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ENHANCEMENT OF BY-PRODUCTS FROM BOVINE INDUSTRY: SYNTHESIS AND STUDY OF LIPOPHENOL

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Abstract

Nowadays, the industries attention is more and more for by-products reuse, instead of their disposal, because they represent a source of nutrients and posses important technological and sensory properties. The aim of this research is the synthesis of lipophenol starting from the lipid fraction of bovine meat by-products, in order to test this new molecule in a real food system as an antioxidant. Characterizing with innovative chromatographic techniques the lipidic fraction of four different bovine meat by-products, storage fat, subcutaneous fat, bone marrow and bone fat; storage fat was evaluated as the most suitable for the aim, in particular for the high concentration of oleic acid. Tyrosol was chosen as phenolic fraction for the lipophenol and its alkysuccinilation with different succinic anhydrides was tested, to improve the emulsifier and antioxidant potential. The alkylsuccinilation between tyrosol and anhydrides with longest alkyl chain display superior performance in stabilizing emulsions and inhibiting lipid oxidation. Finally, tyrosyl oleate, the molecule synthesized using oleic acid and tyrosol was tested in tarallini, to test its antioxidant effectiveness. The lipid oxidation was tested in samples with different concentration of tyrosyl oleate (1, 4 and 7%) and in a control sample, without it, at different storage time (immediately after the formulation, T0; after 15 days, T15; after 30 days, T30; after 37 days, T37 and after 45 days, T45). Accelerated oxidation analysis using the Oxitest® instrument, shown that the control sample had an IP value of 6.10 h; instead the samples with 1, 4 and 7% of lipophenol reported IP value of 13.58, 22.34 and 25.28 h, respectively. Peroxide value allowed to disciminate the different samples, Control sample and the sample with 1% of tyrosyl oleate exceeded the legal limit of 20 meqO_2/kg of fat after 30 days of storage, instead the other two samples after 45 days of storage. Determination of oxidized fatty acids (OFA) showed significant differences; control sample registered significant higher ($p < 0.05$) OFA content rather than all the samples with lipophenol. Considering the volatile compounds originated from lipid oxidation their concentration increases with the increasing of shelf life, but tarallini made with lipophenol shown a significantly lower concentration of these compounds than control sample for all the storage time. All the analyses carried out confirm the effectiveness of tyrosyl oleate, it can counteract the lipid oxidation.

Keywords: bovine meat by-products, chromatographic techniques, alkylsuccinilation, lipophenol, antioxidant potential, lipid oxidation.

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1. Introduction

1.1 Food waste and by-products: the European context

From the beginning of the third millennium, consumers have acquired more and more a central role within the agri-food industry, leading, in some ways, the food industries to divert its own production logic (e.g. the palm oil case). The consumer sensitivity regarding food has been helped by the contribution of the European Community, which drew up for the Member States the fundamental principles that establish food safety standards appropriate to the entire population that live, consume and produce in Europe. Starting from the "White Paper" on Food Safety (2000), a commitment to protect the entire agri-food chain, from field to table, has emerged with the adoption of measures to establish effective alert and control systems (*Regulation (EC) No 178/2002*) and to simplify and unify labeling rules *(Regulation (EU) No 1169/2011*).

The development of the food sector is changing the social, political and economic scenario. The consumer assurance and the simultaneous development of a protected internal market has pushed the institutions of the European Union to draw up an economic vision no longer based on a *linear* economic growth model, unsuitable for satisfying the needs of a globalized planet. The European Commission (2015) explains that "the transition to a more *circular* economy, where products value, materials and resources is kept as long as possible and waste production is minimized, it is an indispensable component of the efforts made by the European Union to develop an economy that is sustainable, releases few carbon dioxide emissions, uses resources in efficient way and remain competitive". Already in 2011, the Commission for agriculture and rural development emphasized that "in Europe an increasing amount of healthy and edible food is lost in all the rings of the agri-food chain, sometimes even up to consumer, turning into waste ". A study published by the Commission estimates the annual production of food waste, in the 27 European Member States, is approximately 89 million tons, that is 179 kg per capita. The highest per capita household losses are recorded in the highest income countries: United Kingdom (110 kg), Italy (108 kg), France (99 kg), Germany (82 kg) and Sweden (72 kg) (*Perrone & Sorice, 2017*). According to the European Commission (2015), the generation of food waste takes place at every stage of the value chain - during production and distribution, in shops, restaurants, catering facilities and homes - with a net waste related to domestic area (47 million tons, which determined the 72% of EU food waste - *Perrone & Sorice, 2017*).

The European Commission has drawn up many key points of its commitment to reduce food waste:

- develop a common methodology for quantifying food waste and defining its indicators; European Commission will create a platform that will represent a meeting point for Member States and stakeholders;

- take measures to clarify EU legislation on waste, food and feed and facilitate the safe use of food and by-products from food chain in the production of feed;
- improve the use by consumers of the date of consumption and its understandability for consumers, particularly as regards the term "to be consumed preferably within".

1.1.1 Directive 2008/98/EC - Waste Framework Directive

The "*Waste Framework Directive*" is a document that "lays down measures to protect the environment and human health by preventing or reducing the adverse impacts of the generation and management of waste and by reducing overall impacts of resource use and improving the efficiency of such use" (*art. 1*). This Directive enshrine the management requirements of food waste and clarifies the concept of "by-product".

Often, the terms "by-product" and "waste" are confused or used as synonyms, but they represent different categories of substances and products. *Article 3* and *Article 5* of this Directive provide the following definitions:

- **Waste**: "any substance or object which the holder discards or intends or is required to discard" (*art. 3*)
- **By-product**: "a substance or object, resulting from a production process, the primary aim of which is not the production of that item, may be regarded as not being waste referred to article 3 but as being a by-product only if the following conditions are met:
    a) further use of the substance or object is certain;
    b) the substance or object can be used directly without any further processing other than normal industrial practice;
    c) the substance or object is produced as an integral part of a production process;
    d) further use is lawful, i.e. the substance or object fulfils all relevant product, environmental and health protection requirements for the specific use and will not lead to overall adverse environmental or human health impacts." (*art. 5*)

In addition, *Waste Framework Directive* excludes from the scope "waste waters, animal by-products including processed products covered by Regulation (EC) No 1774/2002, except those which are destined for incineration, landfilling or use in a biogas or composting plant; carcasses of animals that have died other than by being slaughtered, including animals killed to eradicate epizootic diseases, and that are disposed of in accordance with Regulation (EC) No 1774/2002; waste resulting from prospecting, extraction, treatment and storage of mineral resources and the working of quarries covered by Directive 2006/21/EC" (*art. 2*).

An important step is that one regarding the "End-of-waste status" in *Article 6*, which states that "certain specific waste ceases to be waste [...] when it has undergone a recovery, including recycling, operation and complies with specific criteria to be developed in accordance with the following conditions:

a) the substance or object is commonly used for specific purposes;

b) a market or demand exists for such a substance or object

c) the substance or object fulfils the technical requirements for the specific purposes and meets the existing legislation and standards applicable to products;

d) the use of substance or object will not lead to overall adverse environmental or human health impacts".

Furthermore, as the directive itself explains, "there should be no confusion between the various aspects of the waste definition, and appropriate procedures should be applied, where necessary, to byproducts that are not waste, on the one hand, or to waste that ceases to be waste, on the other hand" (*whereas 22*). This clarification is important for technological and scientific aspects: food by-products may still contain high value substances that can be used and/or transformed into useful products such as ingredients to enrich or store foods.

The set of european and national standards point out the benefits achievable from a proper management of food waste, like the **enhancement of by-products;** this expression means new uses in different fields such as feeding, cosmetics, pharmaceuticals, bioenergy and the recovery of useful ingredients in food enrichment and preservation.

According to *Waste Framework Directive*, in *Figure 1* is reported the decision tree to allocate the term "waste" or "by-product" to a substance originated in a production chain. Summarize, the substance is considered a production residue when it is not produced intentionally

during a production process; after that the substance is considered a waste when its use is not safe, it is not possible to use it only after transformation process and when its production is not integral part of the production chain. On the other hand, a product residue represents a by-product when its use is safe, it is possible to use without transformation process and when its production is integral part of the production chain.

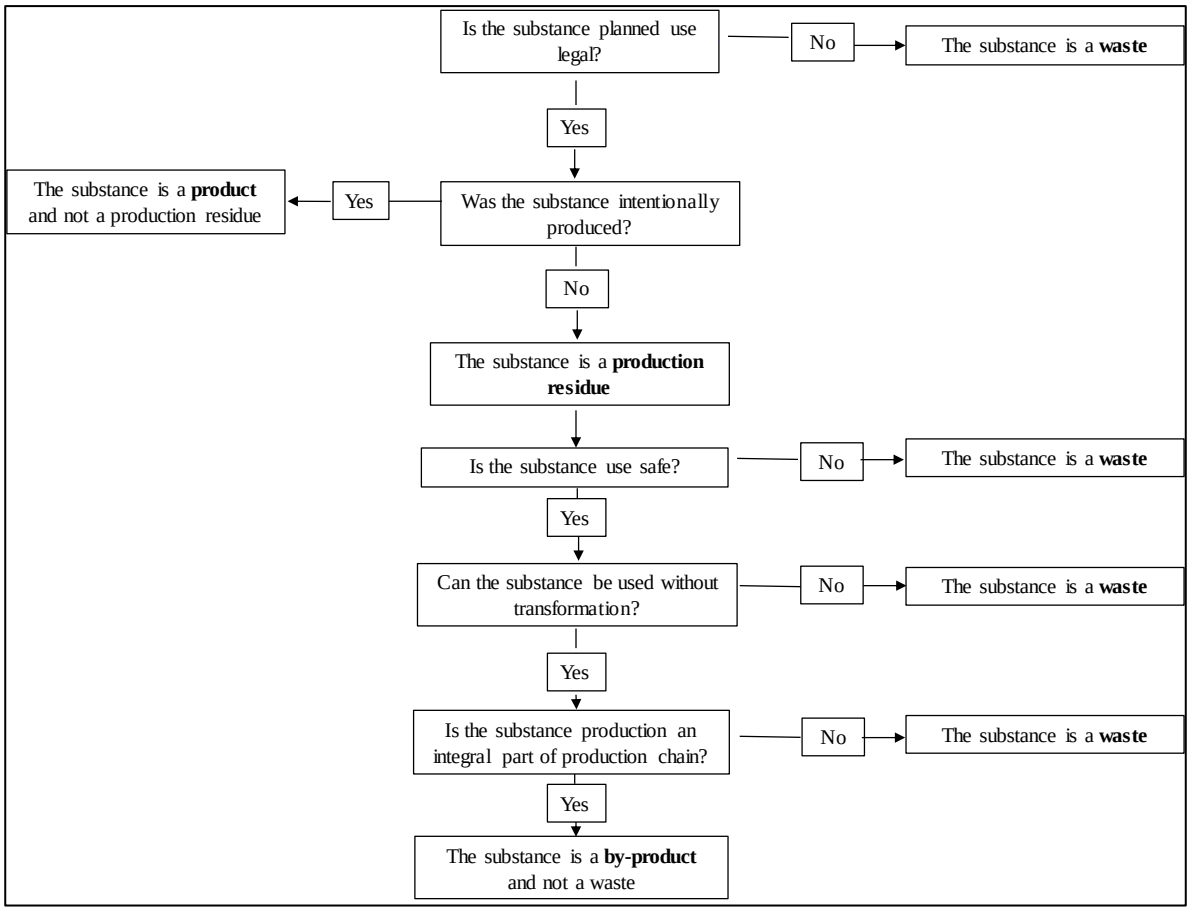


Figure 1. *Decision tree to allocate the term "waste" or "by-product" according to Directive 2008/98/CE and D. Lgs 205/2010*

1.1.2 Regulation (EC) No. 1069/2009 on animal by-products

Waste Framework Directive is an important step towards the distinction between what needs to be disposed and what can be used again, but it does not complete the food by-product topic, since the directive concerns waste and by-products in the widest sense of the term. In fact, in 2009, the European Community issued *Regulation (EC) No 1069/2009* that "lays down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation)". This document specifies the main characteristics of animal by-products: it "lays down public health and animal health rules for animal by-products and derived products, in order to prevent and minimise risks to public and

animal health arising from those products, and in particular to protect the safety of the food and feed chain" (*art. 1*).

It also defines (*art. 3*):

1. **'animal by-products'** means entire bodies or parts of animals, products of animal origin or other products obtained from animals, which are not intended for human consumption, including oocytes, embryos and semen;
2. **'derived products'** means products obtained from one or more treatments, transformations or steps of processing of animal by-products;
3. **'products of animal origin'** means products of animal origin as defined in point 8.1 of Annex I to Regulation (EC) No 853/2004;

According to the *Regulation 1069/2009*, "animal by-products arise mainly during the slaughter of animals for human consumption, during the production of products of animal origin such as dairy products, and during the disposal of dead animals and during disease control measures. Regardless of their source, they pose a potential risk to public and animal health and the environment. This risk needs to be adequately controlled, either by directing such products towards safe means of disposal or by using them for different purposes, provided that strict conditions are applied which minimise the health risks involved" (*whereas 2*). In this regard, *Article 7* resumes and extends the existing categorization in the *Regulation (EC) 1774/2002*, dividing animal by-products according to their level of risk for public and animal health:

- **Category 1 material** (*art. 8*). Among these materials, which must be destroyed by incineration or co-incineration, are included
    a) entire bodies and all body parts, including hides and skins, of the following animals:
        i. animals suspected of being infected by a TSE in accordance with Regulation (EC) No. 999/2001 or in which the presence of a TSE has been officially confirmed;
        ii. animals killed in the context of TSE eradication measures;
        iii. animals other than farmed and wild animals, including in particular pet animals, zoo animals and circus animals;

iv. animals used for experiments as defined by Article 2(d) of Directive 86/609/EEC without prejudice to Article 3(2) of Regulation (EC) No 1831/2003;

v. wild animals, when suspected of being infected with diseases communicable to humans or animals;

b) entire bodies or parts of dead animals containing specified risk material at the time of disposal;

c) animal by-products derived from animals which have been submitted to illegal treatment as defined in Article 1(2)(d) of Directive 96/22/EC or Article 2(b) of Directive 96/23/EC;

d) animal by-products containing residues of other substances and environmental contaminants listed in Group B (3) of Annex I to Directive 96/23/EC, if such residues exceed the permitted level laid down by Community legislation or, in the absence thereof, by national legislation;

e) animal by-products collected during the treatment of waste water required by implementing rules adopted under point (c) of the first paragraph of Article 27

f) catering waste from means of transport operating internationally;

g) mixtures of Category 1 material with either Category 2 material or Category 3 material or both

- **Category 2 material** (*art. 9*). These materials, which must be destroyed by incineration or co-incineration, or which can be used to produce fertilizers, biogas or compost, include:

a) manure, non-mineralised guano and digestive tract content;

b) animal by-products collected during the treatment of waste water;

c) animal by-products containing residues of authorised substances or contaminants exceeding the permitted levels as referred to in Article 15 (3) of Directive 96/23/EC;

d) products of animal origin which have been declared unfit for human consumption due to the presence of foreign bodies in those products;

e) products of animal origin, other than Category 1 material, that are:

i. imported or introduced from a third country and fail to comply with Community veterinary legislation for their import or introduction into the Community except where Community legislation allows their import or introduction subject to specific restrictions or their return to the third country; or

ii. dispatched to another Member State and fail to comply with requirements laid down or authorised by Community legislation except where they are returned with the authorisation of the competent authority of the Member State of origin;

f) animals and parts of animals, other than those referred to in Article 8 or Article 10;

g) mixtures of Category 2 material with Category 3 material;

h) animal by-products other than Category 1 material or Category 3 material.

- **Category 3 material** (*art. 10*). These are by-products for which the health risk is less or even null and are therefore destined to be destroyed by incineration or co-incineration, if eliminated as it stands; eventually they can be disposed in an authorized landfill if they have been transformed into high-temperature treatment plants; they may also be used for the production of petfood or fertilizer production in licensed plants or the production of technical products such as tanned leather, string for musical instruments, paints, or biogas and compost production. Among these materials are included:

a) carcases and parts of animals slaughtered or, in the case of game, bodies or parts of animals killed, and which are fit for human consumption in accordance with Community legislation

b) carcases and the parts originating either from animals that have been slaughtered in a slaughterhouse and were considered fit for slaughter for human consumption following an ante-mortem inspection or bodies and the following parts of animals from game killed for human consumption in accordance with Community legislation:

c) animal by-products from poultry and lagomorphs slaughtered on the farm as referred to in Article 1 (3) (d) of Regulation (EC) No. 853/2004, which did not show any signs of disease communicable to humans or animals;

d) blood of animals which did not show any signs of disease communicable through blood to humans or animals

e) animal by-products arising from the production of products intended for human consumption, including degreased bones, greaves and centrifuge or separator sludge from milk processing;

f) products of animal origin, or foodstuffs containing products of animal origin, which are no longer intended for human consumption for commercial reasons or due to problems of manufacturing or packaging defects or other defects from which no risk to public or animal health arise

g) petfood and feedingstuffs of animal origin, or feedingstuffs containing animal by-products or derived products, which are no longer intended for feeding for commercial reasons or due to problems of manufacturing or packaging defects or other defects from which no risk to public or animal health arises;

h) blood, placenta, wool, feathers, hair, horns, hoof cuts and raw milk originating from live animals that did not show any signs of disease communicable through that product to humans or animals;

i) aquatic animals, and parts of such animals, except sea mammals, which did not show any signs of disease communicable to humans or animals;

j) animal by-products from aquatic animals originating from establishments or plants manufacturing products for human consumption;

1.2 By-products from the food industry

The agri-food industry generates thousands of tons of by-products, mainly consisting of skins, seeds, leaves, bones and other parts that are not edible or discarded due to unsuitable sensory characteristics (*Pérez-Jiménez & Viuda-Martos, 2015*). These by-products also exist because the market has adapted to the increasing consumers requests, which have increased demand for affordable food but with well-defined nutritional characteristics *(Chandrasekaran, 2012*), causing even more wastes. The by-products can be different, depending on the processing phases

concerned: in the category of waste from the agricultural sector there are the stalks of cereals, stems, leaves and residues of different industrial and horticultural crops. The by-products deriving from the industrial transformation of plant and animal production, however, include olives, tomato skins and other processing crops, and finally by-products of animal origin in general (*Soldano & Garuti, 2014; Murugan et al., 2013*).

By-products should not be minimized, since they can generate many value-added products with high commercial potential (*Chandrasekaran, 2012*). These biomolecules, such as catalytic enzymes, pigments, flavors, functional ingredients, micronutrients, nutraceuticals, active ingredients for pharmaceuticals, phytochemicals, biofuels and biomaterials, could be obtained from food processing by-products which are often simply disposed (*Chandrasekaran, 2012*). The recovery of high-value components from food waste can help to promote the variability and diversity of rural and urban economies, in this way the environmental impact of the agro-industrial sector would also be reduced (*Nollet & Toldrá, 2011*).

1.2.1 Food Waste and Food Loss: definitions, differences, origin and problems

In mid-high-income countries, some wastes are present already in the early stages of the food chain, even though the major waste are generated from the final consumer; on the other hand, in low-income countries, there is less waste at consumer level, but there are high losses in the early stages of the food supply chain (*Gustavsson et al., 2011*). This phenomenon is due to the lack of technological means for proper storage and transportation of raw materials that, instead, exist in developing countries.

Data from 2011 estimated the national production of vegetable waste about 13.3 million tons of dry matter, of which more than 6 million were considered available for possible forms of exploitation (*Soldano & Garuti, 2014*). Other data reported that 3.7 million tons of by-products are produced from the industrial processing of vegetable and 9.9 million tons are from animal origin; of the latter, 93% was made from diary products.

Regarding the division between production and waste phases two definitions were proposed first from FAO and then from European Commission (*Perrone & Sorice, 2017*):

Food waste, that is the set of discarded products from the agri-food chain, which, for economic and aesthetic reasons or proximity to the expiration date, though still edible, are directed towards elimination or disposal, producing negative effects for environmental and economic sectors (*Perrone & Sorice, 2017*). These are typical materials from industrial transformation, distribution and final consumption phases and which depend on the technical and structural limits of

transformation/production and distribution systems, as well as errors in the preservation *(Gustavsson et al., 2011)*. This kind of waste is related to the most industrialized countries (*Galanakis, 2012*).

Food loss, food losses occurring during agricultural production, post-harvest and food processing, and which depend on agricultural techniques, climatic and environmental factors and lack of proper conservation. Often, due to their characteristics, these losses are unavoidable or unmanageable, as in the case of adverse natural events and are characteristic of developing countries (*Galanakis, 2012*).

Food waste and food loss are considered only for products intended for human consumption and therefore do not include, in the definition, feed or parts unedible *(Gustavsson et al., 2011*). In the agri-food world, the production of by-products is high as a result of mechanical damage, microbial attacks and incorrect manipulation from consumers (*Galanakis, 2012*). In addition, the use and disposal of by-products is difficult due to their inadequate biological stability, potentially pathogenic nature, high residual water content, rapid oxidation potential and high enzymatic activity (*Jayathilakan et al., 2012*).

In *Table 1* are reported the principal losses and wastes in the different production chain phases for vegetal and animal products.

Table 1. Food supply chain steps that generate losses and wastes

	Agricultural production	**Manipulation post-harvest and storage**	**Transformation**	**Distribution**	**Consume**
Vegetable products	Mechanical damages during the harvest and selections in field	Loss and wasting during manipulation, storage and transportation	Loss and wasting during industrial or domestic transformations: washing, peeling, slicing, boiling	GDO System (wholesale, supermarket, retailers and greengrocers)	Losses from domestic consumption
Animal products	Animal dead during the gestation; losses during fishing; productive losses due to mastitis (milk sector)	Animal dead during the transport to the slaughterhouse; wasting during conditioning, storage and transport; wasting during transport (milk sector)	Cuts during the transformation; losses during packing and smoking; losses during pasteurisation and cheese making	GDO System (wholesale, supermarket, retailers and greengrocers)	Losses from domestic consumption

Disposal of waste from the food supply chain can be difficult for some reasons listed by *Jayathilakan and co-workers* (2012):

- **Microbial stability and potential pathogenic growth.** Some types of wastes and by-products may contain a high microbial concentration, or they may be strongly altered by a microbial attack, with both visual (mold formation) and olfactory (proteolysis with off-flavor formation) consequences.
- **High water content.** In food, the water content can be over the 95% of the weight. The water removal, in order to decrease the transport cost and increase the shelf life, can lead to high levels of organic material in wastewater.
- **Rapid autoxidation.** It is a waste and by-products characteristic that posses a high lipid content which can release the typical rancid odor.
- **Changes due to enzymatic activity.** Many discarded food matrices have a remarkable activity due to endogenous enzymes that can promote chemical and microbiological degradation reactions.

In *Table 2* is shown briefly the different origin of the most common waste originated from food industry.

Table 2. Type of waste and their origin in the food industry

Type of waste	Origin of waste
Waste from different animal food chain (meat, fish and others)	Slaughter house, butcher shops, fish, egg and tallow processing plants
Waste from different agricultural food chain (fruit, vegetables, grain, edible oil, coffee)	Fruit and vegetable processing plant, starch manufacturers, malt houses, grist and husting mill (…)
Waste from sugar production	Sugar manufacturers
Waste from milk production	Dairies
Waste from baked foods and sweets production	Bakeries, confectioners, candy producers
Waste from beverages production (alcoholic and non-alcoholic)	Breweries, wineries, liqueur producers, distilleries, fruit juice producers

1.2.2 Enhancement of by-products from food industry

Valorisation is a relatively new concept in the industrial waste management field and promotes the principle of sustainable development (*Chandrasekaran, 2012*). For a long time, food by-products have been used for low-value activities (*Jiménez & Martos, 2015*), such as feed and fertilization of plots (*Jayathilakan et al., 2012*). Food wastes are considered a low-cost source of valuable ingredients since modern technologies have allowed the recovery of target ingredients and their reintegration into food chain with additive functions in many products *(Galanakis, 2012*).

The use of by-products leads to several advantages such as positive effects on environmental impact, low cost availability of nutrients, giving added value to the main product; on the other hand, there are some limits to their use. In fact, these food by-products may contain high amounts of fiber, processing residues and components such as pesticides and heavy metals.

One of the purposes of enhancing food by-products is the recovery of precise molecules and valuable metabolites production, through chemical and biotechnological way, for use them in products, processes or commercial novelties (*Chandrasekaran, 2012*). Many systems for food by-products enhancement, including chemical and thermo-chemical conversions, anaerobic digestion and composting (*Gowthaman et al., 2012*), as well as alternative methods for molecules recovery (*Jiménez & Martos, 2015*) are already studied.

The chemical conversion of by-products is widely used in different sectors of the food industry and consists of converting a product into another form through hydrolysis, hydrogenation or oxidation, so it can be reused (*Berdanier, 2005*). Thermo-chemical conversion, whose main methods are combustion, pyrolysis and gasification, leads to the production of chemical and energy products with different properties. Combustion, in fact, has the purpose of transforming the chemical energy of an organic substance into heat, releasing water and carbon dioxide *(Pap et al., 2014*); pyrolysis, which consists in applying heat to a product in the absence of oxygen, results in coal production (*Gowthaman et al., 2012*). Finally, gasification is the partial oxidation of organic matter carried out at high temperatures with the aim of transforming it into a mixture of combustible gases consisting predominantly of carbon monoxide, hydrogen, methane and carbon dioxide (*Pap et al., 2014*). Anaerobic digestion is a process involving microorganisms which decompose organic materials, such as food waste, in the absence of oxygen resulting in the production of solid residues and biogas (methane and carbon dioxide) which can be used as an energy source (*Gowthaman et al., 2012*). Interesting is, also, the application of composting treatments, a biological process in which organic materials are subjected to aerobic fermentation more or less controlled, to form a material similar to humus, with agronomic applications (*Schaub & Leonard, 1996*).

Domingues et al., (2015) studied the enzymatic activity on anaerobic digestion on lipids in dairy waste for biogas production; *Merci and co-workers* (2015) conducted a study in order to produce microcrystalline cellulose from soy shells based on reactive extrusion; *Manara et al.* (2015) nvestigated the use of various pre-processing (microwave and high-temperature aqueous organic solvents) on typical Mediterranean area food waste, olives, marc, grape seed and hazelnuts.

In particular, fat obtained from animal by-products can be fused and used in numerous cosmetic applications, such as body lotion formulation or other bath products. The fatty acids are instead used in numerous chemical processes including the polymerization of rubber and plastic, softeners production, lubricants and plasticizers. Collagen, the gelatin and glycerin are used as ingredients for antifreeze agents, surfactants, paints, adhesives, detergents formulation, as well as for pharmaceuticals (*Toldrá et al., 2012*). Usually, among possible uses of animal by-products, including those derived from meat (*Figure 2*), one of the most re-use way is the hydrolysis of proteins, contained in large quantities in different by-products such as blood, collagen, various organs, etc. These proteins are subjected to hydrolysis by specific commercial proteases such as papain, bromelain, thermolisin, proteinase K (*Vercruysse et al., 2005*). Proteolysis is conducted for several hours in specific reactors by an ultrafiltration throught membranes and product purification and fractionation by filtration or chromatographic techniques. In this way it is possible to obtain a higher number of peptides. Free peptides and amino acids can be generated also through endogenous proteolytic activity because meat by-products usually contain endogenous enzymes such as calpaines and catepsins capable to fragmenting proteins. Among the peptides that are produced by proteolysis, endogenous or exogenous, some of them have beneficial effect on consumer health, these are called bioactive peptides and carry out antihypertensive, antioxidant, antithrombotic, immunomodulatory, antimicrobial activities and may also be involved in the prevention of tumors (*Mora et al., 2014*).

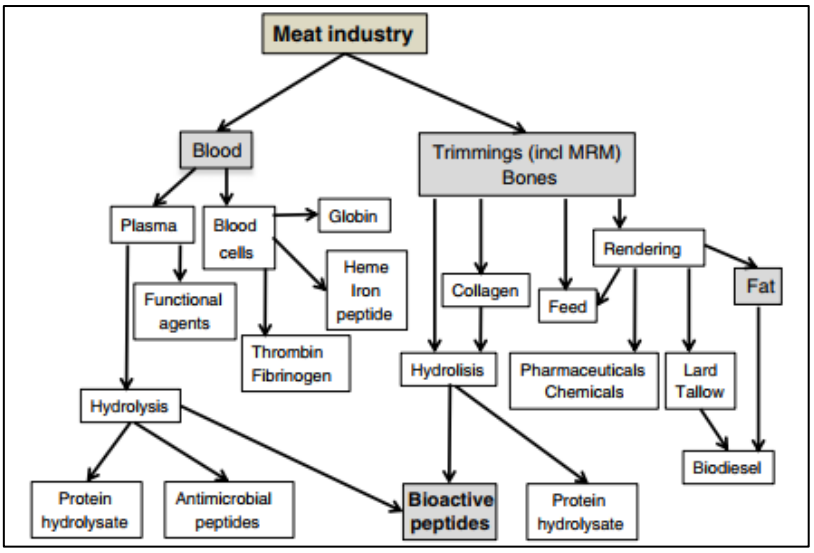


Figure 2. *Flow diagram of main routes for value-addition to meat by-products (Mor et al., 2014)*

The bases on which all the traditional and emerging technologies are based are: maximize the yield of target compounds; meet the needs of industrial transformation; separate the high added value ingredients from impurities and toxic compounds; avoid deterioration and loss of functionality during processing and ensure the final product quality. The technologies and operational steps typical of a by-products valorisation approach are shown in *Figure 3* (*Galanakis, 2012*).

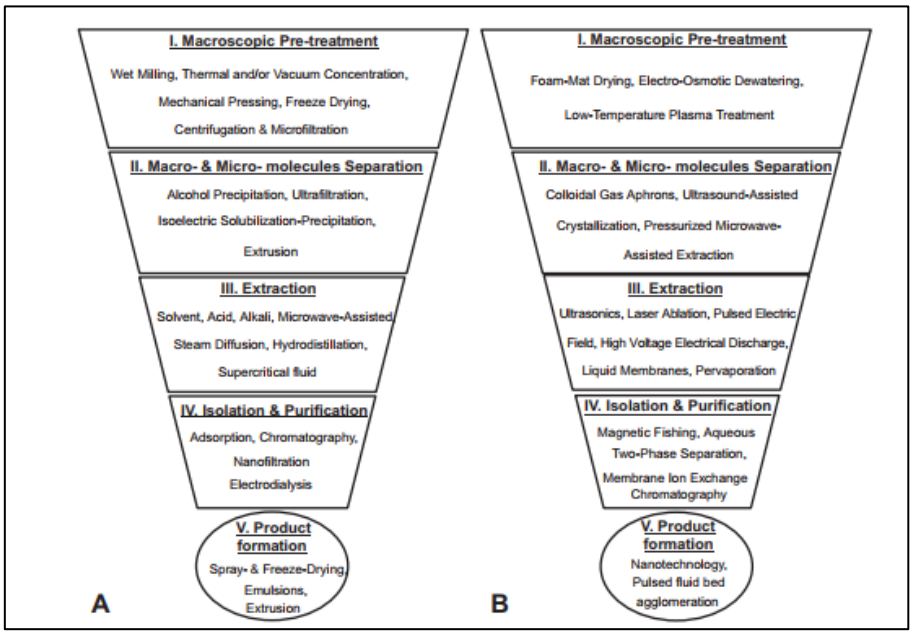


Figure 3. *Recovery stages of high-added value component sfrom food wastes. Established (A) and emerging technologies (B) (Galanakis, 2012)*

There are five major recovery operations. The first one, *macroscopic pretreatment*, has the purpose of regulating the food matrix according to the water content, the enzymatic activity and the tissue permeability. The second step, *separation of macro and micromolecules*, provide simple techniques like alcohol precipitation and complex techniques as the ultrasonic crystallization. The third stage, *extraction*, is well documented with many methodologies that fit into characteristics of molecules of interest, such as solubility or volatility. The fourth phase of recovery has the purpose to *isolate* and *purify* target compounds from the impurities that were extracted along with molecules of interest. The last step in the process is the *product formation*, which is not a real recovery operation, but should be considered as such (*Galanakis, 2012*).

1.2.3 By-products from olive oil production chain: olive mill wastewaters

The recovery of precious ingredients from food by-products concerns today more than ever the enhancement of the wastewaters from olive oil (*Olea europaea* L.) production chain *(Almeida et al., 2017*), which are an important source of phenols *(Ballus et al., 2014; Galanakis, 2012; Hernández-Chávez & Guemes Vera, 2015*). In fact, only 2% of the total phenol contained in the olives pass into the oil, while the rest is divided between wastewaters (about 53 %) and solid residue (about 45%) (*Delisi et al., 2016*).

In general, it can be stated that more than 30 million cubic meters of by-products (*Galanakis, 2016*) originate from the oil production chain, grouped into two main and distinguishable types according to their physical phase. The solid residue derives from the olive pulp, while the liquid wastewaters consist essentially of washing waters and process water, as well as from the acqueous fraction of drupa (*Apat, 2007*).

Olive mill wastewaters derive from the use of continuous batch in the mill, which provide for the initial separation of the olive pomace from olive paste and, subsequently, to the vegetation water removal from the oil. The vegetation waters have a dark color *(Ghanbari et al., 2012*) and are characterized by a strong and intense odor that derives from the drupa. They consist of an aqueous solution of organic substances (reducing sugars, organic acids, polyalcohols) and minerals (potassium, phosphorus, calcium) which may contain suspended solids (*Apat, 2007*). Vegetation waters are rich in antioxidant compounds, in particular hydroxytyrosol derivatives (*Krishna & Chandrasekaran, 2012*), depending on the variety of olives, the maturation state, the type of storage and degradation that the olives may have incurred between harvesting and pressing. The phenols in wastewaters are present in a range between 0.5 and 24 g/L (*Chasekioglou et al., 2017*), depending

on the system used for the oil production *(Aggoun et al., 2016*). The most important phenols identified in wastewaters are hydroxytyrosol, tyrosol, oleuropein, p-hydroxybenzoic acid, verbascoside, 4-methylcatechol, catechol, caffeic acid, vanillic acid and siring acid (*Figure 4*) (*Fava et al., 2017*). For many years the wastewaters and mill residues in general have been considered as low-value by-products. Nowadays, however, the focus has been on the recovery of bioactive components from these materials, such as phenols, dietary fibers and other useful compounds that can be used in both food and cosmetics fields (*Galanakis & Kotsiou, 2017*). Low-humidity residues can be used as an energy source (bioethanol) or for the recovery of other components (such as xylitol, oligosaccharides and antioxidants); high-water residues, however, are normally used in agriculture, but they are also used as a polyphenols source (*Negro et al., 2017*).

Oil production process	Press process			3-Phases process			*p* value		
Olive variety	Azerraj ($n = 4$)	Chemlal ($n = 5$)	Sigoise ($n = 3$)	Azerraj ($n = 7$)	Chemlal ($n = 6$)	Sigoise ($n = 4$)	Variety	Process	Interaction
Vanillic acid	45.0 ± 16.9	35.2 ± 15.1	66.6 ± 19.5	31.9 ± 12.8	15.1 ± 13.8	64.4 ± 16.9	0.069	ns	ns
Hydroxytyrosol	1.0 ± 2.5	5.3 ± 2.2	2.5 ± 2.9	0.1 ± 1.9	0.4 ± 2.0	0.0 ± 2.5	ns	ns	ns
Caffeic acid	33.3 ± 24.7^{b}	35.0 ± 22.1^{b}	187.0 ± 28.5^{a}	26.2 ± 18.6^{b}	35.2 ± 20.1^{b}	95.4 ± 24.7^{ab}	<0.001	0.098	ns
Luteolin	105.1 ± 32.2	95.9 ± 28.8	72.2 ± 37.2	112.8 ± 24.3	104.5 ± 26.3	88.9 ± 32.2	ns	ns	ns
Tyrosol	282.4 ± 107.5	212.8 ± 96.1	231.8 ± 124.1	103.3 ± 81.3	104.9 ± 87.8	239.9 ± 107.5	ns	ns	ns
4-Hydroxyphenylacetic acid	2.7 ± 2.5^{ab}	5.5 ± 2.2^{ab}	9.3 ± 2.9^{ab}	1.4 ± 1.9^{b}	3.0 ± 2.0^{ab}	11.9 ± 2.5^{a}	0.007	ns	ns
3,4-Dihydroxyphenylacetic acid	28.4 ± 14.5^{ab}	28.4 ± 13.0^{ab}	49. 7 ± 16. 8^{ab}	13.9 ± 11.0^{b}	31.1 ± 11.9^{ab}	81.7 ± 14.5^{a}	0.014	ns	ns
Oleuropein	12.4 ± 740.1	23.1 ± 662.0	56.3 ± 854.6	6.4 ± 559.5	256.4 ± 604.3	2723.2 ± 740.1	ns	0.105	ns
Apigenin	3.3 ± 1.4	3.8 ± 1.3	2. 8 ± 1.6	5.71 ± 1.1	3.3 ± 1.2	1.8 ± 1.4	ns	ns	ns
Chlorogenic acid	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	ns	0.041	0.106
Luteolin-7-O-glucoside	32.0 ± 22.1	34.2 ± 19.8	66.2 ± 25.6	49.1 ± 16.7	33.7 ± 18.1	93.2 ± 22.1	0.109	ns	ns
Gallic acid	4.7 ± 1.2	3.5 ± 1.1	3.0 ± 1.4	3.7 ± 0.9	3.3 ± 1.0	6.3 ± 1.2	ns	ns	ns

Values are LS means ± standard error.
a, b: Different letters in a row mean significant differences ($p < 0.05$) (Tukey–Kramer's test).
ns = non-significant difference ($p > 0.05$).

Figure 4. *Phenolic compounds present in olive mill wastewaters (Aggoun et al., 2016)*

The by-products obtained during olive oil production chain, in fact, are the largest potential source of phenols (*Krishna & Chandrasekaran, 2013*). *Galanakis & Kotsiou* (2017) treated wastewater in order to convert oleuropein in hydroxytyrosol, before the phenol extraction with solvents or other technologies. Polyphenols obtained from olive mill wastewaters can exert antimicrobial action against pathogenic species (*Listeria monocytogenes* or *Escherichia coli*), but also microbial selection against those microorganisms that are not pathogenic but degrading foods (*Veneziani et al., 2017*). In addition, several studies have confirmed the tyrosol potential as antimicrobial, anti-inflammatory and antioxidant *(Aissa et al., 2017; Bernini et al., 2015; Bernini et al., 2008*).

1.2.4 By-products of the meat production chain: the tallow

Animal by-products represent a valuable resource from which new functional products and ingredients can be obtained, with significant added value, which can be used in the production of food. Almost all the waste in the meat sector is produced during slaughter. By-products such as

blood, liver, lung, kidney, brain, spleen, and tripe have a good nutritional value (*Chandrasekaran, 2012*) because they are sources of useful compounds such as essential amino acids, minerals, vitamins and others that possess important properties (flavouring, emulsifiers, etc.) (*Toldrá et al., 2012*). In fact, for example, the liver has high levels of vitamin A, iron, zinc, vitamin B, vitamin C and D, copper and fatty acids; the heart contains high doses of iron, as well as selenium, zinc, phosphorus, niacin, riboflavin; the brain is rich in niacin, phosphorus, vitamins B12 and C (*Marti et al., 2012*). Due to the low apparent quality, much of the by-products from the meat sector are considered waste or they are used for animal feed or for agronomic purposes (*Rathinaraj & Sachindra, 2013*). Some wastes are already used in the production of biodiesel and biogas, in dietary and cosmetics products *(Jayathilakan et al., 2012*).

The tallow is the fat substance obtained from adipose tissues fusion from bovine animals (*Jayathilakan et al., 2012*). In general, the tallow has a white/yellow color, with a characteristic odor due to short chain fatty acids. *Table 3* shows the fatty acid composition of this kind of by-product (*Bonaga & Frega, 1997*). Due to consumer demands, the tallow is often heated and deodorized before use in foods and then it can be used in margarines production (*Jayathilakan et al., 2012*). Fats extracted from bovine animals are commonly used in the confectionery and chewing gum industries, in lubricants, soaps, glycerines, medicinal products productions (*Marti et al., 2011*). In recent years the studies on the alternative use of this by-product have been multiplied: *Heshmati & Khodadadi*, (2009) have shown that is possible to lower the cholesterol and free fatty acids content in the tallow after raffination and deodorization.

Table 3. Fatty acid composition of tallow (Bonaga & Frega, 1997)

Fatty acid	Concentration in tallow (%)
Capric acid (C10:0)	0.1
Lauric acid (C12:0)	0.2
Miristic acid (C14:0)	2.5-4.5
Palmitic acid (C16:0)	25-29
Palmitoleic acid (C16:1)	2.5-3.5
Stearic acid (C18:0)	20-28
Oleic acid (C18:1)	30-35
Rumenic acid (C18:2)	3-3.5

1.3 Lipid oxidation

Lipid oxidation is a complex sequence of chemical changes that result from the interaction of lipids with oxygen-active species (*Frankel, 1998*). Oxidative chemical modifications are undesired modifications. In general, lipids are subjected to oxidation and it is directly related to lipid food shelf life. Oxidation damages foods from the nutritional point of view (subtracting some components), organoleptic (that changes the taste and the smell) and finally from the point of view of preserving the quality characteristics of products.

Fat substances interact with themselves or with the surrounding environment, but never with the aqueous environment because they are lipophilic or hydrophobic molecules. Adversity of the fat substances occurs by hydrolysis (enzymatic or chemical) and by oxidation (microbial, enzymatic and chemical way). With the increasing of fatty acids concentration increase the oxidation phenomena; because free fatty acids are small molecules with higher kinetics when compared to other molecules.

The oxidation process, from a chemical point of view, consists in transferring an electron from an atom to another and occurs normally in living organisms, where oxygen acts as the ultimate accepter of electrons with energy production. However, this molecule can give rise to highly reactive chemical species which are predominantly radicals (*Sakihama et al., 2002*), when transferring unrelated individual electron. Free radicals are species characterized by an unpaired electron in the outer orbit, which exist as independent species, and therefore, to reach an electronic configuration with major stability, tend to subtract an electron from other molecules by attacking essential cell components. Molecular oxygen has two unpaired electrons in external orbitals and, therefore, tends to react very easily with electrons donor molecules. Following the monoelectronic reduction performed by such molecules, ROS (oxygen reactive species) are generated: hydroxyl radical ($OH\cdot$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), ozone (O_3) and singlet oxygen ($^1O_2$) (*Lerker & Capella, 1997*).

Oxidation is affected by numerous factors such as fatty acid composition, content and activity of pro-oxidants and antioxidants, irradiation, heating, oxygen pressure and surface area in contact with oxygen.

Lipid oxidation products can be produced by free radical mechanism (autoxidation), by the action of singlet oxygen activated by light (photooxidation), by the catalytic action of enzymes (lipoxygenase pathway) or by the catalytic action of metals in oil or food containing oil and fats. Lipid oxidation may occur during the isolation of oils and fats from the raw material, during food

preparation and also during storage (*Sikorski & Kolakowska, 2010*). The result is a pattern of undesirable effects, as shown in *Figure 5*.

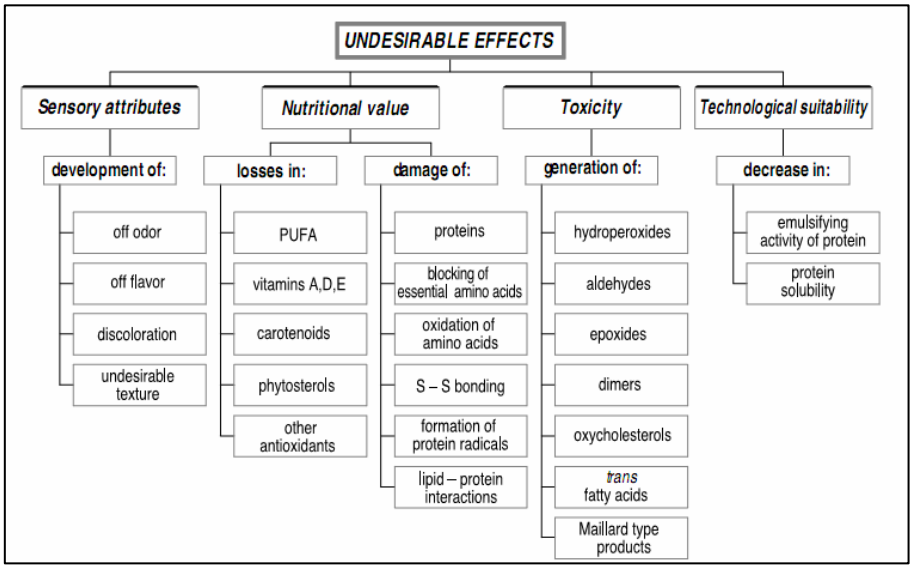


Figure 5. *Undesirable effects caused by food lipid oxidation (Sikorski & Kolakowska, 2010)*

1.3.1 Oxidation trend

The *Figure 6* shows the oxidation trend in a fat matrix. Starting from zero time, as time goes by there is oxygen consumption ("pure lipid" curves). When oxidative kinetics is in an advanced stage, different products are formed: peroxides, which, together with hydroperoxides, represent the primary oxidation products and polymers and volatile compounds that are, instead, secondary oxydation products.

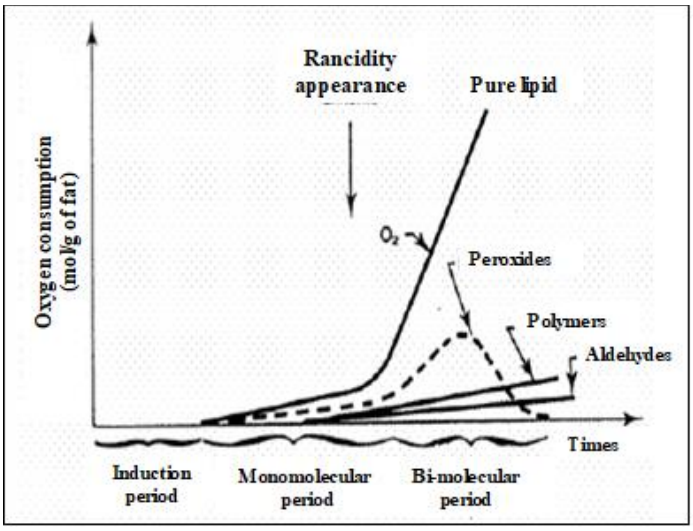


Figure 6. *Oxygen consumption during oxidative process*

The lipid oxidation process is caused by a series of chain reactions that can be summarized in three main phases: initiation, propagation and termination (*Kamal-Eldin, 2003*).

Initiation. $RH \rightarrow R\cdot + H\cdot$

The hydrogen removal from a fatty acid and replace it with oxygen requires a huge amount of energy. The initiation phase occurs because of molecules that promote this mechanism or even situations that facilitate this reaction. These fatty acid radicalization mechanisms, which are not entirely known, cause the hydrogen loss from the fatty acid forming a lipid radical R·.
This phase is accelerated by temperature, light or the presence of iron and copper traces that act as catalysts at very low concentrations.

Propagation. During the propagation two phases are distinguished: monomolecular and bimolecular. These are two steps in which the oxidation reaction leads to the formation of a single radical (monomolecular phase), while the reaction between two hydroperoxides leads to the formation of two distinct radicals (and water) which will in turn act as radicals against fatty acids (bimolecular phase).

Monomolecular phase:

$R\cdot + O_2 \rightarrow ROO\cdot$

$ROO\cdot + RH \rightarrow ROOH + R\cdot$

Bimolecular phase:

$2\ ROOH \rightarrow ROO\cdot + RO\cdot + H_2O$

$ROO\cdot + RH \rightarrow ROOH + R\cdot$

$RO\cdot + RH \rightarrow ROH + R\cdot$

The bimolecular phase proceeds much faster than the monomolecular one and with a different reaction kinetics, as it occurs only when the concentration of hydroperoxides reaches a critical value. This promotion phase is linked to the ever-increasing concentration of free radicals that, at first, originate from the radicalization of fatty acid, and subsequently come from the radical interaction of neutral hydroperoxides. Thus, alkoxy radicals ($RO^{\cdot}$) and peroxy radicals ($ROO^{\cdot}$) are formed, with considerable reactivity, which in turn produce volatile demolition products (aldehydes and carbonyl compounds) responsible for the characteristic odors and flavors of rancid (*Lerker & Capella, 1997*).

Termination. With the termination phase radicals cease to exist.

$R\cdot + R\cdot \rightarrow R\text{-}R$

$RO\cdot + R\cdot \rightarrow ROR$

$ROO\cdot + R\cdot \rightarrow ROOR$

$ROO\cdot + RO\cdot \rightarrow ROO\text{-}R$

$2\ ROO\cdot \rightarrow ROO\text{-}OOR$

The peroxides degradation is not a positive aspect, since they lead to the complete qualitative deterioration of food. The peroxide value is a law parameter that must be kept below of 20 meqO_2 / kg fat (*EU Regulation 1348/2013*).

1.4 Lipid oxidation prevention: antioxidants compounds

Antioxidants compounds can significantly influence oxidation. Antioxidants, in fact, are substances that can delay the onset of the typical reactions of the oxidative process and contrast its propagation (*Cabras & Tuberoso, 2014*). Antioxidants compounds can counteract free radicals and increase shelf life of foods, delaying the process of lipid oxidation during storage (*Gülçin, 2012*). Vitamins A, E, C; minerals such as selenium, manganese, copper; flavonoids and coenzyme Q10 can act as natural antioxidants

Antioxidants are generally classified in three categories according to their own mechanism of action:

- **First type antioxidant.** Also called *chain breakers* (AH), they inactivate free radicals by donating electrons and converting high-energy lipid radicals into thermodynamically more stable products (*Shahidi & Naczk, 1995*). Scavenging the free radicals (ROO·, RO·) they can interrupt the propagation step and form an antioxidant radical (A·) with low reactivity that no further reaction with lipids can occur.

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

  $RO\bullet + AH \rightarrow ROH + A\bullet$

This group includes tocopherols and polyphenols. There are natural and synthetic antioxidants, naturally present in food or added in formulation. Polyphenols are very active in this radical scavenging mechanism. Aromatic amines inhibit the autoxidation through the same electron-transfer mechanism. Polyphenols can also inhibit enzymatic lipid oxidation inducted by lipoxygenase. Quinones (vitamin K, ubiquinone, α- tocopheryl quinone) are also chain-breaking inhibitors (or antioxidants) of autoxidation acting as electron-acceptor antioxidants by competing with oxygen for alkyl radicals. This competitive reaction would only become important at low oxygen pressures (e.g. elevated temperatures), because alkyl radicals react extremely rapidly with oxygen under atmospheric conditions.

- **Second type antioxidants.** Also called *metal scavengers*, they form free radicals by blocking oxidation catalysts metals (chelation). They act before the propagation phase, preventing the reduction of activation energy in initiation reactions. They include citric acid, ascorbic acid, EDTA and some phenols. Tocopherols and carotenoids are physiscal quencher of the excited states of pigment molecules, as well as of singlet oxygen and thus can prevent oxidation (*Yanishlieva-Maslarova et al., 2001*).
- **Third type antioxidants.** They are environmental factors, (*Capella & Lercker, 1997*); this type includes temperature, partial oxygen pressure, light, moisture and they can vary the lipid oxidation reaction rate.

1.4.1 Phenolic compounds

Phenolic compounds are plants secondary metabolites, essential for growth, reproduction and defence (*Cabras & Tuberoso, 2014*).

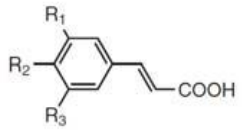
For a long time, the interests concerning polyphenols has been increased due to the recognition of their antioxidant properties, their great abundance in our diet and their probable role in the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases are well known (*Wightman & Heuberger, 2015*).

Polyphenols are a ubiquitous class of non-volatile secondary plant metabolites, characterised by one or more hydroxyl groups attached to an aromatic ring. There are probably upwards of 5000 total plant polyphenols known and several hundred are found in edible plants.

Phenolic compounds may be classified into different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another. Thus, they can be divided into several classes: hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes, and lignans. The main subclasses that are important for a human health perspective are: phenolic

acid, flavones, flavonols, flavan-3-ols, isoflavones, flavanones, anthocyanidins and lignans (*Hooper & Cassidy, 2006*). The structures of these compounds differ primarily in terms of substitution patterns, such as the number and position of hydroxyl or methoxyl groups on a basic skeleton. In nature, many of these structures are present in glycosylated or conjugated forms, which further increases their complexity (*Macheix & Fleuriet, 1990*).The most important classes of phenolic compounds are shown in *Figure 7* and *Figure 8* (*Shahidi, 2004*)

Acid		R_1	R_2	R_3
p-Hydroxybenzoic	4-Hydroxybenzoic	H	OH	H
Protocatechuic	3,4-Dihydroxybenzoic	OH	OH	H
Vanillic	4-Hydroxy-3-methoxybenzoic	OCH_3	OH	H
Syringic	3, 5-Dimethoxybenzoic	OCH_3	OH	OCH_3
Gallic	3,4,5-Trihydroxybenzoic	OH	OH	OH



Acid		R_1	R_2	R_3
p-Coumaric	4-Hydroxycinnamic	H	OH	H
Caffeic	3,4-Dihydroxycinnamic	OH	OH	H
Ferulic	4-Hydroxy-3-methoxycinnamic	OCH_3	OH	H
Sinapic	4-Hydroxy-3,5-dimethoxycinnamic	OCH_3	OH	OCH_3

Figure 7. *Phenolics acid found in food (Shahidi, 2004)*

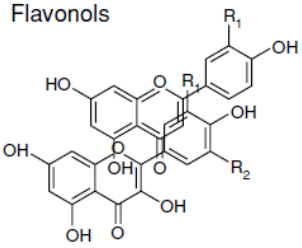
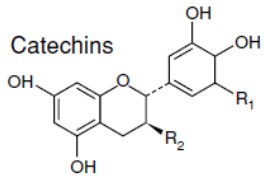
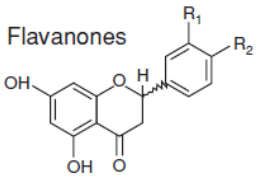
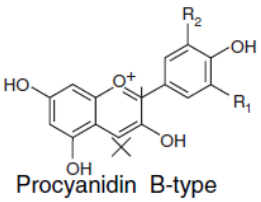
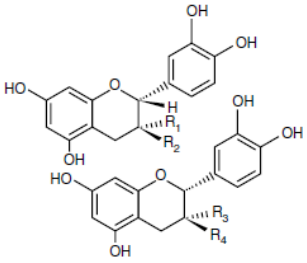
Flavonoid Subclass	Compound
Flavonols 	Quercetin ($R_1 = OH$, $R_2 = H$) Kaempferol ($R_1 = R_2 = H$) Myricetin ($R_1 = R_2 = OH$)
Flavones	Apigenin ($R_1 = H$) Luteolin ($R_1 = OH$)
Catechins 	(+)-Catechin (C) ($R_1 = H$, $R_2 = OH$) (−)-Epicatechin (EC) ($R_1 = H$, $R_2 = H$) (−)-Epigallocatechin (EGC) ($R_1 = OH$, $R_2 = OH$) (−)-Epicatechingallate (ECG) ($R_1 = H$, $R_2 = -OC-Ph(OH)_3$) (−)-Epigallocatechingallate (EGCG) ($R_1 = OH$, $R_2 = -OC-Ph(OH)_3$) (−)-Gallocatechin (GC) ($R_1 = -OC-Ph(OH)_3$, $R_2 = H$)
Flavanones 	Hesperetin ($R_1 = OH$, $R_2 = OMe$) Naringenin ($R_1 = H$, $R_2 = OH$)
Anthocyanidins 	Delphinidin ($R_1 = R_2 = OH$) Cyanidin ($R_1 = OH$, $R_2 = H$) Pelargonidin ($R_1 = R_2 = H$) Petunidin ($R_1 = OMe$, $R_2 = OH$) Peonidin ($R_1 = OMe$, $R_2 = H$) Malvidin ($R_1 = R_2 = OMe$)
Procyanidin B-type dimers 	B1: R1 = OH; R2 = H; R3 = H; R4 = OH B2: R1 = OH; R2 = H; R3 = OH; R4 = H B3: R1 = H; R2 = OH; R3 = H; R4 = OH B4: R1 = H; R2 = OH; R3 = OH; R4 = H

Figure 8. *Flavonoids subclasses (Shahidi, 2004)*

Regarding the aim of this research project the most interesting phenolic compounds are those contained in oil. Trysorol and hydroxytyrosol are two phenyl ethyl alcohols present in olive oil and also in the by-products originated from olive oil production chain. They are present as free form or conjugated with secorroid or anglicones *(Miró-Casas et al., 2003*). These two phenyl ethyl alcohols are constituted by a benzene ring in which there is a hydroxyl group in para position

(tyrosol), or there are two hydroxyl groups in meta and para position (hydroxytyrosol) and a carbon chain with another terminal hydroxyl group (*Figure 9*).

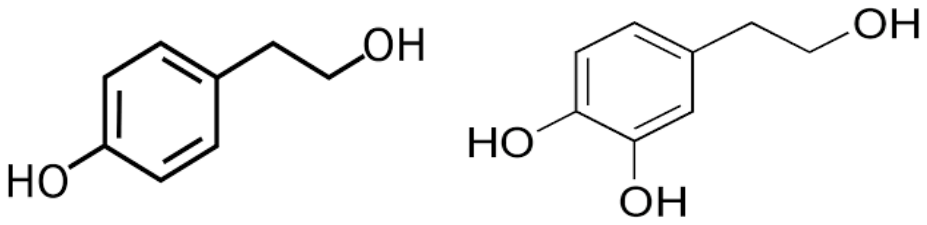


Figure 9. *Tyrosol (left) and hydroxytyrosol (right) structures*

The concentration of tyrosol and hydroxytyrosol increases during the harvest period *(Ghanbari et al., 2012*). It is known that hydroxytyrosol is partially formed from the hydrolysis of oleouropein (the most important biophenol in almost olive cultivars) during the oil extraction esterase action *(Obied et al., 2005*). Furthermore, these compounds are among the most abundant in olive mill wastewaters *(Fava et al., 2017*).

Hydroxytyrosol and tyrosol are important antioxidants from olive oil and are responsible for the antioxidant activity of olive plant components and extracts (*Bernini et al., 2015; Bernini et al., 2013; Ricciutelli et al., 2017; Shahidi & Zhong, 2010*). Recently, tyrosol has attracted the attention of organic chemists and pharmacologists as a versatile and cheap substrate for the synthesis of a variety of esters exhibiting diverse and improved biological effects (*Barontini et al., 2014*). This is partly due to tyrosol being the most abundant biophenol in extra virgin olive oil and its ability to exert protective effects against oxidative injuries in cell systems *(Giovannini et al., 1999*) and improve intracellular antioxidant defense systems *(Di Benedetto et al., 2007*).

1.4.2 Polyphenols antioxidant mechanism

In a work by *Prior and co-workers* (2005), the methods of action of phenolic compounds are listed. They act as Single Electron Transfer (SET) and Hydrogen Atom Transfer (HAT). The most common antioxidants acting as reducing agents and hydrogen donors in addition to the phenols are the tocopherols.

SET are agents that give electrons to another substance:

$R\bullet + AH \rightarrow RH + AH^{\bullet+}$

$X\bullet + H_3O^+ \rightarrow HX + H_2O$

$M\ (III) + AH \rightarrow AH^+ + M\ (II)$

HAT, instead, are hydrogen donor:

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

For example, flavonoid inactivate free radicals forming a phenoxyl radical which is stable because of electronic delocation of aliphatic and aromatic structures (*Figure 10*) *(Bors et al., 1992*)

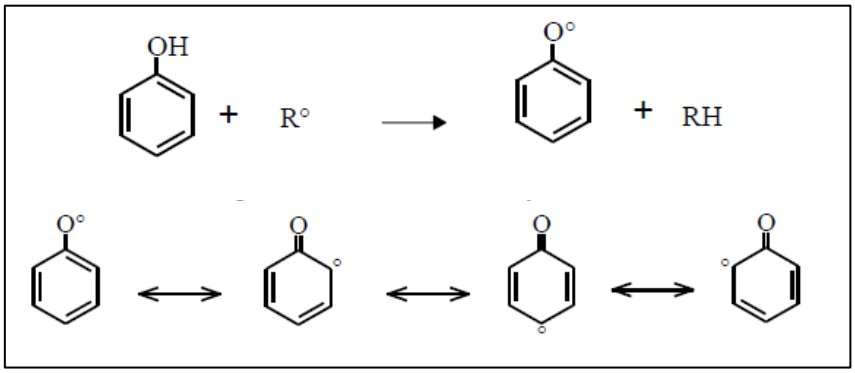


Figure 10. *Antioxidant action of flavonoids (top); radical electronic relocation (bottom)*

A large variety of biologically active phenolic compounds found in plants contain one or more aromatic rings. In fact, in the phenols the active state is found after replacing the ortho- or para-position, increasing the hydroxyl group electronic density and lowering the oxygen-hydrogen energy, increasing the reactivity towards lipid free radicals (*Gülçin, 2012*). In general, the effectiveness of antioxidant capacity of phenolic compounds depends on some factors: the metal chelating potential, which is strongly influenced by the rearrangement of hydroxyl and carbonyl groups around molecules; the presence of hydrogen or electron donor capable to reduce free

radicals and, finally, the number of hydroxyl groups attached on the benzene ring that implements radical scavenger activity (*Kao & Chen, 2013*).

1.5 Lipophilization

Lipophilization is an innovative system that is able to transform polar molecules into more hydrophobic molecules (*Grajeda-Iglesias et al., 2017*). Specifically, lipophilization consists in the esterification of the lipophilic fraction (fatty acids or fatty alcohols) of different substrates in order to obtain molecules where the hydrophilic/lipophilic ratio has been modified (*Villeneuve, 2007*). The focus of the reaction is to modify or improve the functional properties of the original compound and facilitate its incorporation into emulsified or lipid-based media (*Figueroa-Espinoza & Villeneuve, 2005*). This reaction has been successfully applied in different studies over the past years, especially on phenolic compounds such as chlorogenic, rosemary, caffeic and protocatechic acid (*Grajeda-Iglesias et al., 2017*); in order to obtain a series of amphiphilic molecules, known as lipofenols (C*rauste et al., 2016*) or phenolipids, which, depending on the length of the esterified alkyl chain, exhibited remarkable antioxidant properties (*Grajeda-Iglesias et al., 2017*). The use of natural phenols with antioxidant function has exploded in recent years, since many antioxidants available on the market (BHT, BHA, TBHQ) would seem to be totally unsatisfactory due to a suspected role of promoting carcinogenesis (*Mbatia et al., 2011*). Both unsaturated fatty acids and polyphenols are molecules that can be successfully recovered from by-products of the agro-food chain (meat and oil production chains) and their combination lead to a compound of very high added value, in accordance with the recovery and reuse explained in paragraph 1.2.

1.5.1 Purposes and targets of lipophilization

As mentioned, lipophilization is an innovative and interesting method to made available antioxidant molecules in a lipid matrix that can produce beneficial effects for the matrix but which, at the same time, would be difficult to use due to the chemical incompatibility that characterize thsee molecules (*Mbatia et al., 2011*). Oxidation of animal and vegetable fats used in bakery products is a clear example. Examining a bakery product, in which dough coexisting fatty, aqueous and gaseous ingredients, the addition of antioxidants compounds in the formulation may result a good initiative to delay as long as possible the induction phase of oxidation. From chemical point of view, a hydrophilic antioxidant will hardly be able to place at the air-fat interface if it is not adequately modified for this purpose. Lipid oxidation in food systems is a critical factor affecting food quality, nutritional aspects, safety, color and consumer acceptability *(Sørensen et al., 2014*). Oxidative stress is also the cause of the formation of radical species which, are strongly linked to

cardiovascular, neurodegenerative and inflammatory pathologies, which in turn can be counteracted by the action of antioxidants compounds in general *(de Pinedo et al., 2007*). It is also for this reason that most of the studies on lipophilization consider the treatment of natural compound classes with well-defined technological characteristics, such as polyphenols and fatty acids, which, if combined, they have a strong antioxidant function and can be transported inside the lipid fraction to perform the protective function *(Crauste et al., 2014*). The advantage of the conjugation of unsaturated fatty acids and polyphenols is bivalent: it is important to limit the autoxidative phenomenon affecting PUFAs (polyunsaturated fatty acids) due to the double bonds in the carbon chain, thus preserving the health benefits. At the same time, esterification of phenols with unsaturated fatty acids is a good way to mask their polar function and increase the antioxidant properties in a lipophilic medium *(Crauste et al., 2016*).

Oxidized lipids, when absorbed by human organism, are incorporated as lipoproteins and their conversion to low density lipoproteins (LDL) would lead to atherosclerosis *(de Pinedo et al., 2007*). The synthesis of a fatty acid with a phenol can be spendable not only in the food sector, but also in the medical and pharmacological context. Increasing the lipophilicity of phenolic compounds increase cell penetration and bioavailability of the starting polar molecule *(Crauste et al., 2016*). The development of innovative molecules with better antioxidant capacity and less toxicity is desirable for the prevention and treatment of diseases and for proper food storage (*de Pinedo et al., 2007*)

1.5.2 Lipophenol synthesis: the enzymatic way and the cut-off effect

The modification of antioxidants molecules by lipophilization is an important field of research whose purpose is to improve its effectiveness. What characterizes the author's work was a substantial lack of accurate information on the influence of hydrophilicity on antioxidant activity. For this reason, lipophilization strategies have been largely based on empirical evidence where the final result was not optimal for different aspects *(Laguerre et al., 2013*).

In general, the literature agrees that the combination of unsaturated fatty acid and a phenol compound can be obtained chemically, enzymatically or chemo-enzymatically, often by esterification or acylation of the phenolic group -OH with fatty acids (*Crauste et al., 2016*), as shown in *Figure 11*.

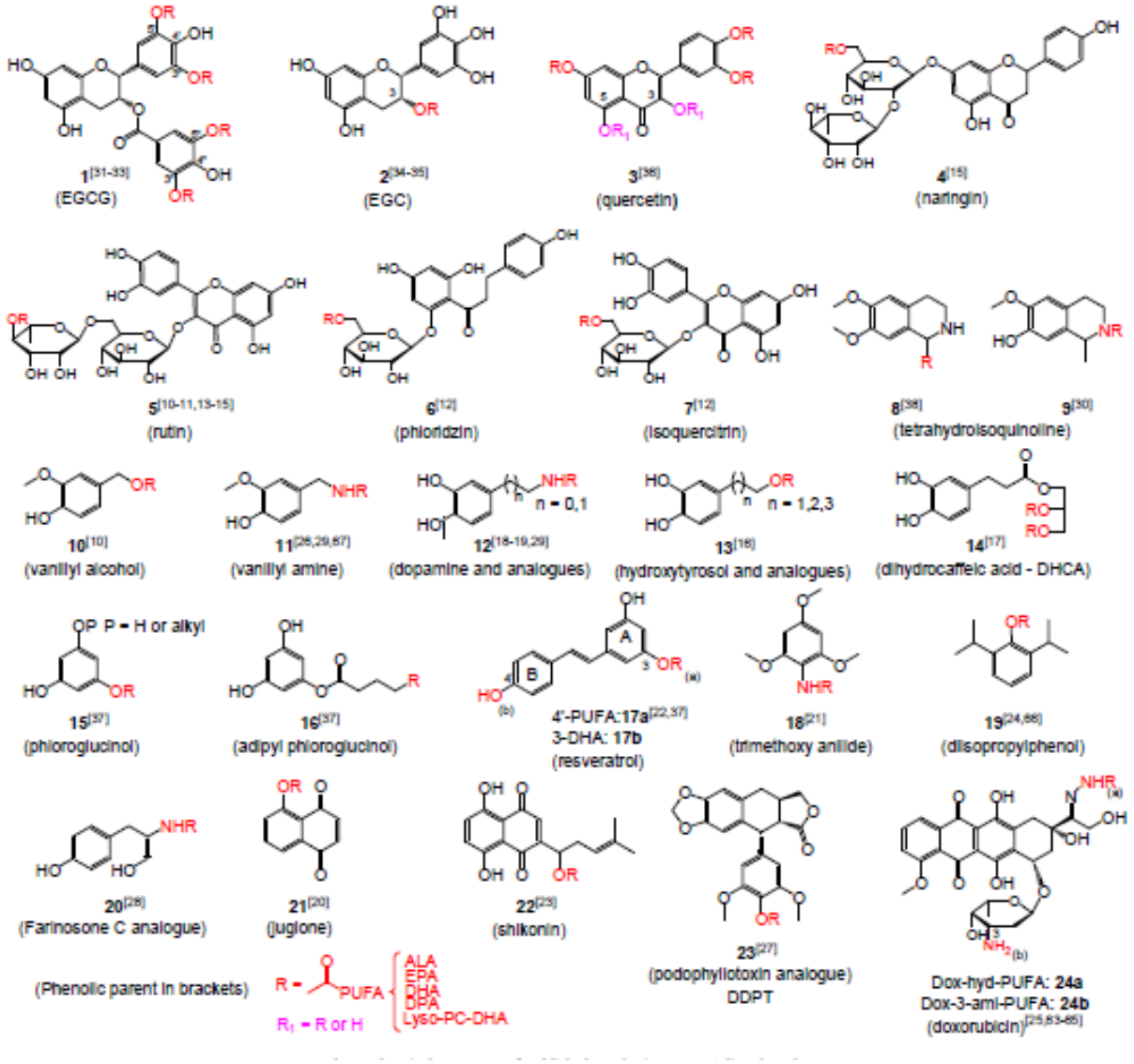


Figure 11. *Chemical structures of lipophilic derivatives obtained with polyunsaturated fatty acids. In red and pink are higlightened acylation sites (Crauste et al., 2016)*

Enzymatic synthesis. The synthesis of phenols and PUFAs is closely related to the phenol structure and some phenols have already been conjugated to α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In the work conducted by *Crauste et al.* (2016), emerges that the enzymatic way, which involves lipases, is always preferable to chemical way for the synthesis between fatty acids and phenols. The high selectivity of the lipase allows only the introduction of a fatty acid residue and the less extreme process conditions prevent the substrate from alterations (*Villeneuve, 2007*). However, the presence of double bonds could negatively affect the specificity of the lipase enzyme; the most studied enzyme is Novozyme 435, commercially an immobilized lipase by *Candida antarctica* (CALB). Esterification and transesterification catalyzed by lipase has been used to produce lipophenolic structures having a

catecholic group such as vanillyl alcohol, dihydrocaffeic acid and hydroxytyrosol. The tests carried out over the years discovered a limitation of lipophilization reactions, that is time: acceptable yields were obtained only after many hours of continuous reaction (even 58 hours); only in one case the times were significantly shortened by the use of vacuum *(Torres de Pinedo et al., 2005; Wang et al., 2015*).

In a study by *Villeneuve* (2007), *C. antarctica* immobilized lipase was used for esterification of hydroxytyrosol (3,4-dihydroxyphenylethanol) with caprylic acid (C8:0) in different solvents: first, dissolved in diethyl ether, an 85% yield conversion in 15 hours at 35 ° C was obtained; subsequently, in more polar solvents such as chloroform, dichloromethane and tetrahydrofuran, conversions were close to 20%. Evaluating antioxidant activity in refined sunflower oil, it was noted that the octanoate ester (synthesis between caprylic acid and hydroxytyrosol) had lost significantly the antioxidant capacity when compared with the starting hydroxytyrosol. This loss appears to be caused by the absence of the primary hydroxide group after esterification with fatty acid, as shown in *Figure 12.*

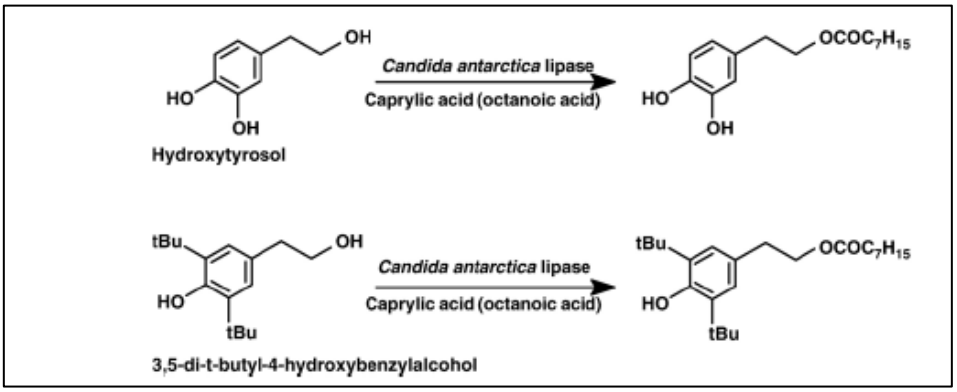


Figure 12. *Esterification catalysed with lipase between hydroxytyrosol and caprilic acid (Villeneuve, 2007).*

According to *Laguerre and collaborators* (2013), antioxidant activity increases with the increasing of compound hydrophobicity up to a threshold, when it is reached any lengthening of the hydrophobic chain leading to a collapse of antioxidant activity. This phenomenon, evidently non-linear, is called *cut-off* effect and has been observed in many biological and physico-chemical systems, such as emulsions (*Laguerre et al., 2013*). Because of this effect, the antioxidant activity of the final compound may not be optimal either when the conjugated aliphatic chain is too short or too long (*Figure 13*).

Homologous series	Medium/oxidant	CCL ± Sd	Chain length tested (* = CCL)	Reference
Chlorogenate alkyl esters (Fig. 2A)	O/W emulsion/AAPH	12 ± 3	0, 1, 4, 8, *12**, 16, 18, 20	[19]
Chlorogenate alkyl esters (Fig. 5)	Fibroblasts (no oxidation inducer)	12 ± 3	0, 1, 4, 8, *12**, 16	[21]
Rosmarinate alkyl esters (Fig. 2B)	O/W emulsion/AAPH	8 ± 3	0, 1, 4, *8**, 12, 16, 18, 20	[20]
Rosmarinate alkyl esters	O/W emulsion (no oxidation inducer)	4 ± 3[a]	0, *4**, *8**, *12**, 18, 20	[29]
Rosmarinate alkyl esters	O/W emulsion/Riboflavin	6 ± 1	0, *4**, *8**, 12, 18, 20	Personal data
Rosmarinate alkyl esters (Fig. 4)	Liposomes (no oxidation inducer)	6 ± 5	0, *4**, 12, 18, 20	[35]
Rosmarinate alkyl esters	Fibroblasts (no oxidation inducer)	10 ± 1	0, 1, 4, 8, *10**, 12, 16, 18	Personal data
Hydroxytyrosol alkyl esters	O/W emulsion (no oxidation inducer)	8 ± 3	0, 2, 4, *8**, 12	[30]
Hydroxytyrosol alkyl esters	Muscle cells/cumene hydroperoxide	7 ± 4	0, 2, *4**, *6**, *7**, *8**, *9**, *10**, 12, 16, 18	[37]
3,4-DHP alkyl esters[b]	Muscle cells/cumene hydroperoxide	6 ± 1	0, 2, 4, *6**, 8, 10, 12, 14, 16, 18	[38]
2,3-DHE alkyl esters[c]	Muscle cells/cumene hydroperoxide	7 ± 2	0, 2, 4, *6**, *8**, 10, 12, 14, 16, 18	[38]
2,3-DHP alkyl esters[d]	Muscle cells/cumene hydroperoxide	7 ± 2	0, 2, 4, *6**, *8**, 10, 12, 14, 16, 18	[38]
Daidzein alkoxyethers	Liposomes/AAPH	12 ± 3	0, 4, 8, *12**, 16	[36]
Ferulate alkyl esters (Fig. 3B)	Liposomes/AAPH	7 ± 2	0, 1, 2, 4, *6**, *8**, 10, 12	[25]
Gallate alkyl esters (Fig. 3A)	Liposomes/AAPH	10 ± 6.5	0, 1, 3, *12**, 18	[25]
Ubiquinol derivatives	Microsomes/Fe^{2+}, ascorbate; Fe^{2+}, NADPH	6 ± 3	0, *6**, 10, 14, 18, 22, 26, 30, 34, 38, 42	[33]
Ascorbate alkyl esters	Caco-2 cells/$FeSO_4$	13 ± 2	0, 10, *12**, *14**, 16, 18	[39]

Italics and asterics represent the critical chain length.
[a] Based upon hydroperoxides measurement only (from hexanal measurement, CCL corresponds to 4 carbon atoms).
[b] 3-(3,4-Dihydroxyphenyl)-1-propanol (3,4-DHP), hydroxytyrosol analogues.
[c] 2-(2,3-Dihydroxyphenyl)-1-ethanol (2,3-DHE), hydroxytyrosol analogues.
[d] 3-(2,3-Dihydroxyphenyl)-1-propanol (2,3-DHP), hydroxytyrosol analogues.

Figure 13. *Critical chain lengths (CCL) within homologous series of antioxidants.*

According to *Laguerre et al*. (2013), there are three advanced hypothesis to justify this *cut-off* effect:

- **Reduced mobility.** According to this hypothesis, the mobility of an antioxidant decreases as the alkyl chain increases, decreasing the ability to move in the direction of the numerous oxidation sites. In other words, the frequency of hydrophobic contacts increases for long chain antioxidants compared to medium chains since the former are strongly bounded by hydrophobic interactions to their environment and thus they have a lower freedom degree. This aspect may partially affect the antioxidant activity.
- **Internalization.** The internalization hypothesis is based on the idea that the increasing of carbons chain lenghth can drive the antioxidant away from the interface (where oxidation primarily would occur) toward the lipid core of emulsions and micelles or to internal membrane of the liposomes and cells where it would not fully carry out the antioxidant function.
- **Self-aggregation.** The last hypothesis is based on the concept that the collapse of the antioxidant capacity is due to the compound self-aggregation, since long chain antioxidants exist predominantly as colloidal aggregates. This could lead to two drawbacks: first, self-aggregation would lead to the removal of the antioxidant from the interface in which oxidation is induced; secondly, micellisation would make the long chain antioxidants more bulkier than the free molecules, making them less mobile. This last point shows that the first hypothesis and self-aggregation are complementary; reduced mobility may be a consequence of self-aggregation.

1.5.3 Study cases of antioxidant compounds obtained by lipophilization

Numerous case studies have been published over the last ten years regarding the lipophilization tests of phenolic compounds and fatty acids.

In a study by *Mellou and collaborators* (2006) is evaluated the enzymatic esterification between rutine and naringin with different mono and polyunsaturated fatty acids, catalyzed with *C. antarctica* immobilized lipase in non-toxic solvents. In this study, enzymatic esterification of flavonoids was completely site selective, according to the previous literature. *Torres de Pinedo*, along with his collaborators (2007), has synthesized new compounds with a radical scavenging activity better than the starting antioxidant (hydroxytyrosol). *Viskupicova et al.* (2010) synthesized rutine lipophilic derivatives with saturated and unsaturated fatty acids. The result has shown, in lipophilic derivatives, greater hydrophobicity and fat solubility. Two years after, these derivatives were used to inhibit the action of serine protease *(Viskupicova et al., 2012*). In a study conducted by *Zheng et al.* (2013) the esterification of rutine and naringine with unsaturated fatty acids under ultrasonic pretreatment has been demonstrated: reaction time for esterification in the pretreatment process was 24 shorter than the reaction time in agitation, without evident lipase damage. In a recent work by (*Chen et al., 2017*), lipophilization with free fatty acids of epicatechin in refined camellia seed oil was successfully performed, producing mainly epicatechin oleate and epicatechin palmitate, with enhanced antioxidant capacity. Finally *(Grajeda-Iglesias et al., 2017*) used anthocyanins from *Hibiscus sabdariffa* flowers obtaining lipophilic dyes to be incorporated into foods and cosmetic formulations.

1.5.4 Emulsions

An emulsion is a suspension of an element in a non-miscible phase. In most cases, these are two liquids: the emulsion is characterized by the presence of microscopic drops (0.1 to 10 μm) of one of the two dispersed liquid in the other (*Adrian et al., 2009*). In order to obtain a stable emulsion, it is necessary to form a ternary mixture: the base, the non-miscible element and a third component which allows the suspension maintenance of the initial non-miscible particles. Most foods are a complex matrix that is often found in the form of emulsions in which fat is incorporated (*Sørensen, et al., 2014; Sørensen et al., 2015*). It is frequent, in fact, to come into fat suspensions or hydrophobic elements in an aqueous phase. This is achieved when phospholipids or lipoproteins - having hydrophobic and hydrophilic moieties - cover the fat cells maintaining and stabilizing them in the aqueous phase, as in milk (*Adrian et al., 2009*). In addition to these oil-water emulsions, there are water-oil emulsions (margarine and butter) and also gas-liquid emulsions (whipped cream). Food emulsions are not stable systems and often, during storage, fatty and aqueous phases tend to

separate if they are not properly stabilized with emulsifiers such as casein and natural or synthesized phospholipids (*Caporaso et al., 2016*). The *Figure 14* shows the behaviour of amphiphilic molecules in an emulsion.

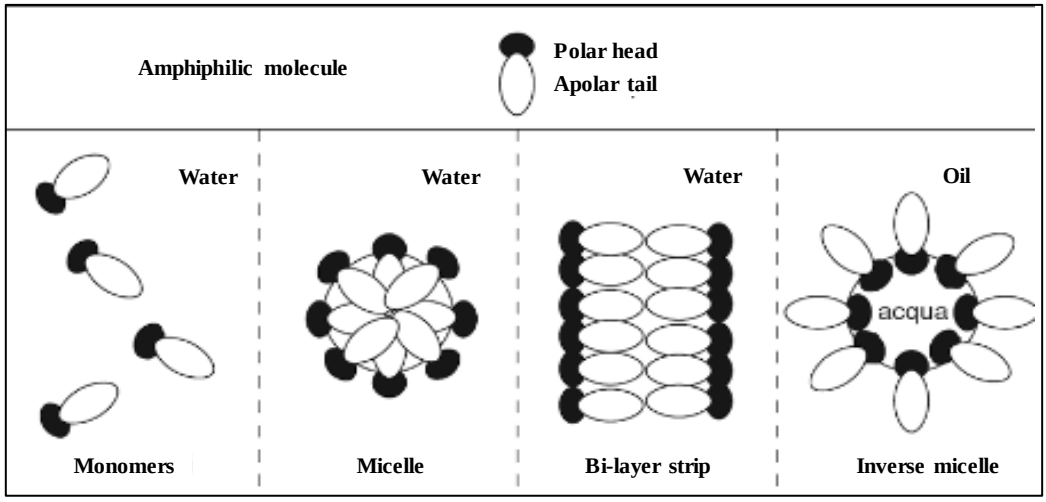


Figure 14. *Emulsions examples starting from amphiphilic molecules*

1.5.5 Antioxidant function in an emulsion: the polar paradox

From physical point of view, emulsions present three phases: an aqueous phase, a lipophilic phase, and an oil-water interface (*Sørensen et al., 2015*). For this reason, since lipid is initiated at the interface, the position of the antioxidants in an emulsion system is crucial for their effectiveness (*García-Moreno et al., 2014*). Depending on their characteristics, including the lipophilicity of the antioxidant and the emulsion composition, antioxidants can be divided into the three different phases.

Many studies over the years have focused on the development of antioxidants molecules that could be transported directly to the oxidation induction site proposing lipophilization. The lipophilization of the phenolic compounds by esterification with fatty alcohols may alter their location in an emulsion and therefore their antioxidant efficacy (*Sørensen et al., 2015*). In addition to the previously mentioned *cut-off* phenomenon (*Laguerre et al., 2013*), the first hypothesis advanced to explain the effectiveness of antioxidant capacity of some compounds in emulsions is called *polar paradox* (*Sørensen et al., 2014*), which says that apolar antioxidants would be more efficient than polar antioxidants. In particular, hydrophilic antioxidants in an oil-water emulsion are distributed in the aqueous phase to which they are chemically more related; however, this may result in lesser protection of the fat matrix by such antioxidants than lipophilic ones, which are oriented on the oil-water interface *(Frankel, 1996; Frankel et al., 1994; Shahidi & Zhong, 2010*).

The lipophilization of phenolic compounds with different lengths alkyl chains would reduce their polarity, altering the distribution in the emulsion phases, also increasing their antioxidant efficacy *(Alemán et al., 2015*). Considering the *cut-off* effect, antioxidants with carbon-length equal to the threshold value are more concentrated at the oil-water interface, while antioxidants with chain length below and above the threshold value are conducted far away from the interface (*Sørensen et al., 2014*).

For now, there are not many studies that have evaluated the efficacy of lipophilic phenolic compounds in real food systems. Good antioxidants have been shown in the emulsions of alkylated caffeids due to their free radical scavenger properties (*Sørensen et al., 2014*). Rutine esters were also studied in milk enriched with fish oil: in particular, lauryl ester (C12) and palmitate (C16) were used by *Sørensen et al.* (2015); which concluded that the best antioxidant action in enriched milk is done by the medium-chain ester (rutine + lauric acid). An interesting study conducted by *Alemán et al.* (2015) demonstrated that lipophilization of caffeic acid with fatty acids at different lengths (C1-C20) had, in milk and mayonnaise enriched with fish oil, higher antioxidant capacity values than the native phenolic molecule. In addition, the efficacy of the caffeids was different for different matrices: short or medium chains (C4, C8 and C12) had greater efficacy in mayonnaise, while the short chain compounds (C1 and C4) showed more antioxidant activity in the enriched milk sample.

The aim of this research project was the synthesi of a lipophenol, starting from molecules resulting from food chain by-products. Four different by-products from bovine meat industry were characterized in order to study their composition to use their lipid fraction in lipophenol synthesis; and, in collaboration with Aarhus University in Denmark, the improvement of the antioxidant activity of tyrosol was studied, in order to use this compound as phenolic fraction. After the synthesis, the lipophenol was tested in a lipid real food system to study its antioxidant effectiveness.

2. Materials & Methods

Section I

Lipid characterization of by-products originated from beef meat industry through innovative chromatographic techniques

I.1 Bovine by-products

A total of four by-products produced by a slaughterhouse in the north of Italy were analysed: storage fat, subcutaneous fat, bone marrow and bone fat. These by-products were obtained from three Friesian bovine categories, differing in age and morphology, that is calf (CA), young bull (YB) and cow (CO). Calf was fed only with milk due to the young slaughter age. Young bull and cow were fed with a traditional diet, based on the use of concentrate and hay (*dry feeding period*) and fresh forage (*fresh forage feeding period*) without silages. The different categories were slaughtered according to slaughter legislation: calves were slaughtered at 5-6 months old, 200-250 kg; young bulls at 16-18 months old, 600-650 kg and cows at 14-18 months old, 420-480 kg. These samples are the result of all the waste obtained at the end of a working day, approximately thousand animals. Before the lipid extraction, in order to obtain a homogeneous sampling and to facilitate the breaking of fat cells, all by-products were dissected and crumble with knives and blade blender and then sonicated at room temperature.

I.2 Extraction of the lipid fraction

Total lipid extraction was performed with the method by *Folch et al.* (1957), with some modifications (*Boselli et al., 2001*). Approximately 20 g of dissected sample were weighed and 200 mL of chloroform:methanol 1:1 (v/v) were added. After 15 minutes of sonication, each Sovirel® bottle containing the sample was placed at 60 °C for 20 minutes. After cooling, 150 mL of chloroform was added and the mixture was again sonicated for 15 minutes and then filtered through Buchner funnel. 70 mL of KCl 1 M were added to the filtered extract and stored in the fridge overnight. The next day, without agitation, the organic phase was recovered through a separating funnel and sodium anhydrous sulphate was added and then left in the dark for 2 hours. Subsequently, the extracts were filtered in 250 mL flasks and dried at 40 °C. The obtained fat was resuspended with a solution of hexane:2-propanol 4:1 (v/v) and stored at -18 °C until analysis. Three extractions were performed for each sample.

I.3 Fatty acids analysis

The fatty acids composition was determined as fatty acid methyl esters (FAMEs) by capillary gas chromatography analysis after alkaline treatment. Methyl tridecanoate (C13:0, 2 mg/mL) was used as internal standard and FAMEs were analyzed on a GC 2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector (FID) and an AOC-20s auto sampler (Shimadzu Corporation), according to *Verardo et al.* (2013) with slight modifications. The FAMEs were analysed using a BPX70 fused silica capillary column (10 m x 0.1 mm i.d. 0.2 µm film thickness; SGE Analytical Science. Ringwood. VIC. Australia). The injector and flame ionization detector temperatures were set at 250°C. Hydrogen was used as carrier gas at a flow rate of 0.8 mL/min. The oven temperature was held at 50°C for 0.2 min, increased to 175°C at 120°C/min, held at 175°C for 2 min and finally increased from 175 to 220°C at 20°C/min. Samples were injected in split mode (0.3 µL) with a split ratio set at 1:100. Peak identification was accomplished by comparing peak retention time with GLC-463 standard mixture from Nu-Check (Elysian. MN. USA) and FAME 189-19 standard mixtures from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and expressed as weight percentage of total FAMEs. FAMEs composition was measured in 2 replicates for each lipid extract (*n=6*) and each analysis lasted 7 minutes.

I.4 Oxidative status: peroxide value (PV)

The method used is the one of the International Dairy Federation, similar to *Shantha & Decker* (1994). The method is based on the determination of Fe^{3+} ions formed by oxidation of Fe^{2+} ions by hydroperoxides in the presence of ammonium thiocyanate (NH_4SCN). Thiocyanate anions (SCN^-) react with Fe^{3+} ions to give a red colored which can be determined spectrophotometrically.

The method for the determination of peroxide value involves the preparation of two solutions that allow quantitative evaluation of such oxidation compounds. The first one is a Fe^{2+} solution, composed by two solutions: solution A was prepared with 0.4 g of barium chloride dissolved in hydrochloric acid (0.4 N) in a 50 mL flask. Solution B was prepared with 0.5 g of ferrous sulphate heptahydrate dissolved in water in a 50 mL flask. 2 ml of 10 N hydrochloric acid and solution B slowly were added to solution A under constant magnetic stirring. The second solution is an ammonium thiocyanate solution: 3 g of ammonium thiocyanate were dissolved in 10 mL of water. 50 mg of fat were weighted and 9.9 mL of chloroform:methanol 7:3 (v/v) were added. After stirring the tube for a few seconds to complete fat solubilization, 50 µl of Fe (II) solution and 50 µl of ammonium thiocyanate solution were added. After agitation, the sample was stored in the dark for 5 minutes and after this time spectrophotometric

reading was carried out at 500 nm. The analysis was repeated twice for each lipid extract (*n=6*). Quantification of peroxides, expressed as meqO_2/kg of fat, was carried out using the following formula:

$$\frac{[ABS - ABS_{Bianco}] - b}{m \times 55{,}84 \times w}$$

Where:

ABS: absorbance value at 500nm;

m: slope of the calibration curve;

b: intercept of the calibration curve;

55.84: atomic weight of iron;

w: fat weight expressed in g

I.5 Triglycerides analysis

Triglycerides (TGs) analysis was carried out with the injection of 1.0 µL of solution (10 mg/mL of fat in hexane) into a GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector (FID) and an AOC-20s auto sampler (Shimadzu Corporation) according to *Guerra et al.*, (2015), with slight modification. TG separation was performed with an Rtx-65 TG fused silica capillary column (30 m x 0.25 mm x 0.10 µm film thickness) with 35% dimethyl, 65% diphenyl polysiloxane (Restek, Chromatography Products, Superchrom Milano, Italy). The initial oven temperature of 140°C was raised to 360°C at a rate of 25°C/min and was held at 360°C for 5 min. The injector and detector temperatures were set at 360°C. The hydrogen flow rate was 3.84 mL/min. The split ratio was 1:30. TGs were identified based on retention time of a standard mixture injected with the same method and from the comparison with the chromatograms reported in literature. The methodology for the analysis was based on the separation of classes of TGs according to the total number of carbon atoms (CN; sum of the three FAs) and each class has been quantified as a percentage of total TG content. TG composition was measured in 2 replicates for each lipid extract (*n=6*) and each analysis had a duration of 26 minutes.

I.6 Diglycerides analysis

To evaluate the diglycerides content, 100 mg of fat were weighted and 70 µl of dihydrocholesterol (1 mg/ml) were added. Everything was recovered with hexane:diethyl ether 80:20 (v/v). Solid Phase Extraction (SPE) columns (Phenomenex, California, USA) were supplemented with anhydrous sodium sulphate and conditioned with 3 mL of hexane; on each column the fat sample was loaded and 5 mL of hexane:diethyl ether 80:20 (v/v) were added discarding the eluate obtained. Then 4 mL of hexane:diethyl ether 1:1 (v/v) and 3 mL of methanol were added, in both cases the eluate obtained was collected. The extract obtained was evaporated and subsequently derivatized by the method reported in *Sweeley et al.,* (1963): 500 µL of silylating mixture (pyridine anhydride: hexamethyldisilazane: trimethylchlorosilane 5:2:1) were added to the sample left for 20 minutes at 40 °C. At the end of the silanization, the solvent was evaporated under nitrogen and then resuspended in 200 µL of hexane and injected.

The separation of the diglycerides was performed by a RTX-65 TG column (Restek, Superchrom Srl, Milan) (30 m x 0.25 mm i.d. x 0.1 µm f.t., Crossbond® 65% diphenyl: 35% dimethyl polysiloxane).

1 µL of each sample was injected using the following parameters:

- Carrier gas: helium;
- Column flow: 3.84 mL/min;
- Split ratio: 1:100 v/v;
- Injector and detector (FID) temperature: 350 °C;
- Temperature program: initial temperature of 240 °C maintained for 0.5 minutes, then 350 °C with a 10 °C/min increase and this temperature was maintained for 15 minutes.

DG identification was obtained by comparing the retention times and GC profiles of standard diglycerides reported in literature. The total content in DG of the samples was expressed as a percentage of total content and for each lipid extract the analysis was carried out twice (*n=6*).

I.7 Cholesterol determination

Cholesterol was collected by cold saponification at room temperature according to *Sander et al.,* (1989) after addition of 500 µL of internal standard (dihydrocholesterol, 2 mg/mL) to 250 mg of fat. The unsaponificable fraction was evaporated by vacuum evaporator, silylated (*Sweeley et al., 1963)* and dried again under gentle nitrogen flow. After redissolution in 500 µL of *n*-hexane, 1 µL was injected

into a GC 2010 Plus gas chromatograph (Shimadzu. Kyoto. Japan), equipped with a flame ionization detector (FID) and an AOC-20i autosampler, according to the method reported by *Guerra et al.* (2016). A Rxi-5ms fused silica capillary column (10 m x 0.10 mm i.d. x 0.10 µm film thickness) from Restek (Restek Corporation, Bellefonte, USA) was used. Oven temperature was set at 240°C and the injector and detector temperature were 325°C. The column flow of hydrogen was 0.4 mL/min and the injection volume was 0.30 µL, with a split ratio of 1:50. Cholesterol content was measured in 2 replicates for each lipid extract (*n=6*) and was expressed in mg of cholesterol on kg of fat; and each analysis lasted 15 minutes.

I.8 Phospholipids determination

The phospholipid (PL) extraction was made according to *Avalli & Contarini* (2005) by a purification of the lipid extracts with solid-phase extraction (SPE) cartridges. The identification and quantification of PL classes was performed using an Agilent liquid chromatography HP 1200 Series, (HPLC; Agilent Technologies, Palo Alto, CA, USA) combined with an evaporative light-scattering detector (ELSD; PL-ELS1000, Polymer laboratories, Church Stretton, Shropshire, UK). The separation of PLs was achieved using a silica column, 150 mm x 3 mm with 3 µm particle diameter (Phenomenex, Torrance, CA, USA) and applying the method by *Verardo et al.* (2013) with some modifications. The HPLC system was controlled by Agilent Chem-Station software (Agilent Technologies), whilst chromatogram and data processing were assessed by ClarityLite (ver. 2.4.0.190, Data- Apex, Praha, Czech Republic). PLs were identified by comparison with pure standards and quantified with external calibration curves, prepared separately for each phospholipid identified (from 1 to 500 mg/mL of PE, PI, PS, PC and SM); and each analysis lasted 36 minutes. Phospholipids content was measured in two replicates for each lipid extract (*n=6*) and was expressed in mg phospholipid/100g of fat.

I.9 Isolation of oleic acid

For the isolation of oleic acid, 1 g of storage fat was stored in 15 mL of different solvents at 4°C overnight in a mixture of acetone:hexane 1:1 (v/v). The next day a separation between the crystal phase and the liquid phase was observed; the fatty acids composition of liquid phase was analysed with the same method reported in paragraph I.3.

Section II

Enzymatic alkylsuccinylation of tyrosol: synthesis, characterization and property evaluation as a dual-functional antioxidant

II.1 General procedure for lipase-catalyzed synthesis

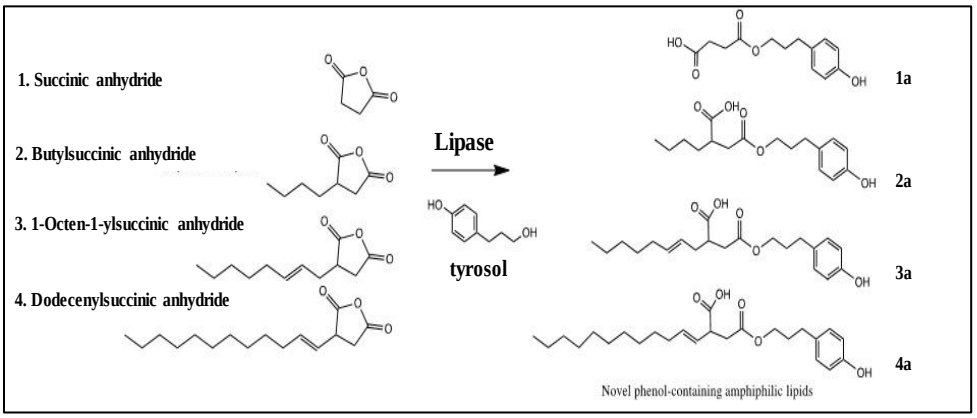


Figure 15. *Scheme of alkylsuccinilation of tyrosol*

The reaction mixture to obtain the different products (*Figure 15*) consisting of 1 mmol of tyrosol, 6 mmol of the respective anhydride and 18 mg molecular sieves (3 Å, activated at 180 °C for 8 h) in 10 mL hexane:2-MTHF (7:3) was incubated at selected temperatures in a 50 mL jacketed glass reactor with temperature controlled by a circulating water bath. After being heated for 30 min to ensure the complete solubility of the reagents, the reaction was initiated by the addition of 15% Novozyme 435 (based on tyrosol weight), with magnetic agitation at 350 rpm for 20 h.

Thin layer chromatography (TLC) analysis was used to monitor the reaction progress. Briefly, aliquots from the reaction mixture were taken at set time intervals, diluted with 300 µL $CHCl_3$: methanol 3:1 (v/v) and analyzed on TLC plates (TLC silica gel 60, 5 cm x 10 cm, Merck, Germany). The plates were developed with diethyl ether:petroleum ether:acetic acid 85:15:1 (v/v/v) until the solvent front moved 8 cm. The plates were then dried and checked under UV lamp at 254 nm. At the end of the reaction, the lipase and molecular sieves were removed by filtration and the solvent evaporated under vaccum. The conversion of tyrosol was estimated with HPLC (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a UV-visible detector (Thermo Fisher Scientific, Waltham, MA, USA) at an emission

wavelength of 280 nm. The column used was a Supelco (250 mm x 4.6 mm i.d., 5 µm particle size) column at a temperature of 35 °C. Two solvents mixtures were used with solvent A being methanol containing 0.1% of H_3PO_4. Solvent B was water:methanol (90:10) with 1.2% of H_3PO_4. Flow rate and injection volume were 1 mL/min and 10 µL respectively. Compound peaks were identified using retention times of tyrosol standards and its acid esters, purified in this work. Area percentage was used as weight for the calculation of the yields of the products. The determinations were performed in duplicates. Optimization of the reaction conditions was done using 2-dodecen-1-yl succinic anhydride as a representation. The parameters for the optimization of were substrate molar ratios, type of solvents and enzymes, reaction temperature, and enzyme loading. Six solvents (hexane, *t*-BuOH, toluene, 2-methyltetrahydrofuran, MTBE and a mixture of hexane:2-MTHF 7:3), three types of enzymes (Lipozyme RM IM, Lipozym 435 and Novozym 435), four substrate molar ratios (alcohol:anhydride, 1:1, 1:2, 1:4 and 1:6), five temperature levels (40 °C, 50 °C, 60 °C, 65 °C and 70 °C) and four levels of enzyme loading (2%, 5%, 10% and 15%) were considered. Each reaction time were sampled twice and the calculate yields were expressed as percentage (%).

II.2 Purification of synthetic compounds

Synthesized compounds were purified on a glass column packed with silica gel using diethyl ether:petroleum:ether:acetic acid 85:15:1 (v/v/v) as elution solvent. Identification of the compounds was carried out by ^{1}H NMR spectroscopic analysis (Bruker Avance III spectrometer) at 400 MHz. MS spectra were obtained on a Bruker Maxis Impact electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-QTOF-MS) (Bruker Daltonic GmbH, Bremen, Germany). FT-IR Spectra were recorded using an ATR-FTIR (PIKE, Madison, WI; Bruker, Ettlingen, Germany).

Synthesis of 4-(3-(4-hydroxyphenyl)propoxy)-4-oxobutanoic acid (1a). 1 mmol of tyrosol and 6 mmol of succinic anhydride were catalyzed by Novozym 435 (15% of total weight of equivalent amount of tyrosol) in 10 mL hexane:2-MTHF 7:3 for 20 h. White liquid; isolation yield: 40.61%, R_f: 0.48; ^{1}H NMR (400MHz, $CDCl_3$, 25 °C, TMS): δ=1.19 (s, 2H, –C**H**$_2$–), 2.58-2.61 (d, J = 12 Hz, 6H, 1×C**H**$_2$–, 2×-C**H**$_2$–), 7.19 (s, 1H, AR-H); MS, m/z calcd for $C_{13}H_{16}O_5$:238.08; found: 261.08 (M + Na^+). (*Appendix 1* and *Appendix 2*).

Synthesis of compound 2-(2-(3-(4-hydroxyphenyl)propoxy)-2-oxoethyl)hexanoic acid (2a). White liquid; isolation yield: 52.03%, R_f: 0.67; ^{1}H NMR (400MHz, $CDCl_3$, 25 °C, TMS): δ=1.00 (s, 3H, –C**H₃**), 1.42 (s, 4H, –C**H₂**–C**H₂**), 1.62-1.76 (m, 2H, –C**H₂**), 1.77- 1.81 (s, 2H, –C**H₂**–), 2.20 (s, 2H, –C**H₂**–), 2.60 (s, 1H, –C**H**–), 2.95 (s, 4H, –C**H₂**–C**H₂**–), 4.37 (s, 2H, –C**H₂**–), 6.89-6.91 (d, J = 8 Hz, 2H, AR-H), 7.13-7.15 (d, J = 8 Hz, 1H, AR-H); MS, m/z calcd for $C_{17}H_{24}O_5$:308.16; found: 317.14 (M + Na^+), 611.28 (2M + Na^+). (*Appendix 3 and Appendix 4*).

Synthesis of compound (E)-2-(2-(3-(4-hydroxyphenyl)propoxy)-2-oxoethyl)dec-4-enoic acid (3a). White liquid, isolation yield: 60.84%, R_f: 0.68; ^{1}H NMR (400MHz, $CDCl_3$, 25 °C, TMS): δ=0.80 (s, 3H, –C**H₃**), 1.19 (s, 6H, alkane chain), 1.89-1.91 (d, J = 8 Hz, 2H, –C**H₂**–), 2.32 (s, 2H, –C**H₂**), 2.34-2.36 (d, J = 8 Hz, 2H, –C**H₂**–), 2.38-2.42 (m, 2H, –C**H₂**–), 2.57-2.62 (m, 2H, –C**H₂**–), 2.76 (s, 1H, –C**H**–), 3.61 (s, 2H, –C**H₂**–), 4.17-4.19 (d, J = 8, 1H, –C**H**–), 5.20-5.24 (t, J = 8 Hz, 1H, –C**H**–); 5.39-5.43 (t, J = 16 Hz, 1H, –C**H**–), 6.67-6.69 (d, J = 8 Hz, 1H, AR–H), 6.94-6.96 (d, J = 8, 1H, AR–H); MS, m/z calcd for $C_{20}H_{28}O_5$:348.19; found: 349.19 (M + H^+), 371.19 (M + Na^+), 719.38 (2M + Na^+). (*Appendix 5* and *Appendix 6*)

Synthesis of compound (E)-2-(2-(3-(4-hydroxyphenyl)propoxy)-2-oxoethyl)pentadec-4-enoic acid (4a). White solid; isolation yield: 71.58 %, R_f: 0.72 ; m.p. 28.23 °C; ^{1}H NMR (400MHz, $CDCl_3$, 25 °C, TMS): δ=0.81 (s, 3H, –C**H₃**), 1.19 (s, 12H, alkane chain), 1.89-1.91 (d, J = 8 Hz, 2H, –C**H₂**–), 2.35-2.40 (m, 2H, –C**H₂**–), 2.78-2.79 (d, J = 4 Hz, 2H, –C**H₂**–), 3.62 (s, 1H, –C**H**–), 4.19-4.23 (m, 2H, –C**H₂**–), 5.22-5.24 (d, J = 8 Hz, 1H, –C**H**–), 5.39-5.45 (m, 1H, –C**H**–), 6.66-6.68 (d, J = 8 Hz, 1H, AR–H), 6.96-6.98 (d, J= 8 Hz, 1H, AR–H); MS, m/z calcd for $C_{24}H_{36}O_5$:404.26; found: 405.26 (M + H^+), 427.25 (M + Na^+), 831.51 (2M + Na^+). (*Appendix 7* and *Appendix 8*)

II.3 Differential scanning calorimetry (DSC)

The thermal properties of compound 1a were analyzed using differential scanning calorimetry on a Pyris 6 DSC system (Perkin-Elmer Cetus, Norwalk, USA). Approximately 8 mg of compound 1a was put into an aluminum pan and placed in the DSC under a purging atmosphere of nitrogen (20 mL/min), while using an empty pan as an inert reference. The heating and cooling profile was: (1) initial

temperature 20 °C; (2) ramp 10 °C/min to 90 °C; (3) isothermal for 5 min; (4) ramp -10 °C/min to -60 °C; (5) isothermal for 5 min; (5) ramp 10 °C/min to 90 °C. The DSC scans were evaluated using MicroCal Origin 8.6 Software.

II.4 Temperature-Ramp Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) was used to determine the packing behavior of the compounds. FT-IR Spectra were recorded using an ATR-FTIR (PIKE, Madison, WI; Bruker, Ettlingen, Germany). The synthesized compounds were dried under vacuum over night, and then pressed onto a ZnSe ATR crystal mounted in a trough plate. The ATR crystal was coupled with an Auto Pro Temperature Controller (Pike Technologies, Madison, WI) for gradual heating of the crystal from 25 °C to 60 °C. Spectra were collected with a spectral resolution of 4 cm^{-1} with 8 scans over the range of 3500-650 cm^{-1}. The FTIR spectra were analyzed by using MicroCal Origin 8.6 software.

II.5 Critical micelle concentration (CMC)

The critical micelle concentrations (CMCs) of the synthesized compounds were determined by pyrene fluorescence using a fluorescence spectrometer (Varian Cary Eclipse, Agilent Technology, California, USA). Sample solutions of different concentrations (1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 mM) were prepared using water previously saturated with pyrene (final concentration of 1 µM). Emission spectra were obtained by exciting the samples at 343 nm. The fluorescence intensity ratio of I_1/I_3 (I_1=373 nm, I_3=383 nm) was plotted against sample concentration. The CMC was determined as the sample concentration at which the I_1/I_3 ratio remained constant after the initial abrupt decrease. Analysis was conducted in triplicate.

II.6 Free radical scavenging activity

The free radical scavenging activity of different products (1a, 2a, 3a, 4a) was determined based on DPPH assay (*Chen et al., 2013*) with slight modifications. Briefly, 1 mL of 0.01 mM DPPH in ethanol was mixed with 0.5 mL surfactant solution in ethanol. After incubation in the dark for 30 min, the absorbance was measured at 517 nm using a UV/VIS-spectrophotometer (Varian, Agilent Technology, California, USA). DPPH radical scavenging activity was expressed as $\left[1-\left(\frac{sample\ absorbance}{blank\ absorbance}\right)\right] x\ 100$ (*Park et al., 2017*). The analysis was conducted in triplicate.

II.7 Inhibition of lipid oxidation in emulsions

Oil-in-water emulsions were prepared by mixing 20% (w/v) lipid phase (fish oil) with 80% aqueous phase containing 1.5 wt% of surfactant. A coarse emulsion premix was prepared by homogenizing the lipid and aqueous phases together using a high-speed homogenizer (PRO250, PRO Scientific, Oxford, USA) at 8,000 rpm for 2 min at room temperature. Then, the droplet size in the premixed emulsions was reduced by sonication for two min using a probe sonicator (Branson sonifier 250, Branson ultrasonics, Danbury, US). Thiobarbituric acid-reactive substances (TBARS) were used to measure lipid oxidation reaction products, particularly malondialdehyde (MDA), as an important auto-oxidation product (*Cai et al., 2013*). A solution of trichloroacetic acid (TCA)-TBA-HCl was prepared by mixing 15 g TCA, 375 mg TBA, 1.76 mL 12N HCl and 82.9 mL H_2O. Two milliliters of the TCA-TBA-HCl solution was mixed with 20 µL emulsion sample and 1mL of distilled water. After mixing, the mixture was heated at 100 °C for 15 min, cooled to room temperature for 10 min using tap water, and centrifuged at 2,000 rpm for 15 min. Absorbance was read at 532 nm using a UV/VIS spectrophotometer (Varian, Agilent Technology, California, USA) and the results were expressed as $\left(\frac{A_1}{A_O}\right) x\ 100$, where A_1 and A_O are absorbance of the sample with synthesized compounds and that of sample with Tween 20 respectively. The analysis was conducted in triplicate.

Section III

Lipophenol: synthesis and evaluation of antioxidant performance in a real system

III.1 Reaction set up

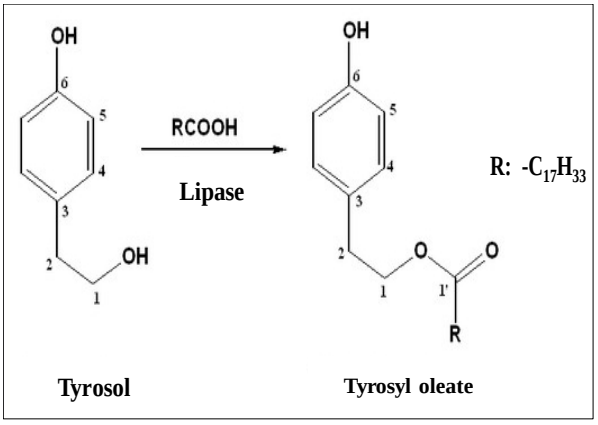


Figure 16. *Lipophilization reaction studied in this work*

The reagent used were standard oleic acid and standard tyrosol and the lipase used was immobilized from *Mucor miehei*. Tests for set up reaction (*Figure 16*) were carried out analyzing different variables and monitoring yield of each reaction using HPLC-UV at 280 nm. The variables considered were:

- *Molar ratio*: tyrosol:oleic acid 1:1, 1:2, 1:4, 1:6
- *Solvent*: hexane, MTBE, hexane: THF 3: 1, *tert*-butanol
- *Temperature*: 40 °C, 50 °C, 60 °C, 70 °C
- *Enzyme Percentage*: 10% based on the tyrosol weight

The reagents, tyrosol and oleic acid, were dissolved in 5 mL of solvent and shaked in a fixed temperature bath on a thermomagnetic plate. When the reagents were solubilized was considered the zero time of the reaction and the enzyme was added. The reaction was carried out for different times up to the maximum yield plateau. At that point, the mixture was filtered through Buchner funnel to

remove the enzyme. The product of the reaction was dried in a rotary evaporator at 40 °C under vacuum.

III.2 Monitoring of reaction by HPLC-UV

In order to monitor the yield of the reaction, 100 µl of samples were dissolved in 300 µl of chloroform: methanol 3:1 (v/v) every hour. The sample was centrifuged for 5 min at 140.000 rpm at 25 °C to facilitate deposition of the enzyme. The supernatant was analyzed by HPLC-UV (HPLC 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) at 280 nm according to *Fernández et al.,* (2012). The compound of interest was quantified using a Gemini - NX C18 column 110 ° (150 mm x 4.60 mm i.d; 5 µm particle size) maintained at 25 °C. Phase A was methanol: acetonitrile 90:10 (v/v) with 0.01% acetic acid and phase B was water with 0.01% acetic acid. The elution gradient was as follows: 0 min 90% A and 10% B; 10 min 70% A e 30% B; 15 min 10% A e 90% B; 30 min 10% A e 90% B; 35 min 90% A e 10% B; 40 min 90% A and 10% B. The flow was 2 mL/min. Each reaction time were sampled twice and the calculate yields were expressed as percentage (%).

III.3 Column purification

In order to separate the reaction product from the excess reagents, a chromatographic column was packed with Silica gel (stationary phase) (Sigma Aldrich, Saint Louis, MO). The sample resulted from the lipophilization reaction, was weighed and mixed with silica gel in equal parts (*w/w*) and inserted into the column for the separation. The solvent that represent the liquid phase was a mixture of hexane:diethyl ether:acetic acid 45:25:1 (*v/v/v*). Below the column were placed numbered glass tubes filled drop by drop with the eluted sample. In order to identify the fraction containing the molecule of interest, tyrosyl oleate, a qualitative analysis of the collected fractions was performed on TLC plates. At least 20 minutes before the analysis, the elution chamber was saturated with 70 mL of the same solvent hexane:diethyl ether:acetic acid 45:25:1 (*v/v/v*). On the plate, 10 µl of each sample in the tubes and 10 µl of standard tyrosol were deposited at 1 cm from the bottom. After deposition, the solvent was left to reach 8 cm from the deposition point. For the detection of the compounds a UV lamp at 254 nm (wavelength for phenolic compounds detection) was used. The fractions corresponding to the product of interest were combined and dried under vaccum at 40 ° C.

III.4 Identification of tyrosil oleate with different techniques

Nuclear Magnetic Resonance (NMR)

In order to identificate the synthesized molecule, 20 mg of compound were dissolved in 800 µl of chloroform-d by a ^{1}H NMR spectroscopic analysis using an Ultrashield 600 Plus NMR (Bruker – Billerica, MA, USA) at 600 MHz.

Identification of tyrosil oleate with FT-MIR

The spectrum of the samples was acquired with a FT-IR spectrophotometer (Fourier transform infrared spectroscopy) Tensor Series 27TM (Bruker Optics, Milan, Italy), in the range between 4000 and 700 cm^{-1} with an FT resolution of 4 cm^{-1}. In particular, 32 scans have been acquired for each sample or background spectrum. About 1-1.5 mL of each sample were placed on the crystal surface and the absorbance spectrum was recorded. Prior to the acquisition of each spectrum, the ATR crystal was cleaned with a hexane and cellulose tissue and then rinsed with acetone to remove any impurities. For each sample 3 spectra were recorded.

III.5 Formulation of tarallini with lipophenol

In order to study the antioxidant activity of the synthesized molecule, different tarallini, tipycal Italian bakery product, were produced with different concentrations of lipophenol. The ingredients used in the formulation of tarallini were:

- Wheat flour type 00 (200 g)
- Water (70 mL)
- Salt (4 g)
- Sunflower oil (40 g)
- Tyrosyl oleate in different percentages based oil weight

The ingredients were weighed and kneaded for 2 and a half minutes in an automatic kneader. Subsequently, the dough was hand blended and tarallini were formed and cooked at 200 °C for 20 minutes. The tarallini were stored in closed glass jars, leaving for each sample the same head space, and stored at room temperature.

Different concentration of lipophenol, based on sunflower oil weight, were tested:

- **Control sample, tarallini without lipophenol (CS)**
- **Tarallini with 1% lipophenol (1L)**
- **Tarallini with 4% lipophenol (4L)**
- **Tarallini with 7% lipophenol (7L)**

Sunflower oil was used because of its chemical unsaturation. Infact, it is well known (*Verardo et al., 2010 and Falade et al., 2017*) that sunflower oil is very subjected to oxidation process; so, using it, it is easily possible study the effect of the synthesized molecule in a real food system. In order to characterize the samples depending on the different percentage of lipophenol added, these were sampled at different shelf life times: at 0 day (T0), after 15 days (T15), 30 days (T30), 37 days (T37) and 45 days (T45) after formulation. Samples were chopped and stored at -18 ° C until analysis.

III.6 Accelerated Oxidation Test: OXITEST®

10 grams of ground tarallini were placed in the appropriate oxidation reactors in OXITEST® (Velp Scientific, Usmate Velate - MB - Italy); the analysis was carried out at 90 °C and 6 bar of oxygen pressure until the instrument did not show a collapse of the pressure, the signal which is the maximum possible oxidation achieved in the sample. The analysis was repeated twice for each sample.

III.7 Extraction of the lipid fraction

Total lipid extraction was performed with the method by *Folch et al.,* (1957), with some modifications (*Boselli et al., 2001*) with the procedure explained in paragraph I.2. For each sample was performed two extractions.

III.8 Oxidative status: peroxide value (PV)

The method used is the one of the International Dairy Federation, similar to *Shantha & Decker* (1994), previously described in paragraph I.4. The analysis was repeated twice for each lipid extract (*n=4*) and peroxide value was expressed as meqO_2/kg of fat.

III.9 Determination of conjugated dienes and trienes

The aim of this analysis is the detection, in the fatty matrix, of the phenomena of slipping of the double bond of unsaturated fatty acid with the formation of a conjugated dienic system caused by an oxidative oxidation of the raw material. For the determination of conjugated dienes and trienes 20 mg of fat were weighted and dissolved in 2 mL isooctane to obtain a 1% (w/v) fat solution. The readings were conducted at 232 nm for the determination of the dienes and at 268 nm for the trienes. The analysis was repeated twice for each lipid extract (*n=4*) and quantified with this formula:

$$K\lambda = l/g \times A \times V/100$$

Where:

l: cuvette thickness

g: fat weight

A: absorbance measured

V: dilution (if dilution 1:10, V = 100).

III.10 Determination of Oxidized Fatty Acids (OFA)

The determination of OFA was carried out throught fast GC-FID with the same method used for the determination of FAME described in paragraph I.3, with a run time of 7 minutes. The identification of the oxidized fatty acids was done according to *Verardo et al.* (2010). The analysis was repeated twice for each lipid extract (*n=4*).

III.11 Analysis of the volatile composition by SPME-GC-MS

To evaluate the secondary lipid oxidation, the volatile composition of tarallini was determined. Determination of volatile compounds was made placed 3 g of ground sample in an amber vial (10 mL capacity) using the SPME (solidphase micro extraction) technique in a CombiPal volatile autosampler, which allows to automate the pre-conditioning of samples, exposure and desorption of the fiber, reducing the error due to the operator. The GC instrument was GC-MS QP2010 Ultra Shimadzu (Kyoto, Japan) with a Restek ZB-WAX column (30 m × 0.25 mm ID, 1 μm f.t.) (Restek Corporation, Bellofonte, USA). The analysis was carried out twice for each sample with the following condition.

GC conditions:

- Carrier gas: helium
- Column flow: 1.5 mL/min
- Split ratio: 1:10
- Injector temperature: 240 °C
- Temperature Program: 40 °C for 10 minutes, then 200 °C for 3 minutes with 3 °C/min increase, then 240 °C with 10 °C/min increment and maintenance at this temperature for 5 minutes
- Run time: 75.33 minutes

MS Conditions:

- Source temperature: 200 °C
- Interface temperature: 240 °C
- Mass range: 30-250 m/z
- Acquisition Mode: scan from 3.5 min to 70 min

SPME conditions:

- SPME fiber: 2 cm x 0.11 μm, coated with 50/30 μm thick divynylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, Bellofonte, PA, USA).
- Incubation time: 30 minutes at 40 °C
- Exposition time: 10 minutes
- Desorption time: 7 minutes

III.12 Determination of tyrosyl oleate in tarallini after cooking

To evaluate the concentration of tyrosyl oleate in the different samples of tarallini after the heat treatment an HPLC-UV (HPLC 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) at 280 nm was used, and the lipid fraction extracted from tarallini was analyzed using the same method previously described in paragraph III.2. The analysis was repeated twice for each lipid extract (*n=4*).

III.13 Synthesis of lipophenol starting from by-products fat

Lipophenol was synthesized starting from the fractionated storage fat as described in paragraph I.9 and standard tyrosol at the optimized conditions for the reaction with standard reagents (paragraph III.1). In order to monitor the yield, the same method described in paragraph III.2 was used with HPLC-UV (HPLC 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) (λ = 280 nm). Each reaction time were sampled twice and the calculate yields were expressed as percentage (%).

III.14 Statistical analysis

Relative standard deviation was obtained, where appropriate, for all data collected. One-way analysis of variance (ANOVA) was evaluated using Statistica 8 software (2006, StatSoft, Tulsa, OK, USA). The differences between the means of data were compared at the 5% level of significance ($p < 0.05$) using Tukey honest significant difference (HSD) test.

3. Results & Discussion

Section I

Lipid characterization of by-products originated from beef meat industry through innovative chromatographic techniques

The bovine slaughter industry generates high quantities of animal origin by-products which, instead of being disposed as waste, can be enhanced through different recovery processes. The disposal of these products represents a cost for the industries and also causes a strong environmental pollution. It is for these reasons that in recent years are being evaluated possible alternative uses of such animal by-products, which are also a source of important nutrients such as essential amino acids, minerals and vitamins as well as possessing appreciable technological and sensory properties. In the first part of this study has been studied and analysed the lipid fraction of four different by-products derived bovine slaughtering, storage fat, subcutaneous fat, bone marrow and bone fat from three different bovine categories: calf, young bull and cow. The characterization of the lipid fraction of by-products as far as the oxidative status, fatty acids, triglycerides, diglycerides, phospholipids composition and cholesterol content, had the purpose of identify, the most suitable by-product for the final aim of the research, the lipophenols synthesis.

I.1. Total lipid content of by-products

The fat content of the different by-products is reported in *Table 4* and it is expressed as percentage (%) on the total fresh weight of the sample.

Table 4. Fat content of the different by-products

LIPID CONTENT (%)			
	CA	**YB**	**CO**
Storage fat	61.4 ± 4.1^{b}	81.6 ± 4.6^{a}	83.5 ± 0.5^{a}
Subcutaneous fat	8.2 ± 0.7^{a}	22.3 ± 8.8^{a}	14.8 ± 7.0^{a}
Bone marrow	70.5 ± 1.1^{a}	85.0 ± 2.7^{a}	89.8 ± 0.9^{a}
Bone fat	46.5 ± 9.7	21.0 ± 1.9	42.6 ± 7.0

Abbreviations: CA, calf; YB, young bull; CO, cow. Data (means ± SD, n=3) are expressed in percentage (%) on the total fresh weight; results of the analysis of variance by Tukey's test are shown: $p<0.05$, lowercase letters on the same row show significantly different values within each by-product for the three animals.

Comparing the lipid content of the different by-products analyzed, the highest fat percentage was recorded in storage fat and in bone marrow. Storage fat shown a lipid content highest in the cow sample of about 83.5%, followed by storage fat of young bull with a percentage of 81.6% and lastly calf, with a significantly lower value of 61.4% (*Table 4*). These results are in line with what

was expected because they reflect the different characteristics of the three animals regarding their age, morphology and feeding (*Bittante et al., 2005*) in addition to the cut and trimming degree (*Scollan et al., 2006*). The high lipid content found in the bone marrow of the animals agrees with what has already been reported for the bovine species where the percentage of lipids on 100 g of bovine bone marrow is about 89.9% (www.bda-ieo.it). In particular, cow bone marrow reported the highest percentage about 89.9%, followed by young bull (85.0%) and calf (70.5%), but without any significant differences (*Table 4*). On the contrary, in the subcutaneous fat, the lipid fraction covers a very low percentage. This result is also in line with what was expected since the chemical composition of bovine skin is represented by 64% water, 33% protein, 2% fat, 0.5% minerals and 0.5% of other substances (www.istitutoconciario.com). In this case, the percentage of lipid was greater than what is reported in literature, oscillating from 8.2 to 22.3% (*Table 4*) but without any significant differences ($p < 0.05$) among the animal samples. For this by-product, the repeatability of the data was poor probably due to the non-homogeneous matrix. In fact, the standard deviations of these data were very high, causing a flattening of the differences between samples. Even bone fat has exhibited a sample heterogeneity due to the presence of tendons and ligaments. As shown in *Table 4*, this results in a lower fat content compared to storage fat and a content variability that determines a high standard deviation of the data. Nonetheless, young bull shown a significant lower content (21.1%) than those recorded in calf and cow, whose values are statistically similar oscillating in the range of 43-47%.

I.2. Oxidative status: peroxide value (PV)

The number of peroxide (PV) is a chemical parameter that can give information about the oxidative status of the lipid matrix: peroxides, in fact, are markers of the oxidation, as they are the first products of the oxidation reaction. In particular, the peroxide number is an index that evaluates the amount of oxygen absorbed by lipid fraction and is therefore expressed in milli-equivalents of active oxygen per kg of fat (EU Regulation 1348/2013).

Table 5. Peroxide value of the different by-products

PEROXIDE VALUE (PV) – meq O_2/ kg of fat			
	CA	**YB**	**CO**
Storage fat	$0.31 \pm 0.03^{a, D}$	$0.14 \pm 0.02^{a, D}$	$0.47 \pm 0.22^{a, C}$
Subcutaneous fat	$8.56 \pm 1.41^{a, C}$	$10.15 \pm 0.83^{a, A}$	$5.49 \pm 0.04^{b, A}$
Bone marrow	$18.45 \pm 0.64^{a, A}$	$4.06 \pm 0.58^{b, B}$	$5.43 \pm 0.67^{b, A}$
Bone fat	$13.50 \pm 2.98^{a, B}$	$0.70 \pm 0.21^{c, C}$	$1.61 \pm 0.40^{b, B}$

Abbreviations: CA, calf; YB, young bull; CO, cow. Data (means ± SD, n=6) are expressed in meq O_2/ kg of fat; results of the analysis of variance by Tukey's test are shown: $p<0.05$. Lowercase letters on the same row show significantly different values within each by-product for the three animals. Capital letters on the same column show significantly different values within each animal for the four by-products

Considering that the legal limit is 20 meq O_2/ kg of fat, it is possible to observe in *Table 5* that all the samples registered a peroxide value under it. In particular, the storage fat shown the significant ($p < 0.05$) lowest peroxide value than all the other by-products, but without significant differences ($p < 0.05$) among the animals. In fact, storage fat shown a peroxide value of 0.31, 0.14 and 0.47 meq O_2/ kg of fat for calf, young bull and cow respectively. Subcutaneous fat shown the highest peroxide value in young bull (10.15 meq O_2/ kg of fat) followed by calf but without significant ($p < 0.05$) differences (8.56 meq O_2/ kg of fat) and cow, instead, with significantly lower value (5.49 meq O_2/ kg of fat). Also compared to the other young bull by-products, the subcutaneous fat had the significant highest value followed by bone marrow, bone fat and storage fat (4.06, 0.70 and 0.14 meq O_2/ kg of fat respectively). The bone marrow of calf shown the highest peroxide value among all the values but, anyway, under the legal limit (18.45 meq O_2/ kg of fat); followed, with significant different values ($p < 0.05$), by bone marrow in cow and young bull, 5.43 and 4.06 respectively, that did not show significant differences between them. Finally, bone fat shown the highest peroxide value in calf (13.50 meq O_2/ kg of fat), followed, with significant differences, by cow and young bull (1.61 and 0.70 meq O_2/ kg of fat). Significative differences among the different by-products are visible in *Figure 17.*

Compared to other studies on beef meat, due to lack of literature about beef by-products, our results are in line with *Ferioli et al.* (2008) that studied the oxidative status of minced beef under oxygen-enriched atmosphere and obtained a peroxide value ranging from 1.2 to 12.1 meq O_2/ kg of fat. *Boselli et al.* (2009), instead, studied the lipid oxidation of cow meat stored in unmodified and modified atmosphere, obtained in both cases a higher peroxide value than ours. In fact, they obtained a peroxide value from 13.8 to 34.8 meq O_2/ kg of fat under unmodified atmosphere and from 15.7 to 32.8 meq O_2/ kg of fat under modified conditions, so over the legal limit.

The peroxide value and significant differences are also reported in *Figure 17.*

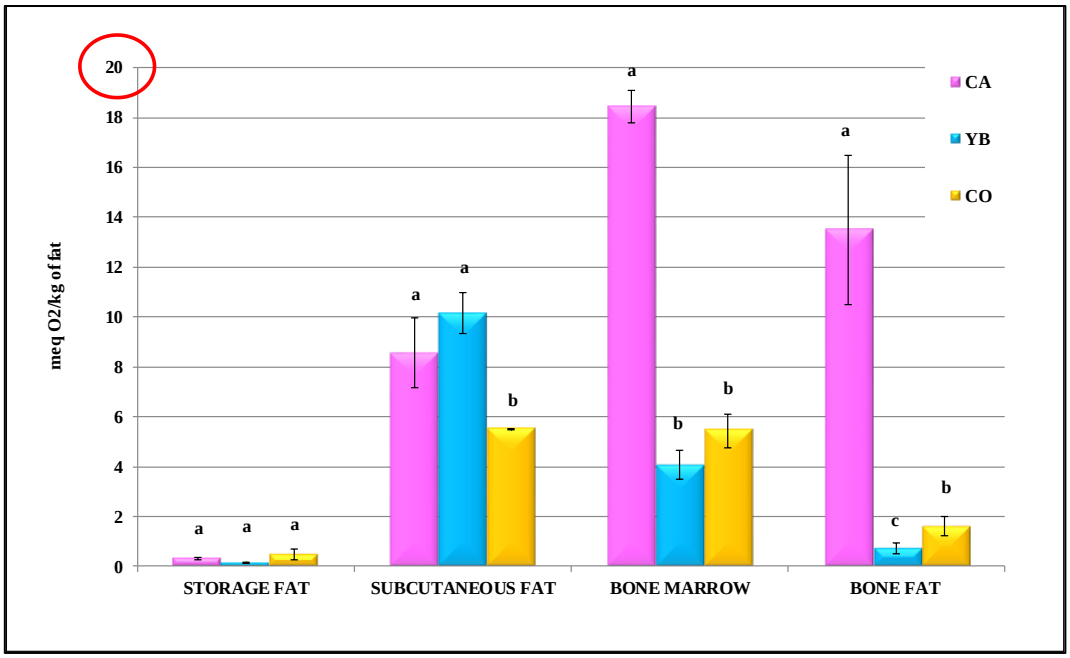


Figure 17. *Graphic representation of the peroxide value in different by-products for the three animals. Calf (CA), young bull (YB) and cow (CO), expressed in meq O2/ kg of fat. The bars with different letters within each by-product are significantly different (p≤0.05).*

I.3 Fatty acid composition (FAME) of by-products

The characterization and quantification of the total fatty acids present in the different by-products were carried out by methylation of the compounds and injection into FAST GC-FID. This innovative chromatographic technique allows to determine FAME with a run time less than 7 minutes; a very low run time compared to several studies (*Aharoni et al., 2004; Indurain et al., 2006; Moreno et al. 2008*).

In all the samples, 25 fatty acids have been identified and average values are reported in *Appendix 9* and expressed in mg FA/100 mg of FAME. The results obtained shown that in all the by-products and animals, the preponderant fatty acid was the oleic acid (C18:1 cis9) ranging from about 32 to 46%. Palmitic acid (C16:0) constituted the second major fatty acid detected (~ 20-28%), followed by stearic acid (C18:0, ~ 10-19%), palmitoleic acid (C16:1 cis, ~ 2-5%), myristic acid (C14:0, ~ 2-4%) and linoleic acid (C18:2 n6, ~ 2-10%). The same acidic pattern was found in previous studies on beef meat (Chow, 2007). Fatty acids were classified in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

In the storage fat, SFA were present in significantly ($p < 0.05$) greater amounts in calf and young bull (46.76 % and 47.62 %) compared to cow storage fat (40.80 %), which instead shown a significantly higher content in MUFA ($p \leq 0.05$), in the amount of 55.70% (calf and young bull shown 48.03 and 48.57 % respectively). Polyunsaturated fatty acids (PUFA) were present in low concentrations in all animal samples, accounting for 5.21 %, 3.81 % and 3.50%, for CA, YB and

CO respectively (*Figure 18*). These trends reflected those of the main individual fatty acids (*Appendix 9*), where the cow had the highest content of oleic acid (44.78%) and palmitoleic acid (5.13%), whereas YB and CA presented the main content of stearic acid (16.64%) and palmitic acid (26.44%), respectively. The calf sample, moreover, had the highest concentration of C18:2 *n6*.

According to *De Smet et al.* (2000) and *Brugiapaglia et al.* (2014) about the investigation on the fatty acid profile of *longissimus thoracis* muscle from different Belgian and Italian young bulls, SFA was the predominant class followed by MUFA and PUFA. For SFA class our results (range between 40.80% in cow and 47.62% in young bull) were slightly lower than their (*De Smet et al.* reported 43.62-48.51 % and *Brugiapaglia et al.* 46.05-49.25 %); instead for MUFA and PUFA we registered higher and lower concentrations respectively rather than these two works. For MUFA we had a range from 48.03 %, in calf, to 55.70 %, in cow (*De Smet et al.* reported 33.94-38.42 % and *Brugiapaglia et al.* 32.08-42.08 %); and for PUFA we had a range between 3.50, in cow, and 5.21 %, in calf (*De Smet et al.* reported 15.04-20.48 % and *Brugiapaglia et al.* 10.69-21.87 %).

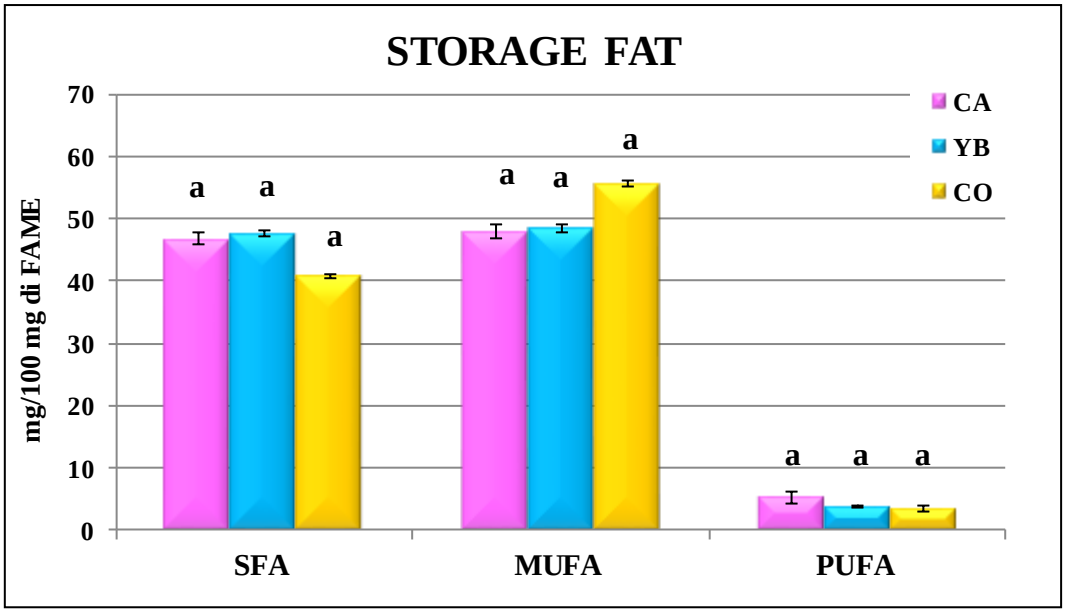


Figure 18. *Graphic representation of the composition of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of storage fat. Calf (CA), young bull (YB) and cow (CO) expressed in mg/100 mg of FAME. The bars with different letters within each class (SFA, MUFA, PUFA) are significantly different (p≤0.05).*

In subcutaneous fat, the principal classes were SFA and MUFA showing not significant differences between the three bovine categories analyzed and with a range between 45.52 and 49.59% and between 45.37 and 49.73% respectively. PUFA were present in considerably smaller amounts and with a significantly ($p < 0.05$) higher content in calf (7.20 %) than the other two animals (4.70 and 5.04 in young bull and cow respectively), as was observed for storage fat *(Figure 19* and *Appendix 9*).

In fact, calf subcutaneous fat reported more linoleic acid (C18:2 *n6*) than young bull and cow, which was 4.69%. These results agree with the study of *Indurain et al.* (2006) on the fatty acid profile of subcutaneous adipose tissue in young bulls. Other investigations were carried out on the fatty acid compositions of bulls and heifers. *Schiavon and co-workers* (2016) showed a similar MUFA content in beef subcutaneous fat, whereas SFA and PUFA were present in higher and lower amount compared to our results. *Noci et al.* (2005) have observed low SFA concentration in heifers and MUFA and PUFA with higher values than our samples.

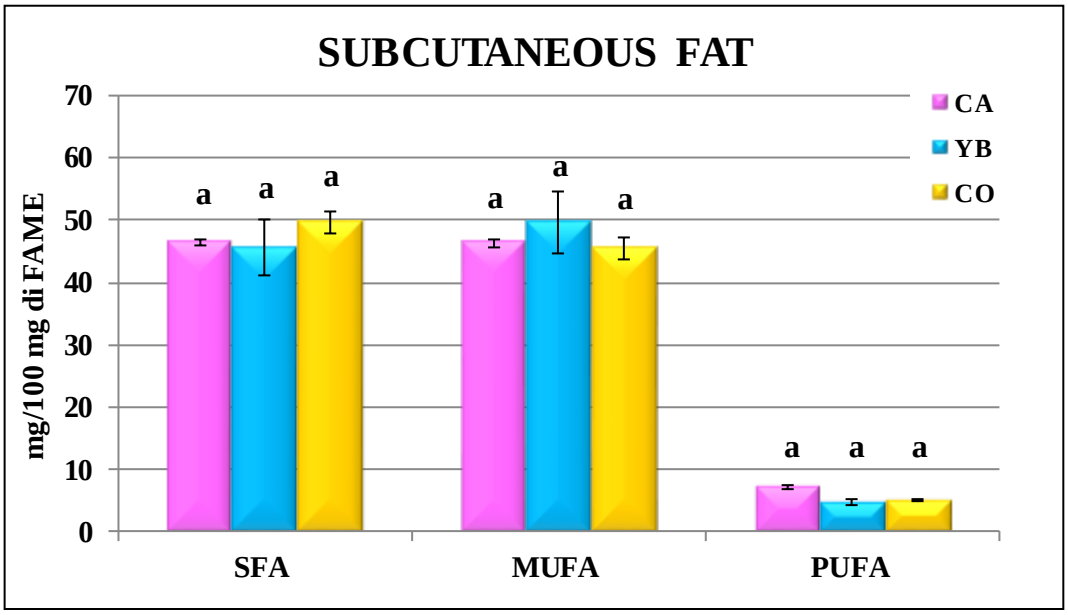


Figure 19. *Graphic representation of the composition of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of subcutaneous fat. Calf (CA), young bull (YB) and cow (CO) expressed in mg/100 mg of FAME. The bars with different letters within each class (SFA, MUFA, PUFA) are significantly different ($p \leq 0.05$).*

In the bone marrow, animals showed more variability in SFA and MUFA contents (*Figure 20* and *Appendix 9*). SFA were present in higher amount in YB sample, with an average content of 53.42 %, followed with significantly lower values by CO (45.29 %) and CA (35,08 %). On the other hand, MUFA were more abundant in CA (53.05 %), followed by CO (49.63 %) and YB (41.72 %). As already seen in the previous two by-products, PUFA presented low concentrations

and CA was the sample with the highest percentage (11,87 %), due to the important amount in linoleic acid (10.56 %) compared to YB (4.86 %) and CO (5.09 %).

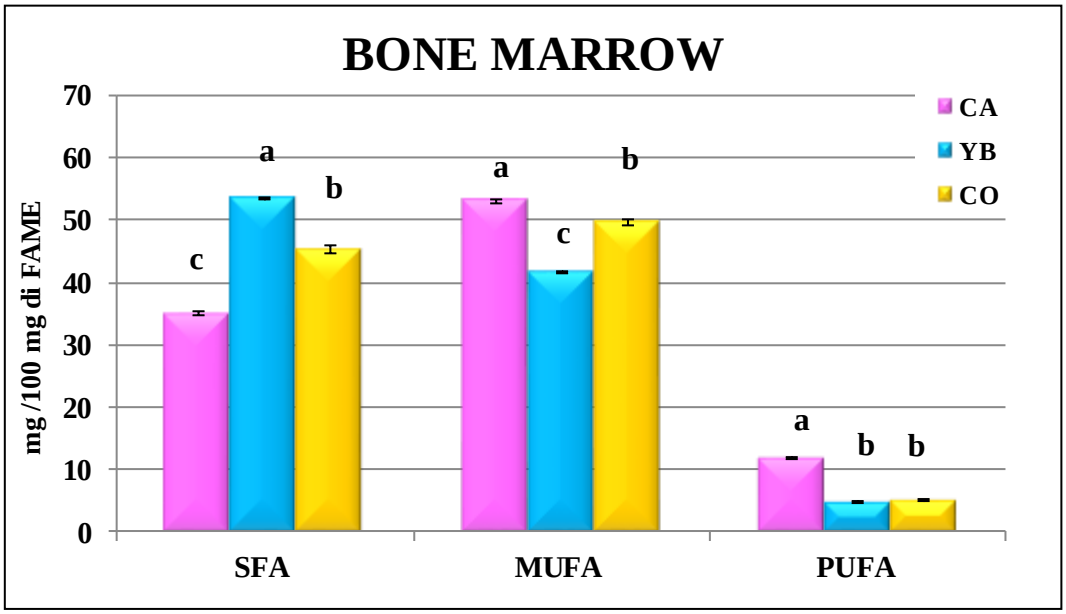


Figure 20. *Graphic representation of the composition of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of bone marrow. Calf (CA), young bull (YB) and cow (CO) expressed in mg/100 mg of FAME. The bars with different letters within each class (SFA, MUFA, PUFA) are significantly different (p≤0.05).*

Finally, in the bone fat SFA were contained in significantly higher amount in young bull ($p \leq 0.05$) with a percentage of 44.57% compared to calf that shown a 38.88 % and cow that shown a 38.45 %; while MUFA, similarly to storage fat, were present in greater concentration in the cow, reaching the 57.35% of the total fatty acids identified (CA and YB had 50.41 and 50.46 % respectively) (*Figure 21* and *Appendix 9*). PUFA, like in all previous by-products, have a significantly higher percentage ($p \leq 0.05$) in calf sample, equal to 10.88%. Followed by, in descending order, calf (4.96%) and cow (4.20%).

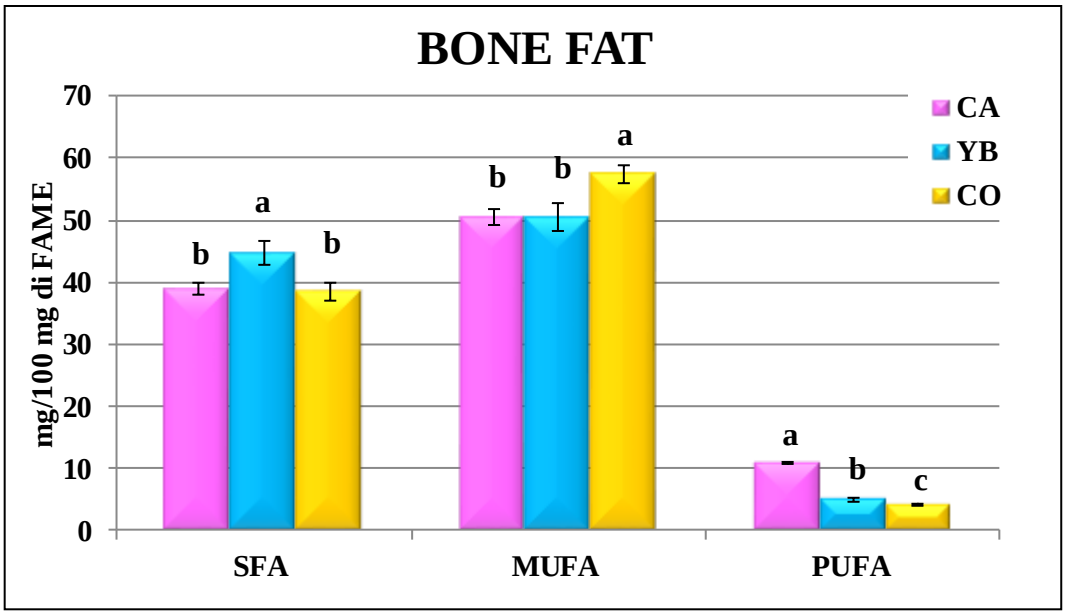


Figure 21. *Graphic representation of the composition of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of bone fat. Calf (CA), young bull (YB) and cow (CO) expressed in mg/100 mg of FAME. The bars with different letters within each class (SFA, MUFA, PUFA) are significantly different ($p \leq 0.05$).*

Considering each animal, SFA in calf were contained in higher percentage in storage and subcutaneous fat, while the lowest concentration was recorded in the bone marrow, in which the concentration of MUFA was determined as the highest. As mentioned above, PUFA in calf were present in significantly higher concentration ($p \leq 0.05$) than the other two animals, reaching values greater than 10% in the bone marrow and bone fat (11.87 and 10.88 % respectively). The cow had the highest content in SFA in the subcutaneous fat (49.59%) and the lowest in bone fat (38.45%) where the MUFA concentration was the highest, equal to 57.35%. The young bull, finally, showed a more balanced concentration of different fatty acid classes, with a greater content in MUFA than SFA, except for the bone marrow that contained the highest amount in SFA (53.42%) and the lowest in MUFA (41.72%). In general, low PUFA content of the analysed cattle's by-products confirm what it is already report in literature. Bovines are ruminants and the rumen microflora conduct a hydrogenation process of unsaturated fatty acids making them saturated, increasing the total SFA amount *(Mapiye et al., 2012*).

I.4 Triglycerides (TG) composition of by-products

Because of fatty acids make up the bulk of a triglyceride molecule, this study also focused on triglycerides (TG) composition of samples, using a fast GC-FID analysis with a run time of less than 13 min. As reported in *Appendix* **10***10*, six classes of triglycerides were identified and quantified in all by-products, corresponding to TG with 42 to 52 carbon number (CN).

All the by-products showed a high content of long-chain TGs (CN48, CN50 and CN52), with CN50 as the most abundant class, expressing about the 50 % of the total TGs in samples. Following the TGs with CN48 were present with a content of about 18-26 % and in decreasing amount the CN52 (~ 12-19 %), CN46 (~ 5-13 %), CN44 (~ 1-4 %) and CN42 (~ 0.2-0.9 %) classes. These results reflect a close relationship between the fatty acids and TGs content of samples. Indeed, a high content in TGs CN50 and CN48 was coincident with high levels of C18:1, C18:0, C16:0 and C14:0.

In storage fat, the three animals did not show significant differences in TGs content from CN42 to CN48 (*Figure 22*). Whereas for the long-chain TGs, CN50 and CN52, CA samples presented a significantly lower (42.11 %) and higher (17.21 %) content, respectively, than the other two animals; young bull shown 43.00 % and 16.30 % for CN50 and CN52 respectively and cow had percentages of 43.46 % and 15.63 % for CN50 and CN52, respectively.

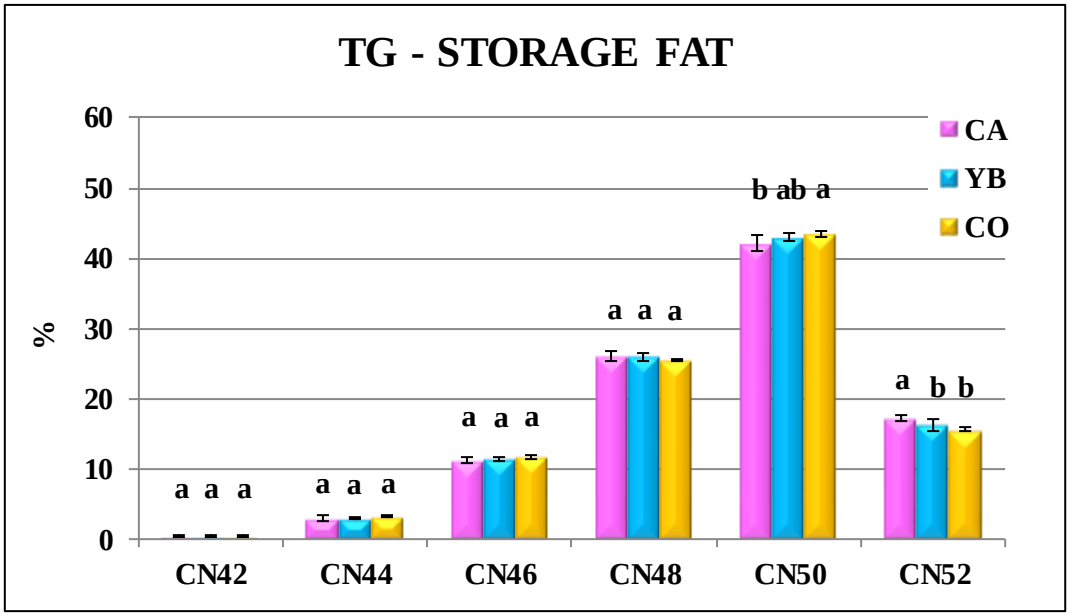


***Figure 22**. Graphic representation of triglyceride content divided in classes: CN42, CN44, CN46, CN48, CN50 and CN52 of storage fat in calf (CA), young bull (YB) and cow (CO) expressed in % on the total triacylglycerols in the sample. The bars with different letters, within each TG class, are significantly different (p≤0.05).*

More variability was observed for the TG content in subcutaneous fat and mainly for the long-chain classes, CN50 and CN52

In subcutaneous fat, more variability was observed for the TG content in the three animals, except for class CN48. In particular, for the class CN44 the young bull showed a significantly higher value (2.49 %) than cow and calf (2.27 and 2.32 %, respectively); while for the class CN46 the cow had a significantly lower ($p \leq 0.05$) concentration, about of 9.00 % (calf and young bull showed a concentration about 9.64 and 9.47 % respectively). However, the biggest differences were recorded for class CN50, the most concentrated, and class CN52. In the first case, the cow showed a significantly ($p < 0.05$) highest content (51.17 %) followed by young bull (48.74 %) and calf (44.15 %), whereas the CN52 class was more concentrated in calf (18.76 %) than young bull (14.15 %) and cow (12.62 %) (*Figure 23*).

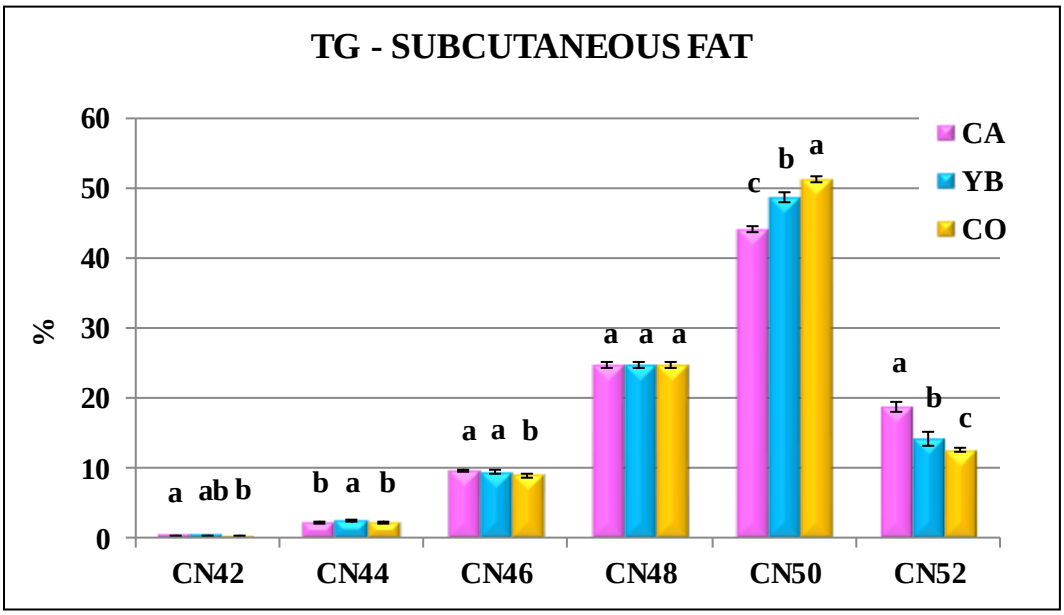


Figure 23. *Graphic representation of triglyceride content divided in classes: CN42, CN44, CN46, CN48, CN50 and CN52 of subcutaneous fat in calf (CA), young bull (YB) and cow (CO) expressed in % on the total triacylglycerols in the sample. The bars with different letters, within each TG class, are significantly different (p≤0.05).*

The TG distribution in the bone marrow samples were significantly different among the three bovine categories (*Appendix* **10***10* and *Figure 24*). TGs medium chain trend was similar among the three animals, while the trend varied for CN50 and CN52. TGs content from CN42 to CN48 was significantly higher ($p \leq 0.05$) in young bull followed by calf and cow. On the contrary, TGs with CN50 were present in high amount in cow (56.11%), the highest recorded in all samples, compared to calf and young bull that recordered 44.47 and 44.67 %, respectively. CN52 concentration was significantly higher in calf (26.16 %), followed by cow (19.00 %) and calf (13.11 %).

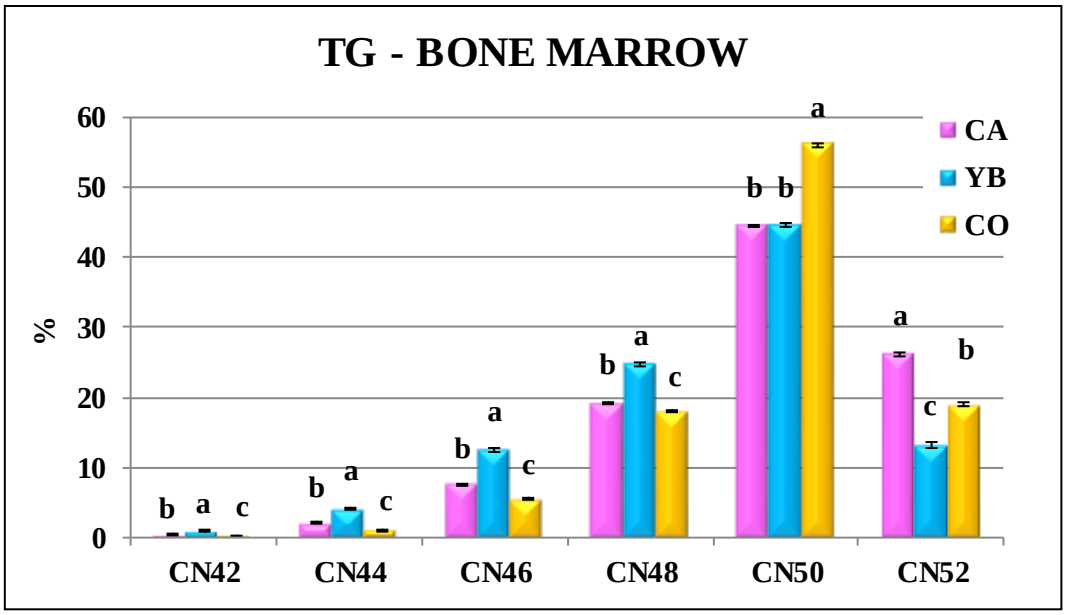


***Figure 24**. Graphic representation of triglyceride content divided in classes: CN42, CN44, CN46, CN48, CN50 and CN52 of bone marrow in calf (CA), young bull (YB) and cow (CO) expressed in % on the total triacylglycerols in the sample. The bars with different letters, within each TG class, are significantly different (p≤0.05).*

In bone fat, except for short-chain TGs such as CN42 and CN44, the other classes had a similar trend to that found in subcutaneous fat. In this case the major significant differences were recorded for classes CN50 and CN52 ($p \leq 0.05$), where in the first case the cow shown the highest content (49.05%) and the calf shown the lowest (41.33%); while in the second case the calf shown a higher percentage of about 20.03 %, followed by young bull (17.88 %) and cow that shown the lowest concentration (16.50%) (*Figure 25* and *Appendix 10*).

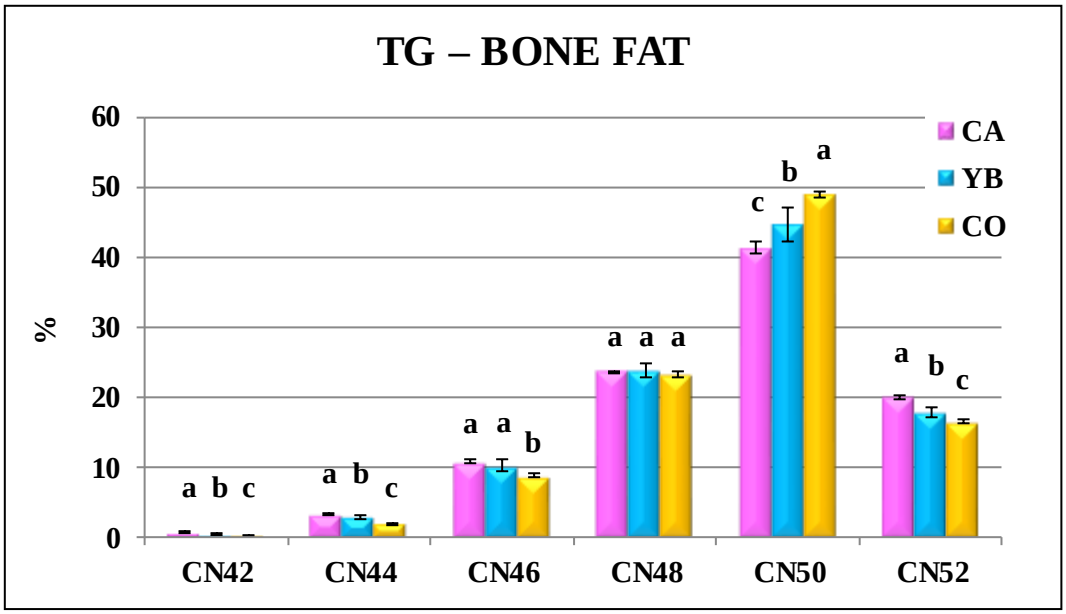


***Figure 25**. Graphic representation of triglyceride content divided in classes: CN42, CN44, CN46, CN48, CN50 and CN52 of bone fat in calf (CA), young bull (YB) and cow (CO) expressed in % on the total triacylglycerols in the sample. The bars with different letters, within each TG class, are significantly different (p≤0.05).*

Examining each individual animal, the calf had a significantly higher content ($p \leq 0.05$) in short chain TGs (CN42 and CN44) in bone fat, while medium chains TGs (CN46 and CN48) in storage fat and CN50 and CN52 in the bone marrow. The cow, on the other hand, had a significantly higher content ($p \leq 0.05$) of medium and short chain TGs storage fat and of long chain TGs in the bone marrow, as for the calf. Conversely, the young bull had a more varied distribution of the different classes of TGs in by-products. In particular, unlike cow and calf, the CN42, CN44 and CN46 were more concentrated in the bone marrow, while CN50 and CN52 had a significantly higher value ($p \leq 0.05$) respectively in subcutaneous fat and in bone fat.

I.5 Diglycerides (DG) composition of by-products

In the different samples, albeit in low concentrations, were present also diglycerides (DGs). These compounds generally result from the cleavage of a fatty acid from the original triglycerides. They are the lipolysis product, enzymatic or chemical, of triglycerides: often this kind of degradation is correlated with the conservation or poor quality of raw material, along with the presence of free acidity. However, DGs, unlike free acidity, do not can be easily eliminated, so they remain as lipolysis tracers.

Analysis through FAST GC-FID was carried out for the identification and quantification of DGs and, as for TGs, they have been grouped into classes depending on the total number of carbon atoms of the fatty acids from which they are composed, and they have been subdivided into 6 classes: CN30, CN32, CN34, CN36, CN38 and CN40. Each class has been quantified as percentage of the total content of diglycerides. In addition, long chain classes, D38 and D40, have only been identified in the subcutaneous fat of the three animals.

In storage fat, the main class of DG was the CN34 where calf and young bull shown significantly ($p \leq 0.05$) higher values, 41.84 and 42.60 % respectively, than cow, 40.36 %. The CN36 class shown slightly lower values that oscillated between 33.93 % in cow and 37.34 % in calf, and for which calf had a significant higher content than the other two animals ($p \leq 0.05$). In lower quantities than the previous classes, CN30 and CN32 groups with some significant differences between animals ($p \leq 0.05$) were identified. Calf in both cases shown the lowest content (5.47 and 15.34% resepctively) and the cow the highest (7.99 and 17.71 % respectively) (*Figure 26* and *Appendix 11*).

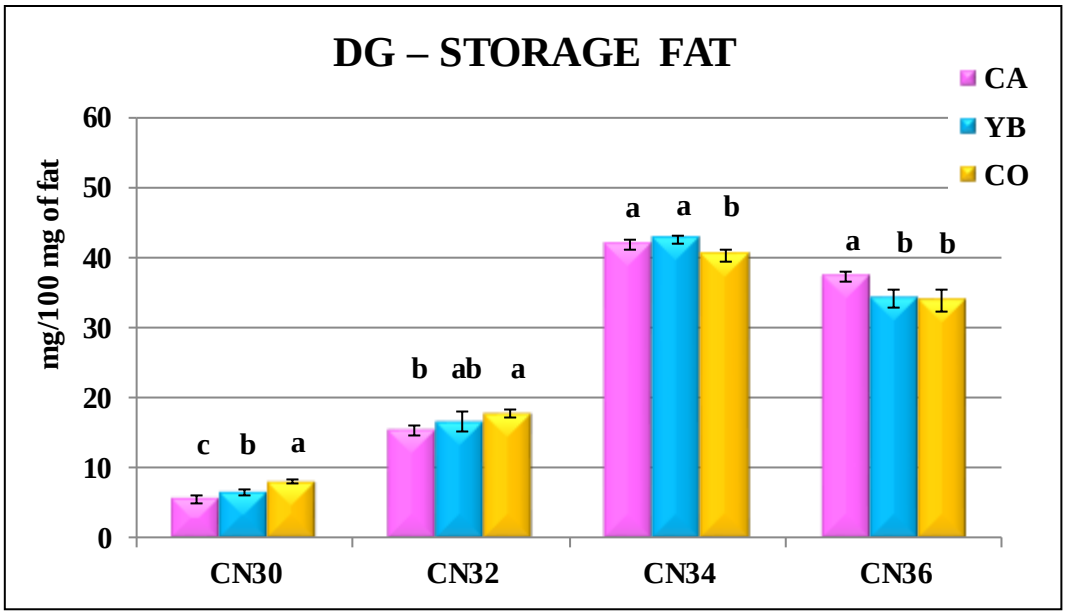


***Figure 26**. Graphic representation of diglyceride content grouped in D30, D32, D34 and D36 classes of storage fat in calf (CA), young bull (YB) and cow (CO) expressed in % of the total diglycerides present in the sample. The bars with different letters, within each DG class, are significantly different (p≤0.05).*

As mentioned above, two more long chain groups of DG have been identified in subcutaneous fat, the CN38 and CN40. This by-product was characterized by a strong heterogeneity that involved in a high variability of the data among the different samples that shown significantly different contents ($p \leq 0.05$) (Graph 4.14). The most present classes were CN34, CN36 and CN38. In particular, the young bull contained a significantly higher content of short chain DG classes, CN30, CN32 and CN34 (7.64, 7.96 and 36.65 % respectively), while shown the lowest content for the CN38 (15.63%). The cow, on the other hand, had the highest content for the CN40 (10.08 %) and the CN38 (26.36%) with a no statistically different value from calf (29.63%), which in turn had the highest percentage of CN36 class (30.87%) (*Figure 27* and *Appendix 11*).

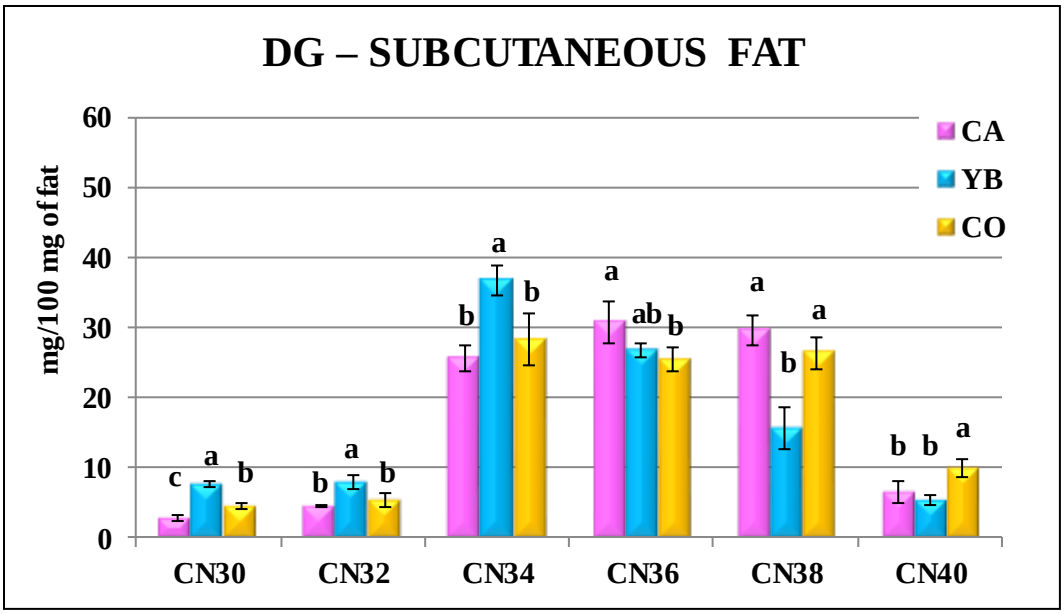


Figure 27. *Graphic representation of diglyceride content grouped in D30, D32, D34 and D36 classes of subcutaneous fat in calf (CA), young bull (YB) and cow (CO) expressed in % of the total diglycerides present in the sample. The bars with different letters, within each DG class, are significantly different (p≤0.05).*

In the bone marrow, as the other byproducts, the most present DG class was the CN34, with significantly higher values ($p \leq 0.05$) in cow (46.76 %) and young bull (45.01 %) compared with those of calf (36.85 %). They were followed by the CN36, CN32 and CN30 with significantly different values between animals. In particular, the young bull shown the higher values for short chain DGs, CN30 (9.41 %) and CN32 (18.53 %) and lower values for the CN36 (26.60 %), where the calf had the highest content (42.57 %) (*Figure 28* and *Appendix 11*).

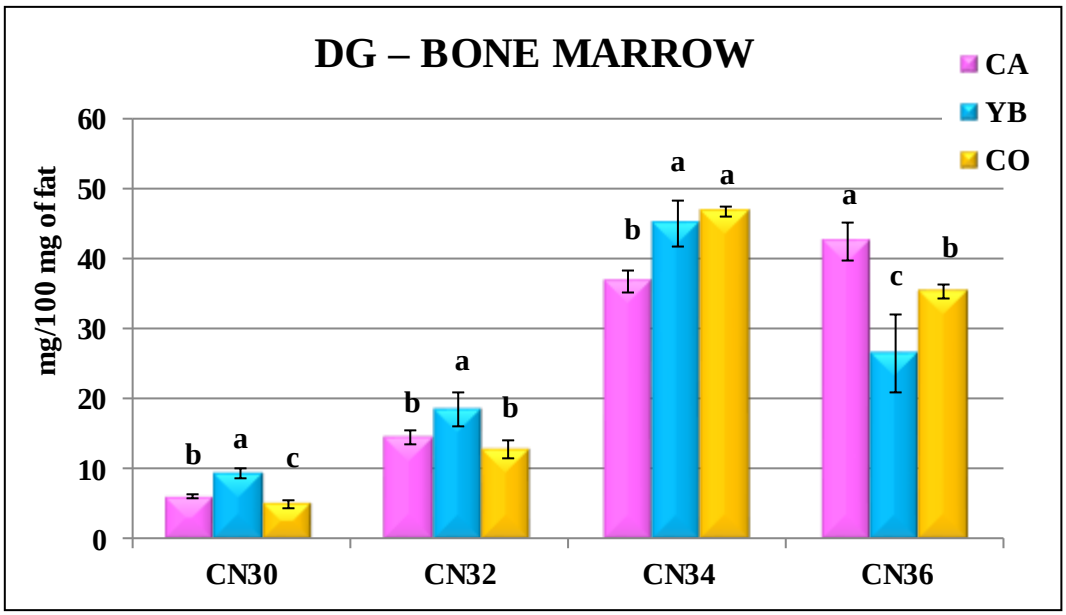


Figure 28*. Graphic representation of diglyceride content grouped in D30, D32, D34 and D36 classes of bone marrow in calf (CA), young bull (YB) and cow (CO) expressed in % of the total diglycerides present in the sample. The bars with different letters, within each DG class, are significantly different (p≤0.05).*

Bone fat had the same concentration trend of DG classes as found in other by-products. Particularly, class CN34 was present in significantly higher quantities ($p \leq 0.05$) in young bull (38.92 %) and cow (39.99 %), as well as the CN32 (18.17 % in young bull and 17.76 % in cow) and the CN30 (7.46 % in youg bull and 6.61 % in cow). For the CN36, as already found for other by-products, the calf exhibited the highest concentration, equal to 41.77% of the total content in DG (*Figure 29* and *Appendix 11*).

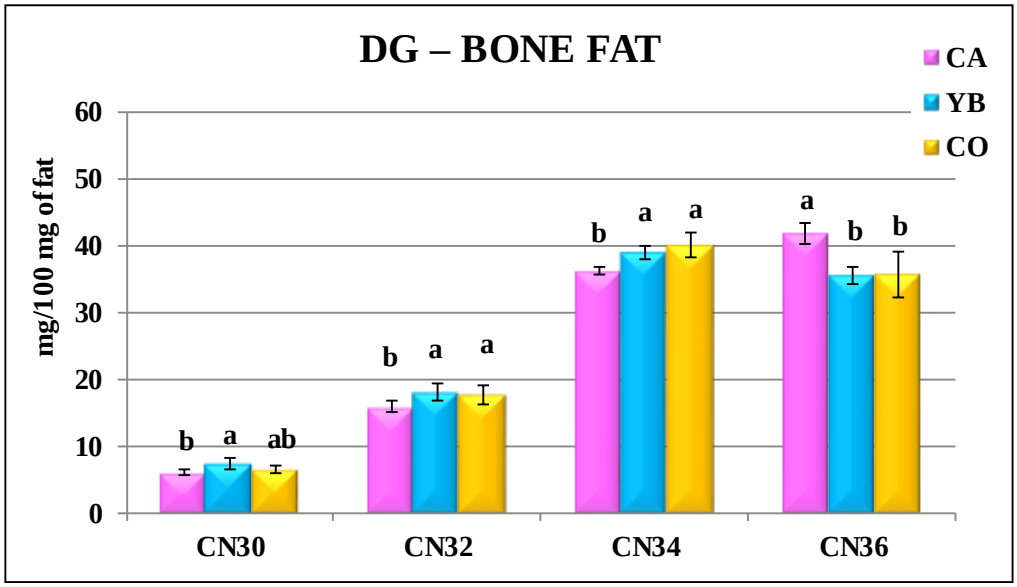


Figure 29. *Graphic representation of diglyceride content grouped in D30, D32, D34 and D36 classes of bone fat in calf (CA), young bull (YB) and cow (CO) expressed in % of the total diglycerides present in the sample. The bars with different letters, within each DG class, are significantly different ($p \leq 0.05$).*

Considering each individual animal, subcutaneous fat of calf shown the presence of the two more long chain groups, this involves in a significantly lower concentration ($p \leq 0.05$) of the other classes (CN30-CN36), registering the lowest values rather than the other products. In general, the other three by-products did not show high differences in content. The same trend was also found in the cow, where subcutaneous fat had the lowest values for all classes of DG in common with other by-products; except for the CN30 class that shown the lowest content even in the bone marrow, with a no statistically different value ($p \leq 0.05$) than that of the subcutaneous fat. Finally, also the young bull shown lower contents for classes from CN30 to CN36 in subcutaneous fat but with values not always statistically different from all other by-products, as was found for cow and calf (*Appendix 11*).

As mentioned at the beginning of the paragraph, diglycerides are the lipolysis product, enzymatic or chemical, of triglycerides so they are correlated with the conservation or poor quality of raw material, along with the presence of free acidity. The total amount of diglycerides, reported

in *Appendix 3* and shown in *Figure 30*, was low for all the different samples analyzed and oscillated between 0.02 and 0.09 mg/100mg of fat. For all three animals, a significantly higher content in DG was registered ($p \leq 0.05$) in storage fat. In addition, both in storage fat and subcutaneous fat, the three animals did not have significant differences between them ($p \leq 0.05$); in bone marrow and bone fat, instead, calf reported a significantly higher content than the cow and calf (*Appendix 11*).

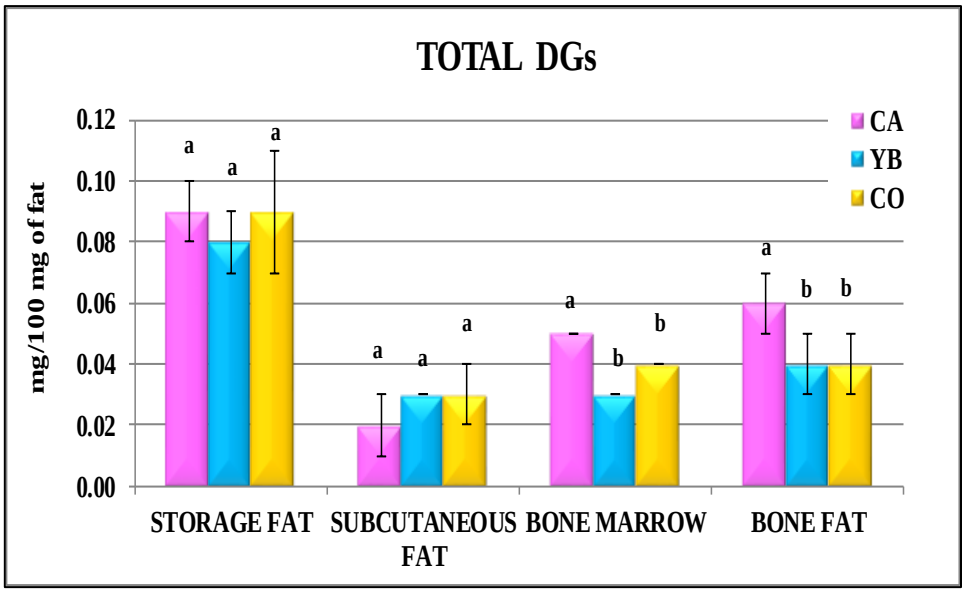


Figure 30. *Total DGs in the different by-products in calf (CA), young bull (YB) and cow (CO) expressed in % of the total diglycerides present in the sample. The bars with different letters, within each by-product, are significantly different (p≤0.05).*

I.6 Cholesterol content of by-products

Cholesterol is a sterol from animal origin that belong to lipid unsaponificable fraction. It is predominantly localized in the membrane of blood cells and it can affect their fluidity *(Boselli et al., 2009*). The required daily amount corresponds to 1-1.4 g and almost comes exclusively from the biosynthesis carried out by organism itself, only 10-20% comes from the diet's contribution. The amount of plasma cholesterol is maintained constant by varying the balance between intestinal absorption of the exogenous cholesterol introduced with diet, endogenous production and elimination cholesterol itself (*Ros, 2000*). For this for the reason and for the important physiological functions it performs, the cholesterol biosynthesis is inversely proportional to the amount introduced with the diet. Meat, including beef meat, has a cholesterol content around 100 mg/100 g of fresh meat. Some parts of the animal, especially the brain and spinal cord, can present a higher quantity of 20-30 times that contained in meat and fat (*Bittante et al., 2005*). For this reason, in order to characterize the by-products, samples were analyzed for their total content in cholesterol, by injection into the FAST GC-FID of the silanized unsaponificable fraction. *Table 6* and the

following figures (*Figure 31, Figure 32, Figure 33* and *Figure 34*) show cholesterol contents, expressed in mg/kg of fat, of the three animals depending on the type of by-product.

Table 6. Cholesterol content expressed in mg cholesterol/kg of fat in the different by-products

CHOLESTEROL CONTENT – mg cholesterol/kg of fat			
	CA	**YB**	**CO**
Storage fat	862.2 ± 101.4 ^{aB}	732.4 ± 14.5 ^{aB}	739.7 ± 31.0 ^{aB}
Subcutaneous fat	5192.4 ± 899.6 ^{aA}	3431.2 ± 109.2 ^{aA}	2937.6 ± 157.7 ^{aA}
Bone marrow	990.8 ± 38.4 ^{aB}	963.1 ± 168.4 ^{aB}	1160.3 ± 168.4 ^{aB}
Bone fat	1055.1 ± 149.9 ^{aB}	1865.6 ± 574.9 ^{aAB}	1415.6 ± 390.4 ^{aAB}

Data (means ± SD, n=6) are expressed in mg cholesterol/kg of fat. Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters on the same row show significantly different mean values within each by-product (storage fat, subcutaneous fat, bone marrow, bone fat) for the three animals (CA, calf; YB, young bull; CO, cow); capital letters on the same column show significantly different mean values within each individual animal (CA, YB, CO) for their respective four by-products (storage fat, subcutaneous fat, bone marrow, bone fat).

In particular, for each by-product no differences ($p < 0.05$) were carried out among the three animals, whereas each animal showed a significantly higher cholesterol content in subcutaneous fat compared to the storage fat, bone marrow and bone fat. This result was plausible being cholesterol a leading and fundamental component of cell membranes. In the other three by-products no significant differences were found, ranging from 732.4 mg/kg in the storage fat of young bull to 1865.6 mg of cholesterol/kg of fat in the bone fat of young bull (*Table 6*). Despite the lack of literature about cholesterol content in beef by-products, our results are higher than other studies. Several authors (*Almeida et al., 2006; Baggio & Bragagnolo, 2006; Cifuni et al., 2004; Muchenje et al., 2009*) investigated the cholesterol amount on beef meat and all of them had lower cholesterol content than our by-products. In fact, *Cifuni et al.* (2004) investigated the cholesterol content in Podolian young bulls obtained values ranging from 460.76 to 490.69 mg cholesterol/kg; *Almeida et al.* (2006) obtained a cholesterol content ranging from 510.97 to 630.02 mg cholesterol/kg in beef meat; *Baggio & Bragagnolo* (2006) studied the cholesterol content on processed beef meat products obtained values from 300 to 340.4 mg cholesterol/kg and, finally, *Muchenje et al.*, (2009) found a cholesterol content from 360.30 to 410.5 mg cholesterol/kg in beef meat from different cattle breeds.

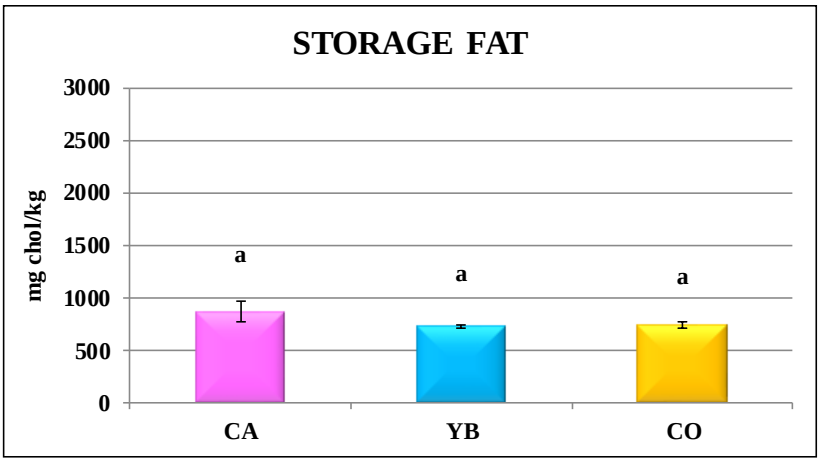


***Figure 31**. Graphic representation of total cholesterol content in storage fat of calf (CA), young bull (YB) and cow (CO) expressed in mg cholesterol/kg of fat. The bars with different letters are significantly different (p≤0.05).*

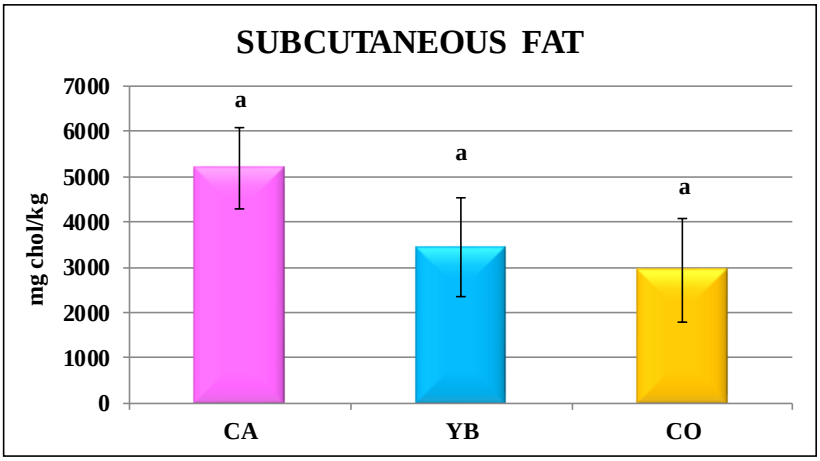


***Figure 32**. Graphic representation of total cholesterol content in subcutaneous fat of calf (CA), young bull (YB) and cow (CO) expressed in mg cholesterol/kg of fat. The bars with different letters are significantly different (p≤0.05).*

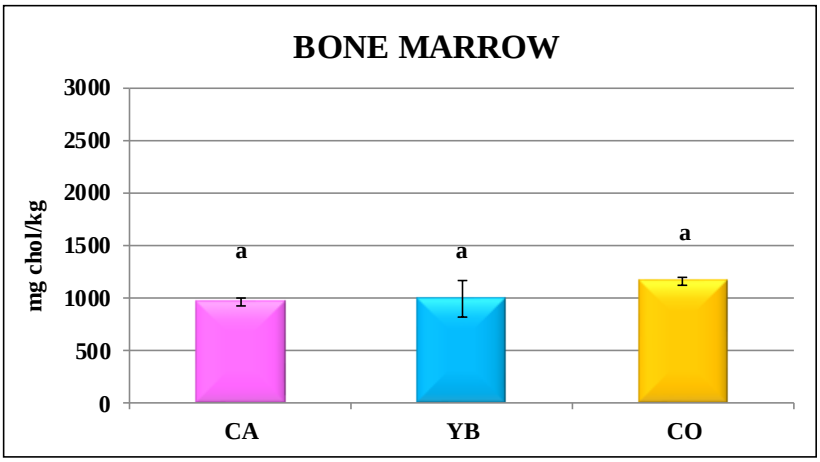


***Figure 33.** Graphic representation of total cholesterol content in bone marrow of calf (CA), young bull (YB) and cow (CO) expressed in mg cholesterol/kg of fat. The bars with different letters are significantly different (p≤0.05).*

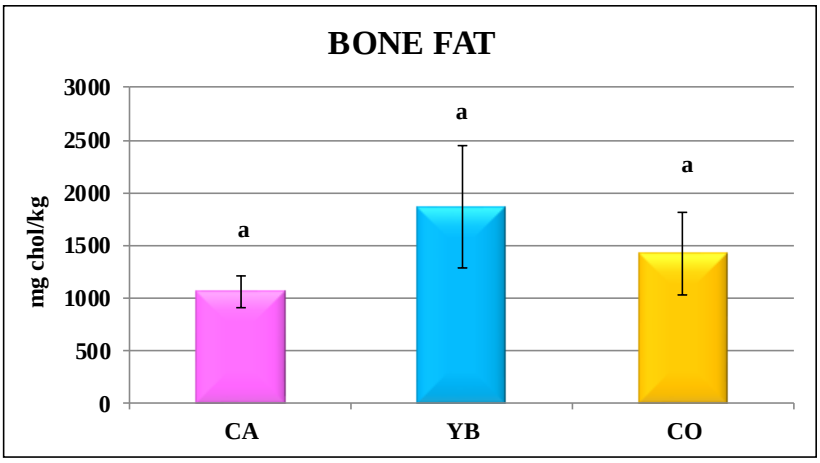


Figure 34. *Graphic representation of total cholesterol content in bone fat of calf (CA), young bull (YB) and cow (CO) expressed in mg cholesterol/kg of fat. The bars with different letters are significantly different (p≤0.05).*

I.7 Phospholipids content of bone marrow

The phospholipid content and the distribution of individual phospholipid species were determined only in the bone marrow of the three different bovine categories. Two phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM), were detected in all the samples. According to literature *(Dannenberger et al., 2007; Descalzo et al., 2003*), PC was the major phospholipid in all the three animals, accounting about the 60 % of the total phospholipid content, because it is the key building blocks of membrane bilayers; CA, YB and CO registered a PC value of 27.97, 16.22 and 17.62 mg/100g of fat, respectively (*Table 7*).

Table 7. Phospholipids content expressed in mg/100g of fat in the bone marrow of the bovines

PHOSPHOLIPIDS CONTENT – mg phospholipid/100g of fat			
	CA	**YB**	**CO**
PC	27.97 ± 2.79 ^{a}	16.22 ± 0.51 ^{b}	17.62 ± 1.49 ^{b}
SM	21.18 ± 0.78 ^{a}	10.80 ± 0.57 ^{c}	12.40 ± 0.82 ^{b}
TOTAL	49.15 ± 3.52 ^{a}	27.14 ± 1.01 ^{b}	30.03 ± 2.08 ^{b}

Data (means ± SD, n=6) are expressed in mg phospholipid/100 g of fat. Abbreviation: CA: calf, YB: young bull, CO: cow, PC: phosphatidylcoline, SM: sphingomyelin. Results of the analysis of variance by Tukey's test are shown: p<0.05, the different letters in the same row represent significantly different values within the same phospholipid (p≤0.05).

In all samples, SM was present in lower amount compared to PC but it plays an important role in animals because it is a substitute for PC as a building block of membranes. Calf reported the highest SM content (21.18 mg/100g of fat) and young bull and cow registered significant lower

content, 10.80 and 12.40 mg/100g of fat, respectively. Besides, CA's bone marrow showed the highest content in total phospholipids (49.15 mg/100g of fat) compared to YB (27.14 mg/100g of fat) and CO (30.03 mg/100g of fat) samples. The results are shown also in *Figure 35*. This result can be expected since calf is a young growing animal and its cell membranes need more phospholipids, critical to cells' ability to function and grow *(Wood et al., 2008*).

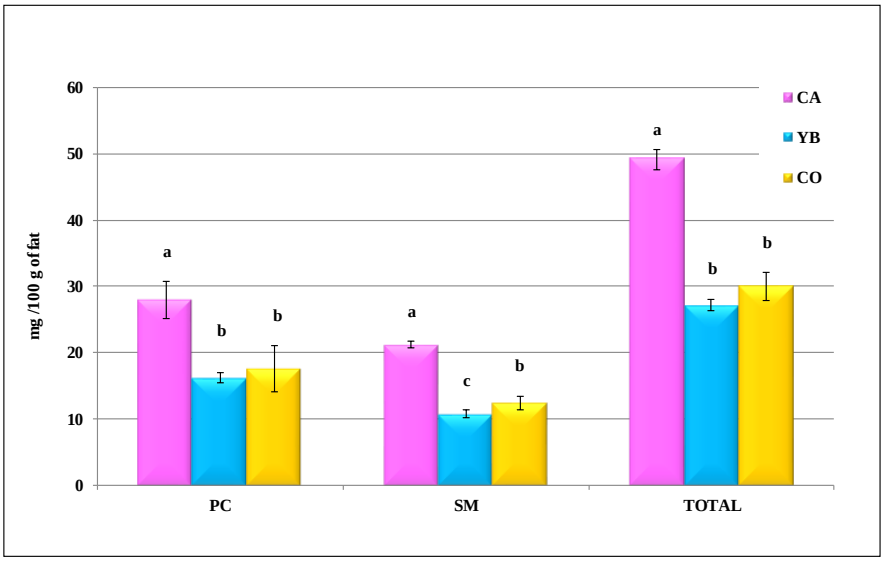


Figure 35. *Graphic representation of phospholipids content in bone marrow of calf (CA), young bull (YB) and cow (CO) expressed in mg/100 g of fat. PC: phosphatidylcoline, SM: sphingomyelin. The bars with different letters represent significantly different values within the same phospholipid (p≤0.05).*

Because of the lack of literature on the lipid quality of meat by-products, this first part of the research represents a first screening of bioactive lipids in different wastes produced by bovine meat industry. The obtained results underline that subcutaneous fat is a non-homogeneous waste with a highly variable composition. Nevertheless, as expected, subcutaneous fat showed the highest cholesterol content compared to the other by-products, because cholesterol is involved in the structure of the membranes that are present were the storage fat is low. On the other hand, the storage fat and especially the bone marrow presented a high fat amount and they were characterized by a low cholesterol content and important concentration of some essential fatty acids (linoleic acid, α and γ linolenic acid, eicosadienoic acid and arachinodic acid). In particular, calf showed the maximum content of oleic acid, linoleic acid and phospholipids, but also the highest content of cholesterol in the subcutaneous fat rather than young bull and cow. Conversely, young bull presented the lowest cholesterol amount and cow the highest TG content, especially CN48 class. The lipid fraction showed different concentrations depending on the type of by-product and was more present in storage fat and in bone marrow, with a percentage from 60 to 90%. The samples, on

the other hand, of subcutaneous fat and bone fat contained lower quantities, according to what has already been reported in the literature. Also, these two by-products also showed a strong non-homogeneity of the sample, due to the presence of connective tissue and hair in case of subcutaneous fat and tendons and ligaments in bone fat. The fatty acid composition was similar from a qualitative point of view for all three animals and the corresponding by-products. In particular, all of them had a higher saturated fatty acids (SFA) and monounsaturated (MUFA) contents compared to polyunsaturated (PUFAs), which are hydrogenated and converted in SFA in animal rumen. The results also showed how the calf had a significantly higher ($p \leq 0.05$) content in PUFA than cow and young bull, in all the by-products. This is probably related to the young age of calf when it is slaughtered, after only 6 months of life. In this period the rumen can not completely develop and therefore the PUFA do not undergo the hydrogenation process. Triglycerides (TGs) were the most contained compounds in the lipid fraction of analysed by-products, the most abundant were medium and long chain TGs (CN48, CN50 and CN52). The major differences between three animals were found for long chain TG, where the cow by-products contained a significantly higher ($p \leq 0.05$) content in CN50, while for CN52, where the cow had the lowest content, the calf reported the greater concentration than the other two animals. The diglyceride (DG) analysis, present in small quantities but with a fundamental importance for a quality evaluation of product, has allowed to identify 4 main classes: CN30, CN32, CN34 and CN36. Only in the subcutaneous fat of the three animals have been detected also the classes CN38 and CN40. This by-product also exhibited a strong non-homogeneity resulting in high variability of the data obtained and significant differences in content between the three animals. Nonetheless, the CN34 and CN36 classes showed the highest content in all the samples studied. Finally, the evaluation of cholesterol content showed a low concentration of this compound in all by-products of calf, cow and beef. Only the subcutaneous fat showed a significantly higher ($p \leq 0.05$) content than the other products for all three the animals. However, cholesterol content was significant not different from the three bovines for each by-product considered.

According to this first evaluation, it could be assumed a future application of these bovine by-products for our aim, synthesis of lipophenols. For the characteristics described above storage fat was chosen as the lipid fraction of lipopohenol.

I.8 Isolation of oleic acid

Hexane:acetone 1:1 (v/v) was used as solvent in order to isolate the maximum concentration of oleic acid from storage fat of the different bovine categories. After the night at 4°C, storage fat

showed clearly two different phases. As shown in *Table 8*, was impossible to isolate only the oleic acid but the separation between saturated fatty acids and the polyunsaturated fatty acids was possible. The percentage of oleic acid, however, was the highest among all the fatty acids because it was the most preponderant from the beginning. In fact, the enrichment in oleic acid was above 50 % for the storage fat of calf and young bull, 50.05 and 52.03 %, respectively and 27.49% in cow.

Even if the concentration of saturated fatty acid (C16:0 and C18:0), that were preponderant in the original analyzed samples, decreased after the isolation, their presence was relevant, but at laboratory level this was the best yield we could obtain.

Consequently, the concentration of saturated fatty acid decreased after the isolation, from 41.47, 41.56 and 36.27 mgFA/100 mg FAME to 31.72, 35.64 and 33.15 mgFA/100 mg FAME for CA, YB and CO, respectively. On the other hand, the concentration of monounsaturated and polyunsaturated fatty acid increased. In the case of MUFA, that is more relevant for the oleic acid presence, the concentration increased about 36.63, 40.17 and 22.38% in CA, YB and CO, respectively (*Table 8*).

Table 8. Fatty acid composition of storage fat of the three bovine categories after the tentative isolation of oleic acid

FATTY ACID (FAME) – mgFA/100 mg FAME (%)									
FA	**Before isolation**			**After isolation**			**Enrichment (%)**		
	CA	**YB**	**CO**	**CA**	**YB**	**CO**	**CA**	**YB**	**CO**
C14:1c	0.81	0.94	2.12	0.87	0.76	1.76	7.41	-19.15	-16.98
C15:1c	0.10	0.14	0.14	0.10	0.15	0.14	0.00	7.14	0.00
C16:0	26.44	24.92	25.51	22.12	22.52	24.44	-16.34	-9.63	-4.19
C16:1t	0.29	0.24	0.20	0.22	0.12	0.19	-24.14	-50.00	-5.00
C16:1c	3.62	3.71	5.13	5.39	4.51	6.01	48.90	21.56	17.15
C17:1	0.68	1.11	0.94	1.00	1.33	1.05	47.06	19.82	11.70
C18:0	15.03	16.64	10.76	9.60	13.12	8.71	-36.13	-21.15	-19.05
C18:1t	8.52	5.14	1.22	7.33	4.74	0.62	-13.97	-7.78	-49.18
C18:1 c9	**31.65**	**35.42**	**42.43**	**47.49**	**53.85**	**54.09**	**50.05**	**52.03**	**27.49**
C18:2 tt	0.31	0.55	0.39	0.63	1.06	0.97	103.23	92.73	148.72
C18:2 n6	3.81	1.85	1.40	4.51	2.33	1.66	18.37	25.95	18.57
C18:3 n3	0.24	0.18	0.20	0.23	0.22	0.21	-4.17	22.22	5.00
SFA	41.47	41.56	36.27	31.72	35.64	33.15	-23.51	-14.24	-8.60
MUFA	45.67	46.70	52.18	62.40	65.46	63.86	36.63	40.17	22.38
PUFA	4.36	2.58	1.99	5.37	3.61	2.84	23.17	39.92	42.71

Section II

Enzymatic alkylsuccinylation of tyrosol: synthesis, characterization and property evaluation as a dual-functional antioxidant

In this Section II of the study is reported the synthesis and characterization of an array of novel dual functional amphiphilic lipids based on tyrosol with both surface active and antioxidant properties (*Figure 36*). Tyrosol was chosen because it will be the phenolic fraction in the lipophenol synthesis. Tyrosol (2-(4-hydroxyphenyl) ethanol) is a well-known monophenolic antioxidant present in large amount in olive oil and can be extracted from olive mill waste water (*Fki et al., 2005*). Recently, tyrosol has attracted the attention of organic chemists and pharmacologists as a versatile and cheap substrate for the synthesis of a variety of esters exhibiting diverse and improved biological effects (*Barontini et al., 2014*). This is partly due to tyrosol being the most abundant biophenol in extra virgin olive oil and its ability to exert protective effects against oxidative injuries in cell systems *(Giovannini et al., 1999*) and improve intracellular antioxidant defense systems *(Di Benedetto et al., 2007*). However, all naturally occurring antioxidants are strongly hydrophilic and this makes their incorporation into fat and oil matrices difficult. This problem is being approached by the preparation of lipophilic antioxidants. Because of the limited solubility of these biophenols in lipid media, the search for new lipophilic derivatives with enhanced properties is of great interest. On the other hand, anhydrides are excellent electrophiles, which readily react with nucleophiles (e.g. alcohols), anhydrides were preferred for alkyl succinylation of tyrosol. In addition, previous works *(Anankanbil et al., 2017* and *Falkeborg & Guo, 2015*) showed that the ring opening mechanisms of anhydrides with various alcohols yield compounds with multi-functional properties including surface activity. Starting from these two assumptions, a new group of amphiphilic lipid derivatives by-chemoenzymatic succinylation of tyrosol was synthesized.

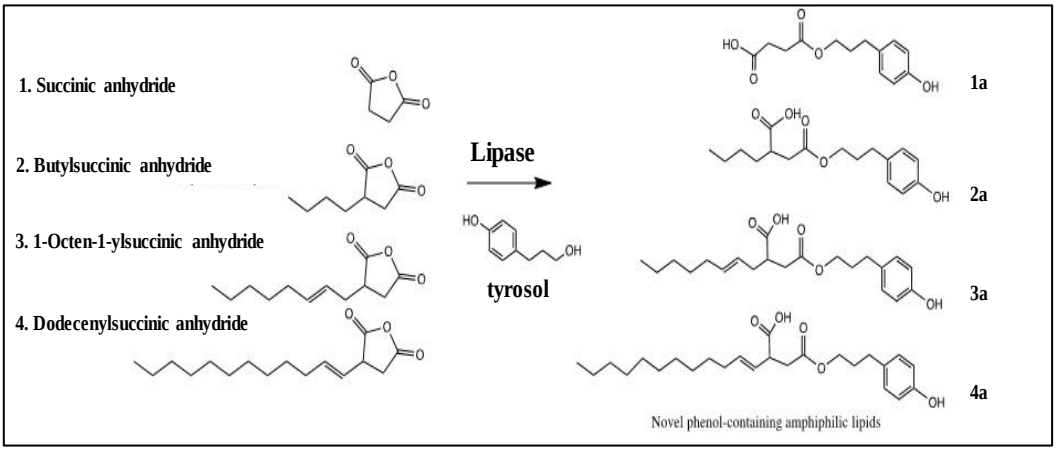


Figure 36. *Synthetic route for alkylsuccinilation of tyrosol*

II.1. Lipase-catalyzed synthesis of amphiphilic lipid derivatives

Given that the activity of lipases is affected by several operational parameters, we investigated the effect of different solvent systems, temperature, substrates ratios, and dosage of enzyme on the conversion of tyrosol (%) by succinylation with 2-dodecen-1-yl succinic anhydride. The effects of lipase type were elucidated by carrying out the succinylation in 10 mL of hexane, using 10% of enzyme and a substrate ratio of 1:1. From *Figure 37*, the highest conversion of 41% ± 1.39 was observed for Novozyme 435 followed by Lipozyme 435 and then Lipozyme RM-IM (38% ± 1.66 and 15% ± 0.73, respectively).

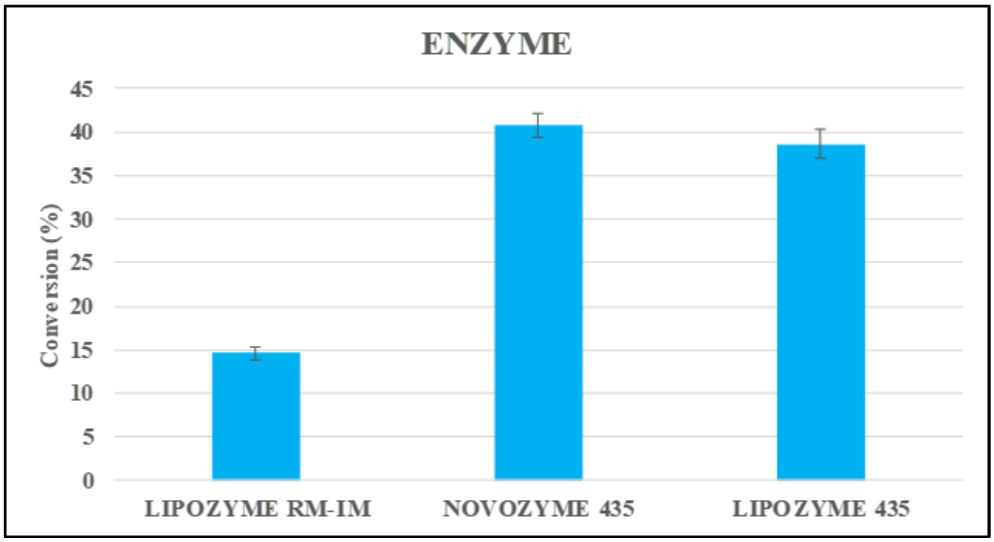


Figure 37*. Effect of lipases on the lipase catalyzed synthesis of compound 4a. Reaction conditions: hexane, 60 ° C, alcohol:anhydride 1:1, 10 % enzyme*

Solvent systems impact on lipase mediated catalysis and consequently, we investigated the effect different solvents may have on the conversion of the phenolic alcohol. The order of conversion of tyrosol in various organic solvents was 2-methyl THF<tert-butanol<MTBE<Toluene<Hexane<Hexane:2-methyl THF (*Figure 38*). In general conversions were lower in the more polar solvents (e.g. 2-methyl THF) compared to the less polar or mixed solvents systems (hexane and hexane:2-methyl THF). Polar solvents have been postulated to strip water from enzymes leading to enzyme inactivation (*Gorman & Dordick, 1992*) and that could explain the lower conversions of tyrosol in 2-methyl THF compared to hexane/less polar solvents employed. Succinnylation in hexane on the other hand was expected to give the highest conversion due to its high hydrophobicity compared to the other tested solvents. However, conversions of tyrosol were limited in hexane due to poor solubility of the more polar anhydride. Using mixed solvents systems of hexane and 2-methyl THF significantly ($p<0.05$) improved the conversion rates of tyrosol.

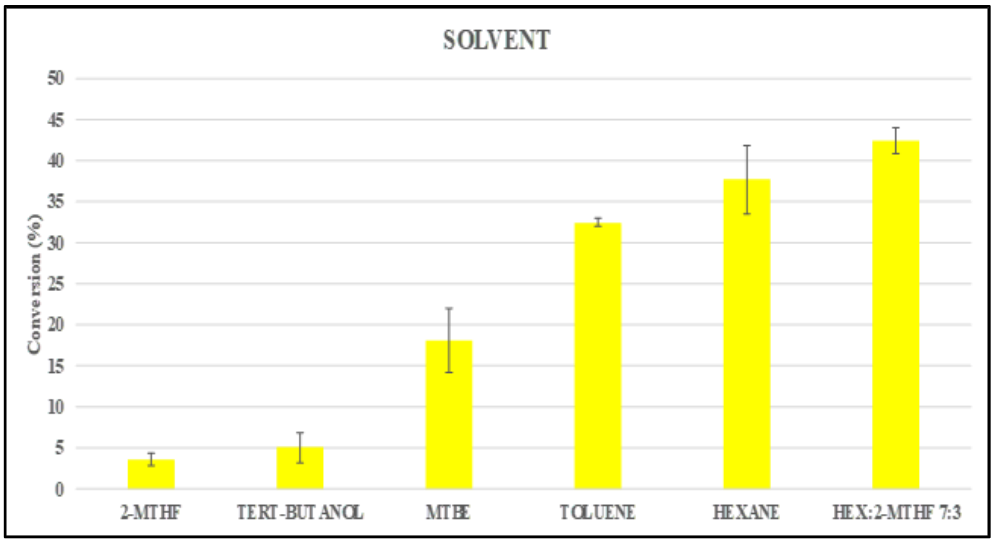


Figure 38. *Effect of solvent on the lipase catalyzed synthesis of compound 4a. Reaction conditions: Novozym 435, 60 °C, alcohol:anhydride 1:1, 10 % enzyme.*

In addition, mole ratios between substrates affect equilibrium of reactions. Therefore, the mole ratio between tyrosol and 2-dodecen-1-yl anhydride were investigated using mole ratios of 1:1, 1:2, 1:3, 1:4, and 1:6 of tyrosol: 2-dodecen-1-yl succinic anhydride in 10 mL of hexane:2-methyltetrahydrofuran (7:3) and 10% of Novozyme 435. The conversion rates increased with increasing number of moles of 2-dodecen-1-yl succinic anhydride (5.85%, 12.09%, 24.23% and 40.89% respectively for 1:1, 1:2, 1:4 and 1:6) relative to the number of moles of tyrosol (*Figure 39*). The substrate ratio which allowed the highest yield of succinylated tyrosol was 1:6.

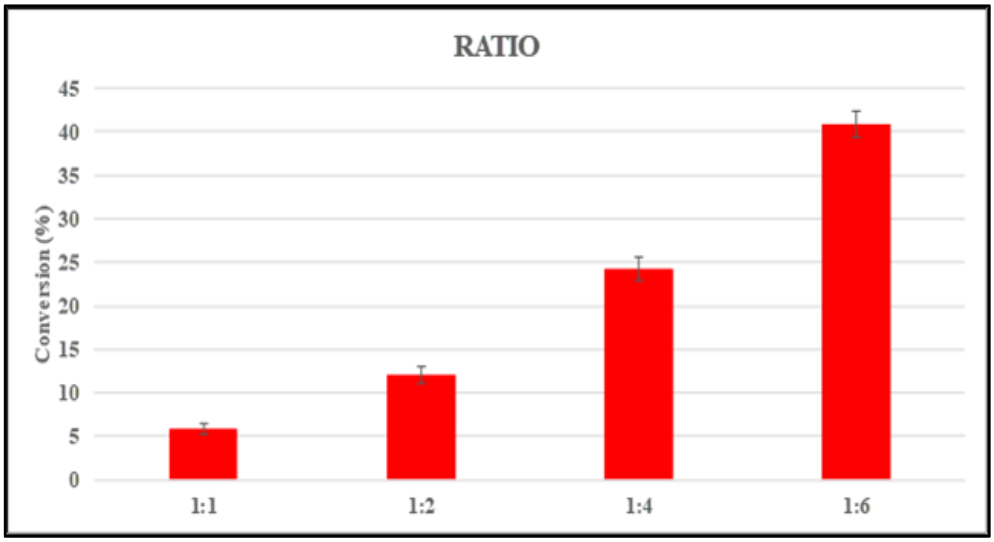


Figure 39*. Effect of substrates ratio on the lipase catalyzed synthesis of compound 4a. Reaction conditions: Novozym 435, 60 °C, hexane:2-MTHF 7:3, 70 °C, 10 % enzyme.*

Furthermore, lipases being biocatalysts have their temperature optimum at which catalysis is more efficient. To that end, the effect of temperature on conversion of tyrosol at five temparature levels (40 °C, 50 °C, 60C°C, 65 °C and 70 °C) was investigated for Novozyme 435. A significant

difference ($p<0.05$) between the conversions at 40 °C, 50°C and 60 °C (23, 38 and 40 % respectively) and the conversions at 65 °C and 70°C (79 and 82%) was observed (*Figure 40*). Thus, a temperature of 70 °C was considered the best even if the conversion did not show significant difference with the conversion at 65 °C ($p>0.05$).

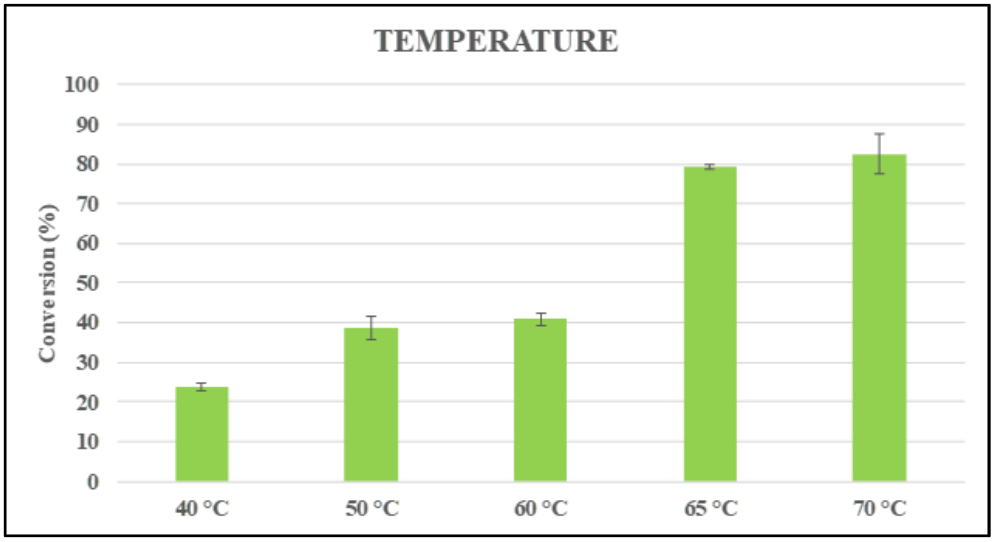


Figure 40. *Effect of temperature on the lipase catalyzed synthesis of compound 4a. Reaction conditions: Novozym 435, 60 °C, hexane:2-MTHF 7:3, alcohol:anhydride 1:6, 10 % enzyme.*

Moreover, the effect of enzyme dosage on the conversion of tyrosol by Novozyme 435 was performed by succinylation of tyrosol with 2-dodocen-1-yl anhydride in hexane:2-methyl THF using a mole ratio of 1:6 (tyrosol:anhydride). As shown in *Figure 41*; 2% enzyme loading (base on weight of tyrosol) gave the lowest conversion of 39% followed by 5% enzyme loading then 10% enzyme loading and 15% enzyme loading (conversions of 89%, 94% and 96% respectively). Hence 15% enzyme dosage was selected as the ideal in this case.

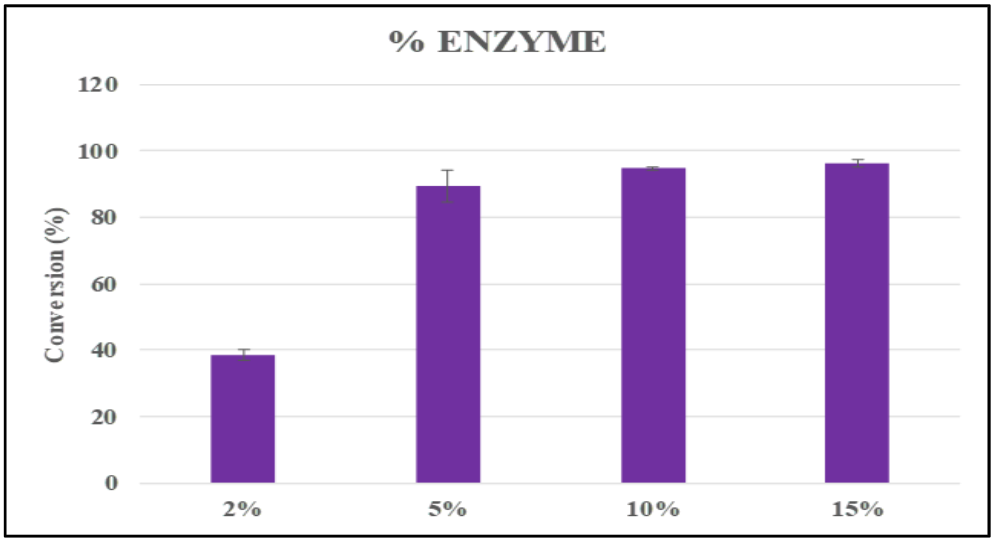


Figure 41. *Effect of amount of enzyme on the lipase catalyzed synthesis of compound 4a. Reaction conditions: Novozyme 435, hexane:2-MTHF 7:3, 70 ° C, alcohol:anhydride 1:6.*

Finally, the reaction time course demonstrated that equilibrium was essentially attained after 20 h of reaction. The time courses of all reactions are reported in *Figure 42*. The conversion of the tyrosol decreased with the increase of time with a concomitant increase in product yield as expected. As shown in Fig. 2A, the conversion of the tyrosol from the reaction with succinic anhydride (**1a**) was 88% at 20h and essentially equilibrated afterwards while the conversion from the reaction with butyl succinic anhydride (**2a**) was around 80%. That from 2-octen-1ylsuccinic anhydride (**3a**) was 95% and the highest conversion of tyrosol was obtained from the reaction with 2-dodecen-1-yl succinic anhydride (**4a**) (96%). The reactions of tyrosol with anhydrides of long chain lengths (**3a** and **4a**) had significantly higher conversions ($p<0.05$) due to improved solubility in the mixed solvent of hexane:2-methyl THF.

In summary, the optimal conditions thus established by single parameter study for the succinylation of tyrosol with 2-dodecen-1-yl succinic anhydride were as follows: Novozyme 435 as enzyme at 15% dosage base on weight of tyrosol; a mixed solvent system of hexane and 2-methyl THF in a ratio of 7:3; mole ratio of 1:6 between tyrosol and respective anhydrides; a reaction temperature of 70°C and a reaction time of 20 h. The above conditions were extended to the esterification of tyrosol with different chain length succinic anhydrides, thus yielding compounds **1a**, **2a**, **3a** and **4a**.

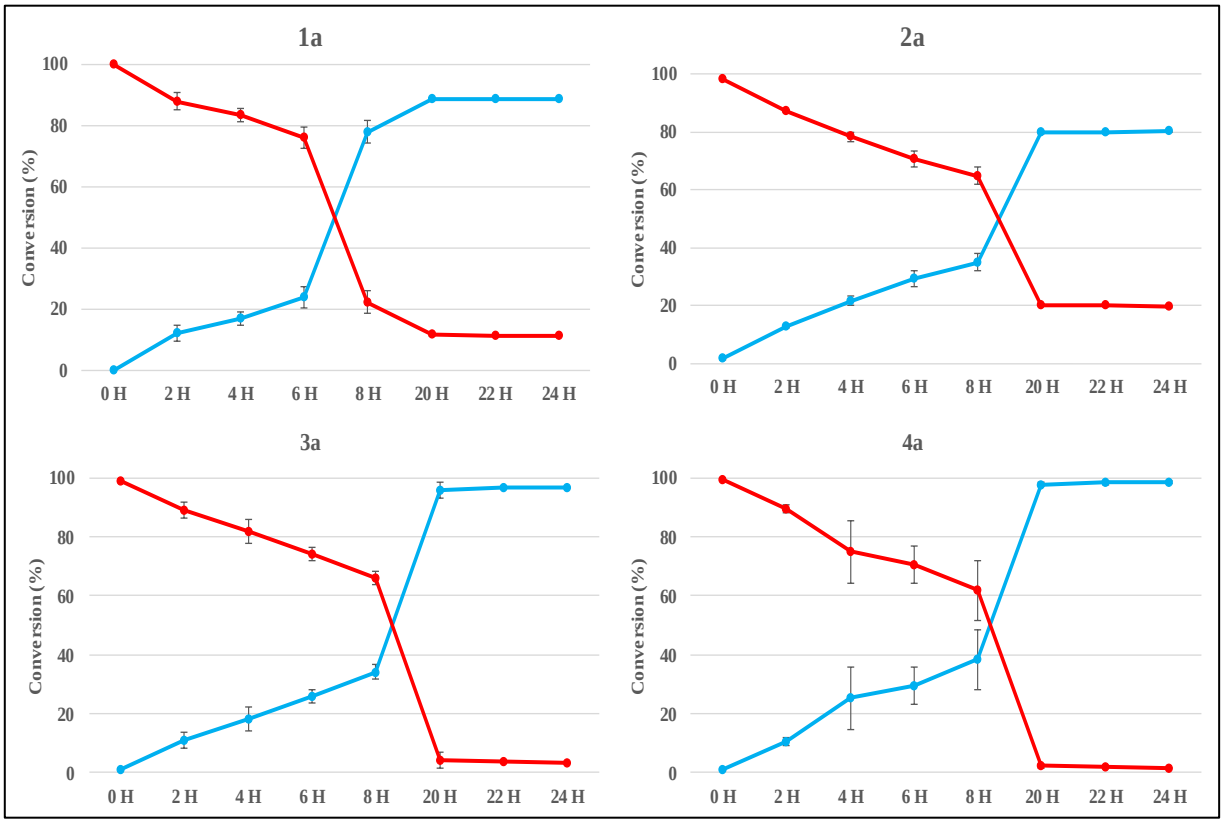


Figure 42. *Time course for the formation of compound 1a, 2a, 3a and 4a. The reaction conditions for all time courses were: Novozym 435, hexane:2-MTHF 7:3, 70 °C, alcohol:anhydride 1:6, 15 % enzyme.*

To the best of our knowledge, this work is the first systematic study on enzymatic succinylation of tyrosol to give new dual functional amphiphilic lipid derivatives. Therefore, characterization of physicochemical properties is of fundamental interest for potential applications of these compounds in food, cosmetics and pharmaceutical products. The compounds in this series were characterized by Differential Scanning Calorimetry to determine their thermal properties. Fourier transform infra-red spectroscopy was also used to monitor the packing behavior of lipid alkyl chains.

II.2 Differential Scanning Calorimetry (DSC) characterization

Characteristic thermal parameters such as melting points from DSC reflect the intra- and inter- molecular interactions of amphiphilic lipids (*Wei et al., 2016*).

The thermal behavior was investigated for compound **4a** because it was solid (melting point at 28.23 °C). Compounds **1a**, **2a** and **3a** were all liquid. Similar works observed decreased melting points for amphiphilic lipids when diacetyltartaric or succinic anhydrides were introduced into sugar alcohol esters (*Wei et al., 2015*). The decreased melting points of compounds **1a**, **2a** and **3a** therefore suggest loose intermolecular interactions perhaps due to repulsions from the carboxylic and ester functional groups present in the amphiphilic lipids *(Pérez et al., 2017*). However, this repulsion was overcomed by van der Waals interactions when the alkyl chain length was increased to 12 carbons (*Pérez et al., 2017*).

II.3 Fourier transform infrared spectroscopy

To study the molecular conformations of the synthesized compounds, FTIR spectroscopy was performed. According to *Moore & Rerek* (2000), FT-IR can predict the transition between fully extended all-trans hydrocarbon chains to disordered chains packing upon conformational changes since this yield a frequency increase in both symmetric and asymmetric stretching, at ~2850 and ~2920 cm $^{-1}$, respectively. As shown in *Figure 43* all synthesized molecules displayed both peaks at ~2850 and ~2920 cm $^{-1}$. Compound **4a** exhibit lower frequencies at both wave numbers than the other synthesized compounds (**1a**, **2a** and **3a**), implying it has a less random organization compared to the other compounds. This was corroborated by thermal analysis from DSC which show a low melting point of 28°C. In addition, FT-IR spectroscopy can yield information about orthorhombic, hexagonal or fluid packing of lipids. The most densely packed is orthorhombic packing, where all alkyl chains display an all-trans conformation organized in a highly dense rectangular crystalline lattice (*Boncheva et al., 2008*). The entire series of synthesized compounds exhibit hexagonal packing by virtue of the single peak at approximately 720-725 cm $^{-1}$ (*Figure 43*) meaning the

synthesized compounds were already disorganized at room temperature. The results from FT-IR analysis corroborated thermal analysis data by use of DSC.

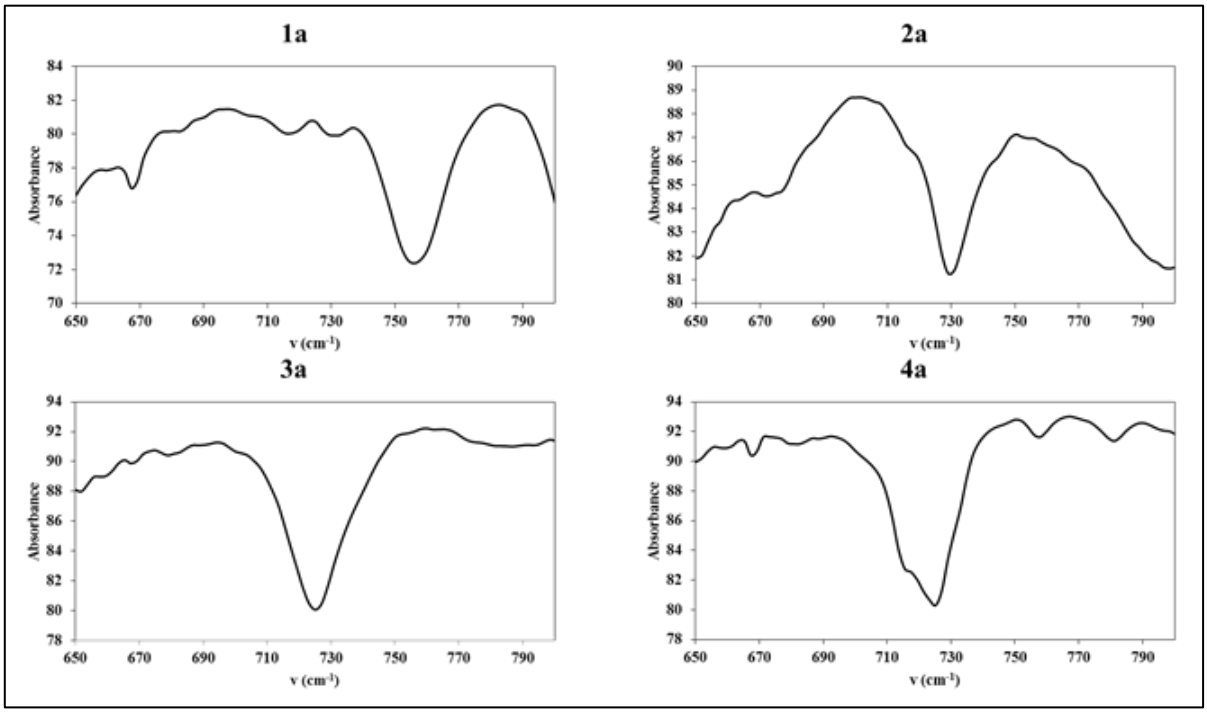


Figure 43. *Second derivate (Savitzky-Golay) of FTIR spectra in the region of 790-650 cm-1 of compounds 1a, 2a, 3a and 4a at room temperature*

II.4 Critical micelle concentration (CMC)

Amphiphilic compounds form micellar or bilayers structures in aqueous environments through self-assembly when they are present above certain threshold concentrations, called the critical micelle concentration (*Sek et al., 2002*). CMC is one of the most useful physicochemical characteristics of a surfactant and provides highly useful information on the surface activity of surfactants in aqueous solutions (*McClements, 2015*). At the CMC surface tension is independent of sample concentration (*Park et al., 2017*). The minimum concentration to form micelles (critical micelle concentration, CMC) was measured for the synthetic compounds (*Figure 44*) CMC depends on intrinsic factors such as the structures of the hydrophobic and hydrophilic parts of the amphiphilic molecule, and external factors such as medium temperature and composition (ionic strength, dielectric constant and pH) (*Ahmad & Xu, 2015*). It was well documented that for surfactants with similar head groups, CMC decreases as alkyl chain length of hydrophobic tails increases (*Chandler, 2007; McClements, 2015*). In this work, CMC was observed to decrease as the alkyl chain increased. The CMC value of all compounds were in a low range (0.5-10 µM), which means they tend to form micelles at very low concentrations. Compounds **3a** and **4a** presented the lowest CMC values (0.5 and 1 µM respectively), followed by compound **2a** (CMC value of 5 µM)

and then compound **1a** (10 µM). The latter two compounds have a shorter side chain, are more polar and hence higher CMC values than compounds **3a** and **4a** which are relatively less polar *(González-García et al., 2004*). The CMC data in this study corroborates findings by *Lucas and collaborators* (2010) who reported that lipophilic phenolic antioxidants show adequate surfactants properties with CMC values only when phenolic alcohols are esterified with long chain fatty acids (between 8 and 11 carbons).

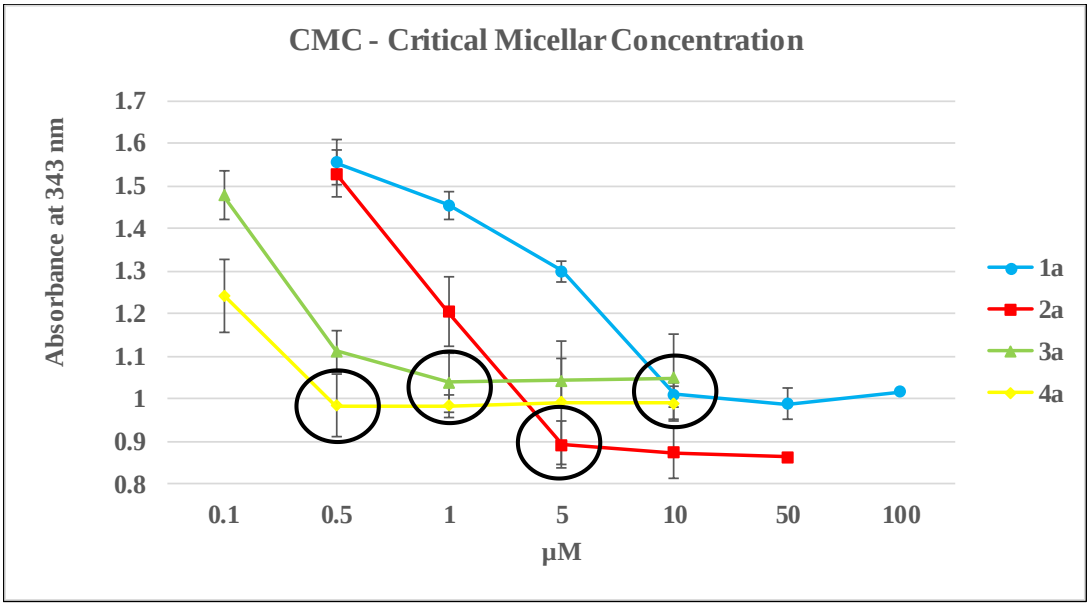


Figure 44. *CMC (Critical Micellar Concentration) value synthetic compounds 1a, 2a, 3a and 4a obtained at 343 nm (n=3)*

II.5 Antioxidant properties of amphiphilic lipids

II.5.1 DPPH free radical scavenging activity

The antioxidant activities of tyrosol, and compounds **1a**, **2a**, **3a** and **4a** were determined using the DPPH assay. It has been reported that the antioxidant activity of the phenolic compounds depends on their molecular structure, especially on their hydrogen-donating ability and subsequent stabilization of the formed phenoxy radical (*Silva et al., 2000*). As shown in *Table 9*, the DPPH activity decreased significantly ($p<0.05$) from the tyrosol (13.77%) to compounds **1a**, **2a** and **3a** (6.49, 0.74 and 9.50 % respectively) and increased significantly for compound **4a** (16.01%) ($p<0.05$). It is interesting to note that derivatization of tyrosol with 2-dodecen-1-yl succinic anhydride (compound **4a**) significantly ($p<0.05$) improved antioxidant activity compared to free tyrosol (*Figure 45*).

Table 9. Free radical scavenging activity (DPPH) of the synthetic compounds

	DPPH (%)
Tyrosol	13.77 ± 0.35 [b]
1a	6.49 ± 0.45 [d]
2a	0.74 ± 0.02 [e]
3a	9.50 ± 0.4 [c]
4a	16.01 ± 0.25 [a]

Data are represented as mean ± SD, n = 3.

The antioxidant activity of the phenolic antioxidants is dependent on the balance between the electron donating effect of the substituents and the steric crowding around the phenolic OH groups which is related to the position of the substituents *(Amorati et al., 2007*). Any substituent destabilizing the ground-state phenolic antioxidants, and/or stabilizing the phenoxy radical form of the antioxidants, reduces the O–H bond strength. The increase in activity in compound 4a rather than free tyrosol is because of the substituents; alkyl chain improves stabilization of the antioxidant radicals and increase radical scavenging activity (*Bernini et al., 2012; and Choe & Min, 2009*). *Kaki et al.* (2012) observed an improved antioxidant activity of a lipoic acid ester of tyrosol compared to the antioxidant activity of free tyrosol. This is in accordance with the observed improved antioxidant activity of compound **4a**. The derivatization of tyrosol with 2-dodecen-1-yl succinic anhydride may have stabilized the resulting phenoxy radical leading to the improved antioxidant capacity.

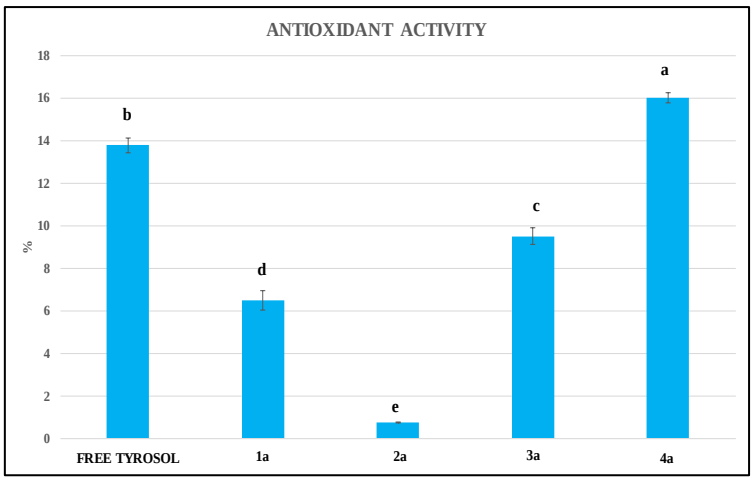


Figure 45. *Antioxidant activity of synthetic compounds 1a, 2a, 3a and 4a obtained with DPPH assay. The bars with different letters are significantly different (p≤0.05). Data are represented as mean ± SD, n = 3.*

III.5.2 Inhibition of Lipid Oxidation in emulsions

The oxidative profile of the emulsions was determined by TBARS; lipid molecules take up oxygen during the early stages of oxidation, forming the primary oxidation products called lipid peroxides, which are converted to TBARS, mainly MDA, secondary oxidation products (*Friberg & Larsson, 2003*). The formation of TBARS was measured over a period of 9 days to assess the evolution of lipid oxidation in fish oil-in-water emulsions. As shown in *Table 10*, the TBARS obtained from emulsion stabilized by tyrosol were significantly higher ($p<0.05$) than the TBARS from emulsions stabilized by compounds **3a** and **4a** from 0 days to 9 days.

Table 10. Antioxidant activity (TBARS) of synthsized compounds in emulsion system

	TBARS (%)			
	0 days	**3 days**	**6 days**	**9 days**
Tyrosol	76.63 ± 0.31	80.40 ± 5.83	86.98 ± 1.46	89.69 ± 0.17
3a	42.32 ± 4.30	48.56 ± 0.40	56.68 ± 3.52	54.29 ± 0.44
4a	21.57 ± 2.61	25.61 ± 1.11	31.11 ± 2.14	25.56 ± 0.05

Data are represented as mean ± SD, n = 3. The data of TBARS for compounds 1a and 2a are not reported because it was impossible create an emulsion with these two compounds.

As expected, TBARS of compound **3a** was significant higher than **4a** ($p<0.05$), because the antioxidant activity of the compound **3a** was significantly lower than that of compound **4a**. The inhibition of oxidation in emulsions is related to the surface activity of antioxidants; compounds **3a** and **4a** were very surface active and that means their concentrations at the oil-interface were higher and therefore they inhibited lipid oxidation better than compounds **1a** and **2a** which were less surface active. Compounds **3a** and **4a** showed a higher inhibition of lipid oxidation in emulsions (42.32, 21.57 % at 0 days) compared to free tyrosol (lipid oxidation of 76.63 % at 0 days) (*Figure 46*). Compare to *Kaki et al.* (2012), who used 5mg/ml of fish oil, compounds **3a** and **4a** exhibited lower TBARS values (42.32, 21.57 % respectively) compared to the synthetic compound from tyrosol and lipoic acid (50.13%).

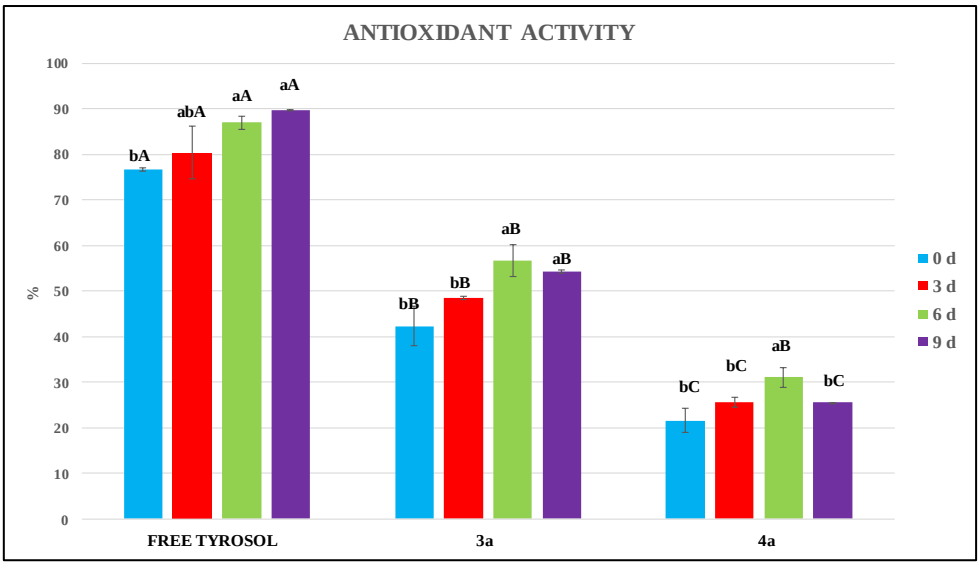


Figure 46. *Antioxidant activity of emulsions created with synthetic compounds 3a and 4a obtained with TBARS assay during 9 days of storage at 4 °C. The bars with different letters are significantly different (p≤0.05).*

Prior to these analyses, the particle size distribution and zeta potential of oil droplets in emulsion stabilized by the synthesized compounds were determined using dynamic light scattering. Measuring particle size and its distribution is important for optical properties and rheology because droplet size affects emulsion stability (*McClements, 2010*). Only compounds **3a** and **4a** formed stable emulsions. Stable emulsions could not be formed by compounds **1a** and **2a**, or by tyrosol. The average droplet size and zeta potential are reported in *Table 11*. As shown the particle size of tyrosol emulsions was very high (3677.67 nm), and that explain why it was impossible to create emulsions with only tyrosol. Furthermore, tyrosol is hydrophilic with less lipophilicity and hence is expected to have a low surface activity. The droplet size decreased significantly in emulsions stabilized by compounds **3a** and **4a** (745.77 and 679.10 nm respectively) but there were not significant differences in terms of emulsion stabilities between emulsions from these two compounds.

As depicted in *Table 11*, oil droplets of emulsions stabilized by the new amphiphilic lipids had negative surface charges (negative zeta potential) due to the presence of COOH which could be deprotonated as negatively-charged COO^- in water. These negatively charged surfaces provide repulsive forces which prevent fusion and aggregation of droplets *(Song et al., 2013*).

Table 11. Particle size, PDI and zeta potential of emulsions prepared with the synthetic compounds

	***Size* (*d*, nm)**	**PDI**	**Zeta potential (mV)**
Tween 20	795.73 ± 13.28	0.57 ± 0.07	-45.07 ± 0.81
Tyrosol	3677.67 ± 195.27	0.53 ± 0.38	-25.60 ± 1.08
3a	745.77 ± 25.74	0.63 ± 0.02	-35.57 ± 2.18
4a	679.10 ± 79.62	0.45 ± 0.04	-32.80 ± 0.87

Data are represented as mean ± SD, n = 3.

This part of the study is the first on that report the enzymatic succinylation of tyrosol into dual functional molecules with both surface active and antioxidant properties. The structures of the new compounds were identified by LC-MS and ^{1}H NMR analyses. A systematic physico-chemical characterization was carried out with focus on the thermal properties, antioxidant activity by DPPH and TBAR assays, surface activity, and molecular packing behavior by means of DSC, FT-IR, and measurement of CMC. The synthetic amphiphilic lipids were applied for the preparation of emulsions, which showed that compounds **3a** and **4a** could form stable emulsions compared to free tyrosol. In addition, the antioxidant activity increased significantly compared to free tyrosol, even though only in the case of compound **4a**. At the same time, a better inhibition of lipid oxidation in emulsions stabilized by compounds **3a** and **4a** rather than emulsions stabilized by free tyrosol was observed. In all, the outcomes from this work not only add new members to the library of lipid materials, which could potentially be used in food, but also elucidate the structure–property–function relationship between hydrophobic alkanyl, and hydroxyl moieties assembled in one molecule, which is of a general instructive value for the design and engineering of new lipid materials.

Section III

Lipophenol: synthesis and evaluation of antioxidant performance in a real system

This section outlines the results of the experiment conducted in order to synthesize a lipophenol reacting tyrosol and oleic acid. Specifically, a first part of the chapter will illustrate and discuss the data obtained optimizing the reaction; secondly, the focus will shift towards the analysis of tarallin samples with different formulations, with a survey of primary and secondary oxidation trends.

III.1. Reaction set up

According to *Aissa et al.*, (2012), a homologous procedure was adopted in this study in order to lipophilize tyrosol and oleic acid to obtain tyrosyl oleate (*Figure 47*).

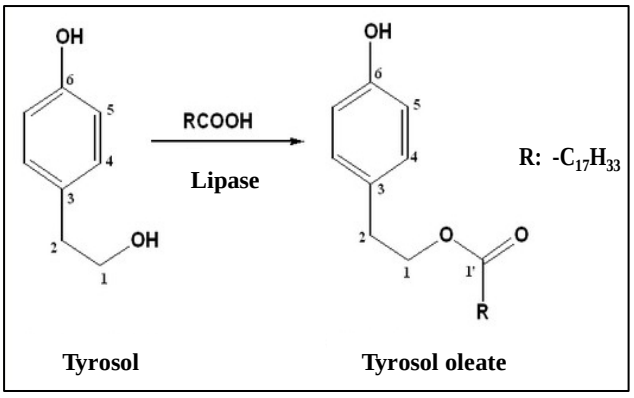


Figure 47. *Structure of tyrosyl oleate*

Lipozyme® enzyme immobilized by *Mucor miehei* was used as the catalyst for the reaction, although the literature has repeatedly reported that excellent yields have been found using Novozyme 435 lipase from *C. antarctica* (*Aissa et al., 2017; Torres de Pinedo et al., 2005; Villeneuve, 2007*). Initially, the purpose of the experiment was to find the best process conditions for achieving the maximum reaction yield. The variables considered were: molar ratio, solvent, temperature and enzyme percentage on the weight of the tyrosol. The variables were evaluated separately, reaching the maximum yield for each of them. The same condition was subsequently applied by modifying a new variable and so on. To have a complete picture of the reaction, the batches were sampled every hour and analyzed with HPLC-UV: this step allowed to calculate the percentage yield of each variable.

Molar ratio

The reagents used for the synthesis of lipophenol were tyrosol and oleic acid. As described in the introductory chapter, tyrosol is one of the major phenols present in oil by-products and, together with the oleic acid recovered from meat by-product, is an excellent substrate to be enhanced. Initially, standard reagents were used to allow the best process conditions to be used for a future test that uses reagents from by-products. For this reason, experimentation was carried out using oleic acid, preponderant fatty acid present in the storage fat. However, many authors *(Torres de Pinedo et al., 2007; Selmi et al., 1998*) have placed attention on the possibility of obtaining higher yields by lipophilizing tirosol with short or medium chain fatty acids, rather than with a long chain fatty acid (*Aissa et al., 2017; Aissa et al., 2012; Laguerre et al., 2013*). The same *Laguerre et al.* (2013) also make it a matter of mobility of the molecule, but already *Selmi and co-workers* (1998) studied that different alkyl chain lenght would ingluence the hydrophobicity, emphasizing that short-chain fatty acids have the best water solubility characteristics.

In the study the molar ratio evaluated were:

- **Tyrosol: oleic acid 1: 1**
- **Tyrosol: oleic acid 1: 2**
- **Tyrosol: oleic acid 1: 4**
- **Tyrosol: oleic acid 1: 6**

These first reactions have been completed under the same conditions, in a thermomagnetic bath at 60 ° C, with MTBE as solvent that allowed dissolve the tyrosol in oleic acid and with 10% of lipase enzyme calculated on the tyrosol weight.

The yields (%) obtained from reaction between tyrosol and oleic acid in different molar ratio are shown in *Table 12*.

Table 12. Yields (%) of the lipophilization reaction using differetn molar ratio between tyrosol and oleic acid, 60 °C, MTBE as solvent and 10% of enzyme based on tyrosol weight

TIME (h)	YIELD (%)			
	1:1	**1:2**	**1:4**	**1:6**
0	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}
1	21.0 ± 1.2 ^{b}	23.8 ± 0.6 ^{a}	21.6 ± 7.3 ^{ab}	9.4 ± 1.2 ^{c}
2	26.9 ± 5.8 ^{b}	39.6 ± 4.7 ^{a}	43.2 ± 12.7 ^{a}	48.1 ± 5.9 ^{a}
3	34.2 ± 2.3 ^{c}	57.4 ± 2.8 ^{b}	59.9 ± 11.9 ^{ab}	65.0 ± 2.9 ^{a}
4	35.5 ± 2.8 ^{c}	62.7 ± 2.0 ^{b}	63.8 ± 14.1 ^{ab}	73.5 ± 1.1 ^{a}
5	37.6 ± 3.0 ^{c}	65.1 ± 2.0 ^{b}	68.1 ± 10.8 ^{b}	77.0 ± 0.1 ^{a}
6	36.1 ± 2.9 ^{c}	66.9 ± 1.4 ^{b}	69.9 ± 10.7 ^{ab}	79.7 ± 0.7 ^{a}
7	42.5 ± 3.6 ^{c}	68.4 ± 1.8 ^{b}	72.5 ± 9.9 ^{ab}	80.2 ± 0.8 ^{a}
8	47.6 ± 2.7 ^{c}	77.7 ± 1.4 ^{b}	73.3 ± 9.0 ^{ab}	80.7 ± 0.8 ^{a}

Data are represented as mean ± SD, n = 2. The bars with different letters, within each row, are significantly different ($p \leq 0.05$).

Using molar ratio 1:1 tyrosol:oleic acid, after an initial net increase,the yield increased from the second hour, 26.9 %, until a final yield of 47.6 % after 8 hours, without a proportional increment. However, the trend of the yield from reaction using a molar ratio of 1:2 tyrosol:oleic acid was more homogeneous than the previous ratio and with a greater increase registered at every hour. The final yield after 8 hours is significantly greater than the ratio of 1:1 (77.7 %). In the first hours of reaction incremental percentage were higher, but remain above 2% until the end of eight hours. The trend registered using tyrosol:oleic acid 1:4 was similar to that seen for 1:2. The final yield obtained in this case (73.3 % after 8 hours) was similar to what was obtained in the reaction using 1:2 as molar ratio. Finally, using 1:6 as molar ratio of tyrosol: oleic acid; after a low percentage increase between the first and the second hour, rather the other molar ratio used, the reaction has a remarkable increase, about 500% during the third hour. At the end of the reaction the highest yield was recovered (80.7 %).

The following figure, *Figure 48*, shows the trends of all four tests carried out. The figure shows that 1:6 molar ratio is significantly better than the others, followed by reaction with 1:4, then 1:2 and finally 1:1 molar ratio.

The synthesis using the molar ratio 1:1 was discarded due to low yield. The reaction 1:4 was also discarded because it showed similar trends to reaction 1:2 but with high variability of the data. The products obtained from reactions with molar ratio 1:2 and 1:6 were analyzed by Nuclear Magnetic Resonance (NMR), it allowed to detect a remarkable concentration of unreacted oleic acid, especially in 1:6. So this reaction, despite the highest yield in %, was therefore discarded because it provided a high concentration of unreacted oleic acid. Definitely, the best ratio was **tyrosol:oleic acid 1:2** and was therefore chosen as the base for testing solvents and different temperatures.

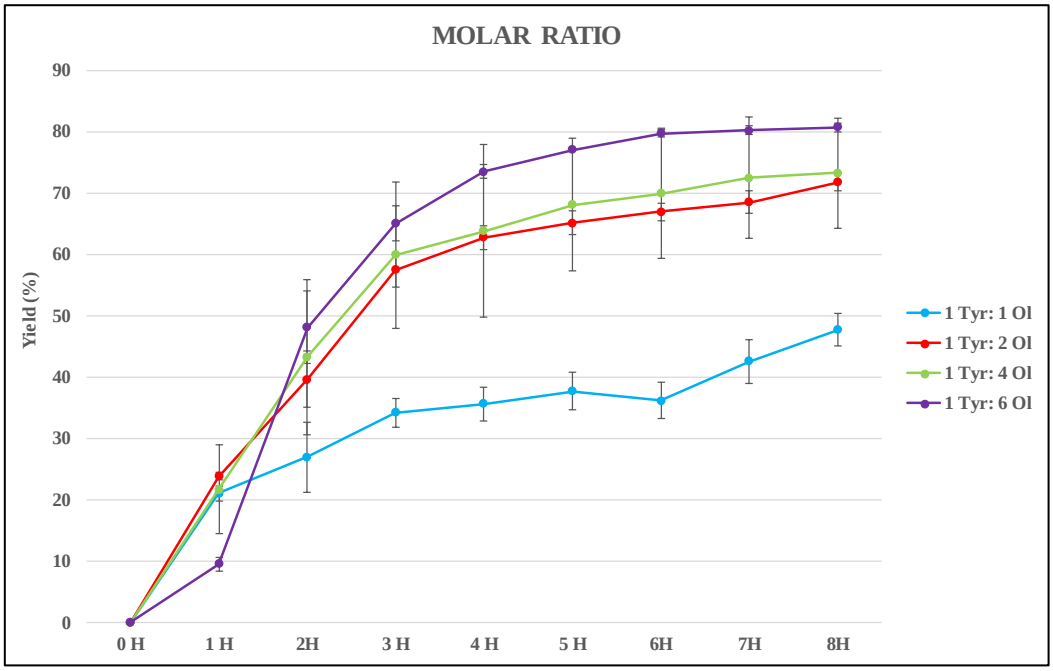


Figure 48. *Comparison of yields (%) of the lipophilization reaction using different molar ratio, 60 °C, MTBE as a solvent and 10 % of enyme based on tyrosol weight. Abbreviation: Tyr:tyrosol and Ol: oleic acid.*

Solvents

The study of different solvents has become necessary since tyrosol and oleic acid could not form a single phase. Methyl-t-butyl ether (MTBE), hexane, a mixture of hexane:tetrahydrofuran (THF) 3:1 and finally *tert*-butanol were tested. At the end of the reaction, the residual solvent was removed with rotavapor, so it does not remain dissolved in the final product. The reaction conditions for these tests were: molar ratio 1:2, 60° C and 10% enzyme based on tyrosol weight.

The yields (%) obtained from reactions using 1:2 as molar ratio and different solvents are shown in *Table 13*.

Table 13. Yields (%) of the lipophilization reaction using different solvents, 1:2 as molar ratio between tyrosol:oleic acid, 60 °C, MTBE as solvent and 10% of enzyme based on tyrosol weight.

TIME (h)	YIELD (%)			
	MTBE	**Hexane**	**Hexane:THF 3:1**	**tert-butanol**
0	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]
1	21.1 ± 1.0 [a]	5.4 ± 0.2 [b]	2.5 ± 0.4 [c]	0.6 ± 0.1 [d]
2	45.0 ± 0.2 [a]	4.3 ± 1.1 [b]	2.1 ± 0.6 [c]	0.8 ± 0.1 [d]
3	59.3 ± 0.3 [a]	5.9 ± 2.0 [b]	2.7 ± 0.5 [c]	0.9 ± 0.2 [d]
4	69.9 ± 0.5 [a]	5.6 ± 1.7 [b]	2.3 ± 0.3 [c]	0.1 ± 0.2 [d]
5	74.1 ± 1.8 [a]	6.6 ± 1.9 [b]	2.3 ± 0.3 [c]	1.1 ± 0.2 [d]
6	74.9 ± 1.4 [a]	5.9 ± 1.9 [b]	2.7 ± 0.4 [c]	1.2 ± 0.2 [d]
7	77.3 ± 0.2 [a]	5.8 ± 1.7 [b]	2.7 ± 0.4 [c]	2.9 ± 0.6 [c]
8	80.0 ± 1.9 [a]	3.1 ± 0.5 [b]	2.7 ± 0.4 [b]	2.9 ± 0.6 [b]

Data are represented as mean ± SD, n = 2. The bars with different letters, within each row, are significantly different (p≤0.05).

The first test was carried out using MTBE as solvent. The performance of yield (%) leads to a final value final of 80 % which is in line with what was obtained in the previous test. None of the other three solvents used (hexane, hexane:THF 3:1 and *tert*-butanol) acted better than MTBE, in fact the yields do not exceed 7 % for every solvent tested. Also, visually, these solvents did not show a complete dissolution of the two reagents. Also from *Figure 49* it is clear the different yield obtained using MTBE and the other solvents. Regarding this test, the variable that was maintained was clearly **MTBE** solvent.

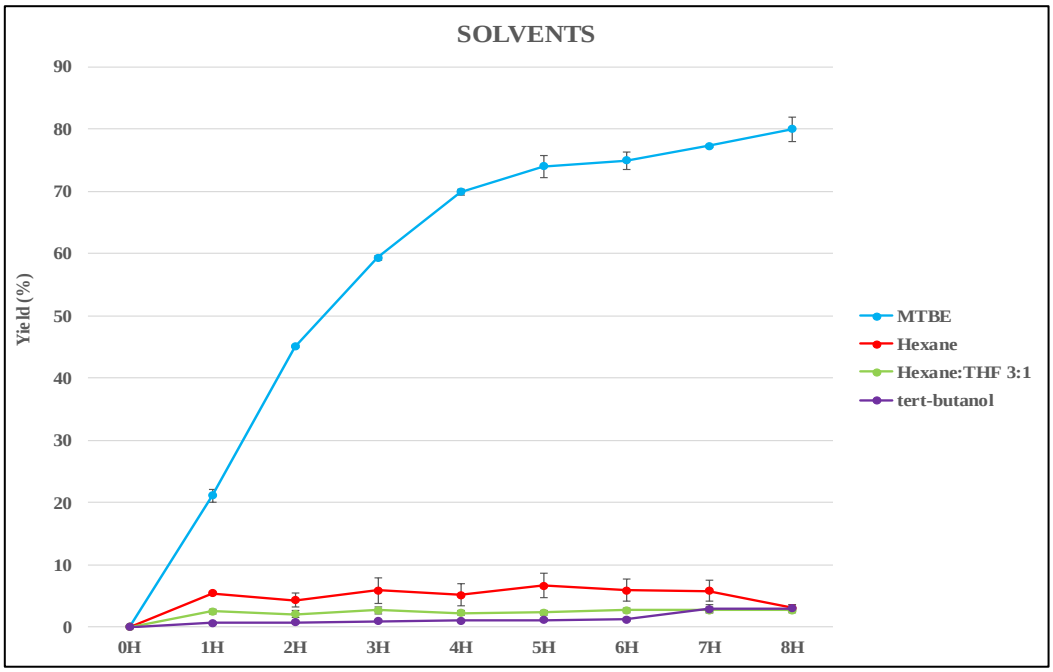


Figure 49. *Comparison of yields (%) of the lipophilization reaction using different solvents, 1:2 tyrosol:oleic acid as molar ratio, 60 °C and 10 % of enyme based on tyrosol weight.*

Temperature

The temperature comparison has become necessary to understand how the yield could increase in relation to the enzyme used. In literature, the temperatures used are very different: *Selmi, Gontier, Ergan, & Thomas* (1998) carried out work at 80 °C; *Aissa et al.,* (2017) and *Aissa et al.,* (2012) proposed a lipophilization reaction at 45° C; in the review work conducted in by *Villeneuve* (2007) has been reviewed numerous works where reaction temperatures vary in a very wide range, up to 81 °C. In the present study, the temperatures tested were 40 °C, 50 °C, 60 °C and 70 °C. The reaction conditions were tyrosol:oleic acid 1:2 as molar ratio, MTBE as solvent, 10% enzyme based on tyrosol weight and different temperatures. The different yields are reported in *Table 14.*

Table 14. Yields (%) of the lipophilization reaction using different temperatures, 1:2 as molar ratio between tyrosol:oleic acid,MTBE as solvent and 10% of enzyme based on tyrosol weight.

TIME (h)	**YIELD (%)**			
	40 °C	**50 °C**	**60 °C**	**70 °C**
0	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}
1	14.0 ± 3.3 ^{b}	9.8 ± 2.3 ^{c}	26.5 ± 0.4 ^{a}	2.8 ± 0.4 ^{d}
2	32.8 ± 3.0 ^{b}	31.9 ± 3.4 ^{b}	55.1 ± 4.2 ^{a}	2.4 ± 0.2 ^{c}
3	75.4 ± 0.3 ^{a}	48.4 ± 8.9 ^{c}	69.5 ± 1.6 ^{b}	2.3 ± 0.3 ^{d}
4	74.2 ± 0.5 ^{a}	54.6 ± 10.3 ^{b}	74.3 ± 0.8 ^{a}	2.4 ± 0.2 ^{c}
5	71.7 ± 0.8 ^{b}	61.4 ± 10.9 ^{b}	75.9 ± 0.3 ^{a}	3.3 ± 0.0 ^{c}
6	71.1 ± 1.5 ^{b}	60.4 ± 13.9 ^{b}	77.5 ± 0.0 ^{a}	3.2 ± 0.0 ^{c}
7	72.0 ± 1.3 ^{b}	63.5 ± 10.9 ^{b}	79.2 ± 0.4 ^{a}	4.3 ± 0.1 ^{c}
8	72.8 ± 1.7 ^{b}	66.9 ± 6.1 ^{b}	79.4 ± 0.4 ^{a}	4.6 ± 0.2 ^{c}

Data are represented as mean ± SD, n = 2. The bars with different letters, within each row, are significantly different (p≤0.05).

The first temperature tested was 40 °C. This temperature is the optimum for the enzyme: the maximum yield is reached after only three hours (75.4 %). In the reaction conducted at 50 °C the final yield is less than the reaction conducted at 40 °C, with a value of 66.9 % after 8 hours. The yield obtained from the reaction conducted at 60 °C was the same showed in *Table 13*. Finally, the yields of reaction conducted at 70 °C were very low and never exceeded 5%, probably because at this temperature the enzyme is not able to work and undergo a strong inhibition due to thermal conditions applied.

Figure 50 shows the yields percentage comparison at different temperatures. Excluding the reaction conducted at 70 °C, the other three temperatures have similar performance, but the test at 60 °C is better than the other two.

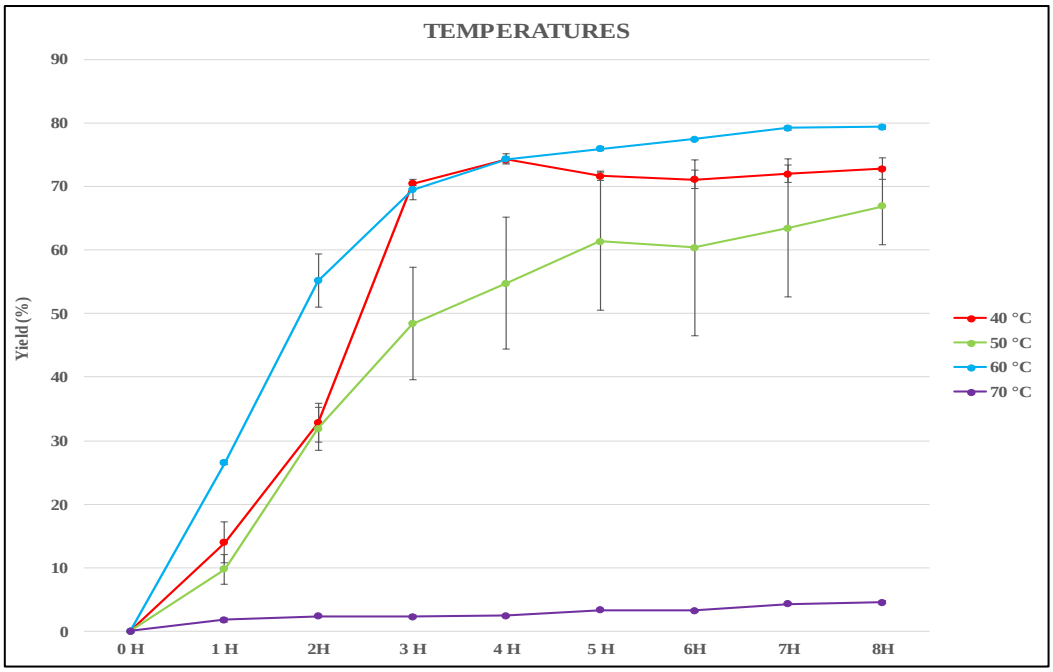


Figure 50. *Comparison of yields (%) of the lipophilization reaction using different temperatures, tyrosol:oleic acid 1:2 as molar ratio, MTBE as solvent and 10 % of enyme based on tyrosol weight.*

The method of lipophilization, therefore, results optimized with the following operating conditions:

- **8 hours total reaction time**. *Villeneuve* (2007) reports reactions time about 2.5 hours, while *Aissa et al.*, (2012) studied a reaction with a duration of 72 hours and *Selmi et al.*, (1998) for 6 hours.
- **Molar ratio between tyrosol and oleic acid of 1:2.** One of the most interesting works in literature regarding the lipophilization of tyrosol is by *Aissa and collaborators,* (2012), who perform 72-hour trials with a ratio tyrosol:fatty acid of 1:8. Despite this, the yields obtained in this work are encouraging.
- **MTBE as solvent**. In literature, many authors do not cite any solvents in the reaction tests they perform, probably because there is a better miscibility of the reagents than the one found in this experiment.
- **60 °C as temperature.** Also in relation to this variable, there are discordant references in the literature, with a wide applied thermal range.
- **10% of the enzyme based on tyrosol weight.** In literature, the recurrent enzyme is Novozyme 435 lipase from *C. antarctica*. As far as percentages of use are concerned, *Villeneuve* (2007) reports authors' works employing different percentages, from 1.5% to 5% p/p. The lipase employed in this thesis (Lipozyme® immobilized by *Mucor miehei*) was not

found for this kinf of reaction, but the percentage of use in combination with other optimized variables can be considered satisfactory.

III.2 Purification of tyrosyl oleate with chromatographic column

Once the product was obtained with the optimized conditions, the purification was carried out with chromatographic column, in order to separate the unreacted tyrosol and oleic acid from the compound of interest, tyrosyl oleate (*Figure 51*).

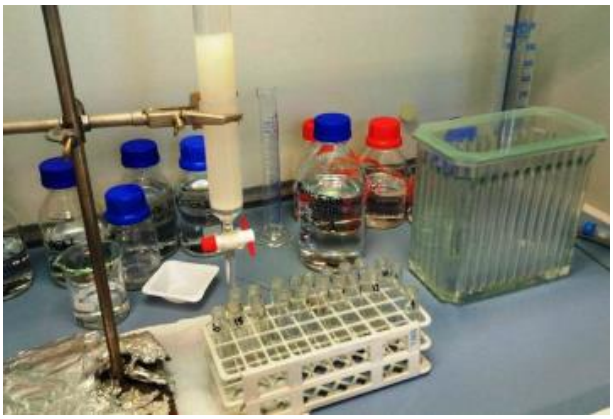


Figure 51. *Chromatographic column packed with Silica gel with tubes for the recovery of tyrosyl oleate and elution chamber for TLC plates*

Figure 52 shows the TLC plates with standard tyrosol (on the left) and pure tyrosyl oleate (on the right). In the plate on the left there is not the elution of tyrosyl oleate but only the elution of standard tyrosol, very close to the baseline, so it was not present, in those aliquots, our molecule. In these plates the oleic acid could not be seen, it was placed at the front of the solvent and, not having in its chemical structure an aromatic ring, it was not visible under the UV lamp but only after a carbonization of the plate in oven.

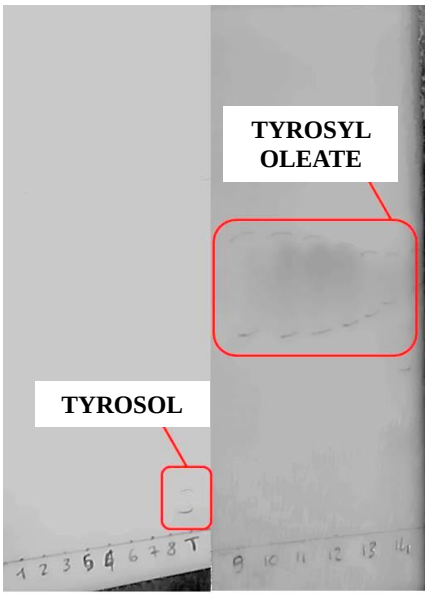


Figure 52. *TLC plates with standard tyrosol (on the left) and TLC plate with tyrosyl oleate (on the right)*

III.3 Identification of tyrosyl oleate with different techniques

Nuclear Magnetic Resonance (NMR)

After purification with chromatographic column, the identification of the pure tyrosyl oleate was carried out.

Figure 53 shows the NMR spectrum of tyrosyl oleate; where it possible to identificate the entire alkyl chain (1-17) of oleic acid, also the carbons relative to the double bond (9-10), and the aromatic ring of tyrosol (23, 24, 26, 27).

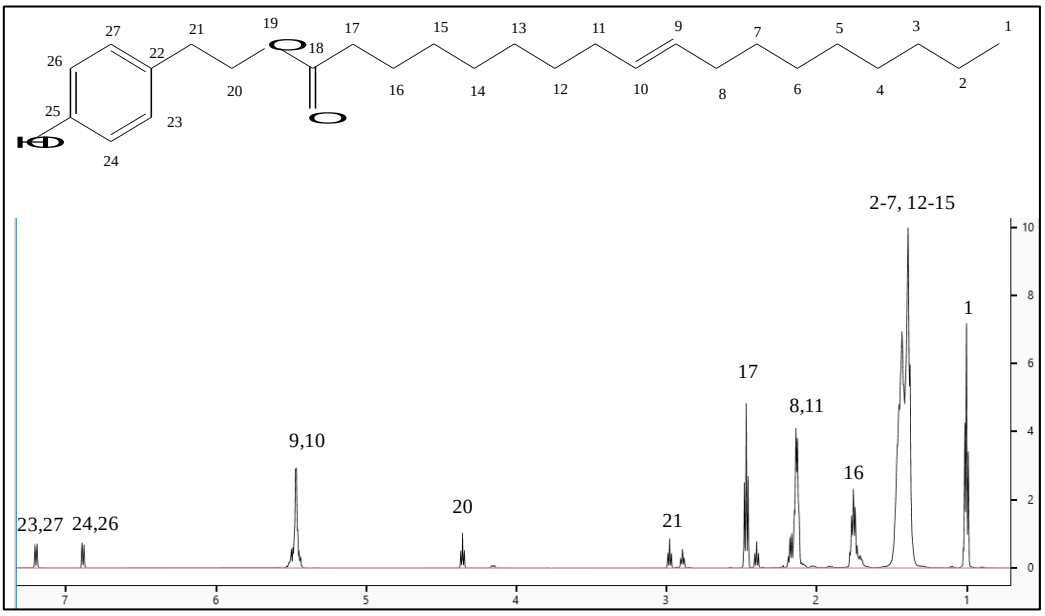


Figure 53. *NMR spectrum of pure tyrosyl oleate*

FT-MIR – Infrared Spectrometer

FT-IR is a spectroscopic analysis that allow to identify a molecule of interest, knowing its chemical structure, displaying the functional groups present in the molecule. All the spectrum were acquired in the range of wavelength between 4000 and 700 cm^{-1} with an FT resolution of 4 cm^{-1}. *Lozano et al. (2017)* operate a reading of the resulting interferogram according to a division of the same into two main sections: the first, comprised of 3750 and 2750 cm^{-1}, would correspond to the resonances of the bonds containing atoms of hydrogen, C-H, O-H and N-H; the range between 1900 and 400 cm^{-1} would be associated with the deformation vibrations of C=C, C=O, C-C, C-N, C-O and C-O. In agreement with the literature and with the chemical structure of tyrosyl oleate, the following wavenumber (cm^{-1}) were investigated within the interferogram:

- **Carbon chain**: 3000 - 2850 cm^{-1} *(Lozano et al., 62017)*
- **Esters**: 1750 - 1735 cm^{-1} *(Lozano et al., 2017)*
- **Benzene ring**: 1600 - 1500 cm^{-1} (*Largo-Gosens et al., 2014*)
- **Carboxylic acid**: 1720-1705 cm^{-1} *(Zhao et al., 2015)*

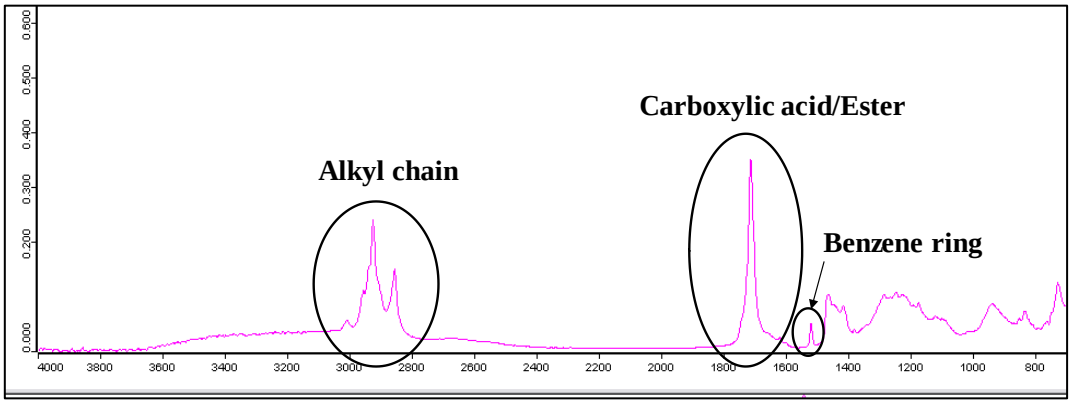


Figure 54. *Interferogram of tyrosyl oleate with FT-MIR spectroscopy*

As shown in *Figure 54*, all the the functional group of the molecule were found in the interferogram. Peaks within the range of the alkyl chain were not very defined as the peak between 1750 and 1705 cm^{-1}. The peaks detected from 1500 cm^{-1} up to 700 cm^{-1} can be traced back to the unreacted oleic acid fraction that was present in the final product of the lipophilization reaction: as also reported by *Zhao et al.* (2015), these are aliphatic chains and other atomic bonds typical of fatty acids such as C=C (~ 719 cm^{-1}), CH (~ 966 cm^{-1}, ~ 1230 cm^{-1}, ~ 1417 cm^{-1} ~ 1463 cm^{-1}) and triglycerides ester (~ 1161 cm^{-1}).

HPLC-UV

Finally, also HPLC-UV allowed to identify tyrosyl oleate injecting the purified tyrosyl oleate with the same method used for the determination of yield. *Figure 55* shows a typical chromatogram obtained from HPLC-UV where are clearly visible both tyrosol and tyrosyl oleate.

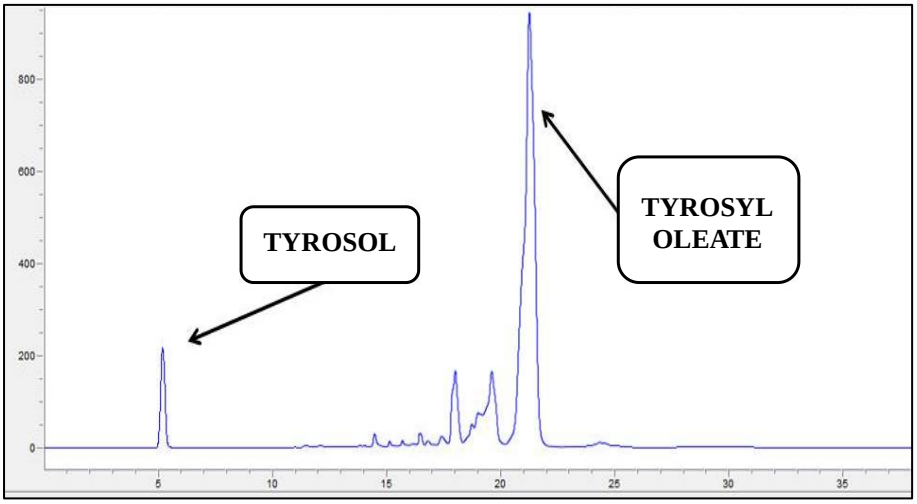


Figure 55. *HPLC-UV chromatogram of the lipophilization reaction: on the left the peak of tyrosol (area of which falls progressively); on the right the peak of tyrosyl oleate which increases with the reaction time. The ratio between the two areas was calculated as the percentage yield to define the best process conditions.*

III.4 Formulation of tarallini with synthesized lipophenol

According to literature, lipophilic phenolic derivatives have been used to facilitate the miscibility of aqueous and fat phases in emulsions. In *Aissa et al.*, (2017) several lipophilized tyrosol derivatives were tested in emulsions: the synthesized molecules were incorporated with emulsifying and antioxidant purpose and the results were so positive that the same authors subsequently replaced the butyl-hydroxytoluene (BHT), classical antioxidant employed in emulsions.

Also in this study, the product obtained from the lipophilization reaction was incorporated in a real system. Confirmations of what is obtained are found in the literature: *Aissa et al.* (2017)*; Lucas, et al.* (2010)*;* and *Souilem et al.* (2014) achieved positive results in the use of esterified derivatives of phenolic molecules with medium chain fatty acids.

Four types of tarallini with different concentration of tyrosyl oleate, based on sunflower oil weight, were made; the different concentrations were:

- **Control without lipophenol (CS);**
- **1% lipophenol (1L);**
- **4% lipophenol (4L);**
- **7% lipophenol (7L).**

Figure 56 shows tarallini sample before and after cooking; they were stored in a glass jar at room temperature with the same head space. In order to characterize the tarallini samples according to the different percentage of lipophenol added, these were analyzed at different shelf life times: immediately (T0), after 15 days (T15), after 30 days (T30), after 37 days (T37) and after 45 days (T45) from the formulation.

Figure 56. *On the left, raw tarallini before cooking; on the right, cooked tarallini*

III.5 Oil oxidative stability by OXITEST®

Considering a product such as tarallini a rapid determination of lipid oxidation as a marker of the quality of food during shelf life is fundamental. Formulation can play an important role in food quality: in fact, interaction of lipid oxidation products with Maillard's sugar, proteins or reaction products significantly affects the development of oxidation in lipid foods (*Frenkel*, 1984).

In order to assess rapidly if the use of tyrosyl oleate in formulation had some influence in the resistance to the oxidative stability, the four samples were subjected to accelerated oxidation analysis using the Oxitest® instrument. This instrument acts as a quali-quantitative analysis without sample preparation. The instrument consists of two thermostated reactors, which can be adjusted at different temperatures, where oxygen is pressurized under pressure. The collapse of oxygen pressure inside the reactors is expressed as an induction period (IP) which is theoretically defined as the time required to obtain a continuous oxidation cycle in the oxidation process; it is measured as the time required for a sudden and rapid change in the oxidation rate (*Frankel, 1998*).

Regarding the control sample (CS), formulated without lipophenol, the accelerated oxidation trend is shown in *Figure 57*. This sample completed the oxidation process after 6.10 hours from the beginning of the test.

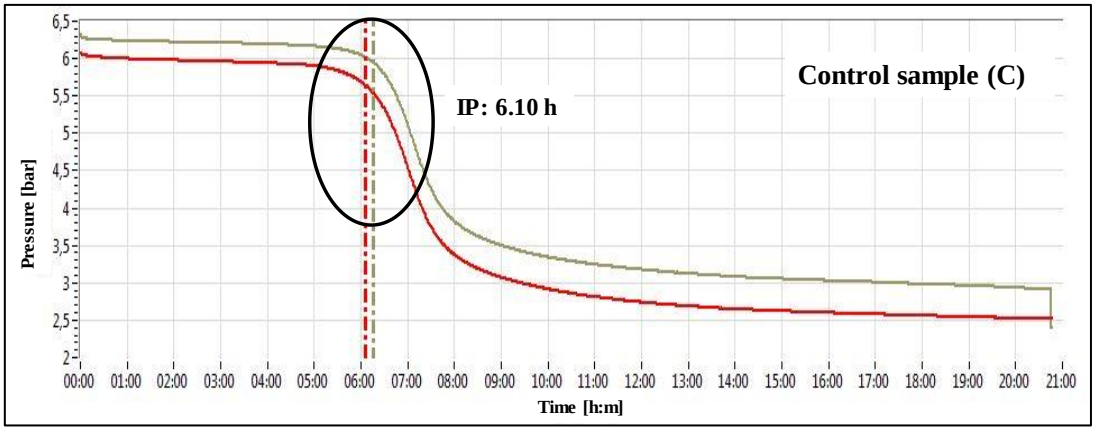


Figure 57. *IP values of Control sample (CS)*

The purpose of Oxitest® is to be able to evaluate how long a food containing fat can counter a continuous oxidative stress until the oxygen pressure collapse, when considering the food unable to accumulate and consume the oxygen supplied. Considering the formulation of the control sample, consisting only of sunflower oil as a lipid source, is clear that there is no added element that can counter the oxidation induced. It is, therefore, normal to find an IP values too lower than that reported in literature on sunflower oil, *Comandini et al.*, (2009) reports a comparison between extra virgin olive oil and sunflower oil, the latter presents a IP value of 9.16 hours at the same conditions of pressure and temperature. The differences between an oil and a bakery product formulated with the same oil can be explained with matrix effect *(Frenkel, 1984; Verardo et al, 2010*). Similarly, the taralli with spicy chili peppers tested under the same conditions by *Caruso et al.* (2017) have showed an IP value of 42.33 hours: in the formulation of the samples, in fact, in addition to spicy chili, a natural source of antioxidant compounds, olive oil and palm oil were used, notoriously less susceptible to oxidative stress due to the greater presence of fatty acids saturated rather than sunflower oil.

In tarallini formulation, sunflower oil was chosen because we did not know the antioxidant power of tyrosyl oleate in formulation and the use of an easily oxidized oil would eventually return the values significantly different IP between samples 1L, 4L and 7L compared to CS.

Already in the tarallini sample formulated with 1% of lipophenol the objective has been fully centered. In *Figure 58* the action of tyrosyl oleate is clearly visible, in fact the IP value was

significantly higher than Control sample. The final value was almost 14 hours (13.58 h), 126% more than the Control sample formulated without lipophenol.

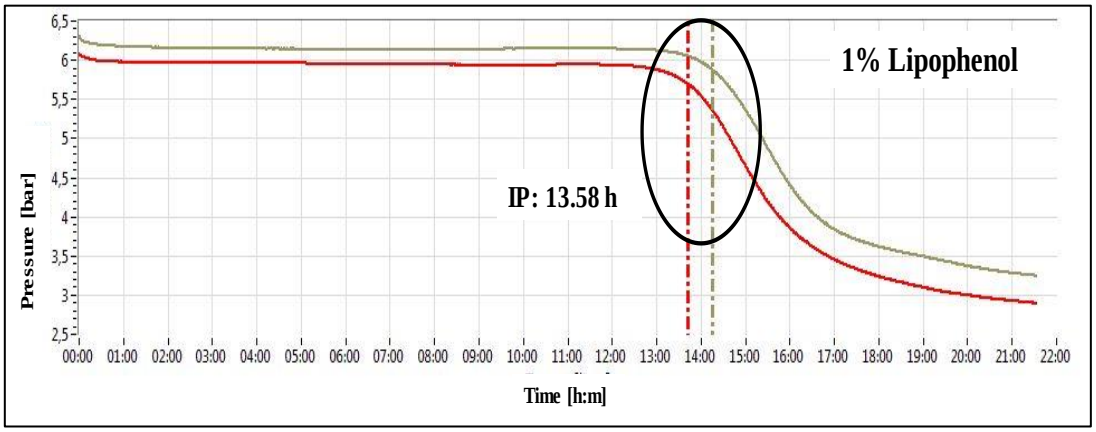


Figure 58. *IP values of sample with 1% of lipophenol*

In the samples formulated with 4% and 7% of lipophenol the IP value still increase. Between the sample with 1% and the sample with 4% there was an increase of 61.53% of IP value, with the final value of the sample with 4% of lipophenol equal to 22.34 hours (*Figure 59*). Comparing Control sample and sample with 4%, the IP value increased from 6.10 hours to 22.34 hours, with a net increase of 266%. The sample with 4% of tyrosyl oleate took more than three and a half times, in terms of time, to oxidize completely compared to Control sample with only sunflower oil.

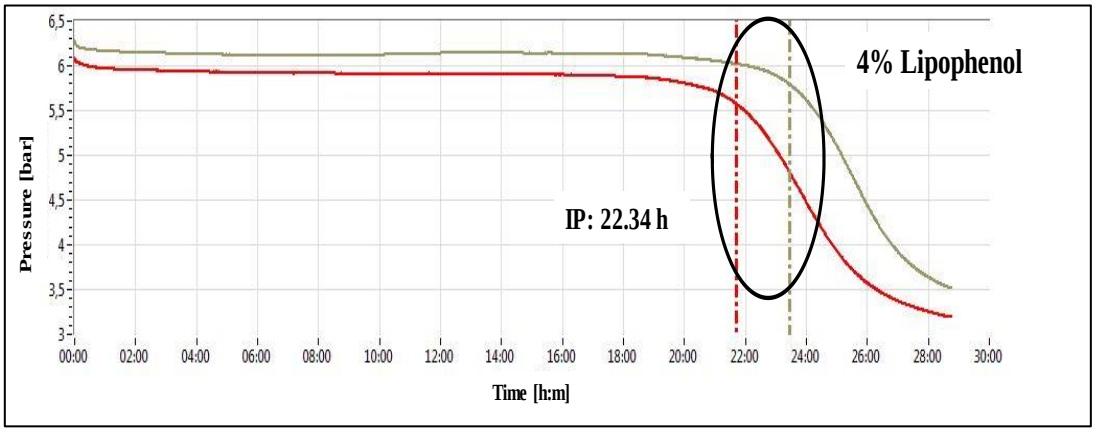


Figure 59. *IP values of sample with 4% of lipophenol*

Finally, the 7% lipophenol sample had a final IP value of 25.28 hours (*Figure 60*) but it was not significantly different from 4L sample. Compared with CS, the sample with 7% of tyrosyl oleate had an IP value higher of 313%, which means that, in terms of time, it took more than 4 times compared to the control sample.

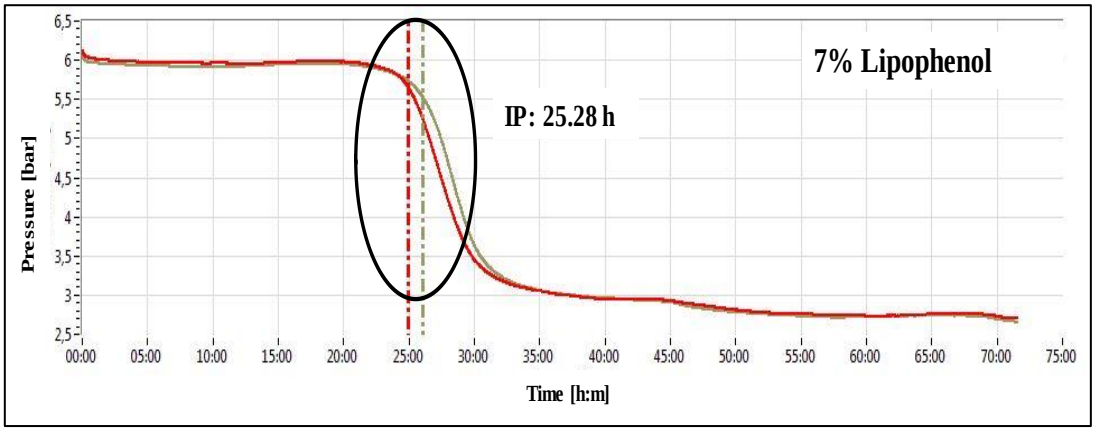


Figure 60. *IP values of sample with 7% of lipophenol*

In summary, the best performance was provided by tarallini made with 4% and 7% of lipophenol even if they did not show significant differences ($p < 0.05$), but already the sample with 1% of lipophenol had a significantly higher IP value than Control sample (*Figure 61*). The test with Oxitest® was satisfactory and it represent an excellent analytical screening of the oxidative stability.

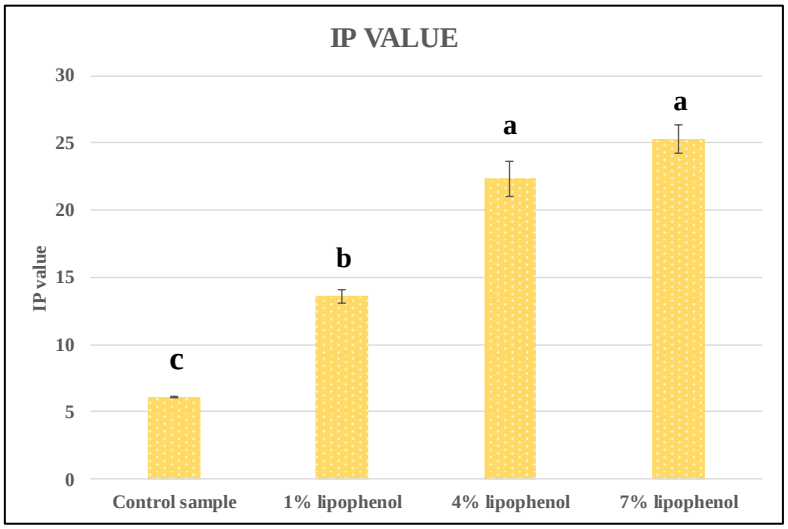


Figure 61*. Graphic rapresentation of IP values recordered for the different samples. Different letters represent significantly different values (p < 0.05).*

III.6 Evaluation of the oxidative state: peroxide value (PV)

The analytical evaluation of oxidative kinetics has as its aim the tracking of different products: from a situation where triglycerides and free fatty acids coexist, peroxides and hydroperoxides are formed, as primary oxidation products (products from direct reaction of oxygen and fatty acids) *(Giarnetti et al., 2012*). The number of peroxide (PV) is a chemical parameter that can give information about the state of preservation of the lipid matrix: peroxides, in fact, act as markers of a fat oxidative status, as they are the first oxidation products. In particular, peroxide number is an index that evaluates the amount of oxygen absorbed by lipid fraction and it is expressed in milli-equivalents of active oxygen per kg of fat and the legal limit is 20 meqO_2/kg of fat (EU Regulation 1348/2013).

Table 15 shows the peroxide value of the different samples at different storage time. It is evident that the Control sample and the sample with 1% of lipophenol exceed the legal limit for peroxide value after 30 days of storage; on the other hand, the samples with 4% and 7% of lipophenol exceed the limit law after 45 days of storage.

Table 15. Peroxide value (PV) of the samples expressed in meqO_2/kg of fat

PEROXIDE VALUE (meqO_2/kg of fat)					
	T0	**T15**	**T30**	**T37**	**T45**
Control sample	15.1 ± 1.2^{aB}	18.2 ± 2.4^{aB}	79.6 ± 15.8^{aA}	-	-
1% lipophenol	15.0 ± 0.2^{aB}	15.7 ± 0.6^{aB}	49.0 ± 6.4^{bA}	-	-
4% lipophenol	15.3 ± 0.7^{aB}	17.0 ± 0.4^{aB}	17.3 ± 2.1^{cB}	18.4 ± 0.3^{aB}	70.0 ± 2.0^{aA}
7% lipophenol	15.1 ± 0.3^{aC}	15.6 ± 0.4^{aC}	16.1 ± 0.7^{cBC}	17.2 ± 0.6^{aB}	69.0 ± 0.3^{aA}

Data are expressed as mean ± SD, n = 4. Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters on the same column show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation); capital letters on the same row show significantly different mean values within each sample (Control sample, 1%, 4%, 7% lipophenol)

The peroxide number was also evaluated on the sunflower oil used in tarallini formulation: it had a significantly lower value than the samples analyzed, equal to 1.44 ± 0.06 meqO_2/kg of fat. This gap so wide between the starting oil and the ready-to-eat tarallini is because of in refined oils, like sunflower oil, free fatty acids are removed during the neutralization process and the hydroperoxide are degraded due to the catalytic activity of decolorising and high temperatures reached during the refining phase (*Giarnetti et al., 2012*). In addition, *Caponio and collaborators*

(2009) have shown that the kneading phase is the cause of a significant increase of primary and secondary oxidation compounds (due to forced oxygen ingestion in the dough), as well as one consequential decrease of the content in polyunsaturated fatty acids (PUFA). The cooking also catalyzes the onset of lipid oxidation, very fast in the samples containing sunflower oil, despite the presence of antioxidant substances *(Verardo et al., 2010*).

Immediately after the tarallini formulation (T0) the peroxide value did not show any significant differences ($p < 0.05$) among the different samples, in fact CS, 1L, 4L and 7L reported a peroxide value of 15.14, 14.95, 15.33 and 15.08 $meqO_2$/kg of fat, respectively.

After 15 days of storage (T15), the peroxide value increased, without exceeding the legal limit, in all the samples than T0, but without any significant differences ($p < 0.05$); the highest increment, 20.30 %, was registered for CS, as we expected, with a peroxide value of 18.21 $meqO_2$/kg of fat. Also among the different samples there were not any significant differences ($p < 0.05$), the peroxide values for the samples added with lipophenol were 15.74, 17.04, 15.59 $meqO_2$/kg of fat for 1L, 4L and 7L.

After 30 day of storage (T30), Control sample and samples added with 1% of lipophenol exceed the legal limit for peroxide value; in fact, the samples registered values of 79.63 and 48.99 $meqO_2$/kg of fat, respectively; of course, with significant differences than T0 and T15. It means that for this two samples the shelf life, at environmental condition, last one month. For the other two samples, instead, the peroxide value increased again but without significant differences ($p < 0.05$) than the values registered at T15; the sample with 4% of lipophenol registered a value of 17.28 $meqO_2$/kg of fat, instead the sample with 7 % had a value of 16.11 $meqO_2$/kg of fat.

After that the shelf life of the last two samples, was studied every week instead of every 15 days. After 37 days of storage the peroxide value of the samples added with 4% and 7% of tyrosyl oleate, increased than T30 but, again, without significant differences ($p < 0.05$) and under the legal limit; 18.35 and 17.20 $meqO_2$/kg of fat, respectively.

Finally, after 45 days of storage, both samples with 4 % and 7 % of lipophenol, exceed the legal limit for peroxide value. Tarallini with 4% of lipophenol registered a peroxide value of 70.04 $meqO_2$/kg of fat, more than 280 % than T37 and of course, with significant difference ($p < 0.05$). The trend for samples with 7 % of lipophenols was similar, infact the peroxide value increased until 69.01 $meqO_2$/kg of fat, more than 300 % than the value registered at T37.

Compared all the shelf life time (*Figure 62*) is evident that the peroxide value increase during storage, but the addition of tyrosyl oleate allows the sample to counter, in a significant way ($p < 0.05$), the advancement of primary oxidation products. Generally, storage time causes a significant increase of the level of primary oxidation products (*Caponio et al., 2009*). They,

studying the influence of processing and storage time on the lipid fraction of taralli, shown that in taralli made with palm oil, cooked at 230 °C for 20 min and stored in modified atmosphere the PV increase during time but exceed the legal limit only after 6 months. *Verardo et al.* (2010), instead, studied the difference of lipid oxidation in tarallini made with extravirgin olive oil and sunflower oil. Tarallini made with sunflower oil (SO) had a higher PV at 0, 3 and 6 months than tarallini made with extravirgin olive oil (EVO); at 0 month the sample had a PV of 6.9-8.8 (EVO) and 17.5-26.2 (SO), at 3 months 23-35.5 (EVO) and 45.7-47.8 (SO) and at 6 months 24.3-50.5 (EVO) and 45.4-56.1 (SO). Shelf life of bakery products, therefore, are strongly influenced by the type of fat present in the formulation (*Verardo et al., 2010*).

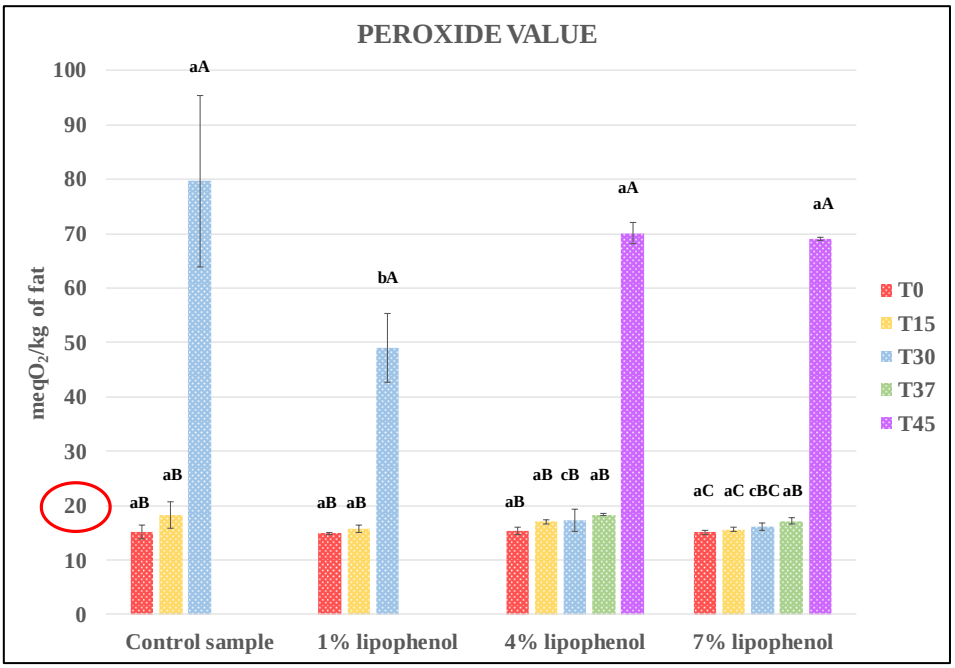


Figure 62. *Graphical representation of peroxide value (PV) of the samples expressed in meqO_2/kg of fat. Results of the analysis of variance by Tukey's test are shown: $p<0.05$, lowercase letters show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation); capital letters show significantly different mean values within each sample (Control sample, 1%, 4%, 7% lipophenol).*

III.7 Determination of conjugated dienes and trienes

In this study the extent of oxidative deterioration through conjugated dienes and trienes were evaluated. The determination of conjugated dienes and trienes has the purpose to identify, in lipid matrix, the shift of double bond of unsaturated fatty acid with the formation of a conjugated dienic system caused by an oxidative deterioration of the raw material.

Table 16 shows that immediately after tarallini preparation, at T0, there were not any significant differences ($p < 0.05$) among the samples, infact CS, 1L, 4L and 7L reported 24.36, 19.50, 29.50 and 19.08 conjugated dienes respectively. After 15 days of storage (T15) the samples

reported the highest values for dienes among all the shelf life times: 50.62, 49.95, 46.89 and 49.61 conjugated dienes for CS, 1L, 4L and 7L, respectively; without significant differences ($p < 0.05$) among the samples, but significantly different form tha values registered at T0. At T30 the values of conjugated dienes in all the sample decreased significantly ($p < 0.05$) than T15. Control sample, 1%, 4% and 7% of lipophenol samples registered 25.79, 12.10, 26.22 and 11.46 conjugated dienes, respectively; here there are some significant differences.

After 37 and 45 days only, the samples with 4 % and 7 % of lipophenol were studied because the other two samples had a PV value over the legal limit. In both cases the concentration of conjugated dienes kept on decreasing with significative differences in the sample with 4 % of lipophenol and without significative differences in the sample with 7% between T45 and T37. In the sample with 4 % of lipophenol at T37 the concentration decreased significantly ($p < 0.05$) reaching a value of 15.87 (about 65 % less) from T30 and, again, at T45 decreased from T37 until 9.39 (69 % less) but without significative differences. Also in the sample with 7% of lipophenol the concentration of dienes decreased from T30 to T37 reaching a value of 10.95 (4 % less) and, again, decreased at T45 until a value of 9.29 (17 % less).

Table 16. Conjugated dienes recorder in the diferent samples

CONJUGATED DIENES (εl cm (λ 232 nm))					
	T0	**T15**	**T30**	**T37**	**T45**
Control sample	24.4 ± 0.5 ^{aB}	50.6 ± 2.0 ^{aA}	25.8 ± 11.8 ^{aB}	-	-
1% lipophenol	19.5 ± 3.6 ^{aB}	50.0 ± 2.2 ^{aA}	12.1 ± 0.5 ^{bC}	-	-
4% lipophenol	29.5 ± 7.7 ^{aB}	46.9 ± 3.7 ^{aA}	26.2 ± 5.1 ^{aB}	15.9 ± 1.0 ^{aC}	9.4 ± 0.5 ^{aC}
7% lipophenol	19.1 ± 8.7 ^{aB}	49.6 ± 1.0 ^{aA}	11.5 ± 1.6 ^{bBC}	11.0 ± 0.7 ^{aBC}	9.3 ± 0.7 ^{aC}

Data are expressed as mean ± SD, n= 4. Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters on the same column show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation); capital letters on the same row show significantly different mean values within each sample (Control sample, 1%, 4%, 7% lipophenol).

As can be seen in *Figure 63* it is evident that the concentration of dienes increases significantly from T0 to T15 and, for the rest of shelf life, the concentration decrease; this trend is the same for all the samples. The chemical nature of fat determines protection against oxidation, in *Verardo et al.* (2010) tarallini formulated with sunflower oil showed higher values of conjugated dienes than those homologues formulated with extra virgin olive oil. A study by *Silva and co-*

workers, (2010) shown that the content of conjugated dienes after product transformation is lower when extra virgin olive oil is used, because of the greater protection against oxidation rather than sunflower oil. Infact lipid oxidation should be faster in cooked product rather than fresh product because cooking process induces an acceleration of oxidative processes as a result of the high temperature reached, destruction of cellular structures and interaction of lipids and prooxidants (*Silva et al., 2010*). At the same time, storage promotes the growth of conjugated dienes in all bakery products containing sunflower oil (*Verardo et al., 2010*). Probably, the decrement of conjugated dienes registered after 30 days of storage is related to the degradation of dienes with the formation of other different compounds; in any case from this analysis we achieved that the addition of lipophenol did not have any effect on oxidation of lipid.

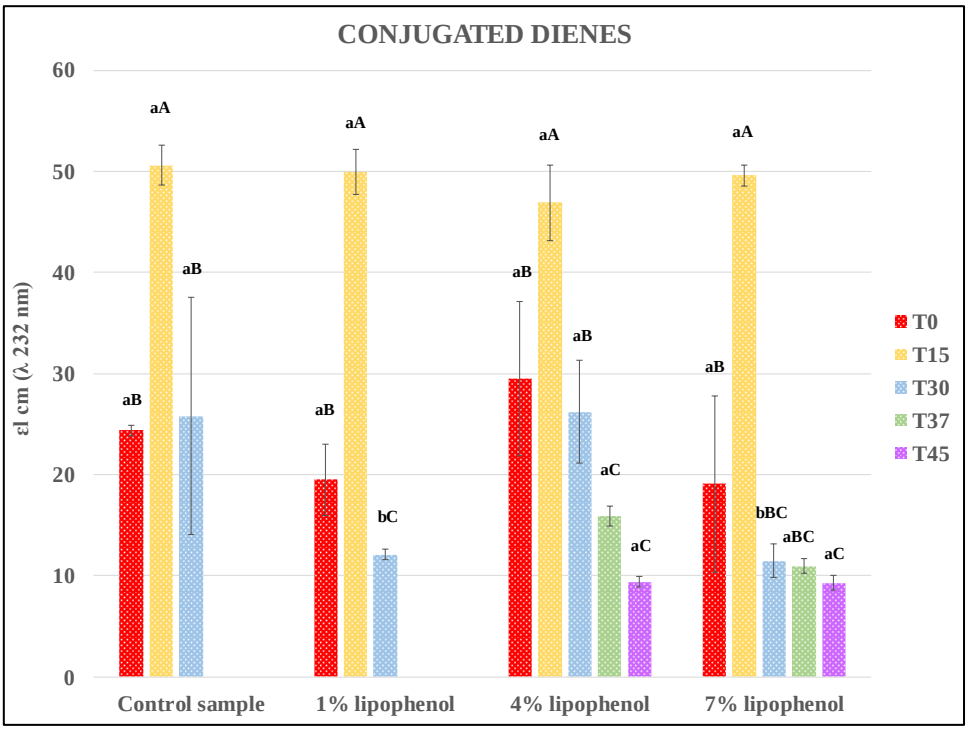


Figure 63. *Graphical representation of conjugates dienes of the samples. Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation); capital letters show significantly different mean values within each sample (Control sample, 1%, 4%, 7% lipophenol).*

Table 17 shows the conjugated trienes trend determined in the samples. Immediately after the formulation (T0) the samples showed different concentration of trienes, but without significative difference ($p < 0.05$); 11.59, 10.14, 14.88 and 10.10 for CS, 1L, 4L and 7L, respectively. Increasing time increased also the concentration of trienes, but, again, without any significative differences ($p < 0.05$). Infact for CS, starting from a concentration of 11.59 at T0, reached values of 12.69 and 12.60 for T15 and T30 respectively. In 1L sample the concentration at T0 was 10.14 and reached values of 12.62 and 12.21 for T15 and T30. In tarallini with 4 % of lipophenol in the formulation

the trienes had a value of 14.88 at T0 and reached values of 18.89, 16.99, 17.50 and 20.59 for T15, T30, T37 and T45, respectively. Finally, in 7L sample at T0 the concentration was 10.10 and 14.68, 13.88, 19.55 and 18.32 at T15, T30, T37 and T45, respectively (*Table 17*).

Table 17. Conjugated trienes recorded for the different samples

CONJUGATED TRIENES (εl cm (λ 268 nm))					
	T0	**T15**	**T30**	**T37**	**T45**
Control sample	11.6 ± 0.1 ^{aA}	12.7 ± 0.8 ^{bA}	12.6 ± 2.2 ^{abA}	-	-
1% lipophenol	10.1 ± 1.8 ^{aA}	12.6 ± 1.0 ^{bA}	12.2 ± 1.8 ^{bA}	-	-
4% lipophenol	14.9 ± 3.6 ^{aA}	18.9 ± 4.4 ^{aA}	17.0 ± 2.7 ^{aA}	17.5 ± 1.6 ^{aA}	20.6 ± 0.2 ^{aA}
7% lipophenol	10.1 ± 4.3 ^{aA}	14.7 ± 0.5 ^{aA}	13.9 ± 1.9 ^{abA}	19.6 ± 0.9 ^{aA}	18.3 ± 0.1 ^{bA}

Data are expressed as mean ± SD, n = 4. Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters on the same column show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation); capital letters on the same row show significantly different mean values within each sample (Control sample, 1%, 4%, 7% lipophenol).

Figure 64 shows that 4L and 7L samples had higher values of trienes than CS and 1L. This result can be interpreted in two ways: first, in agreement with *Verardo et al.* (2010), the value of conjugated trienes is high in bakery products formulated with sunflower oil due to the high PUFA content that chemically characterizes this lipid matrix. In addition, tyrosyl oleate has one unsaturation in the alkyl chain derived from the oleic acid, so it can increase the production of primary oxidation compounds in samples formulated with 4% and 7% oif lipophenol in which it was quantitatively more present.

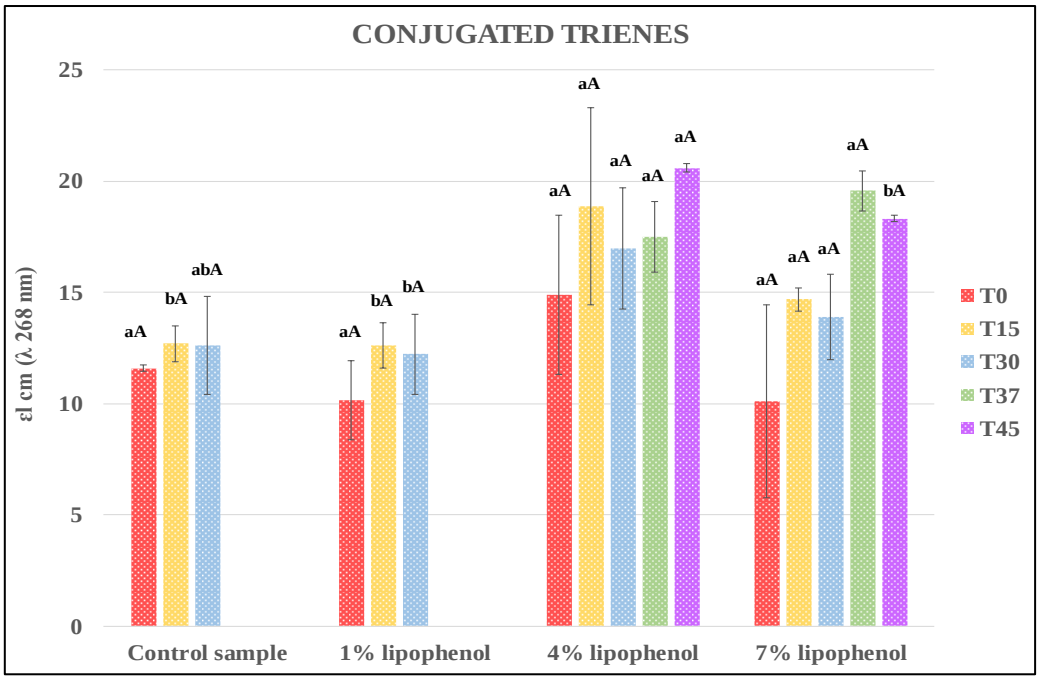


Figure 64. *Graphical representation of conjugates trienes of the samples. Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation); capital letters show significantly different mean values within each sample (Control sample, 1%, 4%, 7% lipophenol).*

III.8 Secondary oxidation products: oxidized fatty acids (OFA)

Oxidized fatty acids (OFA), secondary oxidation products, were analyzed in all tarallini samples to investigate if the addition of lipophenol in the formulation can reduce their formation. There are three types of OFA, hydroxy-FAMEs, that correspond to hydroxy derivates of methyl oleate and linoleate (*Marmesat et al., 2008*), ketoFAMEs corresponding to linoleic and oleic keto-derivatives and epoxy-FAMEs (*Verardo et al., 2010*).

Table 18 shows that the OFA concentration in the samples increased with the increasing of storage time, but the concentration in tarallini made with lipophenol addition was significantly lower than the one registered in the control sample made without lipophenol, starting from T0.

Table 18. OFA concentration in the different tarallini samples

OXIDIZED FATTY ACIDS (OFA) – mg FA/100 mg FAME					
	T0	**T15**	**T30**	**T37**	**T45**
Control sample	0.20 ± 0.01 ^{aC}	0.38 ± 0.00 ^{aB}	0.60 ± 0.00 ^{aA}	-	-
1% lipophenol	0.14 ± 0.01 ^{bC}	0.23 ± 0.00 ^{bB}	0.41 ± 0.01 ^{bA}	-	-
4% lipophenol	0.13 ± 0.01 ^{bD}	0.21 ± 0.03 ^{bC}	0.37 ± 0.01 ^{bB}	0.46 ± 0.01 ^{aA}	0.46 ± 0.01 ^{aA}
7% lipophenol	0.12 ± 0.01 ^{bE}	0.23 ± 0.03 ^{bD}	0.31 ± 0.01 ^{cC}	0.41 ± 0.01 ^{bB}	0.45 ± 0.01 ^{aA}

Data (mean ± SD, n= 4) are expressed in mg FA/l'' mg of FAME. Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters on the same column show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation); capital letters on the same row show significantly different mean values within each sample (Control sample, 1%, 4%, 7% lipophenol).

At T0 the OFA concentration in CS was 0.20 mgFA/100mg FAME, significantly higher than the other samples at the same time, 0.14, 0.12 and 0.13 mgFA/100mg FAME in 1L, 4L and 7L, respectively. At T15 the concentration increased but with the same trend, CS had a OFA concentration of 0.38 mgFA/100mg FAME, significantly higher than the other samples, 0.23, 0.21 and 0.23 mgFA/100mg FAME in 1L, 4L and 7L, respectively. At T30 was reached the highest concentration of OFA in CS, 0.60 mgFA/100mg FAME, significantly higher, again, than the other samples. Finally, OFA concentration registered for samples 4L and 7L at T37 and T45 was lower than the one registered for control sample at T30.

The significant differences among the different samples is evident also in *Figure 65*.

OFA concentration is strongly related to the type of oil used, in fact *Verardo et al.* (2010) studied the lipid oxidation in tarallini made with extravirgin olive oil and sunflower oil and they show that OFA concentration was higher in tarallini made with sunflower oil. This was explained by the different PUFA/total fatty acid ratio that is 0.6 for sunflower oil and 0.1 in extravirgil olive oil. In this study sunflower oil, very unsaturated oil, was used to test the effectiveness of tyrosyl oleate and it is evident that tyrosyl oleate can counteract lipid oxidation.

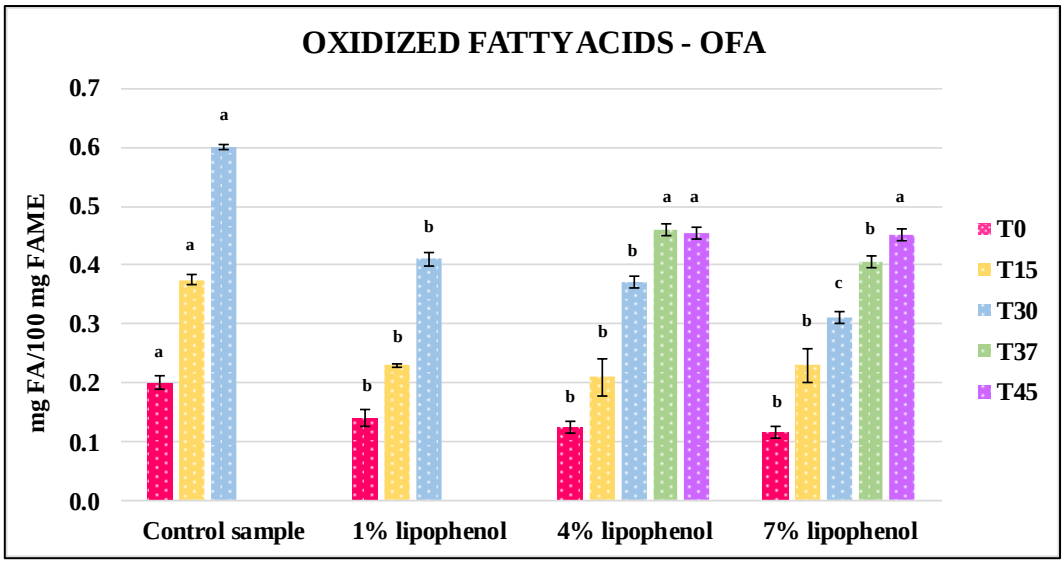


Figure 65. *Oxidized fatty acids (OFA) expressed as mgFA/100mg FAME. in the different samples (Control samples, tarallini made with 1, 4 and 7% of lipophenol) at different storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation). Different letters show significantly different mean values within each storage time (T0, T15, T30, T37, T45).*

III.9 Secondary oxidative state assessment: volatile composition by SPME-GC-MS

The analysis of volatile compounds by SPME-GC-MS has allowed to identify a different number of molecules depending on the conservation time and sample typology. Maillard reaction, caramelization and lipid oxidation mainly contribute to volatile profile in bakery product (*Giarnetti et al., 2012*). The decomposition free radicals and peroxides, formed as a consequence of lipid oxidation, can result in the formation of different classes of volatile compounds, alcohols, aldehydes, ketones, among which many have an unpleasant flavor; which decrease product shelf life (*Wąsowicz et al., 2004*).

Immediately after tarallini formulation and cooking (T0) 40 compounds were found in the control sample, 43 compounds in the sample with 1% of lipophenol; while 48 compounds were found in the sample added with 4 % of tyrosyl oleate and 44 compounds in the sample with 7 % of lipophenol.

Increasing the storage time of 15 days (T15) increase also the number of compound determined: infact the founded compounds were 55, 56, 56 and 50 for Control sample, 1 %, 4 % and 7 % samples, respectively. On the contrary at T30 the number of compound decrease: 30, 36, 41 and 43 for Control sample, 1 %, 4 % and 7 % samples, respectively. After 30 days of storage the number of compounds identified decrease in all the samples, in fact they were 28, 38, 43, 45 for Control sample, sample wit 1%, 4% and 7% of lipophenol, respectively. For T37 and T45 the volatile profile was evaluated only for the samples with 4% and 7% of lipophenol. In the first case

were determined 41 and 45 compounds for the sample with 4% and 7% respectively; in the second case, instead, the compounds identified were 48 for both sample.

The Maillard reaction and the lipid oxidation are the mainly responsible processes of the developing of the final flavor in bakery products *(Giarnetti et al., 2012*).

In *Table 19* are reported the volatile compounds derived from Maillar reaction, according to *Poinot et al*., 2008; *Giarnetti et al*., 2012 and *Caponio et al*., 2013.

Table 19. Mean area values of volatile compounds originated from Maillard reaction in different samples of taralliNI

COMPOUNDS ORIGINATED FROM MAILLARD REACTION															
	Control sample			**1% lipophenol (1L)**			**4% lipophenol (4L)**					**7% lipophenol (7L)**				
Compund	**T0**	**T15**	**T30**	**T0**	**T15**	**T30**	**T0**	**T15**	**T30**	**T37**	**T45**	**T0**	**T15**	**T30**	**T37**	**T45**
2,3-butanedione	1.00 ± 0.00d	0.17 ± 0.00d	0.18 ± 0.01d	1.05 ± 0.00c	0.31 ±0.00c	0.43 ± 0.01c	1.29 ± 0.00b	0.89 ± 0.01b	1.05 ± 0.00b	12.29± 0.08b	14.08± 0.40b	1.38 ± 0.00a	1.58 ± 0.07a	1.55 ± 0.00a	18.92± 0.86a	17.34± 0.71a
2-methyl-butanal	0.57 ± 0.21c	0.49 ± 0.08b	n.d.	1.34 ± 0.11c	0.38 ± 0.17d	n.d.	2.19 ± 0.24b	1.04 ± 0.02a	n.d.	n.d.	n.d.	2.87 ± 0.06a	0.43 ± 0.00c	n.d.	n.d.	n.d.
3-methyl-butanal	1.82 ± 0.24a	1.31 ± 0.11a	n.d.	1.92 ± 0.46a	1.22 ± 0.25a	n.d.	1.62 ± 0.15a	1.11 ± 0.08b	n.d.	n.d.	n.d.	0.97 ± 0.18b	0.86 ± 0.00c	n.d.	n.d.	n.d.
Methylpyrazine	n.d.	0.53 ± 0.09b	n.d.	0.73 ± 0.00a	0.78 ± 0.03a	0.71 ± 0.02a	0.32 ± 0.00c	0.29 ± 0.02c	n.d.	n.d.	n.d.	0.43 ± 0.00b	0.47 ± 0.01b	0.41 ± 0.02b	.	0.35 ± 0.00a
1-hydroxy-2-propanone	1.34 ± 0.10a	0.79 ± 0.06c	n.d.	1.17 ± 0.26a	1.24 ± 0.06a	1.60 ± 0.12b	1.15 ± 0.26a	1.41 ± 0.14a	1.41 ± 0.02c	1.14 ± 0.03b	1.18 ± 0.02b	0.92 ± 0.43a	0.99 ± 0.07b	2.13 ± 0.07a	2.64 ± 0.07a	7.09 ± 0.42a
Furfural	n.d.	n.d.	n.d.	n.d.	0.43 ± 0.00b	n.d.	n.d.	0.37 ± 0.00c	n.d.	n.d.	n.d.	n.d.	0.51 ± 0.05a	n.d.	n.d.	n.d.
2-furanmethanol	2.53 ± 0.21b	2.35 ± 0.09b	2.28 ± 0.00c	3.28 ± 0.37a	3.06 ± 0.25a	3.85 ± 0.37a	1.76 ± 0.34c	2.38 ± 0.04b	2.34 ± 0.00b	1.73 ± 0.01b	1.36 ± 0.02b	1.13 ± 0.16d	1.50 ± 0.10c	2.16 ± 0.02d	2.45 ± 0.05a	4.36 ± 0.10a
Maltol	3.69 ± 0.51a	3.24 ± 0.14a	n.d.	n.d.	3.38 ± 0.54a	2.69 ± 0.14a	n.d.	2.08 ± 0.10b	2.19 ± 0.00c	5.48 ± 0.07b	7.45 ± 0.22b	n.d.	1.62 ± 0.03c	3.00 ± 0.00b	6.21 ± 0.51a	13.79 ± 0.55a

Data (mean value ± SD, n=2) are expressed with peak area (area x 10^5). Results of the analysis of variance by Tukey's test are shown: $p<0.05$, lowercase letters on the same row show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation).

Among volatile compounds derived from Maillard reaction and/or sugar degradation there are 2-methylpropanal, 2-methylbutanal and 3-methylbutanal; these compounds are Strecker's aldehydes coming from valine, isoleucine and leucine, respectively (*Giarnetti et al., 2012*). In our samples 2-methylpropanal was not identified; 2-methylbutanal and 3-methylbutanal, instead, were founded in all the samples but only in T0 and T15 storage time. For both compounds the concentration decreased with increasing time; for 2-methylbutanal the highest concentration was founded, at T0, in the sample with 7% of lipophenol that was significantly ($p < 0.05$) higher than all the other samples. Sample with 1% of lipophenol registered the highest concentration of 3-methylbutanal at T0, significantly higher than all the the other samples. Probably these compounds were destroyed during storage time with the formation of other compounds. These aldehydes are responsible for malty and chocolate taste (*Giarnetti et al., 2012*).

Moreover, 2,3-butanedione and 2,3-pentanedione are responsible for a butter taste, related to rancidity (*Giarnetti et al., 2012 and Caponio et al., 2013*). The first one is a Maillard reaction product according to *Wnorowski & Yaylayan* (2000) and the second one is a sugar degradation derived product (*Duckham et al., 2002 and Mohsen et al., 2009*). In our sample only the 2,3-butanedione was detected in all the storage times considered. The concentration of this compound increased with the increasing of storage time; for all the different storage times the sample 7L registered a significantly ($p < 0.05$) higher concentration rather than the other samples. During the last two storage time, T37 and T45, the concentration was registered only in 4L and 7L samples; in the other two samples the volatile composition was not analyzed because the oxidation has already exceeded the legal limit of 20meqO_2/kg of fat for peroxide value. The increment of 2,3-butanedione concentration is related to the sensory evaluation, in fact the rancid odor in the samples stored for 37 and 45 days was higher than the same sample stored for 0, 15 and 30 days.

Pyrazines are Maillard reaction products that have an important impact of food flavor. In our samples methylpyrazine was the only pyrazine detected and the trend was the same in all the samples; its concentration increased between T15 and T0 and then decreased until the end of shelf life, but the concentration is, in general, low. This is due to the oil used, in fact *Giarnetti et al.*, (2002) show that the concentration of pyrazine was higher in taralli made with olive oil than taralli made with refined palm oil. This can be related to the different unsaturation degree of oils that cause changes in viscosity and in the mass transfer between water and lipid phase (*Negroni et al., 2001*); this explain also because in our samples the pyrazine concentration is law using sunflower oil, a very unsaturated oil.

Other compounds from Maillard reaction were detected: 1-hydroxy-2-propanone, a keton, furfural, an aldehyde, 2-furanmethanol and maltol, two alcohols. The concentration trend of 1-

hydroxy-2-propanone was the same for all the samples with lipophenol, it increased with the increasing of storage time with the highest value in 7L sample after 45 days of storage. On the other hand, in the control sample this compound is present only at T0 and T15 but without significant ($p < 0.05$) differences in the concentration at T0 than the samples with lipophenol. The concentration of furfural is very low and detected only in T15 in the samples with lipophenol; the concentration shown significant differences between the samples, it was significantly higher in 7L sample and, in descending order, 1L and 4L. Finally, two alcohols were detected, 2-furanmethanol and maltol. 2-furanmethanol showed a constant decrement in CS, on the contrary its concentration in 1L and 7L samples increased. In 7L it increased until T30 and then started to decrease at T37 and T45. The concentration of maltol in the control sample and in the sample with 1% of lipophenol decreased with increasing of storage time; in the control sample was detected at T0 and at T15, instead in 1L sample it was not detected at T0, but only starting from T15. In the last two samples, maltol was not detected at T0 but only starting from T15, increased with the increasing of storage time reaching the highest concentration at T45 in the sample with 7% of lipophenol.

The compounds originated from lipid oxidation are the most important for the aim of this study. In *Table 20* are reported all the products originated from lipid oxidation according to *Poinot et al.* (2008); *Giarnetti et al.* (2012) and *Caponio et al.* (2013).

Table 20. Mean area values of volatile compounds (%) originated from lipid oxidation in different samples of taralli

COMPOUNDS ORIGINATED FROM LIPID OXIDATION (%)															
	Control sample			**1% lipophenol (1L)**			**4% lipophenol (4L)**					**7% lipophenol (7L)**				
Compund	T0	T15	T30	T0	T15	T30	T0	T15	T30	T37	T45	T0	T15	T30	T37	T45
2-butanone	n.d.	2.09 ± 0.09a	n.d.	1.24 ± 0.00a	0.41 ± 0.02c	n.d.	0.50 ± 0.00b	1.06 ± 0.09b	1.11 ± 0.00a	12.59 ± 0.00a	n.d.	n.d.	1.09 ± 0.0b2	0.43 ± 0.00b	n.d.	n.d.
Pentanal	9.43 ± 0.83a	40.30 ± 2.95a	164.60 ± 28.76a	1.88 ± 0.18c	4.58 ± 0.17c	8.46 ± 0.72b	2.03 ± 0.06b	4.38 ± 0.10c	8.92 ± 0.72b	6.06 ± 0.51a	8.06 ± 2.17a	1.63 ± 0.11d	5.06 ± 0.09b	7.53 ± 0.98c	5.85 ± 0.17b	7.41 ± 0.26b
Hexanal	39.71 ±1.42a	496.87 ±30.72a	2086.60± 393.58a	20.35 ±0.54c	40.57 ± 1.12d	74.42 ±6.73d	22.36 ± 1.09b	48.35 ± 2.92c	84.60 ±1.15c	95.27 ± 5.39b	137.50 ±22.64b	19.35 ± 2.12d	52.70 ±2.81b	114.45 ± 8.38b	141.26 ± 5.89a	167.53 ± 3.27a
2-heptanone	5.80 ± 0.85a	6.55 ± 0.22a	17.98 ± 2.72a	4.65 ± 0.34b	0.63 ± 0.03b	0.88 ± 0.00c	3.80 ± 0.38c	0.69 ± 0.07b	0.74 ± 0.33d	1.06 ± 0.13a	1.78 ± 0.00a	3.51 ± 0.09c	0.66 ± 0.08b	0.91 ± 0.03b	1.03 ± 0.61a	1.34 ± 0.18b
1-pentanol	27.67 ± 0.24a	63.89 ± 2.07a	215.71 ± 29.91a	7.39 ± 0.52c	9.21 ± 0.45c	13.14 ± 1.58c	11.75 ± 0.05b	9.86 ± 0.14c	13.41 ± 0.44c	11.21 ± 0.41b	15.80 ± 2.80b	7.68 ± 0.59c	14.17 ± 0.56b	17.16 ± 2.08b	15.21 ± 0.31a	19.01 ± 0.05a
1-hexanol	n.d.	1.12 ± 0.05a	4.51 ± 0.63a	n.d.	0.30 ±0.00d	n.d.	0.47 ± 0.03a	0.70 ± 0.03b	n.d.	0.67 ± 0.09b	1.90 ± 0.03a	0.45 ± 0.05a	0.52 ± 0.04c	1.20 ± 0.00b	0.89 ± 0.04a	1.74 ± 0.24a
Nonanal	3.03 ± 0.23a	2.40 ± 0.28b	6.98 ± 1.17a	1.61 ± 0.06c	1.47 ± 0.55c	1.98 ± 0.21d	1.67 ± 0.35c	2.89 ± 0.53ab	2.86 ± 0.12c	4.00 ± 0.49b	4.53 ± 0.93b	2.05 ± 0.06b	3.18 ± 0.33a	3.30 ± 0.14b	5.00 ± 0.38a	5.94 ± 0.77a
3-octen-2-one		0.72 ± 0.02a	2.91 ± 0.86a	n.d.	0.30 ± 0.04c	1.10 ± 0.26b	n.d.	0.34 ± 0.05c	0.66 ± 0.00d	1.21 ± 0.40a	1.41 ± 0.19a	0.71 ± 0.00a	0.47 ± 0.03b	0.83 ± 0.06c	1.32 ± 0.17a	1.55 ± 0.30a
2-octenal	2.12 ± 0.14a	13.63 ± 0.47a	47.94 ± 6.87a	0.92 ± 0.02d	1.34 ± 0.08d	2.60 ± 0.08c	1.34 ± 0.04b	1.83 ± 0.04c	2.07 ± 0.28d	2.86 ± 0.12b	3.98 ± 0.93a	1.05 ± 0.01c	2.10 ± 0.10b	3.18 ± 0.23b	4.58 ± 0.19a	4.99 ± 0.68a
1-octen-3-ol	4.22 ± 0.06a	5.77 ± 0.51a	17.05 ± 2.06a	1.18 ± 0.13c	1.61 ± 0.20d	3.30 ± 0.41b	1.82 ± 0.22b	2.26 ± 0.01c	2.47 ± 0.21c	3.38 ± 0.22b	4.36 ± 1.14b	1.45 ± 0.25bc	3.32 ± 0.04b	3.96 ± 0.36b	5.60 ± 0.25a	6.13 ± 0.14a
Benzaldehyde	0.97 ± 0.06a	0.95 ± 0.03a	n.d.	0.62 ± 0.00b	0.62 ± 0.08b	n.d.	0.52 ± 0.10c	0.65 ± 0.04b	0.68 ± 0.02b	4.28 ± 1.59a	2.19 ± 0.23b	0.54 ± 0.05c	0.73 ± 0.04b	1.12 ± 0.00a	3.15 ± 0.10b	9.45 ± 0.80a
2-nonenal	0.93 ± 0.08a	0.87 ± 0.09a	n.d.	0.67 ± 0.17a	0.60 ± 0.02b	0.85 ± 0.00b	0.84 ± 0.09a	0.91 ± 0.13a	0.66 ± 0.13c	1.02 ± 0.38b	1.60 ± 0.02a	0.95 ± 0.04a	0.83 ± 0.06a	0.92 ± 0.03a	1.88 ± 0.03a	1.34 ± 0.05b
1-octanol	0.82 ± 0.03a	0.84 ± 0.05a	4.09 ± 0.00a	n.d.	0.42 ± 0.03c	n.d.	0.78 ± 0.00b	0.54 ± 0.04b	n.d.	44.20 ± 10.16a	15.61 ± 1.15b	0.59 ± 0.06c	0.51 ± 0.02b	0.82 ± 0.05b	25.46 ± 3.24b	45.66 ± 6.68a
2,4-decadienal	2.90 ± 0.00a	2.42 ± 0.46b	n.d.	1.65 ± 0.08c	2.62 ± 0.07b	2.58 ± 0.58b	2.38 ± 0.29b	2.86 ± 0.17b	1.99 ± 0.29b	4.66 ±0.00b	5.08 ± 0.71b	1.95 ± 0.19b	4.00 ± 0.71a	3.95 ± 0.58a	7.44 ± 0.71a	7.80 ± 0.74a
Hexanoic acid	n.d.	22.99 ± 3.16a	136.48 ± 21.73a	n.d.	2.43 ± 0.03c	5.17 ± 0.26b	3.34 ±0.00a	3.53 ± 0.36b	4.52 ± 0.17c	5.78 ± 0.87b	8.15 ± 1.83b	2.16 ± 0.42b	3.69 ± 0.39b	4.83 ± 0.25c	9.95 ± 1.14a	10.78 ±0.80a

Data (mean value ± SD, n=2) are expressed with peak area (area x 10^5). Results of the analysis of variance by Tukey's test are shown: $p<0.05$, lowercase letters on the same row show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation).

Among the compounds originated from lipid oxidation process, the hexanal was the preponderant. Hexanal comes from the degradation of linoleic acid (*Angerosa et al., 2004*) and since the sunflower oil is rich in linoleic acid this result was expected. Considering this compound, it is evident that the addition of lipophenol in tarallini formulation could reduce and retard the lipid oxidation. In fact, at T0 the hexanal concentration was significantly higher in CS without lipophenol than the samples with lipophenol in the formulation Increasing the storage time, increase also the hexanal concentration; at T15 the concentration in CS was significantly higher than the concentration registered in 1L, 4L and 7L. The same trend was registered for T30, in fact CS had a significantly higher value, again, than the other samples. Finally, after 37 and 45 days of storage (T37 and T45) the concentration of hexanal decreased in 4L and 7L.

Other relevant compounds are pentanal, 1-pentanol, 2-octenal and hexanoic acid. The concentration of all these compounds in the control sample was significantly higher than the concentrations in the samples with lipophenol. Again, this shows that the lipid oxidation is lower and retarded in the sample with lipophenol. The concentration of pentanal in CS and 1L sample increased with the increasing of storage time, while in 4L and 7L samples it increased from T0 to T30 and then decreased after 37 and 45 days of storage, probably because pentanal run into degradation.

As regard 1-pentanol, the concentrations increased with the increasing of storage time; they were significantly ($p < 0.05$) higher, for T0, T15 and T30, in CS than the samples with lipophenol in the formulation.

Concentration of 2-octenal increased with the increasing of storage time in all the samples considered, again with significantly ($p < 0.05$) higher concentrations in CS.

Finally, hexanoic acid was not detected at T0 in CS and 1L; but the concentration increased with the increasing of shelf life showing significant higher value in the control sample at T30 than all the other samples.

Maillard reaction and lipid oxidation are strong related because these two processes have common intermediates and polymerization mechanism, so they can produce similar product. In fact, lipid oxidation products may polymerize and produce brown-colored oxypolimers and oxidative reactions can be termined by reactions with compounds other than those originating from oxidation of the lipid substrate, antioxidants and/or proteins (*Giarnetti et al., 2012 and Caponio et al., 2013*). Reaction of lipid oxidation products with amines, amino acids and proteins has long been related to the browning observed in many fatty foods during processing and storage (*Zamora et al., 2008 and Adams et al., 2011*).

In *Figure 66* is reported the total concentration of the products originated from Maillard reaction in the different samples. It is evident that in the control sample the concentration is significantly lower, for T15 and T30, than the other samples and it decreases with the increasing of shel life.

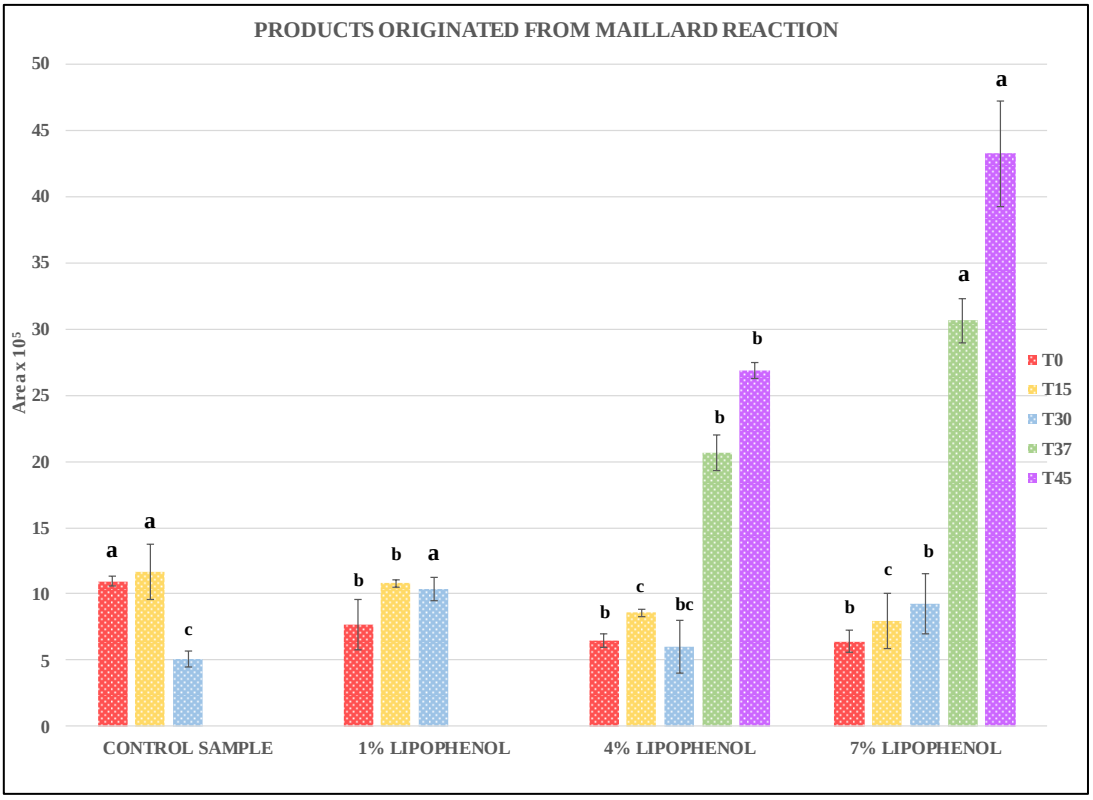


Figure 66. *Products originated from Maillard reaction in the different samples (Control samples, tarallini made with 1, 4 and 7% of lipophenol) at different storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation). Different letters show significantly different mean values within each storage time (T0, T15, T30, T37, T45).*

In *Figure 67* are reported the total concentrations of products originated from lipid oxidation. The control sample had a significant higher concentration of these products than the sample with lipophenol in all the storage time. The majority is represented by hexanal, that was the compound with the highest concentration.

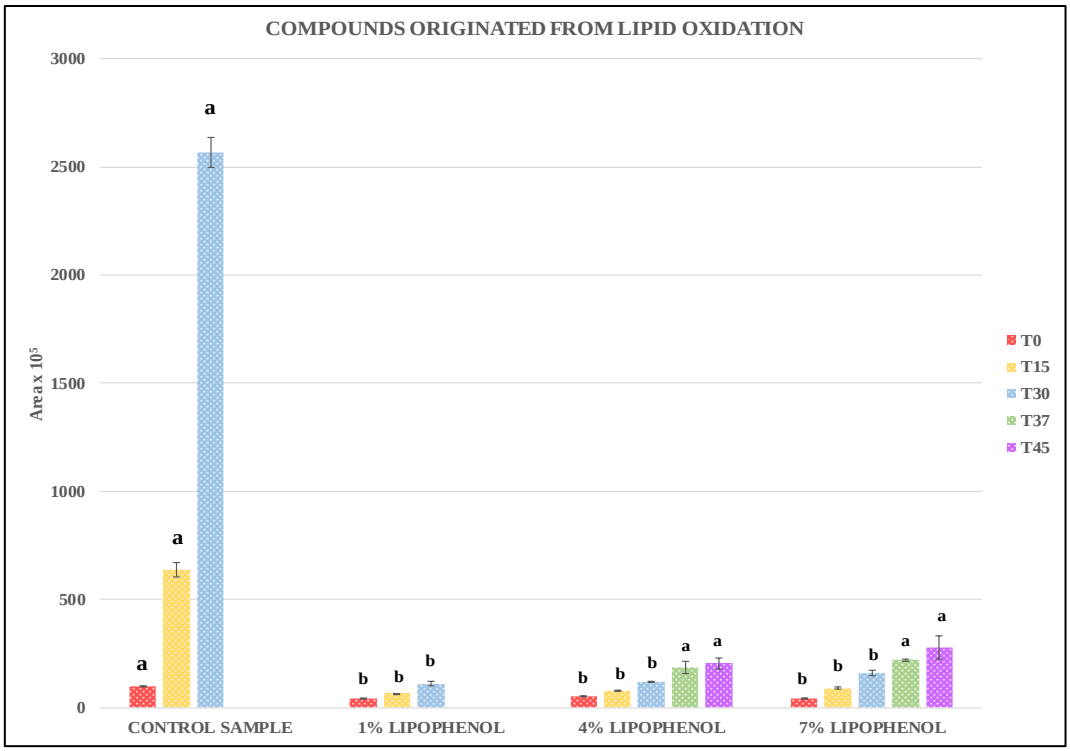


Figure 67. *Products originated from lipid oxidation in the different samples (Control samples, tarallini made with 1, 4 and 7% of lipophenol) at different storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation). Different letters show significantly different mean values within each storage time (T0, T15, T30, T37, T45).*

According to *Mastrocola & Munari* (2000) and *Brudzynski & Miotto* (2011), Maillard reaction products are able to decrease the lipid oxidation rate; but very few data are available on the interaction between the Maillard reaction and lipid oxidation when they take place simultaneously in a food system during storage after heating. This relationship can be explained considering the lipophenol structure because in the sample with lipophenol the compounds from Maillard reaction were greater than the control sample, without lipophenol. Tyrosyl oleate oxidises instead of the lipid fraction of tarallini giving rise to aldehydes from the alkyl chain; aldehydes then react with aminoacides to form Maillard products.

III.10 Determination of tyrosyl oleate in tarallini after cooking

To test the resistance of tyrosyl oleate to heat treatment the samples were analyzed by HPLC-UV with the same method used for the reaction set up.

Table 21 and *Figure 68* show the concentration of tyrosyl oleate in the samples added with it after the heat treatment tha was the cooking at 200 °C for 20 minutes. Using 1, 4 and 7 % of tyrosyl oleate based on the sunflower oil that was 40 g, the initial concentration of tyrosyl oleate was 1000, 4000 and 7000 mg/100g of fat, respectively. For all the samples the concetration of tyrosyl oleate decreased drastically after the cooking but it remains constant during the shelf life.

Table 21. Tyrosyl oleate content after heat treatment in the different samples

TYROSYL OLEATE AFTER COOKING – mg/100g of fat					
	T0	**T15**	**T30**	**T37**	**T45**
1% lipophenol	30.0 ± 2.2 [a]	30.7 ± 0.3 [a]	29.6 ± 0.6 [a]		
4% lipophenol	41.7 ± 6.6 [a]	42.9 ± 7.8 [a]	44.1 ± 1.9 [a]	41.0 ± 3.0 [a]	38.3 ± 6.6 [a]
7% lipophenol	58.3 ± 6.3 [a]	59.2 ± 0.9 [a]	53.2 ± 1.0 [a]	52.2 ± 5.7 [a]	52.8 ± 8.2 [a]

Data (mean ± SD, n = 4) are expressed in mg/100 g of fat. Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters on the same row show significantly different mean values within each storage time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation).

All the samples shown a drastical decrement from the initial concentration, but the concentration in the different storage time did not show significant differences ($p < 0.05$); even if the decrement is relevant after cooking the residual concentration is enough to counterat the lipid oxidation process.

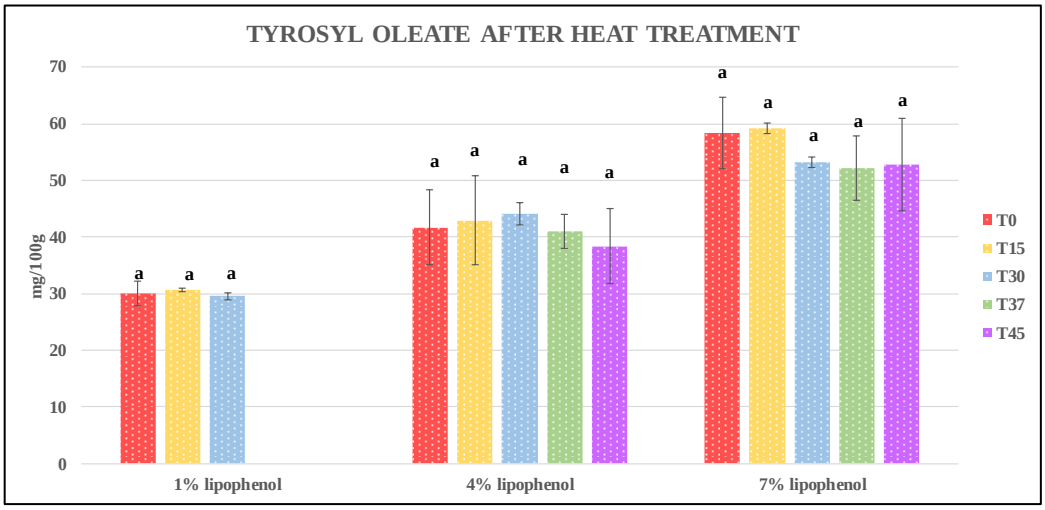


Figure 68. *Concentration expressed as mg/100g of tyrosyl oleate after the heat treatment in the different samples during shelf life time (T0, after 0 day; T15 after 15 days; T30 after 30 days; T37, after 37 days and T45, after 45 days from the formulation).*

III.11 Synthesis of lipophenol starting from by-products fat

According the optimized condition studied and identificated as reported in paragraph III.1, the reaction between tyrosol and fractionated storage fat from calf, young bull and cow (paragraph I.9) was carried out. As reported in that paragraph with the fractionating of storage fat it was not obtained only oleic acid but a mixture of unsaturated fatty acid where the oleic acid was the preponderant.

The reaction condition were: MTBE as solvent, 60 °C, molar ratio tyrosol:fractionated storage fat 1:2 and 10% of enzyme based on the tyrosol weight.

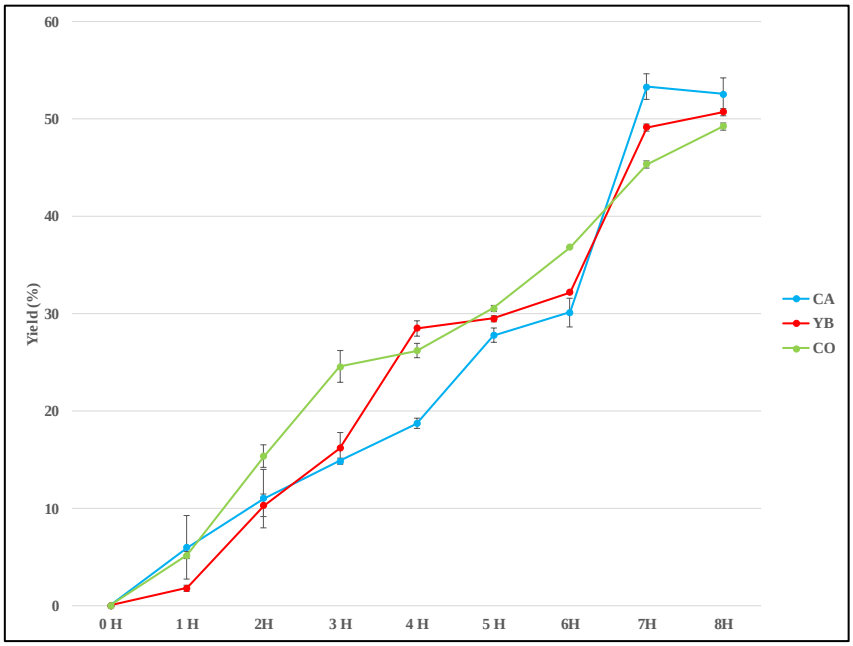


Figure 69. *Comparison of yields (%) of the lipophilization reaction using tyrosol:fractionated storage fat 1:2 as molar ratio, 60 °C, MTBE as a solvent and 10 % of enyme based on tyrosol weight. CA, calf; YB, young bull, CO, cow.*

As shown *Figure 69*, the fractionated storage fat of the three bovine categorie allowed to obtain a good yield considered that in this fat there was not oleic acid. The yields recovered, after 8 hours of reaction, were 52.59, 50.75 and 49.26% using fat from calf, young bull and cow.
The yields recovered were lower that the ones obtained using standard reagents, but they can be considered good results, for the fist time.

Next steps will be to test this compound obtained reusing bovine meat by-products to confirm the antioxidant potential demonstrated for tyrosyl oleate. Then, to continue this study will be tested other reactions using different both lipid and phenolic fraction. In nature exist a lot of phenolic compound that can exert the same potential in a lipid matrix if they become more amphiphilic.

4. Conclusions

Nowadays, industries are becoming more and more careful and inclined about the recovery and the enhancement of by-products originated from their production chain. This is a growing interest because by-products represent an excellent source of nutrients, including essential amino acids, mineral and vitamins, and, also, posses important technological and sensory properties. In addition, the reuse of by-products, instead of their disposal, would increase industry profits and have beneficial effects on environmental pollution that would be reduced.

The aim of this research was to find a reuse for by-products originated from bovine meat production; and, because of the interest in antioxidant food from consumers, the way to reuse these by-products goes in this direction.

A new way to synthesized antioxidant compound is the lipophilization, that consists in the esterification of the lipophilic fraction (fatty acids or fatty alcohols) of different substrates in order to obtain molecules where the hydrophilic/lipophilic ratio has been modified. The focus of the reaction is to modify or improve the functional properties of the original compound and facilitate its incorporation into emulsified or lipid-based media. In this way a phenolic compound, with antioxidant potential, can exert its funcionality in a lipid matrix.

Four different by-products from bovine meat industry were considered, storage fat, subcutaneous fat, bone marrow and bone fat; each obtain from three different bovine categories, calf, young bull and cow. A quali-quantitative characterization of lipid fraction, with innovative chromatographic tecniques that allow fast analyses, of these by-products were carried out in order to identify which one was suitable for the synthesis of new lipophenols.

After several analyses, peroxide value, fatty acids, tryglicerides, dyglicerides and phospholipids composition and cholesterol content; only the storage fat was considered suitable for the aim of the study. First of all, storage fat, shown the lowest peroxide value, so a low lipid oxidation. It represents a good starting point in view of their use in the synthesis of antioxidant molecules to test later in a real food system. The obtained results underline that subcutaneous fat is a non-homogeneous by-product with a highly variable composition. Nevertheless, as expected, subcutaneous fat showed the highest cholesterol content compared to the other by-products, because cholesterol is involved in the structure of the membranes that are present were the storage fat is low. On the other hand, the storage fat and especially the bone marrow presented a high fat amount and they were characterized by a low cholesterol content and important concentration of some essential fatty acids (linoleic acid, α and γ linolenic acid, eicosadienoic acid and arachinodic acid). In particular, calf showed the maximum content of oleic acid, linoleic acid and phospholipids, but also the highest content of cholesterol in the subcutaneous fat rather than young bull and cow.

Conversely, young bull presented the lowest cholesterol amount and cow the highest TG content, especially CN48 class. According to this evaluation, it could be assumed a future application of storage fat for lipophilization. In addition, appropriate research and development activity can help to convert the other animal by-products in bioactive components for nutritional properties and other non-food applications like pharmaceuticals, cosmetics or energy.

In collaboration with the Department of Engineering – Lipid Biotechnology and Engineering – at Aarhu University in Denmark the succinylation of tyrosol was studied. Tyrosol was choosen as molecule to use in the lipophilization for its antioxidant potential and because is a by-product originate from olive oil production and olive mill wastewaters are rich in tyrosol. The succinylation with four different succinic anhydrides was carried out to study how to make tyrosol more amphphilic so that it can exert its antioxidant potential in a lipid food; because free tyrosol can not do that.

The study reports the enzymatic succinylation of tyrosol into dual functional molecules with both surface active and antioxidant properties. The succinilation was carried ou using four different anhydrides, succinic anhydride, butylsuccinic anhydride, 2-octen-1-ylsuccinic anhydride and dodecenylsuccinic anhydride, which differ for the alkyl chains length that consist of 0, 4, 8 and 12 atoms of carbon.

The structures of the new compounds were identified by LC–MS and ^{1}H NMR analyses. A systematic physico-chemical characterization was carried out with focus on the thermal properties, antioxidant activity by DPPH and TBAR assays, surface activity, and molecular packing behavior by means of DSC, FT-IR, and measurement of CMC. The synthetic amphiphilic lipids were applied for the preparation of emulsions, which showed that compounds with the longest alkyl chain could form stable emulsions compared to free tyrosol. In addition, the antioxidant activity increased significantly compared to free tyrosol, even though only in the case of the compound obtain from the reaction between tyrosol and the longest succinic anhydride, dodecenylsuccinic anhydride. At the same time, a better inhibition of lipid oxidation in emulsions stabilized by compounds, again, with the longest alkyl chains rather than emulsions stabilized by free tyrosol was observed.

The outcomes from this part of the research not only add new members to the library of lipid materials, which could potentially be used in food, but also elucidate the structure–property–function relationship between hydrophobic alkanyl, and hydroxyl moieties assembled in one molecule, which is of a general instructive value for the design and engineering of new lipid materials.

In the last part of the study, numerous experimental trials have been carried out on the synthesis of a lipophenol, tyrosil oleate, by a lipophilization reaction between oleic acid recovered from bovine meat by-product and tyrosol. Then, the application of the lipophenol in a real food system, i.e. tarallins with different concentrations of lipophenol in the formulation, was studied to see if this new molecule can counteract the lipid oxidation.

The reaction between tyrosol and oleic acid was a lipophilization catalyzed by Lipozyme® lipase immobilized by *Mucor miehei*. Many reactions between reagents, solvents and reaction temperatures have been tested with the aim of achieving maximum final yield. The structure of the synthesized compound, tyrosyl oleate, was confirmed by ^{1}H NMR and FT-MIR. The study of the yields has led to the definition of tyrosol: oleic acid 1:2 as molar ratio, MTBE as solvent, 60 °C as temperature and 10% enzyme lipase based on tyrosol weight as the best process conditions. The final product was purified by column chromatography to remove unreacted tyrosol and oleic acid.

Then a typical italian bakery product, taralli, was made according to the traditional recipe using the tyrosyl oleate in the formulation; to test the antioxidant effectiveness of tyrosyl oleate sunflower oil was chosen as lipid fraction because of its high concentration of unsaturated fatty acid so subjected to lipid oxidation. Four tarallini samples were formulated: a control sample (CS) without tyrosil oleate, a sample with 1% of lipophenol (1L), a sample with 4% of lipophenol (4L) and a sample with 7% lipophenol (7L), the percentage of tyrosyl oleate was based on sunflower oil weight used. The tarallini, after cooking, stored at room temperature and the lipid oxidation process analysed at different storage time, T0, T15, T30, T37 and T45 after 0, 15, 30, 37 and 45 days, respectively. Several analyses to study the lipid oxidation were carried out: accelerated oxidation analysis by Oxitest® on the samples at T0, peroxide value (PV) to determine the primary oxidation products, determination of conjugated dienes and trienes and oxidized fatty acids (OFA) to determine the secondary oxidation products and; finally, the volatile composition using SPME-GC-MS.

Analysis by Oxitest® allowed to discriminate between the control sample and the three samples with tyrosyl oleate; in fact, already in 1L sample the IP value was more than twice (13.58 h) of the control sample (6.10 h); in addition, in the 4L and 7L samples the IP value reached time 22.34 h and 25.28 h, respectively. This first analysis shows that tyrosyl oleate can exert its antioxidant potential and counteract lipid oxidation.

The determination of peroxide value (PV), first oxidation products, allowed, again, to discriminate the different samples. At T0 and T15 all the samples did not show significant differences ($p<0.05$) having the peroxide value under the legal limit of 20 meqO_2/ kg of fat. At T30,

instead, CS and 1L exceed the legal limit (79.6 and 49.0 meqO_2/ kg of fat, respectively), while 4L and 7L registered a peroxide value of 17.3 and 16.1 meqO_2/ kg of fat, without any significant differences among these two samples. These two samples exceed the legal limit after 45 days of storage (T45); so, in the worst condition (sunflower oil and storage at room conditions) the addition of tyrosyl oleate can slow down the lipid oxidation process. According to this analysis, all the other determination on CS and 1L were carried out until T30, instead for samples 4L and 7L until T45.

Determinations of conjugated dienes and trienes were not decisive for the study because, in general, did not show any particular significative differences among the different samples.

Determination of oxidized fatty acids (OFA), instead, showed significant differences. CS registered significant higher ($p < 0.05$) OFA content rather than all the samples with lipophenol. Among tarallini made with tyrosyl oleate, 7L sample showed the significantly lowest values than 1L and 4L at T30 (0.41, 0.37 and 0.31 mgFA/100 mg FAME, respectively).

Evaluation of volatile profile with SPME-GC-MS showed interesting results. Considering the total volatile compound originated from Maillard reaction, the samples with lipophenol in the formulation showed significantly higher concentration than the control sample. In the control sample the concentration of these compounds decrease with the increasing of storage time, while in the other samples the trend is the opposite, the concentration increases with the increasing of shelf life.

Considering the volatile compounds originated from lipid oxidation their concentration increases with the increasing of shelf life. Tarallini made with lipophenol shown a significantly lower concentration of these compounds than control sample for all the storage time. Hexanal was the preponderant compound from lipid oxidation present in the samples, the major representative compound of lipid oxidation and it influences the total concentration of this class of compound. Also from this determination the effectiveness of tyrosyl oleate is clear, it can counteract the lipid oxidation.

Considering that the tarallini were made with one of the most oxidable oil, sunflower oil, and stored at room temperature without any modified atmosphere; the presence of lipophenol allow to extend the shelf life of tarallini added with it.

Finally, testing the same reaction starting from lipid fraction of bovine by-products good yields has been obtained, even if the fat fractionating process must be improved. In the future, different phenolic and lipid fraction will be tested in order to synthesize other lipophenol and test them in different lipid matrix to evaluate their antioxidant activity.

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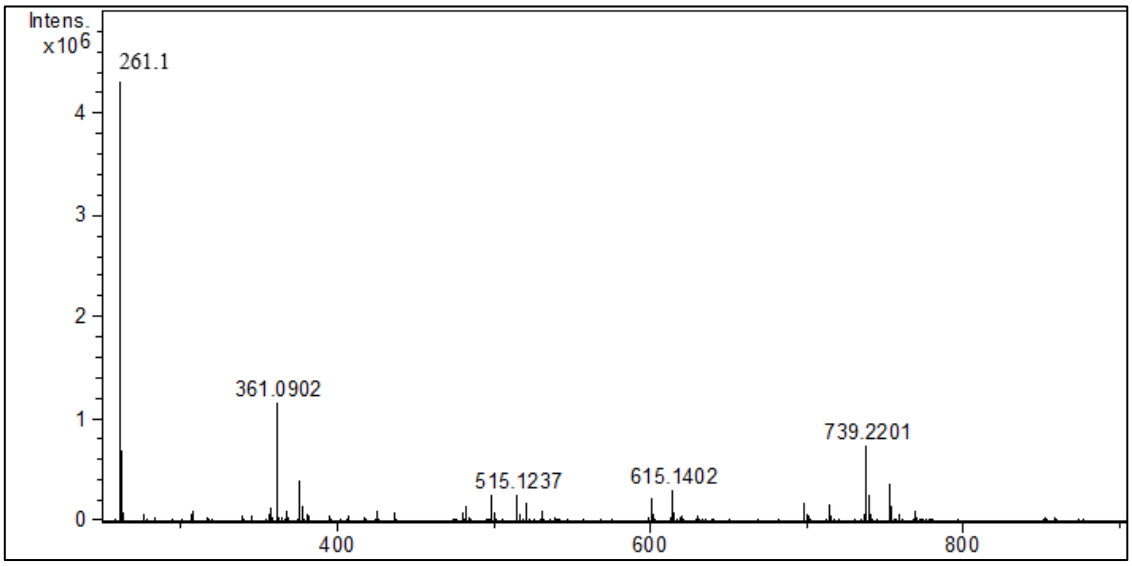
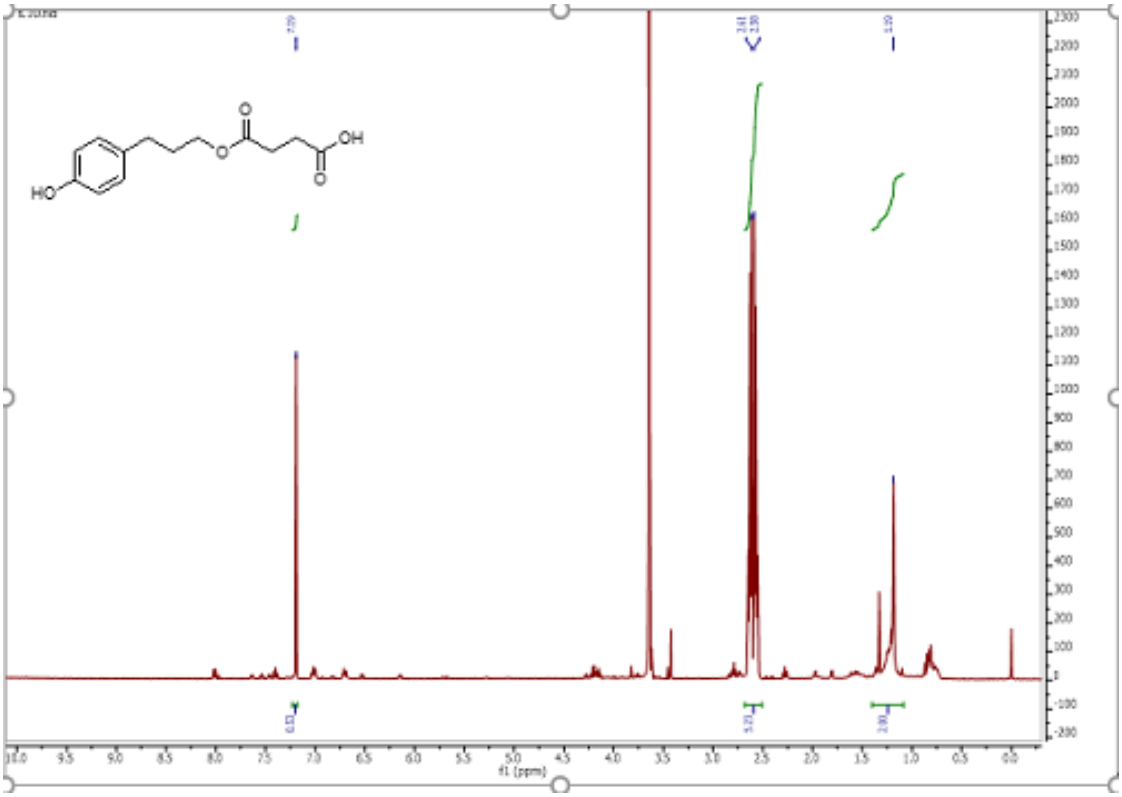
Appendices

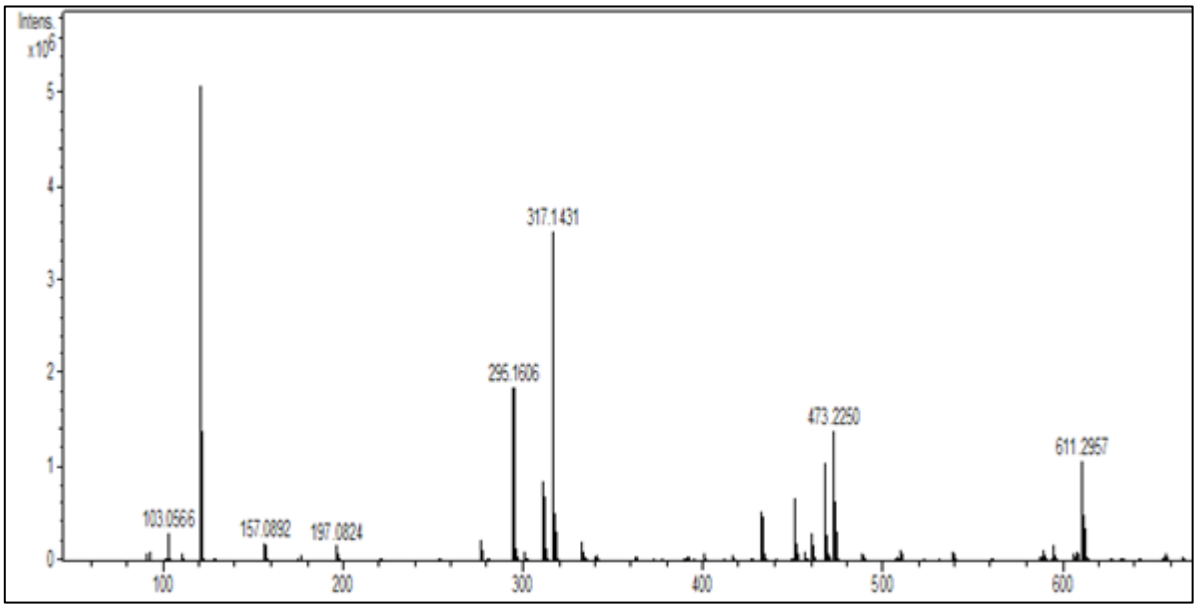
Appendix 1. HPLC-MS spectrum of compound 1a



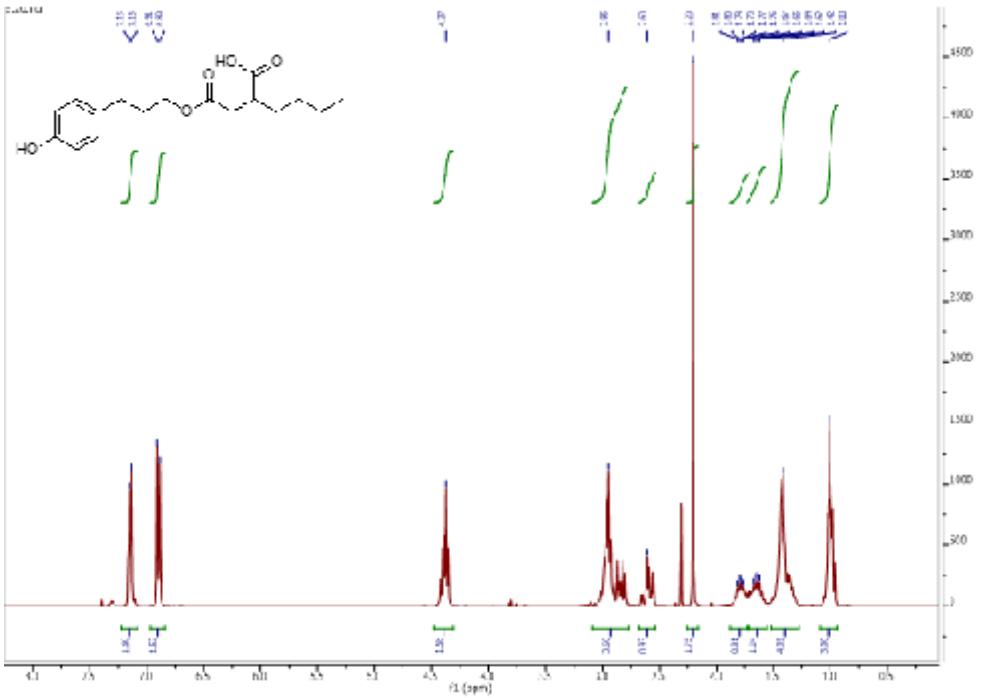
Appendix 2. ^{1}H NMR of compound 1a in $CDCl_3$ at 400 MHz



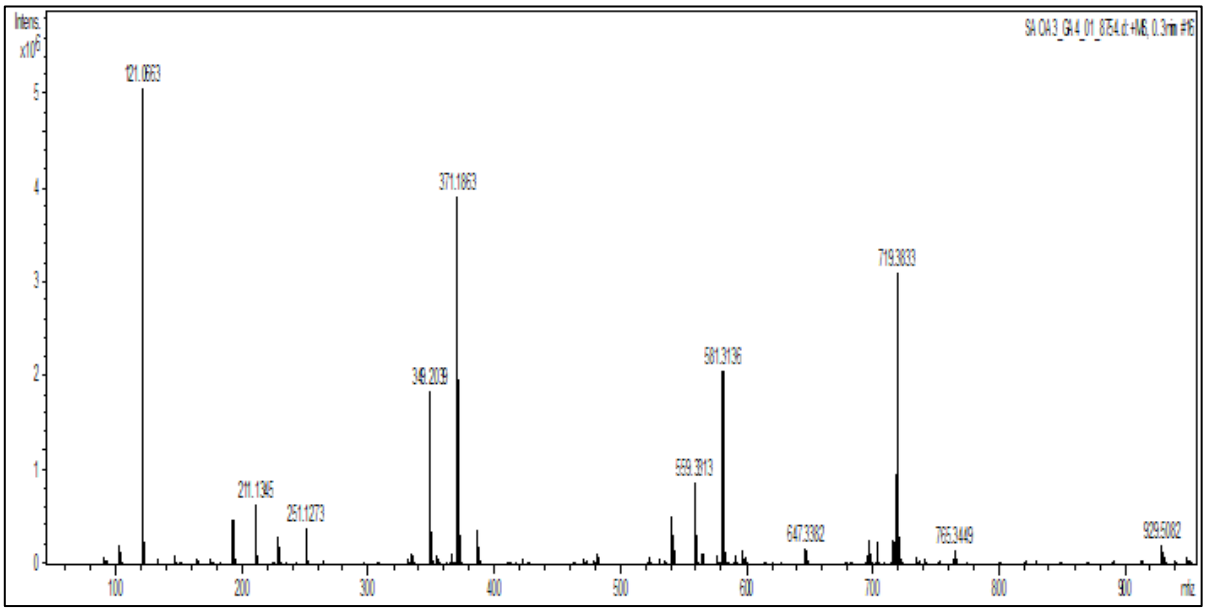
Appendix 3. HPLC-MS spectrum of compound 2a



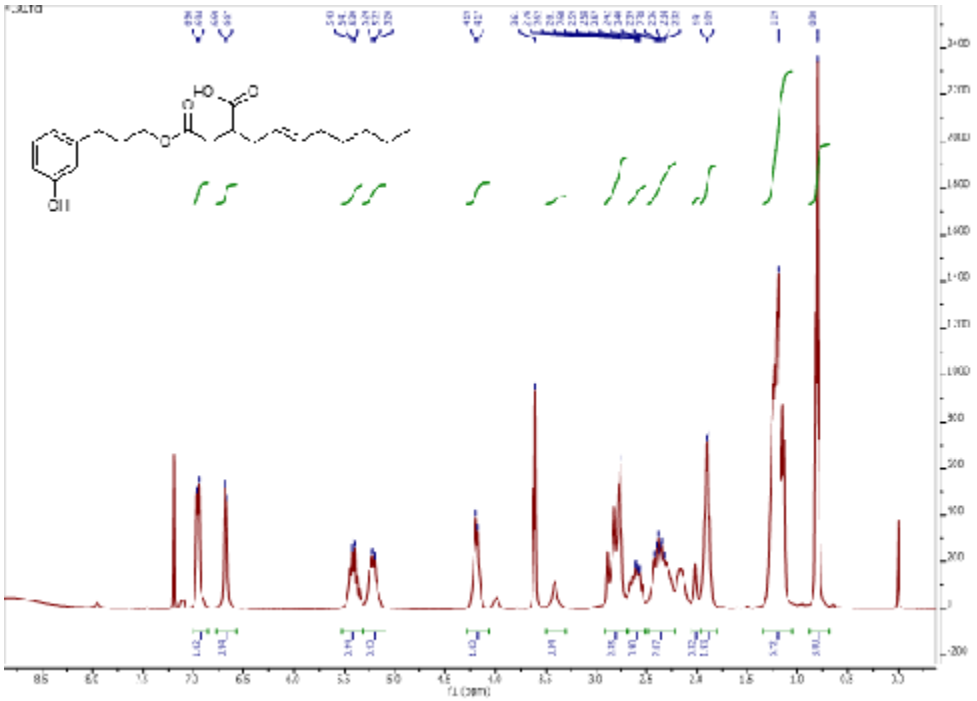
Appendix 4. ^{1}H NMR of compound 2a in $CDCl_3$ at 400 MHz



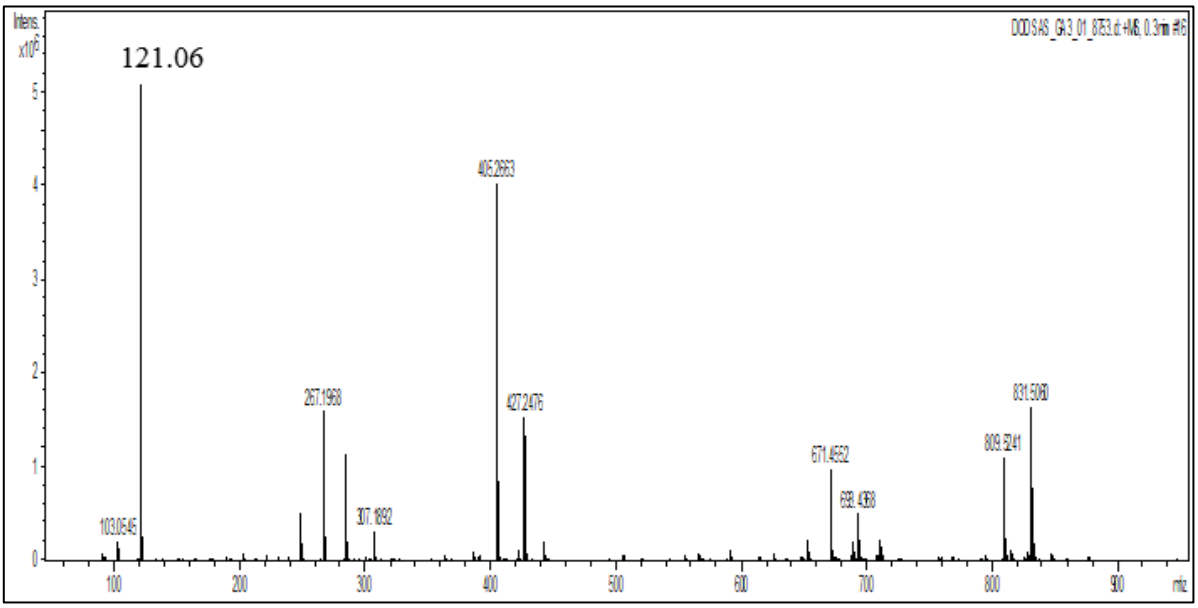
Appendix 5. HPLC-MS spectrum of compound 3a



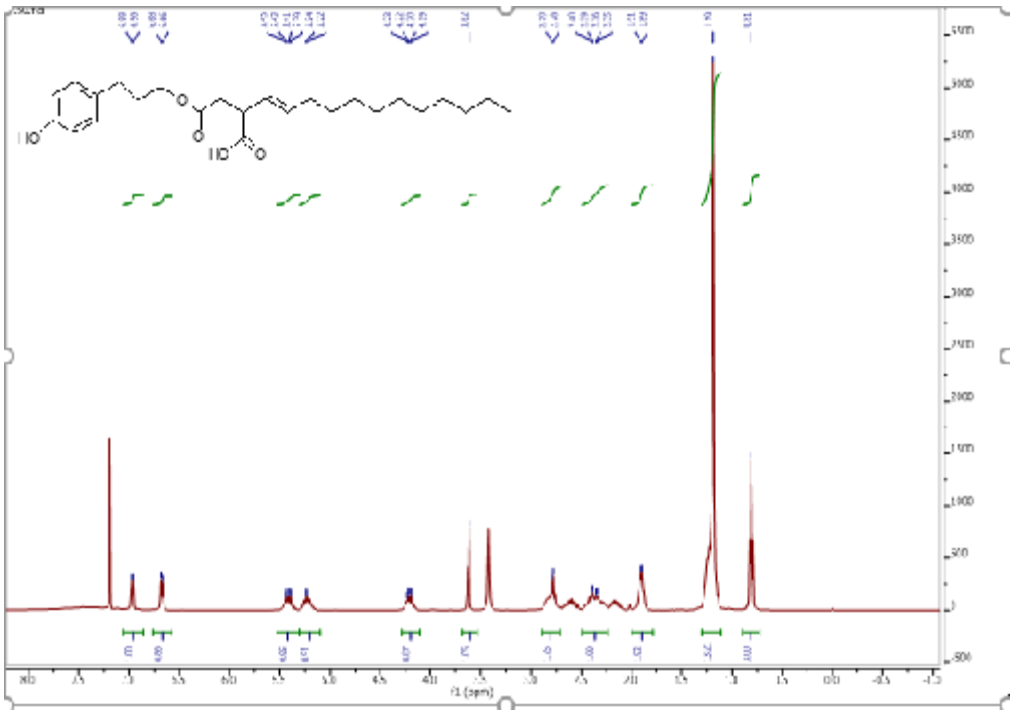
Appendix 6. ^{1}H NMR of compound 3a in $CDCl_3$ at 400 MHz



Appendix 7. HPLC-MS spectrum of compound 4a



Appendix 8. ^{1}H NMR of compound 4a in $CDCl_3$ at 400 MHz



Appendix 9. Total Fatty Acid values (FAME) of the samples expressed in mg FA/100 mg FAME (%).

TOTAL FATTY ACID (FAME) – mg FA/ 100 mg FAME (%)

FA	STORAGE FAT			SUBCUTANEOUS FAT			BONE MARROW			BONE FAT		
	CA	YB	CO	CA	YB	CO	CA	YB	CO	CA	YB	CO
C12:0	0.18 ± 0.14 a, C	0.11 ± 0.01 a, B	0.09 ± 0.02 a, A	0.11 ± 0.01 a, C	0.07 ± 0.01 b, C	0.07 ± 0.00 b, B	0.42 ± 0.03 a, B	0.19 ± 0.01 b, A	0.07 ± 0.01 c, B	0.55 ± 0.06 a, A	0.11 ± 0.02 b, B	0.08 ± 0.00 b, AB
C14:0	3.57 ± 0.52 a, A	3.73 ± 0.11 a, B	3.04 ± 0.03 b, A	2.81 ± 0.05 a, B	2.36 ± 0.10 b, D	2.35 ± 0.15 b, B	2.95 ± 0.03 b, B	4.23 ± 0.09 a, A	1.61 ± 0.03 c, D	4.01 ± 0.18 a, A	3.23 ± 0.30 b, C	2.20 ± 0.07 c, C
C14:1	0.81 ± 0.06 c, B	0.94 ± 0.06 b, B	1.87 ± 0.06 a, A	0.81 ± 0.05 b, B	1.12 ± 0.19 a, A	0.81 ± 0.09 b, C	0.72 ± 0.02 b, B	0.91 ± 0.03 a, B	0.52 ± 0.04 c, D	1.09 ± 0.08 b, A	1.16 ± 0.08 ab, A	1.29 ± 0.14 a, B
C15:0	0.48 ± 0.03 b, A	0.66 ± 0.03 a, A	0.38 ± 0.01 c, B	0.41 ± 0.02 b, B	0.55 ± 0.13 a, AB	0.56 ± 0.03 a, A	0.17 ± 0.01 c, D	0.66 ± 0.01 a, A	0.54 ± 0.01 b, A	0.23 ± 0.01 c, C	0.52 ± 0.06 a, B	0.39 ± 0.02 b, B
C15:1	0.10 ± 0.01 b, B	0.14 ± 0.01 a, C	0.15 ± 0.03 a, B	0.12 ± 0.01 b, A	0.28 ± 0.03 a, A	0.27 ± 0.01 a, A	0.05 ± 0.01 c, C	0.23 ± 0.01 b, B	0.26 ± 0.01 a, A	0.06 ± 0.01 c, C	0.22 ± 0.02 a, B	0.17 ± 0.01 b, B
C16:0	26.44 ± 0.66 a, A	24.92 ± 0.65 b, B	25.63 ± 0.19 ab, B	25.03 ± 0.46 c, B	27.06 ± 0.58 b, A	28.46 ± 0.71 a, A	20.10 ± 0.03 c, D	27.73 ± 0.21 a, A	24.13 ± 0.25 b, C	23.13 ± 0.13 b, C	24.88 ± 0.37 a, B	24.48 ± 0.33 a, C
C16:1t	0.29 ± 0.05 a, C	0.24 ± 0.02 ab, C	0.20 ± 0.06 b, B	0.32 ± 0.03 a, C	0.25 ± 0.05 b, BC	0.26 ± 0.01 b, B	0.57 ± 0.05 a, A	0.40 ± 0.03 b, A	0.36 ± 0.04 b, A	0.45 ± 0.03 a, B	0.31 ± 0.05 b, B	0.25 ± 0.03 b, B
C16:1c	3.62 ± 0.36 b, AB	3.71 ± 0.17 b, A	5.13 ± 0.10 a, A	3.31 ± 0.08 a., BC	3.90 ± 1.17 a, A	3.26 ± 0.49 a, B	3.20 ± 0.10 a, C	2.12 ± 0.09 b, B	1.62 ± 0.11 c, C	3.89 ± 0.23 b, A	3.57 ± 0.14 b, A	5.08 ± 0.52 a, A
C17:0	1.06 ± 0.06 b, A	1.57 ± 0.04 a, A	0.85 ± 0.03 c, B	0.94 ± 0.04 b, B	1.15 ± 0.26 ab, B	1.34 ± 0.09 a, A	0.51 ± 0.01 c, C	1.16 ± 0.01 b, B	1.37 ± 0.01 a, A	0.50 ± 0.02 c, C	0.96 ± 0.04 a, B	0.85 ± 0.08 b, B
C17:1	0.68 ± 0.07 c, A	1.11 ± 0.02 a, A	0.95 ± 0.04 b, A	0.60 ± 0.02 c, B	0.81 ± 0.07 a, B	0.67 ± 0.02 b, C	0.42 ± 0.03 c, C	0.50 ± 0.01 b, D	0.65 ± 0.04 a, C	0.43 ± 0.01 c, C	0.72 ± 0.02 b, C	0.86 ± 0.04 a, B
C18:0	15.03 ± 0.79 b, B	16.64 ± 0.52 a, AB	10.82 ± 0.11 c, B	17.21 ± 0.39 a, A	14.33 ± 4.03 a, B	16.81 ± 0.86 a, A	10.93 ± 0.16 c, C	19.44 ± 0.31 a, A	17.57 ± 0.38 b, A	10.46 ± 0.66 b, C	14.87 ± 1.17 a, B	10.45 ± 1.01 b, B
C18:1t	*8.52 ± 1.19* a, A	5.14 ± 0.14 b, A	1.21 ± 0.06 c, D	3.38 ± 0.19 a, B	1.59 ± 0.34 b, D	1.72 ± 0.11 b, B	1.26 ± 0.16 c, C	3.52 ± 0.31 a, B	2.57 ± 0.19 b, A	1.19 ± 0.07 b, C	2.53 ± 0.42 a, C	1.44 ± 0.13 b, C
C18:1c	32.22 ± 1.13 c, D	36,81 ± 0,61 b, B	44,78 ± 0,63 a, B	37,13 ± 0,54 b, C	40,90 ± 3,70 a, A	37,57 ± 1,24 ab, D	45,97 ± 0,41 a, A	33,41 ± 0,25 c, C	43,00 ± 0,58 b, B	42,49 ± 0,94 b, B	22,12 ± 0,78 c, D	47,11 ± 0,69 a, A
C18:2tt	0.31 ± 0.06 c, A	0,55 ± 0,02 a, B	0,40 ± 0,02 b, B	0,32 ± 0,04 a, A	0,33 ± 0,02 a, C	0,29 ± 0,01 a, C	0,00 ± 0,00 c, C	0,64 ± 0,04 a, A	0,44 ± 0,00 b, A	0,17 ± 0,02 c, B	0,56 ± 0,02 a, B	0,40 ± 0,02 b, B

FA	STORAGE FAT			SUBCUTANEOUS FAT			BONE MARROW			BONE FAT		
	CA	**YB**	**CO**	**CA**	**YB**	**CO**	**CA**	**YB**	**CO**	**CA**	**YB**	**CO**
C18:2 n6	3.81 ± 0.87 $^{a, D}$	1,85 ± 0,12 $^{b, B}$	1,41 ± 0,09 $^{b, C}$	4,69 ± 0,26 $^{a, C}$	2,31 ± 0,35 $^{b, A}$	2,53 ± 0,08 $^{b, B}$	10,56 ± 0,09 $^{a, A}$	2,39 ± 0,03 $^{c, A}$	3,08 ± 0,22 $^{b, A}$	9,34 ± 0,24 $^{a, B}$	2,44 ± 0,13 $^{b, A}$	2,38 ± 0,18 $^{b, B}$
C18:3n6 + C19:0	0.12 ± 0.01 $^{b, B}$	0.27 ± 0.02 $^{a, B}$	0.35 ± 0.09 $^{a, A}$	0.23 ± 0.06 $^{b, A}$	0.33 ± 0.03 $^{a, A}$	0.27 ± 0.02 $^{ab, B}$	0.17 ± 0.04 $^{b, B}$	0.20 ± 0.02 $^{b, C}$	0.27 ± 0.03 $^{a, B}$	0.16 ± 0.01 $^{b, B}$	0.23 ± 0.02 $^{a, C}$	0.24 ± 0.01 $^{a, B}$
C18:3 n3	0.24 ± 0.09 $^{a, B}$	0.18 ± 0.01 $^{a, D}$	0.18 ± 0.03 $^{a, D}$	0.17 ± 0.02 $^{b, B}$	0.41 ± 0.06 $^{a, C}$	0.44 ± 0.02 $^{a, A}$	0.50 ± 0.00 $^{b, A}$	0.68 ± 0.02 $^{a, A}$	0.32 ± 0.02 $^{c, B}$	0.53 ± 0.01 $^{a, A}$	0.47 ± 0.02 $^{b, B}$	0.24 ± 0.01 $^{c, C}$
CLA	0.37 ± 0.05 $^{c, B}$	0.58 ± 0.03 $^{a, C}$	0.48 ± 0.06 $^{b, B}$	0.60 ± 0.02 $^{a, A}$	0.59 ± 0.04 $^{a, C}$	0.52 ± 0.02 $^{b, B}$	0.23 ± 0.02 $^{c, C}$	0.70 ± 0.03 $^{a, B}$	0.60 ± 0.02 $^{b, A}$	0.21 ± 0.01 $^{c, C}$	0.76 ± 0.03 $^{a, A}$	0.50 ± 0,02 $^{b, B}$
C20:1	0.27 ± 0.05 $^{b. B}$	0.21 ± 0.02 $^{b, C}$	0.47 ± 0.04 $^{a, A}$	0.33 ± 0.02 $^{b, A}$	0.43 ± 0.06 $^{a, A}$	0.40 ± 0.03 $^{a, B}$	0.37 ± 0.03 $^{a, A}$	0.29 ± 0.03 $^{b, B}$	0.37 ± 0.02 $^{a, B}$	0.35 ± 0.02 $^{a, A}$	0.27 ± 0.02 $^{c, BC}$	0.30 ± 0.02 $^{b, C}$
C20:2 n6	0.08 ± 0.02 $^{ab, B}$	0.09 ± 0.02 $^{a, A}$	0.05 ± 0.01 $^{b, AB}$	0.09 ± 0.02 $^{a, B}$	0.05 ± 0.01 $^{b, B}$	0.06 ± 0.01 $^{b, A}$	0.17 ± 0.01 $^{a, A}$	0.00 ± 0.00 $^{b, C}$	0.00 ± 0.00 $^{b, C}$	0.14 ± 0.01 $^{a, A}$	0.04 ± 0.01 $^{b, B}$	0.05 ± 0.01 $^{b, B}$
C20:3 n6	0.07 ± 0.01 $^{b, B}$	0.07 ± 0.01$^{b, B}$	0.16 ± 0.06 $^{a, B}$	0.16 ± 0.05 $^{ab, A}$	0.16 ± 0.02 $^{b, A}$	0.22 ± 0.02 $^{a, A}$	0.05 ± 0.01 $^{b, B}$	0.06 ± 0.01 $^{b, B}$	0.12 ± 0,02 $^{a. BC}$	0.05 ± 0.01 $^{c, B}$	0.07 ± 0.01$^{b, B}$	0.10 ± 0.01 $^{a, C}$
C20:4 n6 (AA)	0.07 ± 0.01$^{a, B}$	0.06 ± 0.01 $^{a, C}$	0.06 ± 0.01 $^{a, B}$	0.45 ± 0.06 $^{a, A}$	0.20 ± 0.05 $^{c, A}$	0.31 ± 0.09 $^{b, A}$	0.06 ± 0.01 $^{b, B}$	0.06 ± 0.00 $^{b, C}$	0.08 ± 0.01 $^{a, B}$	0.07 ± 0.01 $^{b, B}$	0.12 ± 0.01$^{a, B}$	0.11 ± 0.01 $^{a, B}$
C22:3 + C22:4	0.07 ± 0.01 $^{a, B}$	0.07 ± 0.01 $^{a, B}$	0.10 ± 0.04 $^{a, B}$	0.18 ± 0.01 $^{a, A}$	0.10 ± 0.02 $^{c, A}$	0.15 ± 0.01 $^{b, A}$	0.04 ± 0.01 $^{b, C}$	0.05 ± 0.01 $^{b, C}$	0.08 ± 0.00 $^{a, B}$	0.04 ± 0.00 $^{b, C}$	0.06 ± 0.00 $^{a, BC}$	0.02 ± 0.01 $^{b, C}$
C22:5n3 (DPA)	0.07 ± 0.01 $^{b, C}$	0.09 ± 0.02 $^{b, B}$	0.30 ± 0.11 $^{a, A}$	0.30 ± 0.06 $^{a, A}$	0.20 ± 0.04 $^{b, A}$	0.23 ± 0.06 $^{ab, AB}$	0.08 ± 0.01 $^{b, C}$	0.08 ± 0.00 $^{b, B}$	0.10 ± 0.01 $^{a, B}$	0.16 ± 0.02 $^{b, B}$	0.21 ± 0.04 $^{a, A}$	0.16 ± 0.03 $^{b, B}$
SFA	46.76 ± 0.97 $^{a, A}$	47.62 ± 0.53 $^{a, B}$	40.80 ± 0.28 $^{b, C}$	46.51 ± 0.47 $^{a, A}$	45.52 ± 4.50 $^{a, B}$	49.59 ± 1.79 $^{a, A}$	35.08 ± 0.20 $^{c, C}$	53.42 ± 0.19 $^{a, A}$	45.29 ± 0.61 $^{b, B}$	38.88 ± 0.98 $^{b, B}$	44.57 ± 1.93 $^{a, B}$	38.45 ± 1.45 $^{b, D}$
MUFA	48.03 ± 1.23 $^{a, C}$	48.57 ± 0.64 $^{a, A}$	55.70 ± 0.50 $^{b, A}$	46.29 ± 0,69 $^{a. D}$	49,73 ± 4.92 $^{a, A}$	45.37 ± 1.76 $^{a, C}$	53.05 ± 0.29 $^{a, A}$	41.72 ± 0.17 $^{c, B}$	49.63 ± 0.53 $^{b, B}$	50.41 ± 1.23 $^{b, B}$	50.46 ± 2,15 $^{b, A}$	57.35 ± 1.41 $^{a, A}$
PUFA	5.21 ± 0.93 $^{a, D}$	3.81 ± 0.13 $^{b, B}$	3.50 ± 0.43 $^{b, C}$	7.20 ± 0.28 $^{a, C}$	4.70 ± 0.49 $^{b, A}$	5.04 ± 0.11 $^{b, A}$	11.87 ± 0.12 $^{a, A}$	4.86 ± 0.03 $^{b, A}$	5.09 ± 0.25 $^{b, A}$	10.88 ± 0.26 $^{a, B}$	4.96 ± 0.23 $^{b, A}$	4.20 ± 0.17 $^{c, B}$

Mean ± SD, n= 6. Abbreviation: CLA (Conjugated Linolenic Acid) SFA (saturated fatty acids), MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids), CA (Calf), YB (Young Bull), CO (Cow). Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters on the same row show significantly different mean values within each by-product (storage fat, subcutaneous fat, bone marrow, bone fat) for the three animals (CA, YB, CO); capital letters on the same row show significantly different mean values within each individual animal (CA, YB, CO) for their respective four by-products (storage fat, subcutaneous fat, bone marrow, bone fat).

Appendix 10. Triglycerides content in the samples (Calf, CA; Young Bull, YB; Cow, CO) expressed in g TG/100 g of fat (%)

TRIGLYCERIDES (TG) – g TG/100 g of fat (%)

TG	STORAGE FAT			SUBCUTANEOUS FAT			BONE MARROW			BONE FAT		
	CA	YB	CO	CA	YB	CO	CA	YB	CO	CA	YB	CO
CN42	0.50 ± 0.16 $^{a, B}$	0.43 ± 0.02 $^{a, BC}$	0.49 ± 0.03 $^{a, A}$	0.41 ± 0.05 $^{a, B}$	0.38 ± 0.04 $^{ab, C}$	0.34 ± 0.03 $^{b, B}$	0.42 ± 0.02 $^{b, B}$	0.85 ± 0.07 $^{a, A}$	0.21 ± 0.00 $^{c, C}$	0.76 ± 0.06 $^{a, A}$	0.52 ± 0.09 $^{b, B}$	0.33 ± 0.01 $^{c, A}$
CN44	2.91 ± 0.48 $^{a, B}$	2.91 ± 0.12 $^{a, B}$	3.23 ± 0.10 $^{a, A}$	2.32 ± 0.06 $^{b, C}$	2.49 ± 0.11 $^{a, C}$	2.27 ± 0.10 $^{b, B}$	2.10 ± 0.03 $^{b, C}$	4.13 ± 0.17 $^{a, A}$	1.16 ± 0.02 $^{c, D}$	3.36 ± 0.16 $^{a, A}$	2.88 ± 0.38 $^{b, B}$	2.02 ± 0.04 $^{c, C}$
CN46	11.28 ± 0.48 $^{a, A}$	11.40 ± 0.33 $^{a, B}$	11.70 ± 0.33 $^{a, A}$	9.64 ± 0.15 $^{a, C}$	9.47 ± 0.33 $^{a, C}$	9.00 ± 0.24 $^{b, B}$	7.61 ± 0.07 $^{b, D}$	12.51 ± 0.32 $^{a, A}$	5.48 ± 0.09 $^{c, C}$	10.82 ± 0.23 $^{a, B}$	10.22 ± 0.85 $^{a, C}$	8.83 ± 0.24 $^{b, B}$
CN48	25.99 ± 0.74 $^{a, A}$	25.95 ± 0.64 $^{a, A}$	25.46 ± 0.23 $^{a, A}$	24.72 ± 0.33 $^{a, B}$	24.80 ± 0.37 $^{a, B}$	24.62 ± 0.41 $^{a, B}$	19.24 ± 0.08 $^{b, D}$	24.74 ± 0.24 $^{a, B}$	18.03 ± 0.23 $^{c, D}$	23.71 ± 0.08 $^{a, C}$	28.81 ± 1.02 $^{a, B}$	23.28 ± 0.44 $^{a, C}$
CN50	42.11 ± 1.19 $^{b, B}$	43.00 ± 0.56 $^{ab, B}$	43.46 ± 0.45 $^{a, D}$	44.15 ± 0.38 $^{c, A}$	48.74 ± 0.69 $^{b, A}$	51.17 ± 0.46 $^{a, B}$	44.47 ± 0.17 $^{b, A}$	44.67 ± 0.34 $^{b, B}$	56.11 ± 0.30 $^{a, A}$	41.33 ± 0.74 $^{c, B}$	44.69 ± 2.38 $^{b, B}$	49.05 ± 0.42 $^{a, C}$
CN52	17.21 ± 0.35 $^{a, D}$	16.30 ± 0.86 $^{b, B}$	15.63 ± 0.17 $^{b, C}$	18.76 ± 0.73 $^{a, C}$	14.15 ± 1.06 $^{b, C}$	12.62 ± 0.38 $^{c, D}$	26.16 ± 0.24 $^{a, A}$	13.11 ± 0.36 $^{c, C}$	19.00 ± 0.34 $^{b, A}$	20.03 ± 0.25 $^{a, B}$	17.88 ± 0.63 $^{b, A}$	16.50 ± 0.31 $^{c, B}$

Means ± SD, n = 6. Results of the analysis of variance by Tukey's test are shown: $p<0.05$, lowercase letters on the same row show significantly different mean values within each by-product (storage fat, subcutaneous fat, bone marrow, bone fat) for the three animals (CA, YB, CO); capital letters on the same row show significantly different mean values within each individual animal (CA, YB, CO) for their respective four by-products (storage fat, subcutaneous fat, bone marrow, bone fat).

Appendix 11. Diglycerides content in the samples expressed in g DG/100 g of fat (%)

DIGLYCERIDES (DG) – g DG/100 g of fat (%)

DG	STORAGE FAT			SUBCUTANEOUS FAT			BONE MARROW			BONE FAT		
	CA	YB	CO	CA	YB	CO	CA	YB	CO	CA	YB	CO
CN30	5.47 ± 0.67 $^{c, A}$	6.54 ± 0.40 $^{b, B}$	7.99 ± 0.24 $^{a, A}$	2.77 ± 0.35 $^{c, B}$	7.64 ± 0.37 $^{a, B}$	4.54 ± 0.53 $^{b, C}$	6.04 ± 0.35 $^{b, A}$	9.41 ± 0.69 $^{a, A}$	5.01 ± 0.52 $^{c, C}$	6.17 ± 0.39 $^{b, A}$	7.46 ± 0.85 $^{a, B}$	6.61 ± 0.54 $^{ab, B}$
CN32	15.34 ± 0.73 $^{b, AB}$	16.60 ± 1.36 $^{ab, A}$	17.71 ± 0.57 $^{a, A}$	4.48 ± 0.10 $^{b, C}$	7.96 ± 1.01 $^{a, B}$	5.38 ± 0.91$^{b, C}$	14.54 ± 0.99 $^{b, B}$	18.53 ± 2.54 $^{a, A}$	12.92 ± 1.27 $^{b, B}$	15.87 ± 0.86 $^{b, A}$	18.17 ± 1.29 $^{a, A}$	17.76 ± 1.45 $^{a, A}$
CN34	41.84 ± 0.67 $^{a, A}$	42.60 ± 0.53 $^{a, A}$	40.36 ± 0.86 $^{b, AB}$	25.76 ± 1.84 $^{b, C}$	36.65 ± 2.15 $^{a, B}$	28.22 ± 3.70 $^{b, C}$	36.85 ± 1.51$^{b, B}$	45.09 ± 3.35 $^{a, A}$	46.76 ± 0.67 $^{a, A}$	36.19 ± 0.66 $^{b, B}$	38.92 ± 1.06 $^{a, B}$	39.99 ± 1.91 $^{a, B}$
CN36	37.34 ± 0.62 $^{a, B}$	34.25 ± 1.25 $^{b, A}$	33.93 ± 1.53 $^{b, A}$	30.87 ± 3.02 $^{a, C}$	26.80 ± 0.94 $^{ab, B}$	25.42 ± 1.74 $^{b, B}$	42.57 ± 2.76 $^{a, A}$	26.60 ± 5.56 $^{c, B}$	35.30 ± 0.98 $^{b, A}$	41.77 ± 1.55 $^{a, A}$	35.45 ± 1.21 $^{b, A}$	35.64 ± 3.32 $^{b, A}$
CN38	n.d.	n.d.	n.d.	29.63 ± 2,28 ^{a}	15.63 ± 2.94 ^{b}	26.36 ± 2.37 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CN40	n.d.	n.d.	n.d.	6.50 ± 1.49^{b}	5.32 ± 0.75^{b}	10.08 ± 1.28^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TOTAL	0.09 ± 0.01 $^{a, A}$	0.08 ± 0.01 $^{a, A}$	0.09 ± 0.02 $^{a, A}$	0.02 ± 0.01 $^{a, C}$	0.03 ± 0.00 $^{a, B}$	0.03 ± 0.01 $^{a, B}$	0.05 ± 0.00 $^{a, B}$	0.03 ± 0.00 $^{b, B}$	0.04 ± 0.00 $^{b, B}$	0.06 ± 0.01 $^{a, B}$	0.04 ± 0.01 $^{b, B}$	0.04 ± 0.01 $^{b, B}$

Means ± SD, n = 6. Results of the analysis of variance by Tukey's test are shown: p<0.05, lowercase letters on the same row show significantly different mean values within each by-product (storage fat, subcutaneous fat, bone marrow, bone fat) for the three animals (CA, YB, CO); capital letters on the same row show significantly different mean values within each individual animal (CA, YB, CO) for their respective four by-products (storage fat, subcutaneous fat, bone marrow, bone