 0.0^{B}
SAL	1.3 ± 0.1^{A}	24.9 ± 1.1^{A}	2.3 ± 0.0^{A}	13.2 ± 0.7^{A}	$0.3 \pm 0.0^{A,B}$	39.8 ± 0.9^{A}	3.0 ± 0.1^{A}	11.3 ± 0.7^{B}	0.6 ± 0.1^{B}	0.9 ± 0.0^{A}	0.5 ± 0.0^{C}	$0.6 \pm 0.1^{A,B}$
SOP	1.3 ± 0.0^{A}	24.0 ± 0.1^{A}	2.3 ± 0.0^{A}	12.9 ± 0.0^{A}	0.3 ± 0.0^{B}	39.1 ± 0.0^{A}	3.0 ± 0.0^{A}	12.9 ± 0.0^{A}	0.8 ± 0.0^{A}	0.9 ± 0.0^{A}	0.6 ± 0.0^{A}	0.7 ± 0.0^{A}
JAM-P	1.2 ± 0.1^{c}	23.7 ± 0.1^{b}	2.0 ± 0.0^{c}	13.6 ± 0.0^{a}	0.3 ± 0.0^{b}	42.6 ± 0.2^{a}	2.9 ± 0.0^{c}	10.0 ± 0.0^{b}	0.6 ± 0.0^{c}	0.9 ± 0.0^{a}	0.5 ± 0.0^{a}	0.6 ± 0.0^{d}
JAM	$1.3 \pm 0.0^{b,c}$	24.2 ± 0.2^{a}	2.5 ± 0.0^{b}	12.0 ± 0.0^{c}	0.3 ± 0.0^{a}	40.8 ± 0.1^{b}	3.2 ± 0.0^{b}	11.7 ± 0.1^{a}	0.7 ± 0.0^{b}	0.8 ± 0.0^{b}	0.5 ± 0.0^{a}	0.7 ± 0.0^{c}
PARMA	1.5 ± 0.0^{a}	$24.0 \pm 0.0^{a,b}$	2.5 ± 0.0^{b}	12.5 ± 0.0^{b}	0.2 ± 0.0^{b}	42.7 ± 0.1^{a}	3.6 ± 0.0^{a}	9.1 ± 0.0^{c}	0.5 ± 0.0^{d}	0.9 ± 0.0^{a}	0.4 ± 0.0^{b}	0.8 ± 0.0^{b}
NORCIA	1.3 ± 0.1^{b}	23.6 ± 0.3^{b}	2.8 ± 0.0^{a}	11.3 ± 0.1^{d}	0.3 ± 0.0^{a}	40.8 ± 0.2^{b}	3.6 ± 0.0^{a}	11.7 ± 0.1^{a}	0.9 ± 0.0^{a}	0.8 ± 0.0^{b}	0.5 ± 0.0^{a}	1.0 ± 0.0^{a}

Different superscript letters denote significant differences ($p < 0.05$) between samples of the same kind (capital letters refer to sausages).

Table 7.3. – Relative amounts, expressed as percentage of total FAME identified, of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in different samples. All the results are expressed as mean ± SD (n = 3).

Sample	SFA	MUFA	PUFA	U/S
CHOR 1	39.0 ± 0.4^{A}	46.2 ± 0.2^{A}	14.3 ± 0.2^{A}	1.5 ± 0.0^{A}
CHOR 2	39.2 ± 0.1^{A}	45.2 ± 0.1^{A}	15.1 ± 0.0^{A}	1.5 ± 0.0^{A}
SAL	40.3 ± 1.9^{A}	46.3 ± 1.0^{A}	12.9 ± 0.8^{B}	1.5 ± 0.1^{A}
SOP	39.1 ± 0.1^{A}	45.5 ± 0.0^{A}	14.7 ± 0.0^{A}	1.5 ± 0.0^{A}
JAM-P	39.1 ± 0.2^{a}	48.7 ± 0.2^{b}	11.6 ± 0.0^{c}	1.5 ± 0.0^{c}
JAM	38.4 ± 0.2^{b}	47.6 ± 0.1^{d}	13.4 ± 0.1^{b}	1.6 ± 0.0^{b}
PARMA	$38.7 \pm 0.1^{a,b}$	50.0 ± 0.1^{a}	10.5 ± 0.0^{d}	$1.6 \pm 0.0^{b,c}$
NORCIA	37.1 ± 0.3^{c}	48.3 ± 0.2^{c}	13.6 ± 0.1^{a}	1.7 ± 0.0^{a}

U/S: unsaturated-to-saturated fatty acids ratio.

Different superscript letters denote significant differences ($p < 0.05$) between samples of the same kind (capital letters refer to sausages).

Table 7.4. – Amount, expressed as g/100 g of fat, of the main FFA and relative composition of FFA fraction.

Sample	C16:0 palmitic	C 18:0 steraric	C18:1 n-9 oleic	C18:2 n-6 linoleic	% SFA	% MUFA	% PUFA	U/S
CHOR 1	0.8 ± 0.2^{B}	0.4 ± 0.1^{B}	2.1 ± 0.4^{B}	1.4 ± 0.2^{B}	24.5 ± 0.3^{B}	44.4 ± 0.1^{B}	29.0 ± 0.2^{B}	3.0 ± 0.0^{A}
CHOR 2	0.8 ± 0.1^{B}	0.4 ± 0.0^{B}	1.9 ± 0.1^{B}	1.4 ± 0.1^{B}	24.7 ± 0.6^{B}	42.9 ± 0.0^{C}	30.2 ± 0.5^{A}	3.0 ± 0.1^{A}
SAL	1.9 ± 0.1^{A}	0.8 ± 0.0^{A}	5.3 ± 0.3^{A}	2.2 ± 0.1^{A}	23.9 ± 0.2^{B}	52.3 ± 0.4^{A}	21.8 ± 0.2^{D}	3.1 ± 0.0^{A}
SOP	0.9 ± 0.0^{A}	0.4 ± 0.0^{B}	1.9 ± 0.1^{B}	1.3 ± 0.1^{B}	26.8 ± 0.5^{A}	42.8 ± 0.1^{C}	27.5 ± 0.4^{C}	2.6 ± 0.1^{B}
JAM-P	3.8 ± 0.4^{a}	1.7 ± 0.2^{a}	7.7 ± 0.9^{a}	3.1 ± 0.4^{a}	30.3 ± 0.2^{a}	48.3 ± 0.2^{a}	19.4 ± 0.0^{d}	2.2 ± 0.0^{b}
JAM	3.1 ± 0.3^{a}	1.2 ± 0.1^{b}	5.5 ± 0.5^{b}	3.0 ± 0.3^{a}	30.5 ± 0.3^{a}	44.2 ± 0.2^{c}	22.9 ± 0.1^{b}	2.2 ± 0.0^{b}
PARMA	2.1 ± 0.1^{b}	0.9 ± 0.0^{c}	4.3 ± 0.1^{b}	2.0 ± 0.1^{b}	28.4 ± 0.6^{b}	46.7 ± 0.5^{b}	20.7 ± 0.2^{c}	2.4 ± 0.1^{a}
NORCIA	1.5 ± 0.2^{b}	0.8 ± 0.1^{c}	2.6 ± 0.3^{c}	1.8 ± 0.2^{b}	30.1 ± 0.3^{a}	38.4 ± 0.3^{d}	26.0 ± 0.2^{a}	2.1 ± 0.0^{b}

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; U/S: unsaturated-to-saturated fatty acids ratio.

Different superscript letters denote significant differences ($p < 0.05$) between samples of the same kind (capital letters refer to sausages).

7.8. LEGENDS TO FIGURES

Figure 7.1. – Gas chromatographic traces of FAME obtained, after a trans-methylation on fat, from samples JAM-P (left trace) and SAL (right trace). Peaks identification: IS (internal standard): C13:0; 1, C14:0; 2, C16:0; 3, C16:1; 4, C18:0; 5, C18:1; 6, C18:1 n-7; 7, C18:2; 8, C18:3; 9, C20:1; 10, C20:2; 11, C20:3 n-3 + C 20:4 + C 22:0. For the analytical conditions, see section Materials and methods.

Figure 7.2. – Gas chromatographic trace of FFA, after SPE performed on fat and methylation with diazomethane, from sample JAM-P (left trace) and NOR (right trace). Peaks identification: IS (internal standard): C13:0; 1, C14:0; 2, C16:0; 3, C16:1; 4, C17:0; 5, C18:0; 6, C18:1; 7; C18:1 n-7; 8, C18:2; 9, C18:3 n-3; 10, C20:1, 11, C20:3 n-3 + C20:4. For the analytical conditions, see section Materials and methods.

Figure 7.3. – Gas chromatographic traces of TMS derivatives of DG collected after SPE from samples JAM-P (left trace) and NOR (right trace). Peaks identification: IS (internal standard), squalane; Ch, free cholesterol; D32, C14-C18 and C16-C16 diglcerices; D34, C16-C18 diglycerides; D36, C18-C18 diglycerides. Peaks beside internal standard were methyl esters of FFA. For the analytical conditions, see section Materials and methods.

7.9. FIGURES

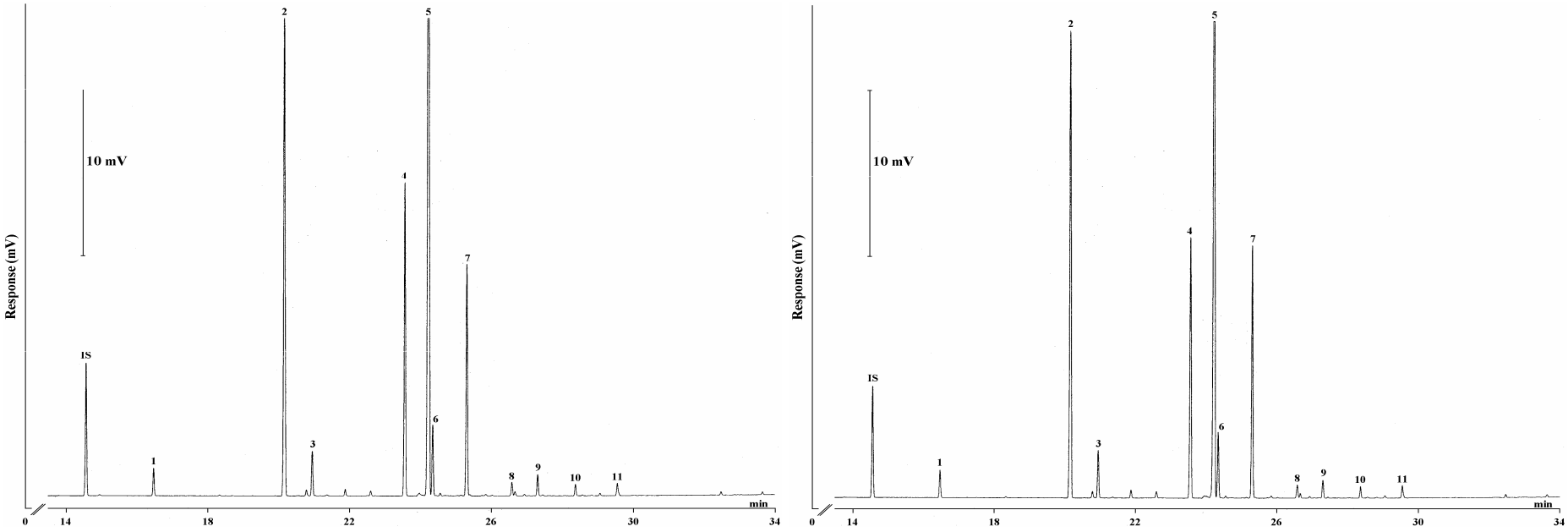


Fig. 7.1.

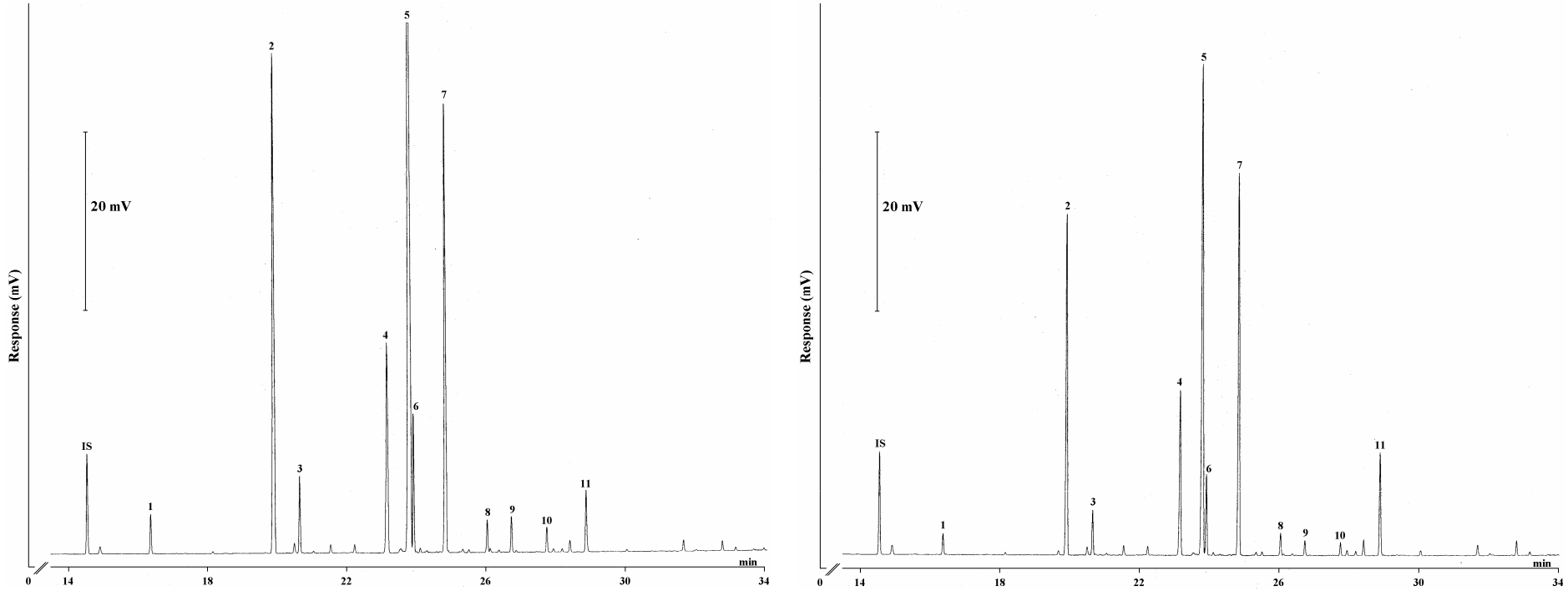


Fig. 7.2.

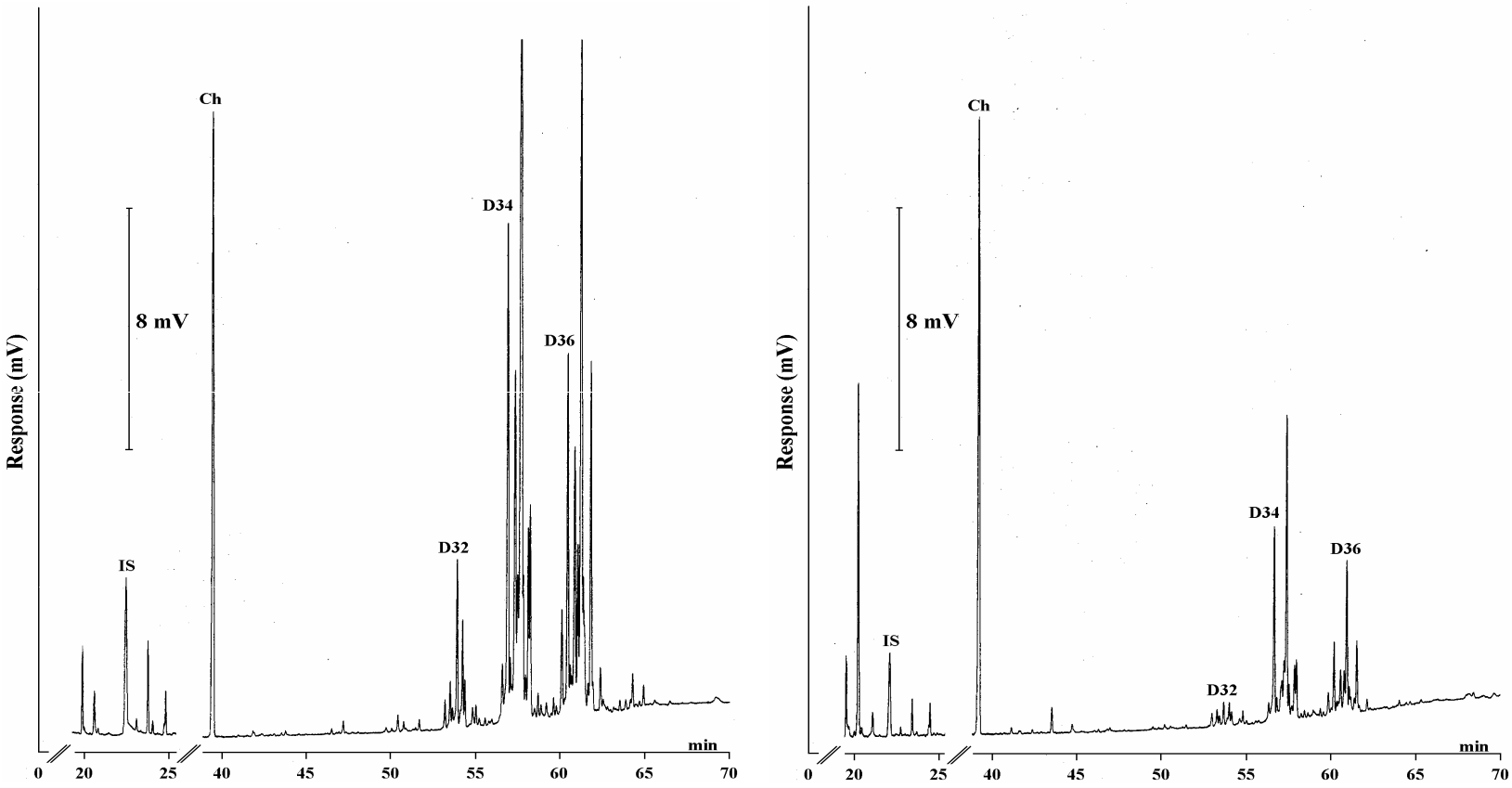

Response (mV)
8 mV
IS
Ch
D32
D34
D36
min
0
20
25
40
45
50
55
60
65
70
Response (mV)
8 mV
IS
Ch
D32
D34
D36
min
0
20
25
40
45
50
55
60
65
70

Fig. 7.3.