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TITOLO TESI

Quality and safety of oils and fats obtained as co-products or by-products of the food chain and destined to animal production

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1. AIM AND DESCRIPTION OF THE PROJET

The traceability in the agro-food chain has become very important, because it must guarantee the consumers' safety and prevent risks from the public health standpoint. A particular case is the meat production chain, where the quality of feeding strongly influences the quality and safety of the final food product. The feeding is often formulated with fats obtained from waste or by-products of the food chain, which can accumulate pollutants and/or degradation substances that directly affect the profile of the animal food products that reach the consumer (Woodgate *et al.*, 2004). The accumulative and persistent undesirable contaminants (such as PCBs, dioxins and dioxin-like compounds) undergo a biomagnification effect, increasing their concentration all along the food chain. These compounds are very liposoluble, poorly degradable and relatively volatile, so animal tissues tend to accumulate them. The main cause of exposure to man of these toxic compounds is the food chain (>95%), whereas 60-80% comes from feeds or plants ingested by livestock (in the form of food of animal origin) and 20-40 % from processed foods.

Other liposoluble compounds that accumulate in food are polycyclic aromatic hydrocarbons (PAHs), which may constitute a significant public health problem. Although their presence is partly due to environmental pollution and plant deposition, the main source of PAHs is food processing. In fact, cereals and oils are the most contaminated food sources, which should encourage studies on the PAHs content in cooking oils and other residual fat materials coming from food industrial activities, where they may form during heating at high temperatures or partially disappear by volatilization. However, PAHs are considered as inevitable contaminants (difficult to eliminate), so the practical management for limiting their presence in those oils and fats must be very different from the limitation of PCBs and dioxins, whose environmental presence can be decreased by regulating different practices or industrial activities.

Fats and oils can also degrade during frying, cooking and industrial processes, generating some toxic compounds such as triacylglycerol dimers, trimers and other polymers, whose presence may reach up to 30% of total oil in re-utilized cooking oils. Some technological processes, such as

fusion, refining and hydrogenation, can increase the content of *trans* fatty acids (TFA) isomers, whose negatives effects on the health are well-known (British Nutrition Foundation, 1995; Kris-Etherton, 1995). On the other hand, many conjugated linoleic acid (CLA) isomers also present the *trans* configuration, but they apparently display an antitumoral activity (Yurawecz *et al.*, 1999). Many studies have demonstrated the effect of the feeding fat quality on the deposit and accumulation of TFA and CLA isomers in meats (Sebedio *et al.*, 1996). Because of a lack of precise information on various aspects of feeding fats obtained from co- and by-products of the food chain, it would be of outmost importance to focus the attention on the following points: a) the rigorous characterization of the composition and the quality of the feeding fats; b) the description of nutritional aspects of interest for various animal production systems; c) the effects on the meat lipid composition; d) the effects on lipid oxidation of meat and other animal tissues; e) the toxicological and physiological effects of some degradation products and undesirable pollutant agents.

The objective of the first part of this study is, therefore, to evaluate the safety of meat production based on recycling of co- and by-products of the food chain. This meat production system should also warrant a low transferring rate of noxious environmental contaminants from feeding fats into meat, so as to improve its nutritional and sensory quality. In particular, the *trans* fatty acid (TFA) and conjugated linoleic acid (CLA) isomers will be assessed in: feeding fats; enriched feeds with different levels of TFA, contaminants (PBDEs, dioxins and dioxin–like PCBs), PAHs and oxidation products; poultry and rabbit tissues (meat and liver) obtained with the enriched feeds. This study is part of a large, collaborative European project (http://www.ub.edu/feedfat/), where other chemical and health parameters will be assessed.

The second part of the thesis will deal with the study of lipid oxidation in washed turkey muscle added with different antioxidants. Oxidative stability of muscle foods will be monitored by using methods that allow to determine fixed and volatile secondary oxidation products. Headspace solid-

phase microestraction (SPME) and thiobarbituric acid reactive substances (TBARs) method will be used for this scope.

Key words: Feeding fats, co-products, by-products, *trans* fatty acids, CLA, meat, liver, poultry, rabbit.

2. INTRODUCTION

The traceability in the agro-food chain has become very important, because it must guarantee the consumers' safety and prevent risks from the public health standpoint. A particular case is the meat production chain, where the quality of feeding strongly influences the quality and safety of the final food product. The feeding is often formulated with fats obtained from waste or by-products of the food chain, which can accumulate pollutants and/or degradation substances that directly affect the profile of the animal food products that reach the consumer (Woodgate *et al.*, 2004). The accumulative and persistent undesirable contaminants (such as PCBs, dioxins and dioxin-like compounds) undergo a biomagnification effect, increasing their concentration all along the food chain. These compounds are very liposoluble, poorly degradable and relatively volatile, so animal tissues tend to accumulate them. The main cause of exposure to man of these toxic compounds is the food chain (>95%), whereas 60-80% comes from feeds or plants ingested by livestock (in the form of food of animal origin) and 20-40 % from processed foods. (http://www.ub.edu/feedfat/)

Other liposoluble compounds that accumulate in food are polycyclic aromatic hydrocarbons (PAHs), which may constitute a significant public health problem. Although their presence is partly due to environmental pollution and plant deposition, the main source of PAHs is food processing. In fact, cereals and oils are the most contaminated food sources, which should encourage studies on the PAHs content in cooking oils and other residual fat materials coming from food industry activities, where they may form during heating at high temperatures or partially disappear by volatilization. However, PAHs are considered as inevitable contaminants (difficult to eliminate), so the practical management for limiting their presence in those oils and fats must be very different due to the limitation of PCBs and dioxins, whose environmental presence can be decreased by regulating different practices or industrial activities.

Fats and oils can also degrade during frying, cooking and industrial processes, generating some toxic compounds such as triacylglycerols dimers, trimers and other polymers, whose presence may

reach up to 30% of total oil in re-utilized cooking oils. Some technological processes, such as fusion, refining and hydrogenation, can increase the content of *trans* fatty acids (TFA) isomers, whose negative effects on the health are well-known (British Nutrition Foundation, 1995; Kris-Etherton, 1995). On the other hand, many conjugated linoleic acid (CLA) isomers also present the *trans* configuration, but they apparently display an antitumoral activity (Yurawecz *et al.*, 1999). Many studies have demonstrated the effect of the feeding fat quality on the deposit and accumulation of TFA and CLA isomers in meats (Sebedio *et al.*, 1996). Because of a lack of precise information on various aspects of feeding fats obtained from co- and by-products of the food chain, it would be of outmost importance to focus the attention on the following points: a) the rigorous characterization of the composition and the quality of the feeding fats; b) the description of nutritional aspects of interest for various animal production systems; c) the effects on the meat lipid composition; d) the effects on lipid oxidation of meat and other animal tissues; e) the toxicological and physiological effects of some degradation products and undesirable pollutant agents.

2.1 FEEDING FAT COMPOSITION AND QUALITY

The problem of the classification of fats for feeding purposes (sampling procedure, sample registration, classification and distribution for evaluation) is a real problem for the European scenario and sometimes represents a barrier for the use of by- and co-products of food industry in animal nutrition. At the same time, this lack of rules and definitions represents a barrier for these companies that need to place in the market high tech products with special properties and composition for particular uses. If these products, which provide an added-value and benefits to the farmers, to the animal wellness and finally to the consumers, are evaluated according to classical rules, they can be sometimes evaluated as out the law, with all problems connected to this definition. For instance, many times in the past mixtures of mono- and diglycerides, widely used in animal and human nutrition, perfectly safe and with high nutritional value, have been considered out of law if assessed as common fats and analyzed for polar content like frying oil. Better

definitions and classification may also mean better control rules and the reduction of *twilight zones,* where everything is allowed:

a) Fat materials obtained from by-products and co-products of the food chain are interesting feedings stuffs for feed producers, but the origin and quality of these fats are quite variable and often not well regulated and controlled (lack of strict quality characterization of these feed fats).

b) Process of producing some of these fat materials (animal fats) are recently more strictly regulated (*Regulation 1774/2002*). However, there is a lack of standardization for the rest.

c) Fat blends as a feeding stuff are not well studied. Nevertheless, they could be an interesting way to balance quality characteristics of fats added to feed.

d) There is usually a high emphasis in the productive aspects, but a lower emphasis in the improvement of the nutritional value of meat fat, related to feed fat formulation.

2.2 PRESENCE OF CONTAMINANT IN FEEDING FATS AND MEAT PRODUCTS

Foods of animal origin contribute 80% to the total dioxin human exposure (*Recomendation 2002/201/CE*), and feedingstuffs are the first contributors (90%) to meat content of contaminants. Fish oils and fish meals are the most heavily dioxin contaminated feedingstuffs, and animal fats are the next in order of dioxin contamination (*Opinion Sci. Comm. Animal Nutrition on dioxin contamination of feedingstuffs and their contribution to the contamination of food of animal origin 06-11-2000*). In contrast, available data on contents of dioxin-like PCBs in feedingstuffs are limited. Dietary intake of PAHs is estimated only in an approximate level, because of the lack of reliable data. Provisional data from EU countries show a level of intake of benzo-a-pyrene from 0.05 to 0.29 µg/day, for adults. The major contributors to PAHs human intake were the oils and fats group (50% for benzo-a-pyrene, 34% for a sum of 11 another PAH), followed by cereals (about 30%).

Fats of animal origin show higher contaminants values than vegetable and great attention should be paid to establish maximum levels and their control in fat blends (*Regulation EC 199/2006*).

It is necessary to generate more data about presence of these compounds in feedingstuffs (*Recomendation 2002/201/CE*).

It is strongly recommended to include dioxin-like PCB in all future studies on food contaminants, because they seem to greatly contribute to the total TEQ intake (*Report on tasks for scientific cooperation: Assessment of dietary intake of dioxins and related PCBs by the population of EU member states 07-06-2000).*

Transfer of dioxins and another contaminants from feed to animal tissues are scarcely known. Transfer factors should be established for each contaminant (*Opinion Sci. Comm. Animal Nutrition on dioxin contamination of feedingstuffs and their contribution to the contamination of food of animal origin 06-11-2000*). Transfer rate depends on bioavailability, which is related to the different level of retention of contaminants by different type of feedingstuffs. Transfer rate also depends on the different ability to be bioaccumulated, depending on the congener (level of chlorine substitution). PCBs as a meat contaminants have not been studied in depth yet.

PAHs are not still included in the list of undesirable substances in feedingstuffs and maximum levels have not been established, but they are in the list of substances to be evaluated (*Opinion of Sci. Comm. On Animal Nutrition on undesirable substances in feed 25-04-2003*).

Levels of dioxins detected in feeding fats are indicated in the (*Regulation EC 199/2006*).

Analytical means for contaminant determinations should be developed according to the aim of the analysis carried out (*Opinion Sci. Comm. Animal Nutrition on dioxin contamination of feedingstuffs and their contribution to the contamination of food of animal origin 06-11-2000*).

2.2.1 Effects of contaminants on animal health

Poultry feeds include between 2 and 10% of added fat as ingredient, whereas those destined to rabbits contain between 1 and 5%. Expected impact of dioxins on animal health should be very low in birds, mammals and fishes, according to usual levels of environmental contamination (*Opinion*

Sci. Comm. Animal Nutrition on dioxin contamination of feedingstuffs and their contribution to the contamination of food of animal origin 06-11-2000).

Toxicological effects reported for dioxins and PCB, a part from carcinogenicity, are: hepatotoxicity, inmunotoxicity, neurotoxicity, dermal toxicity, teratogenicity and embryotoxicity, and enzyme induction. In poultry and rabbit, dioxins and dioxin-like compounds show loss of body weight as a dose-dependent effect. Liver and thymus atrofia are also characteristic. Poultry shows a particular toxicological response to dioxins, the "chick oedema desease" (neck subcutaneous edema, ataxia, ascites, ...). In young chicken, gasping and waddling gait are characteristic toxic symptoms.

Toxicological effects of PAHs are mainly related with carcinoma of upper digestive tract. Other effects observed are hepatic and nephrotoxicity, myelotoxicity, haemolymphatic alterations, inmunotoxicity, mutagenicity and reproduction toxicity (depending on the compound). Some PAHs, like naphthalene, show cataracts as the most characteristic toxic effect on the rabbit.

Toxicological data related to PBDEs are scarce and this does not allow to establish toxicological properties of those contaminants in feed (*Opinion of Sci. Comm. On Animal Nutrition on undesirable substances in feed 25-04-2003*).

2.3 FEEDSTOCKS

(*Reference*: UE-STREP FOOD-CT-2004-07020, Feeding Fats Safety, Deliverable 1 http://www.ub.edu/feedfat/)

2.3.1 Acid oils from chemical refining (AOCHE)

The Acid Oils from Chemical Refining (AOCHE) are a by product of oils and fats refining industry, generated by the removal of the last traces of phospholipids, impurities and free fatty acids from a fatty feedstock, carried out according to the chemical process. Also a variable amount of oil is contained, depending on the efficiency of separation process in use. The following scheme will help the reader to better understand this production technology and the problems related with it. Crude oils containing important amounts of phospholipids are generally pretreated near the crushing facility, in order to remove the main fraction of phospholipids that could cause a sludge settling

during storage and transportation. The complete description of degumming unit operation is reported in the production of lecithin samples (2.3.3).

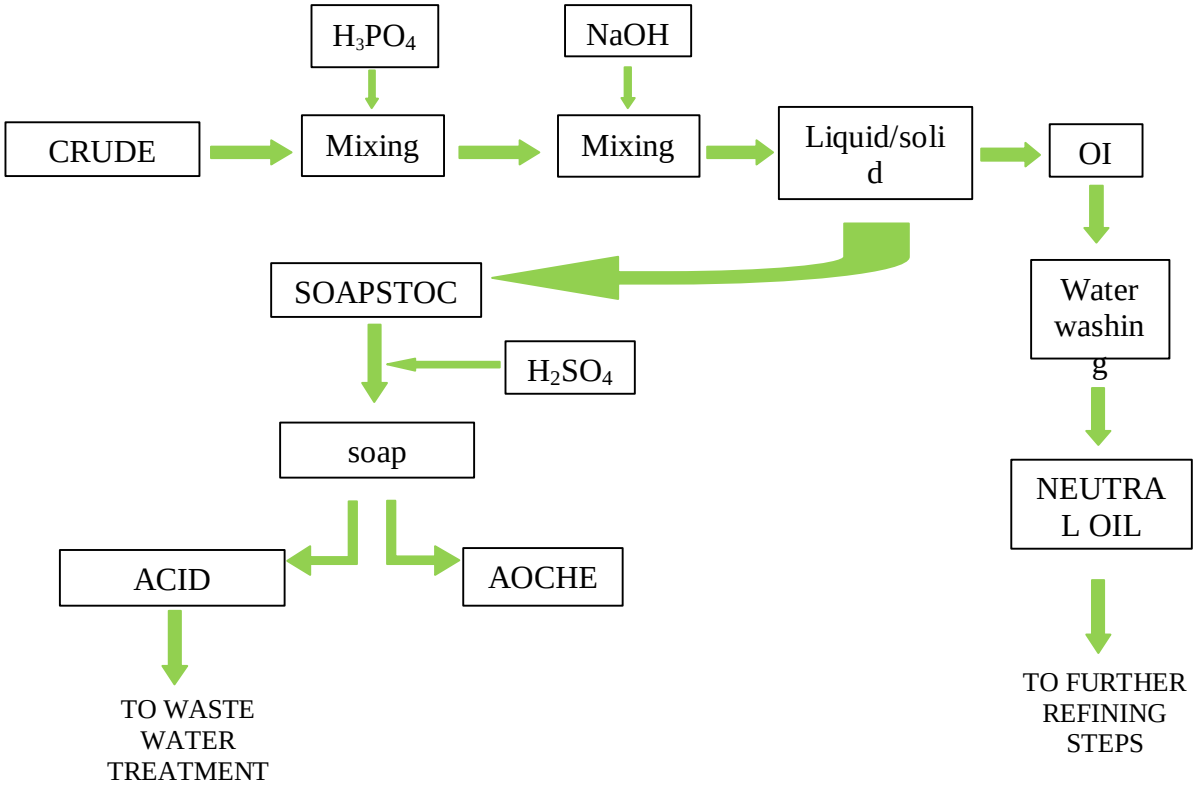

Fig.1 Production of lecithin samples

The crude oil entering into the refining unit is preheated at 60-80°C before the addition of phosphoric acid, having the scope to make easier the removal of last phospholipid traces (from 100 mg/kg down to 10-15 mg/kg, as P) and after a suitable hydration time, an aqueous NaOH solution is added in an amount calculated for the complete neutralization of free fatty acids plus the phosphoric acid previously added.

After a suitable contact time, the obtained mixture is separated to get neutral oil (delivered to a washing unit before continuing its refining trail) and a sludge generally defined as “soapstock” (a semi-solid mixture of soaps, neutral oil, phospholipids and water), which is immediately transferred to an acidic treatment device where, by reacting with sulphuric acid, soaps are splitted and free fatty acids restored. The effect of the acidic treatment is the formation of a liquid/liquid bilayer system, consisting of AOCHE (upper phase) and acid water (lower phase). The quantity of AOCHE

generated from 100 ton of crude oil during the chemical refining process strictly depends on free fatty acid (FFA) content of feedstock, according to the following equation:

$$AOCHE\ (ton) = K \times FFA$$

where K is the refining factor that depends on the FFA content of crude oil (the higher FFA, the higher K) and the separation efficiency of the settling/centrifugation units. Tipical K values range between 1.4 and 2.0. Crude oils with a FFA content higher than 6-8% cannot be processed in this way, because of the high refining losses.

Some factories follow the bad habit to mix the deodistillates (a very small amount, highly contaminated stream coming from the deodorization operation) with AOCHE, with the potential risk of contaminating the feedstock with volatile PAHs and dioxins. This operation should be forbidden if AOCHE are employed in feed formulation

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2.3.2 Acid oils from physical refining (AOPHY)

The Acid Oils from Physical Refining (AOPHY) are a by-product of oils and fats refining industry, generated by the removal of FFA from a fatty feedstock, carried out according to a physical process. A variable amount of natural minor compounds, such as terpenic hydrocarbons, tocopherols, sterols, fatty acid methyl and ethyl esters, are also present in AOPHY. Depending on the efficiency of the separation process, some traces of neutral oil can be recovered. The following scheme will help the reader to better understand this production technology and the problems related with it. Crude oils from the crushing facility are acid treated to remove the remaining fraction of phospholipids, then treated with bleaching earths for color removal before the distillation/neutralization treatment.

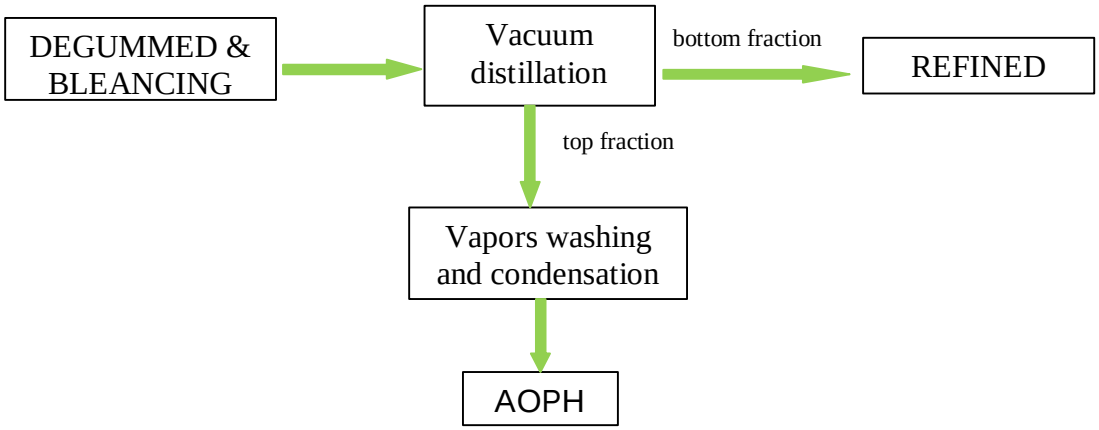

Fig 2. Production of aophy samples

The removal of FFA by distillation is generally carried out at high temperature (200-240 °C) under a low pressure (1-2 mbar of residual pressure) in steam flow and it represents the technical evolution of refining process, allowing savings in energy, water and refining losses.

The quantity of AOPHY usually generated from 100 ton of crude oil during the chemical refining process strictly depends (as in the case of AOCHE) on FFA content of feedstock, according to the following equation:

$$\text{AOPHY (ton)} = K \times \text{FFA}$$

where K is the refining factor depending on the FFA content of crude oil (the higher FFA, the higher K) and the separation efficiency of the settling/centrifugation units. Typical K values range between 1.1 and 1.5. Using the physical refining process, very acidic oils can be also processed with reasonable losses. During the physical neutralization step, the oil deodorization also takes place and traces of contaminants and neo-formed products can be detected in AOPHY samples. For this reason, if the use of AOPHY for feeding purposes can be foreseen, a rigorous selection of incoming materials in terms of environmental contaminants is necessary, in order to avoid the concentration of aforementioned contaminants in the by-product.

A special case is reppresented by AOPHY coming from olive oil refining, where high concentrations (up to 20%) of endogenous terpenic hydrocarbon *squalene* (2,6,10,15,19,23-

hexamethyl-2,6,10,14,18,22 tetracosahexaene, C30H50), along with important amounts of natural methyl and ethyl fatty acid esters can be detected. These ingredients must be taken into account during the feed formulation due to their nutritional added-value.

The by-product generally recognized as lecithin is a mixture of polar lipids (such as phosphatidylcoline, phosphatidylethanolamine, phosphatidyltrimethylethanolamine, phosphatidylinositol) with neutral oil. The common commercial product is a 65:35 mixture of polar and neutral lipids, respectively. LECI are obtained by means of water or steam degumming of lecithin-rich vegetable oils, such as rapeseed oil, sunflower oil, corn oil, soybean oil. Degumming is carried out immediately after the extraction process (screw pressing or solvent extraction). The crude oils from solvent extraction are generally richer in lecithin. The principle of degumming unit operation lays on the different solubility of polar lipids in oil, as related to their hydration degree. In few words, when the crude oil is anhydrous, the polar lipid are dissolved/dispersed in it. On the contrary, when water or steam is added to the oil mass, the polar lipids tend to absorb water and to become oil insoluble. A semi-solid precipitate is formed into the hydration vessel and it can be easily separated by settling or centrifugation. The separated by-product contains water that must be removed, in order to get a stable and technologically useful product. A product containing moisture is not microbiologically stable. The water removal is generally carried out in thin-layer, scraped surface evaporators.

The following scheme will help the reader to better understand this production technology and the problems related with it.

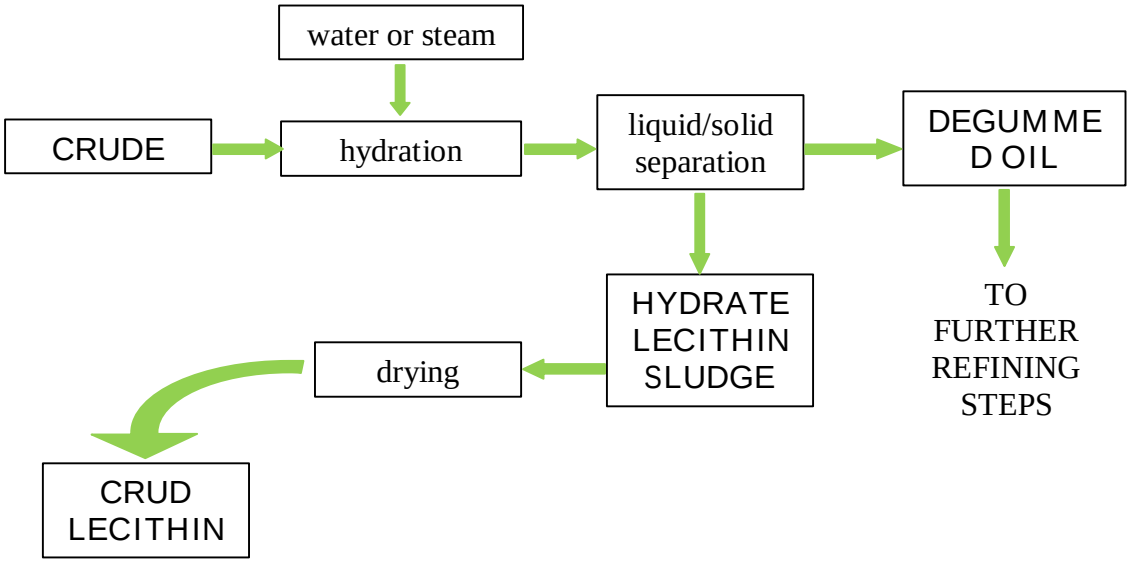

Fig.3. Production of lecithin samples

The yield in LECI during a water/steam degumming unit operation is a function of the original phospholipid content of crude oil. The phospholipid content of an oil is indirectly evaluated by means of phosphorous (P) content. The commonly used factor to evaluate phospholipids from P content is 31. The yield of LECI from 100 ton of crude oil can be roughly evaluated as follows:

$$\text{LECI (ton)} = P(\text{mg/kg}) \times 31/6500$$

LECI is a co-product of processing of numerous conventional oilseeds. LECI cannot be generated from fruit (such as olive, palm, avocado, etc) or animal fats processing.

For the LECI co-product, some problems related to GMO issues may arise. In fact, it is well known that soybean, corn or rapeseed from extra European origin can be of GMO origin. Nevertheless, it was demonstrated that the actually available techniques can detect the presence of GMO lecithin in LECI feedstocks.

2.3.4 Recycled cooking oils (RECY)

The recycled cooking oils (RECY) are a waste product coming from the food chain, generated from deep frying processes. These oils are generally collected from food industries, catering facilities and private houses by authorized actors and concentrated in authorized processing units. This system allows environmental protection from uncontrolled spill in waste waters and sometimes allows energy or chemicals recovery from a discarded product. The use of RECY oils for feed formulation is actually restricted in Europe, in according to the Commission Decision 2003/320/EC

The processing technology after collection is a very simple procedure and it includes the following steps:

a) filtration to remove insoluble impurities (such as part of food and other extraneous bodies);

b) a water washing to remove the water soluble salts and carbohydrates;

c) a final drying to remove the residual moisture in order to make the obtained product more stable towards hydrolysis and microbiological damage.

Additional treatments of RECY oils can be represented by a dry fractionation to obtain a liquid and a solid fraction with different iodine values and different use possibilities, as well as a partial chemical or physical neutralization to remove the main portion of FFA content of feedstock. As stated before, the main part of RECY oil does not come back to the food chain but it is used for technical or energetic applications.

The following scheme will help the reader to better understand this production technology and the problems related with it.

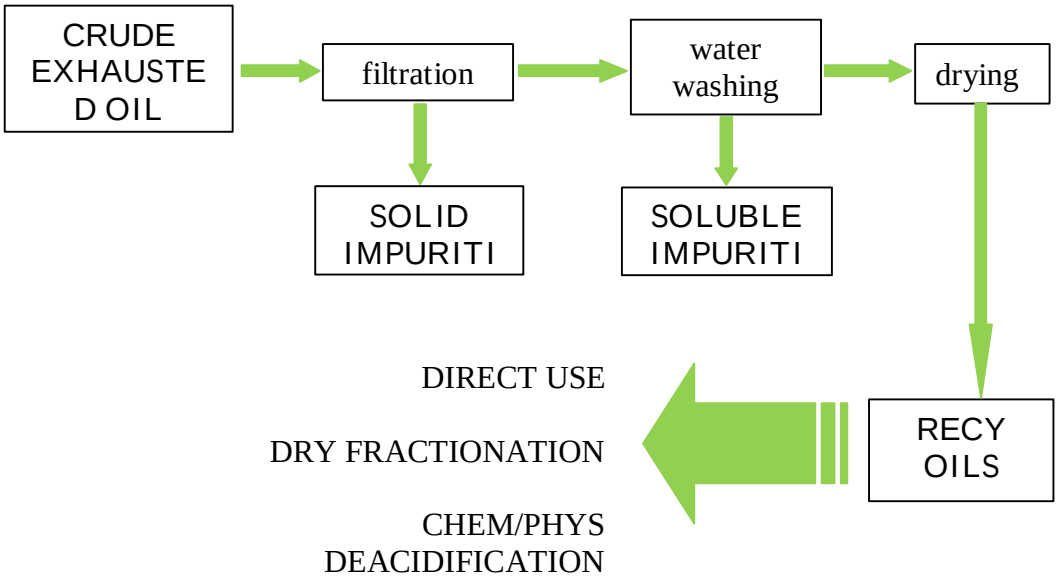

Fig.4 Production of recy samples

2.3.5 Animal fats (ANFA)

The animal fats (ANFA) are the by-product of the meat industry and their production, trade and utilization are actually under the EC regulation 1774/2002. Only ANFA belonging to category 3 (includes materials from animals "slaughtered fit for human consumption"). These materials, when processed to proscribed standards, can be used in a range of applications – including animal feed (Woodgate *et al.*, 2004). The same EC regulation establishes the minimum processing requirements to get a safe product for feed purposes.

The process of fat recovery from animal tissue is called rendering. The starting material is collected from the meat industries and from butcheries and transferred to the transformation plant where, after a vigorous milling to obtain an important size reduction (less than 5 cm as max dimension), they are thermally treated in a autoclave apparatus. The heat treatment (both dry or humid) allows protein denaturization, sterilization of the total mass and fat melting.

After this treatment, the resulting liquid liquor is sent to a separation unit for the production of a fatty phase and an aqueous phase. The residual solid product is treated in a screw press, where fat is recovered, centrifuged and pooled with the stream coming from the treatment of liquid effluent

described above. The solid residue is sent to a dryer and gives rise to the animal meal, which is only allowed for energy production. The following scheme will help the reader to better understand this production technology and the problems related with it.

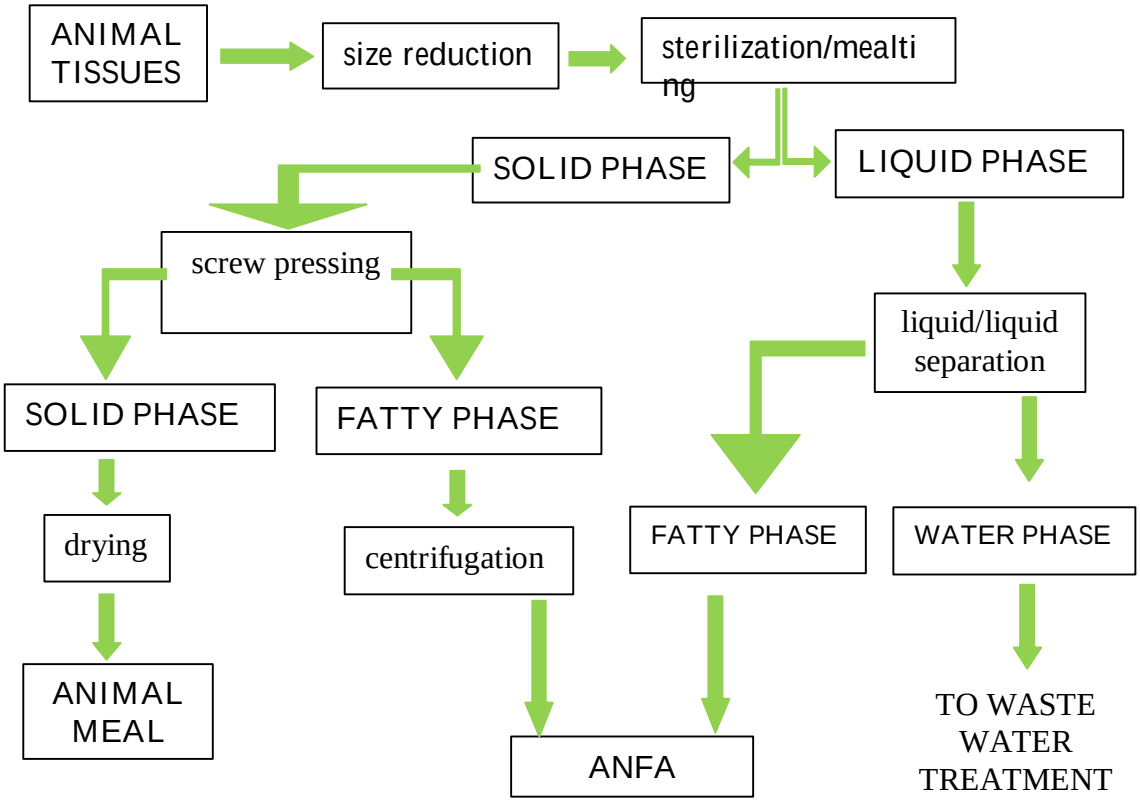

Fig. 5 Production of anfa samples

2.3.6 Oils extracted from exhausted bleaching earths (EBE)

The exhausted bleaching earths are a by-product of oils and fats refining industry and they are generated in an amount ranging between 0.2 to 2% of the processed oil during bleaching.

During this step, the oil is mixed with the bleaching earth at a temperature between 60 and 100°C, at low pressure and vigorous stirring. The typical contact time is 30-60 min. After this time period, the earth containing the adsorbed pigments, oxidized products, trace metals, soaps, phospholipids and some oil, is removed by filtration, blown with steam and compressed air to recover the oil as much as possible and discarded. The dark and stinky solid product is generally disposed off in dumps but, since it still contains 25-40% of oil, in certain particular logistic conditions it could be economically feasible to recover the oil from it. There are some industries in Europe specialized in

this process and the obtained oil, forbidden for food and feed purposes, is generally used for low value technical applications.

The oil recovery from exhausted bleaching earth is generally carried out with hexane, according to the scheme shown below.

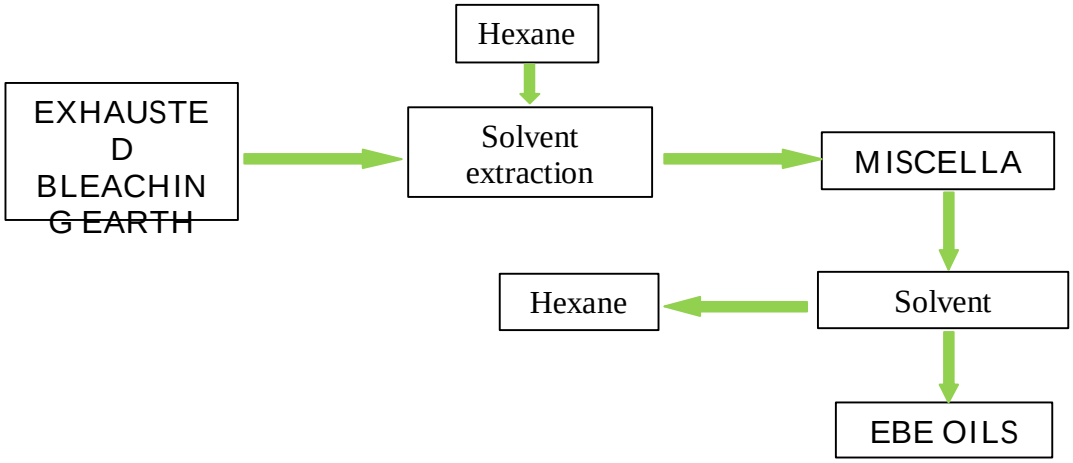

Fig. 6 Production of ebe samples

Bleaching earths that are used in oil technology, are of mineral origin (montmorillonite). The mineral product coming from mines or caves is processed by deep milling in order to obtain a specific particle size; the earth is then acid activated by sulphuric acid treatment in a rotating oven. The mineral acid is decomposed and removed during the oven treatment, resulting in a bleaching earth with a weak acidic behavior. In some special cases, the mineral bleaching earth is used together with activated coal (especially when green colors must be removed or when high PAHs concentrations are present) or synthetic silica. Both materials can be recovered along with their contamination content, after filtration of the sludge.

2.3.7 Fish oils (FISH)

The FISH oil represents a co-product of low value fish processing during fishing or a by-product of fish canning industry, such as canned tuna fish, smoked salmon, salted sardines, etc. In the first case, the whole fish is processed, while in the canning industries only discarded tissues undergo the rendering process for the final preparation of FISH oil and fish meal.

The process for the preparation of oil and meal from fish or fish tissues has many similarities with the one previously described in the case of animal fats and it is shown in the following scheme.

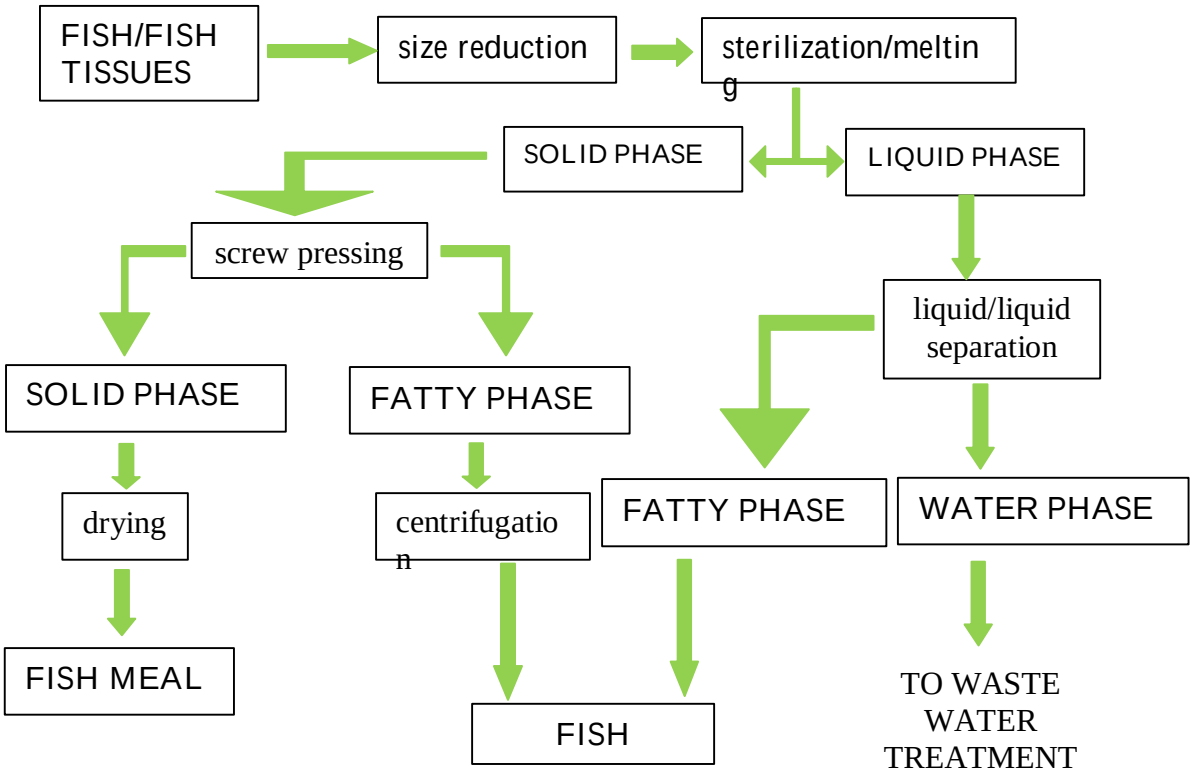

Fig. 7 Production of fish samples

During the past few years, there has been several concerns about the fish oil contamination as related to environmental pollutants. Marine fish acts as a concentrator of lipophilic organic pollutants, such as dioxins, PCBs, PBDEs, etc. The degree of contamination strongly depends on the source, in terms of geographic position where fish was withdrawn, as well as the position of fish species within the food chain. Generally speaking, the higher the fish dimension, the higher the contamination problems.

2.3.8 Hydrogenated fats from by-products (HYBY)

For the production of this special product, AOPHY from palm oil or from animal fats are used as starting material. The main objective of hydrogenation is to completely eliminate the unsaturated fatty acids, in order to obtain a product with iodine value close to zero, high melting point (> 60°C) and very resistant towards the oxidation damage. The saturation of fatty acid double bonds is achieved by means of the catalytic hydrogenation process, a well-known technology widely used in food industry. The hydrogenation reaction is carried out in a gas/liquid reactor, where hydrogen is intimately mixed with the feedstock in presence of metal catalyst supported on activated carbon, metal oxide or metal salt. The most utilized catalyst for this reaction is Ni, supported on different media and at different particle size. Other applications described in the literature, use precious metals (Pt or Pd) as catalyst.

The catalyst is generally removed and, when possible, recycled by means of filtration, which is often carried out in presence of filter aids.

The general scheme for a hydrogenation process is described below.

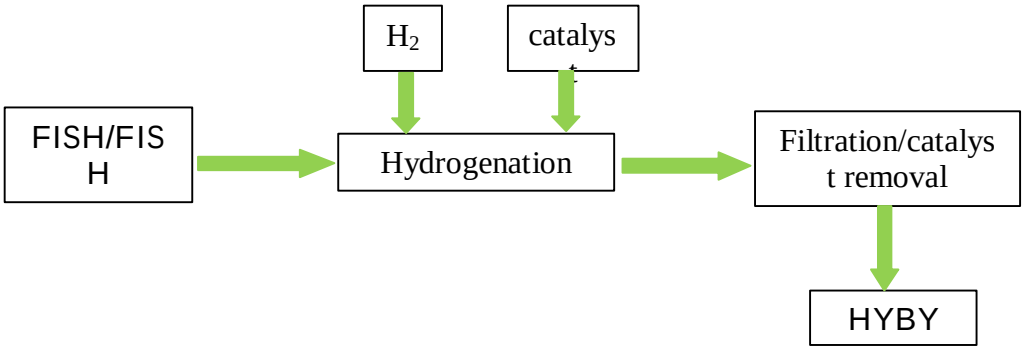

Fig.8 Production of hyby samples

The hydrogenation reaction can be carried out batch wise or continuously, at a temperature ranging between 80 and 150°C, using a hydrogen pressure up to 6 bar, under a strong mixing to ensure the proper gas/oil/catalyst intimate contact and mass transfer. The reaction shows an exothermal behavior and sometimes it is necessary to use cooling devices to keep the temperature under control.

2.3.9 Fatty acid calcium soaps (FACS)

The industrial preparation of fatty acids calcium soaps (FACS) can be carried out from different fatty materials using three different procedures:

1. Direct neutralization of free fatty acids with slaked lime, according to the following reaction:

$$R\text{-}COOH+Ca(OH)_2 \quad (R\text{-}COO)_2Ca+H_20$$

2. By double exchange reaction of fatty acids sodium/potassium salts with calcium chloride:

$$R\text{-}COOH+NaOH \quad R\text{-}COONa+H_20 \qquad R\text{-}COOH+KOH \quad R\text{-}COOK+H_20$$

$$2R\text{-}COONa+CaCl_2 \quad (R\text{-}COO)_2Ca+2NaCl \qquad 2R\text{-}COOK+CaCl_2 \quad (R\text{-}COO)_2Ca+2KCl$$

3. By direct saponification of an oil or fat with slaked lime:

$$2 \text{ triglyceride} + 3\ Ca(OH)_2 \quad 3\ (R\text{-}COO)_2\ Ca + 2\ C_3H_8O_3 \text{ (glycerol)}$$

FACS is a combination of fatty acids and calcium as calcium soaps. The product is a dry, free-flowing solid granule used for inclusion in total mixed rations. The melting point of FACS is so high (> 150°C) that can be used as solid ingredient for feeds. Heating over the melting temperature may lead to a decomposition reaction, where carbon dioxide is released and straight chain and branched hydrocarbons are formed.

Finally, this powder is very sensitive to room moisture, so that a rigorous control of storage temperature and humidity is necessary to maintain FACS as powder or granules.

2.3.10 Miscellaneous products (MIX)

All products that could not be classified in other groups, are included in this group, which can be divided in three categories:

a) Refined soybean oil for human consumption: it is sometimes employed for feed formulation when the international oil prices are convenient. The production technology is the same as the one utilized in the food industry: from the crude oil obtained by screw pressing or solvent extraction, it is possible to obtain a fully refined oil after being subjected to degumming, neutralization, bleaching and deodorization.

b) Hydrogenated oils with high fatty acids content: these special products are prepared from fully refined oils by means of hydrogenation. In this case, a partial hydrogenation was performed, in order to avoid the full saturation of double bond and to promote the *cis-trans* isomerization of olephinic moieties. The obtained product has particular melting and plastic properties and it is particularly used for the preparation of milk replacers for veals and other young animals.

c) Fatty acid mono- and diglycerides: this type of products are prepared by direct esterification of glycerol with free fatty acids in presence of a metallic or metal oxide heterogeneous catalyst. The

reaction can be carried out without catalyst under particular conditions. The amount of mono-, di- and triglyceride can be fixed during the reaction design, by setting the fatty acids/glycerol stechiometric ratio. The reaction is generally carried out at 180-240°C in a stirred reactor at a residual pressure of 100-300 mbar. Water is continuously removed from the reaction vessel and glycerol is condensed and refluxed into the reactor by means of an hot condenser, set at a temperature of approx 80-100°C. The preparation of a mono- and diglycerides mixture may be also carried out by making react refined triglyceridic oil with an amount of glycerol, calculated in a way to get the desired mono-/diglyceride ratio. In this case, the final mixture will also contain free glycerol and triglyceride.

The so called *superglicerination* reaction is generally catalyzed by alkaline reactants, such as sodium methoxyde or ethoxide, sodium or potassium hydroxides. The reaction temperature is generally set around 100-120°C when methoxides are used or at 180-200 °C for hydroxides. In every case, the operation is carried out under vacuum to avoid oxidation. At the end of the reaction, an addition of a weak acid (such as acetic or citric acid) allows catalyst deactivation. The same effect of interesterification can be also obtained using lipases, but this biotechnological procedure is, for the time being, limited to fats for direct human consumption.

2.4 CHEMICAL DEGRADATIONS IN FEEDING FATS

Fats and oils used in animal feeds vary in their physical and chemical properties. Generally, feed fats and oils have either animal or plant origin or both. These fats and oils are mainly made up of triacylglycerols, which are esters of fatty acids and glycerol. Sterols are minor compounds that are present in fats and oils from animal and plant origins. However, sterols are the major portion of the unsaponifiable matter of most fats and oils. Sterols are present as free sterols and/or as esters of fatty acids in addition to sterol glycosides and acetylated. The major phytosterols are sitosterol, stigmasterol, campesterol and brassicasterol; cholesterol is the major animal sterol. Other important group of components of the unsaponifiable matter is known as vitamin E. Vitamin E is the collective name of a fat soluble vitamin group composed of eight naturally occurring forms: á, â, ã, and ä-tocopherols and á, â, ã, and ä-tocotrienols. The structures of the tocopherol and tocotrienol homologs vary by the number and location of methyl groups on the chromanol ring. In addition, tocotrienols have three double bonds on the isoprenoid side chain.

Fats and oils used in animal feeding can undergo different chemical degradations during processing and storage, which are mainly oxidation, polymerization and isomerization. The products generated by these chemical degradations can decrease the fat nutritional value and give rise to toxic compounds.

Lipid oxidation yields both primary and secondary oxidation products, whose structures and amounts depend on the oxidation conditions (time, temperature, and presence of oxygen and substance with prooxidant and antioxidant effects) to which they are subjected. Primary oxidation products are usually evaluated through the peroxide value, which indicates the level of hydroperoxides present in the sample.

Due to their high instability, hydroperoxides are easily converted into different secondary oxidation products (such as aldehydes and ketones), which can be determined by different analytical methods, such as the *para*-anisidine index and the thiobarbituric acid reactive substances (TBARs). In addition, oxidation can also give rise to oxidized fatty acids (OFA), which non-volatile oxygenated

products are having molecular dimensions similar to those of the fatty acids from which they are generated. Under stressed oxidative conditions, dimers and polymers can also be formed by the combination of free radical species. However, polymerization can also occur during frying and hydrogenation.

Lipid oxidation is not only limited to fatty acids, but it can also affect other molecules that have double bonds in their chemical structures, such as sterols. In fact, cholesterol and phytosterols can oxidize and give rise to a wide range of oxidation products (sterol oxidation products, SOPs), some of which are well known for their negative biological effects (i.e. atherogenic, cytotoxic, mutagenic, carcinogenic). Sterols in fats and oils may oxidize during different processing steps, such as heating, degumming, neutralization, bleaching, deodorization, storage and handling.

Another important lipid chemical degradation is the double bond isomerization of fatty acids from the *cis*-isomeric configuration to the *trans*- one, which can take place during refining of edible oils (bleaching and deodorization) or catalytic hydrogenation of unsaturated oils. This gives rise to the formation of *trans* fatty acids (TFA), which are known for their atherogenic effects. TFA can be also produced by biohydrogenation, which are mostly isomers of oleic acid.

However, not all TFA have a negative biological effect. In fact, it has been demonstrated that some conjugated linoleic acid (CLA) isomers are anticarcinogenic and antiatherogenic. CLA isomers can be located in positions 9-11, 10-12 e 11-13 and can display different geometrical configurations (*cis, trans*; *trans, cis*; *cis, cis*; *trans, trans*). Although CLA isomers are mainly present in ruminant fat, they can be also formed in small amounts during oxidation or partial hydrogenation. However, different CLA isomers are preferentially formed according to their origin (natural or induced by heat, e.g.).

2.4.1 Oxidation

Lipid oxidation is accelerated by metals, light, heat and several initiators, and can be inhibited by avoiding pro-oxidant and including antioxidants (natural and/or synthetic). The primary oxidation

products are allylic hydroperoxides. The double bonds remain, though they may have changed configuration and position in the fatty acid chain.

These compounds are not directly responsible for the undesirable flavor and aroma associated with rancid fat but they are labile molecules, which readily undergo a number of secondary reactions including the formation of short chain aldehydes (hexanal, propanal, etc) and other compounds (oxidized fatty acids, OFA).

Autoxidation is a radical chain process, i.e. the intermediates are radicals (odd electronic species) and the reaction involves an initiation step, followed by a propagation sequence and a “termination” phase (fig 9). There is usually an induction period, where oxidation is a slow process, followed by a more rapid reaction phase. It is desirable to extend the induction period (and hence the shelf-life of the product) as long as possible. The detailed nature of the initiation step is not fully understood but any or all of three following reactions may be involved: 1) metal-catalyzed decomposition of hydroperoxydes produces initiating radicals (it is very difficult to obtain olefinic compounds completely free of oxidation products); 2) photo-oxygenation (a very rapid reaction) may be responsible for the first-formed hydroperoxides; 3) thermal initiation is possible in a heated sample. In the propagation phase, given an adequate supply of oxygen, the reaction between alkyl radical (R) and molecular oxygen is fast and the reaction between peroxy radical (ROO) with another olefinic molecule is rate-determining. Autoxidation can be inhibited minimizing the initiation step and/or promoting the termination step, so that the propagation sequence goes through as few cycles as possible.

1. Initiation

$$R\text{-}H \rightarrow R\bullet \quad \text{Initial promoting factor??}$$
$$R\bullet + O_2 \rightarrow ROO\bullet$$
$$ROO\bullet + RH \rightarrow ROOH + R\bullet$$

2 Propagation

Monomolecolar phase

$$R\bullet + O_2 \rightarrow ROO\bullet$$
$$ROO\bullet + RH \rightarrow ROOH + R\bullet$$

Bimolecolar phase

$$2ROOH \rightarrow ROO\bullet + RO\bullet + H_2O$$
$$ROO\bullet + RH \rightarrow ROOH + R\bullet$$
$$RO\bullet + RH \rightarrow ROH + R\bullet$$

3. Termination

$$R\bullet + R\bullet \rightarrow R\text{-}R$$
$$RO\bullet + R\bullet \rightarrow ROR$$
$$ROO\bullet + R\bullet \rightarrow ROOR \text{ (stable products)}$$
$$ROO\bullet + RO\bullet \rightarrow ROOOR \text{ (stable products)}$$
$$ROO\bullet + ROO\bullet \rightarrow ROO\text{-}OOR \text{ (stable products)}$$

Figure 9. Oleifin autoxidation (RH represents an oleific molecule, where H is attached to an allylic carbon atom).

The consumer's preference for meat and meat product is influenced by several factors, such as quality freshness, color, price, aroma, food habits, and particularly, safety (Saba & Di Natale, 1999). Lipid oxidation is one of the most important factors limiting the shelf-life and commercial stability of meat and meat products. Meat oxidation is related to the content of natural antioxidant and the polyunsaturation degree of fatty acids (Morrissey *et al.,* 1998).

2.4.2 Fatty Acids Isomerization

2.4.2.1 Trans Fatty Acid

The relation among dietary intakes of fatty acids (FA), blood cholesterol level, and risk for cardiovascular disease (CVD) remain an important health issue. Currently, it is well-established that dietary saturated FA raises blood total and low density lipoprotein (LDL) cholesterol (LDL-C) concentration compared with linoleic and oleic acids (Kris-Etherton *et al*, 1997; Mensink *et al*, 2003). Over the past 15 years, metabolic studies have evinced that, in addition to saturated (FA), TFA also have a negative effect on human plasma lipoprotein profile and have adverse implication for atherogenesis. The negative effect of TFA on lipoprotein is caused by increasing the total cholesterol, LDL-C, lipoprotein a [Lp(a)], and decreasing high density lipoprotein cholesterol (HDL-C) relative to natural occurring *cis*-unsaturated (FA) (Judd *et al*, 2002). Moreover, the replacement of saturated (FA) by TFA was shown to decrease HDL-C in many studies (Müller *et al*, 1998; De Roos *et al*, 2001). Thus, compared with saturated FA and *cis*-unsaturated FA, the overall effect of increased intake of TFA is a less favorable LDL-C/HDL-C ratio (Ascherio *et al*, 1996), which is additional increasing of cardiovascular disease (CVC). Some (Willett et al, 1993; Clifton et al, 2004) but not all epidemiologic and case-controlled studies (Aro et al,1995; Bolthon-Smith *et al*, 1996) showed that high intake of TFA increased the risk of CVD, which agrees with the observed the effect of TFA rise of blood lipids. The current scientific literature suggests that replacing saturated and TFA with unhydrogenated fat has clear beneficial effects of blood lipids and thus provides an alternative strategy for reducing the risk of CVD.

Although experts have somewhat different interpretations of the scientific evidence on negative health effects of TFA (Judd et al, 2002), the health recommendations to reduce the risk of CVD provided by official research institutions and government organizations (such of the Institute of Medicine, European Atherosclerosis Society, Food and Agricultural Organization (FAO), Canadian Medical Association (Fodor et al, 2000), the Nutrition Committee of the American Heart Association, Joint WHO/FAO Consultation on Diet, Nutrition and the Prevention of Chronic

Diseases, and government health agencies in Denmark, Canada and the United States), usually stress the importance of reducing the intake of foods rich in both saturated and TFA. The food industry is already responding by reducing the TFA content of many products, especially in margarines (Akoh & Lai, 2005).

Recently, the negative health effects of TFA and the phrases "*trans* fat" and "the partial hydrogenated fat" would have been familiar only to lipid researchers, biochemists, fats and oil technologists, and some health professionals. Although group such as the Center for Science in the Public Interest have been warning consumer about the adverse health effects of *trans* fats and the high levels of these in certain commercial food products including the fast foods (Liebman *et al,* 1999), the North American public was largely unaware of the TFA issue in spite of being the highest consumers of TFA in the world.

In a study conducted in Canada to obtain information on consumer attitudes and behavior related to nutrition labeling, 17% of consumers interviewed claimed to understand well the term "*trans* fat", whereas 55% indicated they had no idea about the meaning of the term.

The legislation accepted by the Danish Government on March 11, 2003, prohibits the sale of food containing >2% industrially produced TFA (as percentage of total fat), whereas the publication of the amendments to food-labeling regulations in Canada in January 2003 and the United States in July 2003, require mandatory declaration of TFA level in foods. The subsequent media reports in Canada and the United States on the level of *trans* fats in foods and their potential damaging effect on health brought *trans* fats to the attention of many health-conscious consumers.

In the European Union, the Council Directive on nutrition labeling of foodstuff provides for voluntary nutrition labeling except where a nutrition claim is made. The current rules require that when nutrition labeling is provided, the declaration of energy, protein carbohydrate, and fat, or of energy, protein, carbohydrate, sugars, fat, saturated fats, fiber, and sodium if a claim for any of sugars, saturated fat, fiber or sodium is made. Provision is also made for the inclusion of starch,

polyols, monounsaturated FA (MUFA) and PUFA, cholesterol and specific vitamins and minerals. There is no provision for the declaration of TFA.

On request from the European Commission, the Scientific Panel of the European Food Safety Authority on Dietetic Products, Nutrition and Allergies, recently published an opinion related to the presence of TFA in food and the effect on human heath of the consumption of TFA. The panel concluded that, based on prospective cohort studies, a high intake of TFA is associated with an increased risk of cardiovascular disease and that the effect of TFA were more pronounced that those of saturated fat. On the other hand, the panel also noted that current intakes of TFA were 10 times lower than those of saturated FA, whose intakes in many European countries exceeds dietary recommendations. The Food Standards Agency of the United Kingdom allows claims regarding TFA, so their amount must be declared with nutrition labeling.

In 2003, although it had originally considered implementation of regulations to label foods with TFA content, Denmark chose to limit the amount of *trans* fat in foods as a way to decrease consumers intake of trans fat. Under Order No. 160 of the foodstuff Act, as of June 1, 2003, it is prohibited to sell oils and fats if they contain>2 g of *trans* FA/100 g. as of January 1, 2004, any oils or fats used as ingredients in foods are prohibited from containing >2 g of fat/100 g of the fat or ingredient. The Order does not apply to naturally occurring TFA in animal products. For the purposes of the Order, “TFA” are defined as the sum of all FA isomers with 14, 15, 18, 20, or 22 carbons atoms and one or more double *trans;* in the case of PUFA, however, only those with methylene-interrupted double bonds are included (Akoh &. Lai, 2005).

2.4.2.2 Origin of TFA

The carbon-carbon double bonds (also know as ethylenic bonds) of natural unsaturated FA, which are widely present in all plant materials and animal tissues, are primarily of *cis* configuration. TFA are also unsaturated FA but contain one or more double bands in the *trans* configuration. The hydrogen atoms in the double bond in the *trans* form are located on either side of the carbon atoms,

whereas those in the *cis* form are located in the same side. Some *trans* FA isomers occur naturally, although in much less abundance than the *cis* form. Foods produced from ruminants animals, including meat and dairy products such as milk, butter, and cheese, are the most common natural dietary sources of TFA. These *trans* FA are the result of biohydrogenation of dietary *cis*-unsaturated FA by rumen microorganism of ruminant animals.

Tissues of these animals and the products derived from them, therefore, contain small amounts of TFA isomers. TFA also occur naturally in green level. An example is 3- *trans*-hexadecenoic acid (3*t*-16:1); although it is a minor ingredient, it is a ubiquitous component of all green levels. This is an intermediate component in the biosynthesis of saturated FA. Some seed fats (e.g., tung) may contain up to the 80% *trans* FA, such as 9-*cis*,11-*trans*,13-*trans*-octadecatrienoic acid (9*c*,11*t*,13*t*-18:3), although they are not dietary fats.

TFA are also formed intentionally during the commercial process of hydrogenation that converts liquid vegetable or marine oils rich in *cis*-polyunsaturated FA (PUFA) into solid fats. Hydrogenation, which is performed by doubling hydrogen through the liquids oils in the presence of a metal catalyst such as nickel, is usually not allowed to go to completion (hence termed as partial hydrogenation) and results in the conversion of some of the *cis* double bonds to the *trans* configuration. During the hydrogenation process, double bonds migrate along the carbon chain, resulting in positional isomerization. Additionally, some double bonds change their natural *cis* configuration to the *trans* configuration, which is know as geometrical isomerization.

The melting point of a TFA falls between that of the corresponding *cis* FA and the saturated FA. Food manufacturers prefer partially hydrogenated fats over liquid oils, because they provide a solid fat for the manufacture of a variety of food products.

Nickel catalysts are most commonly used for vegetable hydrogenation in conventional hydrogenation. Nickel catalysts offer high activity, tailored linoleic acid and linolenic acid selectivity, low cost, and easy removal from oils by filtration. The nickel catalyst causes the isomerization of the natural *cis* double bonds to *trans* double bonds.

The TFA content of vegetable oil hydrogenated by precious metal catalysts was lower than that of oil hydrogenated by nickel catalysts. Low hydrogenation temperature using precious metals produces less TFA than the conventional high-temperature hydrogenation using nickel. Nickel in not very active below 120°C. Precious metal catalyst, on the other hand, are active at the low temperature of 70°C. Precious metal catalyst at low temperature decreased the TFA in the hydrogenated vegetable oils. It is generally accepted that the platinum catalyst produces the lowest TFA during hydrogenation.

Hsu *et al*. (1988) also reported that hydrogen pressure has the most significant effect on the formation of TFA during hydrogenation with palladium black. Berben *et al.* (2000) produced hydrogenated oil with low saturated fatty acids and TFA by adding ammonia to the catalyst as a reaction modifier. The addition of ammonia to a palladium catalyst formed a very low amount of saturated fatty acids. The addition of ammonia to a platinum catalyst greatly decreased both saturated fatty acids and TFA. The amount of TFA and saturated fatty acids in hydrogenated oil using a combination of ammonia and platinum on alumina support were only 6.6 and 6.8%, respectively.

Solid fats are essential in making good pastry, cakes, crackers, donuts, and man other bakery products because they contribute tenderness and help incorporate air into the dough or butter. In addition, solid fats are less prone to rancidity; therefore, foods made from solid fats can be stored for a longer period then those made from liquid oil.

Partially hydrogenated fats were development in part to replace the highly saturated solid animal fats such as butter, tallow, and lard previously used for this products. The use the partially hydrogenation vegetable oil (PHVO) in margarines, shortening, deep frying, bakery products, snacks, fast foods and other processed foods, provide a more healthful alternative to animals fats because they contain no cholesterol have less cholesterol-raising saturated FA.

During partial hydrogenation, process parameter such as pressure, catalyst type, concentration, and temperature can be controlled to decrease the amount of TFA in the final product; however, to date,

no process conditions, other than full hydrogenation, have successfully produced *trans*-free (<0.5%) or low-*trans* (<5%) oil blend (Allen *et al.*, 1998). Recently, fully hydrogenated fats were blended with oil to produce fat mixture with no *trans* acids for different food applications. However, the high amount of SFA in these oil-fat blends can still make them a less desirable ingredient for healthy food applications.

The edible-oil industry modifies fats and oils to increase their stability and functionality for a range of application areas and nutritional benefits. Blends of fats and oils may yield a fat mixture with different characteristics than that of the individual components.

The range of food applications of the fats and oil or their blends may be increased by application of a modification technique or combination of modification techniques (Allen *et al*, 1998). Several processes are used to modify the properties of fats and oils. The most common processes are hydrogenation, interesterification, and fractionation.

Interesterification has found many applications in the area of low or no-*trans* fatty acid-containing oils and fats especially for the production of margarines, spread, and shortenings; however, the process is ~ 20% more expensive than regular shortening production based of hydrogenation (Akoh & Lai, 2005).

2.4.2.3 TFA Effects on Health

Within-population studies that link TFA intake from hydrogenated vegetable fats to cardiovacular heart disease (CHD) risk include the Nurse's Health Study (Willett et al., 1993) and a case-control study on man and women with coronary artery disease (Ascherio *et al*., 1994). The Nurse's Study, a prospective cohort study, reported positive relations with relative risk (P<0.001) at 1.5 time in the highest quintile group of TFA intake (5.7 g/d), compared with those in the lowest quintile of intake (2.4 g/d). The positive association was attributed to an intake of partially hydrogenated vegetable fats rather than isomers from ruminant sources. After adjusting for total energy consumptions, the

relative risk for developing CHD was 50% greater in the highest quintile (P = 0.001), compared with those in the lowest quintile of TFA consumer.

Ascherio *et al*. (1994) established the relative risk of myocardial infarction at 2.4 times (P<0.001) for the highest quintile of TFA intake (6.5 g/d), compared with the lowest quintile of TFA intake (1.7 g/d). In both studies, dose-response relations were difficult to establish for the intermediate quintiles.

Methodological limitations in the studies such as the use of semi quantitative food-frequency questionnaires, as well as food databases without comprehensive TFA content, led to difficulties in establishing a dose-response relation.

In contrast, Kromhout *et al*. 1995 reported for the seven Countries Study, a cross-population study involving 12,763 men 40-59 year old, a significant correlation (r = 0.78; P<0.001) between TFA intake, TC levels, and 25 year coronary artery disease mortality. Food data were collected by the weighed food method, and the records were used to recreate foods that were subsequently analyzed for their individual FA composition. A confounding effect was the presence of SFA, which prevented establishing an independent effect for TFA. A prospective case-control study in a subgroup of the EURAMIC study established that those in the highest quartile of TFA intake from partially hydrogenated fish oil (PHFO) experienced five times the risk of myocardial infarction compared with those in the low-est quartile of intake (Aro *et al*., 1995). This study linked deleterious alterations in the low-density lipoprotein (LDL): high-density lipoprotein (HDL) ratio to thrombogenesis. Another case-control study established a link between high TFA content in human erythrocyte membranes to cardiac arrhythmias and sudden death (Katz, 2002).

Follow-up cohort studies in subject with coronary disease indicate associations between TFA intake and the recurrence of cardiac events as well as mortality. Data collection depended again on food-frequency questionnaires or 24-h recalls. Tavani *et al*. (1997) found that margarine could explain ~ 6% of myocardial infarction in 429 women with relative risk varying between 1.0 for a low or no intake and 1.5 for a medium or high intake. A positive association between *trans* 18:2 content of

partially hydrogenated-soybean oil (PHSO) and adipose tissue to increased risk of nonfatal myocardial infarction (P<0.001), was reported recently in adult Costa Ricans (Baylin *et al.,* 2003).

2.4.2.4 CLA Sources and Human Studies

Conjugated linoleic acid (CLA) refers to a class of position and geometric conjugate dienoic isomers of linoleic acid; *cis*-9, *trans*-11 (*c*9,*t*11) CLA and *trans*-10, *cis*-12 (*t*10,*c*12) CLA are the main isomers. CLA is the naturally present in the milk and meat of ruminants due to its production by anaerobic bacteria in the rumen of these animals; it can also be produced industrially by isomerization of linoleic acid (LA) CLA concentration in ruminant-derived products range from 3 to 7 mg CLA/g fat depending on source and processing of products (Chin *et al,* 1994). The estimated average daily intake of CLA from these dietary sources ranges from 0.19 to 1.0 g CLA (Fritsche *et al.,* 1998) and varies for different countries (Parodi, 1994).

Various positive health effects are ascribed to CLA consumption, including anticarcinogenic, antiatherogenic, and antidiabetic activity. In addition, animal and human studies suggested that CLA has favorable effects of certain aspects of immune function. CLA was also reported to decrease body fat while increasing lean body mass (LBM) (Delany *et al.,* 1999; Blankson *et al.,* 2000).

Many human intervention studies with a mixture of *c*9, *t*11 CLA and *t*10, *c*12 CLA and 4-isomer preparations from industrial sources were recently published (Malpuech-Brugere *et al.,* 2004). Furthermore, two human intervention studies with single-isomer material were published. Intervention with mixed isomers in doses ranging from 0.7 to 6 g/d, for periods of 8 week to 1 year in various populations [healthy, overweight, obese, noninsulin-dependent diabetes mellitus (NIDDM), syndrome X] investigated clinical outcomes in relation to body composition, immune function, insulin sensitivity, lipid metabolism, and safety (Albers *et al.,* 2003).

2.4.2.5 Natural origin of CLA

CLA is an intermediate in the biohydrogenation of linoleic acid (LA); until recently, it was accepted that CLA in ruminants originated from the incomplete biohydrogenation of LA by rumen bacterial. Complete biohydrogenation of LA in the rumen is a three-step process, leading to the production of C18:0 (fig 10). CLA is formed as the first intermediate of this pathway by the action of LA isomerase, an enzyme of the anaerobic rumen bacterial *Butyrivibrio fibrisolvens* (Kepler *et al*., 1970); it is unusual that the reaction occurs in the middle of a hydrocarbon chain away from any activating functional groups. In addition, it is an absolute substrate requirement for a *c*9, *c*12 diene structure and a free carboxyl group. The enzyme was shown to exhibit maximum activity with the substrates LA and C18:3. Lipid biohydrogenation in the rumen is affected by the type and the amount of fatty acid substrate, the forage to grain ratio, and the nitrogen content of the diet fed to ruminants (Gerson *et al.,* 1983).

Once the explanation for the presence of CLA in ruminant lipids was accepted, it was assumed that the amount of CLA escaping rumen biohydrogenation and being adsorbed was adequate to account for CLA levels in milk and body fat. However, the absence of any reported measurement of the amount of CLA escape and absorption, together with *in vitro* studies showing rapid conversion of CLA, formed by LA isomerase, to vaccenic acid (*t*11-C18:1) has cast considerable doubt on the rumen as the sole source of CLA in tissues and milk fat.

Work by Griinari *et al*. (1997) showed that CLA could be endogenously produced from *t*11-C18:1 in the tissues by $Ä^9$ desaturase. Fig 10 and 11 illustrate the two pathway of CLA biosynthesis, which may account for the high CLA concentrations observed in milk fat even when cow are fed diets low in LA, e.g. pasture feeding or fish oil supplements. It is proposed that *t*11-C18:1 accumulate in the rumen and that a portion escapes further biohydrogenation. After absorption in the digestive tract, *t*11-C18:1 is utilized by different tissues where a portion is desaturated to CLA and incorporated into tissues and milk lipid. This “desaturated hypothesis” was proposed to explain the relatively constant ratio of *t*11-C18:1 and CLA in bovine milk fat across a range of diets. The

presence of *t*7,*c*9 CLA and *c*9,*t*13 C18:2 support the role of an active Ä^{9} desaturase, an enzyme that introduce a *cis* double bond between carbons 9 and 10. Evidence suggests that Ä^{9} desaturation of *t*11-C18:1 may be more related to the production of CLA than previously though. Several studies reported a strong correlation coefficient between *t*11-C18:1 and CLA concentration in milk fat (Jahreis *et al.,* 1997).

A close linear relationship between milk fat *t*11-C18:1 and CLA was observed, suggesting a precursor-product association where *t*11-C18:1 is the precursor and CLA in the product. A slope of 0.5 suggests that approximately one third circulating *t*11-C18:1 was desaturatated by Ä^{9} desaturase.

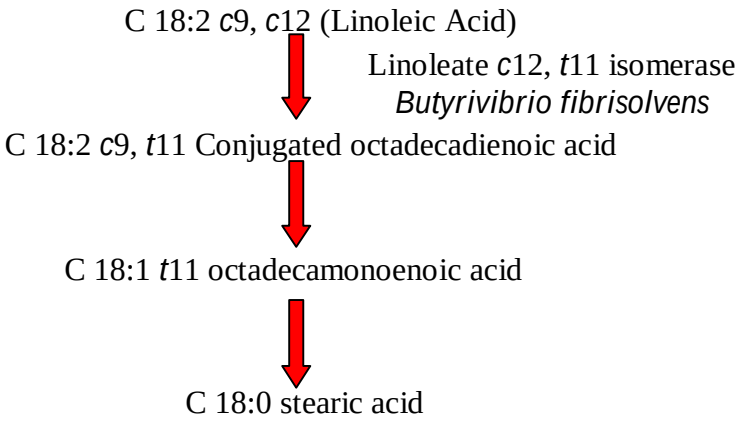

Fig 10. Biohydrogenation of linoleic acid in the rumen

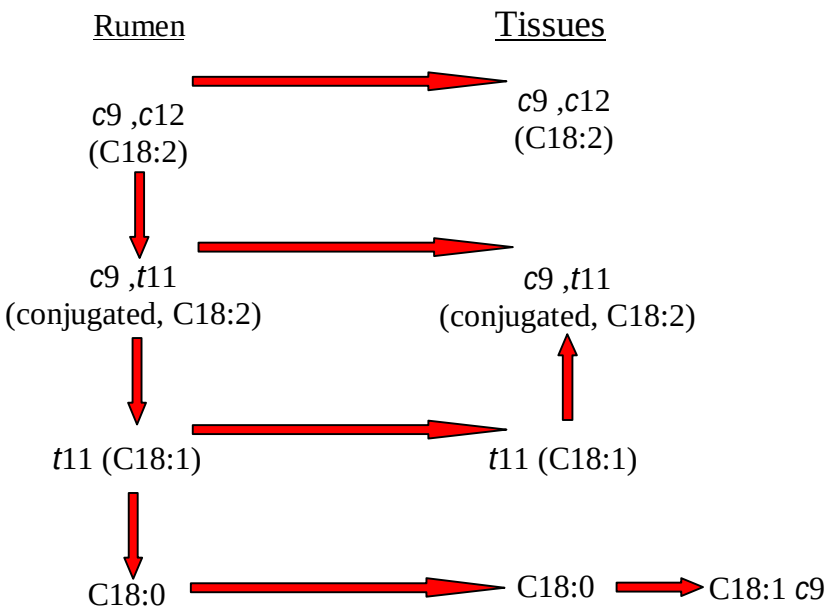

Fig. 11 Pathways of conjugated linoleic acid byosynthesis

A study where *t*11-C18:1 and CLA were fed in equal quantities to mice reported that 12% of the *t*11-C18:1 consumed during 2-week feeding period was recovered in the carcass as CLA. Of the portion of *t*11-C18:1 present in the tissues that was available for bioconversion, 48.8% was desaturated. CLA was found in the carcass only when *t*11-C18:1 or CLA was fed. CLA was found in both triglyceride and phospholipids when CLA was fed, but only in triglyceride when *t*11-C18:1 was fed, suggesting that bioconversion occurred in the adipose tissues (Santora *et al.,* 2000).

CLA in human serum was shown to derive in part from the diet and in part by conversion of dietary TFA. Serum level of CLA varied between 0.17 and 0.43%, when healthy subjects were fed a dairy fat diet for 5 week, followed by either a TFA diet or C18:0 diet for 5 week (Salminen *et al.,* 1998). The levels of CLA in the dairy fat, TFA, and C18:0 diets were 0.37, 0.04, and 0.10% of total fatty acid methyl esters, whereas the corresponding percentages in serum were 0.32, 0.43, and 0.17%, respectively. The difference in the CLA content of serum between subjects fed the dairy fat and C18:0 diets was explained by the different dietary intakes of CLA.

Level of total CLA in various foods range from as low as 0.2 mg/g fat in corn and peanut oil (Chin *et al.,* 1992) to as high as 17 mg/g in beef (Shanta *et al.,* 1995) and 30 mg/g in milk fat; *c*9,*t*11 is the predominant isomer in milk comprising 90% of the total CLA.

Several studies examined the effects of CLA in humans. A review of these studies was reported by Gaullier *et al.* (2002). The daily doses of CLA range from 3 to 7 g and the treatment periods ranged from 4 week to 1 year. The trials were carried out in a wide range of population groups, e.g., body builders, healthy individuals, overweight and obese individuals, individuals with metabolic syndrome, and populations with noninsulin-dependent diabetes mellitus (NIDDM). These studies were categorized according to the isomeric composition of the CLA preparation, due to the fact that it is a major influence on the effects of CLA. As stated before, the CLA preparations containing mainly the *c*9,*t*11 and *t*10,*c*12 isomers in approximately equal proportions, demonstrated to have beneficial effects in human intervention trials.

2.5 EFFECTS OF DIETARY MANIPULATIONS ON LIPID METABOLISM

Recommendations for lipid-lowering diets have traditionally focused on fats quality and composition as a means of lowering total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), which are independent risk factors for CHD. To achieve these end-points, dietary modulation require only a decrease in fat content *per se*, but also a decrease in saturated fatty acid (SFA) and an isoenergetic increase in polyunsaturated fatty acids (PUFA) and/or monounsaturated fatty acids (MUFA). Thus, dietary manipulations require a control over the fat composition in the diet, by striking a balance between the various fatty acids (FA) classes. Given the complexity of lipoprotein metabolism, the approach to dietary modulation for the last 40 year has remained focused on the steady state of LDL-C generation and catabolism, through an understanding that saturated fats promote increased LDL-C production by down regulating LDL receptor (rLDL) activity. Conversely, replacing these saturated fats with unsaturated fats up regulates rLDL activity, thereby increasing the catabolism of LDL. This approach was substantiated by considerable evidence from human and animal studies.

However, it has also been documented that most lipid-lowering diets designed to decrease in TC and LDL-C also lead to lower high-density lipoprotein cholesterol (HDL-C). Thus, traditional dietary advice concentrates on the endogenous fat transport pathways, whereas it ignores the exogenous fat transport and reverses cholesterol transport systems, which are also components of lipoprotein metabolism.

The ratio of TC and HDL-C is considered a more specific marker of CHD risk than LDL-C alone. This is in line with evidence correlating an increase in HDL-C concentration with a lower risk of CHD.

Another aspect to consider about lipid-lowering diets is that the premise on which recommendations are based, i.e., the predictive regression equation, accounts for the behavior of only limited fat variables and FA, over simplifies lipoprotein response to these variables. The wider matrix today includes the adverse effects of *trans* FA and the recognition that plasma HDL-C and triacylglycerol

(TAG) also constitute risk for CHD, especially when there is a constellation of risk factor for “low metabolic capacity”.

2.5.1 Individual FA and LDL-C Metabolism

Direct evidence from animal studies was provided on increasing rLDL activity by feeding unsaturated instead of SFA (Dietschy *et al.,* 1993). These studies examined individual FA and LDL metabolism in relation to the regulatory mechanisms governing LDL concentrations, i.e., hepatic LDL fractional clearance rate, LDL production rate, rLDL activity, and the hepatic free cholesterol/cholesteryl ester pool. Daumerie *et al.* (1992) fed hamsters C8:0, C14:0, and C18:1; relative to C8:0, C14:0 increased LDL-C and reduced hepatic cholesteryl ester (CE). There was an associated decrease in rLDL activity and increased LDL-C production rate but switching to C18:1 had the opposite effect. Woollett *et al*. (1992), also using the hamster model, substantiated that relative to a control, C6:0-C10:0 were neutral, whereas C12:0-C16:0 were negative regarding LDL-C concentrations, rLDL-C activity, LDL-C production rate, or the hepatic cholesterol pool. Only C18:0, although reducing hepatic cholesterol to the same extent as C16:0, continued to unregulated rLDL activity and therefore had no effect on LDL-C concentrations. Ohtani *et al*. exchanging C18:2 and C16:0 at 5% by weight, with and without dietary cholesterol in a hamster study, showed that C16:0 augmented the effect of dietary cholesterol in elevating LDL-C through suppressing rLDL receptor activity. However, C18:2 diminished both the effect of C16:0, as well as the addition of dietary cholesterol.

Other study approaches have used single vegetable oils and fats, with diverse FA profiles or oil blends with a dominant FA profile, to evaluate LDL metabolism. It is difficult to attribute the true outcome of individual FA in these studies because they were less well-controlled for C18:1 and C18:2 content. Observations in these categories found that C12:0 + C14:0 had the most negative effect on LDL metabolism, compared with C16:0 and C18:0 *via* reduced LDL fractional clearance rate and rLDL number (Fernandez *et al.,* 1993). Plasma LDL-C concentrations were shown to be

reduced in hamsters fed either a C12:0 + C14:0-rich hydrogenated coconut oil diet or a C18:2-rich safflower oil diet at 0, 0.06, and 0.12% dietary cholesterol (Horton *et al.,* 1993). Changes in LDL-C were associated with the 0.12% cholesterol diet, with the hepatic rLDL activity at 30% in the SFA diet and 77% in the PUFA diet.

The different pathways of FA assimilation from the gastrointestinal system are hypothesized to explain the differential effect of FA chain length on LDL-C metabolism. These include the following: a) A rapid absorption of C6:0, C8:0, and C10:0 and entry into portal circulation, conversion into acetyl CoA, and entry into oxidative and synthetic pathway; b) a sequence of changes in TAG structure for C12:0, C14:0, and C16:0 during micellar solubilization in the gut, resterification and incorporation into chylomicrons leading to accumulations of these FA in the liver, and subsequent down regulation of rLDL activity (Wollet *et al*., 1992); c) the reason C18:0 does not enter either pathway is unclear, but it is further hypothesized that signal transduction, alternate metabolic pathways, or processes of membrane fusion are implicated; d) the fast rate of conversion of C18:0 into C18:1 (Bonanome *et al.,* 19929).

2.5.2 Individual FA and HDL-C Metabolism

Observation in humans studies note the replacement of SFA (C12:0-C16:0) with C18:0 or an unsaturated FA not only has a cholesterol-lowering effect, but is also linked to a decrease in HDL-C concentrations. Numerous animal studies, all using the hamster model, generally agree with data from human studies, but in addition, have provided only limited information on HDL-C metabolism.

Terpstra *et al*. (2000) observed that decreasing dietary fat saturation did indeed lower HDL-C in hamsters, and this was associated wit increased HDL binding in the liver. The hamsters were fed cholesterol-enriched diets with corn oil (predominantly C18:2, P/S = 4.77), olive oil (predominantly C18:1, P/S = 0.84), and palm oil (predominantly C16:0, P/S = 0.26) exchanged at ~19% dietary energy, but this study reported only P/S rations without individual FA value. Substituting C18:2

content for C16:0 or C18:1 lowered ($P<0.05$) plasma TC, TAG, HDL-C, and non-HDL-C fractions. Although the C18:1 diet tended to lower value compared with C16:0, the difference was not significant. The same study examined the mechanism by which increasing fat saturation lowered plasma cholesterol concentrations, particularly HDL-C through measured hepatic lipase, cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP), and lecithin cholesterol acyltransferase (LCAT) activities. Dietary fat saturation did not significantly affect plasma LCAT and CETP activities, but PLTP activity increased with increasing saturation. There was a significant correlation between PLTP activity and the non-HDL-C fraction, as well as with the HDL-C fraction.

The effect of a single FA on HDL metabolism was also evaluated in hamsters by Loison *et al*. (2002). By feeding hamsters C14:0 in varying concentrations, it was shown that C14:0 modulated HDL-C *via* a regulation in SR-B1 expression. Increasing the amount of C14:0 in the diet was the most important factor, causing increasing HDL-C concentrations, and this effect was linked to a decrease in the amount of SR-B1 in the liver.

2.6 ESSENTIAL FATTY ACID METABOLISM OF SELF-HEALING AGENTS.

Healthful lipids include two different types-those we eat and those the body makes. Both types require wise management to maintain good health. The enzymes in our body readily make new lipids from the excess food we eat, and an important health principle for all foods is “eat no more than needed”. Each meal we eat usually as more food energy that is needed at that precise moment, and the “excess” food energy is converted into the into the hydrocarbon chains of fatty acids (FA) that are stored primatily as triacylglycerol esters (fat), which might be needed to supply energy at a later time. The time scale within which food is needed and when it gives benefits or harm is defined and interpreted by the dynamics of many different steps in lipid metabolism. Unfortunately, those metabolic steps are not tightly linked to the neural sensors regulated appetite, satiety, or food intake.

Once food enters the body under the motivation of appetite, metabolic transformations proceed from the molecular collision of the digest nutrients with multiple enzymes and cofactors, which give either needed energy or stored products (mainly fat).

Fat provides 9 kcal/g or 4000 kcal/lb when metabolized to carbon dioxide and water. Many Americans carry 25-40% of their body mass in the form of stored fat, which has large proportions of oleic (C18:1-n9) and palmitic acid (C16:0).

These nonessential fatty acid are abundant in most diets, and they also are made by metabolism of the carbohydrate and protein in foods. Such non essential FA compete in metabolic processes with the essential FA (EFA) that vertebrate cannot make form carbohydrate and protein. Competitive interaction of the mixture of FA with many indiscriminate of promiscuous enzymes of lipid metabolism allow the relative supply of n-3 and n-6 EFA in the diet to have a large influence of the proportion of EFA that are maintained in tissues. Those food-induced proportions strongly influence several aspects of human health. Choosing food fats wisely remains an important way of preventing disease.

The current epidemic of cardiovascular disease among Americans (along with its associated disorder, obesity) comes from a public health environmental that has failed to educate people adequately about when food gives benefits or harm. Good health requires preventing excessive ingested food being converted into unneeded structures, uncontrolled energy, or imbalanced signals. These two readily prevented imbalances cause vascular damage that begins in childhood (Zieske *et al.,* 2002) and slowly progress toward the near-fatal onset of cardiovascular signs and symptoms that causes people to seek expensive treatments to extend their lives. Disease caused by such imprudence can be prevented with better lifelong advice to the public about foods and lipid metabolism. Current treatments that fail to correct the dietary imbalances that cause disease leave patients vulnerable to a continued lifelong dependence on expensive medications and treatments.

Essential polyunsaturated FA are like vitamins that must by taken regularly in small amounts relative to the larger amounts of food eaten to provide energy.

Like all FA, EFA are metabolized and stored in tissues lipids and oxidized to CO_2 and H_2O. The importance of EFA becomes evident after they are converted into important tissues structures and tissues signals. The time scale within which EFA give benefit or harm depends on the dynamics of the specific action involved. Tissues structures needed in the body often develop over house, days, and weeks with low-lasting effects, whereas tissue signaling actions occur transiently within seconds and minutes. The relative proportions of n-6 (ù-6) and n-3 (ù-3) types of EFA derivatives stored in tissues lipids come from foods eaten at an earlier time. Rapid conversion of the stored EFA to hormone-like autacoids (auto=self, akos=healing) is part of normal tissues adjustment to changing conditions, but the amplifying dynamic actions sometimes go beyond desired healthy intensities. Although both n-3 and n-6 types participate in the transient tissue signaling that regulate body responses to stress, signaling by the n-6 derivatives can sometimes be more intense than that by n-3 derivatives (Akoh & Lai, 2005).

Excessive n-6 eicosanoid signaling occurs in inflammatory/immune vascular disorder, thrombotic heart attacks, and cardiac arrhythmic event, as well as arthritis, asthma, cancer proliferations, and other chronic disorders of serious concern to aging adults. Billions of dollars are spent every year to develop and market pharmaceuticals that moderated imbalanced actions of these potent hormone-like autacoids, but little publicity is given to the fact that the sole source of the precursors for these autacoids is the food that people eat. Altering the food eaten can alter the intensity of the signaling in tissues. Here descript how the fat and FA that we eat and make are transported, stored in tissues, and made into hormone-like agents by metabolic processes that include the following: (a) remodeling of glycerolipid molecules, (b) converting food energy to other forms in tissues, (c) rearranging the EFA into signaling lipids during self-healing events, (d) preventing imbalances when choosing foods. (Akoh &.Lai, 2005).

2.6.1 Digestion and transport of fats and phospholipid metabolism

Lipids in the food we eat are digested by a relatively efficient and indiscriminate hydrolysis of the ester bond in the intestine. Triglyceride lipase and phospholipase are capable of hydrolyzing the lipid ester bonds. However, docosahexaenoic acid (DHA; C22:6n-3) esters are cleaved a bit more slowly, presumably due to steric hindrance by the presence of the Ä^4 double bond too close to the carboxyl ester. In addition, faster lipase hydrolysis of the 1- and 3-ester bonds (compared with the more hindered 2-ester) yields many FA soaps and 2-monoglycerides, which are efficiently absorbed by intestinal epithelial cells. Long-chain soaps are there converted into coenzyme A thiol esters (acyl-CoA), mainly esterified into triglycerides (fats), secreted into the lymph, and eventually moved through the thoracic duct into bloodstream as fat-rich chylomicron lipoprotein particles. After every meal, the blood plasma remains“milky” as the plasma carries elevated amounts of fat on lipoproteins being distributed to various tissues.

In the bloodstream flowing through tissues capillaries, the triglycerides on plasma lipoprotein are hydrolyzed by lipoprotein lipase, and the nonesterified FA (NEFA) related enter the tissue, where they again form acyl-CoA esters that transfer acyl chains onto glycerolipid acceptors, forming diglycerides, triglycerides, and phospholipids in the tissues. A continual formation cleavage of ester in tissues rearrange the different FA according to their relative abundance and their efficacy in competing for the small amounts of the vitamin-based cofactor, CoA, in tissues.

Each molecule of this cofactor is used and reused billions of times as various acyl chain esterify to it briefly during metabolism. Fig 12 notes that acetyl-CoA esters can be made longer and more unsaturated as the 18-carbon n-3 and n-6 acids form 20- and 22-carbon highly unsaturated FA (HUFA).

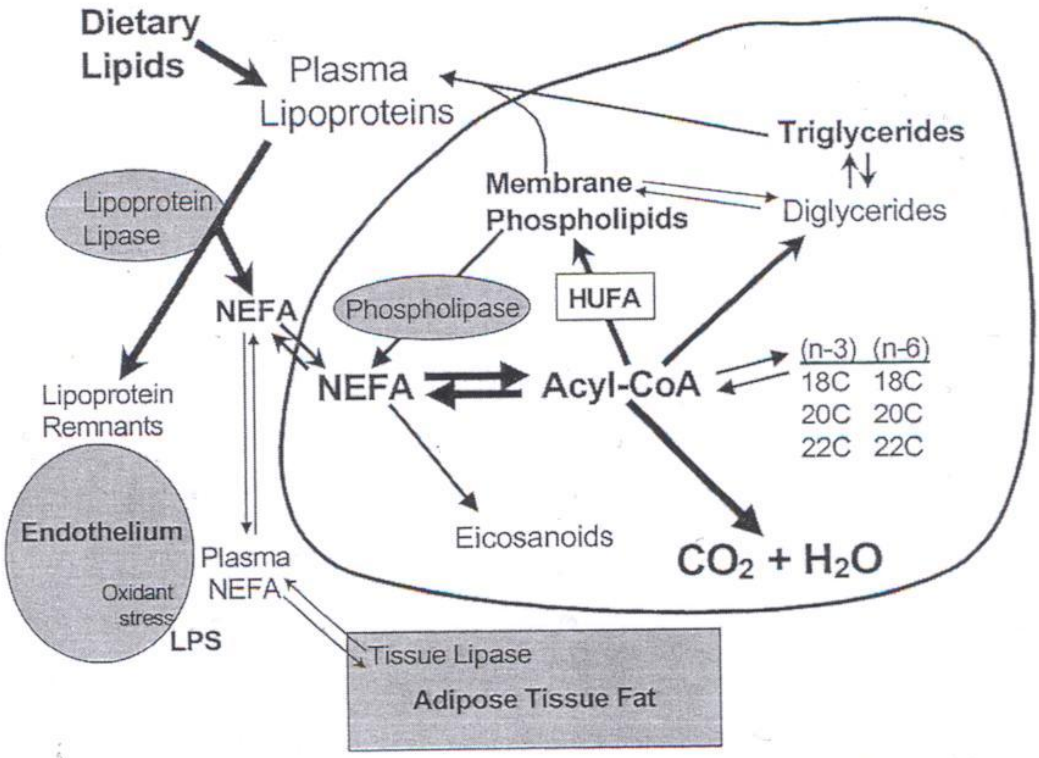

Fig. 12. Metabolism of FA. FA from circulating lipoproteins enter cells and form acyl-CoA esters that can be elongated, desaturated, transfer to glycerolipids, or oxidized to CO_2. NEFA, nonesterified FA; HUFA, highly unsaturated FA; LPS, lipopolysaccharide. (Akoh & Lai, 2005).

When FA are in the liver in large amount, their acyl-CoA esters form tryglicerides (fat), which are packed into fat-rich very low dendity lipoproteins (VLDL) and secreted into to circulating blood plasma. The process parallels the intestinal cell secretion of fat-rich chylomicron lipoproteins into lymph and plasma after every meal.

In the blood, lipoprotein lipase hydrolyzes the fat in VLDL, releasing NEFA and forming smaller, more dense low density lipoproteins (LDL). Unfortunately, high level of NEFA and LDL can increase oxidative stress to vascular endothelial tissue, especially when lipopolysaccharide (LPS) enters from the gut and triggers inflammatory responses (Fig 10.1). Such conditions with overabundant NEFA and LDL occur transiently after every meal. Eating less food energy per meal may help prevent cumulative vascular endothelial injury from such repetitive transient postprandial stress.

The enzymes transferring the acyl chain from acyl-CoA to form phospholipid and triglyceride esters have general selectivities (Fig. 12) that tend to put saturated acids (C16:0, C18:0) at the 1-position and unsaturated acids (C18:1n-9, C18:n-6) at the 2-position. The 3-position of triglycerides also tends to have unsaturated FA, giving typical fats in humans comprised of 33% saturated acids and 66% unsaturated acids, whereas phospholipids tend to have 50% saturated acids and 50% unsaturated acids. These trend are less evident when a great abundance of substrate is present to "push" enzyme into faster reactions with whatever acids are available (as occurs after every large meal). However, during periods between meal, these general selectivities eventually influence the net composition of tissue lipids (Akoh & Lai, 2005).

High-carbohydrate, low-fat meals induce high rate of FA synthesis from excess acetyl-CoA, and the Ä^{9}desaturase forms large amounts of n-7 and n-9 non essential FA (C16:1n-7, C18.1n-7, C18:1n-9) *via* palmitate and stearate (C16:0 and C18:0, respectively). In this condition, the acetyl-CoA pool has more abundant nonessential FA than essential n-3 and n-6 acids.

When the percentage of food energy (en%) as fat is <20%, the proportion of n-7 FA in tissue lipids is inversely related to the en% of fat intake. As a result, the elongation and desaturation enzymes form n-7 and n-9 HUFA (rather than n-3 and n-6 HUFA from dietary 18-carbon EFA).

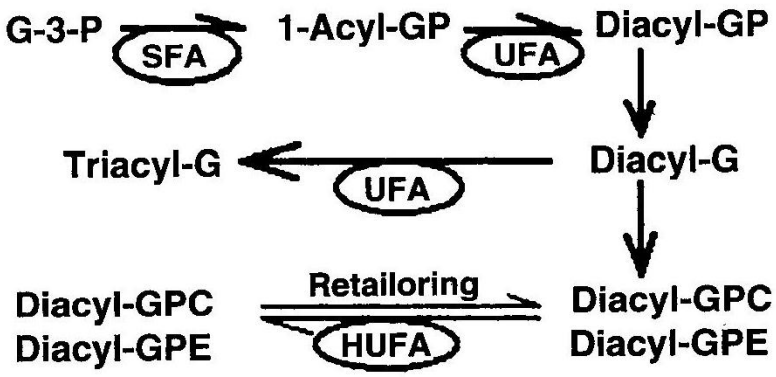

Fig 13. General selectivity in forming tissue lipids. In general, the 1-pisition has much palmitic acid (C16:0) and the 2-position has much oleic acid (C18.1n-9). G-3-P, glycerol-3-phosphate; SFA, saturated fatty acids; UFA, unsaturated FA; HUFA, highly unsaturated FA. (Akoh & Lai, 2005).

Alternatively, the very low levels of the n-9 HUFA, C20:3n-9, typical of many human studies, indicate a strong competition from n-3 or n-6 fats (or both). In fact, the almost undetectable level of C20:3n-9 in Americans can be regarded as preliminary evidence of excessive dietary supplies of either n-3 or n-6 EFA. Inattention to this important metabolic marker leads to another serious flaw in current dietary advice from the many committees attempting to advise Americans about “healthy eating” (Akoh & Lai, 2005).

3. MATERIAL AND METHOD

3.1 GUIDELINES FOR THE CLASSIFICATION OF DIFFERENT FAT FEEDSTOCKS

Scope of this document is the presentation of tentative rules for the classification of fats for feeding purposes. Starting from technological, compositional, degradation and contamination data we tried to create nine different fatty categories and we tried to find out an easy way for a preliminary classification, allowing the correct choice for analytical methods for their evaluation.

As all generic rules this is far to be perfect, but nevertheless it represents a very important starting point for the correct evaluation of different materials. During the practical realization of – Characterization of Feeding Fats, in reality ten different categories were created, but the last one "Mixed Products – MIX" is a collection of feedstocks that cannot be classified in one of the previous categories. The presence of this last category of product must be carefully taken into account because it represents a collection of specially designed products mentioned above.

The final scope of this document is to promote the logical process of evaluation described below.

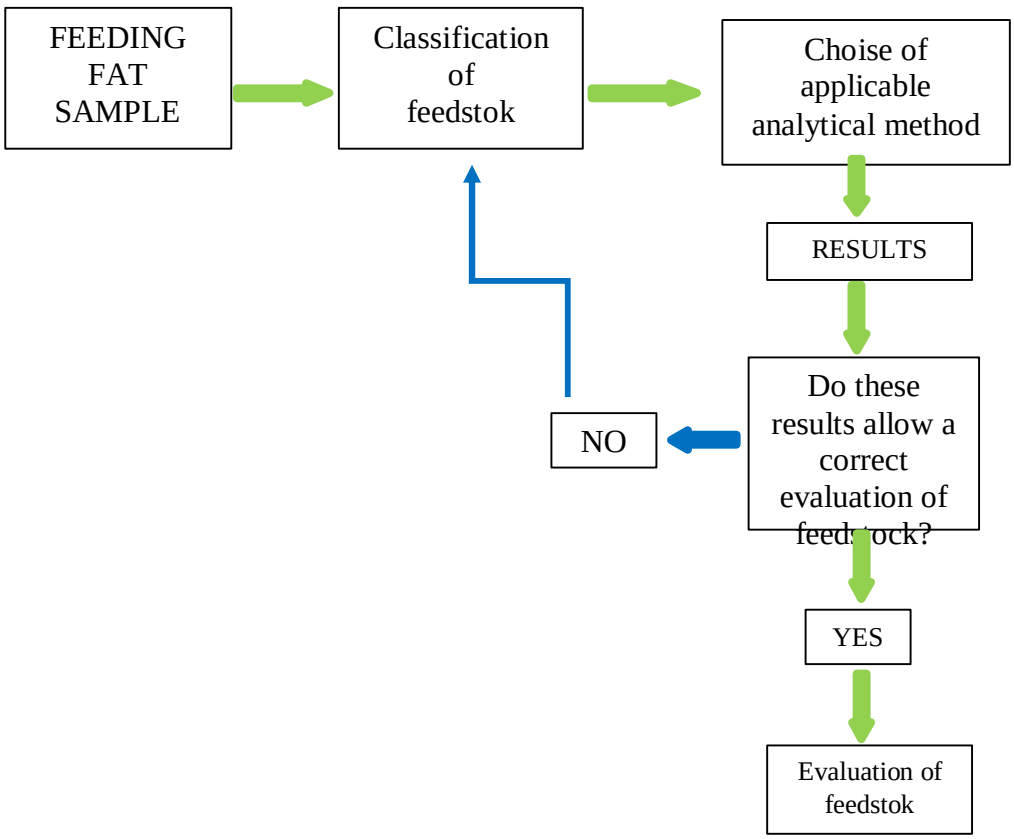

Fig. 14 Experimental design for sample collection

3.2 SAMPLE COLLECTION

During the experimental design care was paid in trying to get a rigorous sampling to achieve a real representativeness of sample, to obtain useful and standardized information from sample suppliers and to avoid random contamination. The following actions were performed to do so:

a. *Sampling procedure*: a sampling procedure extracted from ISO 5555 and adapted for the scope of Feeding Fats Safety project was prepared and distributed.

b. *Sampling form:* a sampling form, specially designed to get the maximum of information about the source, the starting material and the production technology from each sample provider was prepared. It is contained in Sampling Procedure along with delivery instructions for suppliers.

c. *Sampling container*: after preliminary discussions it was agreed that, in order to avoid casual contamination, all sampling containers should have the same properties (and the same

contamination background, if present). To achieve this task SSOG purchased 150 PE-HD (polyethylene, high density) food containers having a capacity of 10 litres. These containers were delivered to the participating partners who used them for their own sampling operations. In few cases only, where container delivery was difficult or non economic, the samples were collected in containers directly provided by sample supplier. In these cases the samples, immediately after reception in SSOG Institute, were transferred into the standard containers and this unusual procedure was recorded on the sampling form.

All partners began to ask for samples in their own Country and when each sample was ready, it was delivered along with the corresponding sampling form, to SSOG Institute, in charge for sample collection, classification and distribution. All samples were identified by means of a temporary code, indicating the providing partner and a progressive number, regardless of the different origin of sample, clearly recorded into the corresponding sampling form.

3.3 SAMPLE FINAL CLASSIFICATION:

Once arrived at the final destination the samples were recorded and classified, according to the nature of the feedstock and the production technology, as described below. Each sample was identified by an acronym and a progressive number.

Acronym list

- Acid oils from chemical refining AOCHE
- Acid oils from physical refining AOPHY
- Lecithins LECI
- Recycled cooking oils RECY
- Animal fats ANFA
- Exhausted bleaching earths oils EBE
- Fish oils FISH

• Hydrogenated fats from by-product fats HYBY

• Miscellaneous interesterification products MIX

• Fatty acids calcium soaps FACS

Contemporarily, each sample was recorded in a .xls file, prepared by WP1 co-ordinator and used to monitor the state of sampling procedure in terms of quantity, quality, origin and source. Other sheets of the same file were organised for data input by each partner. The standardised data input was designed to make easier the data handling and correlation at the end of analytical work.

A number of 124 (one hundred twenty four) samples was distributed among the research team. The sub-samples were supplied at all partners in new dark glass containers of a previously agreed size, properly sealed and labelled.

The enclosed series of pictures describes what was done since samples arrival in SSOG till delivery of sub-samples to partners.

Figure 15 – Sample arrival in SSOG

Figure 16 – Sample heating in water bath (approx. 20 °C above the melting point)

Figure 17 – Sub-sample preparation after homogenization

Figure 18 – Filling the dark glass bottles

Figure 19 – Labelled samples ready for delivery to WP1 partners

3.4 COLLECTED SAMPLES

The situation of samples collection and delivery can be summarised looking at the graphs, prepared for each category, where target and actual number of samples are compared.

A short description of the production technology for each product is enclosed. A complete description of each process is reported in deliverable D1 *"Document about chemical composition, level of contamination, presence of degradation compounds"*.

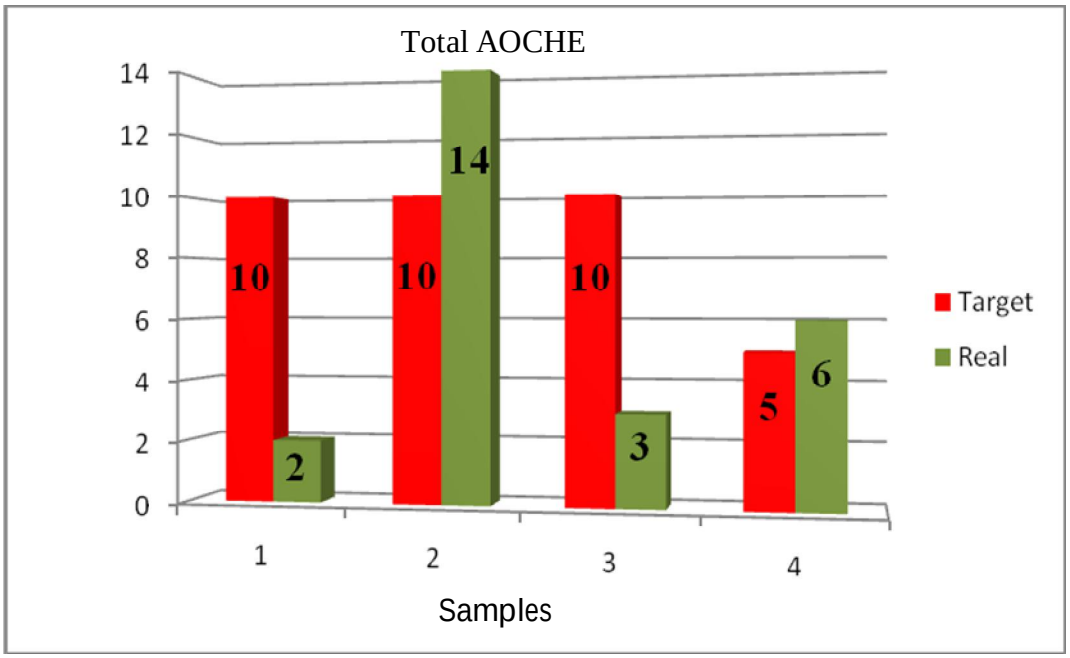

Fig.20: 1–from animal fats; 2-from vegetable oils; 3–from tropical oils; 4 – from olive oil

3.4.1 Acid Oils from Chemical Refining (AOCHE)

These feedstocks were collected in oils and fats refineries and they represent the first by-product of chemical refining process.

After a pre-treatment with a reduced amount of diluted acid such as phosphoric acid, citric acid, etc., the crude oil is treated with a 10-20 % NaOH aqueous solution (in quantity sufficient to neutralise the existing free fatty acid content, the mineral acid added, increased of a slight excess for a complete acidity removal and to ensure a suitable fluidity of obtained soaps). At the end of neutralisation step the formed soaps are removed by settling or centrifugation and the resulting neutralised oil is washed with water to remove the last traces of alkali. The obtained soap fraction, generally recognised as “soapstock” is transferred to a splitting section where, after treatment with mineral acid, the fatty mixture constituted of free fatty acids, oil and impurities (acid oil from chemical refining - AOCHE) is recovered from the oil/water emulsion. The sampling activity was easy to carry out for both vegetable oils and olive oils by-products, while difficulties were found in obtaining by-products from tropical oils because of the reduced number of European factories processing these oils.

Furthermore the actual European situation about BSE disease made a dramatic reduction of factories refining animal fats: for this reason the only two samples collected in this sub-category are of animal origin, one from pork and the other from fish. The real impact of this sub-category on the total economy of fats for feeding purposes (actual and future) can be regarded as negligible.

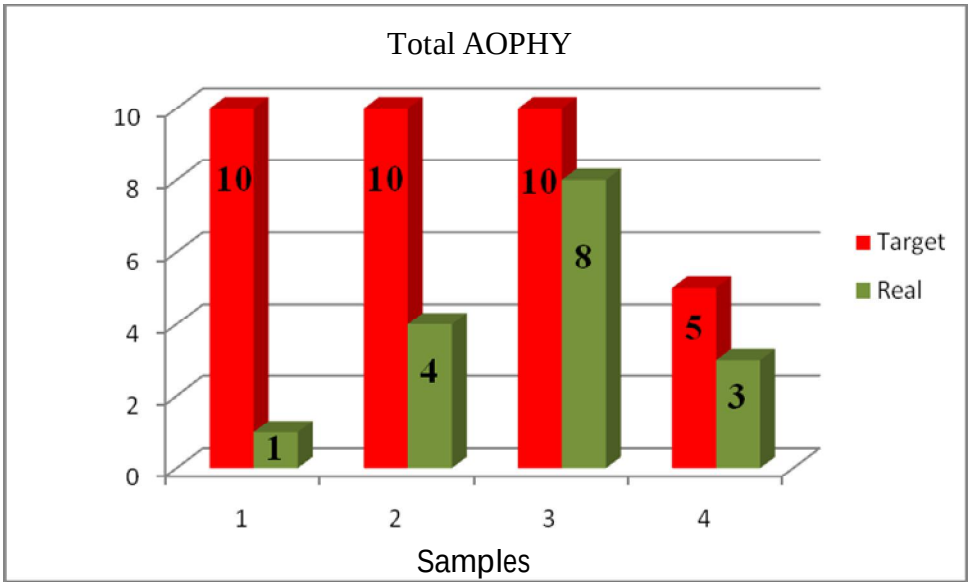

Fig. 21: 1– from animal fats; 2- from vegetable oils; 3– from tropical oils; 4 – from olive oil

3.4.2 Acid Oils from Physical Refining (AOPHY)

These feedstocks were collected in oils and fats refineries and they represent the last by-product of physical refining process (dewaxing excluded). The physical neutralisation step is carried out on acid washed, bleached oils. By means of this operation the free fatty acids are removed by steam/vacuum distillation. During this step also deodorization of oil takes place and the resulting oil, after the final cooling, represents the refined product. The acid oils from physical refining (AOPHY), recovered inside the distillation unit and consisting of free fatty acids, components of the unsaponifiable fraction, odorous compounds, etc, represent the by product of interest for our research project.

The situation for AOPHYs can be considered very similar to the AOCHEs one, but the number of collected samples was lower, because the European factories actually are mainly equipped for chemical refining. No samples derived from animal fats were collected. In this case also the real impact of this last sub-category on the total economy of fats for feeding purposes (actual and future) is close to zero.

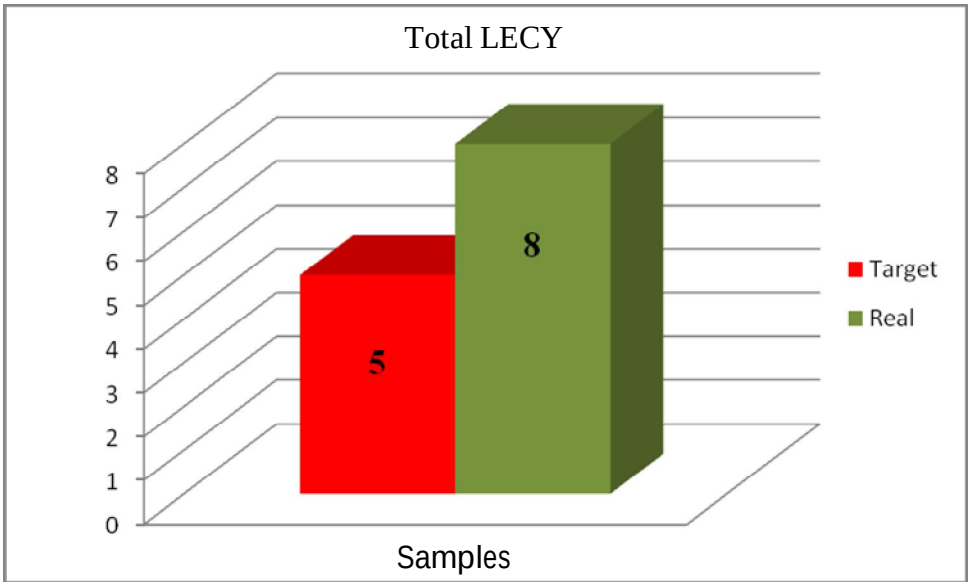

Fig. 22. Profile of sampling from Lecy

3.4.3 Lecithins (LECI)

The lecithin fraction is a by-product of crushing factories. Crude oils coming from both mechanical and solvent extraction are treated with hot water or steam This addition provokes and hydration effect of phospholipids that become insoluble in oil. The formed sludge is then separated by centrifugation and sent to a drying unit to remove the added water. The honey-like dark fluid obtained in this way is called lecithin.

A number of samples exceeding the target value was collected in this case. Lecithins represent a very interesting feedstock for feed preparation because apart from the nutritional value they have also some functional properties, such as the ability to link water, to reduce the powder content of feeds reducing the explosion hazard, etc.

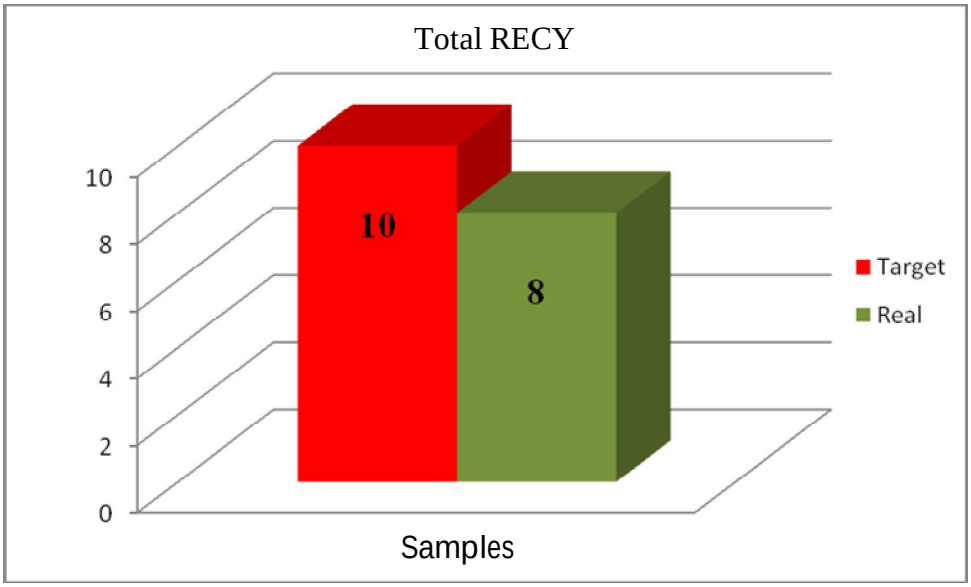

Fig. 23. Profile of sampling from Lecy

3.4.4 Recycled Oils (RECY)

These oils were collected in factories specially devoted to oils and fats recycling for industrial purposes. The exhausted oils discarded by food industries and catering kitchens are generally collected by authorised companies who process these products using simple treatments before reselling theme. The processing in general consists of a filtration to remove solid particles, water washing to reduce the ash content (mainly NaCl coming from fried foods) and a final drying step to remove the residual water. In some cases also a dry cold fractionation step is carried out to obtain a liquid and a solid fraction of recycled oils.

We must remind that actually the use of RECY oils for feeding purposes is restricted in Europe at a total amount of 450.000 tonnes that can be blended with animal fats because of concerns about dioxin content, according to the EU Directive 2003/57/EC

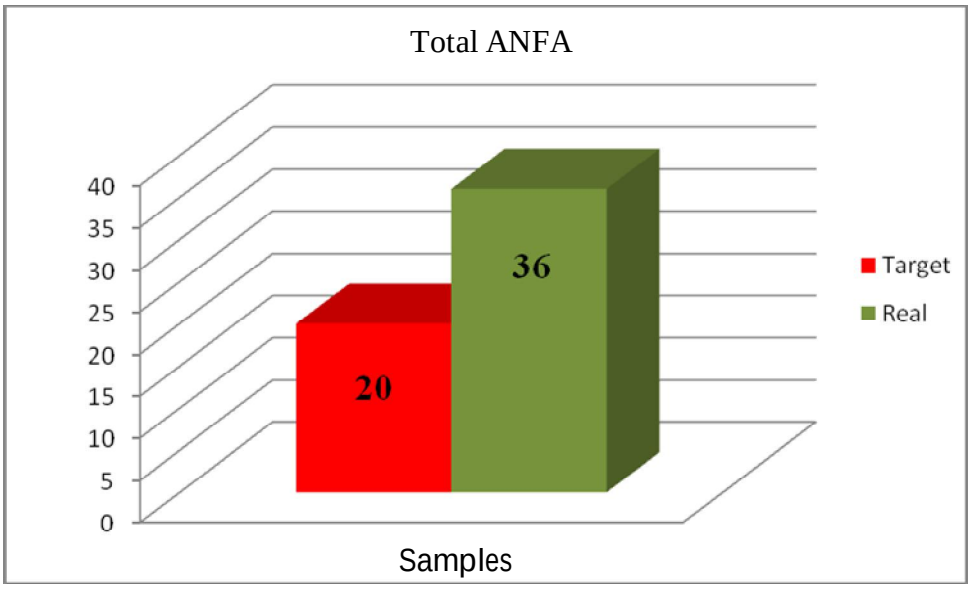

Fig. 24. Profile of sampling from ANFA

3.4.5 Animal Fats (ANFA)

A number of crude animal fats for feeding purposes was collected in every participating Country, exceeding the target value. This higher value could in some way compensate the reduced value for animal AOCHEs and AOPHYs. Animal fats are generally recovered from animal tissues by cold or hot rendering, consisting of an heat treatment of raw material, in presence of steam or water with the double scope of protein denaturation and fat melting. After cooking the material is passed through a screw press for the solid/liquid separation. The liquid fraction obtained from pressing is then centrifuged for the separation of fatty and aqueous fractions. The solid residue, after drying, represents the animal meal. Trade and use of animal fats are regulated by EC Regulation 1774/2002, issued to counteract the diffusion of BSE. In this moment the consumers in several European Countries are against the use of animal fats for feeding purposes because of ethical, religious and healthy issues. For this reasons an accurate system for animal fats detection in feeds is strongly needed.

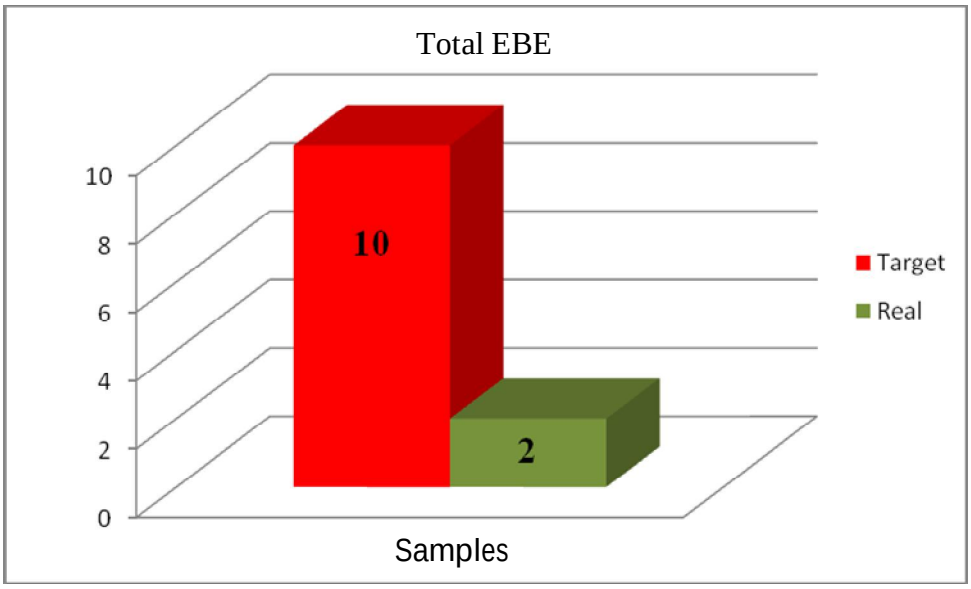

Fig. 25. Profile of sampling from EBE

3.4.6 Oils recovered from Exhausted Bleaching Earth (EBE).

The name of this by-product can be used as a descriptor of this category. The exhausted bleaching earths coming from both chemical and physical refining of oils and fats are collected by specialized companies who carry out a solvent recovery of the oil contained in it. The generally utilised solvent is hexane and the obtained oils are delivered for low added value industrial applications. Because of the poor value of the obtained oil not all EBEs are processed as described above. The main part of it is burnt in oven for the production of concrete, in order to recover the residual energy, or directly disposed in dumps.

In this case only two Italian samples were collected, from the same factory. Probably the target number of samples was over dimensioned with respect to the real availability situation and with the consideration that the use of EBE oils for feeding purposes is actually forbidden. Nevertheless the study of EBE samples was continued for classification purposes, in order to find the peculiar properties of this category. The final scope of this action must be in this case the protection of the feeding fats chain from unwanted, accidental or voluntary, additions of EBE oils at feeding fats.

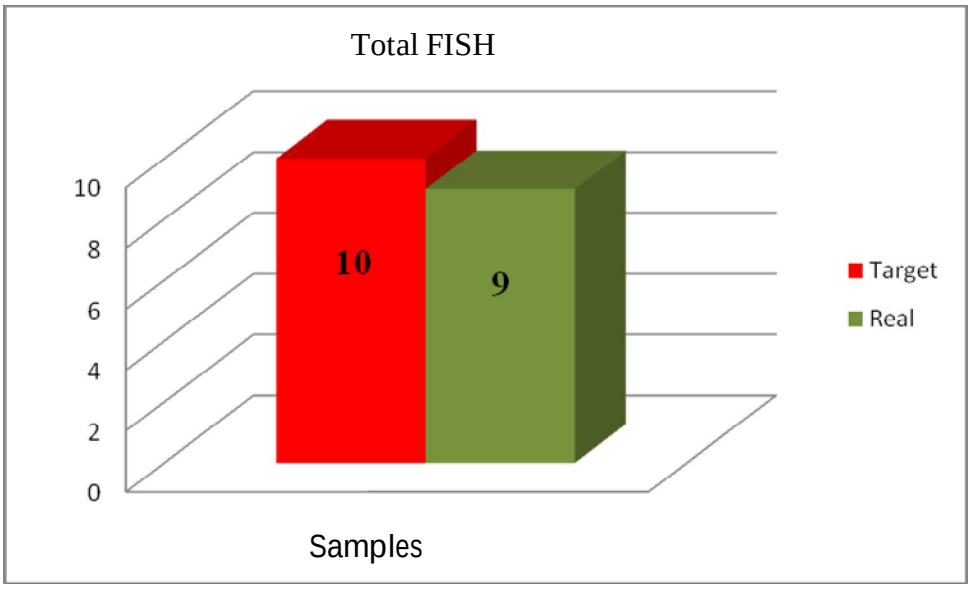

Fig. 26. Profile of sampling from FISH

3.4.7 Fish oils (FISH)

The fish oils are obtained using the same process described above for animal fats. The target value looks adequate in consideration of the distribution and of the economic impact. One barrier for the use of fish oils in feeds is represented by their high oxidability which can lead to a strong smell. This fishy flavour can be partly transferred to the meat when fish oils are used in feeds in high amounts. Several concerns about fish oils are also represented by the concentration effect of lipophilic pollutants such as dioxines, PCBs, PBDEs, etc. in fish tissues living in polluted sea.

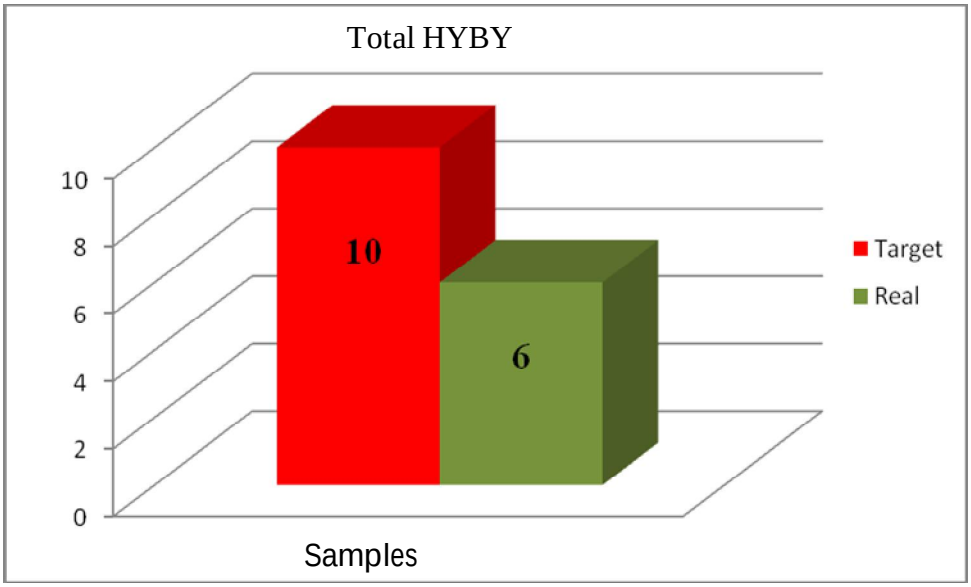

Fig. 27. Profile of sampling from HYBY

3.4.8 Hydrogenated By-products (HYBY)

This category, with samples collected from Malaysia and Spain is represented by Hydrogenated Palm Fatty Acid Distilled (HPFAD) of Malaysian origin and of hydrogenated fatty acids of animal origin. The fatty acid fraction coming from the physical refining process is hydrogenated for the nearly complete saturation of fatty acids double bonds. The obtained product shows a very high melting point and are in use for special applications such as rumen by-pass fats or protector for dietary supplements.

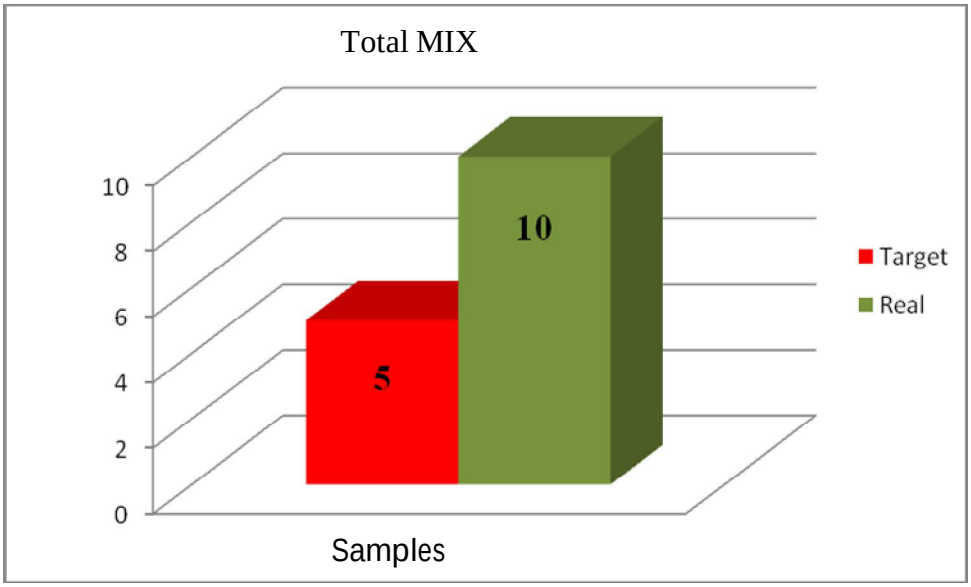

Fig. 28. Profile of sampling from Mix

3.4.9 Miscellaneous (MIX)

In this group all samples that cannot be classified in other groups are comprised. The six collected samples (exceeding the target value) are all coming from Italy. The sample MIX 6 is represented by refined soybean oil for human consumption, while the other MIX representatives are high tech products prepared from vegetable oils by hydrogenation (high trans fatty acid content, used for milk replacer preparation) of by superglycerination (mono- and diglycerides to cover special nutritional requirements of monogastric animals). This category is of growing interest for this project, for modern animal nutrition and for the interesting added values of these products bonded to processing.

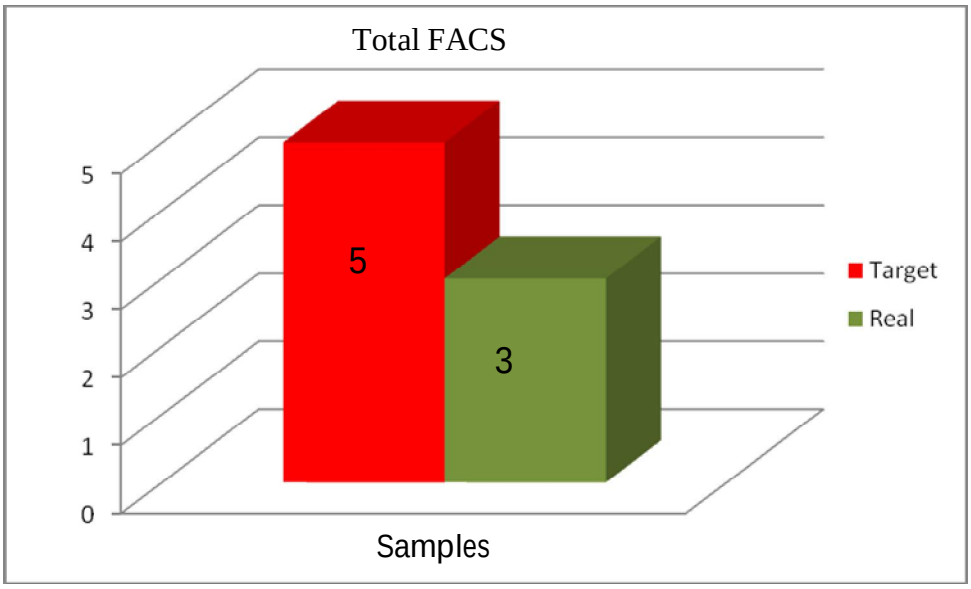

Fig. 29. Profile of sampling from FACS

3.4.10 Fatty Acids Calcium Soaps (FACS)

These products appeared on the market during the early ’80, after the results of the basic researches on the effect of protected fats in production performances were published in Europe. FACS are an insoluble product that is not attacked by rumen bacteria but that is readily available in acidic environment, such as in abomasus, leading to a quantitative energy transfer accompanied by the possibility of feeding the animal with a specific fatty acid blend, avoiding the buffering effect of microbial hydrogenation and isomerisation taking place in rumen with animals fed with classic feeds. The industrial preparation of FACS can be carried out using three different techniques: *i)* by direct neutralisation of distilled fatty acids with slaked lime [$Ca(OH)_2$], *ii)* by double exchange reaction of fatty acid sodium/potassium soaps with Calcium chloride [$CaCl_2$] and *iii)* by direct saponification of a triglyceridic fat with slaked lime [$Ca(OH)_2$].

3.5 CHARACTERISATION OF EXPERIMENTAL FEEDS

Feed samples were taken 3 times during the experimental period for analysis. The following parameters have been determined:

• Dry matter, ashes, crude protein, ether extract and crude fibre following the AOAC (1995) procedures

• Gross energy by adiabatic calorimetric bomb (IKA-C4000, Jankel-Kunke, Staufen, Germany)

The composition and the average nutrient analyses of experimental feeds for broiler chickens are shown in Table 1.

Table 1. Ingredients and average nutrient composition of the broiler chicken diets

Ingredient (%)	value	Nutrient Composition	value
Corn	52.7	Gross Energy (kcal/kg)	4968
Soybean Meal (47% of CP)	30.0	Dry Matter, %	90.8
Full-fat Soybean	6.0	Ash, %	6.5
Added Fat Material	6.0	Crude Protein, %	21.1
HCl L-Lysine	0.3	Ether Extract, %	9.5
DL-Methionine (99%)	0.2	Crude Fibre, %	3.8
Dicalcium Phosphate	2.5		
Calcium Carbonate	1.3		
Salt	0.5		
Vitamin and Mineral Premix1	0.5		

1Composition of vitamin and mineral premix (1 kg of feed contained): Vitamin A: 6000 UI; Vitamin D3: 1200 UI; Vitamin E: 10 mg; Vitamin K_3: 1.5 mg; Vitamin B_1: 1.1 mg; Vitamin B_2: 4 mg; Vitamin B_6: 1.5 mg; Vitamin B_{12}: 9 µg; Folic acid: 4 mg; Biotin: 50 µg; Pantothenic acid: 6 mg; Nicotinic acid: 21 mg; Choline: 360 mg; Mn: 75 mg; Zn: 50 mg; I: 0,18 mg; Fe: 30 mg; Cu: 6 mg; Se: 0.2 mg; Co: 0.2; Ethoxiquin: 16 mg. Addition of Choline Chloride 15 mg.

3.5.1 Sampling and processing of samples

In order to asses the risks related to the use of recycled fats in the feed industry and animal production, we have selected different diagnostic tools as daily controls and records of incidences including deaths during the growing period, post-mortem inspection and the evaluation of different biochemical, microbiological or histological parameters in animals housed on floor pens and sequentially slaughtered. The effect of age was also considered.

The post-mortem inspection was made in all animals by a pathologist in order to evaluate any pathological signs derived from the treatments. Muscular tissue, liver, spleen, thymus, Fabricio's bursa and digestive tract were inspected. The parameters evaluated in these tissues and organs were: consistency, size, colour and the presence of macroscopic lesions. Table 2 shows the types and number of samples recorded during the experimental procedure.

Table 2. Summary of samples colleted and analytical determinations

Sample	Analytical Determination	Sampling days (days old)	Samp./Treat in each exp.	Analyzed samples
Blood	Haemolysis rate TBARs[1]	9, 13, 21 and 37	8*	256
Cecal content	Microbiota Diversity (t-RFLP) SCFA[2] production	9, 21 and 37	8*	192
Excreta	Oocists of *Eimeria* (*coccidia* McMaster determination)	18 and 37	4 **	64
Jejunum Portion	Histomorphology: Villous height Crypt depth Intraepithelial lymphocytes	9 and 21	8	128

* Day 9: pool of samples of 2 birds for constituting analytical samples (16 animals). ** pool of excreta per replicate.
[1]TBARs: thiobarbituric acid reactive substances. [2]SCFA: short chain fatty acids.

Samples were collected and stored as follows:

• Blood: 1 ml of blood sample per bird was collected in heparin coated tubes (VenosafeTM VF-054SHL). Blood samples were stored at 4ºC until analysis.

• Caecal content: Samples of caecal content were collected in eppendorf tubes of 1.5 ml and were immediately frozen at –80 ºC until analysis.

• Excreta: A representative sample of excreta was collected from each pen. Samples were stored at 4 ºC until *coccidia* determination.

• Jejunum portion: A jejunum portion (10 cm long), close to the caecum, was tied off, opened and collected. Samples for histological study were stored in formalin at room temperature.

3.6 FEED MANUFACTURING

Experimental feeds for chicken and rabbits were formulated according to their different nutritional needs (NRC, 1994 for broilers; De Blas and Mateos, 1998 for rabbits) and including 6% or 3% (for chickens and rabbits, respectively) of the appropriate fat. The included fats were:

• "*Trans* fatty acids" experiment: a palm fatty acid distillate and its corresponding hydrogenated palm fatty acid distillate, respectively with low and high *trans* fatty acid content

• “Dioxins and PCBs” experiment: two fish oils, one with very low levels of dioxins/PCBs and another with quite high levels, after enrichment by addition of standards from Wellington Laboratories, 1 ml of EPA-1613PAR (PCDDs/PCDFs) and 3.6 ml of WP-STK (PCBs) in 100 kg, because the batch of this fish oil provided for feed manufacturing was not so high in contaminants as expected according the previous analysis

• “PAHs” experiment: two acid oils from chemical refining of olive oil and olive pomace oil, respectively with low and high PAHs content

• “Lipid oxidation” experiment: a vegetable oil, fresh or recycled after using in a commercial frying process and then heated at 165-170 ºC during 8 hours, respectively with low and high level of lipid oxidation

A total of 16000 kg of chicken and rabbit experimental feeds were manufactured in December 2005, January 2006 and April 2006. Feeds for chicken were sent to the experimental farm located in UAB. In the case of rabbit feeds, robenidine was included as coccidiostatic and, in the “PAHs” and “lipid oxidation” experiments, neomycin, oxytetracycline and tiamulin were also included for preventing Epizootic Rabbit Enteropathy (ERE), because very high mortality rate with ERE signs occurred in early experiments on “*trans* fatty acids” and “dioxins and PCBs”. Batches of rabbit feeds without coccidiostatic and antibiotics were also prepared and distributed during the last fattening week in all cases.

3.7 DEVELOPMENT AND CONTROL OF RABBIT TRIALS

For measuring growith performance with 2 diets (Low and High) in each experiment, weaned (28-day old) three-way crossbred rabbits were housed in individual cages (width=27cm, length=44cm, and height=32cm), with water and feed provided *ad libitum*. Feed intake, weight gain and feed efficiency were recorded between 28 and 63-day old, with an intermediate control at 56-day old, coinciding with the change to withdrawal feeds. From December 2005 to July 2006, a total of 920 weaned rabbits were used (230 animals/experiment, 115 animals/diet). During this period, 5 series

of rabbits were fattened: 3 series for the "*trans* fatty acids" and "dioxins and PCBs" experiments (without antibiotics, with in water enrofloxacin during five days after weaning, and with in water enrofloxacin+neomycin during two weeks after weaning followed with in water apramycin+tylosin during the fourth fattening week, respectively) and 2 series for the "PAHs" and "lipid oxidation" experiments (with in feed antibiotic treatment above described). Because of very high mortality rate in the two earlier series (higher than 70%) and in order to have enough data for obtaining statistically consistent results, an additional series with 212 animals were controlled in September-October 2006 for the "*trans* fatty acids" and "dioxins and PCBs" experiments (106 animals/experiment, 53 animals/diet; with the in water antibiotic treatment described for the third series).

On the other hand, a total of 96 six-week old rabbits, allocated in individual metabolic cages (width=29cm, length=48cm, and height=32cm), were used for measuring apparent digestibility of nutrients (24 animals/experiment, 12 animals/diet). A European reference method (Perez *et al*., 1995) was used, with an adaptation period of seven days followed by feed intake control and total faecal collection during four days, water and feed being always provided *ad libitum*.

Finally, an experiment was performed in order to test the possible effects of the factors under studying in the used fatty recycled materials on the hepatic and renal functions, and on the caecal ambient. A total of 128 weaned 28-day old rabbits (16 animals per diet, 8 diets) were allocated in collective cages and fed ad *libitum* on the experimental diets. At slaughtering, at 63-day old, blood and caecal digesta samples were taken from a total of 60 healthy surviving animals (7-9 animals per diet).

All the trials were carried out at the Universidad Politécnica of Valencia and were subject to agreement of Animal Protocol Review Committee of the Institution. The protocol, housing, husbandry and slaughtering conditions agreed to current European Union guidelines.

3.8 CHARACTERISATION OF EXPERIMENTAL FEEDS

Feed samples were taken 3 times during all the experimental periods for analysis. The following parameters have been determined:

• Dry matter, ashes, crude protein, ether extract and crude fibre following the AOAC (1995) procedures

• Gross energy by adiabatic calorimetric bomb (IKA-C4000, Jankel-Kunke, Staufen, Germany)

• Detergent fibres according Van Soest *et al.* (1991)

The composition and the average nutrient analyses of experimental feeds for rabbits are shown in Table 3.

Table 3. Ingredients and average nutrient composition of the rabbit diets

Ingredient (%)	value	Nutrient Composition	value
Barley	10.0	Gross energy (kcal/kg)	3908
Beet Pulp	30.0	Dry matter, %	89.5
Sunflower Meal (30% of CP)	20.0	Ash, %	8.5
Alfalfa Hay	34.0	Crude protein, %	13.1
Added Fat Material	3.0	Ether Extract, %	4.2
HCl L-Lysine	0.35	Crude Fibre, %	20.1
DL-Methionine (99%)	0.2	Neutral Detergent Fibre	35.4
L-Threonine	0.15	Acid Detergent Fibre	22.7
Dicalcium Phosphate	1.3	Acid Detergent Lignin	4.5
Salt	0.5		
Vitamin and Mineral Premix[1]	0.5		

[1]Composition of vitamin and mineral premix (1 kg of feed contained): Vitamin A: 8375 UI; Vitamin D3: 750 UI; Vitamin E: 20 mg; Vitamin K_3: 1 mg; Vitamin B_1: 1 mg; Vitamin B_2: 2 mg; Vitamin B_6: 1 mg; Nicotinic acid: 20 mg; Choline Chloride: 250 mg; Mg: 290 mg; Mn: 20 mg; Zn: 60 mg; I: 1.25 mg; Fe: 26 mg; Cu: 10 mg; Co: 0.7; BHA+Ethoxiquin: 4 mg.

3.8.1 Sampling and processing of samples

Animals were electrically stunned, then slaughtered and blood samples directly taken into 10 ml centrifuge tubes. After clotting at ambient temperature, serum was obtained by centrifugation at 1500xg during 10 minutes, being stored in eppendorfs vials of 2 ml at –80 ºC until analysis for testing the hepatic and renal functions.

After, the caecum was separated and its content was collected into a flask for measuring the pH (pH-meter GLP 21, CRISON, Alella, Spain). Aliquots of about 1 g of caecal content were weighted into 5 ml centrifuge tubes and added with 3 ml of 2% sulphuric acid solution or 2 ml of 2% ortophosphoric acid for analysing NH3 and short chain fatty acids (SCFA), respectively. Samples for SCFA analysis were centrifuged at 10000xg during 10 minutes and the liquid phase was collected into eppendorfs vials of 0.5 ml. Finally, all samples were stored at –80 ºC until analysis. The rest of caecal content was stored at –20 ºC until dry matter analysis.

Table 4. Summary of samples colleted and analytical determinations

Sample	Analytical Determination	Analyzed samples
Serum	Gamma glutamyl transferase (GGT) Glutamic oxaloacetic transaminase (GOT) Glutamic pyruvic transaminase (GPT) Alkaline phosphatase (ALP) Urea Creatinine	60
Cecal content	pH Dry matter NH_3 concentration SCFA concentration	60

3.8.2 Feeding manufacturing

Experimental feeds for chicken and rabbits were formulated according to their different nutritional needs (NRC, 1994 for broilers; De Blas and Mateos, 1998 for rabbits) and including 6% or 3% (for chickens and rabbits, respectively) of the appropriate fat. The included fats were:

• "*Trans* fatty acids" experiment: a palm fatty acid distillate and its corresponding hydrogenated palm fatty acid distillate, respectively with low and high *trans* fatty acid content

• "Dioxins and PCBs" experiment: two fish oils, one with very low levels of dioxins/PCBs and another with quite high levels, after enrichment by addition of standards from Wellington Laboratories, 1 ml of EPA-1613PAR (PCDDs/PCDFs) and 3.6 ml of WP-STK (PCBs) in 100 kg,

because the batch of this fish oil provided for feed manufacturing was not so high in contaminants as expected according the previous analysis

• “PAHs” experiment: two acid oils from chemical refining of olive oil and olive pomace oil, respectively with low and high PAHs content

• “Lipid oxidation” experiment: a vegetable oil, fresh or recycled after using in a commercial frying process and then heated at 165-170 ºC during 8 hours, respectively with low and high level of lipid oxidation Consequently, chicken and rabbit feeds obtained for the four different experiments had low or high level of *trans* fatty acids, dioxins/PCBs, PAHs or lipid oxidation, respectively.

The remaining feed was made of an identical basal mix (based in corn, soybean meal and extruded full-fat soybean in chicken feeds; based in alfalfa, sugar beet pulp, barley and sunflower meal in rabbit feeds). Samples of these seven raw materials were sent to CSIC and to UBORD to confirm their low dioxins/PCBs and PAHs/PBDEs contents respectively. To minimize dietary variations other than the added fat: i) the same batches of each one of these seven raw materials were used in all experimental feeds, ii) batches of basal mix were prepared and then aliquoted to obtain identical participation of each batch in each experimental feed.

In the case of rabbit feeds, robenidine was included as coccidiostatic and, in the “PAHs” and “lipid oxidation” experiments, neomycin, oxytetracycline and tiamulin were also included for preventing Epizootic Rabbit Enteropathy (ERE), because very high mortality rate with ERE signs occurred in early experiments on “*trans* fatty acids” and “dioxins and PCBs”. Batches of rabbit feeds without coccidiostatic and antibiotics were also prepared and distributed during the last fattening week in all cases.

3.9 CHARACTERIZATION OF EXPERIMENTAL FEEDS AND OILS

Feed samples were taken 3 times during all the experimental periods for analysis. The following parameters have been determined:

• Dry matter, ashes, crude protein, ether extract and crude fibre following the AOAC (1995) procedures

• Gross energy by adiabatic calorimetric bomb (IKA-C4000, Jankel-Kunke, Staufen, Germany)

• Detergent fibres according Van Soest *et al.* (1991) The composition and the average nutrient analyses of experimental feeds for broiler chickens and rabbits are shown in Tables 1 and 2, respectively.

Table 5. Ingredients and average nutrient composition of the broiler chicken diets

Ingredient (%)	value	Nutrient Composition	value
Corn	52.7	Gross Energy (kcal/kg)	4968
Soybean Meal (47% of CP)	30.0	Dry Matter, %	90.8
Full-fat Soybean	6.0	Ash, %	6.5
Added Fat Material	6.0	Crude Protein, %	21.1
HCl L-Lysine	0.3	Ether Extract, %	9.5
DL-Methionine (99%)	0.2	Crude Fibre, %	3.8
Dicalcium Phosphate	2.5		
Calcium Carbonate	1.3		
Salt	0.5		
Vitamin and Mineral Premix[1]	0.5		

[1]Composition of vitamin and mineral premix (1 kg of feed contained): Vitamin A: 6000 UI; Vitamin D3: 1200 UI; Vitamin E: 10 mg; Vitamin K_3: 1.5 mg; Vitamin B_1: 1.1 mg; Vitamin B_2: 4 mg; Vitamin B_6: 1.5 mg; Vitamin B_{12}: 9 µg; Folic acid: 4 mg; Biotin: 50 µg; Pantothenic acid: 6 mg; Nicotinic acid: 21 mg; Choline: 360 mg; Mn: 75 mg; Zn: 50 mg; I: 0,18 mg; Fe: 30 mg; Cu: 6 mg; Se: 0.2 mg; Co: 0.2; Ethoxiquin: 16 mg. Addition of Choline Chloride 15 mg.

Table 6. Ingredients and average nutrient composition of the rabbit diets

Ingredient (%)	value	Nutrient Composition	value
Barley	10.0	Gross energy (kcal/kg)	3908
Beet Pulp	30.0	Dry matter, %	89.5
Sunflower Meal (30% of CP)	20.0	Ash, %	8.5
Alfalfa Hay	34.0	Crude protein, %	13.1
Added Fat Material	3.0	Ether Extract, %	4.2
HCl L-Lysine	0.35	Crude Fibre, %	20.1
DL-Methionine (99%)	0.2	Neutral Detergent Fibre	35.4
L-Threonine	0.15	Acid Detergent Fibre	22.7
Dicalcium Phosphate	1.3	Acid Detergent Lignin	4.5
Salt	0.5		
Vitamin and Mineral Premix[1]	0.5		

[1]Composition of vitamin and mineral premix (1 kg of feed contained): Vitamin A: 8375 UI; Vitamin D3: 750 UI; Vitamin E: 20 mg; Vitamin K_3: 1 mg; Vitamin B_1: 1 mg; Vitamin B_2: 2 mg; Vitamin B_6: 1 mg; Nicotinic acid: 20 mg; Choline Chloride: 250 mg; Mg: 290 mg; Mn: 20 mg; Zn: 60 mg; I: 1.25 mg; Fe: 26 mg; Cu: 10 mg; Co: 0.7; BHA+Ethoxiquin: 4 mg.

Tables 7 and 8 show respectively some distinguishing traits and the fatty acid profile of different fats used in the trials. In Tables 9 and 10, the degree of alteration or contamination of broiler chicken and rabbit feeds respectively is presented.

Table 7. Level of alteration or contaminants of added fats

Experiment	Treat.[1]	Added Fat	Alteration or contaminant level of the added fat
1. *Trans* Fatty Acids (T)	HT	Hydrogenated Palm FA Fatty distillate	12.30 % Total *Trans* Fatty Acids [A]
	LT	Palm FA distillate	0.71 % Total *Trans* Fatty Acids [A]
2. Dioxins and PCBs (C)	HC	Fish oil	28.8 pg WHO-TEQ PCDD/Fs + DL-PCBs/g oil [B]
	LC	Fish oil	9.64 pg WHO-TEQ PCDD/Fs + DL-PCBs/g oil [B]
3. PAHs (P)	HP	Olive-pomace acid oil	5290 ng PAHs/g oil + PBDEs < 2 ng/g oil [C]
	LP	Olive acid oil	<18 ng PAHs/g oil + PBDEs < 2 ng/g oil [C]
4. Lipid Oxidation (O)	HO	Sunflower/olive oils (70:30 v/v) after frying	6.61 % Polymers, 67.43 p-anisidine value [D]
	LO	Sunflower/olive oils (70:30 v/v)	0.35 % Polymers, 2.74 p-anisidine value [D]

[1] H, L = high or low level of degradation or contaminants

[A] Analyses realized in the Department of Food Science, Bolognia University (Italy); [B] WHO-TEQ = toxic equivalent concentration; PCDD/Fs = polychlorinated dibenzo-dioxins/furans; DL-PCBs = dioxin likepolychlorinated biphenyls; Analyses realized in the Laboratory of Dioxins, Department of Ecotechnology IIQAB-CSIC (Spain); [C] PAHs = polycyclic aromatic hydrocarbons (20 compounds); PBDEs = polybromodiphenylethers (4 compounds); Analyses realized in the Physic & Toxico-Chemist Laboratory, Bordeaux University (France); [D] Analyses realized in the Nutrition and Bromatology Department, Barcelona University (Spain).

Table 8. Fatty acid profile of added fats (% of total fatty acids)

Experiment	Fatty Acids (T)		Dioxins and PCBs (C)		PAHs (P)		Lipid Oxidation (O)	
Fatty Acids*	HT	LT	HC	LC	HP	LP	HO	LO
SFA	84.8	54.1	23.2	30.3	19.0	18.9	14.2	12.4
MUFA	15.2	36.9	37.5	31.2	71.2	71.3	44.9	39.2
PUFA	0	9.0	31.8	33.2	9.8	9.9	40.9	48.4

* SFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.

Table 9. Level of alteration or contaminants of broiler chicken

Experiment	Treat.[1]	Alteration or contaminant level of the experimental feed
1. *Trans* Fatty Acids (T)	HT	5.7 % Total *Trans* Fatty Acids [A]
	LT	0.6 % Total *Trans* Fatty Acids [A]
2. Dioxins and PCBs (C)	HC	1.75 pg WHO-TEQ PCDD/Fs + DL-PCBs/g oil [B]
	LC	0.59 pg WHO-TEQ PCDD/Fs + DL-PCBs/g oil [B]
3. PAHs (P)	HP	33.97 ng PAHs/g oil + PBDEs < 2 ng/g oil [C]
	LP	2.07 ng PAHs/g oil + PBDEs < 2 ng/g oil [C]
4. Lipid Oxidation (O)	HO	15.62 p-anisidine value [D]
	LO	4.32 p-anisidine value [D]

[1] H, L = high or low level of degradation or contaminants

[A] Analyses realized in the Department of Food Science, Bolognia University (Italy); [B] WHO-TEQ = toxic equivalent concentration; PCDD/Fs = polychlorinated dibenzo-dioxins/furans; DL-PCBs = dioxin likepolychlorinated biphenyls; Analyses realized in the Laboratory of Dioxins, Department of Ecotechnology IIQAB-CSIC (Spain); [C] PAHs = polycyclic aromatic hydrocarbons (20 compounds); PBDEs = polybromodiphenylethers (4 compounds); Analyses realized in the Physic & Toxico-Chemist Laboratory, Bordeaux University (France); [D] Analyses realized in the Nutrition and Bromatology Department, Barcelona University (Spain).

Table 10. Level of alteration or contaminants of rabbit feeds

Experiment	Treat.[1]	Alteration or contaminant level of the experimental feed
1. *Trans* Fatty Acids (T)	HT	5.7 % Total *Trans* Fatty Acids [A]
	LT	0.5 % Total *Trans* Fatty Acids [A]
2. Dioxins and PCBs (C)	HC	0.86 pg WHO-TEQ PCDD/Fs + DL-PCBs/g oil [B]
	LC	0.33 pg WHO-TEQ PCDD/Fs + DL-PCBs/g oil [B]
3. PAHs (P)	HP	13.87 ng PAHs/g oil + PBDEs < 2 ng/g oil [C]
	LP	2.71 ng PAHs/g oil + PBDEs < 2 ng/g oil [C]
4. Lipid Oxidation (O)	HO	Not analysed
	LO	Not analysed

[1] H, L = high or low level of degradation or contaminants

[A] Analyses realized in the Department of Food Science, Bolognia University (Italy); [B] WHO-TEQ = toxic equivalent concentration; PCDD/Fs = polychlorinated dibenzo-dioxins/furans; DL-PCBs = dioxin likepolychlorinated biphenyls; Analyses realized in the Laboratory of Dioxins, Department of Ecotechnology IIQAB-CSIC (Spain); [C] PAHs = polycyclic aromatic hydrocarbons (20 compounds); PBDEs = polybromodiphenylethers (4 compounds); Analyses realized in the Physic & Toxico-Chemist Laboratory, Bordeaux University (France).

3.10. EXPERIMENTAL DESIGN

The feeding trials with broiler chickens were performed at the Experimental Farms of the University Autònoma of Barcelona (UAB) and the rabbit feeding trials were carried out at the University Politécnica of Valencia (UPV).

In both species, growth and digestibility trials were conducted. For each species, the same experimental protocols were followed in all the experiments. These protocols received prior approval from Animal Protocol Review Committee of both institutions. The protocols, housing, husbandry and slaughtering conditions conformed to current European Union guidelines.

3.10.1. Growth trials

Poultry and rabbit trials were scheduled in parallel to start on December 1st 2006. Four growth trials were performed in each species, according the following calendar:

• Broilers:

- Trial 1, *Trans* Fatty Acids (T): 1st December 2005 - 16th January 2006
- Trial 2, Dioxins and PCBs (C): 19th January - 6th March 2006
- Trial 3, PAHs (P): 4th May - 21st June 2006
- Trial 4, Lipid Oxidation (O): 18th May – 4th July 2006

• Rabbits: Trials 1 to 4 were carried out from December 2005 until October 2006

The growing period lasted from 7 to 47 days of life for broiler chickens while the experimental period for rabbits lasted from weaning at 28-day old until 63-day old.

Briefly, the common protocol of growth trials was as follows:

3.10.2. Broiler chicken trials

In each experiment, 64 female Ross 308 broiler chickens of 7 days were randomly distributed into the 2 dietary treatments, High (H) and Low (L), with 8 replicates each. The animals were housed in

groups of 4 in 16 cages (width=65cm, length=60cm, and height=40cm), under standard conditions of temperature, humidity and ventilation.

The experimental period covered from 7 to 47 days of life of chickens. Feed and water were provided *ad libitum* during the trials. The animals were sacrificed in commercial slaughterhouse (Gimave, S.A., Ripollet, Barcelona, Spain).

Daily, room temperature, ventilation, sanitary state and possible losses were strictly controlled.

Feed intake and body weight were controlled at 7, 22 and 47-day old. Average daily feed intake (ADI), average daily weight gain (ADG) and feed efficiency (FE), were estimated from these data. At the end of the experiments, animals were slaughtered and body weight (BW), abdominal fat weight and carcass weight were recorded. The yield carcass (CY, as a % BW) was calculated.

3.10.3. Rabbit trials

For measuring growth performance with 2 diets (Low and High) in each experiment, weaned (28-day old) three-way crossbred rabbits were housed in individual cages (width=27cm, length=44cm, and height=32cm), with water and feed provided *ad libitum*. Feed intake, weight gain and feed efficiency were recorded between 28 and 63-day old, with an intermediate control at 56-day old, coinciding with the change to withdrawal feeds (without coccidiostatic and antibiotics).

From December 2005 to July 2006, a total of 920 weaned rabbits were used (230 animals/experiment, 115 animals/diet). During this period, 5 series of rabbits were fattened: 3 series for the "*trans* fatty acids" and "dioxins and PCBs" experiments (without antibiotics, with in water enrofloxacin during five days after weaning, and with in water enrofloxacin+neomycin during two weeks after weaning followed with in water apramycin+tylosin during the fourth fattening week, respectively) and 2 series for the "PAHs" and "lipid oxidation" experiments (with in feed antibiotic treatment above described). Because of very high mortality rate in the two earlier series (higher than 70%) and in order to have enough data for obtaining statistically consistent results, an additional series with 212 animals were controlled in September-October 2006 for the "*trans* fatty acids" and

"dioxins and PCBs" experiments (106 animals/experiment, 53 animals/diet; with the in water antibiotic treatment described for the third series).

3.11. DIGESTIBILITY TRIALS

3.11.1. Broiler chicken trials

From experiments 2nd (dioxins and PCBs), 3rd (PAHs) and 4th (lipid oxidation), two digestibility balances were performed in each of them in order to calculate the coefficient of apparent digestibility of dry matter, organic matter and ether extract.

The first digestibility balance was performed from day 14th to 16th of age and the second digestibility balance, from day 37th to 39th of age. During 48 hours, all the faeces excreted were collected by replicate, and their weights were registered. Figure 30 describes the chronogram of the trials. Once the excreta were homogenized, they were frozen at -20°C, lyophilized (Alpha I- 6, Christ ®, 336 Osterode/Halz) and stored until analysis following the AOAC (1995) procedures. Apparent digestibility of dry matter, organic matter and ether extract were calculated as the difference between intake and excretion, and expressed as a percentage of the intake.

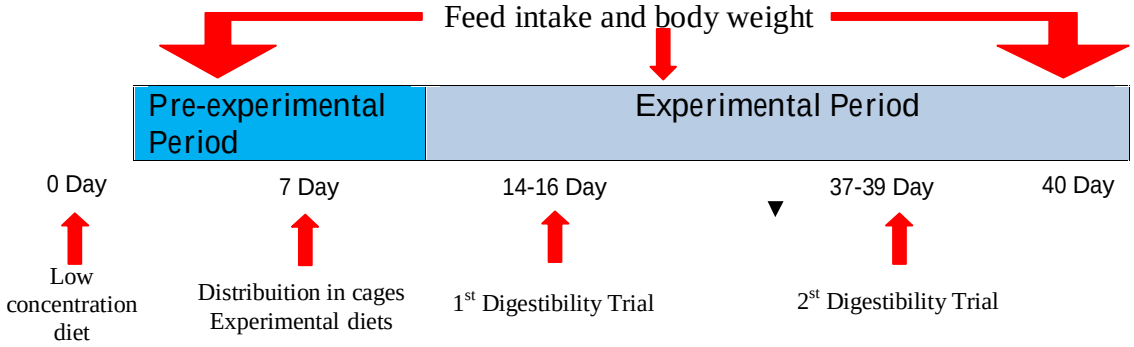

Fig. 30. Chicken trials

3.11.2. Rabbit trials

A total of 96 six-week old rabbits, allocated in individual metabolic cages (width=29cm, length=48cm, and height=32cm), were used for measuring apparent digestibility of nutrients (24 animals/experiment, 12 animals/diet). A European reference method (Perez *et al.*, 1995) was used,

with an adaptation period of seven days followed by feed intake control and total faecal collection during four days, water and feed being always provided *ad libitum*. Figure 31 shows the diagram of the trials. Faeces were frozen at -20°C, oven-dried, ground and analysed.

Coefficients of apparent digestibility of dry matter, organic matter, gross energy, crude protein, ether extract, neutral and acid detergent fibre were determined as the difference between intake and excretion, and expressed as a percentage of the intake. For ether extract (except in 1st experiment, on *trans* fatty acids), neutral and acid detergent fibre no individual faeces but pools of them were analysed and only the average coefficients were obtained.

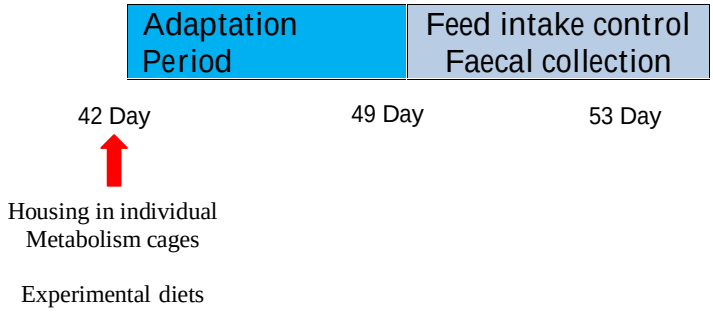

Fig. 31.Rabbits trials

3.11.3. Final remarks

The results presented in this Deliverable allow us to conclude that:

The use of hydrogenated palm fatty acid distillate with 12.4% of total *trans* fatty acids had some negative implications on productive performance or digestibility. But the fact that this fat source also contains a high percentage of saturated fatty acids does not allow us to conclude a net effect of *trans* fatty acid content on the impaired parameters.

The use of fish oils with different content in dioxins and PCBs (9.64 pg or 28.8 pg WHO-TEQ PCDD/Fs + DL-PCBs/g oil) did not cause differences in productive performance but originated some difference in nutrient digestibility in broiler chickens while the opposite occurred in rabbits.

These effects seem better due to the origin, fatty acid profile and quality of the fish oils than to their level of dioxins and PCBs.

The use of acid oils with different PAHs levels (5290 ng or < 18 ng/g oil) did not cause differences in productive performance but originated some differences in nutrient digestibility in broiler chickens and rabbits. These differences could be better due to the origin, fatty acid profile and quality of the acid oils than to their level of PAHs.

The use of sunflower/olive oils before or after frying, with different levels of polymers (6.61% vs. 0.35%, respectively) and p-anisidine values (67.43 vs. 2.74, respectively), did not cause differences in the productive performance or digestibility in both broiler chickens and rabbits.

3.12 EXPERIMENTAL DESIGN

Feed manufacturing and the characterization of oils and experimental feeds.

3.12.1. Broiler chicken trials

In parallel to the development of the feeding trials described in the Deliverable 5, four experimental tests, one for each study, were performed.

Trial 1: *Trans* fatty acids

Trial 2: Dioxins and PCBs

Trial 3: PAHs

Trial 4: Lipid oxidation

All the experiments followed the same experimental protocol and were performed at the Experimental Unit of the Universitat Autònoma of Barcelona and received prior approval from Animal Protocol Review Committee of the Institution. The protocol, housing, husbandry and slaughtering conditions agreed to current European Union guidelines.

Eighty ROSS 308 female broiler chickens five days old, housed on pen floor in groups of 10, were used in each trial. The pens were randomly distributed into 2 treatments of 4 replicates each (4

replicates x 10 animals x 2 treatments, High and Low). The animals were sequentially slaughtered at 9, 13, 21 and 37-day old (that correspond to 4, 8, 16 and 32 in experimental days). Thus, 4 birds per pen (16 animals per treatment) were randomly slaughtered at 9 day (1st sacrifice), and 2 birds per pen (8 animals per treatment) at the others sampling days (13, 21 and 37-day old).

Feed and water were provided *ad libitum*. Temperature was kept initially at 29ºC and was decreased 1ºC every 3 days until 24 days of age, when it was maintained at 21ºC. Animals were maintained on a photoperiod of 23:1.

The experimental feeds were the same as described in Deliverable 5.

3.12.2. Rabbit trials

After finishing the feeding trials described in the Deliverable 5, an experiment was performed in order to test the possible effects of the factors under studying (levels of trans fatty acids, dioxins and PCBs, PAHs or lipid oxidation) in the used fatty recycled materials on the hepatic and renal functions, and on the caecal ambient.

The trial was carried out at the Universidad Politécnica of Valencia and was subject to agreement of Animal Protocol Review Committee of the Institution. The protocol, housing, husbandry and slaughtering conditions agreed to current European Union guidelines.

A total of 128 weaned 28-day old rabbits (16 animals per diet, 8 diets) were allocated in collective cages and fed ad *libitum* on the experimental diets (described in Deliverable 5). At slaughtering, at 63-day old, blood and caecal digesta samples were taken from a total of 60 healthy surviving animals (7-9 animals per diet).

3.12.3. Sampling and processing of samples

Animals were electrically stunned, then slaughtered and blood samples directly taken into 10 ml centrifuge tubes. After clotting at ambient temperature, serum was obtained by centrifugation at

1500xg during 10 minutes, being stored in eppendorfs vials of 2 ml at –80 ºC until analysis for testing the hepatic and renal functions.

After, the caecum was separated and its content was collected into a flask for measuring the pH (pH-meter GLP 21, CRISON, Alella, Spain). Aliquots of about 1 g of caecal content were weighted into 5 ml centrifuge tubes and added with 3 ml of 2% sulphuric acid solution or 2 ml of 2% orto-phosphoric acid for analysing NH3 and short chain fatty acids (SCFA), respectively.

Samples for SCFA analysis were centrifuged at 10000xg during 10 minutes and the liquid phase was collected into eppendorfs vials of 0.5 ml. Finally, all samples were stored at –80 ºC until analysis. The rest of caecal content was stored at –20 ºC until dry matter analysis.

3.13. SAMPLE PREPARATION OF TURKEY

100 gr of meat turkey into a 500 ml beaker with 300 ml of buffer solution, and mixed for 2 min and followed for 15 min.After time was filtered with a cotton tissue..The meat was twice washed with a buffer solution pH = 5.6. (5,67g\L of NaH2PO4 and 0,365g\L of Na2HPO4 in tridistilled water) a ratio of 1gr/3ml of solution.

After that 50 g of meat was placed into a baker in an ice bath and homogenized for 30 sec using a Ultraturax. Then the sample was tranfered into ULTRA 80 centrifuge at 12000 rpm for 20 min at 4°C. (Through electronic spreadsheet considering that A = 541 (radius of the port capsules) is getting so that 15263 = 11720.11 that is 12000 rpm).The sample obtained was kept into the plastic vacuum bags at –80°C until analysis. (Eric W. Grunwald & mark P. Richards, 2006)

3.14. EXTRACTION OF LIPID FRACTION

The extraction of lipid fraction was done according to a modified version of the method described by Folch *et al*, 1957. They were extracted samples from the freezer; meet and liver were weighed by technical balance. Then they were added 200 ml of solution chloroform: methanol (1:1, v/v) in a

glass bottle sovitel from 500 ml with a screw cap, then homogenized for three minutes.The bottle was kept closed at 60 °C for 20 minutes.

After that the bottle was cold and then was introduced 100 ml chloroform and homogenized using ultra-turrax for 2 minutes. The content of bottle was filtered in Buchner with vacuum. The filtrate was mixed with a 1M KCL solution and left overnight at 4°C in order to obtain phase searation. The lower phase was collected and dried with a rotary evaporator at 40°C. The fat content was determined gravimetrically, then was added a solution of hexane: isopropanol (4:1 v / v). The sample was stored at -20°C until the analysis.

3.15 METHYLATION AND -METHYLATION

The methylation was done by adding a small amount of diazomethane, and then the sample was dry under nitrogen flow. Then fatty acid methyl esters were prepared by reaction the sample with 20 of a 2 N methanolic solution of KOH at room temperature (IUPAC standard method 2.301) and centrifuging at 2000 rpm for 5 min. Therefore added 50 µL of Standard (tricadenoic acid ester methyl) and centrifuging at 1800 rpm for 4 min, then the upper layer, containing the FAMEs, was injected into CG.

The GC instrument consisted of a HRGC series (instruments Carlo Erba, Milan, Italy), with flame ionization detector (FID), and interfaced with a computerised system to capture data (Turbocrom, Perkin Elmer). A RESTEK 2330 (10% cianopropilfenilsilossano, 90% biscianopropil-polisilossano) capillary column: silica, (30 m x 0.25 mm i.d. x 0.2 mm thick film) was used. Helium was used as a gas carrier with a flow rate at 1.25 mL / min; flow split: 46 mL / min, flow purge: 5mL/min; split: 1/42, injector and detector temperature: 240 °C. Column temperature was programmed from 60°C, maintained for 1 min and then increased to 240 °C, with temperature ramp of 4 °C / min. Volume injected: 1 µl.

Figure 32 – Gaschromatography Carlo Erba

The GC instrument used GC8000 series (MFC 800–fision instruments) and Autosampler (AS800–fision instruments) (instruments Carlo Erba, Milan, Italy), equipped with flame ionization (FID), interfaced with a computerised system to capture data (Turbocrom, Perkin Elmer). A CP.SIL 88 (10% cianopropilfenilsilossano, 90% biscianopropil-polisilossano) capillary column: silica was used, (100 m x 0.25 mm i.d. x 0.2 mm thick film); Helium was used as a gas carrier with a flow rate at 1.27 mL / min, split: 1/51, flow split: 65.2 mL/min, flow purge: 5.9 ml/min, injector and detector temperature: 250 °C. Column temperature was programmed from 173°C maintained for 85 min and then increased to 225 °C, a rate of 25 °C/min. Volume injected: 1 µl.

Figure 33 – Gaschromatography

3.16 THIN LAYER CHROMATOGRAPHY, TLC SILVER

The identifaction of *trans* fatty acids from *cis* was done using a TLC silver. This method is based on the different interaction with silver ions, deposited chased to activation of TLC plates through exposure to a solution is AgNO3.

The TLC silver plate (20 cm x 20 cm) were introducted in an aqueous solution of AgNO3 (10% w/v), for 30 sec. After the plate were dryed at 110°C for 30 min. Therefore 2 mg of fat were loaded on the TLC. The mobile phase was a mixtured of n-hexane:diethyl ether (90:10,v/v). The band of *trans* fatty acids was visualized by spraying with a 0.2% ethanolic solution of 2'7'-dichlorofluorescein sodium salt, under UV light (254 nm). The band was then scraped off and the extraction of *trans* fatty acids from the silica was carried out twice with chloroform. The solvent

was finally evapored under nitrogen flow at room temperature and the *trans* fatty acids were then dissolved in *n*-hexane and injected into a GC.

Figure 34 – Room UV light

3.17 TOTAL FATTY ACIDS

Carried out according to-house GLC method. All samples were first methylated with diazomethane (Fieser and Fieser, 1967) and then transmethylated with 2N KOH/methanol (EC. 2002. Enclosure X.B. Regulation L 128/14). Samples were then added with tridecanoic acid methyl ester as internal standard. The derivatized samples were then injected into a GLC instrument, coupled to a 30 m polar fused silica capillary column. The GLC analytical conditions are similar to those described in the official methods. Correction factors for the different FID detector response for fatty acids were calculated by using a commercial fatty acid methyl esters (FAME) standard mixture. This determination was necessary for the quantitation of the octadecanoic acid methyl ester, which was used as internal standard for the quantitation of *trans* fatty acids (TFA) and the conjugated linoleic acid (CLA) isomers. The fatty acid calcium soap (FACS) samples displayed a low solubility in the

solvent used in this method, so they had to be solved in a mixture of acetic acid: chloroform (3:2, v/v) in order to be derivatized and injected in GC as methyl esters.

3.18 FATTY ACIDS (TFA)

Carried out according to an in-house silver ion GLC-TLC method (method Lab 7) described in Buchgraber and Ulberth, 2001. This method only allows the quantification of the C18 *trans* isomers; the evaluation of the *trans* isomers of long-chain polyunsaturated fatty acids in fish oil samples, was not feasible with this analytical technique. All samples were first methylated with diazomethane (Fieser and Fieser, 1967) and then transmethylated with 2N KOH/methanol (EC. 2002. Enclosure X.B. Regulation L 128/14). The transmethylated samples were injected in GLC before and after silver-ion TLC fractionation. The TLC bands of the saturated and *trans* monounsaturated fatty acids were joined and injected in GLC, so that the octadecanoic methyl ester could be used as internal standard for the quantitation of TFA. The low solubility problem of the FACS samples was solved as described in the determination of the total fatty acids.

3.19 CONJUGATED LINOLEIC ACID (CLA) ISOMERS.

Carried out according to a modified version of the GC method suggested by Kramer et al., 1998. All samples were first methylated with diazomethane (Fieser and Fieser, 1967) and then transmethylated with 2N KOH/methanol (EC. 2002. Enclosure X.B. Regulation L 128/14). The GLC analytical conditions are similar to those described by Kramer et al., 1998. The octadecanoic acid methyl ester was used as internal standard for the quantitation CLA. The low solubility problem of the FACS samples was solved as described in the determination of total fatty acids.

Quantification of the fatty acid (C 18:0) trans isomers:

$$\text{In GC instrument used HRGC: mg/100 g sample} = \frac{\text{Area C 18:0 (GC-B) x (mg I.S.) x 100000}}{\text{(P) x (Kr) x Area I.S.}}$$

Quantification of the fatty acid (C 18:1, C18:1, C18:2, C18:3) trans isomers and CLA isomers:

In GC instrument used GC8000:

$$\text{mg/100 g sample} = \frac{\text{Area isomers (GC-A)}}{\text{Area C18:0 (GC-A)}} \text{ X } \frac{\text{Quantification C18:0 (GC-B)}}{\text{mg/100g sample}}$$

Quantification of the fatty acid (C 18:1) trans isomers next TLC-Silver:

$$\text{Area correct C18:1 trans isomer (GC-A)} = \frac{\text{Area C18:1 (B) (GC-A)}}{\text{Area C18:0 (T) (GC-A)}} \text{ X Area C18:0 T(GC-A)}$$

$$\text{mg/100 g sample} = \frac{\text{Area correct C18:1 trans isomer (GC-A)}}{\text{Area C18:0 (T) (GC-A)}} \text{ X } \frac{\text{Quantification C18:0 (GC-B)}}{\text{mg/100g sample}}$$

(I.S.) = mg tricadenoic acid ester methyl;
(P) = mg fat
(Kr) = K di risposta;
(T) = sample not purification (metyl-ester);
(B) = sample band extractioned in TLC
(GC-A)=GC-8000. A CP.SIL 88 (10% cianopropilfenilsilossano, 90% biscianopropil-polisilossano) capillary column: silica was used, (100 m x 0.25 mm i.d. x 0.2 mm thick film)
(GC-B) = HRGC. A RESTEK 2330 (10% cianopropilfenilsilossano, 90% biscianopropil-polisilossano) capillary column: silica, (30 m x 0.25 mm i.d. x 0.2 mm thick film)

3.20 OXIDIZED FATTY ACIDS (OFA)

Carried out according to a modified version of the HPLCUV method suggested by Rovellini and Cortesi, 2004 for the analysis of extra virgin olive oil. Since many samples were solid at room temperature, it was necessary to dissolve them in chloroform before being filtered in a paper filter containing sodium sulfate. One ml of the filtered sample was methylated with diazomethane (Fieser and Fieser, 1967), added with two internal standards (tricaproine and triheptadecanoine) and benzylated.

The sample is placed in oven at 70°C for 1 hour. 500 mg of fat was placed into a flask with chloroform and filtered, then a small amount of sodium sulfate anhydrous was added. Therefore 1 ml of the solution was dried under flow of nitrogen at 40°C.

The methylation was done by adding a small amount of diazomethane, and then the sample was dry under nitrogen flow. After 1ml of *n*-hexane and then 1ml of internal standard was added. The

benzilation was donde by reaction with 50µl of benzyl solution (1M in alcohol) 15 min at room temperature. Afeter time 10 µl of glacial acetic acid was added to block the reaction. Centrifugation was done at 3000 rpm for 10 min, the solvent was evaporated under flow of nitrogen at 40°C. Then 1 ml of isopropanol was added a solution, and filtered with a nylon filter of 0.45µm.

To avoid sample interference problems and overloading of the HPLC column, the derivatized sample was filtered with a 25-µm nylon filter before being injected into HPLC-UV. Since some samples can give some false positive OFA data, the correct identification of OFA has been

The HP Hewlett Packard series 1050 instrument and Autosampler series 1100 (instruments Sigma-Aldrich Chemical Co., St.Louis MO, USA) was used, equipped with UV spectrophotometric detector at 254 nm or photodiodes, interfaced with a computerised system to capture data (HPCORE ChemStation Copy right © Agilent Technology 1999-2000). A Sphere Clone 5µ ODS (2) column was used;size 250 x 4,6 mm; flow rate 1 mL; detection 254 nm; injection 1 µL; back pressure 1302 Psi (phenomenex, 411 Modarid Avenue, Toronce, CA 90501-1430, USA). Gradient binary linear flow with 1 mL / minute

T = 0 minuti	Acqua/Acetonitrile	40/60 v/v
T = 50 minuti	Acqua/Acetonitrile	0/100 v/v
T = 70 minuti	Acqua/Acetonitrile	0/100 v/v
T = 71 minuti	Acqua/Acetonitrile	40/60 v/v
T = 85 minuti	Acqua/Acetonitrile	40/60 v/v

Standard. Internal solutions: Accurately weigh 0,010 g (+ / - 0,001) of tricaproina (IS 1) and 0,020 g (+/-0,001) of trieptadecanoina (IS 2) in a flask dsa 50 mL. Make up to volume with hexane by chromatography.

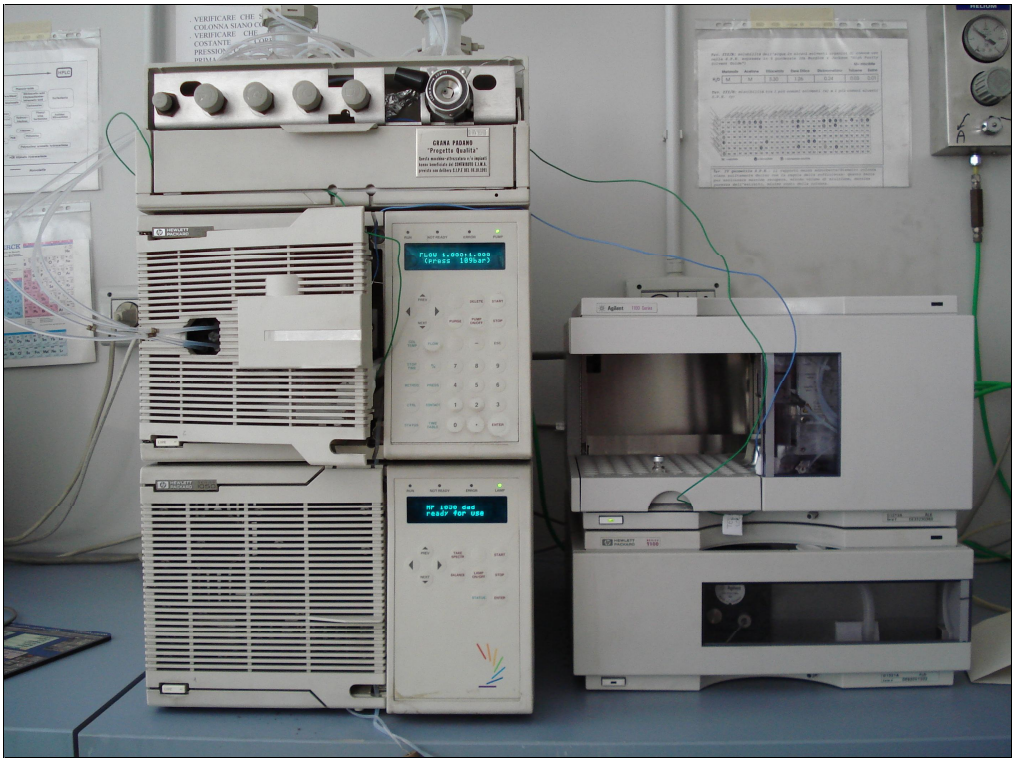

Figure 35 – HPLC

The control of complet reaction of transterification was done by RRF between RF

benzilcaproin(I.S-1) and RF benzilptadecaoin (I.S-2) in fuction of:

a) $\mu g \text{ injected benzilcaproina} = \frac{\mu g/ml \text{ tricaproina} \times 3 \times \text{F.W. benzilcaproina} \times 20\ \mu l}{\text{F.W. tricaproina} \times 1000\ \mu l/ml}$

b) $\mu g \text{ injected benzileptadecanoina} = \frac{\mu g/ml \text{ trieptadecanoina} \times 3 \times \text{F.W. benzileptadecanoina} \times 20}{\mu l}$

F.W. = Formula Weight
20 µL = volumn injected

RF (I.S. 1) = Area benzilcaproina/µg injected benzilcaproina
RF (I.S. 2) = Area benzileptadecanoina/µg injected benzileptadecanoina

c)

RRF = RF (I.S. 1)/ RF (I.S. 2)

This value is constant as weel as the blank and sample.

d) % (w/w) of total fatty acid

% (mg/100 mg) = ((A) x 100 x (V))/(RF (I.S. 2)) x (W) x 20

(A) = Area OFA
100 = %
(V) = volume of solvent
(W) = mg of fat
20 = µL injected in HPLC.

3.21 DETERMINATION OF VOLATILE COMPOUND IN SPME

Weight 0.7 g of turkey meat washed previously with a mix of antioxidats,100 µl of streptomyn in buffer solution, 150 µl of EDTA [1nM], trolox [163.4 µM] and propyl gallate [0.5 µM]; then the sample was stirred for a few seconds. Then 50 µl of NaCL [171 µM] was added for the chromatography analysis.

3.21.1 Calibration curve of propanal :

Seven concentration of propanal (0, 1, 2, 3, 5, 10, 15 µM/mL) were prepaire and injected for preparation of a standard curve by GC-2014 Shimadzu, Tokyo, Japan. Tree injection were carried out for each concentration and curve was obteint by ploting the overeice concentration against average area. A high correlation coefficient (R^2=0.999) was obtain and the contenct of propanal was calculate from the regression equation of the standard curve.

3.21.2 Calibration curve of hexanal:

Sex concentration of hexanal (0, 0.1, 0.2, 0.3, 0.4, 0.5, µM/mL) were prepaire and injected for preparation of a standard curve by GC-2014 Shimadzu, Tokyo, Japan. Tree injections were carried out for each concentration and curve was obteint by ploting the overeice concentration against average area. A high correlation coefficient (R^2=0.995) was obtain and the contenct of hexanal was calculate from the regression equation of the standard curve.

The GC-2014 Shimadzu, (Tokyo, Japan instrument was used, equipped with flame ionization (FID), interfaced with a computerised system to capture data. Autosampler: (AOC-5000 Auto injector Shimadzu), Tokyo, Japan, equipped with SPME Fiber Assembly: 50/30 µm

DVB/Carbonex/PDMS Stable Flex, for Auto Holder gran, (Supelco, Bach: P363859, Catalog Number: 57329-U, 595 North Harrison Rood. Bellefonte, PA 16823-0048 USA. Phone: 814-359-3441). A Supelco Fused Silica Capil-Column (30 m x 0.32 i.d x 1µm film thickness). Helium was used as a gas carrier with a flow rate 28 mL/min; column flow: 2.56 mL/min, Flow purge 5mL/min; split ratio 1/8, pressure 80.1 kPa, Linear Velocity: 40 cm/sec; Injector at: 270 °C and detector at 300°C. The column temperature was 45°C maintained for 2 min, then increased to 255°C a rate of 15°C/min and maintained for 1 min. The injection volume was 1 µl.

Figure 36 – The Shimadzu UV/VIS spectrophotometer, model UV 2101-PC from Shimadzu Scientific Instruments, Colombia, MD, USA.

3.22 DETERMINATION OF TBARs

Weight 0.7 g of turkey meat washed previously with a mix of antioxidats,100 µl of streptomyn in buffer solution, 150 µl of EDTA [1mM], trolox [163.4 µM] and propyl gallate [0.5 µM]; then the sample was stirred for a few seconds. Then 3 ml of TCA-TBA, TBA [1.3%], TCA [50%] solution was added, the sample was left in a water bath at 65°C for 1 hr. After time the sample was placed into a refrigerator at 4°C for 1 hour.

The centrifugation was done at 2000 rpm for 8 min, then was misured at 532nm with spectrophotometer, using as a reference the blank.

(Eric W. Grunwald and mark P. Richards, 2006)

Quantification of TBARs

MDA [M] = A/ $^{o}_{ë}$

$$\text{MDA (mol/g sample)} = \frac{(\text{MDA [M]}) \times 3}{P \times 1000}$$

MDA nmol/kg sample = (MDA mol/g x 10^{12})

3 = ml TBA-TCA solution
1000 = mL
$^{o}_{ë}$ = 1.56 x 10^{5} M^{-1} cm^{-1}coefficient of quenching molar

3.23 EQUIPMENT LABORATORY

(Ultraturax mod. T25 Janke & Kunkel, Germany); auctions steel; analytical balance (amendment E42, Libertini, Italy), 0.1 mg sensitivity; stove; balance technique (amendment L610, Sartorius Laboratories, Germany), sensitivity 10 mg; rotary evaporator (amendment R110, Buchi, Switzerland); centrifuge (amendment ALC 4236); termoblock (mod A120, Falc, Italy); agitator type Vortex (ST5 amendment, Janke & Kunkel, Germany); centrifuge (mod. ALC 4236, Italy); microsiringhe by 10, 25, 100 and 500 µ L (Hamilton, Bonaduz, Switzerland); Gilson by 20 µ L; crimpatrice. For the thin-layer chromatography (TLC) plates of silica gel (20 cm x 20 cm) of 0.25 mm (Merck, Germany); chamber development; plastic trays; nebuliser glass; lamp ultraviolet light

with wavelength from 254 nm; blotting paper. For extraction of lipids was used the following glassware Class A: sovirel bottles from 500 mL equipped with a screw cap; balls with emery neck from 250 mL; stoppers for glass balls with ground glass joint; glass funnels; erlenmajericami codate from 500 mL; funnels of Buchner; funnels separators from 500 mL, 250 mL magnet; cylinder from 10 mL; Pasteur pipettes; pipettes graduate from 5 mL and 10 mL; Sovirel type tubes fitted with a screw cap (diameter = 18 mm). For the methylation and transmetilazione: centrifuge tubes glass with plastic caps with fins; troncoconiche tubes with plastic stoppers; clear glass vial from 10 ml (Labocest), with a cap with an aluminium disc SIL / PTFE from 20 mm (Labocest).

3.24 SOLVENT AND REAGENTS

For lipid extraction solution was used chloroform: methanol, aqueous solution of KCL 1M with KCL, hexane solution: isopropanol 4:1. For the methylation and transmetilazione was used diazomethane prepared with ethyl ether not stabilized (Carlo Erba, Milan), diazal (Sigma, Missouri, USA) and dietilen glycol-monoetil-eter (Sigma, Missouri, USA), according to the method suggested by Fieser and Fieser (Fieser, LF, M. Fieser 1967). The 2N KOH solution in methanol was prepared with methanol (Lichrosolv reagents Carlo Erba, Milan) and KOH (Prolabo, France). The standard reference consists of Tridecanoic Acid Methylester, ca 98% (Sigma, Missouri, USA) lot 20K2504. Were used the following solvents and reagents: Chloroform for analysis (reagents Carlo Erba, Milan); Methanol by analysis (reagents Carlo Erba, Milan); 2-propanol Lichrosolv (reagents Carlo Erba, Milan); Hexane for analysis (reagents Carlo Erba, Milan); Anhydrous sodium sulfate (BDH, England); Potassium chloride; Sodium chloride; AgNo3 pure analysis; Double-distilled water; EDTA Disodium salt, Buch: 1923A47, Catalog Number: 423-383 (Curtin Matheson Scientific, Inc., Houston, Texas, USA); n-Propyl Gallate (3,4,5-Trihydroxybenzoic acid n-propyl ester), Buch: 113H0258, Catalog Number: P-3130, (Sigma Chemical Co. St. Louis, MO 63178 USA 314-771-5750); TBA (2-Thiobarbituric acid, minimum 98%), Buch: 034K0605, Catalog Number: T-5500-100G, (Sigma-Aldrich, Inc., 3050 Spruce Street, St. Louis, MO 63103 USA 314-

771-5765); TCA (Trichloroacetic acid, 99%, extra pure, flakes), Buch: A0240193, Catalog Number: 152130010, (ACROS ORGANICS, New Jersey, USA: 1-800-ACROS-01); Sodium Phosphate Monobasic Anhydrous (NaH_2PO_4), Buch: 016K0039, Catalog Number: S0751-1KG, (Sigma-Aldrich, Inc., 3050 Spruce Street, St. Louis, MO 63103 USA 314-771-5765); Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4), Buch: 053680, Catalog Number: S374-1, (Fisher Scientific, Fair Lawn, New Jersey 07410 (201)796-7100, USA); Potassium Ferricyanide ($K_3Fe(CN)_6$), Buch: 705031, Catalog Number: P-232, (Fisher Scientific Company, Fair Lawn, New Jersey, USA); Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%), Buch: 04426AH, Catalog Number: 238813-1G, (Sigma-Aldrich, Inc., 3050 Spruce Street, St. Louis, MO 63103 USA 314-771-5765); Hexanal ($C_6H_{12}O$), Buch: 77H2511, Catalog Namber: H9008, (Sigma Chemical Co. St. Louis, MO 63178 USA 314-771-5750); Propanal (Propionaldehyde 97% (C_3H_6O)), Buch: A013809401, (ACROS ORGANICS, New Jersey, USA: 1-800-ACROS-01); Streptomycin Sulfate (C42H84N14O36S3), Bach: GC268, Catalog Number: ST135, (Spectrum Chemical MFG. CORP. 14422 S. Pedro St., Gardena, CA 90248, USA)

3.25 STATISTICAL ANALYSES

Data were analysed using Statistica 6.0 software (2001, Statsoft, Tulsa, OK, USA). Two-way analysis of variance ANOVA was performed in order to study the influence of fat supplementation and the technological steps on several quality parameters (CLA, TRANS, OFA, TBARS and volatile compounds) Differences between treatments were analysed through a, considering the cage (broiler chickens) or animal (rabbits) as the experimental units. Tukey-Kramer significant multiple comparision was carried out, in order to determine statistical differences among samples ($p<0.05$).

4. RESULTS AND DISCUSSION

4.1 EVALUATION OF FEEDING FATS COMPOSITION AND THEIR EFFECTS ON MEAT AN LIVER COMPOSITION OF RABBIT AND POULTRY

4.1.1 Feeding fats quality and composition

A total of 123 samples of feeding fats were analyzed, which were classified into 10 categories according to their nature, origin and technological process. Table 11 reports the average contents of TFA, CLA and OFA of the feeding fats. The level of TFA, CLA and OFA significantly varied among the feeding fat categories, which mainly depended on the nature and quality of the raw materials, as well as on the technology and processing conditions used for their production. In general, the total TFA content of the analyzed fats was positively correlated to the total C18:1 *trans* isomers level, whereas the OFA content was related to the level of unsaturated fatty acids, in particular the octadecenoic fatty acids.

The TFA content of the AOCHE samples varied largely, ranging from 99 to 1525 mg/100 g sample. Most samples were below 800 mg/100 g sample, except for four samples. AOCHE prepared from vegetable oils exhibited a larger variation than those obtained from other sources, which may depend on the unsaturation degree of the oils and the applied temperature/time refining conditions. The refining process mainly produced *trans* isomers of the C18 monoenoic fatty acids.

The CLA content of the AOCHE samples varied largely, ranging from 0 to 310 mg/100 g sample. No CLA was detected in 50% of the AOCHE samples, though. AOCHE prepared from vegetable oils exhibited a larger variation than those obtained from other sources; however, it must noted that all the sub-categories displayed a wide range of isomerization of the linoleic acid, which may depend on the unsaturation degree of the oils, as well as on the applied temperature/time refining conditions. It is wellknown that oxidation of linoleic and linolenic acids can actually lead to the formation of little amounts of 9*c*-11*t* CLA isomer. In this fat category, the most abundant CLA

isomers were 9*c*-11*t* and 10*c*-12*t* CLA; this is a relevant result, since the 9*c*-11*t* CLA isomer is the most active from the biological standpoint.

In general, OFA in the AOCHE sample category varied largely in different levels. In fact, OFA ranged from 0,5 to 17,4 mg/100 g sample. The extent of oxidation of AOCHE samples greatly varied regardless of the source from which they were obtained. However, it must be noted that a larger data widespreading was observed within AOCHE obtained from vegetable oils, which may be related to the different contents of linoleic and linolenic acids in these oils.

The TFA content of the AOPHY samples varied largely, ranging from 0 to 4060 mg/100 g sample. Most samples were below 800 mg/100 g sample, except for two samples; in fact, the TFA content of the AOPHY obtained from animal fat was particularly high (4060 mg/100 g sample). AOPHY prepared from vegetable and tropical oils exhibited a larger variation than those obtained from other sources, which may depend on the quality of the raw material, the unsaturation degree of the oils and the applied temperature/time refining conditions. In most cases, the refining process mainly produced *trans* isomers of the C18 monoenoic fatty acids.

The CLA content of the AOPHY samples varied largely, ranging from 0 to 211 mg/100 g sample. No CLA was detected in 63% of the AOPHY samples, though. AOPHY prepared from vegetable and olive oils exhibited a larger variation than those obtained from the tropical oils, which may depend on the quality of the raw material, the unsaturation degree of the oils and the applied temperature/time refining conditions.

OFA in the AOPHY sample category ranged from 0,0 to 7,4 mg/100 g sample, except for one sample (12,8 mg/100 g sample). OFA were found in most samples, regardless of the source from which they were obtained. However, it must be noted that a greater data widespreading was observed within AOPHY obtained from vegetable oils, which may be related to the different contents of linoleic and linolenic acids in these oils. In addition, the overall OFA average value of AOPHY samples was lower than that of AOCHE ones.

The TFA content of the LECI samples ranged from 37 to 162 mg/100 g sample, having an average value equal to 104 mg/100 g sample. *Trans* isomers of the C18 monoenoic fatty acids were those present in higher amounts. The LECI samples actually displayed the lowest degree of isomerization among the fat categories.

The CLA content of the LECI samples ranged from 0 to 30 mg/100 g sample, having an average value equal to 9 mg/100 g sample. No CLA was detected in 63% of the LECI samples, though. The LECI samples actually displayed the lowest amount of CLA among the fat categories.

In the LECI category, the OFA varied from 1,8 to 10,2 mg/100 mg sample, having an average value of 6,2. Only one sample displayed a low OFA value compared to the other samples. A great variation on the amount of non-volatile oxygenated products of this type of fats is observed, which may depend on the extraction conditions used, as well as on the different contents of linoleic and linolenic acids of these oils.

RECY samples displayed a TFA content that ranged from 643 to 4200 mg/100 g sample, having an average value of 1436. *Trans* isomers of the C18 monoenoic and dienoic fatty acids were both present in noticeable amounts. The wide degree of isomerization observed may depend on the quality of the raw material and the unsaturation degree of the oils.

RECY samples displayed a CLA content that ranged from 0 to 276 mg/100 g sample, having an average value of 120 mg/100 g sample. These differences on the amount of CLA may depend on the quality of the raw material, the unsaturation degree of the oils and the type of processing technology used for their production. Since these fat samples were obtained from oils subjected to stressed thermal conditions, it is highly possible that oxidation of linoleic and linolenic acids have led to the formation of little amounts of 9*c*-11*t* CLA isomer.

RECY samples displayed an OFA content that ranged from 3,6 to 8,5 mg/100 g sample, having an average value of 6,2. These levels of non-volatile oxygenated products agree with the nature of the samples and the type of processing technology used for their production, since they were obtained from oils subjected to stressed thermal conditions.

ANFA samples displayed a TFA content that varied largely, ranging from 304 to 4956 mg/100 g sample; the actual average value was equal to 1496 mg/100 g sample. *Trans* isomers of the C18 monoenoic fatty acids were those present in higher amounts. A wide degree of isomerization was observed in this fat category, which may depend on the quality and the unsaturation degree of the raw material, as well as on the rendering conditions used for their production.

ANFA samples displayed a CLA content that varied largely, ranging from 0 to 459 mg/100 g sample; the actual average value was equal to 220 mg/100 g sample. No CLA was detected in 2 ANFA samples (1 and 11), though. In this fat category, the most abundant CLA isomers were 9*c*-11*t* and 10*c*-12*t* CLA. In fact, CLA is usually present in fat from ruminant origin, which is an important source of the 9*c*-11*t* CLA isomer; the latter is actually considered the most active CLA isomer from the biological standpoint. In addition, a small part of these CLA isomers may derive from oxidation of linoleic and linolenic acids, which is strictly related to the quality and the unsaturation degree of the raw material, as well as to the rendering conditions used for the production of these fats.

All ANFA samples showed a very low level of OFA, varying between 0,0 and 3,5 mg/100 g sample. Despite rendering, these samples apparently do not display a great degree of oxidation. However, it must be noted that the OFA parameter does not consider the non-volatile oxygenated products from long-chain polyunsaturated fatty acids; in addition, it is not able to detect volatile oxidation products, which might be generated during the thermal treatments.

EBE samples displayed a similar TFA content (909 and 1047 mg/100 g sample). The nature and quality of the oils, as well as their unsaturation degree, may have influenced the isomerization degree of this fat category.

The two EBE samples displayed a CLA content equal to 132 and 315 mg/100 g sample. The nature and quality of the oils, as well as their unsaturation degree, may have influenced the formation of CLA. The thermal treatments may have favoured oxidation of linoleic and linolenic acids, leading to the formation of little amounts of 9*c*-11*t* CLA isomer.

The EBE category has two samples, which displayed a high level of OFA (6,8 and 9,9 mg/100 g sample). The effect of the thermal treatments can be observed from the entity of non-volatile oxygenated products, which was greater in EBE 2 than EBE 1.

In the FISH category, TFA content ranged from 375 to 1381 mg/100 g sample; the average TFA content was 950 mg/100 g sample. These differences on the isomerization level may depend on the rendering process used for their extraction, as well as on the unsaturation degree of the fats. However, it must be noted that the analytical method used did not allow the evaluation of the *trans* isomers of long-chain polyunsaturated fatty acids, so it is therefore possible that the TFA content of this fat category may be even higher.

In the FISH category, CLA content ranged from 86 to 334 mg/100 g sample; the average CLA content was 241 mg/100 g sample. These differences on the amount of CLA may depend on the rendering process used for their extraction, as well as on the unsaturation degree of the fish fats. Since these samples were subjected to rendering, it is highly possible that oxidation of linoleic and linolenic acids have led to the formation of little amounts of 9*c*-11*t* CLA isomer.

All FISH samples showed a very low level of OFA, varying between 0,0 and 0,8 mg/100 g sample. Despite rendering, these samples apparently do not display a great degree of oxidation. However, it must be noted that the OFA parameter does not consider the non-volatile oxygenated products from long-chain polyunsaturated fatty acids, which are characteristic of these oils; in addition, it is not able to detect volatile oxidation products, which might be generated during the thermal treatments.

HYBY displayed a TFA content that varied largely, ranging from 1856 to 9378 mg/100 g sample; the actual average value was equal to 5900 mg/100 g sample. A complex profile of *trans* C18 monoenoic fatty acids was observed, which is typical from hydrogenated oils; these *trans* isomers were, in fact, those present in higher amounts. A wide degree of isomerization was observed in this fat category, which depends on the hydrogenation conditions (temperature, time, pressure and catalizer) used for their production.

The CLA level of all HYBY samples was so low, that it was not possible to quantify them because they were present below the detection limit of the analytical system. This result could have been expected, because hydrogenation drastically reduces the level of unsaturation of fatty acids.

The OFA level of all HYBY samples was so low, that it was not possible to quantify them because they were present below the detection limit of the analytical system. This result could have been expected, because hydrogenation drastically reduces the level of unsaturation of fatty acids, thus limiting the formation of non-volatile oxygenated products.

The TFA content of the FACS samples ranged from 225 to 666 mg/100 g sample, having an average value equal to 381 mg/100 g sample. It must be noted that two samples had a TFA level below 250 mg/100 g sample. *Trans* isomers of the C18 monoenoic fatty acids were those present in higher amounts. The degree of isomerization observed was quite low as compared to the other fat categories.

The CLA content of the FACS samples ranged from 0 to 55 mg/100 g sample, having an average value equal to 26 mg/100 g sample. It must be noted that one sample had a CLA level below the detection limit of the analytical system. The amount of CLA was quite low as compared to the other fat categories.

The OFA content of the FACS samples was not determined, due to solubility problems.

Table 11. *Average contents of TFA and CLA of different categories of feeding fats obtained from waste or by-products of the food chain. All results are expressed as mean ± SD*

SAMPLE	*N*	TFA (mg/100 g sample)	CLA (mg/100 g sample)	OFA (% w/w on total FA)
AOCHE vegetable	14	484.8±450.8	ND	10.3±4.9
AOCHE tropical	3	384±369.2	ND	1.6±0.9
AOCHE olives	6	302.3±242.5	ND	6.0±2.1
AOCHE animal	2	936.1±168.3	156.3±42.4	2.3±2.3
AOPHY vegetable	4	386.3±296.2	ND	8.7±3.6
AOPHY tropical	8	405±276.5	ND	3.7±1.8
AOPHY olives	3	397.6±260.3	108.5±106.0	3.0±1.3
AOPHY animal	1	4059.9±0.0	179.2±0.0	0.7±0.0
ANFA	36	1675.6±1256.2	220.4±126.8	2.1±0.8
RECY	8	1436.5±1150.2	119.7±96.2	6.4±1.8
FISH	9	950.3±315.2	241.1±89.7	0.4±0.4
MIX	10	8276.5±14190.9	ND	2.1±1.0
EBE	2	978.6±97.9	223.6±129.8	8.4±2.2
LECI	8	104.4±42.9	ND	5.9±3.0
HYBY	6	5900.6±2593.4	ND	ND
FACS	3	380.7±247.5	ND	ND

Abbreviations:
ND = not detected
(OFA LOD = 0,09% in lipids; OFA LOQ = 0,1% in lipids)
(TFA and CLA LOD = 72 mg/100 g sample; TFA and CLA LOQ = 73 mg/100 g sample)

4.1.2 Composition of feeding fats for animal trials

This first part of the research led to the selection of a series of fat samples, which were characterized by low and high levels of TFA, environmental contaminants (dioxins, dioxin-like and PCBs), PAHs and oxidation products. These fats were then utilized to prepare feedings with three enrichment degrees (high, medium and low) of the compounds of interest and were latter sumministered to poultry and rabbits. Table 12 reports the average contents of TFA, CLA and OFA of the feedings.

Table 12 *Average contents of TFA, CLA and OFA of the preselected fats used for feedings formulation. All results are expressed as mean ± SD*

Trials		TFA (mg/100g sample)	CLA (mg/100g sample)	OFA (% on total FA)
	High	12220.5±118.1	ND	ND
	Low	725.8±20.3	ND	ND
	High	1285±36.9	77.8±2.0	0.2±0.1
	Low	904.2±2.4	55.4±2.9	0.5±0.0
	High	677.1±11.1	tr	2.2±0.2
	Low	129.8±1.7	tr	3.1±0.0
	High	153.2±44.5	ND	3.4±0.3
	Low	159.8±40.2	ND	3.3±0.6

Abbreviations: tr, traces; ND, not detected.

Table 12 shows that the highest TFA level was found in hydrogenated palm oil used for the formulation of the high TFA-enriched feeding, whereas the non-hydrogenated palm oil employed for the low TFA trial had less than 1% TFA. These results confirm that hydrogenation deeply influenced the TFA content of fats.

The lowest TFA level was found in vegetable acid oils from chemical refining used for the formulation of the low PAHs-enriched feeding. Most of fats added to the feeds had <1% TFA, except for one of the fish oils used for the high "DIOXIN" trial; low TFA values can be attributed to fatty acid isomerization during refining or oxidation. No differences were observed on the TFA content of the sunflower-olive oil blend (70:30, v/v) ("OXIDATION" trial) before and after being subjected to a commercial frying process, though.

In addition, the feeding fats showed different TFA profiles between and within each trial (high vs. low level), according to the fat origin and the treatment to which they were subjected. In fact, the fish oils used for the "DIOXIN" trial are the only fats that presented *trans* isomers in all fatty acids with 18 carbons atoms having 1, 2 and 3 double bonds (C18:1, C18:2, C18:3). The palm fatty acid distillates ("TRANS" trial) had TFA isomers of C18:1 and C18:2, whereas acids oils from chemical refining ("PAH" trial) and the sunflower-olive oil blends ("OXIDATION" trial) contained only TFA isomers of C18:1 and C18:2, respectively.

Regarding CLA, the content of these fatty acid isomers in fats used for trials also reflected the fat origin and was affected by the technological treatment. No CLA was detected in most fats used for feeds. CLA was only detected in fish oils used for the contaminant-enriched diets; these data agree with those reported in literature (0.1-0.9 mg/g of fat) (Chin *et al.,* 1992). In fact, fish oils and acids oils from chemical refining showed low levels of CLA, which can be attributed to fatty acid isomerization during refining or oxidation. However, no CLA was detected in the oil used for the oxidation trial (sunflower/olive oils, 70:30, v/v, before and after frying chips), because mild frying conditions were employed, thus avoiding a large formation of conjugated dienes, in particular CLA isomers.

The highest content in OFA was detected in oils used for the oxidation trials. It must be noticed that the OFA level did not change before and after frying, because this analytical determination permits to quantify OFA of the octadecenoic fatty acids (C18:1, C18:2 and C18:3) and, therefore, it does not provide an overall picture of the secondary oxidation products of long-chain polyunsaturated fatty acids. No OFA were found in fats used for the “TRANS” trial, due to the already high level of saturated FA in the palm fatty acid distillate, which was further increased after hydrogenation

4.1.3 Composition of feedings enriched with fats with different levels of TFA, contaminants, PAHs and oxidation products

Table 13 reports the average content of lipids, TFA, CLA and OFA of the feedings sumministered to poultry and rabbit.

Table1 13. *Average content of lipids, TFA, CLA and OFA of the TFA-, contaminant-, PAHs- and oxidation products-enriched feedings sumministered to poultry and rabbit*

SAMPLE	N	Lipid content (%)	TFA (mg/100 g sample)	CLA (mg/100 g sample)	OFA (% on total FA)
Rabbit High Trans	2	5.8	331.9	ND	7.2
Rabbit Medium Trans	2	4.8	154.3	ND	10.2
Rabbit Low Trans	2	4.7	25.5	ND	6.8
Poultry High Trans	2	11.9	677.7	ND	5.2
Poultry Medium Trans	2	11.8	331.8	ND	7.1
Poultry Low Trans	2	8.4	50.3	ND	3.7
Rabbit High Dioxin	2	4.8	17.4	1.8	7.5
Rabbit Medium Dioxin	2	4.3	27.7	2.1	6.7
Rabbit Low Dioxin	2	4.8	24.8	1.8	5.7
Poultry High Dioxin	2	7.6	50.1	5.9	4.1
Poultry Medium Dioxin	2	7.1	47.7	3.9	3.6
Poultry Low Dioxin	2	7.4	28.9	2.7	2.5
Rabbit High PAHs	2	4.4	18.3	ND	8.0
Rabbit Medium PAHs	2	3.9	11.8	ND	8.9
Rabbit Low PAHs	2	4.5	5.8	ND	14.3
Poultry High PAHs	2	8.3	38.7	ND	5.6
Poultry Medium PAHs	2	9.5	34.0	ND	6.8
Poultry Low PAHs	2	9.2	15.4	ND	7.1
Rabbit High Oxidation Products	2	4.5	12.8	ND	15.8
Rabbit Medium Oxidation Products	2	4.6	10.0	ND	16.2
Rabbit Low Oxidation Products	2	4.4	10.4	ND	12.5
Poultry High Oxidation Products	2	9.3	20.1	ND	10.2
Poultry Medium Oxidation Products	2	7.4	18.1	ND	10.7
Poultry Low Oxidation Products	2	7.8	17.3	ND	12.1

Abbreviations: ND, not detected.

Lipid content of poultry feeds is about twice as much that of rabbits, due to their different fat metabolic requirements. In fact, the rabbit feeds were added with only 3% fat, whereas chicken feed were added with 6% fat. This diet formulation difference is mainly due to the ciecotrophy phenomenon present in rabbits, which is actually absent in poultry. In addition, the latter require a higher amount of lipids because part of them are necessary for feather growing .

Regarding the formulation of the TFA-enriched feedings, the TFA contents of the poultry and rabbit feedings corresponded to those of the fats used for the feeding preparation. In fact, the TFA content was higher for the “TRANS” trial, followed by “DIOXIN”, “PAH” and “OXIDATION”. However, the experimental feeds were supplemented with soybean oil, which provided TFA originated from soybean oil refining and, thus, influenced the TFA level of the feeds. This is particularly evident for the “PAH” and “OXIDATION” trials, whose fat sources only presented C18:1 and C18:2 *trans* isomers, respectively, whereas the corresponding feeds exhibited *trans* isomers in all TFA categories here studied.

In general, the chicken and rabbit feeds showed different TFA profiles between and within each trial (different levels), that corresponded to the TFA profile of the various fat sources. In most feeds, C18:1 TFA were the most abundant, followed by C18:2 TFA and C18:3 TFA, except for the “DIOXIN” feed where C18:3 TFA > C18:2 TFA. As aforementioned, the effect of the soybean oil supplementation, however, is noticeable in the TFA profile of all feeds.

CLA was detected only in the “DIOXIN” feeds, because the fish oils used for their preparation were the only fats that originally contained quantitatively relevant levels of CLA.

OFA were detected in TFA- and contaminant-enriched feeds for both poultry and rabbit, being 50% higher in those prepared for rabbits. The OFA levels corresponded to those found in fats used for the feeding formulation. Chicken and rabbit feed samples were taken at the beginning and at the end to control oxidation during the experiment. Although OFA slightly varied during this experimental period, no significant oxidation was observed in feeds. The feeds used for the “OXIDATION” trial displayed the highest OFA content, followed by those of the “PAH”, “TRANS” and “DIOXIN”. This trend is slightly different as compared to the one observed in the fat sources, due to the feed supplementation with soybean oil; this effect was particularly evident on the “TRANS” trial, whose fat sources did not presented OFA. No differences were observed on the OFA content of the feeds used for the “OXIDATION” trial, though. On the other hand, it might be possible that the actual

OFA level of the "DIOXIN" feeds was underestimated, since this analytical method is not able to detect OFA from long-chain PUFA (C20, C22).

4.1.4 Composition of meat and liver of rabbit and poultry fed with different enriched feedings.

Tables 14-17 show the average content of lipids, TFA and CLA in meat and liver of both poultry and rabbit fed with TFA-, contaminant-, PAHs- and oxidation products-enriched feeds.

Table 14. *Average content of lipids, TFA and CLA in meat and liver of poultry and rabbit fed with TFA-enriched feedings. All results are expressed as mean ± SD*

SAMPLE	N	Lipid content (%)	TFA (mg/100 g sample)	CLA (mg/100 g sample)
Meat Rabbit High Trans	8	3.7±0.7	90.1±26.4[a]	ND
Meat Rabbit Medium Trans	8	3.6±0.7	58.3±14.2[b]	ND
Meat Rabbit Low Trans	8	3.8±1.1	23.4±6.8[c]	ND
Liver Rabbit High Trans	8	5.1±0.6	72.2±13.0[a]	ND
Liver Rabbit Medium Trans	8	5.2±0.7	52.2±10.1[b]	ND
Liver Rabbit Low Trans	8	5.4±0.4	25.8±8.5[c]	ND
Meat Poultry High Trans	8	11.0±1.3	381.0±58.3[a]	ND
Meat Poultry Medium Trans	8	11.9±0.9	244.6±32.1[b]	ND
Meat Poultry Low Trans	8	12.0±0.6	84.1±17.1[c]	ND
Liver Poultry High Trans	8	5.5±1.0	88.2±25.0[a]	ND
Liver Poultry Medium Trans	8	5.2±0.7	56.3±10.3[a]	ND
Liver Poultry Low Trans	8	4.7±1.2	19.5±9.6[b]	ND

Abbreviations: ND, not detected.

Different superscript letters in the same column within each type of sample denote significant differences due to different levels of TFA supplementation, according to Tukey's test ($p < 0.05$).

Table 15. *Average content of lipids, TFA and CLA in meat and liver of both poultry and rabbit fed with contaminant-enriched feedings. All results are expressed as mean ± SD*

SAMPLE	N	Lipid content (%)	TFA (mg/100 g sample)	CLA (mg/100 g sample)
Meat Rabbit High Dioxin	8	3.8±0.7	43.3±11.0[a]	ND
Meat Rabbit Medium Dioxin	8	3.5±0.7	34.0±9.4[a]	ND
Meat Rabbit Low Dioxin	8	2.9±0.4	21.0±2.7[b]	ND
Liver Rabbit High Dioxin	8	5.5±0.5	27.6±2.4[a]	ND
Liver Rabbit Medium Dioxin	8	6.3±1.0	23.3±4.2[a]	ND
Liver Rabbit Low Dioxin	8	6.8±0.9	22.1±7.5[a]	ND
Meat Poultry High Dioxin	8	17.9±4.4	161.2±29.0[a]	5.8±1.6[a]
Meat Poultry Medium Dioxin	8	16.5±3.4	129.2±36.3[a,b]	5.1±1.5[a]
Meat Poultry Low Dioxin	8	19.3±5.0	113.3±20.4[b]	4.7±2.0[a]
Liver Poultry High Dioxin	8	10.1±3.0	46.3±13.2[a]	ND
Liver Poultry Medium Dioxin	8	10.9±4.4	49.6±26.2[a]	ND
Liver Poultry Low Dioxin	8	11.3±4.0	40.9±19.9[a]	ND

Abbreviations: ND, not detected.

Different superscript letters in the same column within each type of sample denote significant differences due to different levels of dioxin supplementation, according to Tukey's test ($p < 0.05$).

Table 16. *Average content of lipids, TFA and CLA in meat and liver of both poultry and rabbit fed with PAHs-enriched feedings. All results are expressed as mean ± SD*

SAMPLE	N	Lipid content (%)	TFA (mg/100 g sample)	CLA (mg/100 g sample)
Meat Rabbit High PAHs	8	3.4±0.6	16.5±4.0[a]	ND
Meat Rabbit Medium PAHs	8	3.0±0.7	12.9±4.7[a,b]	ND
Meat Rabbit Low PAHs	8	3.2±0.7	10.7±1.3[b]	ND
Liver Rabbit High PAHs	8	4.3±0.2	27.6±4.2[a]	ND
Liver Rabbit Medium PAHs	8	4.5±0.4	26.1±7.8[a]	ND
Liver Rabbit Low PAHs	8	4.3±0.3	21.3±4.8[a]	ND
Meat Poultry High PAHs	8	16.2±1.2	63.9±14.4[a]	ND
Meat Poultry Medium PAHs	8	16.5±2.0	52.3±18.0[a,b]	ND
Meat Poultry Low PAHs	8	15.6±1.5	36.6±10.7[b]	ND
Liver Poultry High PAHs	8	6.6±0.9	35.3±8.2[a]	ND
Liver Poultry Medium PAHs	8	6.6±1.4	25.2±9.1[a,b]	ND
Liver Poultry Low PAHs	8	6.3±2.2	19.1±7.1[b]	ND

Abbreviations: ND, not detected.

Different superscript letters in the same column within each type of sample denote significant differences due to different levels of PAHs supplementation, according to Tukey's test ($p < 0.05$).

Lipid content of poultry meat is about twice as much that of rabbits, which is due to the fact that poultry meat samples included both meat and skin, as well as to the different feed formulation.

In general, the level of TFA and CLA in meat and liver mainly varied according to those originally found in the feeds and fats added to the feeds. It must be pointed out, though, that TFA and CLA accumulation was different for the two animal species, as well as for the two types of tissues. The level of TFA in poultry meat was 2-3 times higher than in liver. In the rabbit, the TFA content of meat was slightly higher than in liver in the "TRANS" and "DIOXIN" trials, whereas the liver

showed a higher TFA content than meat in the "PAHs" and "OXIDATION" trials. The TFA level detected in meats obtained with TFA-enriched diets, was higher than those usually observed in both poultry and rabbit meat (0-1% of total lipids) (Casimir *et al*., 2005).

In the "PAHs" and "OXIDATION" trials, the higher TFA values observed in rabbit liver as compared to meat, are due to both the continous feeding stress, as well as the ciecotrophy digestion mechanism; the latter starts with degradation at blind by bacterial flora that, together with the $NaHCO_3$ buffer action, gives rise to the production of volatile fatty acids.

It is possible to confirm that the levels of TFA in rabbit are lower than those found in the chicken tissues, because rabbit feed undergoes a stronger degradation as a result of its metabolism. Considering this fact, rabbits and poultry received different diets, where the rabbits' one had the highest percentage fiber (20.1% vs 3.8% of chicken diet) and the lowest amount of added fat (3% vs 6% of chicken diet).

Table 17. *Average content of lipids, TFA and CLA in meat and liver of both poultry and rabbit fed with oxidation products-enriched feedings. All results are expressed as mean ± SD*

SAMPLE	N	Lipid content (%)	TFA (mg/100 g sample)	CLA (mg/100 g sample)
Meat Rabbit High Oxidation Products	8	3.7±0.7	14.6±3.9[a]	ND
Meat Rabbit Medium Oxidation Products	8	3.6±0.6	13.8±3.0	ND
Meat Rabbit Low Oxidation Products	8	3.4±0.6	13.6±1.3[a]	ND
Liver Rabbit High Oxidation Products	8	5.5±1.4	20.5±7.9[a]	ND
Liver Rabbit Medium Oxidation Products	8	4.5±1.0	17.8±7.7[a]	ND
Liver Rabbit Low Oxidation Products	8	4.9±1.2	12.4±1.5[a]	ND
Meat Poultry High Oxidation Products	8	16.4±3.2	61.3±8.8[a]	ND
Meat Poultry Medium Oxidation Products	8	15.3±1.2	61.0±11.6[a]	ND
Meat Poultry Low Oxidation Products	8	15.7±2.4	55.7±11.1[a]	ND
Liver Poultry High Oxidation Products	8	7.9±1.1	25.3±5.7[a]	ND
Liver Poultry Medium Oxidation Products	8	7.5±1.4	27.8±6.1[a]	ND
Liver Poultry Low Oxidation Products	8	7.7±1.6	37.7±10.1[a]	ND

Abbreviations: ND, not detected.

Different superscript letters in the same column within each type of sample denote significant differences due to different levels of oxidation products supplementation, according to Tukey's test ($p < 0.05$).

In most chicken and rabbit meat samples, C18:1 TFA were the most abundant, followed by C18:2 TFA and C18:3 TFA, except for the "DIOXIN" trial where C18:3 TFA > C18:2 TFA.

The TFA content was higher for the “TRANS” trial, followed by those of the “PAH”, “DIOXIN” and “OXIDATION” trials. This trend, however, was not identical to that of feeds, where the TFA content varied as follows: “TRANS” > “DIOXIN” >“PAH” > “OXIDATION”.

In the “TRANS” and “OXIDATION” trials for chicken liver samples, C18:1 TFA were the most abundant, followed by C18:2 TFA and C18:3 TFA, whereas C18:3 TFA > C18:2 in the “DIOXIN” and “PAH” trials. In the majority of rabbit liver samples, C18:1 TFA were the most abundant, followed by C18:2 TFA and C18:3 TFA, except for the “DIOXIN” trial where C18:3 TFA > C18:2 TFA.

CLA was only detected in the chicken meat samples of the “DIOXIN” trial, whose feeds and fat sources originally exhibited the highest CLA content.

No CLA was detected in the rabbit meat samples. Although CLA was present in the fat sources and feeds utilized for “DIOXIN” trial, no CLA was found in the corresponding meat.

No CLA was detected in the chicken and rabbit liver samples. Although CLA was present in the fat sources and feeds utilized for “DIOXIN” trial, no CLA was found in the corresponding liver.

Although CLA was found in fish oils, it only deposited in poultry meat, being lower than the amount originally found in the feeding. This is due to the fact that poultry are monogastric animals and, therefore, are not able to produce CLA as ruminants (Szymczyk *et al*., 2001), so the CLA detected in their meat is directly related to the diet (Bolukbasi, 2006). On the other hand, no CLA was found in rabbit meat and liver. This might be due to the fact that rabbit is not a strict monogastric animal and its digestive apparatus has a native microflora that may metabolize CLA coming from diet, thus leading to a lack of accumulation in the various rabbit tissues. In fact, some recent studies have evinced that even CLA-enriched diets (0.5% CLA in feed) give rise to scarce CLA accumulation (0.2% CLA in meat lipids) (Corino *et al*., 2006).

As already mentioned, part of the differences are due to the ciecotrophy digestion mechanism in rabbits. This phenomenon also leads to greater CLA degradation in rabbit as compared to that observed in chicken.

On the other hand, it must be noted that poultry metabolism was also a key factor for feeding preparation and consequent absorption of the compounds of interest. In fact, since poultry absorb more easily polyunsaturated fatty acids than the other fatty acids classes (Turner *et al.,* 1999), "TRANS" feeding was formulated with hydrogenated palm oil, which is highly saturated and thus favor the TFA bioaccumulation. In addition, young chicks mainly assimilate PUFA and hardly absorb saturated FA, but the fat digestibility and absorption displays a gradual improvement with increasing age due to the rise in the production of bile juices and intestinal lipases (Al-Marzooqi & Lesson, 2000). In fact, during animal breading in this study, TFA-enriched feeds were given to poultry after 14-16 days and 37-39 days from birth, thus allowing greater TFA assimilation.

4.2 STUDY OF LIPID OXIDATION IN WASHED TURKEY MUSCLE ADDED WITH DIFFERENT ANTIOXIDANTS

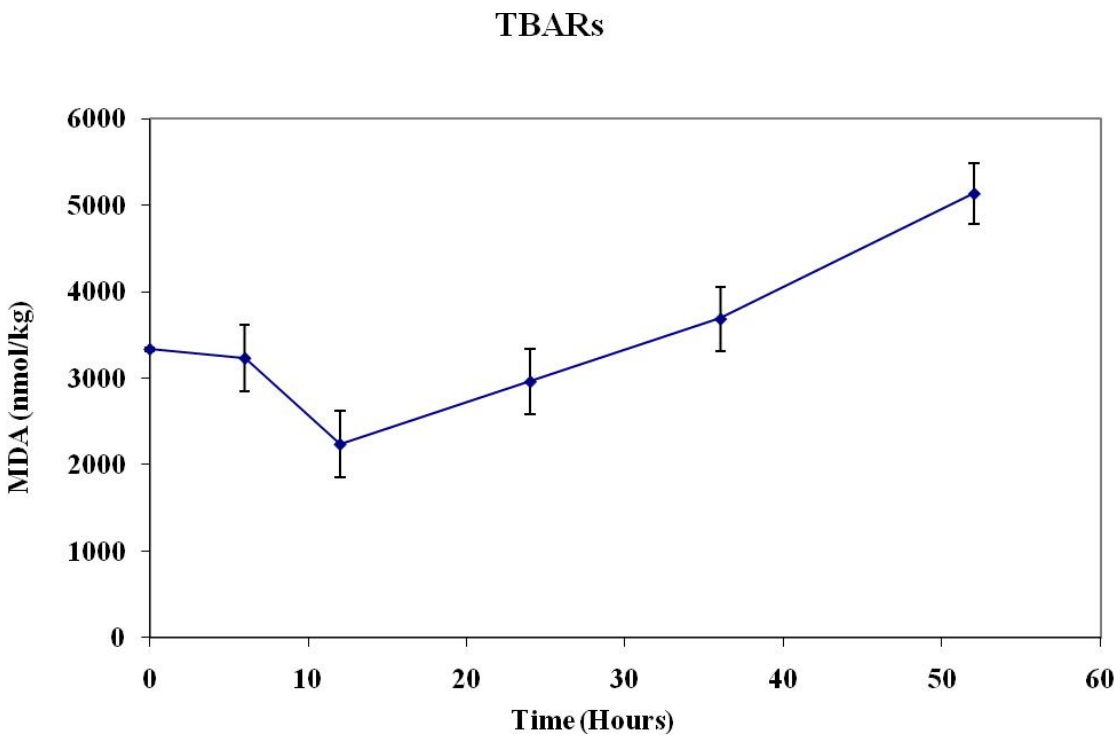

Figure 37. Time course of TBARS in washed turkey muscle stored at 4°C

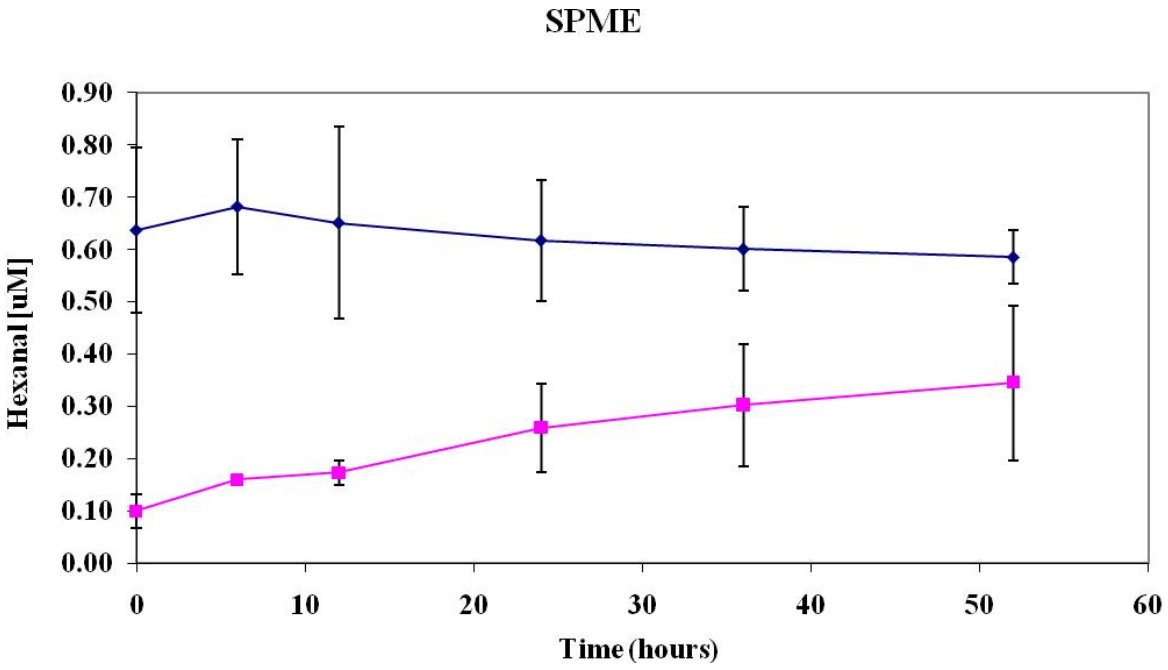

Figure 38. Time course of hexanal (pink) and propanal (blue) in washed turkey muscle stored at 4°C.

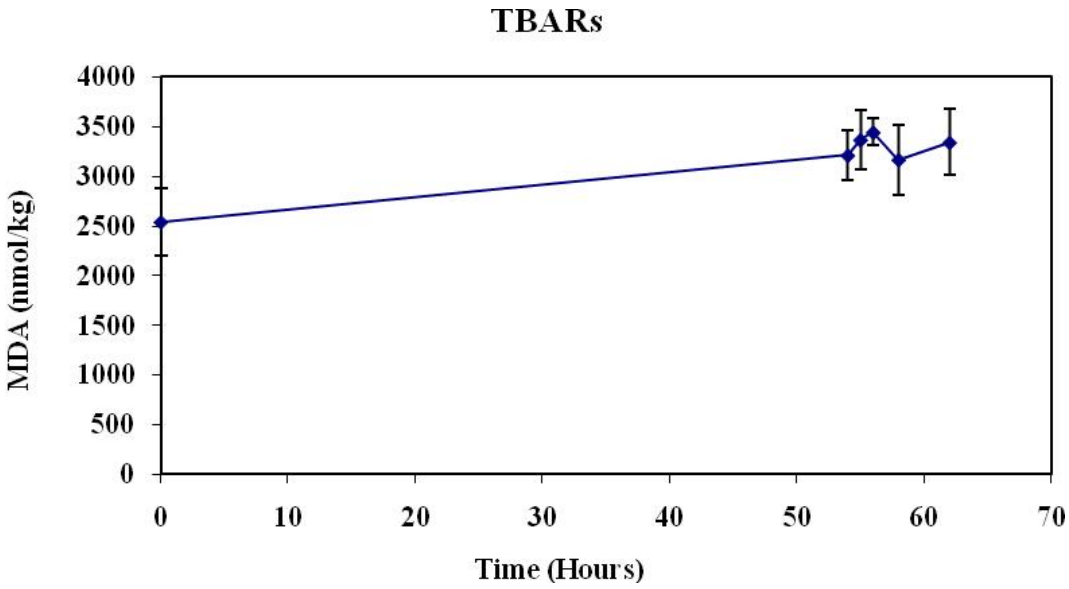

Figure 39. Effect of the addition of the antioxidants after 54 h on TBARS time course

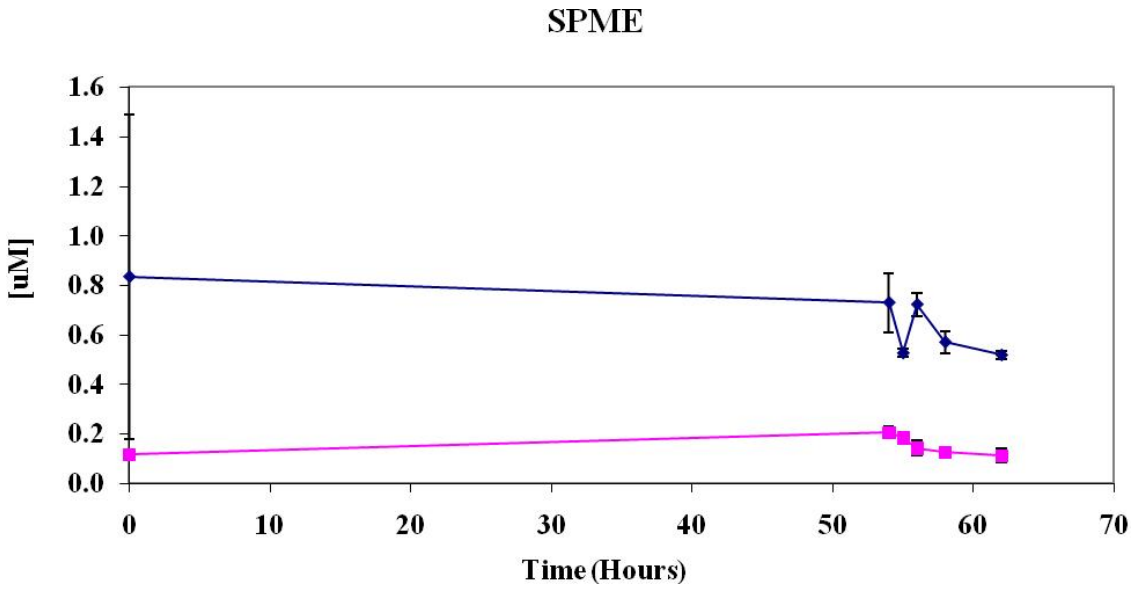

Figure 40. Effect of the addition of the antioxidants after 54 h on hexanal (pink) and propanal (blue) time course

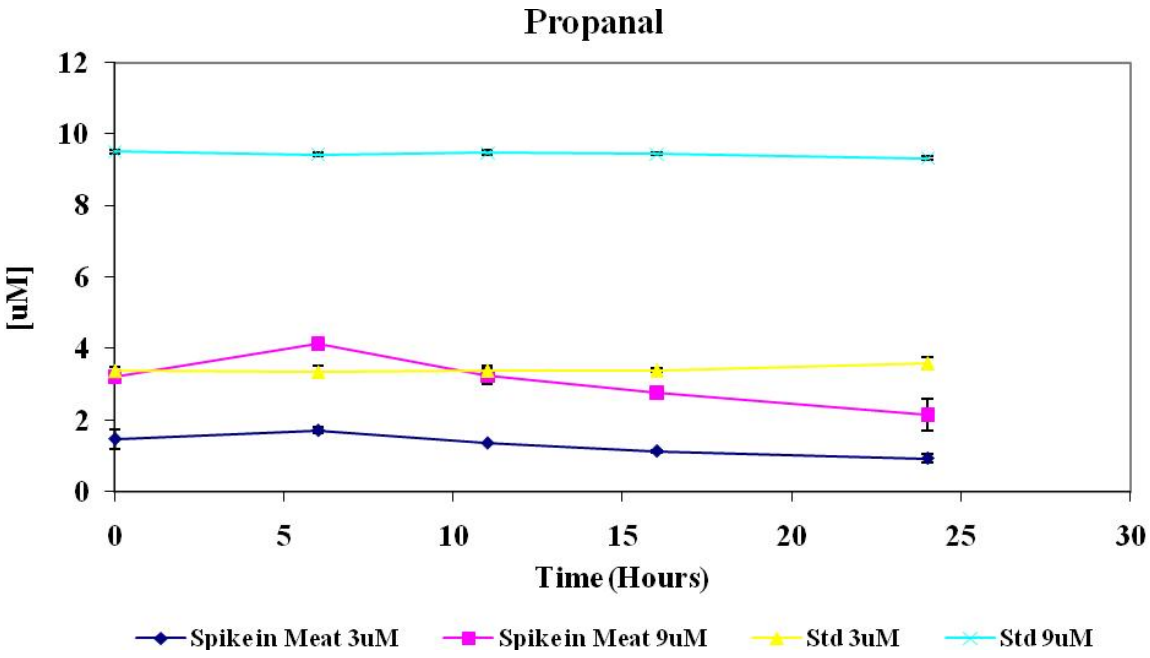

Figure 41. Propanal time course of 2 standard additions levels (3 µM and 9 µM) in aqueous media and in washed turkey meat

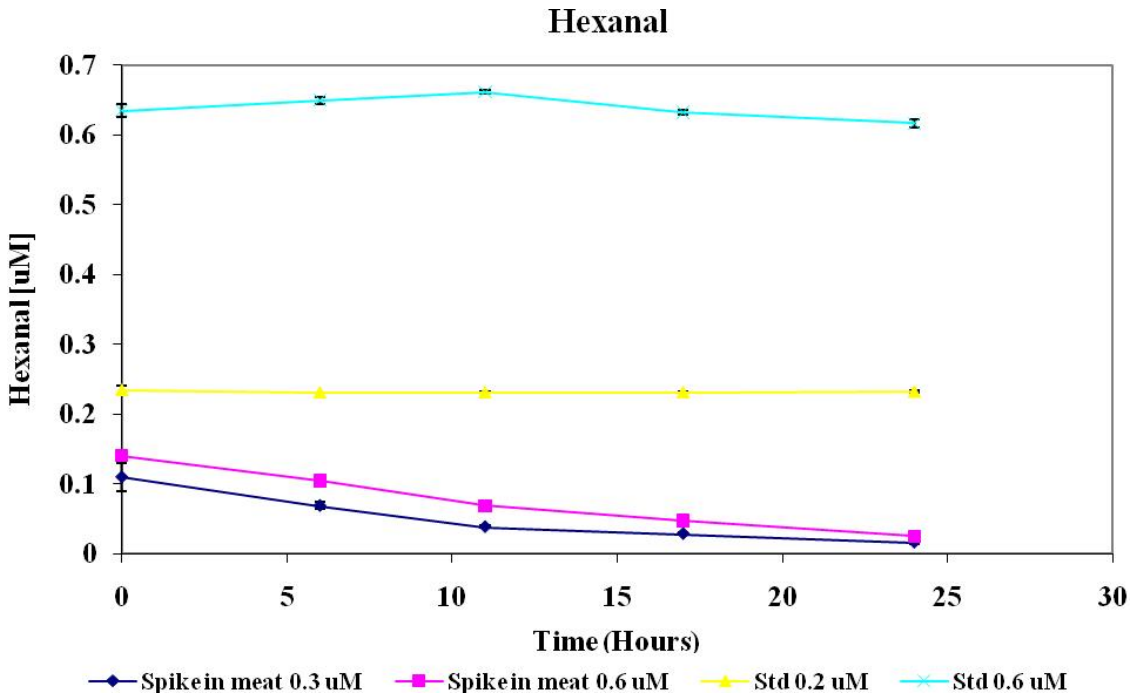

Figure 42. Hexanal time course of 2 standard additions levels (0.2 µM and 0.6 µM) in aqueous media and in washed turkey meat

Figures 37 and 38 show the TBARs and volatile aldehyde kinetics in washed turkey meat stored for different times at 4°C.

Several authors verified a good correlation between hexanal values and other measures of off-flavour development, such as TBARS and sensory scores (Brunton, *et al,* 2000). Nevertheless, TBARS is relatively non specific and does not measure volatile compounds that specifically contribute to warmed over flavor (WOF) (Pearson *et al.,* 1977).

Figures 39 and 40 show the volatile aldehyde kinetics in washed turkey meat stored at 4°C for 54 (hours).

After 54 hours of storage, a mixture of antioxidants, specifically EDTA, ascorbic acid and propyl gallate, was added. Successively, TBARS and SPME determinations were performed after 1, 2, 3, 4 and 8 hours since antioxidant addition. Antioxidants consistently affected TBARS evolution, since no significant increase took place from 54 to 62 hours of storage. A different trend was instead observed for volatile compounds and a remarkable decrease in hexanal and propanal amounts was noticed in the same share of time. Antioxidants proved to be effective in retarding lipid oxidation in meat as illustrated by Nam *et al.* (2003). In that experimentation, addition of antioxidants inhibited hydrocarbon and aldehyde formation in irradiated beef. The fall in volatile amount noticed in the present work appeared as a consequence of aldehyde interaction with free amino acids or free amino groups of proteins, giving rise to Schiff bases (García-Llatas *et al*., 2006).

To confirm this (fig.41 and 42) finding, matrix effect was assessed by adding known amounts of saturated aldehydes (hexanal and propanal) to turkey meat previously washed. The same amounts of standards were added in empty vials without meat as control. Antioxidants were immediately added to meat and control samples spiked with aldehydes. Both types of samples were stored at 4°C and analyzed after 0, 6, 12, 18 and 24 hours since antioxidant addition. The most striking aspect was the matrix effect and the decrease that occurred in volatile amount over storage.

5. CONCLUSION

This thesis work was focused in two main aspects:

a) Evaluation of feeding fats composition and their effects on meat an liver composition of rabbit and poultry

b) Study of lipid oxidation in washed turkey muscle added with different antioxidants

In the first part of this study, it was evinced that not all fats and oils obtained from co- and by-products of the food chain are eligible to feed livestock and then to a livestock production quality while respecting human health and the animal.

In general, the level of TFA and CLA in meat and liver mainly varied according to those originally found in the feeds and fats added to the feeds. It must be pointed out, though, that TFA and CLA accumulation was different for the two animal species, as well as for the two types of tissues.

These differences on TFA and CLA accumulation are mainly due to the metabolic differences between the two species, which lead to diverse qualitative and quantitative assimilation of lipids. On one hand, different lipid amount was added to the poultry (6%) and rabbit (3%) feeds, whereas, on the other hand, fats with a low PUFA content were used to favor TFA accumulation; the latter was actually verified in the results previously reported.

Lipid content of poultry meat is about twice as much that of rabbits, which is due to the fact that poultry meat samples included both meat and skin, as well as to the different feed formulation.

The low content of TFA and the absence of CLA in rabbit tissues are a consequence of a more aggressive feed digestion, accomplished by both enzymes and bacteria. In addition, rabbits display the ciecotrophy process, which leads to a more efficient feed degradation and to a lower compound accumulation in the tissues, especially in meat.

On the other hand, TFA content of livers from both animal species are quite similar because livers from diverse animal species do not exhibit large metabolic differences.

In addition, the higher TFA values observed in rabbit liver as compared to meat in“PAHs” and “OXIDATION” trials, are due to both the continous feeding stress, as well as the *ciecotrofia* digestion mechanism.

CLA was only detected in the chicken meat samples of the “DIOXIN” trial, whose feeds and fat sources originally exhibited the highest CLA content. Upper levels of CLA in feeding fats would have been necessary in order to observe a marked biocaccumulation, especially in rabbit.

The results of the project can contribute to improve standardization in the following aspects:

- Create a database that contains the various composition and degradation parameters for the different feeding fat categories. This will provide an overall picture of the quality of feeding fats used in Europe, which will be helpful to fat and meat producers from the technological standpoint.

- Achieve a more strict regulation of standard and acceptable values of composition and degradation parameters for these fat materials.

- Establish systematic qualitative and quantitative controls in companies dealing with recycling, preparation, manipulation and trade of these fat materials.

- Promote more advanced implementation of integrated control systems for the whole chain of agents involved in meat production (ensuring traceability), from the start point (fat producer) until the end (production of meat).

- Promote collaboration between scientific institutions and companies, from different European countries, for the improvement of standardization of all recycled fat materials for animal feeding, as well as for the development of rapid and simple methods to assess quality and safety of the use of these fats in meat production.

Regarding the study of lipid oxidation in washed turkey muscle added with different antioxidants, secondary lipid oxidation products were monitored by TBARs and volatile aldehyde determinations. It was evinced that the evaluation of aldehydes can be assessed with greater reliability by means of TBARs, even though the latter is not able to distinguish the behaivour of the single aldehydes as the SPME. The kinetic trend of secondary oxidation products, originally attributed to the antioxidant activity, was mainly due to the matrix interaction with aldehydes, which was evinced by a highly sensitivite SPME method. This model system can be applied in the future to evaluate the effect of the heme group on lipid oxidation process.

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