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Food lipids characterization: a tool for food quality assurance and authenticity

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Abstract

Fats and oils represent one of the macro constituents in various foodstuffs that show a strategic role in the technological process during the production of industrial goods. Fats in food influence directly their rheological properties and the evolution of final products during the shelf life, leading to a direct influence on consumer satisfaction.

Lipids could have animal or vegetable origin and constitute a heterogeneous group of biological compounds that can be divided in two different matters: *i)* saponifiable matter, also named glycerol fraction and *ii)* unsaponifiable matter, represented by a wide range of different compounds.

From a chemical point of view, the glycerol fraction is composed of molecules like *tri-hydroxylic alcohols* that can generate tri-esters with one, two or three different fatty acids. Depending on the extent to which the three former hydroxyl groups of glycerol are replaced with fatty acids, the resulting compounds are called *mono-, di-* or *tri* - acylglycerol.

Considering the function of lipids in food products, the most important physical properties related to glycerides are polymorphism, crystal structure and melting point, which affect the melting behavior of fats matters. *Mono, di-* and *tri-* glycerides are polymorphic, i.e. they crystallized in different forms; physics properties are directly affected by fatty acids composition and their distribution on glycerol backbone. The distribution of fatty acids is peculiar and give a lot of information regarding to the type, origin and manipulation of fats matter. Acylglycerol fraction can be crystallized in different form denoted as α form with hexagonal system, β form that shows a triclinic system and β^I forms with orthorhombic system.

The unsaponifiable matter is composed of minor not glyceride components that are present in parts per million levels (*ppm*) and belong to different chemical classes. Unsaponifiable fraction that includes several compounds such as tocopherols and tocotrienols, sterols and sterol esters, volatile and non–volatile compounds, color compounds (chlorophylls and carotenes – hydrocarbons group) and metals (in traces).

Concentration and modification of those patterns during some technological processes have a great interest from analytical and nutritional points of view; several minor compounds such as tocopherols, phenols, and carotenoids, can limit oxidation of fat matrix by their ability to scavenge free radicals, chelate metal ions, and inhibit the decomposition of hydroperoxides.

Commercial vegetable oils and fats are products obtained by processing certain oleaginous crops (such as fruits or seeds) with an extraction and refining process, which comprises different physical and chemical steps such as degumming, neutralization, blanching, and deodorization. However, refining is mandatory since crude oils are composed of a mixture of triacylglycerols, minor components, undesirable pigments, oxidation products, metals, and a huge part of solvent. The principal purpose of the refining process is to remove unwanted impurities and preserve a suitable level of desirable minor components, providing a final product with a stable shelf life. Due to the need to change physical behaviors and structural proprieties, fat matter can be undergoing some manipulation processes. There are three main modification technologies

usually implemented in the edible oils industry: *fractionation*, *hydrogenation* and *interesterification*. In the last decades, a new technique has been deeply investigated, such as the *texturization* or *organogelation* that provides oleogels formation, a viscoelastic, anhydrous and self-supporting materials.

During all steps of the supply chain of fat matter production, starting from harvesting, continuing to extraction, refining, and modification process (but also during transport and storage), lipids must be protected against deterioration and adulteration. The chemical parameters for quality assurance of raw materials are well known and based on official international methods (AOCS, COI, UNI EN ISO and others). These methods assay some particular parameter like *Iodine Value (IV)*, *Free Fatty Acids – FFAs* and *Peroxide Value (PV)*, with common analytical techniques. At the same time, other powerful chemical analyses have been developed in the last years to evaluate the quality and safety of fats matters, like the determination of *mineral oil saturated hydrocarbons (MOSH)* and *aromatic hydrocarbons (MOAH)*, the evaluation of *3-monochloropropane 1,2-diol (3-MCPD)* and *glycidyl esters (GE).*

The aim of the present PhD project has been to investigate different aspects of the fats and oils supply chain from a chemical and technological point of view. The research activities have been organized into two different parts.

In the first part, after a deep study of literature, the research has been focused on the improvement of chromatographic techniques to investigate the acylglycerol and unsaponifiable profile (sterols and oxysterols). In particular, the activity has been focused on the development and implementation of an innovative analytical method to ensure new parameters for quality and authenticity of fats matters as well as a safer working environment for operators. In deep, triacylglycerols regioisomers were identified as key aspects to obtain fats with desired properties in the confectionary sector and to estimate the presence or absence of interesterified fats. The study investigated the use of *n*-heptane as an alternative and less toxic mobile phase to replace the most used *n*-hexane for triacylglycerols analysis using a silver ion high-performance liquid chromatography (Ag^+-HPLC). The impact of column temperature (in the 5°C - 35°C range) on the retention and resolution of five pairs of selected regioisomers has been also considered. The study confirmed the equivalence of *n*-hexane and *n*-heptane for regioisomers separation in Ag+-HPLC and the definition of side considerations regarding the resolution of this challenging technique.

Finally, regarding to the unsaponifiable matter, phytosterol oxidation products (POPs) have been evaluated in oils to define food quality and safety. A deep study was carried out on the presence of oxidized phytosterols in three different oils commodities (such as high oleic sunflower oil, palm oil and palm olein) as related to the refining process. Since no commercial standards of POPs are available (except for 7-keto sitosterol) a semi-preparative liquid chromatographic system (LC-Prep) has been used to isolate and collect the main oxidation products generated from β-sitosterol, campesterol, and stigmasterol – 5,6α-epoxy (α-E), 5,6β-epoxy (β-E), 7-keto (7-K), 7α-hydroxy (7α-H), and 7β-hydroxy (7β-H) isomers. Thanks to the isolated pure standards, an HPLC-Orbitrap-HRMS analytical method has been validated and tested on real sample during the whole refining processing and under forced storage conditions (45°C, 16 days). LC-Orbitrap-HRMS method demonstrated to be a useful and valid tool for a robust, precise, accurate, and sensitive determination of POPs in the selected matrix.

The second part of the project has been focused on the rheological modification of durum wheat oil, a by-product obtained during the wheat milling process, characterized by a high content of bioactive compounds (such as phytosterols, tocopherols and carotenoids). The focus of that work has been to investigate the durum wheat oil oleogel-based performances using natural waxes (bee waxes and carnauba waxes) as oleogelators at different ratios (4, 7 and 8%, w/w). Matrices were analyzed for fundamental rheological properties, microstructure and oil loss. Oil samples with 7% and 8% of carnauba waxes displayed higher stability and performance in terms of all considered parameters, also at high temperatures, showing strong networks and reaching the optimum solid-like gel. Durum wheat oil showed good exploitability in the development of oleogels, as an alternative to other common oil raw materials, with optimal performances and stability.

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CHAPTER 1

State of Art

The human diet was building over three macronutrients and several micronutrients. The macronutrients are different kinds of proteins, carbohydrates and fats/lipids (Wolmarans 2009) and food industry is concerned to supply these as primary products or as constituents of a wide range of foods.

The history of fats and oils substances was the history of human being. Humans had used fats matter from vegetable or animal origin for century for different purpose thanks to the specific characteristic.

Fats and oils are an important ingredient in various foods stuff and play a key role in the technological process during production and shelf life. Fats matters lead desirable characteristics and influence the texture and sensory profile of final products. At the same time, adulteration of fatty substances and more generally of food products has history reaching back to ancient times (Ulberth and Buchgraber 2000).

Adulteration of high commercial value increased greatly as a result of modern practices in food production, i.e. the separation of a direct link between food producers and consumers. Advent of the study of food chemistry linked to organic chemistry discipline, as a scientific discipline, has enabled the scientific community to study the composition of fatty substances in depth (Gunstone 2005) making it possible to take appropriate countermeasures to counter fraudulent practices and at the same time follow the shift of consumer demands.

1.1 Basic of oils and fats chemistry

Fats and oils are common defined “lipids”, a scientific name given to a wide range of natural components based on fatty acids and other natural molecule (Belitz et al., 2009). The classification of fatty substance on fats category or oils category is an ancient characterization based on the liquid state of the matter at room temperature. The physical state of fats matter is strictly related to chemical composition of fatty substances. Most animal fats are solid and most vegetable fats are liquid even if there was a lot of vegetable tropical fats that are solid (e.g. cocoa butter, palm kernel and shea butter) (Patel et al., 2020).

The definition of “room temperature” is a much-debated topic; a good example is represented by coconut oil, which is liquid at room temperature in semi-tropical areas during most of the time except for the winter months which is solid at room temperature and might be called “fat” (Gupta 2008).

Chemically, fats and oils are defined like a heterogeneous group of biological compounds that could be categorized in saponifiable fraction or glycerol fraction that large part is composed by triglycerides formed by esters of a molecule of glycerol and three fatty acid molecules, and unsaponifiable fraction (Rios et al., 2014) composed by elements not soluble in aqueous alkali after hydrolysis. Minor components of lipids include phospholipids, phytosterols, tocols (tocopherols and tocotrienols, including vitamin E) and hydrocarbons (Gutfinger and Letan 1974).

In deep, oils and fats for food purpose are a mixtures of more or less complex organic molecules mainly composed of triacylglycerols or triglycerides (96-98%) with lower levels of diacylglycerols or diglycerides, monoacylglycerols or monoglycerides, and free fatty acids plus other minor components that are fats-dispersible or fats soluble compounds (2–4%) which are can be divided in "major non triglycerides component", generally present at high levels in the crude oil and "minor non triglycerides components" (Gupta 2008).

1.1.1 Saponifiable fraction

The glycerol's fraction is composed by several molecules like mono-, di- and tri- ester of glycerol with fatty acids, free fatty acids (FFAs) and phosphate group (e.g. phospholipids).

1.1.1.1 Fatty acids (FAs)

Fatty acids (FAs) with glycerol backbone are the constituent of glyceridic fraction. Over 1000 natural fatty acids have been identified with various chain length (commonly C12–C24), degree of unsaturation (usually in the range 0–6 *cis* olefinic centers) and the presence or absence of other functional groups such as hydroxy or epoxy (Hamm et al., 2013).

The FAs can be divided into groups according to chain length, number, position and configuration of their double bonds, and the occurrence of additional functional groups along the chains. Fatty acids are usually denoted in the literature by a "shorthand description", that show the number of carbon atoms in the acid chain and the number, position and configuration of double bond (Gunstone 2009). Fatty acids in nature are divided in "short chain" (4-8 carbon atoms) "medium chain" (10-12 carbons atoms), and "long chain" (14 or more carbon atoms). Chemically, it's possible to finds *cis* or *trans* isomers; in natural fatty acids, it is common for the adjacent hydrogen atoms (allylic position) involved in the double bond to be on the same side of the chain, indicating as a "*cis* isomer", but some fat modification processes can lead formation of "*trans*-isomers" (e.g. partially hydrogenated fats) (Holm and Cowan 2008).

Fatty acids with short odd shot numbers of carbon atoms such pentadecanoic acids (C15:0) or margaric acid (C17:0) are present in little amount in vegetable oils and fats but they are more typical in animals' fats like cow's butter. The unsaturated fatty acids dominate fats matter from vegetable origin and contained one, two or three allyl groups in their acyl residues. In addition, it might be pointed out that as the number of carbon atoms in the fatty acid chain increases, with the same number of double bonds, as the melting point increases (C4:0 = -5.3°C and C24:0 = 84.2°C); instead the presence of a double bond reduces the melting point (C16 = 62.9°C and C16:1 9*c* = 0.5°C) (Gupta 2008).

Unsaturated fatty acids usually are more susceptible to the oxidation at room temperature; during oxidation aldehydes, ketones, primarily oxidation polymers and cyclic compounds are generated. In contrast, saturated fatty acids are more stable at the presence of air or oxygen but they are more susceptible to high heat with a related production of thermal polymers and toxin such as acrolein (Gunstone 2009).

Degradation of glycerides fraction, within crude oils, can occur via hydrolysis and provides the formation of mono- and diacylglycerols and free fatty acids (FFAs). FFAs production is also spontaneously promoted via "lipase" enzymes (Buchgraber et al., 2004).

1.1.1.2 Glycerol fraction

The glycerol fraction is mainly composed by triacyl glycerides, common referred to "triglycerides" (TAGs) that represent 96-98% of common edible fats matter. TAGs are composed by a glycerol fraction like a tri-hydroxylic alcohol that can generate tri-esters with one, two or three different fatty acids. According to the formula (1) reported below the Z number give the possible different TAG which can occur in a specific fat taking into account the number of possible positional isomers without considering enantiomers, where n is the number of different fatty acids in a fat's matters, on a theoretical base; on the other hand, the possible different TAGs without considering isomers (X) can be also determined (2).

$$Z = \frac{n^3 + n^2}{2}$$

Formula 1

$$X = \frac{n^3 + 3n^2 + 2n}{6}$$

Formula 2

In the study of triglycerides, it is usual to use the stereospecific numbering (*sn*) nomenclature to describe glycerol-bound compounds. In a TAGs molecule, when the acyl residues in position sn-1 and sn-3 are different, a chiral center exist.

In vegetable oils, the *sn*-2 hydroxyl group is esterified almost entirely with unsaturated acids while saturated acids and the remaining unsaturated acids are in the *sn*-1(3) positions (Gunstone 2009).

Depending on the extent to which the three former hydroxyls groups of glycerol are replaced with fatty acids, the resulting compounds are known as:

- ***Mono-acylglycerol*** molecules that are formed when one of the three hydroxyl groups of glycerol is replaced by a fatty acid.
- ***Di-acylglycerol*** molecules that are formed when two of the three hydroxyl groups of glycerol are replaced by a fatty acid.

- ***Tri-acylglycerol*** molecules that are formed when two of the three hydroxyl groups of glycerol are replaced by a fatty acid.

The molecular structure of each individual triacylglycerol can be described by a three basic attributes (Ulberth and Buchgraber 2000):

- total carbon number (CN), the sum of the alkyl chain lengths of each of the three fatty acids,
- degree of unsaturation in each fatty acid,
- position and configuration of the double bonds in each fatty acid.

The most important physical properties affecting glycerides are polymorphism, crystal structure and melting point, which combine in the melting behavior of lipid mixtures. Melting properties of fats depend not solely to the structure of TAGs. Generally, *mono*, *di* and *tri* glycerides are polymorphic, i.e. they crystallized in different forms, denoted as *α form*, that shown a hexagonal system, *β form* that shows a triclinic system, with a parallel arrangement of the carbon chain and *β^I forms* with orthorhombic system (Figure 1) where the carbon chains are perpendicular to each other (Sato 2001).

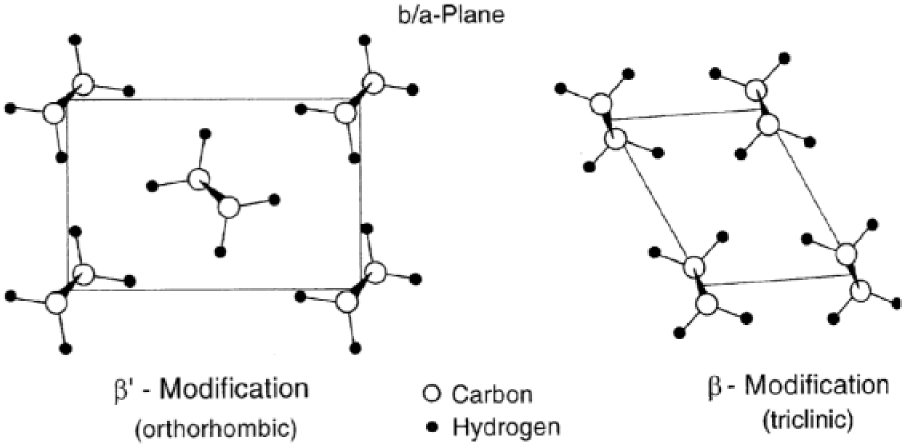

Figure 1 Arrangement of β^I forms with orthorhombic system and β form with a triclinic system of saturated triacylglycerol (Belitz 2009)

Triglycerides physics proprieties are affected by fatty acid composition and their distribution within the glyceride molecule. The distribution of fatty acids on glycerol backbone is peculiar and give a lot information regarding to the type, origin and manipulation of fats matter.

During the cooling of melted acylglycerols on of these three forms is yielded related to the temperature gradient chosen. All crystals forms show different melting and crystallography properties. The change in crystal form is monotropic so the change proceeds in the order of lower to higher stability; *α forms* has the lowest melting point and stability and *β form* is the most stable form with the highest melting point (Marangoni et al., 2020).

1.1.1.3 Phospholipids

Phospolipids are known also like phosphatides or gums, are usually present in fats and oils for food composition divided in five major groups that are follow: phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidic acid.

Chemically, phospholipids are a class of lipids consisting of a glycerol molecule esterified with two fatty acids and a phosphate group (Gunstone 2009).

They have a hydrophilic polar head consisting of the phosphate group and an alcoholic molecule bound to it and a hydrophobic a-polar tail consisting of two fatty acids; phospholipids are therefore amphiphilic molecules.

The level of phospholipids intake in vegetable oils and fats are usually expressed in parts per million (ppm) of phosphorus; the relationship between phospholipids and phosphorus content is $phosphorus\ (ppm) = [phosphatides\ (\%) * 10^4] / 31.7$ (Gupta 2008).

Phospholipids content decrease during the refining process and it is related to the botanical origin of the oils. For instance, phospholipids value in crude and degummed soybean oil are 1-3% and 0.3 – 0.4 %, respectively, whereas a crude palm oil shown a phospholipids content about 0.06 – 0.09 %.

Generally, two types of phospholipids are determined in crude oils before refining process, based on their affinity with water: hydratable phospholipids and non-hydratable phospholipids. Thus, during degumming crude oil – a step of oils refining process – by acid pretreatment heat water treatment more of them are washed off from the oils. The valuable raw product containing phospholipids and other lipid molecules are called “raw lecithin” with high industrial value based on their emulsifier properties (Belitz et al., 2009).

1.1.2 Unsaponifiable fraction

Minor not glyceride components are present in part per million levels (ppm) and represent a wide range of food molecules that are naturally present in vegetable and animal lipids and they included: tocopherols and tocotrienols, sterols and sterol esters, volatile and non – volatile compounds from decomposition of glyceridic fraction, color compound and metals (in traces).

Concentration and modification of profile of these minor components in fats matter, during certain technological processes, have a great interest from an analytical and nutritional point of view. Minor components provide highly specific information about the identity of fats raw materials (Gharby 2022). Minor compounds may be prooxidative by altering the physical properties of emulsion droplets as a result of their surface activity but other some minor components may increase oxidative stability of fats matter in foods stuff (Fernandes et al., 2017). Several compounds, such as tocopherols, phenols, and carotenoids, can limit oxidation by their ability to scavenge free radicals, chelate metal ions, and inhibit the decomposition of hydroperoxides (Hu et al., 2003).

1.1.2.1 Tocopherols and tocotrienols

Tocochromanols (tocopherols plus tocotrienols) or "tocol" are antioxidant compounds that are present in common oils and fats. The *tocol* content in crude vegetable oils ranged from 10 ppm in coconut oil to 1370 ppm in soya beans oil (Belitz 2009). In vegetable oils four tocopherols' forms *α, β, γ, δ,* and four tocotrienols forms *α, β, γ, δ* are reported (Colombo 2010). Tocopherols and tocotrienols have the same basic chemical structure characterized by a long chain linked at the 2-position of a chroman ring. Chemically the difference between tocotrienols and tocopherols is that tocotrienols have unsaturated isoprenoid side chains with three C-C double bonds versus saturated side chains for tocopherols. It is well known that tocopherols and tocotrienols have evolved in the reduction of cardiovascular diseases and cancer, shown a great activity like antioxidant compound. The tocotrienols, though significant in palm oil and in rice bran oil, are less common than the tocopherols, and less is considered about their biological properties even if tocotrienol subfamily possesses powerful neuroprotective, anticancer, and cholesterol-lowering properties that are often not exhibited by tocopherols (Rossi et al., 2007).

1.1.2.2 Sterols

Cholesterol/zoosterol and phytosterols/plant sterols, represent a large group of naturally occurring compounds having a 1,2-cyclopentanophenthren skeleton. They are one of the minor components of the lipids from plants or animals' origin. Phytosterols can be defined as secondary plant metabolites belonging to the triterpene family with a tetracyclic ring and a side chain linked to the central carbon structure (Vanmierlo et al., 2013). They are an endogenous component of all plant-origin food ingredients. Sterols with a double bond in their structure are usually named ''unsaturated sterols'' while the saturated one are called stanols (Rudzinska and Wasowicz 2004). In western diets phytosterols are consumed at levels of 150–400 mg/day and consist primarily of β-sitosterol, campesterol, stigmasterol and brassicasterol (Ryan et al., 2009) most of them coming from diet. Sterols due to the presence of double bond in their structure are undergone to oxidation process trough different pathways (enzymatic or non-enzymatic) (Bush & King, 2009). The sterol oxidation products (SOP) have long been under investigation for possible negative effects on human health.

1.1.2.3 Chlorophylls

Chlorophyll and its magnesium-free derivative (phaeophytin) are not wanted in refined oils because they produce an undesirable green hue and act as sensitizers for photooxidation. The concentration of chlorophylls depends to the type of raw matters. Unrefined olive oil contains about 10–30 ppm of chlorophyll and phaeophytin, whereas sunflower crude oil contains 200–500 ppb of chlorophyll and this value decrease during refining process (<30 ppb) (Gupta 2008).

1.1.2.4 Hydrocarbons

Hydrocarbons compounds are one of minor components of oils and fats with dietary and legislative interest. This group include alkanes, alkenes (such as squalene and carotenes) and polycyclic aromatic hydrocarbons (PAHs).

Alkenes are not likely to be significant in refined oils that have been submitted to high-temperature deodorization. Levels of C13-C33 alkanes in crude oils are usually between 40 and 100 mg/kg (ppm) with lower values for refined oils. There is a preponderance of odd carbon molecules.

Squalene ($C_{30}H_{50}$) a highly unsaturated open-chain triterpene, it is a byproduct mainly used in the cosmetic industry, usually after hydrogenation to squalane ($C_{30}H_{62}$), for this reason squalene has high value on the market.

Carotenes are one of the most important color compounds in vegetable oils, in particularly in crude palm oil. They contain a long chain of conjugated unsaturation and are yellow/orange/red in color after the reaction with oxygen. Crude palm oil contains 500 - 700 ppm of carotenes (Gupta 2008). Carotenes belong to carotenoids family that are divided into carotenes, which are not carrying oxygen, and xanthophylls, oxygenated forms. The major provitamin A active carotenoids are α carotene and β-carotene. The main xanthophylls are lutein, zeaxanthin and β-cryptoxanthin usually found in other vegetable matrix like tomatoes or corn (Franke et al., 2010).

Polycyclic aromatic hydrocarbons (PAHs) present at levels up to 150 µg/kg (ppb) in most crude vegetable oils, though slightly less after refining (<80 ppb). They are removed during bleaching process and somewhat more during deodorization. This holds particularly for the more volatile tri and tetracyclic compounds. The pentacyclic and other less volatile compounds are best removed when activated charcoal is added to the earth during bleaching (Gupta 2008). PAHs have attracted EFSA and FDA authority's attention mostly due to their carcinogenic potential (Tfouni et al., 2014).

1.2 Edible fats and oils markets

Fats commodities source plays a key role in the global market related to world of agriculture, food economy and processing industries. They are "raw material" for production of consumer and technical fats; source of food and feed protein, and some of them, such as cotton and linen; also provide plant fiber industries. Fats and oils have been used throughout the years in food preparation to provide structure, flavors and nutritive value. Geography and agricultural practices have influenced the fat used in food preparation. From the end of 20st century with consumer awareness and the acceptance of new directions in public health, the consumption of animal fats is strongly decreasing in favor of the consumption of vegetable fats. Butter uses decreased of 44% from 1960 to 2000 and the direct use of lard and tallow had decreased of 68% through

1990. On the other hand, the liquid oils consume has more than tripled since 1960 replacing all other fats product losses.

Major plant oil sources of commercial importance out of the 40 documented ones that contain edible fats matter include soybean, sunflower, groundnuts, rapeseed, coconut, cocoa butter, palm oil, palm kernel oil and other tropical plants like shea kokum, illipe, mango and others (Rani et al., 2021). About these oils' commodity, the largest amount comes from seeds of seasonal plants that grown in temperate climates. The second source of vegetable oil is oil-bearing trees like coconut and palm where the fats matter is extracted from the fruit pulp rather than seeds (e.g. palm provides a palm oil from pulp and palm kernel oil from seeds) (Ramadan et al., 2019).

Regarding to animal fats, they are supplied almost by three kinds of domesticated animals: lard from swine, tallow from cow and sheep, and milk fat or butter from cattle.

In the last seventy years there has been a progressive decrease in the production and consumption of animal fats in favor of those of vegetable origin (Zio et al., 2020). The global production of vegetable oils is dominated by two oils commodity, soybean oil and palm oil; Argentina and Brazil, as major producers of soybeans and soybean oil, instead palm oil production is located principally in South East Asia, with Indonesia and Malaysia responsible for the bulk of both (Hamm et al., 2013). According to the Oil World forecast from May 2022 the production of eight main vegetable oils (palm, soybean, rapeseed, sunflower, cotton, peanut, palm kernel and coconut) will amount to 203.5 million tons (Sobczak-Malitka and Sobczak 2023).

1.3 Fats and Oils handling

Vegetable oils and fats are obtained by mechanical extraction or solvent extraction of oleaginous seeds (soybeans, rapeseed, sunflower, etc.) or oleaginous fruit like palm and olive with a primary step called extraction (Hamm et al., 2013). Common fats and oils from vegetable origin, after the extraction by the matrix, must undergo refining process before to be suitable for human consumption. Indeed, several compounds in crude oils show negative effect on the quality and stability of fats matter and the primary goal is to reduce certain undesirable impurities from crude oil to produce high quality edible oil that provides satisfactory results in all application. The crude oils contain several major and minor non-triglycerides impurities and refining process lead a general increase of chemical stability of fats matter during the shelf life. Example of the major impurities are free fatty acids (FFAs), phospholipids, diglycerides and monoglycerides; all of them are undesirable above certain levels. The more usual minor non-glycerides components present in the crude oil are tocopherols, tocotrienols, color compound and sterol; in addition, there are also the "*oil decomposition products*" like polar compounds, polymers, aldehydes, ketones and other volatile and non-volatile compounds (Gharby 2022).

Tocols and sterols fractions with their antioxidant activity are suitable to maintain high oxidative stability of the oil so some precautions are taken to preserve them during the process. Some of the latter are recovered from side streams of the refining process to give commercial products such as phospholipids and tocopherols. Usually, vegetable fats matters can contain some contaminants like pesticides, aflatoxins, dioxins, trace metals (Zio et al., 2020), mineral oil hydrocarbons (MOH), polycyclic aromatic hydrocarbons (PAH) and organic solvent traces (Sánchez-Arévalo et al., 2020; Tfouni et al., 2014). The origin of these pollutants could be attributed to different areas like environment under which oleaginous crops are grown, how seeds are transported and stored, and how crude oils are processed and stored. Refining process is mandatory for crude oils that cannot be consumed as virgin oils, like olive oil, to provide a product odorless and rather neutral in taste, limpid, and colorless, and it must be free of contaminants (Gharby 2022).

1.3.1 Extraction process

Oil content of oleaginous seeds, nut, kernel, or fruit pulps varies between 3% and 70% of the total weight and has chemical structures strictly related to the botanical origin of raw materials and cultivation area. Initial oil content is a major factor that determines the choice of grain processing and extraction methods for the various oil seeds (Reginaldo et al., 2013), the second point is the part that contains the oil, which is the kernel, the seed, or the pulp.

The process of separating lipid from raw material is referred to as “extraction” should carry out through chemical, biochemical, and mechanical techniques in order to maximize yields but minimizing alterations on the quality of the product (Nde and Anuanwen 2020).

Common pre-treatment (Hamm et al., 2013):

- *Decortication*: removal of the fibrous part of the seed that reduces the extraction capacity. This process is always applied in the case of sunflower seed, soya bean and cottonseed processing.
- *Grinding and/or milling processes*: aimed at reducing the size of the matrix in order to make the fatty matrix more available during extraction.
- *Conditioning and/or cooking*: consists of treatment by heating. In the case of conditioning, at temperatures between 60 - 70°C, and in the case of cooking (mainly concerning the cooking of the hulls after rolling as in the case of rapeseed) at temperatures between 90 - 100°C. Thermal pre-processing in general is aimed to increase the availability of the oil before extraction and inhibiting the enzymatic action (lipase) of the matrices.

Two types of extraction should be performed by mechanical or by solvents action. In the first case of mechanical processes, the extraction time is inversely proportional to the plant capacity, hence high costs and low production yields; so, in many cases both type extraction is performed consequentially (Hamm et al., 2013). For raw materials with high level of fats matters (>20% w/w) mechanical extraction can initially be used from which “crude oil” and “pressed cake” (fibrous component still rich in oil) are obtained. The cake is destined for solvent extraction and from this process a liquid component (oil and solvent) and a solid, exhausted cake or flour (after drying solvent, roasted and milling processes) are obtained; both products must be dried by solvent trough evaporation process.

Mechanical extraction (ME) is one of the oldest methods used for oils yield and it is performed by mechanical pressure entails the use of either hydraulic presses or screw presses driven by a motor. There are many publications and patent regarding new advanced technique to perform extraction in most performant way.

The conventional solvent extraction process (CSE) is based on the solvent's ability to dissolve fat matrix by leaching it from the tissue in which it is trapped. CSE process is the most commonly used method in the fats and oils industry and can be performed as a batch process or as a continuous process with high yields. CSE has a 90–98% oil recovery rate but requires high energy, high investments, and the solvent most often used (*n*-hexane or other petrol derivates) is hazardous and can cause health problems with prolonged exposure.

The quality of the raw fats matter and cake depends mainly on the type of solvent used, the reaction temperature and the type of pre-treatment of the matrix. Several studies are currently underway to make the solvent extraction system more environmentally sustainable.

For greater awareness about energy consumption, environment pollution, and health issues have made it imperative for the oil industries to search for better techniques that are suitable, advanced, and eco-friendly than conventional oil extraction methods (Rani et al., 2021). New extraction techniques like enzyme assisted extraction (EAE), ultrasound assisted extraction (UAE), or microwave assisted extraction (MAE), promise to improve production yields while lowering the environmental impact of the fat supply chain (Nde and Anuanwen 2020; Rani et al., 2021).

1.3.2 Refining process

Term “refining” in Europe defined the whole series of processes that will be shortly described in this paragraph, however, in the USA and other country, the terms “refining” it is associated to the step with removal of free fatty acid.

The crude oil is a mixture of triacylglycerols along with the minor components, undesirable pigments, oxidation products, metals and a huge part of solvent. Principal purpose of refining process is removing unwanted impurities and preserve a suitable level of desirable minor component and provide a final product with stable shelf life. The refining process (Figure 2) can be chemically of physically performed. In brief more important chemical refining steps are reported below:

- ***Degumming*** involves treatment with water or acid (phosphoric or citric) solution that led the separation of “gum” containing phospholipids that can be separated with a centrifuge operation. The phospholipids are recovered as crude lecithin with high commercial value. Degumming is usually linked with extraction rather than with the subsequent refining processes.

Most challenging degumming process is related to “non-hydratable phospholipids” (NHP). Various de-gumming process are used in the vegetable oil industry like water de-gumming, acid conditioning, acid de gumming, super de gumming and ultra degumming.

- ***Neutralization*** frequently requires treatment with aqueous alkali to remove FFAs. Some oils are lost along with the soaps in a water stream. Free acids are also removed during deodorization by steam distillation (physical refining). This latter method is preferred to reduce environmental impact (Gupta 2008).
- ***Bleaching***, present only in the chemical refining process, can be performed from 80°C to 180°C with acid-activated bleaching earth; it is the most expensive refining step considering the price of bleaching earth and disposing of spent earth. The process is designed to remove pigmentation composed by carotenes (hydrocarbons group) and chlorophyll. Blanching is a careful process; under acidic conditions free sterols may be dehydrated to steradienes and *cis* bonds in fatty acids may change to the *trans* form. Polycyclic aromatic hydrocarbons (PAH) when present in the oil are not removed by bleaching (Tom Verleyen et al. 2002).
- ***Deodorization*** is the final refining step and requires the oil to be washed with steam at temperature from 170°C to 250°C under vacuum and it is focused on the remove off-flavor. At higher temperatures (more than 240°C) there is isomerization of *cis* to *trans* bonds so highly unsaturated oils should be deodorized at the lowest possible temperature. Deodorized distillate is itself a valuable by-product that serves as a source of sterols and tocopherols (Sabah 2007).

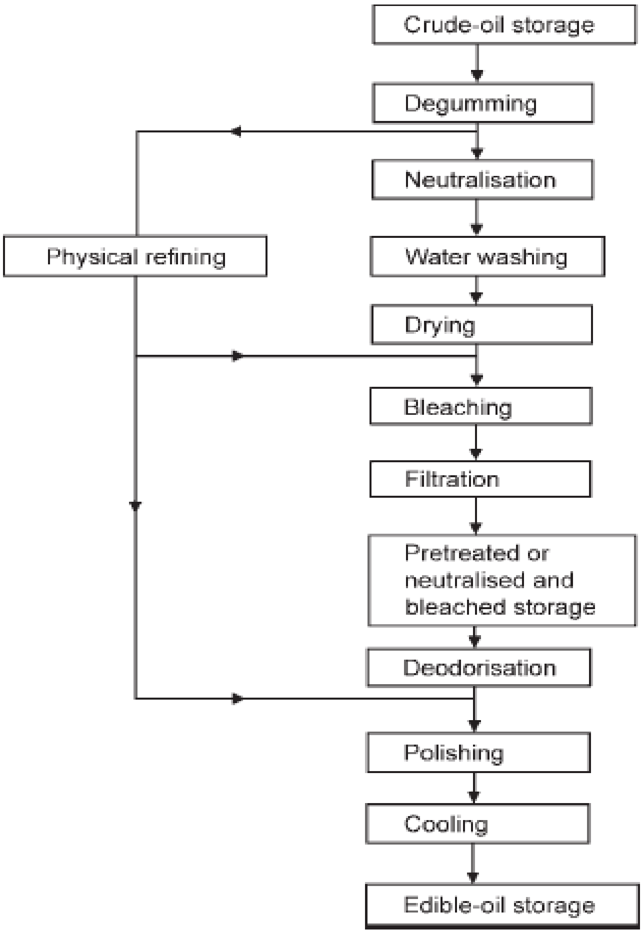

Figure 2: refining process for vegetable fats matters; image edited by Hamm et al., 2013

A recent study showed that high time temperature deodorization process leads the formation of undesirable component named dialkyl ketones (Salgarella et al., 2021) that are usually related to a randomized interesterification events.

1.3.3 Modification of fats matter for food purpose

All oil modification processes are focused on change of physical behaviors and structural proprieties of fats matters, so the modification technique differed from the extraction and refining process because the last is oriented towards improved organoleptic proprieties, nutritional value and increase shelf life.

There are three main modification technologies available in the edible oils industry at present that are usually implemented: the fractionation that is a fractional crystallization of the oil, followed by a phase separation; hydrogenation that involved the reduction of degree of unsaturation on the acyl chains to improve the saturation degree and iodine value and interesterificatio*n*, both chemical than enzymatical, which provide to an intermolecular redistribution of the acyl groups on the glycerol backbone. In the last decades a new technique to modify the physical proprieties of fats matters has been deep evaluated – the texturization or organogelation.

1.3.3.1 Fractionation

Term "fractionation" in oils and fats industry is usually referring to a process involving *a fractional crystallization* to pursuit a specifical separation of some species or fraction based on melting point to focus on reduction of an undesired component or the intentional concentration of another. Although some specific techniques developed and applied in recent decades, among which molecular distillation and supercritical carbon dioxide extraction two main fractionation technologies are used in the 21st century's edible oil industry:

- *Dry fractionation*: the simplest and economist technology allows it to be used for the production of commodity fats and it is based on the use of specific gradient used to cool the matrix and lead the crystallization.
- *Solvent fractionation*: patented in the 1950s, this involves the use of solvents (hexane or acetone) to allow the high-melting components to crystallize in a very low-viscous organic solvent. This can be helpful with respect to the selectivity of the reaction, but mainly offers advantages in the field of phase separation: much pure solid fractions can be obtained, even with a vacuum filtration (Gunstone 2005; Gupta 2008).

1.3.3.2 Hydrogenation

Originally, hydrogenation of edible oils was mainly used to improve the oxidative stability of oils that contained multiple polyunsaturated fatty acids, such as fish oil. The first outcome regarding this process were provide by the Nobel Prize winner *Paul Sabatier* was the first to work out the hydrogenation chemistry of volatile triglycerides and Wilhelm Normann at the beginning of twenty century (Gunstone 2005).

Hydrogenation allows liquid oils to be stored for much longer and the reduction of unsaturation to lead at considerable increase of melting point and thus an increasing transformation of the oil into fat. Hydrogenation reaction consists in a sequence of reactions that allow the adding of hydrogen atoms (proton) to an acid chain. This basic reaction it is not a pure reaction, and different types of reactions can occur when a fatty acid chain (with double bond) adsorbs on the catalyst surface and dissociated hydrogen is present. It possible that the hydrogenation leads a shifting of the double bond down the fatty acid chain (positional isomerism). This isomerization has little impact on the physical behavior of the oil. At the moment of addition of the first hydrogen-atom and the existence of the 'half-hydrogenated' intermediate, the *sp3*-orbital momentarily allows free rotation over the C-C axis. This randomness behavior permits formation of trans fatty acids (geometrical isomerization). A competition exists between these two reactions: saturation and isomerization (Dijkstra

2012). Process parameters such as temperature, pressure, type (nickel or platinum), purity of catalyst and carrier materials (e.g. zeolite) might to influent the ratio between isomerization and saturation (Gupta 2008). In 2020 partially hydrogenated fats were definitively banned also in USA by Food and Drug Administration (FDA) department following the "zero trans fats" policy (Puligundla et al., 2012).

1.3.3.3 Interesterification

Esterification is a common practice of fats and oils industry to change the physical proprieties of fats matter or their mixtures without altering the chemical structure of the fatty acids acylglycerols. Both intra – molecular acyl residue exchanges (e.g. interesterification) and inter – molecular acyl residue exchange (esterification) occur in the reaction until an equilibrium is reached which depends on the structure and composition of the TAGs molecules (Gunstone 2005). Interesterification is performed both from chemical (CIE) or enzymatical (EIE) pathway (Santoro et al., 2018). The chemical interesterification is performed with a catalyst, 0.05% to 0.15% w/w can be an acid, alkaline or metal compound like sodium ethoxide or sodium methoxide (Gupta 2008) that provide to the reaction. Alkali metal alkoxides like Na-methylate is one of more adopted catalyst for this process. Interesterification is usually carried out in a single phase (liquid). With a nonselective catalyst, rearrangement of the fatty acids will then ultimately result in their statistical or random distribution over all possible positions. Enzymatic interesterification (EIE) is a relatively new method based on immobilized lipases column action with very small amount of water dispersed in a continuous organic phase comprising the reactants and a solvent such as hexane. The catalysts provide a random or stereospecific action depending on the type of enzymes that are chosen. The enzymatic process must be more closely controlled to avoid catalyst inactivity (Danthine et al., 2022). Currently, few industries are involved in the development of study of an EIE system and a translation from scale plant to industrial level is not easy. Sodium ethoxide or methoxide catalyzed batch interesterification is applied more widely (Gupta 2008).

The process can be carried out in conventional neutralizing-bleaching equipment provided with adequate facilities for catalyst dosing and oil drying (Holm and Cowan 2008).

1.3.3.4 Texturization

Rheological proprieties of fats are the results of the combined effects of solid fat content (SFC), polymorphism - different spatial arrangements, and fats crystal network microstructures that are related to shape, size and area fraction of crystals fats (Soleimanian et al., 2020). Full saturated fatty acids composed the "hard-stock" in triacylglycerols (TAGs) system. Hard-stock is responsible of the "start" of network structure; so in many cases, it is more difficult or impossible to eliminate this fraction without sacrificing structural and rheological properties (Marangoni et al., 2020). In the last decade, the study of texturization technique like oleogelation for food industry purpose, has been receiving great attention due to both nutritional and legislative aspects. On the other hand, the negative health effects of saturated and trans fatty acids on human health were intensely discussed (Yi et al., 2017). Numerous studies have shown that excessive intake of these dietetic components can increase the risk of cardiovascular diseases (CVDs) by lowering serum levels of high-density lipoproteins and increasing serum concentrations of low-density lipoproteins (Clifton and Keogh 2017). The organogelation or oleogelation is emerged as a new method of plasticization oil's technology (Yilmaz and Öğütcü 2015). Oleogels used in foods are usually composed of a liquid vegetable oil and GRAS (Generally Recognized As Safe) additives (da Silva et al., 2018a). From a physical point of view, oleogels are viscoelastic, anhydrous and self-supporting materials; they have rheological characteristics like a solid but are formed in highest amount (>90%, w/w) of a liquid matter (e.g., vegetable oil). From a thermal point of view, oleogels are structured and thermos-reversible substances. The additives or "oleogelators" lead to the formation of a three-dimensional super-molecular network (Doan et al., 2015). The oleogelators can be divided into two broad categories: low molecular weight oleogelators (LMOGs), small molecules such as fatty acids and waxes and high molecular weight oleogelators (HMOGs) obtained by molecular network of polymers produced by chemical reactions or physical interactions (Palla et al., 2017) Therefore, based on a correct melting and structuring behavior, the oleogels can provide a suitable alternative in different processed foods, such as chocolate and spreads (Patel et al., 2020) , ice cream (Zulim Botega et al., 2013) and biscuits (Hughes et al., 2009).

1.4 Quality parameter in food lipids production

During all steps of supply chain of fat matter production, starting from harvesting, continuing to extraction, refining, and modification process (but also during transport and storage), lipids must be protected against deterioration and adulteration. Since this is most likely to be the consequence of hydrolytic or oxidative change requiring water and oxygen, respectively, these materials should be excluded as far as possible. Such changes are temperature dependent and oxidation can be promoted by light and by some metals, thus oils and fats must therefore always be handled under appropriate conditions (Gunstone 2009). To assay the quality and authenticity of fats and oils a several privates and public agency had promoted a robustness group of regulatory guidelines supported by several official methods that provide to assay all the chemical and physical parameter useful for the commercial categorization of edible oils and fats. The major agencies are reported below:

- AOCS	*American Oil Chemists' Society*
- AOAC	*Association of Official Agricultural Chemists*
- COI	*International Olive Council* – only for olive oil
- IUPAC	*International Union of Pure and Applied Chemistry*
- UNI	*Ente Nazionale Italiano di Unificazione (UNI EN ISO)*

1.4.1 Common quality assurance parameters

Vegetable oil is analyzed during different steps of processing. Each analysis provides specific information to the processor as well as to the users. The most commonly analysis in oil processing plants are reported following with brief descriptions.

- *Iodine Value* (IV) is evaluated to determines the degree of unsaturation in the oil. The results are expressed as grams of iodine absorbed per 100 g of the oil sample. Oils with higher unsaturation show higher *IV* values. To reduce the formation of *trans* fatty acids in hydrogenated fats, only a process that leads an *IV* equal to 0 can provided the absence of fatty acids.
- *Free Fatty Acids* - FFAs are expressed as percent *oleic acid* for seed oils and it is expressed as percent *palmitic acid* for palm oil and palm oil derivatives. For palm kernel or coconut oils it is expressed as percent *lauric acid*. After a refining process the value is up to zero (<0.5%), instead for extra virgin olive oils the value is < 0.8%.
- *Acid Value* for oil is the number of mg of potassium hydroxide required to neutralize the free acid in 1g of the oil.
- *Peroxide Value (PV)* assays the primary oxidation products in oil. The fatty acid can be in free or esterified form. This method measures all substances in the oil, which oxidize potassium iodide and are expressed as milliequivalents of peroxide per 1kg oil or fat.
- *para-Anisidine Value* (*p*AV) measures some of the secondary oxidation compounds of oils and fats generated from the decomposition of the peroxides. Specifically, 2-alkenals and 2,4-dienals are measured by this method. Freshly deodorized oil may have a *p*AV content of 2–6.
- *Solid Fat Index (SFI)* - it is an empirical measure of solids fats content evaluated by dilatometric method which determines the combined volume of solid and liquid in the sample at specific temperatures. The information is used in formulating shortenings, margarines, and spreads.
- *Solid Fat Content (SFC)* - carried out by a Nuclear Magnetic Resonance Spectrometry (NMR) method focused on the estimation of amount of fat solids present in the oil (fat) sample at specific temperatures.
- *Fatty Acid Composition* - this method is focused to the identification of fatty acids in a fat or oil by analysis of fatty acid methyl esters (FAME) by capillary gas–liquid chromatography.
- *Unsaponifiable Matter* - how reported above, the unsaponifiable matter is composed by substances frequently found dissolved in fats and oils, which cannot be saponified by the usual caustic treatment, but are soluble in ordinary fat and oil solvents. Included in this group are high aliphatic alcohols, sterols, pigments, and hydrocarbons. Punctual gravimetric determination of total unsaponifiable matter gives a fast information about the oils and fats raw materials.

However, there are other three unusual analysis that can lead more information about the quality of fats matter and future technological use of raw materials:

- *Determination of triacylglycerols profile* – where TAGs groups having the same carbon number are separated by direct gas/liquid chromatography using a packed column under temperature programmed condition. Based on peak area measurements, TAGs content, by carbon numbers, is expressed as a percentage relative to the total triglycerides content of the sample.
- *Determination of fatty acids sn2 profile* – useful to evaluate the presence of fraudulent admixture. It is a method based on pancreatic lipase reaction, separations on thin layer chromatography (TLC) and analysis by gas liquid chromatography for the determination of the percentage of fatty acid esterified at 2-position of the triacylglycerols (AOCS Official Method Ch 3-91).
- *Determination of Mono- and Di- glycerides* – focused on determination of MAGs and DAGs converted in trimethylsilyl ether and analyzed by capillary GLC solution. The result is expressed like a percentage related to total mass of triglycerides (Gupta 2008).

Other powerful chemical analysis should be performed on lipids to assay quality and safety of fats matters like the evaluation of mineral oil saturated hydrocarbons (MOSH) and aromatic hydrocarbons (MOAH) (Bauwens et al., 2023) originating from petroleum distilled products, the evaluation of 3-monochloropropane 1,2-diol (3-MCPD) and glycidyl esters (GE) content (Sevindirici et al., 2018) or the evaluation of the presence of dialkyl ketones (DAKs) (Mascrez et al., 2021; Santoro et al., 2018) in different industrial fats matter to evaluate in deep the process and the quality of the raw materials and the final refined products.

1.5 Lipids for confectionary industries

The term "*confectionary products*" describe goods with high commercial value usually composed by high percentage of sugar, carbohydrates and lipids. A wide variety of products are included like spreadable cream, chocolates, cookies, bars, gummies, mints, and others. The confectionary market is a billion business that involved several local and international company with a structured supply chain with several stakeholder and business partner. The global confectionery market size was valued at USD 298.4 billion in 2022. It is estimated to reach USD 421.05 billion by 2031, growing at a compound annual growth rate (CAGR) of 3.9% during the forecast period (2023 – 2031) (https://straitsresearch.com/).

Even if the confectionary industry covered a several type of goods, the chocolate derivates represents a biggest percentage of the confectionary market. The global chocolate market size was estimated at USD 119.39 billion in 2023 and is anticipated to grow at a CAGR of 4.1% from 2024 to 2030 (https://www.researchandmarkets.com).

Chocolate industries based their business over the cocoa – *Theobroma cacao* L.– and its derivates like cocoa liquor, cocoa powder and cocoa butter.

The historical uncertainty in the cocoa butter supply and the volatility in cocoa butter prices depending on fluctuating cocoa bean prices forced confectioners to seek other alternatives, which may have a stabilizing influence on the prices of cocoa butter. The continuous need to find new cheap vegetable fats to replace cocoa butter in chocolate and confectionery products has always been a great challenge for the fats supply chain which has always responded with intelligence and cunning. These reformulated fats have become known by the term *hard butters* that were developed using common fats matter like palm kernel, coconut, palm, and other tropical oils such as shea and illipe. The processes involved in producing included hydrogenation, interesterification, solvent or dry fractionation, and blending. The hard butters can be divided into the following three main groups based on their characteristics and raw materials used to produce them:

- *Lauric cocoa butter substitutes (lauric CBS)*: fats that are incompatible with cocoa butter but that have physical properties resembling those of cocoa butter.
- *Non-lauric cocoa butter substitutes (non-lauric CBS)*: these are fats that are partly compatible with cocoa butter chemical composition.
- *Cocoa butter equivalents or extenders (CBE)*: these are fats that are fully compatible with cocoa butter and shows chemical and physical properties similar to those of cocoa butter.

Other terms usually used to describe *hard butters* include *cocoa butter partial replacers, total replacers*, *modifiers*, and *extenders.* All of these categories can be further subdivided into a range of specialty fats, tailored to suit particular purposes (Shukla and International 2005).

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1.6.1 Link

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CHAPTER 2

The aim and structure of the dissertation

As reported above, fat substances world is wide and complex. These raw materials can be potentially affected by countless issues in terms of quality and authenticity, thus raising great interest in the scientific community and private stakeholders (e.g. food companies).

In particular, vegetable oils and fats overtook animal fats in terms of consumption mainly due to material availability, cost, respect for animal welfare and increasing consumer awareness of health and well-being issues.

As explained in Chapter 3, vegetable fatty substances often undergo a whole bunch of different processes (such as refining) and subsequent storage. These processes have a direct impact on their composition and may lead to the neoformation of hazardous compounds which could damage health.

Consequently, fat matrices must fulfill the highest levels of quality, which is routinely tested as elucidated in Chapter 4 with the evaluation of different parameter like peroxide value (PV), acid value and iodine value, but often the investigation require higher-performance analysis.

In details, the research project has been organized in two different parts, after a careful deep review of the official methods and scientific literature:

i) *The development and implementation of an innovative analytical method to ensure new parameters for quality and authenticity of fats matters as well as a safer working environment for operators*

In the first part, the research has been focused on the improvement of chromatographic technique to investigate the acylglycerol and unsaponifiable profile (sterols and oxysterols).

Firstly, triacylglycerols regioisomery has been identified as the key aspect to obtain fats with desired properties and to ensure the presence or absence of interesterification fraction. Secondly, a great study has been carried out on the evolution of the elective analytical technique high-performance liquid chromatography (Ag+-HPLC) coupled with mass spectrometry solution (MS) to transform it into a safer and more usable method (Chapter 3).

Finally, regarding to the unsaponifiable fraction – phytosterol oxidation products (POPs) – have been evaluated to ensure food quality and safety. A depth study has been carried out on the presence of oxidized phytosterols as related to refining process (Chapter 4).

ii) *The evaluation of innovative and possible modification – texturization of uncommon fat substance*

The second part of the project has been focused on new technics for rheological modification of a not well-known fatty substance, i.e. durum wheat oil, from unconventional source (by-product). This has been done by exploiting different oleogelators (bee waxes and carnauba waxes). In fact, oleogelation, as described in Chapter 1 represents the most suitable technique to increase the application field of liquid oils rich in polyunsaturated fatty acids.

CHAPTER 3

Preface

Triacylglycerols, triglycerides or triacyl-sn-glycerols are the main and most abundant class of lipids composed by glycerol moiety in which the three hydroxyl groups are esterified with fatty acids.

Depending on fatty acid structure and position on the glycerol backbone, triacylglycerols naturally present a number of different structures, including regioisomers and/or enantiomers, creating a serious challenge for efforts to determine their molecular and intramolecular structures. Additionally, thermal processes applied for technological reasons e.g., deodorization and some manipulation technique, e.g. interesterification performed through chemical pathway, using a catalyst or enzymatical pathway using enzymes, lead the increase of positional isomers.

Historically, analytical methods developed for regioisomers analysis can be divided into two major groups *(i)* indirect approaches (e.g., methods using a preliminary degradation step by enzymatic hydrolysis) *(ii)* direct approaches (e.g., liquid chromatographic separation with $AgNO_3$ loaded column coupled with mass spectrometric detector).

Since the end of 1980, when the first article was published with an efficient procedure for loading $AgNO_3$ onto a commercial silica gel column, all other "time-consuming methods" have been shelved. Most of published methods used silica columns with 10% AgNO3 loaded and a complex solvent gradient of acetonitrile or ethyl acetate, n-hexane and toluene for resolving regioisomeric TAGs.

Although simple and quick, due to the instruments and safety of the solvents used this technique is not widely used. The interaction between solvent, functionalized stationary phase and analytes has not yet been fully resolved.

Impact of column temperature on triacylglycerol regioisomers separation in silver ion liquid chromatography using heptane-based mobile phases

3.1 Introduction

Triacylglycerols (TAGs) are the most abundant molecules in fats and oils, generally composing more than 95% of the total matrix (Gunstone 2009). Their structure consists of a glycerol backbone esterified with three fatty acids (FAs), and the number of possible FAs combinations on the glycerol skeleton confers to TAGs a very large variability. On a theoretical base, the number of total possible TAG isomers is given by n 3, where n is the number of different FAs, while the total number is reduced to $(n^2+n^3)/2$ without considering enantiomers, and to $(n3+3n^2+2n)/6$ without considering isomers (Rezanka et al., 2023). These numbers are in practice reduced according to the biosynthesis preferences (Indelicato et al. 2017). The regioisomeric configuration of TAGs (i.e., how acyl chains are disposed amongst the sn -1(3) and sn -2 positions of TAGs (Kalo and Kemppinen 2012), is of utmost importance in the food industry, particularly in the confectionery industry, since it affects the crystallization and polymorphic properties of fats, impacting the texture and mechanical properties of the final food product. There have been numerous approaches to TAG regioisomers characterization (Dobson et al., 1995; Momchilova and Nikolova-Damyanova 2022) with high-performance liquid chromatography (HPLC) techniques being the most effective (Indelicato et al. 2017). Among the different HPLC separation mechanisms, particularly widespread for TAG analysis is silver ion (Ag^+) HPLC (Ag^+ -HPLC), which uses columns coated with immobilized silver cation to form weak and reversible complexes with the π-electrons of the double bonds of eluting analytes. Nevertheless, the elution properties and separation capability of this column are highly affected by not only the mobile phase composition but also the elution temperature (Lísa et al., 2013). Comparing the two most frequently used mobile phases, namely chlorinated-based and hexane-based, both added with acetonitrile (ACN) as a polar modifier, it has been shown that only the latter provides resolution of the regioisomers (Lísa et al. 2013). Moreover, although column temperature programming as a control tool for retention times and resolution is rarely used for HPLC, different research groups have studied its potential in the case of Ag^+-HPLC, and have shown that temperature changes could have a strong impact in particular on the retention of polyunsaturated TAGs when hexane-based mobile phase is used (Adlof 2007; Lísa et al. 2013). At present, most of the studies investigating TAGs in Ag^+-HPLC used a hexane-based mobile phase and investigated different polar modifiers (e.g., proprionitrile and/or butyronitrile) (Harfmann et al., 2008; Momchilova and Nikolova-Damyanova 2012). Nevertheless, the attention towards greener analytical chemistry during the last years requires the revision of analytical protocols to minimize or promote the use of more sustainable and less toxic solvents. In this regard, it is desirable to replace hexane with less toxic solvents, such as heptane (Alfonsi et al. 2008). It should be stated that both hexane and heptane are toxic. However, hexane is more volatile, can cause peripheral neuropathy, and is more neurotoxic than heptane (Buddrick et al. 2013). Although the stability of the heptane-ACN mixture as a mobile phase for TAG analysis was comparable to hexane-ACN (Harfmann et al. 2008), the effect of analysis temperature has never been investigated in this alternative mobile phase. The aim of this work was, therefore, to investigate the use of heptane in place of hexane and its effect on the temperature - related behavior in the TAGs elution. The attention is mainly focused on TAGs regioisomers common in the confectionery industry, which have not received much attention previously, and, in particular, on the main TAGs present in cocoa butter, a natural fat of particular relevance because of its unusual and highly valued physical properties and its specific TAG composition (Santoro et al., 2018). Column temperatures ranging from 5 to 35°C were tested analyzing both a TAG standard mixture and a chemically interesterified (CIE) cocoa butter. Furthermore, the suitability of the heptane-based mobile phase was demonstrated on three other vegetable fats: palm olein, CIE palm olein, and CIE shea olein.

3.2 Materials and method

3.2.1 Reagents

n- Heptane, n -hexane, and 2-propanol HiPerSolv CHROMANORM® and anhydrous sodium sulphate AnalaR NORMAPUR® were purchased from VWR (United Kingdom). ACN LiChrosolv® was supplied by Merck (Darmstadt, Germany). All TAG standards (steaoryloleoylsteaoryl glycerol [TAG-$C_{54:1}$, SOS], steaorylsteaoryl-oleoyl glycerol [TAG-$C_{54:1}$, SSO], palmitoyl-oleoyl-steaoryl glycerol [TAG-$C_{52:1}$, POS], palmitoyl-steaoryl-oleoyl glycerol [TAG- $C_{52:1}$, PSO], palmitoyl-oleoyl-palmitoyl glycerol [TAG-$C_{50:1}$, POP], palmitoyl-palmitoyl-oleoyl glycerol [TAG-$C_{50:1}$, PPO], steaoryl- oleoyl-oleoyl glycerol [TAG-C 54:2 , SOO], steaoryl-oleoyl-oleoyl glycerol [TAG-$C_{54:2}$, OSO], palmitoyl-oleoyl-oleoyl glycerol [TAG- $C_{52:2}$, POO], and palmioyl-oleoyl-oleoyl glycerol [TAG-$C_{52:2}$, OPO])) were provided by Larodan (Sweden) (P: palmitic acid, C16:0 ; S: stearic acid, C18:0; O; oleic acid, C18:1n9c). The palm olein, the CIE cocoa butter, the CIE palm olein, and the CIE shea olein samples were provided by the Soremartec Italia srl company.

3.2.2 Sample preparation

The TAG standards, the CIE cocoa butter, the palm olein, the CIE palm olein, the CIE shea olein samples were first heated until melted and then mechanically homogenized. The TAG standard mix was prepared by adding each TAG standard to n-heptane up to a concentration of 10 mg/kg (total TAG concentration being 100 mg/kg). The CIE cocoa butter, the palm olein, the CIE palm olein, and the CIE shea olein samples were dehydrated with Na_2SO_4 and filtered through a Buchner funnel. It was then diluted in n-heptane up to a concentration of 5000 mg/kg.

3.2.3 Silver-ion HPLC–APCI (+) -MS

Silver-ion HPLC–MS experiments were performed on a liquid chromatograph HPLC Ultimate 3000 Agilent (Thermo Fisher Scientific, Milan, Italy) coupled to a linear ion-trap mass analyser LTQ XL (Thermo Fisher Scientific, Milan, Italy) located in the laboratory of Soremartec Italia srl company. Positive-ion APCI was used in the mass range 500 – 1000 Th with the ionization source heated at 450°C. The capillary temperature was set at 250°C, the flow rate of sheath gas and auxiliary gas were set at 35.0 and 15.0 arbitrary units, the capillary voltage was 25.0 V, the source voltage 6.0 kV, and the tube lens 110.0 V. Extracted ion current (EIC) chromatograms of protonated molecules and fragment ions were used to support the identification and data extraction of coeluting peaks. The separation was achieved on a ChromSpher Lipids column (250 mm ×4.6 mm, 5 μm particle size; Agilent Technologies, Italy) in isocratic mode with a mobile phase composed of I) 99.8% (v/v) n- heptane or n -hexane, 0.1% (v/v) 2-propanol, 0.1% (v/v) ACN or II) 99.4% (v/v) n -heptane or n -hexane, 0.3% 2-propanol (v/v) and 0.3% ACN (v/v) and a flow rate of 1 mL/min.. The injection volume was 10.0 μL. HPLC experiments were performed in four replicates at column temperatures of 5, 10, 15, 25, 30, and 35°C for both the standard TAG and cocoa butter sample using the elution I with heptane, while they were performed in triplicate for the comparison of hexane- and heptane-based mobile phases using the standard TAG mixture for the other elution tests, employing either elution I or elution II. The column was conditioned 40 min before each analysis with the same mobile phase and flow, at a column temperature corresponding to one of the following analyses. Blanks (containing n-heptane or n-hexane) were performed following conditioning for each of the given column temperature.

3.2.4 Data elaboration

Data acquisition and analysis were performed using the Thermo Scientific Xcalibur software 4.0. The m/z windows set for the EIC chromatograms were 889.20–890.20 for SOS/SSO, 861.20–862.20 for POS/PSO, 833.20–834.20 for POP/PPO, 887.20–888.20 for SOO/OSO and 859.20–860.20 for POO/OPO. The studied chromatographic parameters for each peak were the retention times (in minutes), the area, the height, the width at 50% height, and the tailing factor (TF) at 10%. The TF was only measured at 5 and 35°C, and a Student's t – test was performed on the results. The resolution was calculated between each couple of regioisomers based on their retention times and peak widths at 50%. Additionally, the number of theoretical plates of the column was calculated for each studied temperature (Cazes and Scott 2002).

3.3 Results and discussion

3.3.1 Hexane- *vs* heptane- based mobile phases

A comparison of hexane- and heptane-based mobile phases was performed for the analysis of a standard mixture of TAG regioisomers and using 0.1 or 0.3% ACN. The amount of ACN was 0.1% as for the optimized internal method, while the value of 0.3% was derived from the calculation of the ACN percentage at the elution time of AOA according to the work of Lísa et al. (Lísa et al. 2013) (the value is an estimation due to the unknown dwell volume of the system used). As can be observed in Tables 1, S1 of the supplementary material, Tables 2 and S2, the retention times (RTs) and resolution among the regioisomers at 10 and 30°C and using 0.1 or 0.3% ACN are relatively alike using hexane or heptane, with resembling chromatograms (Figure 1). In statistical terms, the difference in RTs appears to be significant ($p<0.05$). However, this difference is primarily attributable to the unusually high inter-batch variability (as evidenced by the coefficients of variation of RTs, which range from 15 to 25%, as shown in Table S3 of the supplementary material), despite the relatively low overall intra-batch variability. Since the trials with heptane- and hexane-based mobile phases were conducted on different days, it cannot be excluded that any observed statistical difference is a result of the inter-batch variability rather than a consequence of the change in the mobile phase, which, in any case, guarantee a satisfactory resolution of all the TAGs. In both types of mobile phases, all TAGs were more retained at 30°C than at 10°C, with a temperature effect three times stronger with 0.1% ACN than with 0.3% ACN. Indeed, for 0.1% ACN, on average, the RTs of monounsaturated and di-unsaturated TAGs were 58 and 113% higher at 30°C (than at 10°C), respectively, while these percentages were of 16 and 36% when 0.3% ACN was used (Tables 1 and S1). No measurements of resolution are reported for the systems using 0.3% of ACN as all regioisomers coeluted.

The increase of TAGs' RT at higher temperatures confirms that changing hexane by heptane in the mobile phase does not suppress the increased retention behavior at higher temperatures previously described (Adlof 2007; Lísa et al. 2013). The mechanisms behind this unusual LC retention behavior have not yet been fully understood. It has been suggested that the increased TAG retention behavior could be due to the temperature-dependent stabilities of the silver-ion complexes with unsaturated TAGs and ACN; these two types of complexes being competitively formed during the separation process (Adlof and List 2004).

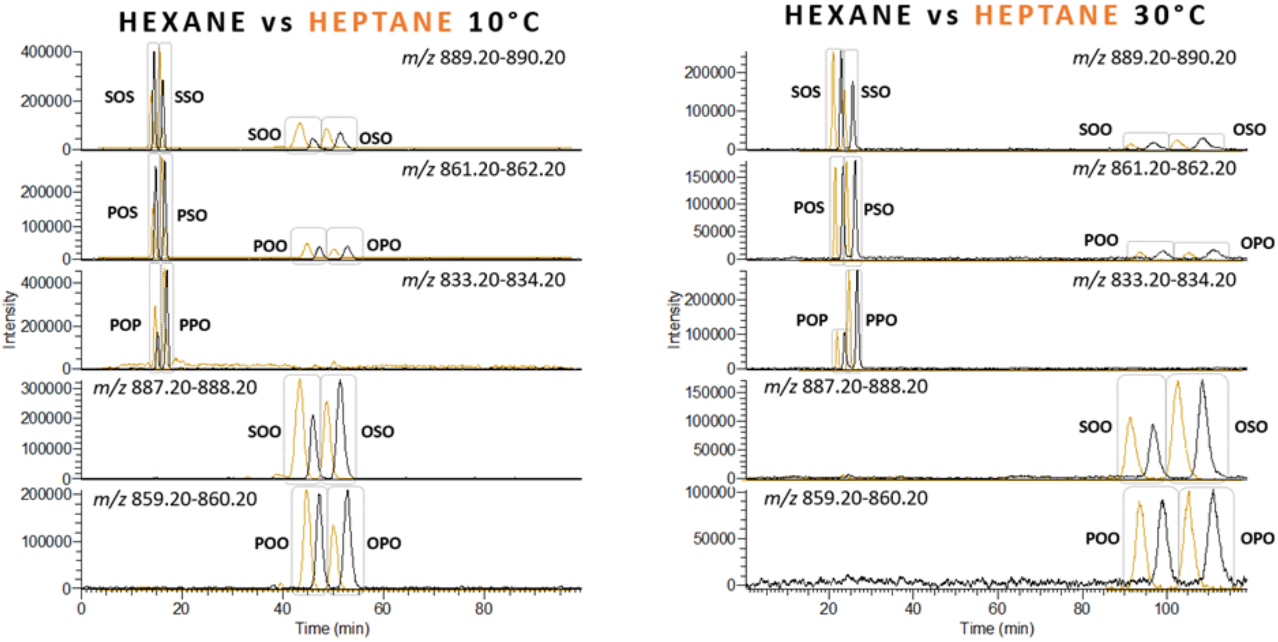
Table 1: Comparison of retention times (RT, in minutes, mean of 3 replicates) of triacylglycerols at different column temperatures and acetonitrile (ACN) concentrations in hexane- (C6) and heptane- (C7) based mobile phases. ΔRT is calculated as (RTC7-RTC6)/RTC6.

	10°C						30°C					
	0.1% ACN			0.3% ACN			0.1% ACN			0.3% ACN		
	C6	C7	ΔRT	C6	C7	ΔRT	C6	C7	ΔRT	C6	C7	ΔRT
SOS	14.5	14.0	**-4%**	4.4	4.7	**6%**	23.4	21.8	**-7%**	4.9	5.6	**12%**
SSO	16.3	15.7	**-4%**	4.4	4.7	**7%**	26.3	24.5	**-7%**	4.9	5.6	**12%**
POS	14.9	14.4	**-3%**	4.4	4.8	**7%**	23.8	22.3	**-7%**	5.0	5.8	**14%**
PSO	16.7	16.2	**-3%**	4.4	5.2	**15%**	26.8	25.2	**-6%**	5.0	5.9	**15%**
POP	15.2	14.8	**-3%**	4.5	4.9	**7%**	24.3	22.8	**-7%**	5.1	6.0	**14%**
PPO	17.1	16.6	**-3%**	4.5	4.9	**7%**	27.3	25.6	**-7%**	5.1	6.0	**14%**
SOO	46.6	43.9	**-6%**	5.4	6.1	**12%**	101.8	91.3	**-11%**	6.8	8.5	**21%**
OSO	52.0	49.4	**-5%**	5.4	6.1	**12%**	114.2	102.1	**-12%**	7.0	9.0	**22%**
POO	47.8	45.3	**-6%**	5.5	6.2	**12%**	103.9	93.6	**-11%**	6.9	8.7	**21%**
OPO	53.5	50.6	**-6%**	5.5	6.2	**12%**	116.8	104.9	**-11%**	7.1	9.2	**22%**

Table 2: Comparison of the resolution (Res, mean of 3 replicates) of triacylglycerol regioisomers in hexane- (C6) and heptane- (C7) based mobile phases at different temperatures. ΔRes is calculated as (ResC7-ResC6)/ResC6.

	0.1% ACN – 10°C			0.1% ACN – 30°C		
	C6	C7	ΔRes	C6	C7	ΔRes
SOS/SSO	2.0	1.7	**-15%**	2.7	2.8	**6%**
POS/PSO	2.0	1.7	**-20%**	2.8	3.2	**14%**
POP/PPO	2.0	1.8	**-11%**	2.6	3.0	**12%**
SOO/OSO	2.1	1.8	**-18%**	2.6	2.3	**-10%**
POO/OPO	2.1	2.0	**-8%**	2.7	2.6	**-5%**

Figure 1: Extracted ions chromatograms of triacylglycerol regioisomers in hexane- (black) and heptane-based (orange) mobile phases (0.1% ACN, 0.1% 2-propanol) at 10 and 30°C of column temperature.

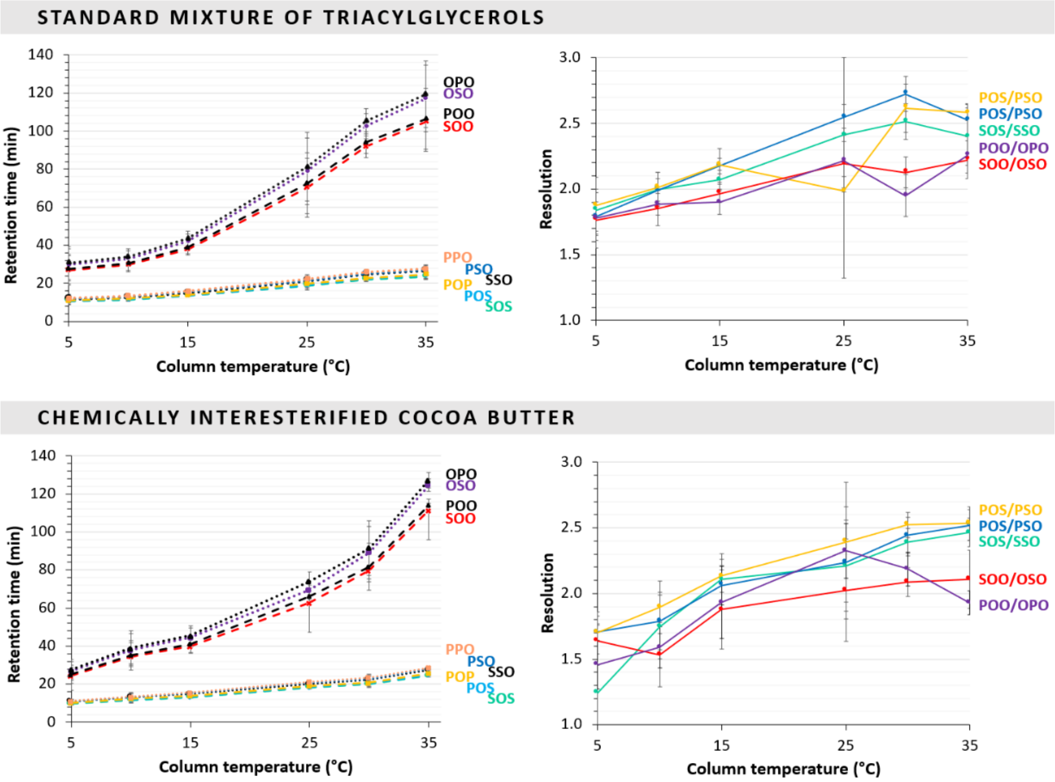


More specifically, the hypothesis made by Adlof and List in 2004 was that Ag$^+$-ACN complex could be exothermic and therefore less stable at higher temperatures (Adlof and List 2004).This hypothesis is supported by the fact that in pure hexane mobile phases TAGs tend to elute faster at higher temperatures (W.W. Christie & X. Han,. 2010). Furthermore, considering the observations made in this study, particularly the results in Tables 1 and S1, it also appears that the strength of the temperature effect is linked to the concentration of ACN in the mobile phase. The change of RTs is indeed higher between two temperatures when a lower (but not zero) ACN concentration is used. This could be explained by the chemical equilibria existing between the ACN found in the solvent and the one complexed to the silver ions (ACN-Ag^+). When the ACN is present in the solvent at low concentrations and the temperature of the system is increased, the dissociation of the exothermic ACN-Ag^+ complex could be more favorable compared to higher ACN concentrations because of the potential antagonistic effect caused by the higher ACN concentration, favoring ACN-Ag^+ complex formation (thus disfavoring its dissociation). This would also explain why in the study of Adlof and List, when they varied the column temperature from 10 to 30°C with 1% of ACN in hexane, POP RTs increased by 11%, whereas in our case, the increase was of 60% with ten times lower ACN concentration (0.1%) and approximately 13% when 0.3% ACN was used. Because of the similar elution behavior of TAGs in both hexane- and heptane-based solvents, it is possible to conclude on the equivalence of heptane and hexane for TAG analysis in Ag^+-HPLC, which means they can be used interchangeably without significantly impacting the separation behavior and performances.

3.3.2 Effect of column temperature in heptane-based solvents

The effect of column temperature on the separation of TAG regioisomers was assessed on a standard mixture of regioisomers and on a CIE cocoa butter sample using heptane-based mobile phase and 0.1% of ACN to assure a satisfactory resolution of all the TAGs of interest. As shown in Figure 2, the increase in column temperature led to higher TAG RTs in the two studied cases. Di-unsaturated TAGs exhibited both higher RTs compared to monounsaturated TAGs and a higher response to temperature change. However, in both types of TAGs, the relationship linking the RT with the temperature resulted exponential, at least in the 5–25°C temperature range. Indeed, when fitting an exponential model to the mean of the mono- and di- unsaturated curves, the obtained $R^2$was 0.998 for both, while it was lower in the case of a linear model (0.985 and 0.949 for mono- and di-unsaturated TAGs, respectively). When it comes to the resolution, the five pairs of regioisomers studied (SOS/SSO, POS/PSO, POP/PPO, SOO/OSO, POO/OPO) were better resolved at 35°C than at 5°C for both the standard and cocoa butter samples (Figure 2 and supplementary Table S4), with an average increase of 41% for monounsaturated TAGs and 29% for di-unsaturated TAGs.

Figure 2: Evolution of retention times and resolution with column temperature for different triacylglycerol regioisomers from a standard mixture and a chemically interesterified cocoa butter sample using a heptane-based mobile phase (0.1% ACN, 0.1% 2-propanol).

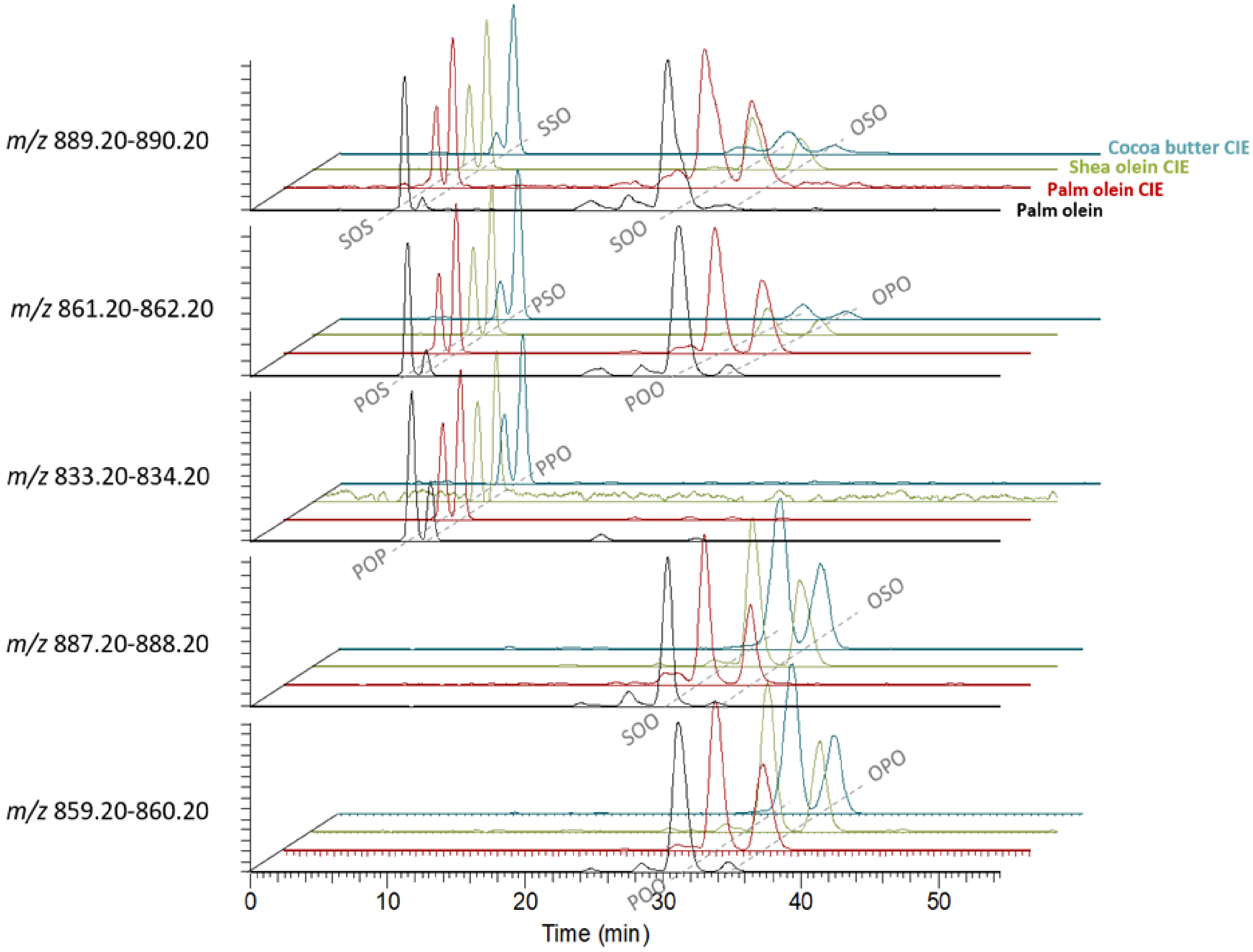


This increase of resolution at higher temperatures was also confirmed by the work of Lísa et al. for a hexane-based mobile phase (Lísa et al. 2013). Nevertheless, the stronger analyte - stationary phase interaction at increasing temperature caused an expected peak broadening that resulted in a reduction of peak height between 5 and 35°C of 55% (with a standard deviation of 3%) and 79% (with a standard deviation of 2%) for mono- and di- unsaturated TAGs, respectively. Obviously, such decrease of height impacts the sensitivity of the method in a negative way. Then, regarding the number of theoretical plates, the value was of ~7100 at 35°C and ~4600 at 5°C (Table S5 in the supplementary material). Finally, the TF was calculated at 5 and 35°C for both samples and was not significantly different between the two temperatures nor between the two samples (Table S6 of the supplementary material).

3.3.3. Analysis of other confectionery fats and oils

As an illustration of the applicability of the method using heptane-based solvents to other matrices, i.e., three additional confectionary fats containing SOS/SSO, POS/PSO, POP/PPO, SOO/OSO and POO/OPO were analyzed, namely CIE shea olein, CIE palm olein and non-interesterified palm olein. All these TAGs eluted in a similar way for the three fat samples, as shown in Figure 3, and there was no substantial difference between the results obtained using standard mixtures of TAGs and fat samples. Furthermore, the mobile phase composed of heptane with 0.1% ACN and 0.1% 2-propanol proved to be well-suited for the separation of the considered regioisomers in confectionery fat matrices.

Figure 3: Extracted ion chromatograms of chemically interesterified (CIE) shea olein, CIE palm olein, CIE cocoa butter and palm olein in a heptane-based mobile phase (0.1% ACN, 0.1% 2-propanol) at 10°C of column temperature. The dashed lines indicate the peaks that are associated to a given TAG



3.4 Conclusion and future perspectives

The present study investigated the replacement of a hexane- by a heptane-based mobile phase in the context of TAGs analysis by Ag^+-HPLC (ChromSpher Lipids column) at different column temperatures. The main focus was on TAGs relevant to the confectionery industry, to which not much attention was previously devoted. A direct comparison of the results obtained with heptane- and hexane-based mobile phases at different temperatures (10 and 30°C) and ACN concentrations (0.1 and 0.3%) allowed to conclude on the equivalence of both solvents when it comes to chromatographic separation of TAGs, thus supporting the use of heptane as a less toxic mobile phase without affecting the chromatographic performance. The particular temperature-related behavior previously observed in hexane-based mobile phases could also be confirmed for heptane-based mobile phases in the present study. In addition, it appeared that the ACN concentration had an influence on the strength of the temperature effect, which was stronger for lower (but not zero) ACN concentrations. What is also interesting is that despite slight differences, probably due to the presence of a low quantity of more polar compounds or to the general poor inter-batch repeatability of the Ag^+-HPLC system, the interesterified cocoa butter sample showed the same general behaviour as the standard mixture. Other studied confectionery fat samples were also tested at 10°C of column temperature, and their regioisomers showed to elute similarly than in the two previous samples. Finally, although an increase in resolution was observed increasing the analysis temperature, it must be underlined that this caused a decrease of the signal intensity due to the peak broadening. Therefore, for the specific TAGs investigated in this study, working at a lower temperature (~10°C) allows for a sufficient resolution between critical pairs and increased sensitivity of the method.

Use of n-heptane as an alternative to n-hexane proved to be a winning choice. The parameters tested, temperature and acetonitrile concentration, show that there is still much to be understood about the interaction between analytes and functionalized stationary phase and elective solvents for this analysis.

The major consideration on analytical evidence leads the awareness regarding the possibility to dismissed the n-hexane (C_6H_{14} CAS n°110-54-3) and replaced with more safety n-heptane (C_7H_{16} CAS n°142-82-5).

This is a stepping stone to prevent the rise of occupational diseases of scientists. Reg. (EC) No. 1272/2008 norms labelling and packaging (CLP) of substances and mixtures, provides a valuable tool to understand the hazardousness of n-hexane, which lead GHS phrase - H372 "*Causes damage to organs through prolonged or repeated exposure*" closely linked to the chemical characteristics of the solvent (e.g. boiling point).

These analytical techniques involve huge consumption of these hazardous solvents, and even in a controlled environment, exposure for the operator is certain. Periodic checks of fume hoods, suction systems and air recirculation with EPA filters must be planned and maintained.

Reducing risk factors protects the analytical operator and the well-being of the laboratory.

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3.6 Supplementary material

Table S1 – Evolution of retention times between 10 and 30°C in different solvent systems (ACN: acetonitrile). $\Delta RT_{10\text{-}30°C} = (RT_{30°C} - RT_{10°C})/\Delta RT_{10°C}$.

	$\Delta RT_{10\text{-}30°C}$			
	0.1% ACN		**0.3% ACN**	
	$\Delta RT_{10\text{-}30°C}$ Hexane	**$\Delta RT_{10\text{-}30°C}$ Heptane**	**$\Delta RT_{10\text{-}30°C}$ Hexane**	**$\Delta RT_{10\text{-}30°C}$ Heptane**
SOS	61%	56%	12%	20%
SSO	61%	56%	12%	19%
POS	60%	55%	13%	22%
PSO	60%	56%	13%	13%
POP	59%	54%	14%	23%
PPO	59%	54%	14%	23%
SOO	118%	108%	26%	39%
OSO	120%	107%	30%	47%
POO	117%	107%	26%	40%
OPO	118%	107%	30%	48%
Mean monounsaturated TAGs	60%	55%	13%	20%
Standard deviation monounsaturated TAGs	1%	1%	1%	4%
Mean diunsaturated TAGs	118%	107%	28%	44%
Standard deviation diunsaturated TAGs	1%	1%	2%	4%
Mean monounsaturated TAGs	**58%**		**16%**	
Standard deviation monounsaturated TAGs	3%		5%	
Mean diunsaturated TAGs	**113%**		**36%**	
Standard deviation diunsaturated TAGs	6%		9%	

Table S2: Tailing factor of triacylglycerol (TAG) regioisomers at 5 and 35°C of Ag-HPLC column temperature. STD: standard TAG sample; CB: chemically interesterified cocoa butter sample.

	SOS		SSO		POS		PSO		POP	
	STD	**CB**	**STD**	**CB**	**STD**	**CB**	**STD**	**CB**	**STD**	**CB**
5°C	1.11	1.76	1.05	1.22	1.16	1.33	1.10	0.95	1.11	1.24
35°C	1.35	1.41	1.04	0.98	1.21	1.40	1.04	0.94	1.25	1.12
T-test p-value	0.1747	0.1134	1	0.01628*	0.6325	0.6404	0.559	1	0.1895	0.4815
	PPO		**SOO**		**OSO**		**POO**		**OPO**	
	STD	**CB**	**STD**	**CB**	**STD**	**CB**	**STD**	**CB**	**STD**	**CB**
5°C	1.10	1.14	1.03	0.95	0.96	0.80	0.94	0.85	0.87	0.85
35°C	1.05	1.07	0.77	0.79	0.84	0.78	0.84	0.81	0.87	0.76
T-test p-value	0.5487	0.4454	0.0046***	0.4561	0.5146	0.752	0.1895	0.7304	1	0.4881

Table S3: Resolution of TAG regioisomers at different column temperatures for a standard and chemically interesterified cocoa butter sample. Mean of 4 replicates.

	Resolution						
	Column temperature (°C)						
	5	**10**	**15**	**20**	**25**	**30**	**35**
SOS/SSO (STD)	1.84	2.00	2.07	1.85	2.41	2.51	2.40
SOS/SSO (CB)	1.24	1.75	2.11	2.07	2.21	2.39	2.47
POS/PSO (STD)	1.79	1.99	2.18	2.05	2.55	2.72	2.52
POS/PSO (CB)	1.71	1.79	2.06	2.47	2.23	2.44	2.52
POP/PPO (STD)	1.87	2.01	2.18	2.04	1.98	2.62	2.58
POP/PPO (CB)	1.70	1.90	2.13	2.54	2.39	2.52	2.53
SOO/OSO (STD)	1.76	1.85	1.97	1.92	2.19	2.13	2.22
SOO/OSO (CB)	1.64	1.53	1.88	1.93	2.03	2.09	2.11
POO/OPO (STD)	1.78	1.89	1.90	1.97	2.22	1.95	2.26
POO/OPO (CB)	1.46	1.59	1.93	1.87	2.33	2.19	1.93

Table S2: Evolution of the resolution among triacylglycerol (TAG) regioisomers between 10 and 30°C in hexane- or heptane-base mobile phases with 0.1% acetonitrile and 0.1% 2-propanol. [A]: batches performed for the comparison study between heptane and hexane, [B]: batches performed for the study of the effect of temperature in heptane-based mobile phases, CIE: chemically interesterified.

	$\Delta\mathrm{Res}_{10\text{-}30°\mathrm{C}}$			
	Standard mixture of TAGs			**CIE cocoa butter**
	Hexane [A]	**Heptane [A]**	**Heptane [B]**	**Heptane [B]**
SOS/SSO	35%	65%	26%	37%
POS/PSO	37%	93%	37%	37%
POP/PPO	34%	69%	30%	33%
SOO/OSO	23%	31%	15%	36%
POO/OPO	28%	32%	17%	38%
Mean monounsat. TAGs	**35%**	76%	31%	36%
Standard dev. monounsat. TAGs	2%	15%	6%	2%
Mean diunsat. TAGs	**26%**	32%	16%	37%
Standard dev. diunsat. TAGs	4%	1%	1%	1%
Mean monounsaturated TAGs	41%			
Standard dev. monounsaturated TAGs	6%			
Mean diunsaturated TAGs	26%			
Standard dev. diunsaturated TAGs	10%			

Table S5: Number of theoretical plates of the ChromSpher Lipids column (250 mm × 4.6 mm, 5 µm) at different temperatures (mobile phase: heptane 99.8%, 0.1% acetonitrile, 0.1% 2-propanol) calculated on a standard mixture of triacylglycerols

Column temperature	**5°C**	**10°C**	**15°C**	**25°C**	**30°C**	**35°C**
Theoretical plates (n=10)	4928	5640	5598	5589	6837	7169

CHAPTER 4

Preface

The refining process of vegetable oils, and their subsequent storage, is necessary to obtain a product that is acceptable to consumers and useful for the food industry. This process entails various stages (degumming, neutralization, bleaching, deodorization etc.); type and intensity of which depend on the type of oil to be treated. In particular, acid earth bleaching and deodorizing at high temperatures can lead important changes in the minor components present in the unsaponifiable fraction. This is the case with sterols, which undergo isomerization with displacement of the double bonds and dehydration with formation of steroidal hydrocarbons of steroidal hydrocarbons (steradienes) and dysteryl ethers. The analysis of these compounds has long been a useful method for detecting the adulteration of virgin olive oils.

Oxidation processes involving phytosterols (β sitosterol, stigmasterol and campesterol) may represent a new tool for evaluating the processes and shelf-life of vegetable fats.

The oxides of phytosterols have long been known and their negative influence on human health has often been linked with the better-studied influence of cholesterol oxides (e.g. 7-keto cholesterol).

Even if the scope and advantage of study of phytosterol oxidation products (POPs) is clear, in particular to manage the better production solution for fats and oils, lipids scientist had been called to resolved huge issue regarding POPs analysis. The lack of official methods and the availability of commercial standards represent the first challenge in the POPs study.

Optimization and validation of an HPLC-HRMS method through semipreparative HPLC system for determining phytosterol oxidation products during refining processing and storage of vegetable oils

4.1 Introduction

Phytosterols can be defined as secondary plant metabolites belonging to the triterpene family with a tetracyclic ring and a side chain linked to the central carbon structure and they are an endogenous component of all plant-origin food ingredients. The phytosterols due to the double bond present in their structure are prone to be oxidized as well as cholesterol (Busch and King 2009a). Several studies hypothesized that cholesterol oxidation products (COPs) play a key role in the development of cardiovascular and neurodegenerative diseases (Malaguti et al. 2019). Others (O'Callaghan et al., 2014; Poli et al., 2013) described several detrimental health effects such as atherogenicity, mitochondrial disfunction, and inflammation due to both cholesterol and phytosterol oxidation. Two main oxidation pathways, enzymatic oxidation and nonenzymatic oxidation are involved in the degradation of phytosterols.

Phytosterol oxides can be exogenously formed through non-enzymatic pathways like autoxidation, that is, by a mechanism involving free radicals and by a non-autoxidation mechanism (Bortolomeazzi et al. 2003). The initial reactions in the process of auto-oxidation of sterols mainly involve the abstraction of a reactive 7-hydrogen-allyl atom forming a free radical, followed by a reaction with molecular oxygen, which gives rise to 3β-hydroxy-5-en-7-peroxyl radicals. These products are stabilized in turn by hydrogen abstraction, producing more stable 7-hydroperoxides. The 7-hydroperoxides can decompose leading to epimeric 7-hydroxysteroids. The epimers of 5,6-epoxysterols are also important secondary oxidation products, but their formation occurs through a bi-molecular interaction involving hydroperoxides and intact sterol (Ryan et al. 2009). Both epimers of 5,6-epoxysterols can be converted to 3β,5α,6β-triol through hydration in an acidic environment. The B-ring of the phytosterols is particularly susceptible to auto-oxidation at high temperatures and gives rise to similar oxidation products such as 7α- or 7β- hydroxytosterols (7α- or 7β-OH), 7-ketosterols

(7-keto) (Soupas et al., 2004), the epoxide derivatives 5α,6α-epoxyphytosterols (α-epoxide) and 5β,6β-epoxyphytosterols (β-epoxide) or their triol end-products (Daly et al., 1983; Soupas et al., 2004). As well reported, mass spectrometry is widely used to determine the POPs; however, since no pure POPs commercial standards except for 7-ketositosterol are available the most common approach used is to correlate the fragmentation of COPs with those of POPs and their relative response (Johnsson and Dutta 2006). Moreover, no study investigated the correlation between COPs and POPs response and the suitability of the above-mentioned approach. Thus, several studies aimed at the synthesis of POPs for qualitative-quantitative aspects. Some authors attempted to couple thermos oxidation of sterols and the chemical synthesis of triol isomers (Menéndez-Carreño et al., 2008). Others, followed the chemical synthesis alone (Dzeletovic et al., 1995); whereas, authors developed an integrated approach using semipreparative liquid chromatography (LC) solution with chemical synthesis focusing just on 7-ketocampesterol, 7-ketositosterol, 7β-hydroxycampesterol, and 7β-hydroxysitosterol (Gao et al., 2013). To determine POPs different analytical techniques were applied. Solid phase extraction (SPE) for isolating POPs, followed by silylation reaction and GC-FID (Scholz et al., 2015) or GC– MS (Hu et al., 2015; Lampi et al., 2002; Menéndez-Carreño et al., 2008) analysis was performed. Again, GC–MS/MS (Alvarez-Sala et al., 2018) was also applied to recognize the POPs, while quantification was performed by GC-FID. Again, the detection of POPs by liquid chromatography coupled to atmospheric-pressure photoionization–mass spectrometry (LC-APPI–MS) was achieved (Grün and Besseau 2016). However, it might be pointed out that above-mentioned approaches display some critical issues. For instance, authors reported a scarce precision (higher than 10%) for SPE purification, which limits the application of the whole analytical procedure when POPs are present at trace levels. Again, the derivatization (silylation) plays a key role in increasing the volatility and sensitivity of the analytes (Halket and Zaikin 2003); however, the yield of the reaction as well as the presence of artifacts must be considered. In fact, to obtain trimethylsilyl (TMS) derivatives at different temperatures (from 40 to 80° C), time of reaction (from 20 to 60 min) as well as different silylating agents were reported (pyridine/hexamethyldisilazane/trimethylchlorosilane mixture; N,O-bis(trimethylsilyl) trifluoracetamide (BSTFA); N-(trimethylsilyl) trifluoroacetamide (MSTFA)) in literature (Cardenia et al., 2012; Hu et al., 2015). On the other hand, LC coupled to low-resolution mass spectrometry (LC–MS or LC–MS/MS) could overcome some critical issues by simplifying the analytical procedure; however, the recognition of untargeted artifacts remains critical. Though, since also high sensitivity, specificity, and robustness are required, LC coupled to high resolution mass spectrometry could lead to better discretize all analytes of interest, especially when complex matrices such as oils and fats are considered. In the present work, the main phytosterols detected in vegetable oils (such as β-sitosterol, campesterol, and stigmasterol) and their main oxidation products were considered. The aim was to isolate pure POPs (5α,6α- epoxy, 5β,6β epoxy, 7-keto, 7α- and 7β-hydroxy isomers of β-sitosterol, campesterol, and stigmasterol) by semipreparative LC system for validating a robust, sensitive, precise and accurate LC-Orbitrap-High Resolution-MS analytical method able to determine POPs. The suitability of the analytical method was then evaluated in palm, palm olein, and high oleic sunflower oils (HOSOs) during the refining processing and storage.

4.2 Experimental procedures

β-Sitosterol (purity >95%; sitosterol) was purchased by Sigma Aldrich (Sigma-Aldrich—St. Louis, US), campesterol (purity >95%), stigmasterol (purity >95%), and 19-hydroxycholesterol (19-HC; purity >99%; internal standard [IS]) were purchased by Larodan (Larodan AB—Retzius, SE). Hyper grade solvent for LC/GC analysis, n-heptane (C_7H_{16}), 2-propanol (C3H8O), and formic acid (HCO_2H) were purchased by VWR (VWR International—Radnor, US); methanol (CH_3OH) was purchased by Supelco (Supelco INC—St. Louis, US). Acetone (CH_3COCH_3), pyridine (C_5H_5N), hexamethyldisilazane (C6H19NSi2), and trimethylchlorosilane (C_3H_9SiCl) were purchased from Sigma-Aldrich (Sigma-Aldrich—St. Louis, US). Low grade methanol (CH_3OH), potassium hydroxide (KOH), citric acid monohydrate ($C_6H_8O_7$ + H_2O) butylhydroxytoluene—BHT ($C_{15}H_{24}O$) and diethylether ((C_2H_5)2O) were purchased from VWR (VWR International—Radnor, US). A commercial mixture of medium chain triglycerides (MCTs) free of sterols and other minor components was supplied from Oleon (Oleon—Ertvelde, Evergem, Belgium).

4.2.1 Preparation of pure phytosterol oxidation products

Five milligrams of pure sitosterol, campesterol, and stigmasterol were separately weighed and dissolved in 5 mL of acetone. The solutions were separately put in open glass petri dishes 5 cm in diameter and dried under a stream of nitrogen at room temperature. That procedure improves the surface area exposure leading to a homogeneous oxidation process. The pure standards were heated in a static oven at 180°C for 90 min without a humidity-controlled system. Then, the samples were cooled in a desiccator containing activated silica gel and dissolved with 4 mL of n-heptane/2-propanol (98:2, vol/vol). The recognition of 5,6α/β-epoxide, 7-keto, and 7α/β-OH isomers in each relative oxidized-sterol mixture obtained by sitosterol (Mix 1), campesterol

(Mix 2) and stigmasterol (Mix 3), respectively, was carried out by HPLC-Orbirtrap-HRMS solution as reported below.

4.2.2 Isolation and collection of POPs

Isolation and collection of selected POPs from oxidized mixtures was carried out by a customized semipreparative liquid chromatography (LC-Prep) system composed of an LC 20AR (Shimadzu—Kyoto, Japan) pump, equipped with five-channel degasser unit, a column hub oven and an autosampler—liquid handler LH-40—that provide injection, collection of fraction, reinjection and a shim-pack UC-Diol II Core Focus as preparative column (250 mm 10 mm ID, 5 µm; Shimadzu,Kyoto, Japan). The LC system was coupled to a photodiode array detector (PDA SPD-M40) and a single quadrupole mass detector (LC–MS 2050) plugged with a DUIS™ probe for online qualification. The best chromatographic separation was reached by injection 1 mL of sample filtered through a 0.22 µm nylon filter and eluted in gradient with n-heptane (solvent A) and2-propanol (solvent B): 0–22.5 min, 1.5% B; at 42– 45 min, 12% B; at 46 min 1.5% B. The POPs UV spectra were acquired in the range 190–370 nm; whereas, about the MS setting, useful to recognize the right fraction to be collected, a positive ionization was performed, and the acquisition was in full scan mode (350–500 m/z) with a desolvation temperature of 250°C. To improve the ionization of analytes, methanol with 0.1% of formic acid (vol/vol) was used. Then, the composition of each collected fraction was evaluated by HPLC-HRMS Orbirtrap® and GC–MS, while the quantification and purity by GC-FID was carried out.

4.2.3 HPLC: Orbitrap—HRMS analysis

Ten microliter of filtered sample (0.22 µm, nylon filter) were injected in an HPLC Ultimate 3000 (Thermoscientific, USA) system coupled with a high-resolution mass spectrometer (HRMS) Exploris 120 (Thermoscientific, USA) with Orbitrap technology equipped with APCI ion source was used. The separation was performed on a Shim-pack UC-Diol II Core Focus column (250 mm 2.1 mm ID, 3 µm, 100 Å pore size; Shimadzu, Kyoto) maintained at 40°C, while the elution was performed using n-heptane (solvent A) and 2-propanol (solvent B) according to the following gradient: 0–15 min 2% B; then at 35 min and up to 40 min 20% B; 40–85 min 2% B. At the end of each chromatographic run, the system was reconditioned for 15 min eluting with n-heptane/2-propanol (98/2, vol/vol). The APCI and ion transfer tube temperature was set at 300°C; the acquisition was in positive and in full scan mode (190–450 m/z) with a resolution of 120,000, 70% of RF lens value and 50% of AGC target value. The MS2 analysis was performed with parallel reaction monitoring (PRM). For MS2 orbitrap resolution was set at 30,000 and the HCD and RF lens are set at 35% and 70%. Precursor ions of POPs were reported in Table 1 and all compounds were qualified by using extracted ion signal. Calibration of the instrument was done by running Calmix® solutions every 24 h of work to improve the accuracy of data.

4.2.4 GC–MS phytosterol oxidation products recognition

From each collected fraction, 200 μL were dried under a stream of nitrogen, silylated (Cardenia et al., 2012), diluted in 1 mL of n-heptane and 1 μL injected (split ratio 1:30) into a Shimadzu GC–MS QP2030 (Kyoto, Japan) equipped with a splitsplitless injector coupled to a single quadrupole mass spectrometer. POPs were qualified according to Leal-Castañeda et al. 2015 with some modifications. The separation was reached by a fused-silica capillary column Restek RTX-5 MS (50 m 0.25 mm I.D. 0.1 μm film thickness; Bellefonte, PA), coated with 95% dimethyl and 5% diphenyl polysiloxane. A deactivated standard split liner (95 mm, 5 mm o.d., 3.4 mm i.d.) was utilized. The oven temperature was programmed from 290 to 330°C at 2C/min and kept for 15 min at 330°C. The injector, ion source, and transfer line temperatures were set at 325, 200, and 325C, respectively. Helium (He) was used as carrier gas at a linear velocity of 37.7 cm/s. The electron energy was set at 70 eV and a mass range from 50 to 600 m/z was scanned at a rate of 3333 amu/s. The acquisition and integration modes were full-scan total ion current (TIC) and single ion monitoring (SIM), respectively. POPs were identified by their retention time and the fragmentation pattern compared with their relative spectra reported in the literature (Dutta and Appelqvist 1997; Johnsson and Dutta 2006).

Table 1: Precursor ions and products ions (m/z) of phytosterols oxidations products (POPs) and their relative abundances (%).

POPs		**Precursor ions (*m/z*)**	**Product ions (*m/z*)**	**Product ions (*m/z*)**
	RT (min)	$M+H^+$	$M-H_2O+H^+$	$M-2H_2O+H^+$
β-sitosterol				
α-Es	12.93	431.3890 **(-)**	413.3785 **(33)**	395.3679 **(100)**
β-Es	16.61	431.3895 **(-)**	413.3792 **(25)**	395.3683 **(100)**
7-Ks	26.01	429.3740 (100)	411.3624 **(45)**	393.3521 **(24)**
7α-Hs	29.63	431.3813 **(-)**	413.3797 **(15)**	395.3685 **(100)**
7β-Hs	30.38	431.3533 (-)	413.3790 **(12)**	395.3682 **(100)**
Campesterol				
α-Ec	15.03	417.3744 **(-)**	399.3638 **(45)**	381.3532 **(100)**
β-Ec	16.95	417.3745 **(-)**	399.3634 **(43)**	381.3539 **(100)**
7-Kc	26.22	415.3578 **(100)**	397.3474 **(45)**	379.3367 **(24)**
7α-Hc	29.77	417.3380 **(-)**	399.3636 **(15)**	381.3526 **(100)**
7β-Hc	30.53	417.3654 **(-)**	399.3627 **(26)**	381.3522 **(100)**
Stigmasterol				
α-Est	15.04	429.3737 **(-)**	411.3633 **(80)**	393.3524 **(100)**
β-Est	16.90	429.3735 **(-)**	411.3630 **(100)**	393.3523 **(75)**
7-Kst	26.26	427.3578 **(100)**	409.3473 **(45)**	391.3366 **(24)**
7α-Hst	29.77	429.3649 **(-)**	411.3628 **(90)**	393.3523 **(100)**
7β-Hst	30.42	429.3645 **(-)**	411.3626 **(73)**	393.3521 **(100)**

Abbreviations: 7-K, 7-keto sterol; 7α-H, 7α-hydroxy sterol; 7β-H, 7β-hydroxy sterol; s, sitosterol, c, campesterol; st, stigmasterol; α-E, α-epoxy sterol; β-E, β-epoxy sterol.

4.2.5 Determination of phytosterol oxidation products purity

The purity and quantification of POPs in each collected fraction were determined by GC-FID according to Cardenia et al. (2012) with some modifications. Five hundred nanoliter of silylated fraction were injected in split mode (1:30) into a Shimazu GC 2010 gas chromatograph (Kyoto, Japan) equipped with a split–splitless injector. The separation was performed under the same analytical conditions reported above. The injector and detector temperatures were set at 325 and 340°C, respectively. Helium (He) was used as the carrier gas at a linear velocity of 37.7 cm/s. No make-up gas was used. The total time of the run was 35 min. The individual fraction was quantified by the internal standard method, comparing the FID response of individual POP to that of the internal standard (19-hydroxycholesterols). The purity was calculated as ratio of single recognized area peak on the total peak area.

4.2.6 Sampling

The suitability of the developed analytical LC-Orbitrap- HRMS method was tested on palm (PO), palm olein (POL), and high oleic sunflower seed oil (HOSO). All raw materials were purchased from an oil industry and stored at 28°C before the process. The POPs content was determined in PO, POL, and HOSO along the whole refining process considering crude oil, degummed and neutralized, bleached and deodorized. The refining process was developed on an internal pilot plant in agreement to Gupta (2007). For PO and POL, the degumming was carried out using phosphoric acid (85% in water) at 0.1% (wt/wt) for 30 min at 85°C; neutralization was performed with NaOH (15% in water) in stoichiometric conditions for acidity plus 10% at 90°C; bleaching was achieved by natural earth (2%, *wt*/*wt*) at 90°C and vacuum (30 mbar); the deodorization was attained at 225°C under vacuum conditions (1.5 mbar) for 2 h. For HOSO, the degumming was performed at 70°C using a solution of phosphoric with citric acid at 2.5% (*wt*/*wt*). The remaining steps of refining were the same as mentioned above. All refining tests were performed in triplicate (n = 3). For the storage study, 300 g of refined PO, POL, and HOSO supplied by a local producer were stored at 45°C in an open vessel for 16 days and the oxidative parameters were monitored on fresh (t0) and after 48 (t2), 96 (t4), 168 (t7), 216 (t9), 336 (t14), and 384 h (t16). Three independent experiments (n = 3) were carried out.

4.2.7 Determination of phytosterol oxidation products in vegetable oils

Five grams of oils were weighed in an amber flask with 10 µL of IS (0.5 mg/mL). Fifty milliliter of 4 N KOH in methanol solution with 5 mg/mL of BHT was added and the cold saponification was performed overnight at room temperature under magnetic agitation. The extraction of unsaponifiable matter was carried out according to AOCS Official Method Ca6b-53. The saponified sample was transferred into a separation funnel and 50 mL of double distilled water and 50 mL of diethyl ether were added. The organic matter was isolated while the water layer was twice washed with 50 mL of diethyl ether. All the ether fractions were pooled and washed at least three times with 40 mL distilled water with citric acid (0.5%, *wt*/*v*) up to reach the neutral conditions (tested by a pH-paper). The neutralized organic layer was filtered on anhydrous sodium sulfate (Na_2SO_4), to remove the presence of water, and dried by a rotary evaporator at 38°C. The unsaponifiable matter was then dissolved in 1 mL of n-heptane/ 2-propanol (98:2, *v*/*v*) and stored at 28°C until analysis.

4.2.8 Evaluation of vegetables oils stability

In addition to the determination of POPs, the quality control of vegetable oils was also assessed by free acidity (AOCS, 1997a; FFA), peroxides value (AOCS, 2017; PV), p-anisidine value (AOCS, 1997b; p-AV), rancimat (ISO 6886, 2016) and K_{232} (AOCS, 2001). Again, the total sterols content was determined (AOCS, 2022 method) on fresh (t0) and stored samples (t16).

4.2.9 Validation and Statistical analysis

For each single POP, a calibration curve in the concentration range 0.001–20.000 µg/mL was built. Seven different concentration levels in triplicates were done. In addition, a calibration curve for 19-HC was built to evaluate the extent of the recoveries. The selectivity of the method was evaluated using the standard addition method. The limit of detection (LOD) and the limit of quantification (LOQ) were determined on a medium chain triacylglycerols (MCT)—based reference matrix appropriately spiked and diluted considering a signal-to-noise ratio (S/N) equal to 3.3:1 and 10:1, respectively. The intraday (R-intra) and interday (R-inter) precision were determined by analyzing a crude HOSO sample (n = 6) on the same day for three different days, respectively. The repeatability was reported as relative standard deviation (%; RSD). To evaluate the matrix effect recoveries were calculated on MCT matrix. The latter, which is free of POPs, was spiked with 1.829 µg/g of pure POPs and 5 µg/g of IS. The recovery was determined according to the following equation:

$$\% = Cf / Ct \times 100$$

where Cf is the POP amount found in the spiked MCT and Ct is the spiked amount; 10 independent replicates (n = 10) were run.

All data were subjected to statistical analysis such as Shapiro–Wilk normality tests; outlier data were discarded based on statistical comparisons based on Dixon and Grubbs tests. The calculation of the uncertainty (U) for the samples used for the validation was carried out with the Horwitz method, checking that the Horrat ratio and a repeatability ≤ 2 U are verified. The mean and standard deviation of independent replicates were calculated. One-way analysis of variance (ANOVA) with Tukey's post hoc multiple comparisons test ($p<0.05$) was applied to evaluate the significant differences between the average values.

4.3 Results and discussion

4.3.1 Semi-preparative HPLC and standards collection

The development of the preparative method was carried out based on our preliminary results performed by HPLC-HRMS. The three mixtures containing the POPs were qualitatively analyzed through an Orbitrap system. Once the elution order and the recognition of each analyte was defined, the analytical conditions were scaled to the preparative HPLC system. Different stationary phases were tested and Sil-Diol Column displayed the best results. In fact, no migration of retention times, a problem usually due to several factors such as solvent moisture, was observed. As shown in Figure 1 under the developed conditions 7α/β-OH, 7-keto and 5,6α/β- epoxy isomers of sitosterol, campesterol, and stigmasterol were fully resolved (R >1) in less than 50 min. The assay of each collected fraction was qualitatively evaluated by comparing the mass spectra (Figure 1) with those reported in the literature (Johnsson and Dutta 2003); GC-FID was used to determine the assay. It might be pointed out, that the present work represents the isolation of 15 different POPs with an assay higher than 90% (Table 2). Authors (Dzeletovic et al., 1995; Menéndez-Carreño et al., 2008) synthesized POPs, however, no data about their purity were reported. Other authors (Gao et al., 2013) reported the purity obtained but following a chemical synthesis and only about some POPs considered. Differently, the present study concerns the total isolation and collection of 15 oxyphytosterols by preparative HPLC system, a faster and certainly less selective method than the chemical route. Moreover, the purity of a substance plays a key role in the accurate and precise determination of analytes.

Figure 1: Extracted ion chromatograms of the phytosterol oxidation products isolated by LC-Prep-MS and obtained from β-sitosterol (A), campesterol (B), and stigmasterol (C). **1** = 5,6α-epoxysitosterol; **2** = 5,6β epoxysitosterol; **3** = 7-ketositosterol; **4** = 7α-hydroxsitosterol; **5** = 7β-hydroxysitosterol; **6** = 5,6α-epoxycampesterol; **7** = 5,6β-epoxycampesterol; **8** = 7-ketocampesterol; **9** = 7α-hydroxycampesterol; **10** = 7β-hydroxycampesterol; **11** = 5,6α-epoxystigmasterol; **12** = 5,6β epoxystigmasterol; **13** = 7-ketostigmasterol; **14** = 7α-hydroxystigmasterol; **15** = 7β-hydroxystigmasterol

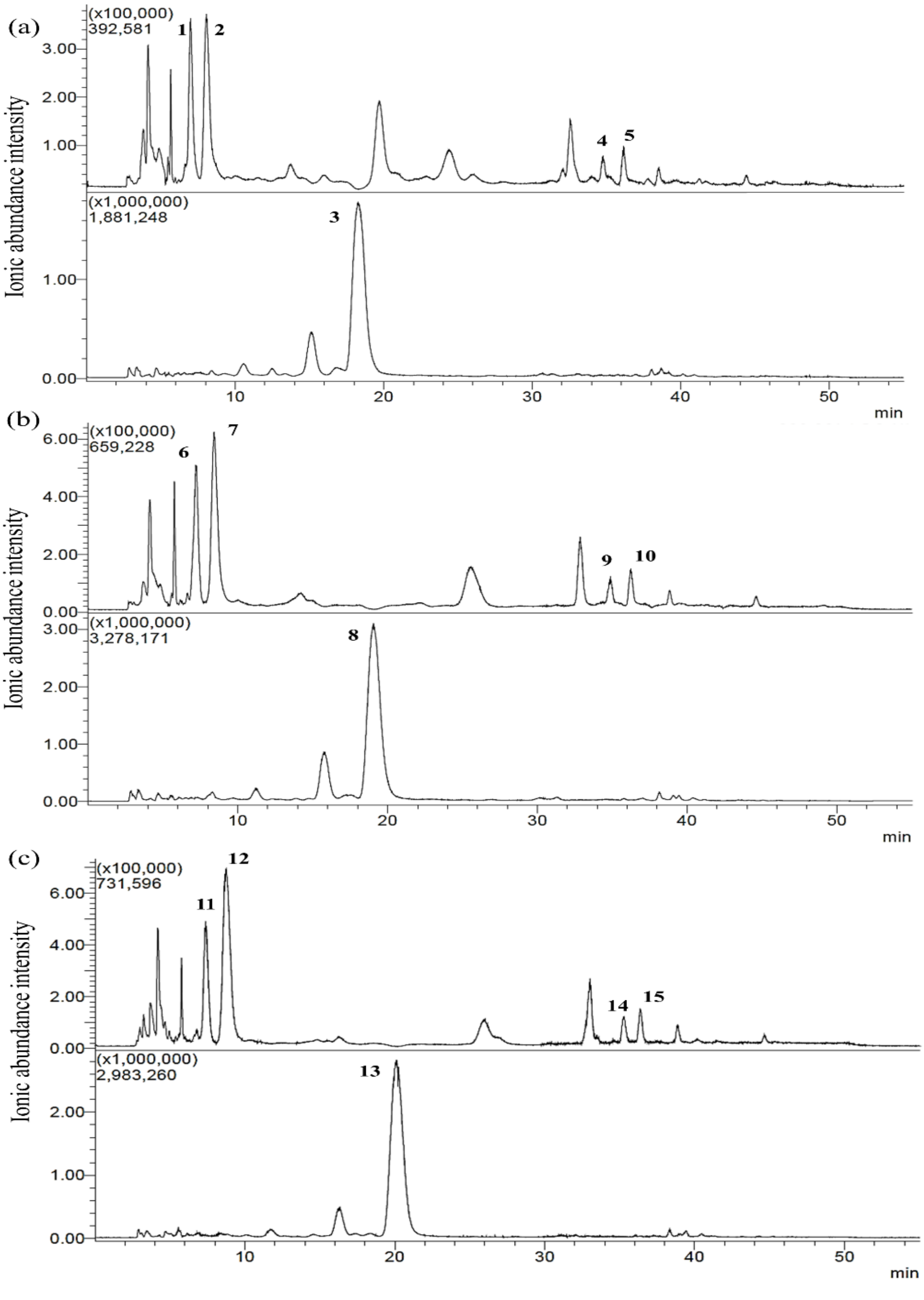


Table 2: Purity grade (%) of isolated phytosterol oxidation products originated from β-sitosterol, campesterol and stigmasterol. Results are expressed as mean of three independent replicates (n=3).

β-sitosterol					**Campesterol**					**Stigmasterol**				
α-Es	*β-Es*	*7-Ks*	*7α-Hs*	*7β-Hs*	*α-Ec*	*β-Ec*	*7-Kc*	*7α-Hc*	*7β-Hc*	*α-Est*	*β-Est*	*7-Kst*	*7α-Hst*	*7β-Hst*
95.3	96.7	94.3	91.7	91.5	90.2	93.1	92.5	96.3	94.8	92.7	91.4	95.3	97.5	91.2

Abbreviations: 7-K, 7-keto sterol; 7α-H, 7α-hydroxy sterol; 7β-H, 7β-hydroxy sterol; s, sitosterol, c, campesterol; st, stigmasterol; α-E, α-epoxy sterol; β-E, β-epoxy sterol.

4.3.2 Validation

Once pure standards were isolated, an LC-Orbitrap- HRMS analytical method was validated. The selectivity of the method was evaluated on MCT (free from sterols) fortified with a known amount of pure oxidized standards, in a range of concentrations similar to that of crude palm oil. The qualitative and quantitative identification of each analyte was based on retention time, full mass spectrum, and MS/MS spectrum (Table 1). Thus, a unique and characteristic identification of each analyte was obtained, avoiding inaccurate recognition due to interferents presence. Linearity was assessed by considering the correlation factors r2 according to the least squares method and verifying that its value was at least 0.99 for all analytes considered. The variability of the angular coefficients was also evaluated considering as a minimum level a variability < 10%. Table 3 reports the validation parameters. The LOD and LOQ confirmed a higher sensitivity of LC-Orbitrap-HRMS with respect to data reported in the literature obtained by GC-FID, GC/MS, or LC–MS/MS (Grün and Besseau 2016; Hu et al., 2015; Menéndez-Carreño et al., 2008). The PRM technique led to carrying on both quantitative and qualitative analysis increasing the signal-to-noise ratio. However, it might be highlighted that the present work represents the first analytical method able to simultaneously determine 15 pure different POP compounds. The intraday and inter-day precision was evaluated on crude palm oil, which contains about 600 mg/kg of free and esterified sterols (Verleyen, Forcades, et al., 2002; Verleyen, Soninska, et al., 2002), respectively.

The intraday and interday precision was lower than 11% and 12% and 7β-OH sitosterol displayed the best performance (Table 4) confirming the robustness of the developed method. Accuracy was assessed by spiking MCT samples with a specific amount of individual oxidized standards to reproduce the theoretical total POPs reported in palm oil (Bortolomeazzi et al. 2003).The sample was evaluated in triplicate. The recoveries (Table 5) were in line with the literature (Menéndez-Carreño et al., 2008). However, in agree with Busch and King

(2009) the lowest recoveries were observed for 7-keto isomers, probably due to its degradation during the saponification; however, further studies are needed to better evaluate that aspect. Busch and King (2009) demonstrated the effect of saponification on the formation of molecular artifacts, probably due to the thermal degradation and alkaline decomposition of some oxysterols. Thus, the presence of those artifacts may invalidate the quantitative analysis. However, since no pure POPs commercial standards are available except for 7-ketositosterol, all studies were conducted on COPs revealing a decomposition of 7-keto and hydration of 5,6-epoxides isomers generating the 3,5-dien-7-one cholesterol and triol, respectively. Based on the fact that both COPs and POPs display similar molecular structures, it is reasonable to hypothesize that a similar behavior could occur on POPs during the saponification. Moreover, in the presence of antioxidant compounds and cold saponification the presence of artifacts is reduced (Busch and King 2009b; Hu et al. 2015; Lampi et al. 2002). Though, thanks to the LC-preparative combined with LC-Orbitrap- HRMS method reported in the present work, deeper studies on POPs behavior as related to the saponification conditions can be carried out in the future, to better evaluate the formation of artifacts.

Table 3: Linearity of calibration curves (R2); limit of detection (LOD) and limit of quantification (LOQ)

Phytosterol Oxidation Products	Calibration curves	R^2	LOD (ng/g)	LOQ (ng/g)
β-sitosterol				
α-Es	$y = 2*10^8 x + 192244$	1.000	0.055	0.183
β-Es	$y = 2*10^8 x - 2*10^6$	0.992	0.074	0.245
7-Ks	$y = 4*10^8 x - 1*10^7$	0.990	0.025	0.083
7α-Hs	$y = 1*10^8 x - 141190$	0.999	0.072	0.239
7β-Hs	$y = 1*10^8 x - 911475$	0.999	0.082	0.273
Campesterol				
α-Ec	$y = 2*10^8 x + 228028$	0.994	0.029	0.098
β-Ec	$y = 2*10^8 x + 1*10^6$	0.991	0.061	0.204
7-Kc	$y = 6*10^8 x - 1*10^7$	0.998	0.012	0.039
7α-Hc	$y = 2*10^8 x - 438783$	0.995	0.033	0.110
7β-Hc	$y= 1*10^8 x + 95970$	0.991	0.093	0.309
Stigmasterol				
α-Est	$y= 1*10^8 x - 186087$	0.990	0.067	0.224
β-Est	$y = 1*10^8 x + 1*10^6$	0.991	0.056	0.185
7-Kst	$y = 4*10^8 x + 8*10^6$	0.990	0.014	0.048
7α-Hst	$y = 1*10^8 x - 468300$	0.993	0.055	0.184
7β-Hst	$y = 1*10^8 x - 470933$	0.998	0.059	0.196

Abbreviations: 7-K, 7-keto sterol; 7α-H, 7α-hydroxy sterol; 7β-H, 7β-hydroxy sterol; s, sitosterol, c, campesterol; st, stigmasterol; α-E, α-epoxy sterol; β-E, β-epoxy sterol.

Table 4: Intraday e interday repeatability (RSD, %) determined on crude sunflower oil

Phytosterol Oxidation Products	Intraday (%)	Interday (%)
β-sitosterol		
α-Es	7%	8%
β-Es	5%	6%
7-Ks	9%	9%
7α-Hs	4%	5%
7β-Hs	2%	5%
Campesterol		
α-Ec	7%	8%
β-Ec	8%	9%
7-Kc	11%	12%
7α-Hc	4 %	4%
7β-Hc	3%	4%
Stigmasterol		
α-Est	4%	9%
β-Est	6%	5%
7-Kst	7%	8%
7α-Hst	2%	2%
7β-Hst	3%	3%

Abbreviations: 7-K, 7-keto sterol; 7α-H, 7α-hydroxy sterol; 7β-H, 7β-hydroxy sterol; s, sitosterol, c, campesterol; st, stigmasterol; α-E, α-epoxy sterol; β-E, β- epoxy sterol.

Table 5: Recovery data obtained determined on MCT

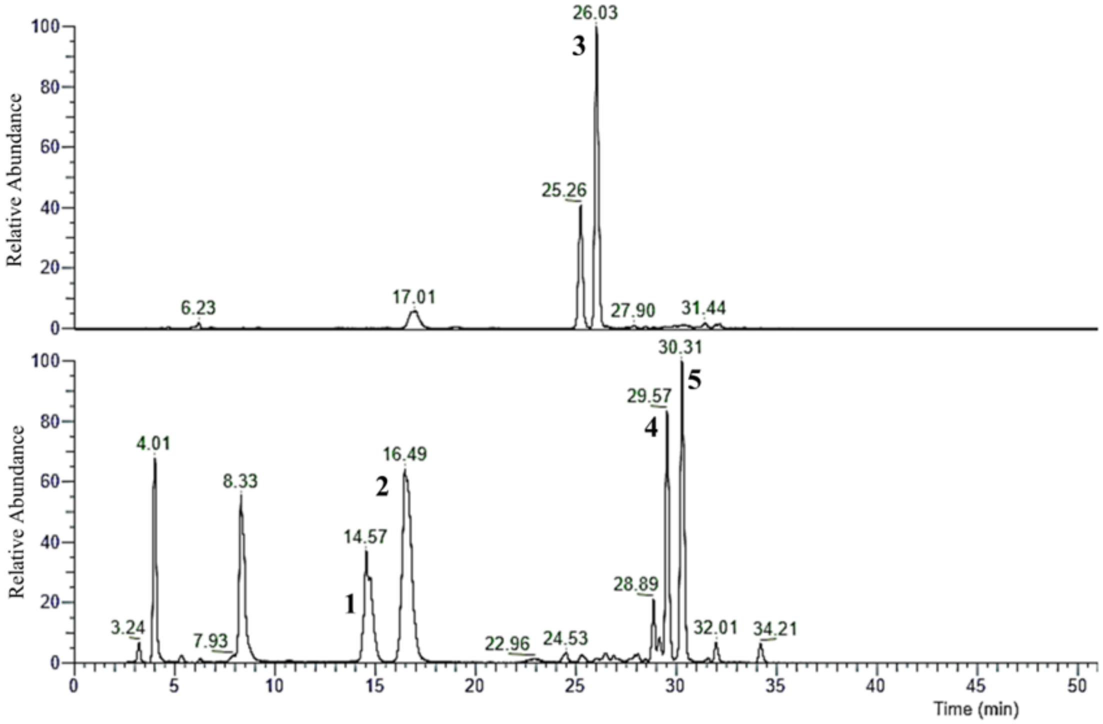
Phytosterol Oxides	Sample (µg/mL)	Spiked sample (µg/mL)	Recovery %
β-sitosterol			
α-Es	0.053	0.057	93
β-Es	0.052	0.059	88
7-Ks	0.144	0.173	83
7α-Hs	0.016	0.018	88
7β-Hs	0.047	0.049	96
Campesterol			
α-Ec	0.099	0.106	93
β-Ec	0.079	0,087	91
7-Kc	0.194	0.238	82
7α-Hc	0.017	0.019	89
7β-Hc	0.062	0.070	89
Stigmasterol			
α-Est	0.155	0.176	88
β-Est	0.179	0.196	91
7-Kst	0.312	0.349	89
7α-Hst	0.066	0.076	87
7β-Hst	0.154	0.157	98

Abbreviations: 7-K, 7-keto sterol; 7α-H, 7α-hydroxy sterol; 7β-H, 7β-hydroxy sterol; s, sitosterol, c, campesterol; st, stigmasterol; α-E, α-epoxy sterol; β-E, β- epoxy sterol.

4.3.3 Determination of POPs

Under the analytical conditions, the normal phase LC method led to properly determining 19-HC, α-epoxy, β- epoxy, 7-keto, 7α-hydroxy, and 7β-hydroxy isomers of sterols. For instance, Figure 2 reports the separation of POPs-sitosterol in crude HOSO. In addition, the epimers displayed similar MS and MS2 fragmentation models. Thus, to properly recognize the sterol oxides a good separation of oxides is needed (Raith et al., 2005). The separation of oxidation products was achieved in less than 40 min and retention times in both standard mixtures and real samples were reproducible. The specific fragmentation and relative ion abundances of the POPs were reported in Table 1. The 7-keto derivates displayed the protonated molecular ion $[M+H]^+$ as the base peak and showed a loss of one and two water molecules only after the MS2 fragmentation. Similar results were also reported for 7-ketocholesterol (Raith et al., 2005). In general, the higher-energy collisional dissociation (HCD) was not able to cleave stable carbon–carbon bonds and the loss of one water molecule $[M-H_2O + H]^+$ (epoxy and hydroxy isomers) or two $[M-2H_2O + H]^+$ water molecules (epoxy and hydroxy groups) were noted. On the other hand, sitosterol, campesterol, and stigmasterol displayed a similar fragmentation behavior with unique differences in their relative abundance of ions in agree with the literature (Kemmo et al., 2007). The quantification of POPs was carried out using the external standard method based on pure compounds in-house collected and isolated. Then, the results were correlated to the amount of 19-HC recovery.

Figure 2: Separation of β-sitosterol POPs in crude high oleic sunflower oil by LC-Orbitrap-HRMS**.** **1** = 5,6α-epoxysitosterol; **2** = 5,6β-epoxysitosterol; **3** = 7-ketositosterol; **4** = 7α-hydroxysitosterol; **5** = 7β-hydroxysitosterol



4.3.4 Determination of POPs in vegetable oil oxidation during refining processing

The suitability of the developed method was tested in vegetable oils subjected to refining process. In addition, to evaluate the efficacy of refining processing FFA, PV and *p*-An value were determined after each step. As expected, for PO, POL, and HOSO, the FFA and PV values significantly ($p < 0.05$) decreased during the refining steps (Egbuna et al., 2007) whereas p-An did not significantly change ($p = 0.057$). The crude PO showed the lowest POPs content (0.344 mg/kg) followed by POL (0.831 mg/kg) and HOSO (11.950 mg/kg). Analyzing the impact of refining on individual oils, it is possible to obtain various preliminary information. In PO, the degumming and neutralization step determined an increase up to 39% of POPs content related to crude oil; whereas, the bleaching led to an increase of 12%. On the other hand, the deodorization reduced the content of POPs and a concentration up to 0.443 mg/kg was found (Table 6). In the preliminary study, the chemical refining of selected vegetable oils under tested conditions determined an overall increase in POPs. The neutralization drastically impacted the stigmasterol oxidation with an increase of 56% in total POPs content, whereas, on sitosterol and campesterol oxidizes an increase of 39% and 31%, respectively, was found. Moreover, the neutralization increased the presence of 7α-Hst content (by 65%). Further, the bleaching affected the presence of 7β-Hc and 7β-Hs. On the contrary, the deodorization decreased the POPs content, in particular 7β-Hc and 7α-Hs dropped by 92% and 67%, respectively. The POL exposed a similar behavior of PO but a different magnitude was observed. The degumming and neutralization determined an increase of 20% in total POPs. The bleaching again promoted the phytosterol oxidation by 8%. Deodorization, as reported for PO, reduced the total POPs up to 0.997 mg/kg. The 7-Ks was the main isomer detected after the neutralization, while after bleaching a less marked increase was denoted. The deodorization reduced the presence of both oxides sitosterol and campesterol,

Table 6: Content of POPs in palm oil (PO), palm olein (POL) and high oleic sunflower oil (HOSO) during the refining process

	Crude	**Degumming and Neutralization**	**Bleaching**	**Deodorization**	***Sign.***
PO					
β-sitosterol					
α-Es	0.114 ± 0.014^{a}	0.160 ± 0.024^{ab}	0.202 ± 0.025^{bc}	0.178 ± 0.017^{bc}	*
β-Es	0.060 ± 0.005^{a}	0.085 ± 0.008^{b}	0.121 ± 0.009^{cd}	0.105 ± 0.010^{cd}	**
7-Ks	0.009 ± 0.002^{a}	0.012 ± 0.002^{a}	0.018 ± 0.004^{ab}	0.016 ± 0.002^{ab}	*
7α-Hs	0.034 ± 0.005^{a}	0.048 ± 0.008^{ab}	0.079 ± 0.008^{bc}	0.062 ± 0.007^{c}	**
7β-Hs	0.014 ± 0.001^{a}	0.017 ± 0.001^{a}	0.024 ± 0.002^{b}	0.008 ± 0.001^{c}	***
ΣPOPs	0.231 ± 0.021^{a}	0.321 ± 0.033^{bd}	0.444 ± 0.041^{cd}	0.370 ± 0.029^{d}	*
Campesterol					
α-Ec	nd	nd	nd	nd	-
β-Ec	nd	nd	nd	0.051 ± 0.009	-
7-Kc	0.023 ± 0.004^{a}	0.030 ± 0.005^{ab}	0.040 ± 0.006^{b}	0.009 ± 0.001^{c}	**
7α-Hc	0.023 ± 0.002^{a}	0.032 ± 0.003^{a}	0.054 ± 0.007^{b}	0.004 ± 0.001^{c}	**
7β-Hc	0.029 ± 0.004^{a}	0.036 ± 0.005^{a}	nd	0.009 ± 0.001^{b}	**
ΣPOPs	0.075 ± 0.015	0.098 ± 0.023	0.094 ± 0.015	0.073 ± 0.011	*ns*
Stigmasterol					
α-Est	nd	nd	nd	nd	-

β-Est	nd	nd	nd	nd	-
7-Kst	0.005 ± 0.001^{a}	0.008 ± 0.001^{a}	nd	nd	*
7α-Hst	0.015 ± 0.001^{a}	0.022 ± 0.001^{b}	nd	nd	*
7β-Hst	0.018 ± 0.002^{a}	0.029 ± 0.002^{b}	nd	nd	*
ΣPOPs	0.038 ± 0.005^{a}	0.059 ± 0.009^{b}	nd	nd	**
ΣPOPs tot	0.344 ± 0.035^{a}	0.478 ± 0.042^{b}	0.538 ± 0.044^{b}	0.443 ± 0.032^{ab}	*
FFA	4.4 ± 0.4^{a}	0.3 ± 0.0^{b}	0.2 ± 0.0^{c}	nd	**
PV	3.71 ± 0.42^{a}	2.52 ± 0.33^{b}	0.62 ± 0.11^{c}	0.12 ± 0.03^{d}	***
p-An	2.7 ± 0.3	4.6 ± 0.6	6.4 ± 0.7	3.9 ± 0.5	*ns*
POL					
β-sitosterol					
α-Es	0.257 ± 0.049	0.287 ± 0.054	0.312 ± 0.049	0.295 ± 0.048	*ns*
β-Es	0.148 ± 0.015^{a}	0.180 ± 0.015^{a}	0.198 ± 0.019^{ab}	0.167 ± 0.020a	*
7-Ks	0.090 ± 0.014^{a}	0.141 ± 0.020^{bc}	0.154 ± 0.020^{c}	0.152 ± 0.020^{bcd}	**
7α-Hs	0.123 ± 0.010	0.137 ± 0.012	0.146 ± 0.012	0.135 ± 0.014	*ns*
7β-Hs	0.150 ± 0.015	0.179 ± 0.018	0.206 ± 0.026	0.201 ± 0.034	*ns*
ΣPOPs	0.768 ± 0.111	0.924 ± 0.184	1.016 ± 0.221	0.949 ± 0.102	*ns*
Campesterol					

α-Ec	nd	nd	nd	nd	
β-Ec	nd	nd	nd	nd	
7-Kc	0.026 ± 0.003	0.029 ± 0.004	0.030 ± 0.005	0.027 ± 0.003	*ns*
7α-Hc	0.008 ± 0.001^{a}	0.010 ± 0.001^{a}	0.011 ± 0.001^{ab}	0.011 ± 0.001^{ac}	*
7β-Hc	0.008 ± 0.001	0.009 ± 0.001	0.010 ± 0.001	0.010 ± 0.001	*ns*
ΣPOPs	0.042 ± 0.008	0.048 ± 0.009	0.051 ± 0.007	0.047 ± 0.005	*ns*
Stigmasterol					
α-Est	nd	nd	nd	nd	*ns*
β-Est	nd	nd	nd	nd	*ns*
7-Kst	nd	nd	nd	nd	*ns*
7α-Hst	0.010 ± 0.002	0.011 ± 0.002	nd	nd	*ns*
7β-Hst	0.011 ± 0.001	0.013 ± 0.001	nd	nd	*ns*
ΣPOPs	0.021 ± 0.005	0.024 ± 0.003	nd	nd	*ns*
ΣPOPs tot	0.831 ± 0.123	0.995 ± 0.125	1.067 ± 0.251	0.997 ± 0.198	*ns*
FFA	5.1 ± 0.6^{a}	0.3 ± 0.0^{b}	0.2 ± 0.0^{c}	nd	***
PV	3.20 ± 0.36^{a}	2.02 ± 0.21^{b}	0.63 ± 0.12b^{c}	0.15 ± 0.03^{bcd}	*
p-An	5.1 ± 0.5	5.4 ± 0.7	6.8 ± 0.8	4.3 ± 0.5	*ns*
HOSO					
β-sitosterol					

α-Es	0.440 ± 0.114^{a}	0.625 ± 0.135^{a}	0.662 ± 0.121^{ab}	0.548 ± 0.111^{a}	*
β-Es	0.710 ± 0.198^{a}	1.043 ± 0.211^{b}	1.043 ± 0.198^{ab}	0.864 ± 0.122^{ab}	*
7-Ks	0.990 ± 0.178	1.111 ± 0.189	1.124 ± 0.225	1.003 ± 0.209	*ns*
7α-Hs	3.520 ± 0.456	3.994a ± 0.501	4.066 ± 0.501	3.650 ± 0.444	*ns*
7β-Hs	2.160 ± 0.307^{a}	2.642 ± 0.331^{a}	2.824 ± 0.347^{ab}	2.507 ± 0.411^{ab}	*
ΣPOPs	7.820 ± 1.322	9.415 ± 1.411	9.719 ± 1.421	8.572 ± 1.344	*ns*
Campesterol					
α-Ec	0.110 ± 0.021	0.146 ± 0.018	0.151 ± 0.022	0.149 ± 0.016	*ns*
β-Ec	0.180 ± 0.026	0.238 ± 0.038	0.246 ± 0.029	0.230 ± 0.032	*ns*
7-Kc	0.130 ± 0.025	0.146 ± 0.035	0.148 ± 0.019	0.142 ± 0.022	*ns*
7α-Hc	0.410 ± 0.109	0.461 ± 0.121	0.504 ± 0.133	0.454 ± 0.127	*ns*
7β-Hc	0.530 ± 0.131^{a}	0.655 ± 0.141^{a}	0.696 ± 0.152^{ab}	0.636 ± 0.161^{ab}	*
ΣPOPs	1.360 ± 0.401	1.646 ± 0.425	1.745 ± 0.511	1.611 ± 0.423	*ns*
Stigmasterol					
α-Est	0.230 ± 0.035	0.304 ± 0.045	0.325 ± 0.047	0.293 ± 0.054	*ns*
β-Est	0.640 ± 0.120	0.776 ± 0.089	0.828 ± 0.187	0.819 ± 0.123	*ns*
7-Kst	0.280 ± 0.071	0.311 ± 0.041	0.347 ± 0.055	0.313 ± 0.062	*ns*
7α-Hst	0.920 ± 0.187	1.023 ± 0.222	1.113 ± 0.295	1.003 ± 0.238	*ns*
7β-Hst	0.700 ± 0.162^{a}	0.849 ± 0.198^{a}	0.933 ± 0.188^{ab}	0.874 ± 0.187^{ab}	*
ΣPOPs	2.770 ± 0.601	3.263 ± 0.666	3.546 ± 0.781	3.302 ± 0.697	*ns*

ΣPOPs tot	11.950 ± 2.514	14.324 ± 2.851	15.010 ± 2.901	13.485 ± 2.669	*ns*
FFA	1.1 ± 0.1^{a}	0.3 ± 0.1^{bc}	0.1 ± 0.0^{cd}	0.1 ± 0.0^{d}	
PV	2.90 ± 0.27^{a}	4.20 ± 0.53^{b}	2.90 ± 0.39^{cd}	0.12 ± 0.02^{d}	
p-An	2.4 ± 0.3	3.6 ± 0.48	4.9 ± 0.6	1.2 ± 0.2	*ns*

Note: POPs are reported as µg/mL; FFA as % Oleic Acid; PV as meqO2/kg oil; p-An as AnV. The results are expressed as mean ± standard deviation (n = 3). Means followed by the same letter (row) are not significantly different at $p < 0.05$ (Tukey's test). n.s. = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Abbreviations: 7-K, 7-keto sterol; 7α-H, 7α-hydroxy sterol; 7β-H, 7β-hydroxy sterol; nd, not detected; Sign., statistical significance; s, sitosterol, c, campesterol; st, stigmasterol; α-E, α-epoxy sterol; β-E, β-epoxy sterol.

while no significant change ($p > 0.05$) was observed for stigmasterol oxides, which are at trace level. The HOSO displayed the highest content of POPs from the crude to the deodorized oil an increase of 13% was detected (from 11.950 to 13.485 mg/kg). The neutralization determined an increase of 20% in the total POPs where campesterol was the most affected followed by sitosterol and stigmasterol. In addition, α-Es and β-Es were in deep affected by neutralization since an increase of 46% and 42% was found, respectively. As reported above, bleaching produced a lower increase (5%) than neutralization; in particular, stigmasterol was mainly oxidized with an increase of 7-Kst, 7α-Hst, and 7β-Hst. Deodorization also in that case led to a reduction of POPs with an overall decrease of 10%. Considering the results obtained, the different refining steps impacted the formation of POPs. The deodorization significantly decreased the content of POPs invegetable oils. As reported in the literature (Verleyen and Forcades et al., 2002; Verleyen and Sosinska et al., 2002) the deodorization process, under certain conditions, can decrease the sterols content in the considered matrix. In a similar way, considering the POPs boiling point similar to those of sterols, it is possible to hypothesize that under tested conditions, the POPs were also stripped during the deodorization. As reported for all minor components, their reduction depends on the refining conditions adopted: temperature, process time, and degree of vacuum (Mariod et al. 2012). It is important to consider that in the present work all the refining tests, including the deodorization step, were carried out in a pilot plant under drastic conditions (225°C; 1.5 mbar; 2 h), which led to a decrease in sterol and POPs content. On the other hand, degumming, neutralization and bleaching steps led to changes in the total POPs content for all considered oils. It is reasonable to assume that under the tested refining conditions a partial oxidation of sterols due to temperature (90°C for neutralization and bleaching) and co-presence of oxygen occurred.

Table 7: POPs content in palm, palm olein and high oleic sunflower oil during the storage (45°C, 16 days)

	t0	*t2*	*t4*	*t7*	*t9*	*t14*	*t16*	*Sign.*
PO								
β-sitosterol								
α-Es	0.134 ± 0.011	0.143 ± 0.014	0.152 ± 0.014	0.157 ± 0.013	0.162 ± 0.016	0.145 ± 0.013	0.139 ± 0.011	*ns*
β-Es	0.067 ± 0.004	0.078 ± 0.006	0.063 ± 0.004	0.072 ± 0.006	0.057 ± 0.005	0.071 ± 0.005	0.076 ± 0.005	*ns*
7-Ks	0.031 ± 0.004	0.029 ± 0.004	0.026 ± 0.003	0.032 ± 0.004	0.035 ± 0.004	0.026 ± 0.003	0.051 ± 0.005	*ns*
7α-Hs	0.008 ± 0.001	0.012 ± 0.002	nd	0.009 ± 0.002	0.011 ± 0.002	0.014 ± 0.002	0.020 ± 0.003	*ns*
7β-Hs	0.016 ± 0.001	0.015 ± 0.001	0.019 ± 0.001	0.022 ± 0.001	0.012 ± 0.001	0.018 ± 0.001	nd	*ns*
ΣPOPs	0.256 ± 0.058	0.277 ± 0.061	0.260 ± 0.041	0.292 ± 0.057	0.277 ± 0.062	0.274 ± 0.047	0.286 ± 0.062	*ns*
Campesterol								
α-Ec	nd	nd	nd	nd	nd	nd	nd	
β-Ec	0.051 ± 0.005	0.054 ± 0.005	0.048 ± 0.005	0.045 ± 0.004	0.055 ± 0.005	0.039 ± 0.004	0.057 ± 0.005	*ns*
7-Kc	0.009 ± 0.001	0.005 ± 0.001	0.012 ± 0.001	0.014 ± 0.001	0.007 ± 0.001	0.008 ± 0.001	0.011 ± 0.001	*ns*
7α-Hc	0.009 ± 0.001	0.006 ± 0.001	0.008 ± 0.001	nd	nd	0.012 ± 0.002	0.005 ± 0.001	*ns*
7β-Hc	0.004 ± 0.001	0.007 ± 0.001	nd	0.009 ± 0.001	nd	0.010 ± 0.001	0.006 ± 0.001	*ns*
ΣPOPs	0.073 ± 0.014	0.072 ± 0.021	0.068 ± 0.023	0.068 ± 0.027	0.062 ± 0.032	0.069 ± 0.041	0.079 ± 0.022	*ns*
Stigmasterol								
α-Est	0.057 ± 0.011	0.061 ± 0.006	0.063 ± 0.012	0.055 ± 0.007	0.049 ± 0.005	0.052 ± 0.005	0.054 ± 0.005	*ns*

β-Est	nd	nd	nd	nd	nd	nd	nd	
7-Kst	0.008 ± 0.001	0.004 ± 0.000	0.009 ± 0.001	nd	0.011 ± 0.001	nd	0.006 ± 0.001	*ns*
7α-Hst	nd	nd	nd	nd	nd	nd	nd	
7β-Hst	0.021 ± 0.003	0.023 ± 0.003	0.031 ± 0.003	0.029 ± 0.001	0.032 ± 0.002	0.033 ± 0.003	0.020 ± 0.001	*ns*
ΣPOPs	0.086 ± 0.017	0.088 ± 0.015	0.103 ± 0.021	0.084 ± 0.018	0.092 ± 0.012	0.085 ± 0.016	0.080 ± 0.014	*ns*
POPs tot	0.415 ± 0.078	0.437 ± 0.081	0.431 ± 0.064	0.444 ± 0.059	0.431 ± 0.071	0.428 ± 0.053	0.445 ± 0.047	*ns*
FFA	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	*ns*
PV	0.12 ± 0.01^{a}	0.24 ± 0.03^{b}	0.31 ± 0.04^{c}	0.50 ± 0.05^{d}	0.58 ± 0.08^{d}	1.49 ± 0.20^{e}	2.01 ± 0.24^{e}	***
p-An	5.9 ± 0.5	6.1 ± 0.7	5.9 ± 0.7	6.3 ± 0.7	6.1 ± 0.8	6.0 ± 0.7	6.2 ± 0.8	*ns*
Rancimat	12.70 ± 0.69	12.40 ± 2.38	11.40 ± 1.97	11.30 ± 1.71	11.20 ± 2.06	10.70 ± 1.77	10.50 ± 1.84	*ns*
K_{232}	2.340 ± 0.293^{a}	2.421 ± 0.307^{a}	2.541 ± 0.282^{a}	2.621 ± 0.372^{a}	2.758 ± 0.370^{a}	2.900 ± 0.357^{a}	3.214 ± 0.405^{b}	*
ΣSterols	527.0 ± 51.6	-	-	-	-	-	602.0 ± 47.0	*ns*
POL								
β-sitosterol								
α-Es	0.198 ± 0.021	0.201 ± 0.039	0.212 ± 0.021	0.209 ± 0.029	0.220 ± 0.035	0.215 ± 0.031	0.229 ± 0.033	*ns*
β-Es	0.141 ± 0.021	0.122 ± 0.011	0.111 ± 0.011	0.099 ± 0.017	0.151 ± 0.023	0.155 ± 0.021	0.161 ± 0.024	*ns*
7-Ks	0.075 ± 0.014	0.080 ± 0.011	0.101 ± 0.015	0.070 ± 0.011	0.094 ± 0.016	0.091 ± 0.013	0.098 ± 0.017	*ns*
7α-Hs	0.065 ± 0.005	0.071 ± 0.006	0.056 ± 0.005	0.069 ± 0.006	0.072 ± 0.007	0.054 ± 0.005	0.077 ± 0.007	*ns*
7β-Hs	0.043 ± 0.004	0.039 ± 0.003	0.037 ± 0.003	0.040 ± 0.004	0.032 ± 0.001	0.041 ± 0.004	0.044 ± 0.004	*ns*

ΣPOPs	0.521 ± 0.082	0.513 ± 0.079	0.517 ± 0.069	0.487 ± 0.068	0.569 ± 0.061	0.556 ± 0.049	0.609 ± 0.063	*ns*
Campesterol								
α-Ec	0.093 ± 0.011	0.060 ± 0.008	0.089 ± 0.014	0.071 ± 0.010	0.099 ± 0.018	0.081 ± 0.014	0.085 ± 0.014	*ns*
β-Ec	0.094 ± 0.013	0.121 ± 0.018	0.087 ± 0.011	0.121 ± 0.014	0.112 ± 0.015	0.117 ± 0.016	0.125 ± 0.020	*ns*
7-Kc	0.031 ± 0.004	0.040 ± 0.006	0.039 ± 0.006	0.029 ± 0.004	0.022 ± 0.003	0.046 ± 0.007	0.039 ± 0.007	*ns*
7α-Hc	0.021 ± 0.002	0.026 ± 0.002	0.018 ± 0.002	0.034 ± 0.003	0.041 ± 0.004	0.030 ± 0.002	0.043 ± 0.004	*ns*
7β-Hc	0.038 ± 0.004	0.040 ± 0.004	0.054 ± 0.006	0.034 ± 0.004	0.034 ± 0.004	0.029 ± 0.004	0.025 ± 0.003	*ns*
ΣPOPs	0.277 ± 0.041	0.287 ± 0.038	0.287 ± 0.043	0.289 ± 0.039	0.308 ± 0.046	0.303 ± 0.051	0.317 ± 0.049	*ns*
Stigmasterol								
α-Est	nd	nd	nd	nd	nd	nd	nd	
β-Est	0.091 ± 0.007	0.082 ± 0.007	0.079 ± 0.007	0.093 ± 0.007	0.080 ± 0.007	0.101 ± 0.011	0.087 ± 0.009	*ns*
7-Kst	0.046 ± 0.005	0.032 ± 0.004	0.009 ± 0.001	0.046 ± 0.006	0.032 ± 0.006	0.011 ± 0.001	0.009 ± 0.001	*ns*
7α-Hst	nd	nd	nd	nd	nd	nd	nd	
7β-Hst	nd	nd	nd	nd	nd	nd	nd	
ΣPOPs	0.137 ± 0.017	0.114 ± 0.009	0.088 ± 0.011	0.139 ± 0.022	0.112 ± 0.017	0.112 ± 0.018	0.096 ± 0.013	*ns*
ΣPOPs tot	0.935 ± 0.198	0.914 ± 0.175	0.892 ± 0.132	0.916 ± 0.141	0.989 ± 0.198	0.971 ± 0.138	1.022 ± 0.144	*ns*
FFA	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	*ns*
PV	0.10 ± 0.02^{a}	0.31 ± 0.04^{b}	0.37 ± 0.04^{b}	0.46 ± 0.07^{c}	0.55 ± 0.06^{c}	2.02 ± 0.32^{d}	2.32 ± 0.39^{d}	**

p-An	11.5 ± 1.5	11.4 ± 1.4	11.4 ± 1.4	11.5 ± 1.3	11.5 ± 1.3	11.5 ± 1.4	11.4 ± 1.5	*ns*
Rancimat	11.27 ± 1.62	11.14 ± 2.14	11.00 ± 2.01	11.00 ± 1.99	10.80 ± 1.88	9.75 ± 1.41	9.40 ± 1.46	*ns*
K_{232}	2.900 ± 0.334^{ab}	3.070 ± 0.390^{ab}	3.250 ± 0.328^{ab}	3.670 ± 0.374^{b}	3.880 ± 0.481^{bc}	4.000 ± 0.532^{bc}	4.100 ± 0.476^{bc}	*
ΣSterols	704.0 ± 71.8	-	-	-	-	-	723.0 ± 78.8	*ns*
HOSO								
β-sitosterol								
α-Es	0.720 ± 0.066	0.750 ± 0.082	0.725 ± 0.071	0.706 ± 0.066	0.680 ± 0.073	0.714 ± 0.074	0.752 ± 0.068	*ns*
β-Es	0.721 ± 0.053	0.751 ± 0.061	0.741 ± 0.057	0.729 ± 0.057	0.730 ± 0.061	0.780 ± 0.056	0.757 ± 0.063	*ns*
7-Ks	1.588 ± 0.238	1.601 ± 0.158	1.772 ± 0.172	1.680 ± 0.171	1.670 ± 0.180	1.767 ± 0.196	1.743 ± 0.190	*ns*
7α-Hs	2.123 ± 0.153	2.129 ± 0.172	2.119 ± 1.526	2.121 ± 0.163	2.125 ± 0.185	2.131 ± 0.170	2.141 ± 0.124	*ns*
7β-Hs	1.086 ± 0.085	1.090 ± 0.077	1.092 ± 0.085	1.088 ± 0.075	1.078 ± 0.091	1.089 ± 0.079	1.071 ± 0.090	*ns*
ΣPOPs	6.238 ± 0.621	6.321 ± 0.611	6.449 ± 0.810	6.324 ± 0.632	6.283 ± 0.638	6.482 ± 0.632	6.464 ± 0.615	*ns*
Campesterol								
α-Ec	0.122 ± 0.013	0.110 ± 0.014	0.101 ± 0.010	0.109 ± 0.009	0.113 ± 0.011	0.120 ± 0.016	0.129 ± 0.012	*ns*
β-Ec	0.098 ± 0.010	0.120 ± 0.014	0.113 ± 0.011	0.111 ± 0.012	0.121 ± 0.013	0.131 ± 0.016	0.136 ± 0.018	*ns*
7-Kc	0.216 ± 0.026	0.210 ± 0.029	0.211 ± 0.030	0.226 ± 0.029	0.216 ± 0.029	0.119 ± 0.018	0.222 ± 0.027	*ns*
7α-Hc	0.341 ± 0.018	0.336 ± 0.026	0.335 ± 0.022	0.330 ± 0.017	0.341 ± 0.022	0.331 ± 0.027	0.351 ± 0.029	*ns*
7β-Hc	0.214 ± 0.017	0.210 ± 0.017	0.208 ± 0.015	0.215 ± 0.014	0.217 ± 0.018	0.211 ± 0.016	0.222 ± 0.018	*ns*
ΣPOPs	0.991 ± 0.142	0.987 ± 0.214	0.969 ± 0.144	0.991 ± 0.162	1.008 ± 0.102	0.912 ± 0.123	1.060 ± 0.214	*ns*

Stigmasterol								
α-Est	0.187 ± 0.019	0.180 ± 0.020	0.182 ± 0.025	0.185 ± 0.021	0.179 ± 0.016	0.170 ± 0.021	0.175 ± 0.018	*ns*
β-Est	0.450 ± 0.032	0.473 ± 0.030	0.460 ± 0.035	0.472 ± 0.039	0.482 ± 0.034	0.478 ± 0.037	0.489 ± 0.043	*ns*
7-Kst	0.368 ± 0.034	0.215 ± 0.021	0.224 ± 0.025	0.350 ± 0.042	0.180 ± 0.018	0.171 ± 0.018	0.191 ± 0.026	*ns*
7α-Hst	0.105 ± 0.006	0.085 ± 0.004	0.109 ± 0.007	0.085 ± 0.004	0.107 ± 0.006	0.089 ± 0.004	0.111 ± 0.006	*ns*
7β-Hst	0.470 ± 0.021	0.481 ± 0.032	0.478 ± 0.024	0.481 ± 0.030	0.474 ± 0.030	0.481 ± 0.021	0.475 ± 0.027	*ns*
ΣPOPs	1.580 ± 0.334	1.434 ± 0.201	1.453 ± 0.321	1.572 ± 0.198	1.422 ± 0.201	1.389 ± 0.231	1.441 ± 0.238	*ns*
ΣPOPs tot	8.809 ± 1.092	8.742 ± 1.117	8.870 ± 1.365	8.887 ± 1.227	8.713 ± 0.988	8.783 ± 0.963	8.965 ± 1.321	*ns*
FFA	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	*ns*
PV	0.14 ± 0.02^{a}	0.20 ± 0.02^{b}	0.30 ± 0.03^{c}	0.80 ± 0.09^{d}	1.43 ± 0.17^{e}	2.48 ± 0.31^{f}	3.10 ± 0.33^{g}	**
p-An	1.6 ± 0.2	1.7 ± 0.2	1.7 ± 0.2	1.8 ± 0.2	1.9 ± 0.3	2.00 ± 0.3	2.00 ± 0.3	*ns*
Rancimat	9.91 ± 1.92	9.91 ± 1.80	9.80 ± 1.60	9.50 ± 1.43	9.20 ± 1.42	8.63 ± 1.17	8.50 ± 1.40	*ns*
K_{232}	1.440 ± 0.180^{a}	1.520 ± 0.208^{ab}	1.610 ± 0.163^{ab}	1.670 ± 0.204^{ab}	1.700 ± 0.194^{b}	1.720 ± 0.212^{b}	1.720 ± 0.234^{b}	*
ΣSterols	1370.5 ± 120.6	-	-	-	-	-	1554.7 ± 132.1	*ns*

Note: POPs are reported as µg/mL; FFA in % Oleic Acid; PV as meqO2/kg; p-An as AnV; Rancimat as IP-hour; K_{232} 1% in isooctane 1 cm); Sterols as µg/mL. The results are expressed as mean ± standard deviation (n = 3). Means followed by the same letter (row) are not significantly different at $p < 0.05$ (Tukey's test). n.s. = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Abbreviations: 7-K, 7-keto sterol; 7α-H, 7α-hydroxy sterol; 7β-H, 7β-hydroxy sterol; nd, not detected; Sign., statistical significance; s, sitosterol, c, campesterol; st, stigmasterol; α-E, α-epoxy sterol; β-E, β-epoxy sterol

4.3.5 Determination of POPs in vegetable oil oxidation during storage

Table 7 reports the content of individual POPs in PO, POL, and HOSO stored at 45°C for 16 days. To better evaluate the oxidative status FFA, PV, p-An, Rancimat, and K_{232} were considered. The PV and K_{232} values were the most parameters that displayed a significant increase ($p < 0.05$) for all three types of oils considered, which, indicates that lipid oxidation is occurring through the primary step of initiation and propagation. On the other hand, the *p*-An remained almost stable ($p = 0.061$) throughout the whole experiment, while the Rancimat data showed a decrease due to the storage effect. In addition, FFA values remain constant over time and under the storage conditions adopted. In PO (Table 7), the main POPs were α-Es and β-Es (0.134 and 0.067 mg/kg, respectively) followed by β-Ec (0.051 mg/kg). Considering the forced storage conditions adopted, it is possible to observe how the total POPs ranged from 0.415 mg/kg (t0) to 0.445 mg/kg (t16). However, no significant differences ($p > 0.05$) were observed for α- Ec, β-Est and 7α-Hst since were at traces level. Overall, a negligible increase ($p = 0.054$) in the total POPs deriving of sitosterol, campesterol, and stigmasterol was observed. Considering the individual oxyphytosterols, it is possible to highlight that the main positive increases concern 7β-Hc with a final concentration of 0.006 mg/kg, followed by 7-Kc (0.011 mg/kg) and 7β-Hs (0.019 mg/kg). It is also possible to notice some decreases during the performed oxidative stress. Specifically, 7α-Hc decreased up to 0.005 mg/kg, α-Est reached a final concentration of 0.054 mg/kg and 7β- Hst displayed a final concentration of 0.020 mg/kg. A specific evaluation of the total sterol content was performed. The reported results correspond to the sterol content before (t0) and after storage (t16) considering the sum of sitosterol, campesterol, and stigmasterol. Again, the so-called oxidation rate of phytosterols (ORP, %) was calculated as content of POPs/content of phytosterols before treatment 100. The ORP (0.006%) confirmed that no significant changes ($p > 0.05$) during the storage of PO occurred. As regards POL, the main POPs were α-Es and β-Es (0.198 and 0.141 mg/kg, respectively), α-Ec and β-Ec (0.093 and 0.094 mg/kg) and β-Est (0.091 mg/kg). During the storage, the total content of POPs increased from 0.935 mg/kg (t0) to 1.022 mg/kg (t16). Besides, it is possible to note how sitosterol and campesterol oxides increased, while those of stigmasterol decreased. On the other hand, after 16 days of storage, the content of 7-Kst and 7β-Hc decreased up to 0.009 and 0.025 mg/kg; respectively. On the other hand, the sterol content did not significantly change ($p > 0.05$) during the storage; however, the ORP (0.012%) was an order of magnitude greater than that found on PO, indicating a greater degradation of phytosterol into POPs with respect to PO. Considering HOSO, the POPs content at time t0 was equal to 8.809 mg/kg, mainly due to sitosterol oxidation products (6.238 mg/kg). That consideration was expected given the prevalence of sitosterol in the sterol composition of HOSO (Firestone, 2013). At t0, the main POP was 7α-Hs (2.123 mg/kg), followed by 7-Ks (1.588 mg/kg), α-Es (0.720 mg/kg), and β-Es (0.721 mg/kg). During the storage, the total POPs content increased reaching a final concentration of 8.965 mg/kg. As regards each POP, both sitosterol and campesterol oxides increased during storage while the stigmasterol oxides decreased reaching a final concentration of 1.441 mg/kg. Again, the greatest increase of β-Ec was found, while a marked reduction of 7-Kst was detected. The ORP was equal to 0.011%, in the same order of magnitude estimated for POL. Moreover, the sterol content determined before and after the storage did not significantly ($p > 0.05$) change. The results obtained shown an ORP in the following order PO (0.006%) < HOSO (0.011%) < POL (0.012%). The latter has to be related to several factors such as composition in terms of fatty acids, degree of unsaturation, presence, or absence of metals that could act as catalysts for oxidative processes, content of natural antioxidants such as tocopherols and tocotrienols and relative content of sterols. All above-mentioned factors affect the formation of POPs. As well reported by Ansorena et al. (2013), the presence of unsaturated fatty acids delays the formation of oxidation products of sterols. In a similar way, higher concentration of unsaturated fatty acids detected in sunflower oil (Firestone, 2013) contributed to the drop off the formation of POPs. Again, the content of tocopherols and tocotrienols, higher in PO than HOSO, counteracted lipid oxidation (Firestone, 2013). Finally, the composition of fatty acids as well as the content of natural antioxidants played a key role in the oxidation phenomenon of tested vegetable oils. The FFA, PV, p-An, Rancimat, and K_{232} data showed the typical trend obtained in oxidative studies conducted in the adopted storage conditions.

The objective of this work was first to describe a new procedure to collect and isolate the major oxidized forms of the major fatty constituent oxysterols of plant nature. The next step was to validate a method in LC Orbitrap- HRMS using the collected forms as analytical standards. Then, it was desired to test that method on two real case studies. The reasoning that can be done, considering the case study of fat phases maintained under forced storage conditions, is also to understand whether or not POPs can be considered descriptive markers of the oxidation processes considered in the present work. To do this, it is of fundamental importance to have monitored, during the oxidative processes considered, a whole series of parameters that to date are used in the oils and fats landscape to evaluate oxidative processes. Assessing the oxidative state of a fat phase is a very complicated procedure. Descriptive parameters based on empirical methods are usually used, the analytical result of which can be affected by a multiplicity of parameters. Only a comparison of the data obtained from so-called freshness analysis and the results in terms of variation in POPs content can return us enough information to understand whether or not oxyphytosterols can be considered markers. Regarding the oxidative process, considering the conditions applied in the present work and in light of the results obtained showing no significant changes recorded for POPs, it is possible to conclude that the latter cannot be considered sufficiently predictive and descriptive markers oxidation processes conducted under the aforementioned conditions. Clearly, that does not represent a conclusive assessment and further investigation is therefore needed by testing different storage conditions by varying the oxidation parameters involved.

4.4 Conclusion

A preparative LC method able to isolate the main pure oxidation products derived from β-sitosterol, campesterol, and stigmasterol, the main phytosterols present in vegetable oils, was developed. Higher purity (>90%) for all α-epoxy, β-epoxy, 7α-hydroxy, 7β-hydroxy, and 7-K isomers of β-sitosterol, campesterol, and stigmasterol was achieved. Based on that, a sensitive, reproducible, and robust LC-Orbitrap-HRMS analytical method was validated. The proposed method led to determining POPs with high accuracy and precision without purification chromatography (e.g., SPE) or derivatization (e.g., silylation) steps. The suitability of the analytical method was assessed in two real systems consisting of the refining chain and storage of palm oil, palm olein, and HOSO. As regards refining processing, it might be highlighted that palm oil displayed the lowest content of POPs, followed by palm olein and high sunflower oil. The neutralization led to a general increase in the oxidation of vegetable oils, while the deodorization induced a general decrease for all matrices considered. However, the present work represents the first study where POPs formation was correlated to refining and since many different parameters affect the refining process, further studies are required. Regarding the evolution of POPs during forced storage, there is a slight increase as related to the fat matrix considered. Thus, further studies on different fats and under slightly different conditions are needed to assess whether POPs could be considered predictive markers of lipid oxidation. However, the fact of having developed a method for isolation of pure POP standards opens the way to new scenarios. The present work represents a preliminary study; however, it could be applied to isolate additional pure compounds (such as brassicasterol or avenasterol oxidation products) necessary to better study the oxidation of different processed vegetable oils.

A powerful validate method now had been developed and fifteen POPs pure standard from three major phytosterols in vegetable oils had been collected. The evolution of POPs profile in different fats matrix had been scouted but the effect of other macromolecules in different model (e.g. semi-finished or finished products) represent the next step to lead the evaluation of POPs at new stage.

Industrial processes such as baking, frying and steam insufflation with the presence of forced factors such as oxygen and high temperatures, can influence the presence of POPs in finished products. The acquired analytical advantage should be used for the study of complex models such as baked goods, French fries, spreadable cream etc. during production and during storage (shelf life).

The qualitative/quantitative approach based on the assumption of the correlation between cholesterol oxidation products (COPs) and phytosterol oxidation products (POPs) should be abandoned and all the literature should be re-evaluated. The assessments should be repeated using the correct POPs standards and further evaluation will be mandatory.

4.5 References

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4.6 Supplementary material

The following data are reported for each oxyphytosterol isolated and collected in the present work:

- structure formulas;
- mass spectra obtained in GC-MS for its recognition;
- full mass chromatogram in LC-Orbitrap-HRMS;
- mass spectra Q1 and mass spectra of the product ion Q3 in LC-Orbitrap-HRMS.

The list of analytes evaluated for sitosterol, campesterol and stigmasterol is as follows:

- 5,6α-epoxy
- 5,6β epoxy
- 7-keto
- 7α-hydroxy
- 7β-hydroxy

5,6α-epoxysitosterol

The structure formula for 5,6α-epoxysitosterol is shown in FIG.1. The fragmentation pattern has been reported in FIG.2. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 502, 412, 394 and 197.

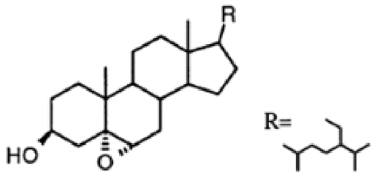


FIG.1 Structure formulas of 5,6α-epoxysitosterol. Image edited by Bortolomeazzi, et al., 2003.

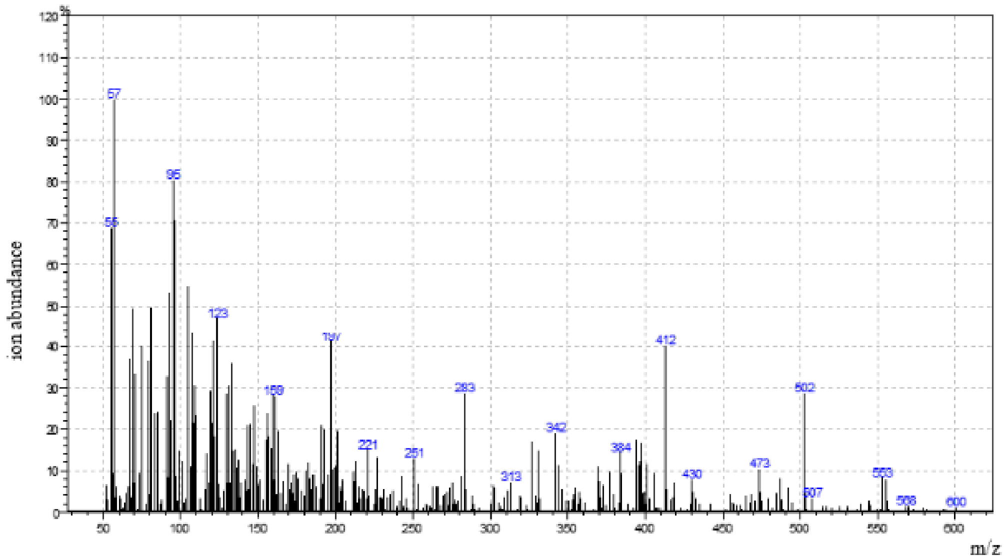


FIG. 2 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 5,6α-epoxysitosterol shows the molecular ion at 502.

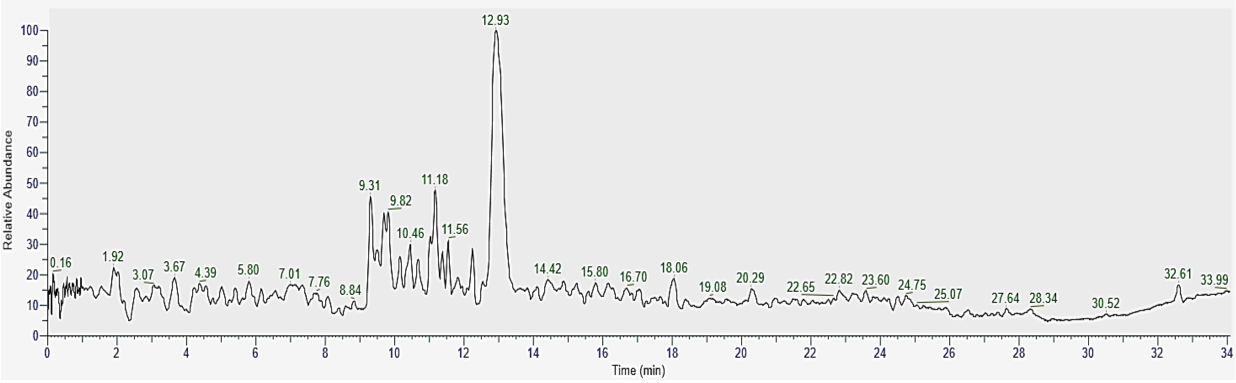


FIG. 3 Full mass chromatogram of 5,6α-epoxysitosterol (R.T. 12.93 min) in LC-Orbitrap-HRMS.

FIG. 4 Mass spectrum Q1 of 5,6α-epoxysitosterol in LC-Orbitrap-HRMS.

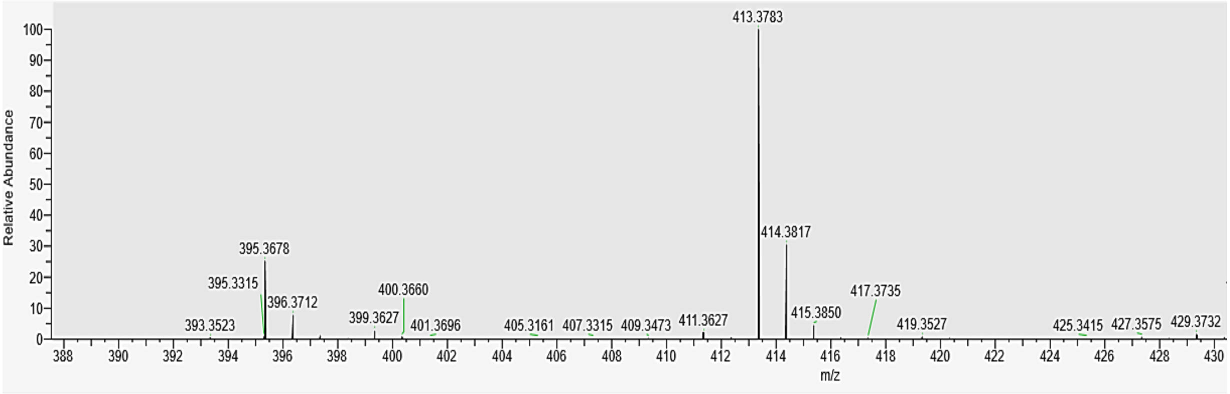
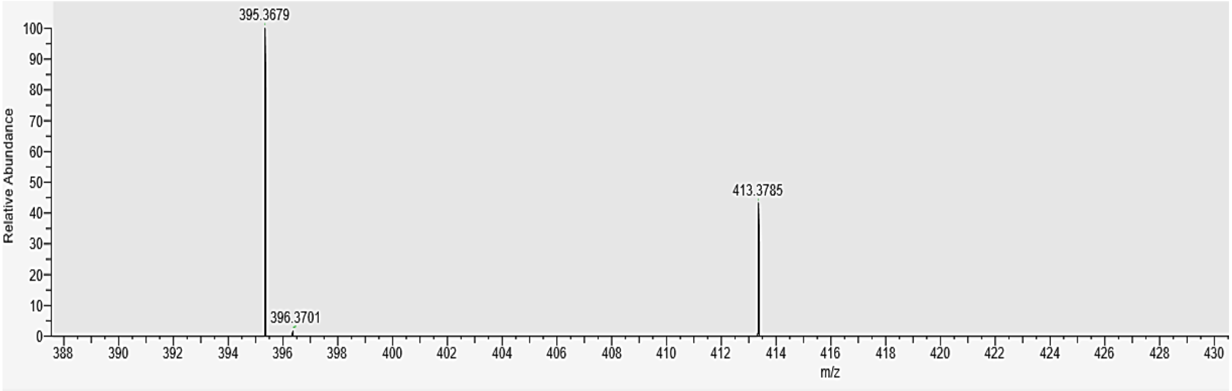


FIG. 5 Mass spectrum of the product ion Q3 of 5,6α-epoxysitosterol in LC-Orbitrap-HRMS.



5,6β-epoxysitosterol

The structure formula for 5,6β-epoxysitosterol is shown in FIG.6. The fragmentation pattern has been reported in FIG.7. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 502, 412, 394 and 197.

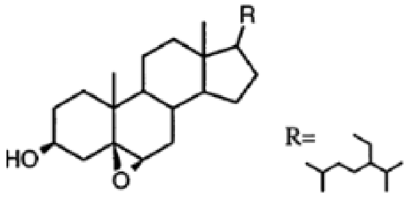


FIG.6 Structure formulas of 5,6β-epoxysitosterol. Image edited by Bortolomeazzi, et al., 2003

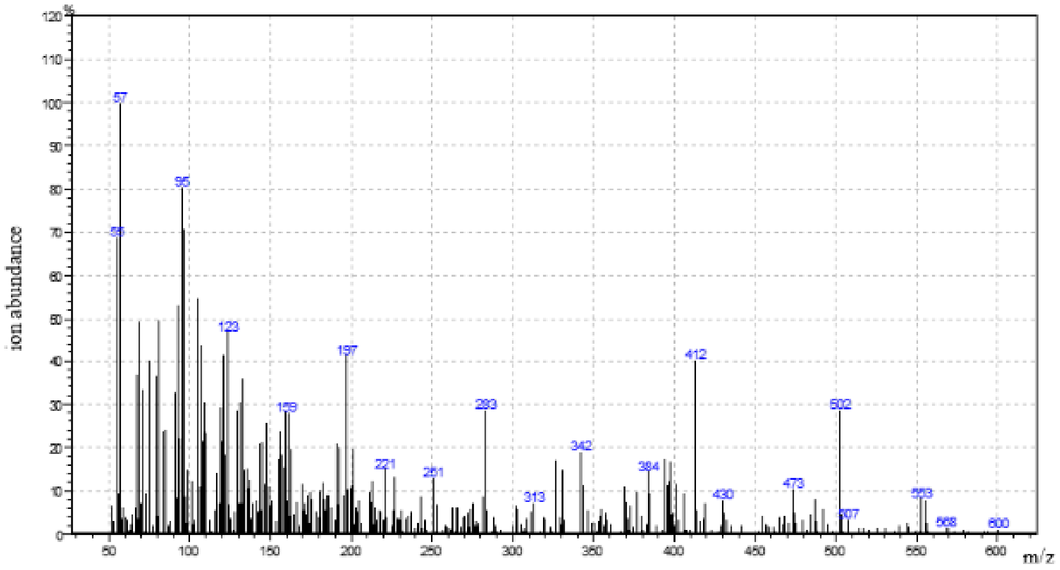


FIG. 7 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 5,6β-epoxysitosterol shows the molecular ion at 502.

FIG. 8 Full mass chromatogram of 5,6β-epoxysitosterol (R.T. 16.63 min) in LC-Orbitrap-HRMS.

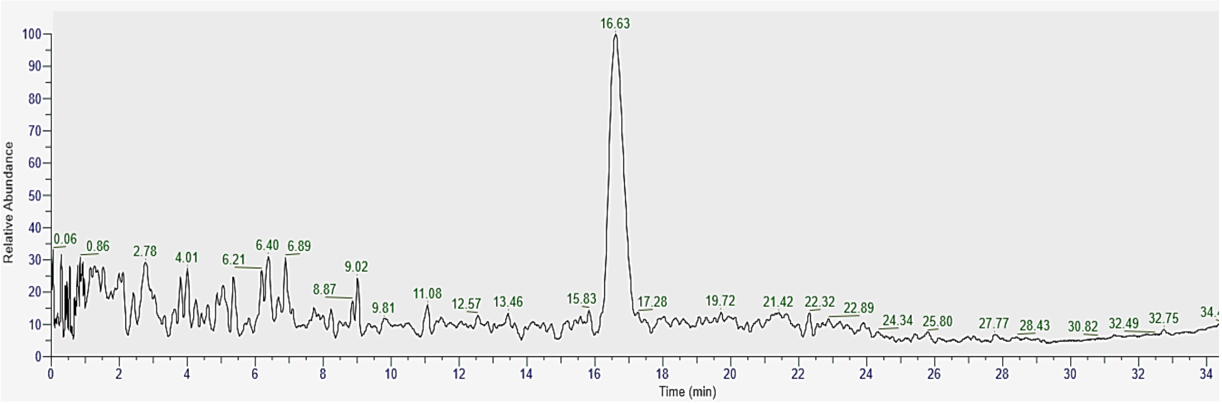


FIG. 9 Mass spectrum Q1 of 5,6β-epoxysitosterol in LC-Orbitrap-HRMS.

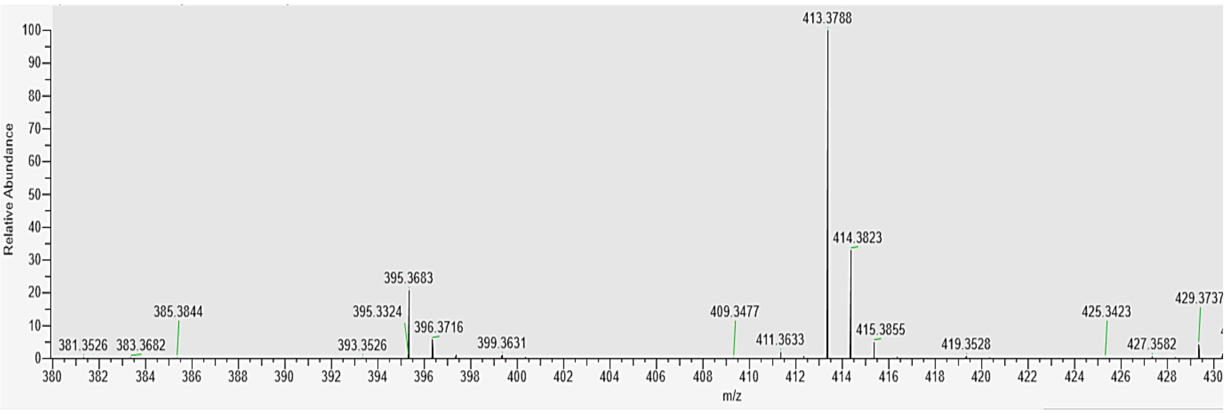
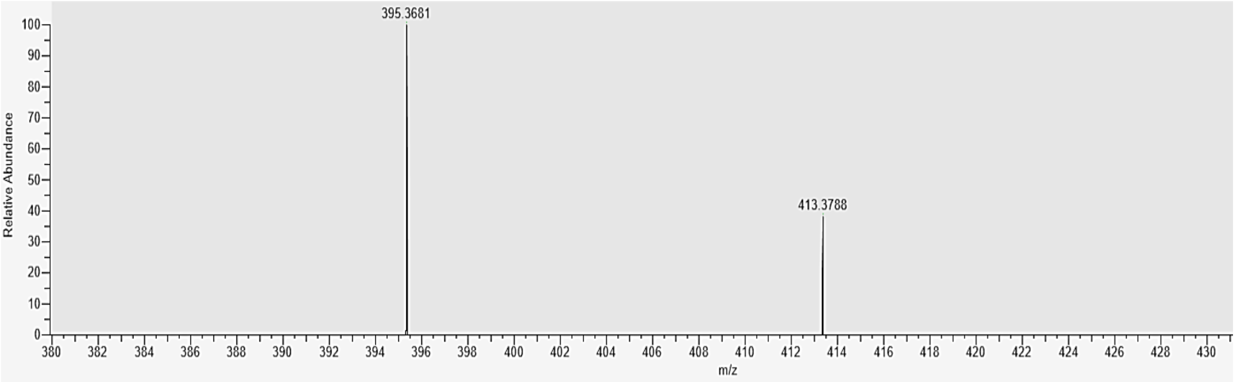


FIG. 10 Mass spectrum of the product ion Q3 of 5,6β-epoxysitosterol in LC-Orbitrap-HRMS.



7-ketositosterol

The structure formula for 7-ketositosterol is shown in FIG.11. The fragmentation pattern has been reported in FIG.12. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 500, 485, 395 and 129.

FIG.11 Structure formulas of 7-ketositosterol. Image edited by Bortolomeazzi, et al., 2003

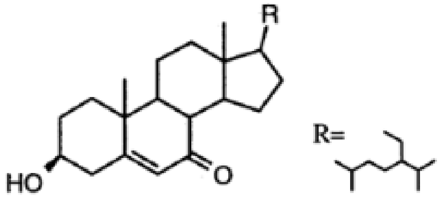


FIG. 12 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 7-ketositosterol shows the molecular ion at 500.

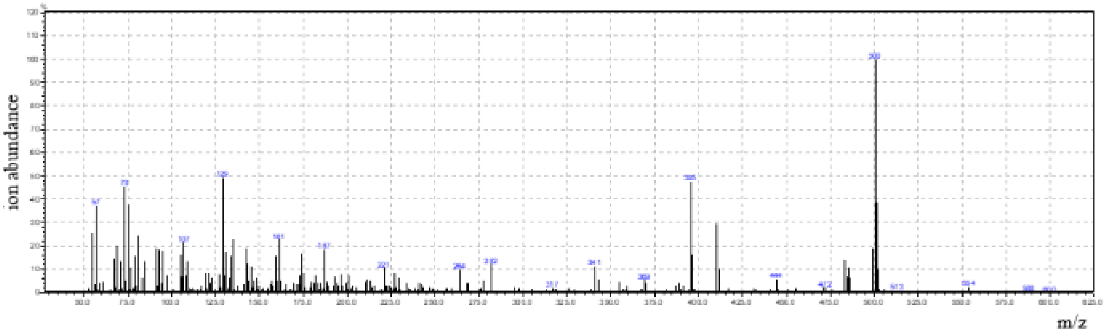


FIG. 13 Full mass chromatogram of 7-keto sitosterol (R.T. 26.01 min) in LC-Orbitrap-HRMS.

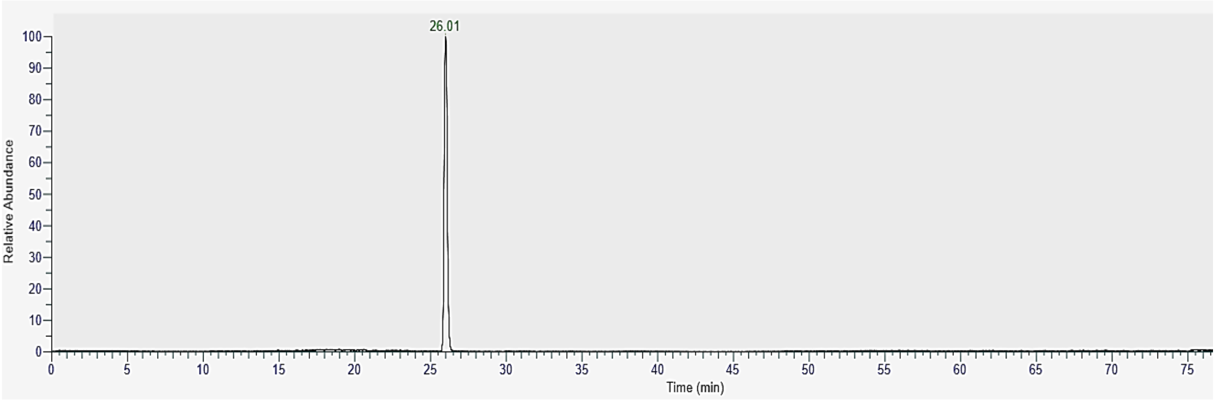


FIG. 14 Mass spectrum Q1 of 7-keto sitosterol in LC-Orbitrap-HRMS.

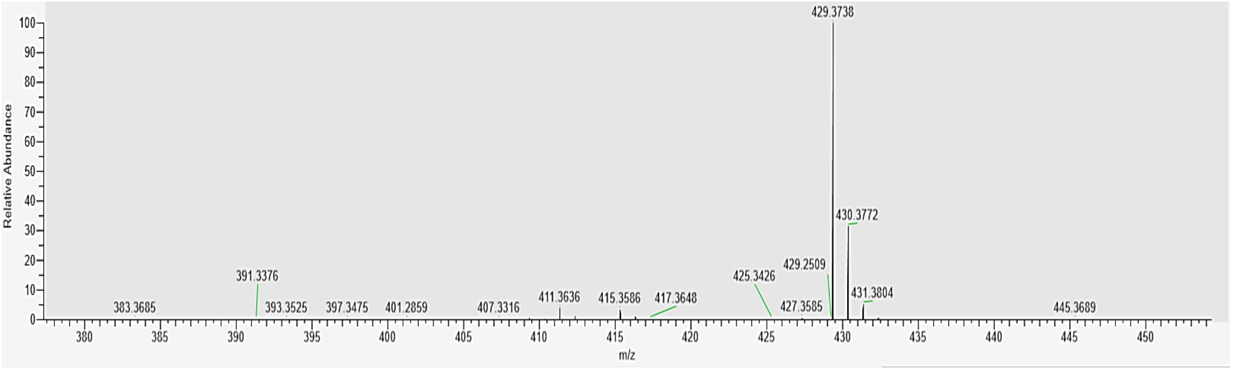
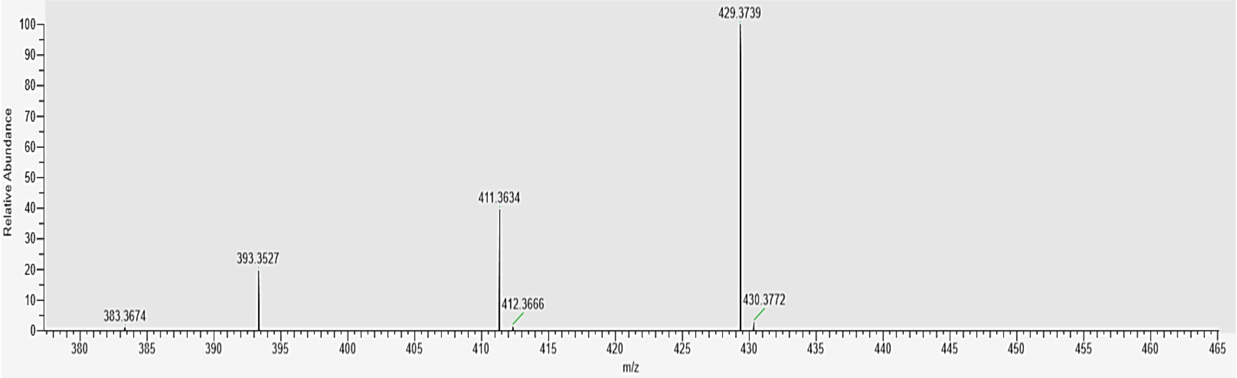


FIG. 15 Mass spectrum of the product ion Q3 of 7-keto sitosterol in LC-Orbitrap-HRMS.



7α-hydroxysitosterol

The structure formula for 7α-hydroxysitosterol is shown in FIG.16. The fragmentation pattern has been reported in FIG.17. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 574, 484, 253, 129.

FIG.16 Structure formulas of 7α-hydroxysitosterol. Image edited by Bortolomeazzi, et al., 2003.

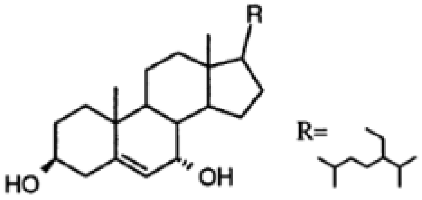


FIG. 17 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 7α-hydroxysitosterol shows the molecular ion at 574.

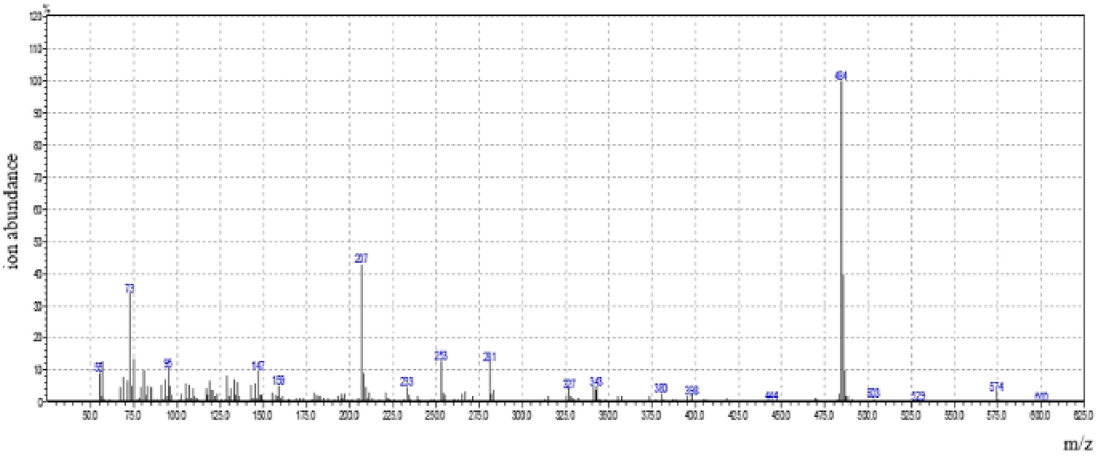


FIG. 18 Full mass chromatogram of 7α-hydroxysitosterol (R.T. 29.63 min) in LC-Orbitrap-HRMS.

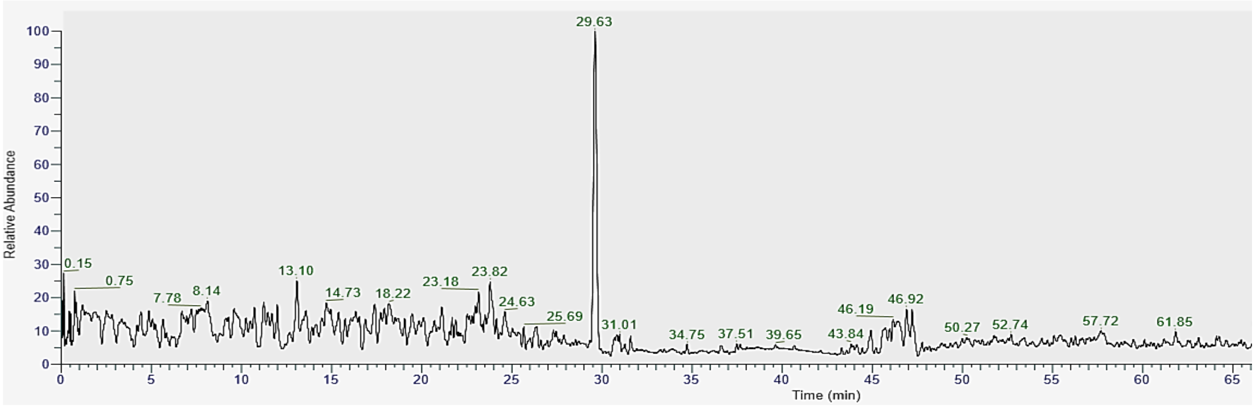


FIG. 19 Mass spectrum Q1 of 7α-hydroxysitosterol in LC-Orbitrap-HRMS.

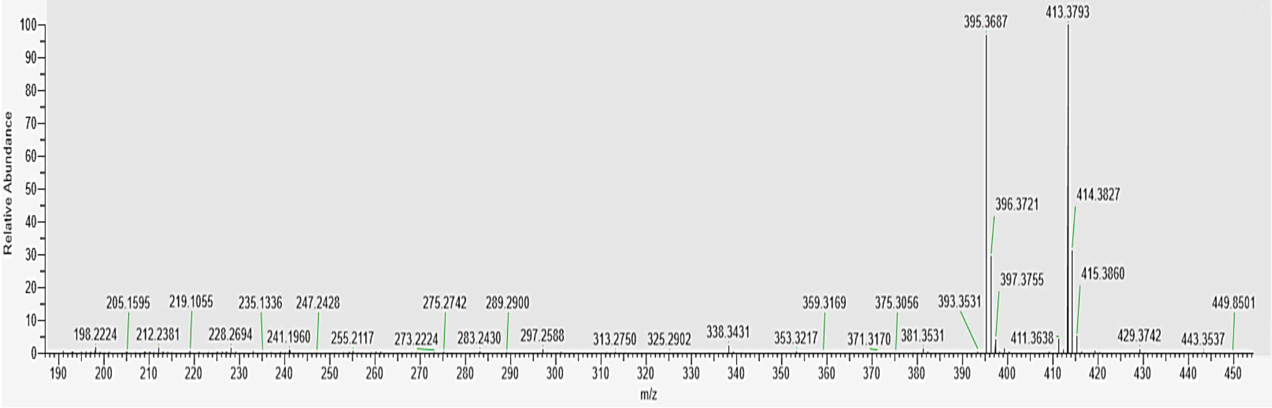
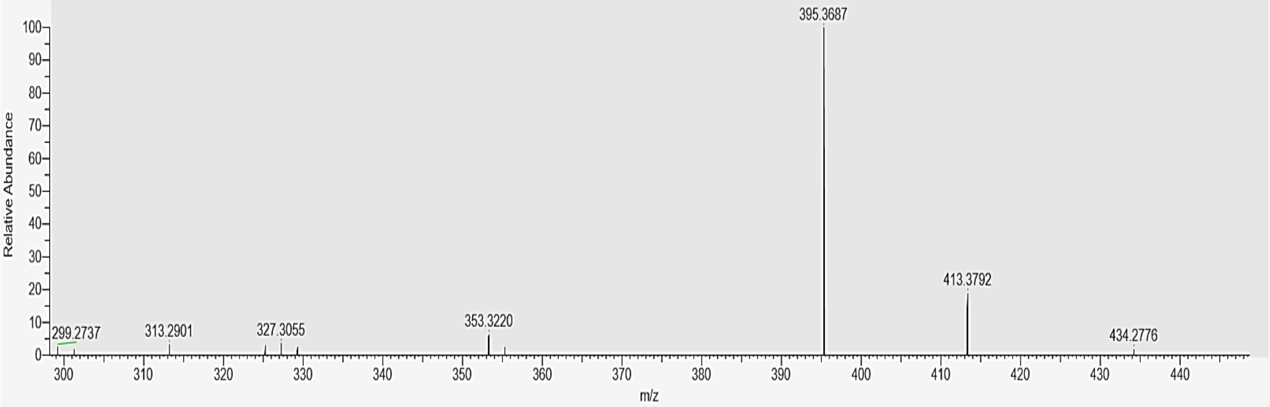


FIG. 20 Mass spectrum of the product ion Q3 of 7α-hydroxysitosterol in LC-Orbitrap-HRMS.



7β-hydroxysitosterol

The structure formula for 7β-hydroxysitosterol is shown in FIG.20. The fragmentation pattern has been reported in FIG.21. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 574, 484, 253, 129.

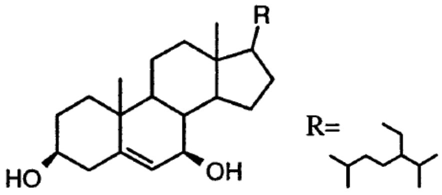


FIG.21 Structure formulas of 7β-hydroxysitosterol. Image edited by Bortolomeazzi, et al., 2003.

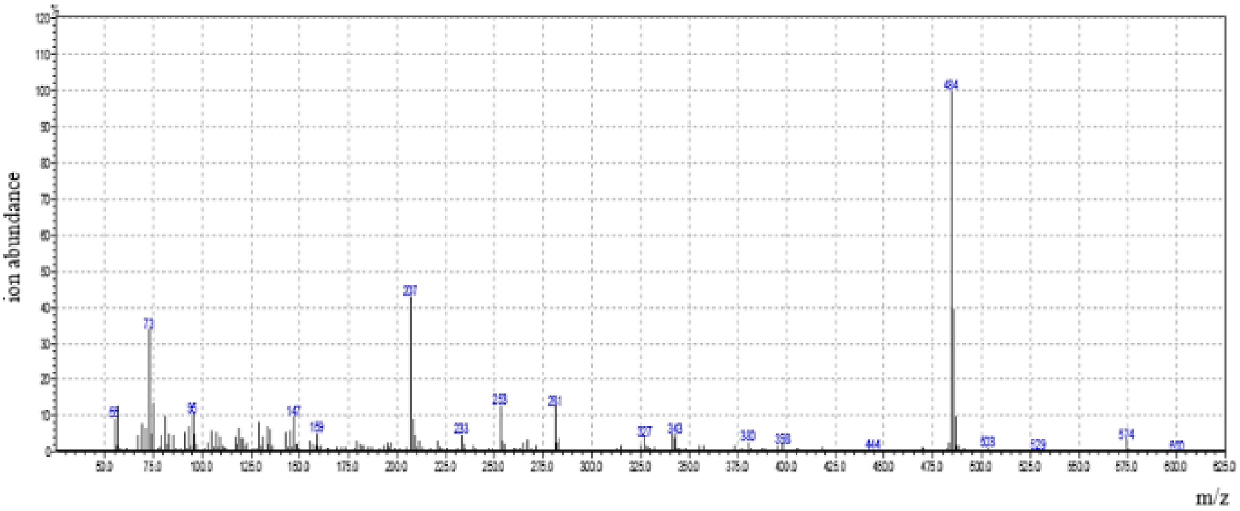


FIG. 22 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 7β-hydroxysitosterol shows the molecular ion at 574.

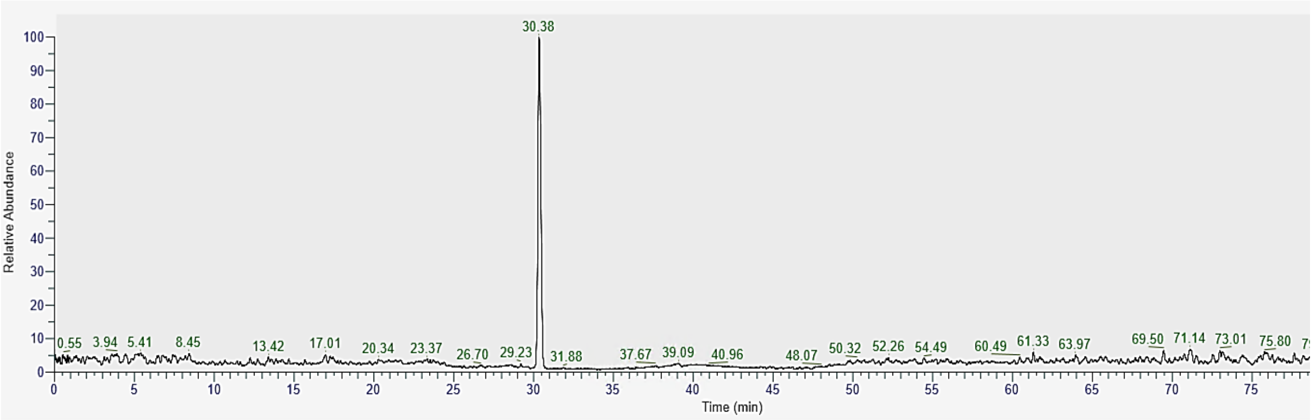


FIG. 23 Full mass chromatogram of 7β-hydroxysitosterol (R.T. 30.38 min) in LC-Orbitrap-HRMS.

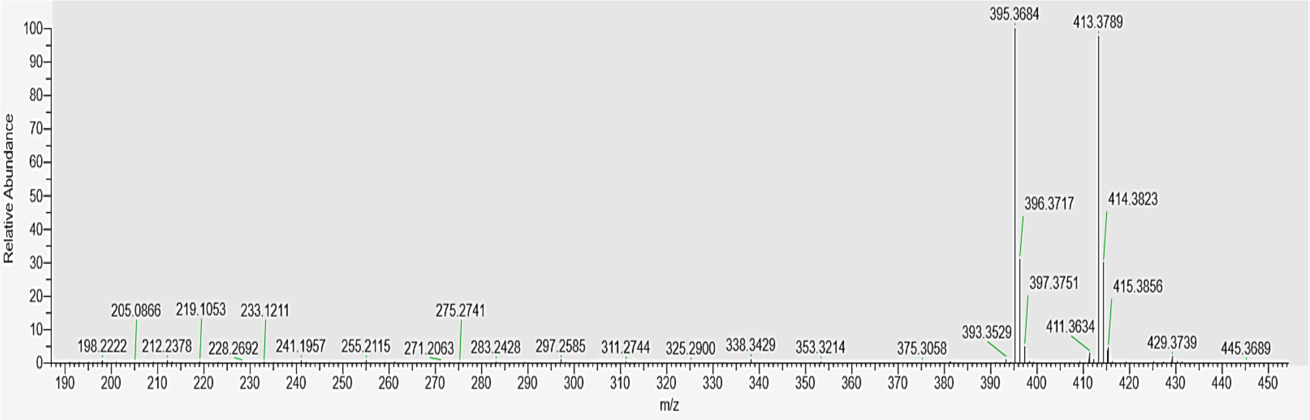


FIG. 24 Mass spectrum Q1 of 7β-hydroxysitosterol in LC-Orbitrap-HRMS.

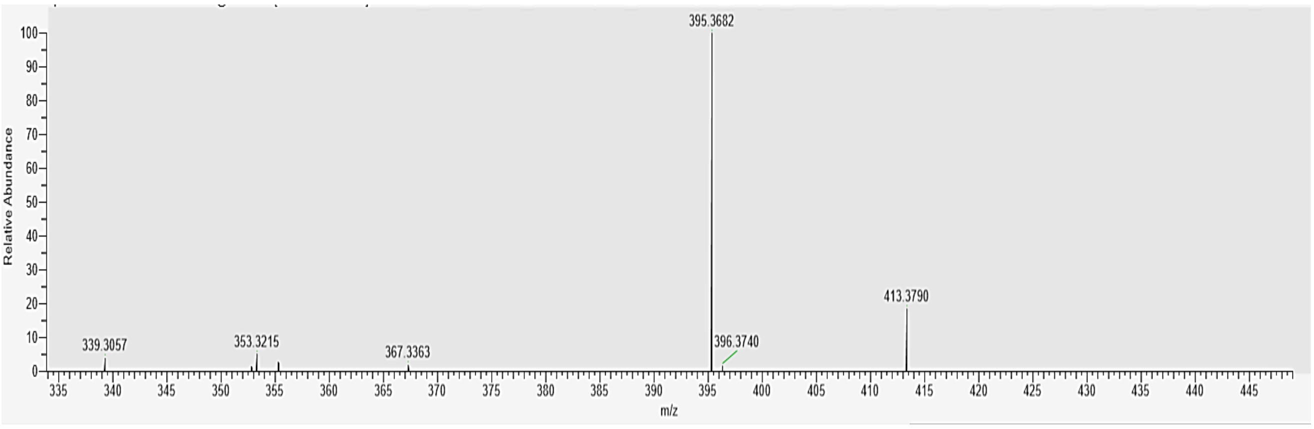


FIG. 25 Mass spectrum of the product ion Q3 of 7β-hydroxysitosterol in LC-Orbitrap-HRMS.

5,6α-epoxycampesterol

The structure formula for 5,6α-epoxycampesterol is shown in FIG.25. The fragmentation pattern has been reported in FIG.26. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 488, 398, 370, 253.

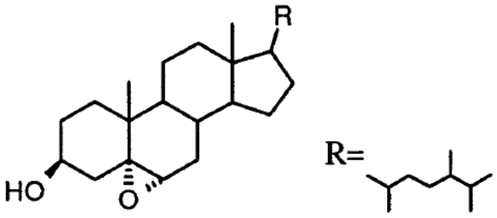


FIG.26 Structure formulas of 5,6α-epoxycampesterol. Image edited by Bortolomeazzi, et al., 2003.

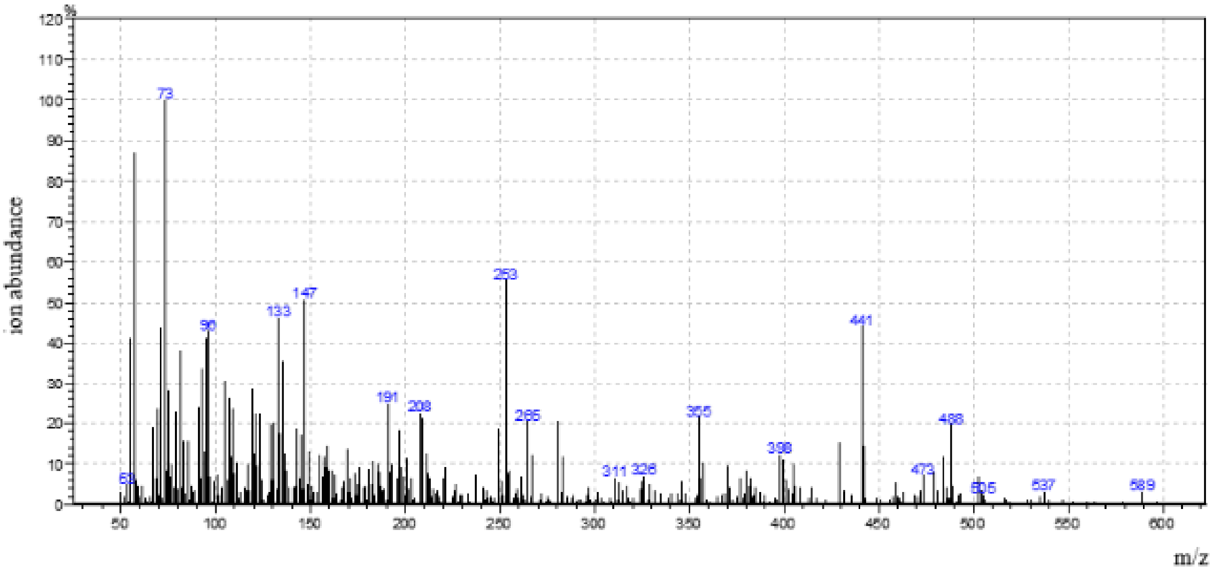


FIG.27 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 5,6α-epoxycampesterol shows the molecular ion at 488.

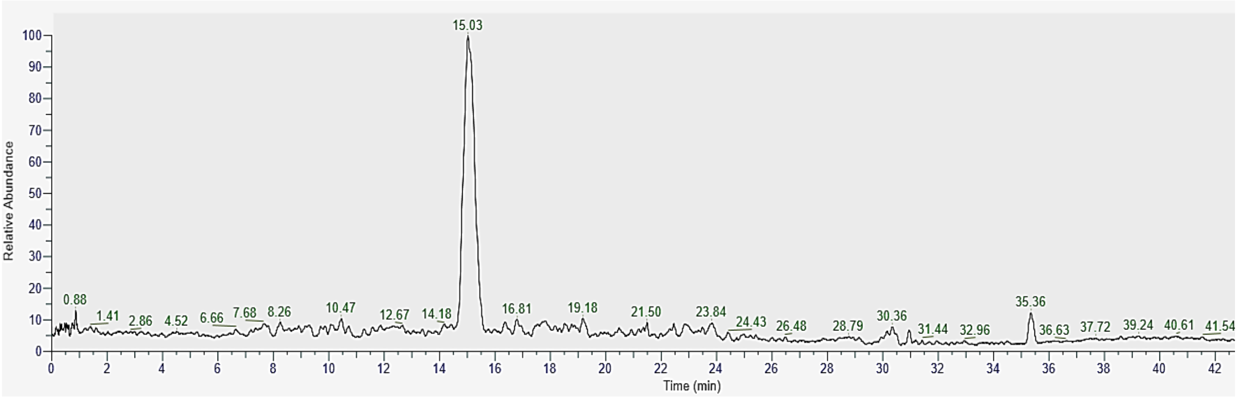


FIG. 28 Full mass chromatogram of 5,6α-epoxycampesterol (R.T. 15.03 min) in LC-Orbitrap-HRMS.

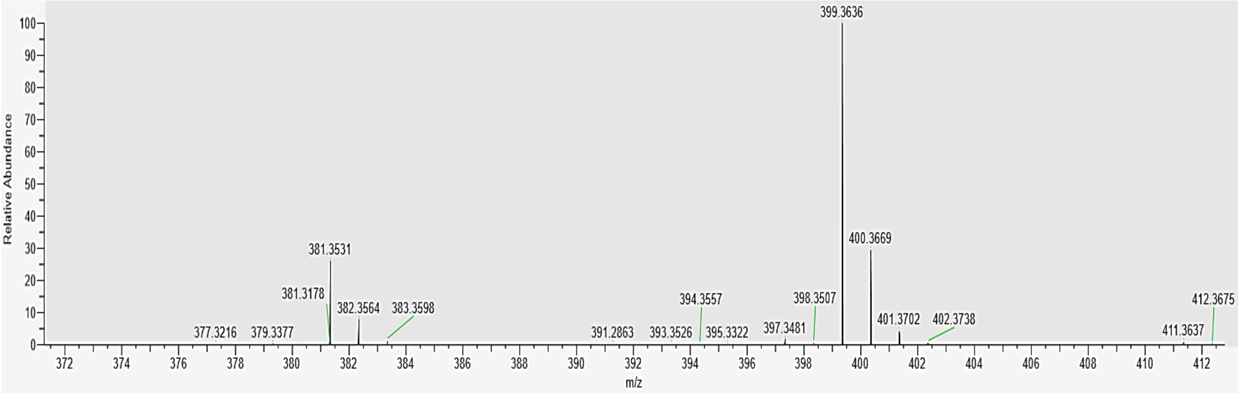


FIG. 29 Mass spectrum Q1 of 5,6α-epoxycampesterol in LC-Orbitrap-HRMS.

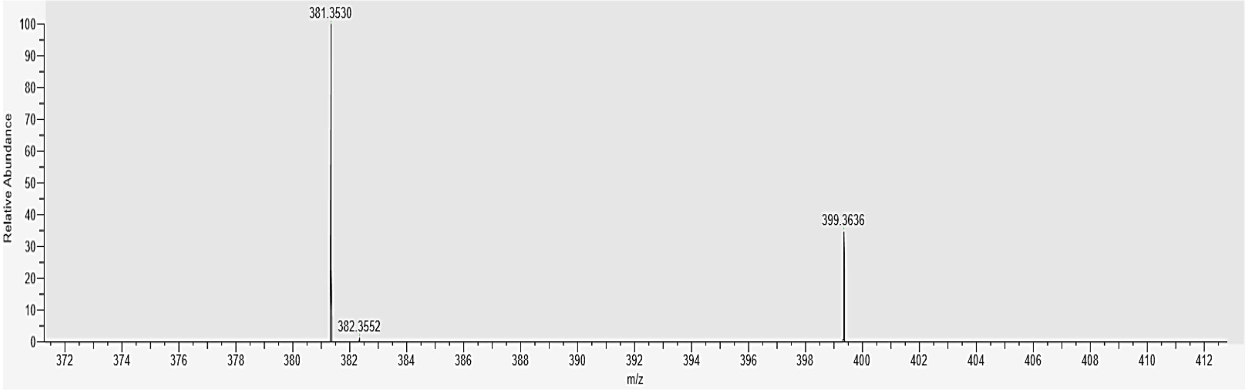


FIG. 30 Mass spectrum of the product ion Q3 of 5,6α-epoxycampesterol in LC-Orbitrap-HRMS.

5,6β-epoxycampesterol

The structure formula for 5,6β-epoxycampesterol is shown in FIG.30. The fragmentation pattern has been reported in FIG.31. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 488, 398, 370, 253.

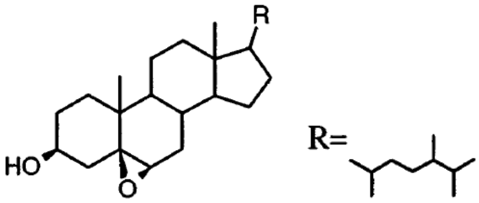


FIG.31 Structure formulas of 5,6β-epoxycampesterol. Image edited by Bortolomeazzi, et al., 2003.

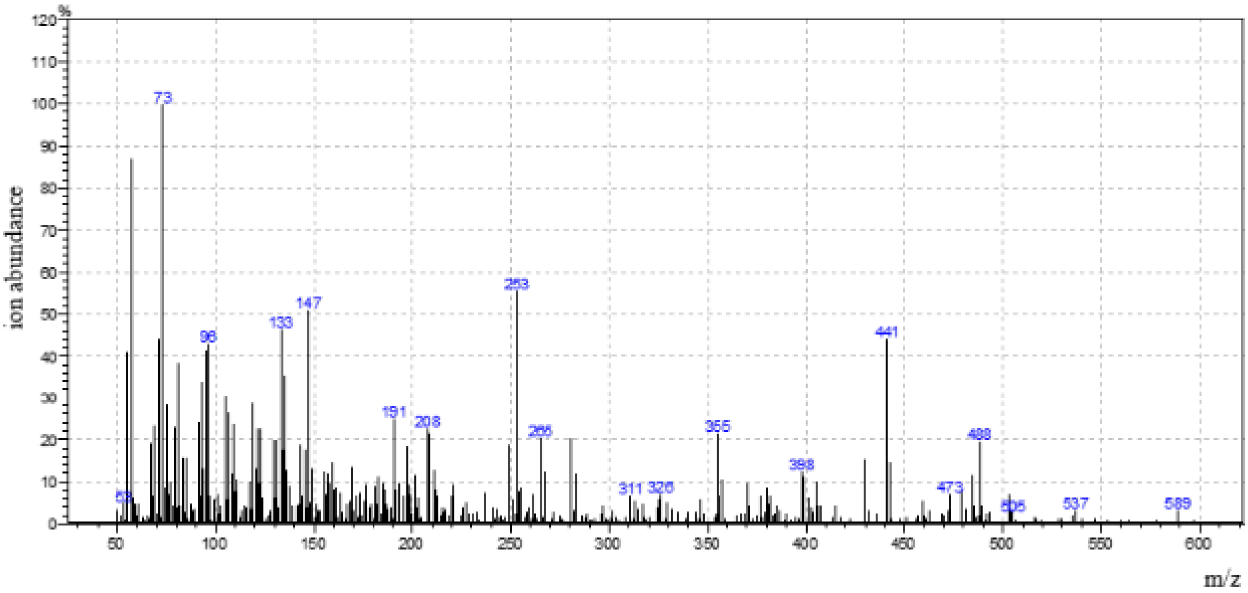


FIG.32 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 5,6β-epoxycampesterol shows the molecular ion at 488.

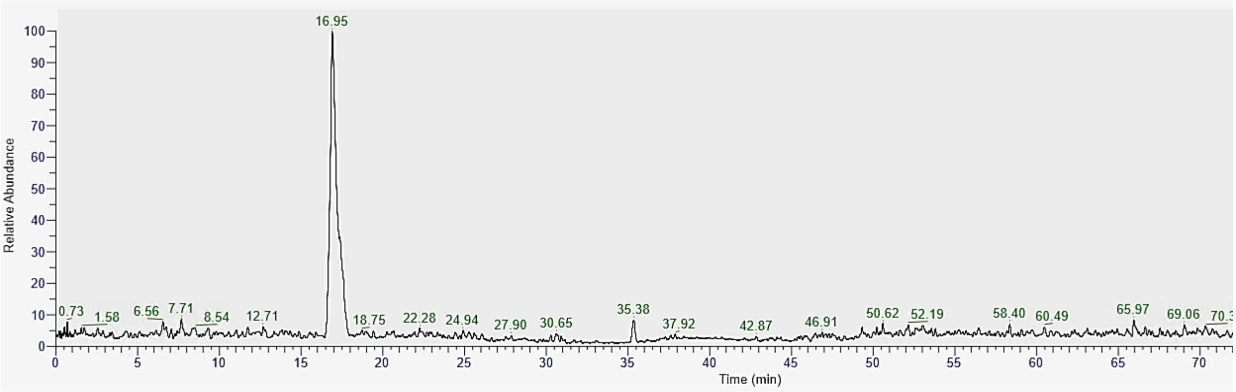


FIG. 33 Full mass chromatogram of 5,6β-epoxycampesterol (R.T. 16.95 min) in LC-Orbitrap-HRMS.

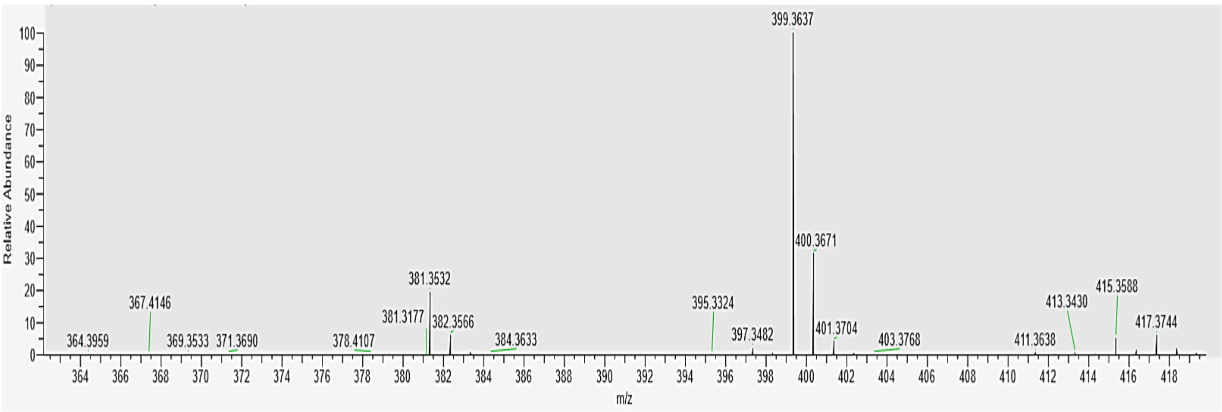


FIG. 34 Mass spectrum Q1 of 5,6β-epoxycampesterol in LC-Orbitrap-HRMS.

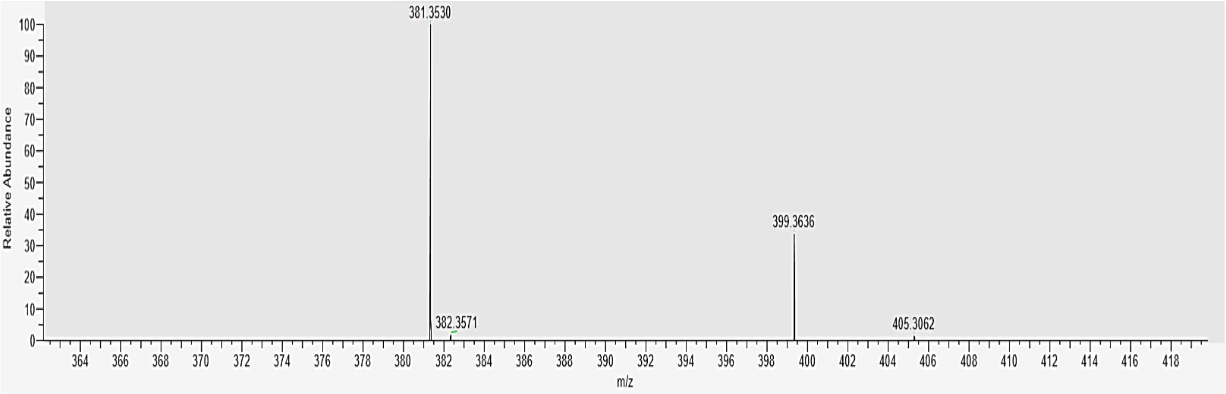


FIG. 35 Mass spectrum of the product ion Q3 of 5,6β-epoxycampesterol in LC-Orbitrap-HRMS.

7-ketocampesterol

The structure formula for **7**-ketocampesterol is shown in FIG.35. The fragmentation pattern has been reported in FIG.36. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 486, 396, 381, 129.

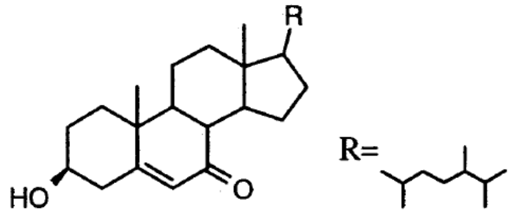


FIG.36 Structure formulas of 7-ketocampesterol. Image edited by Bortolomeazzi, et al., 2003.

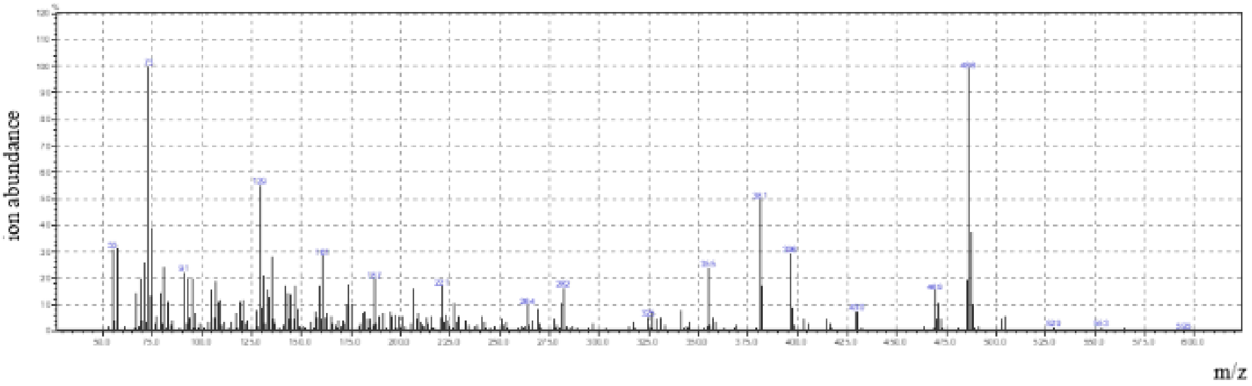


FIG. 37 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 7-ketocampesterol shows the molecular ion at 486.

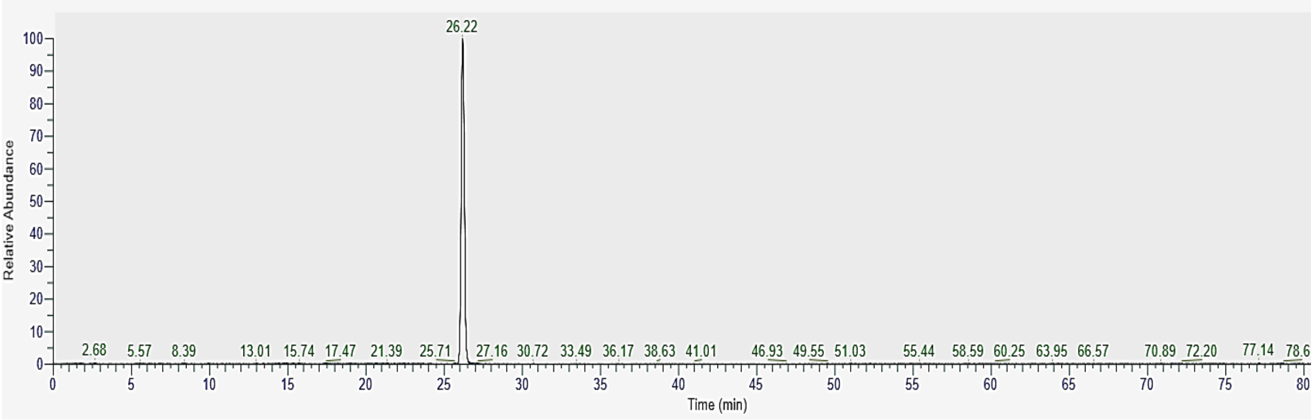


FIG. 38 Full mass chromatogram of 7-ketocampesterol (R.T. 26.22 min) in LC-Orbitrap-HRMS.

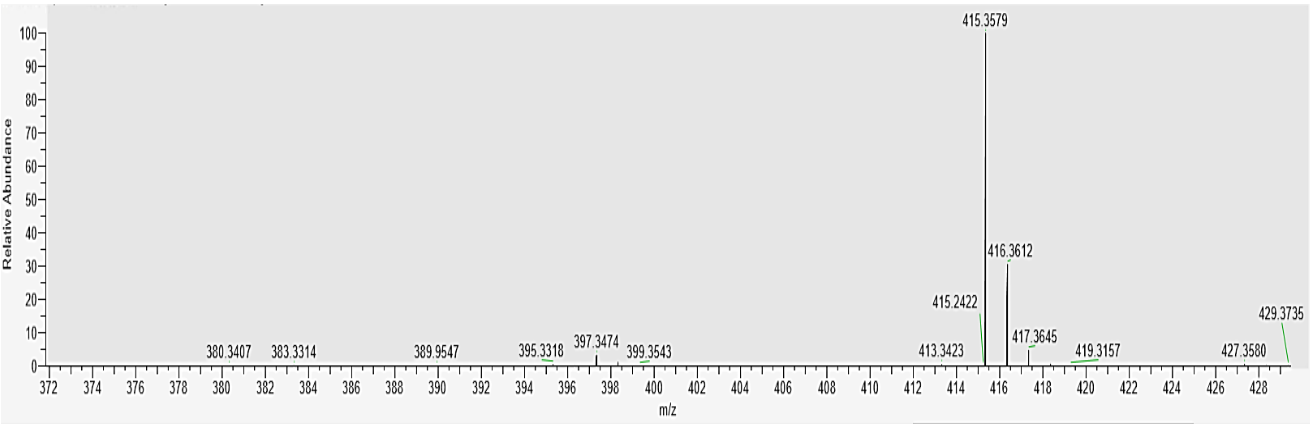


FIG. 39 Mass spectrum Q1 of 7-ketocampesterol in LC-Orbitrap-HRMS.

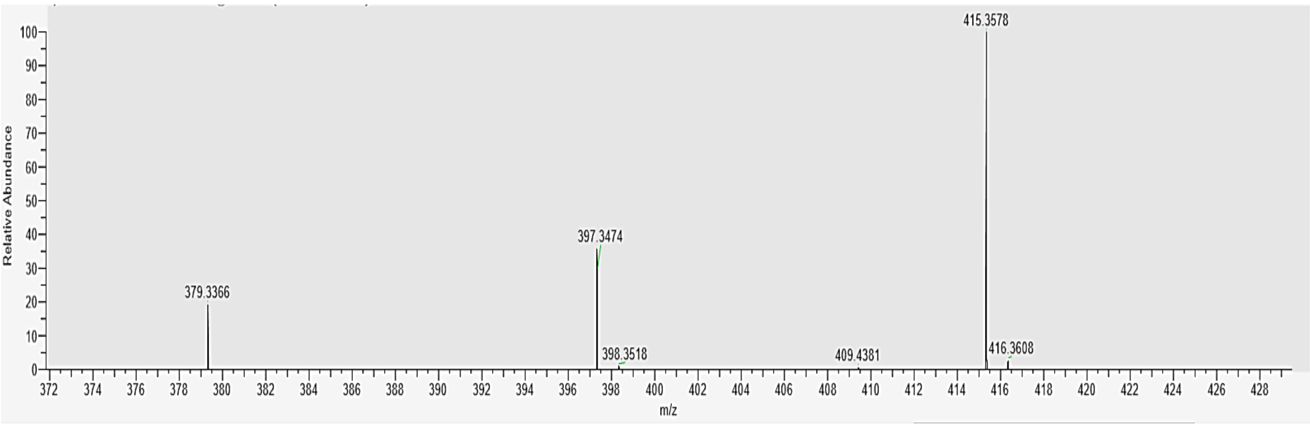


FIG. 40 Mass spectrum of the product ion Q3 of 7-ketocampesterol in LC-Orbitrap-HRMS.

7α-hydroxycampesterol

The structure formula for 7α-hydroxycampesterol is shown in FIG.40. The fragmentation pattern has been reported in FIG.41. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 560, 470, 486, 396.

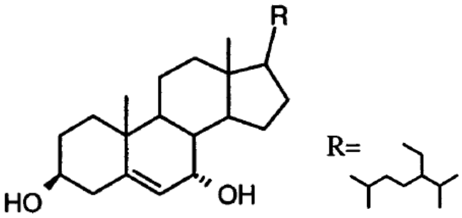


FIG.41 Structure formulas of 7α-hydroxycampesterol Image edited by Bortolomeazzi, et al., 2003.

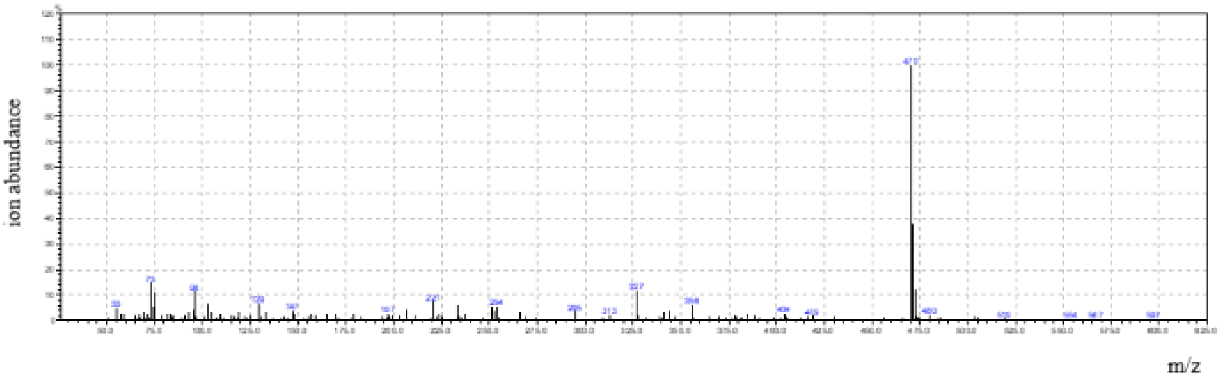


FIG. 42 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 7α-hydroxycampesterol shows the molecular ion at 560.

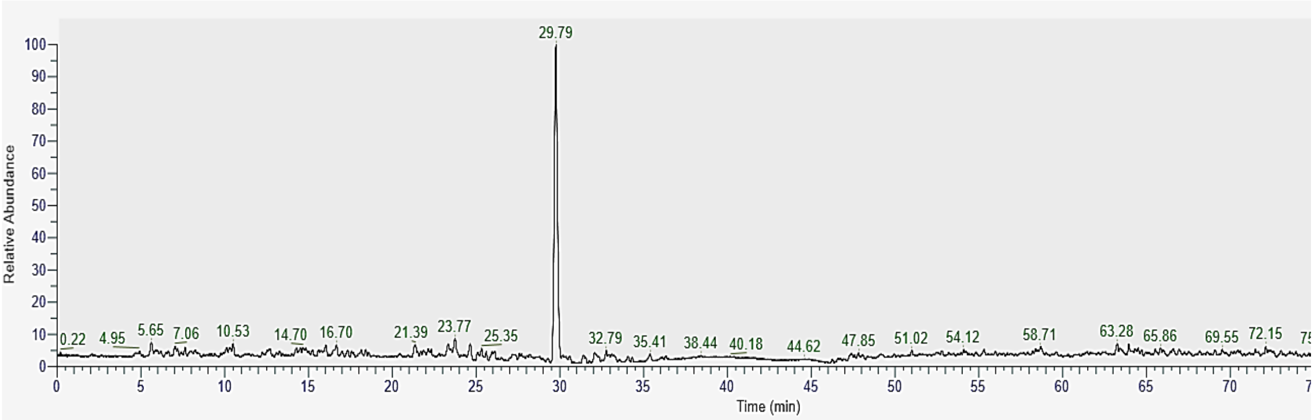


FIG. 43 Full mass chromatogram of 7α-hydroxycampesterol (R.T. 29.79 min) in LC-Orbitrap-HRMS.

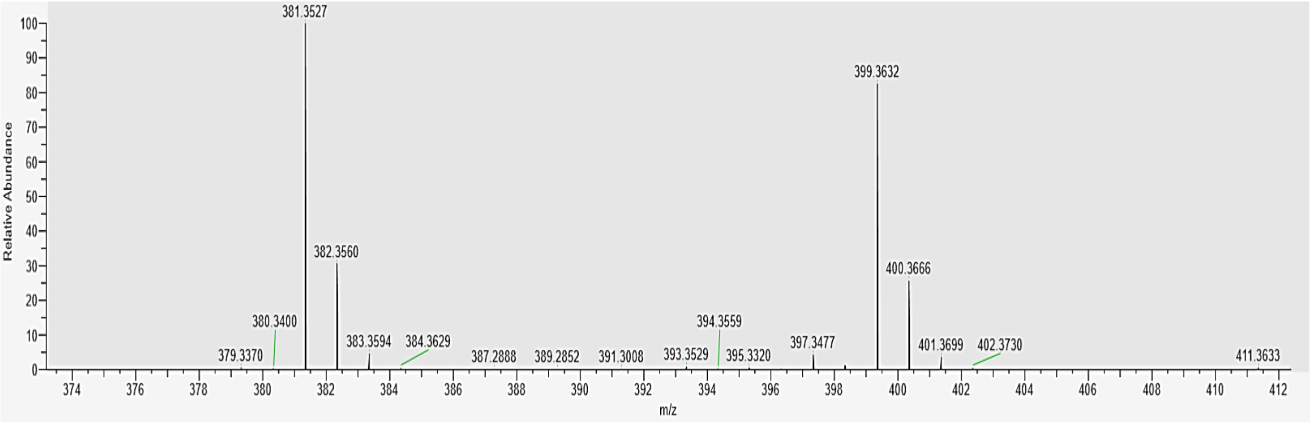


FIG. 44 Mass spectrum Q1 of 7α-hydroxycampesterol in LC-Orbitrap-HRMS.

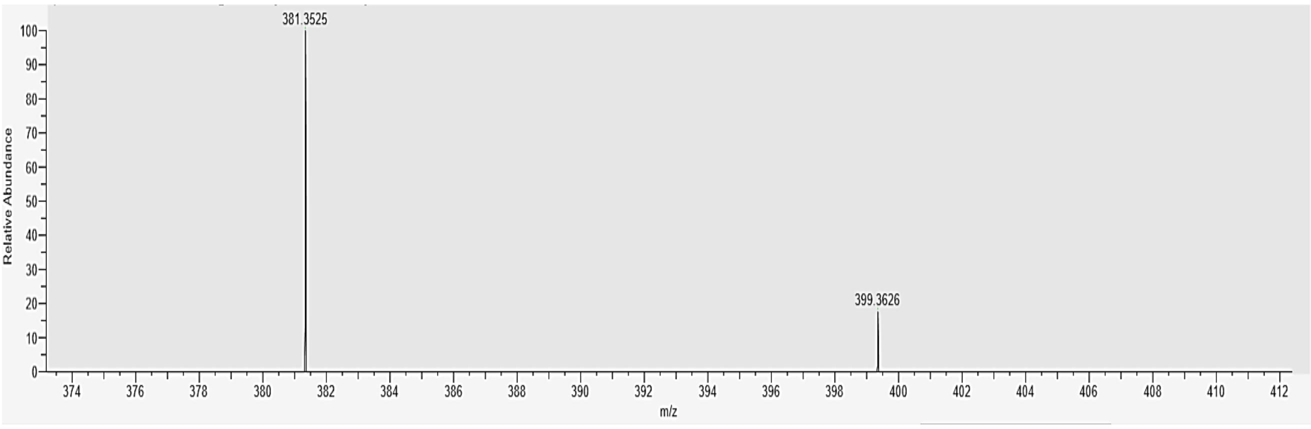


FIG. 45 Mass spectrum of the product ion Q3 of 7α-hydroxycampesterol in LC-Orbitrap-HRMS.

7β -hydroxycampesterol

The structure formula for 7β -hydroxycampesterol is shown in FIG.45. The fragmentation pattern has been reported in FIG.46. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 560, 470, 486, 396.

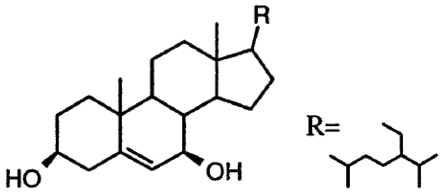


FIG.46 Structure formulas of 7β -hydroxycampesterol Image edited by Bortolomeazzi, et al., 2003.

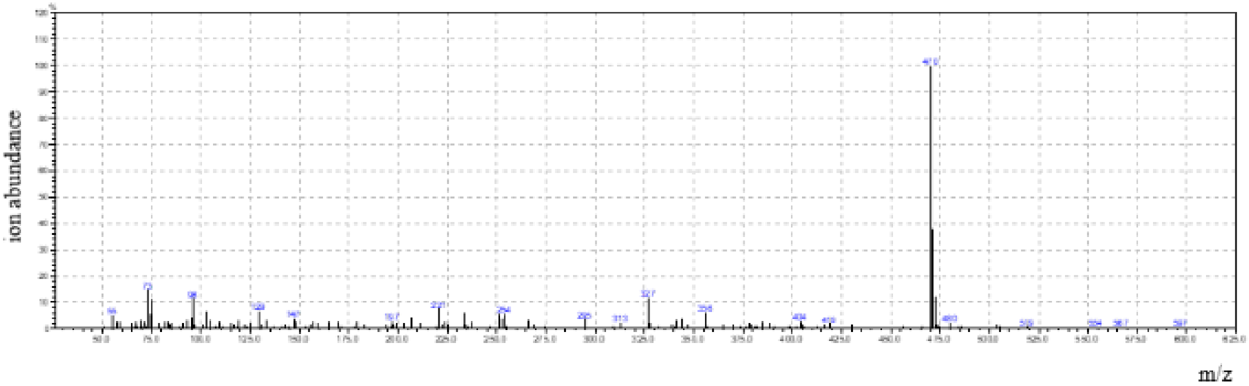


FIG. 47 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 7β-hydroxycampesterol shows the molecular ion at 560.

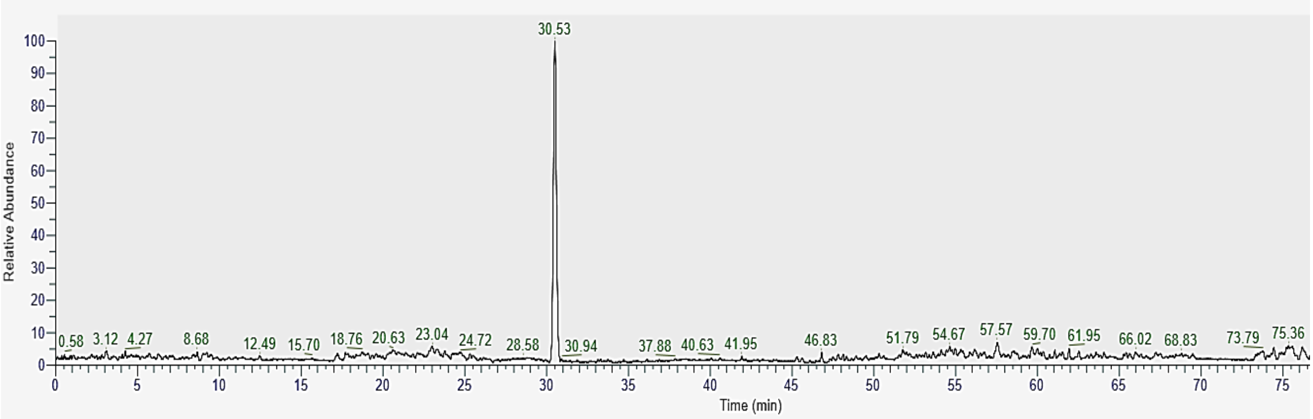


FIG. 48 Full mass chromatogram of 7β-hydroxycampesterol (R.T. 30.53 min) in LC-Orbitrap-HRMS.

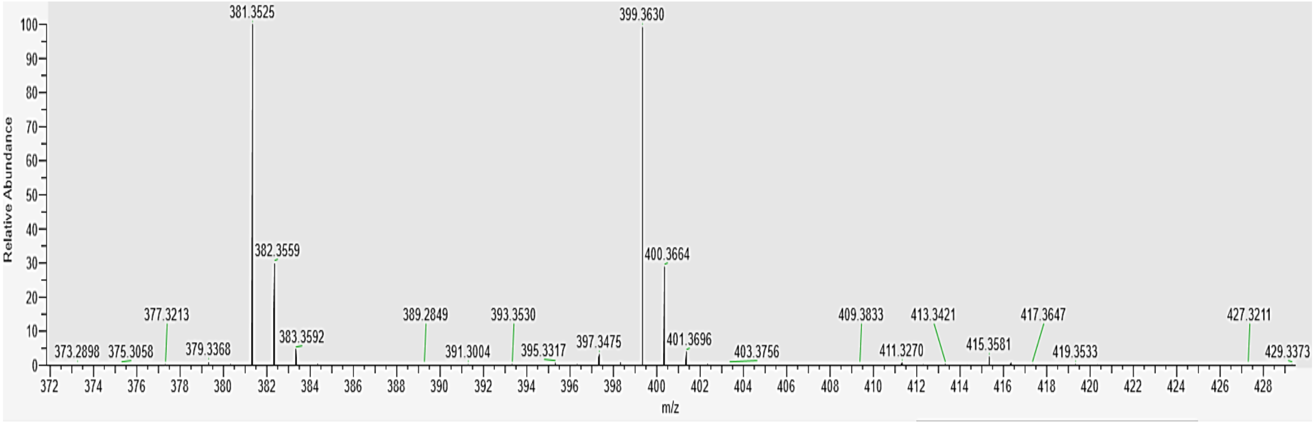


FIG. 49 Mass spectrum Q1 of 7β-hydroxycampesterol in LC-Orbitrap-HRMS.

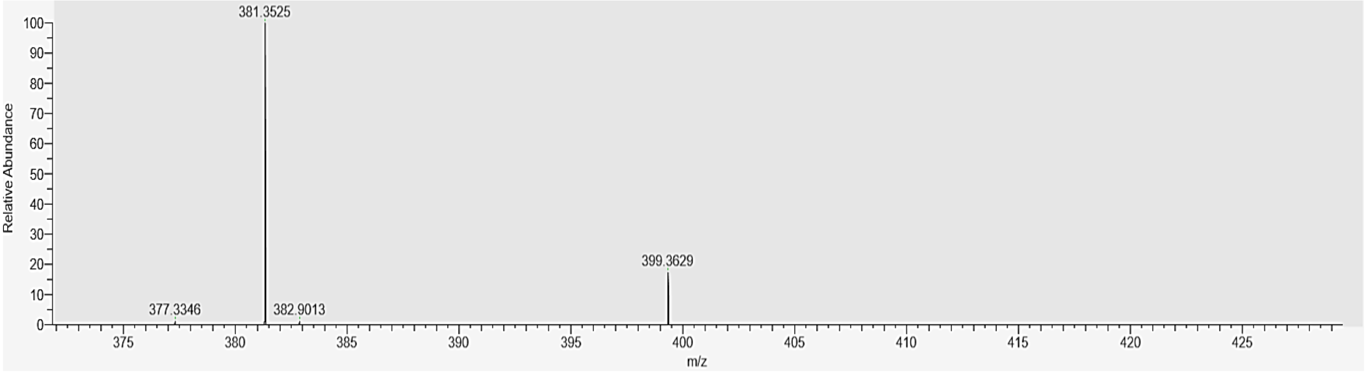


FIG. 50 Mass spectrum of the product ion Q3 of 7β-hydroxycampesterol in LC-Orbitrap-HRMS.

5,6α-epoxystigmasterol

The structure formula for 5,6α-epoxystigmasterol is shown in FIG.50. The fragmentation pattern has been reported in FIG.51. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 500, 410, 349 and 253.

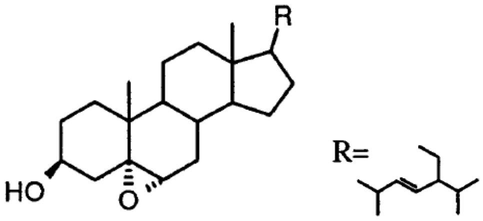


FIG.51 Structure formulas of 5,6α-epoxystigmasterol. Image edited by Bortolomeazzi, et al., 2003.

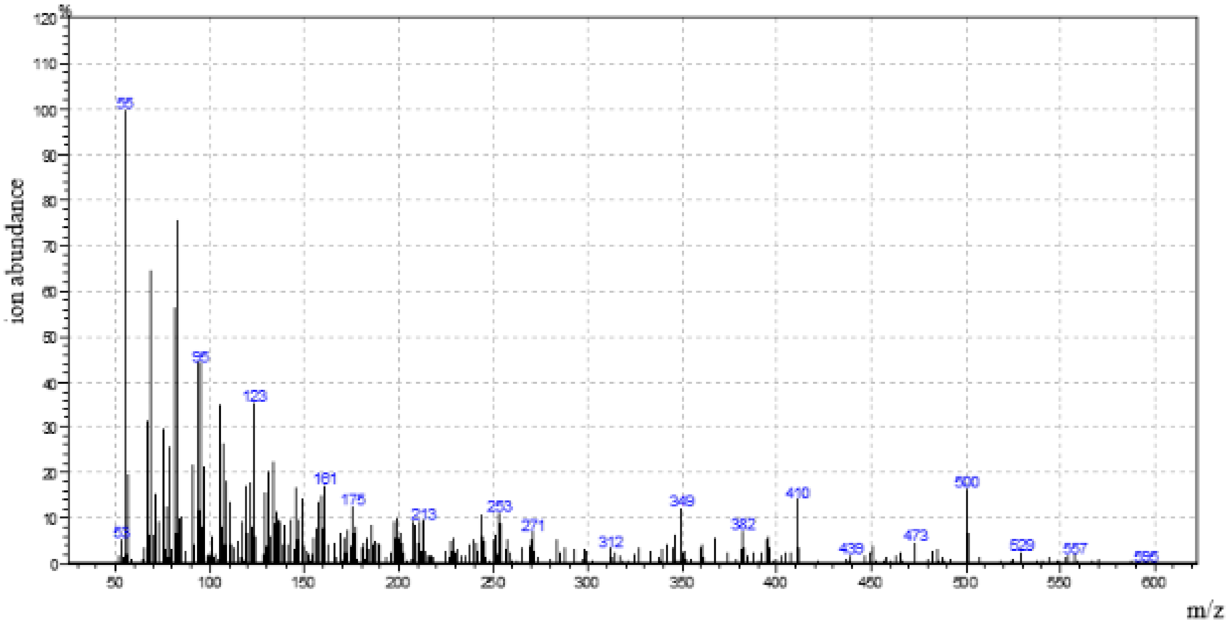


FIG. 52 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 5,6α-epoxystigmasterol shows the molecular ion at 500.

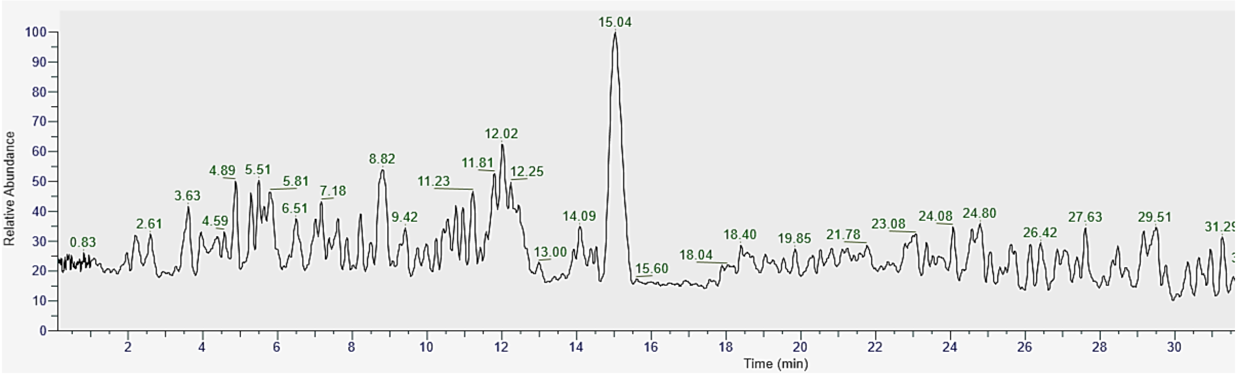


FIG. 53 Full mass chromatogram of 5,6α-epoxystigmasterol (R.T. 15.04 min) in LC-Orbitrap-HRMS.

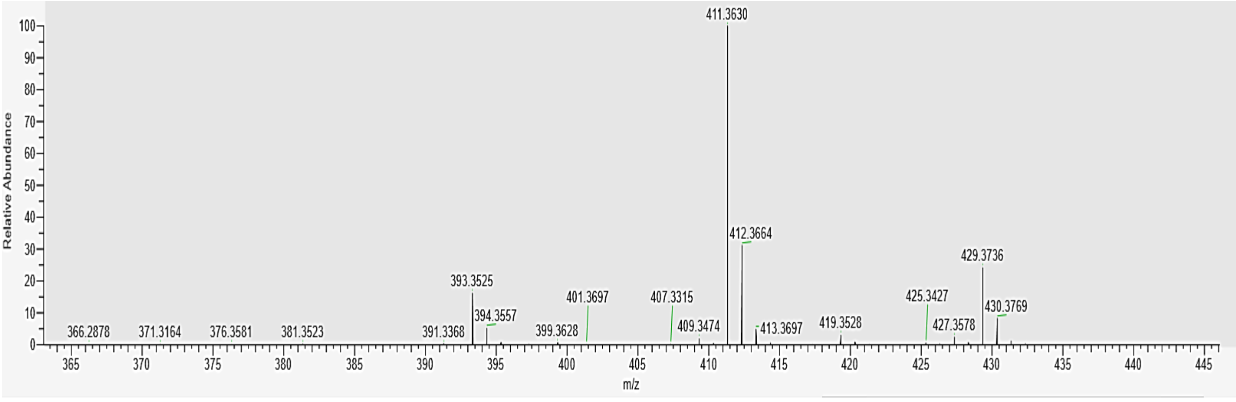


FIG. 54 Mass spectrum Q1 of 5,6α-epoxystigmasterol in LC-Orbitrap-HRMS.

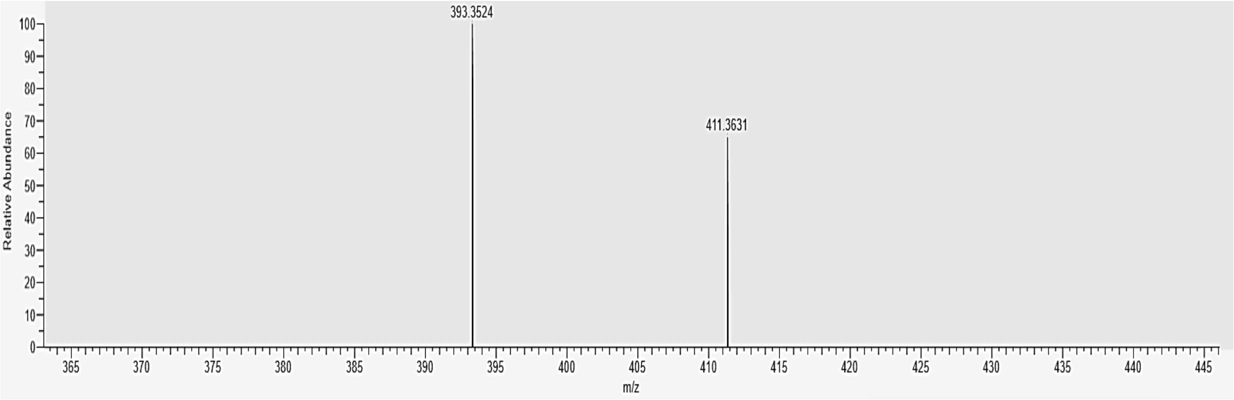


FIG. 55 Mass spectrum of the product ion Q3 of 5,6α-epoxystigmasterol in LC-Orbitrap-HRMS.

5,6β-epoxystigmasterol

The structure formula for 5,6β-epoxystigmasterol is shown in FIG.55. The fragmentation pattern has been reported in FIG.56. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 500, 410, 349 and 253.

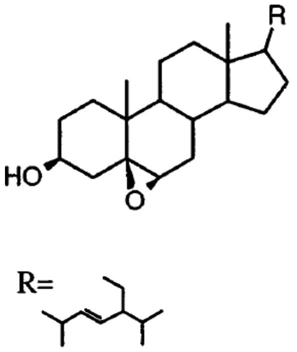


FIG.56 Structure formulas of 5,6β-epoxystigmasterol. Image edited by Bortolomeazzi, et al., 2003.

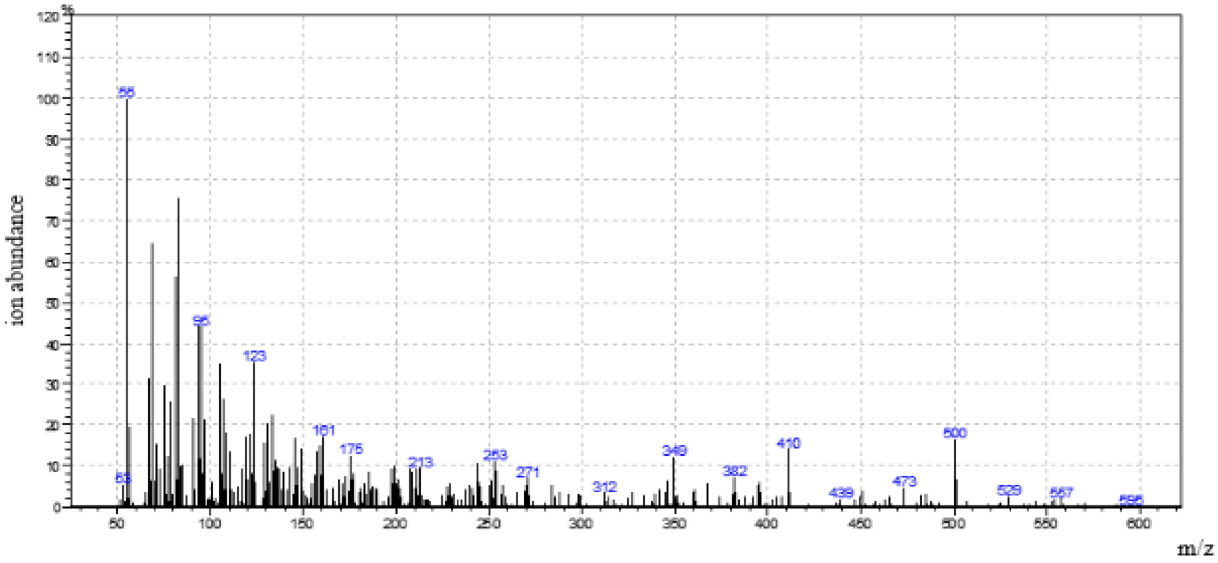


FIG. 57 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 5,6β-epoxystigmasterol shows the molecular ion at 500.

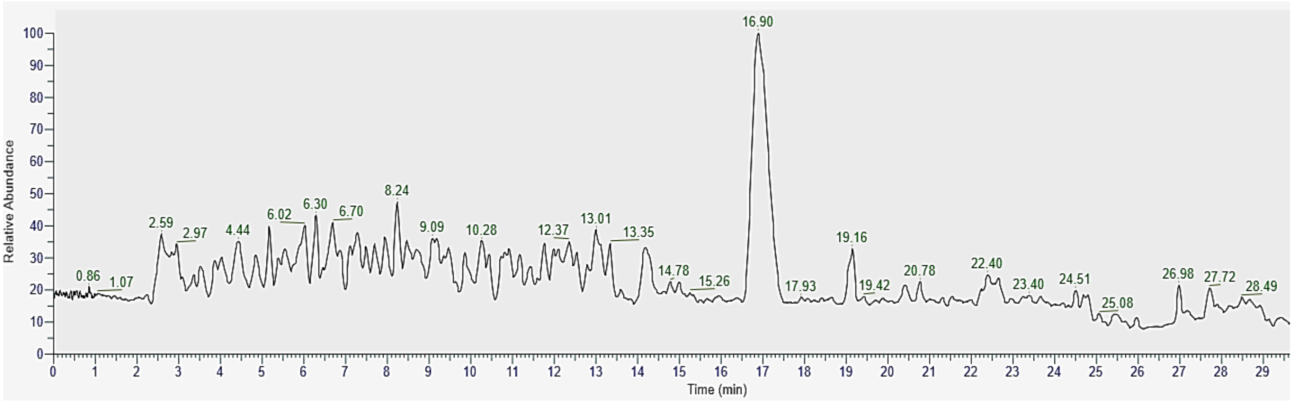


FIG. 58 Full mass chromatogram of 5,6 β-epoxystigmasterol (R.T. 16.90 min) in LC-Orbitrap-HRMS.

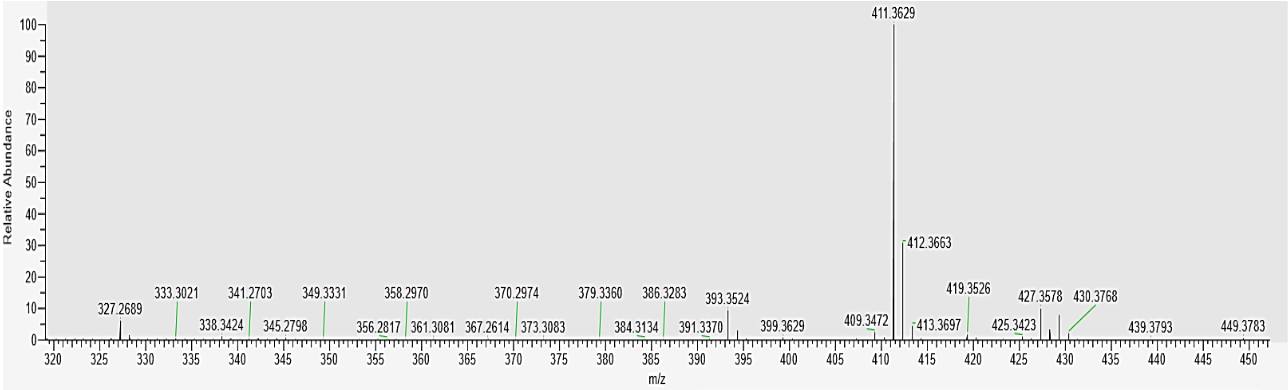


FIG. 59 Mass spectrum Q1 of 5,6β-epoxystigmasterol in LC-Orbitrap-HRMS.

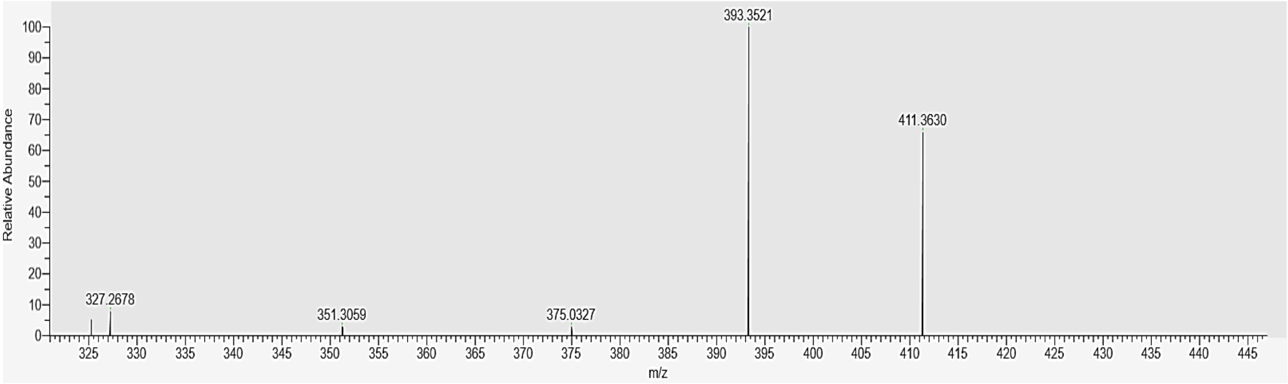


FIG.60 Mass spectrum of the product ion Q3 of 5,6β-epoxystigmasterol in LC-Orbitrap-HRMS.

7-ketostigmasterol

The structure formula for **7**-ketocampesterol is shown in FIG.60. The fragmentation pattern has been reported in FIG.61. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 498, 386, 357 and 129.

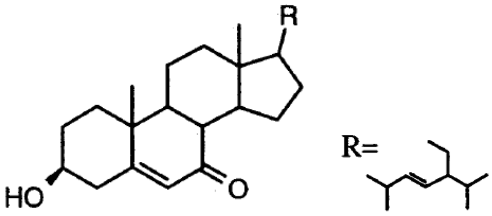


FIG.61 Structure formulas of 7-ketostigmasterol. Image edited by Bortolomeazzi, et al., 2003.

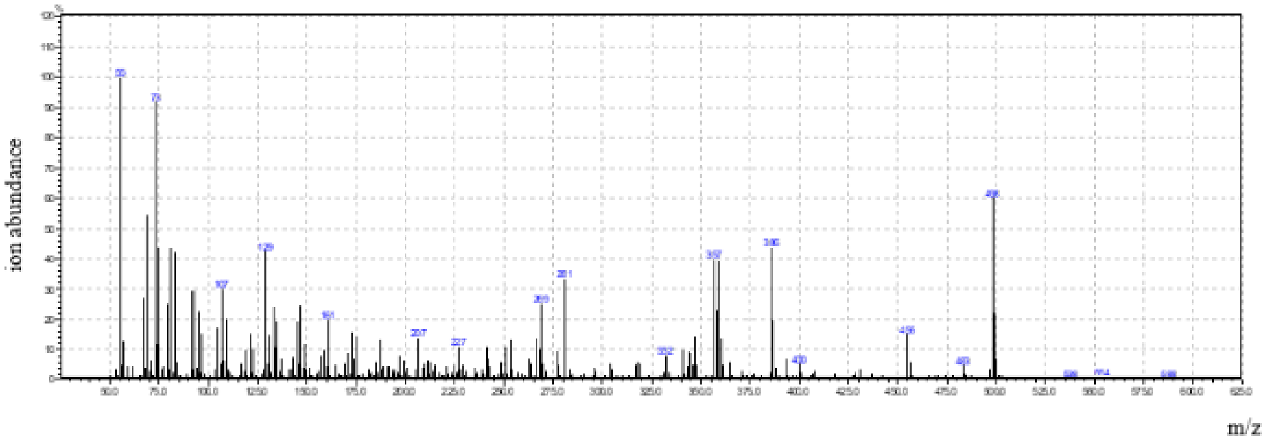


FIG. 62 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 7-ketostigmasterol shows the molecular ion at 498.

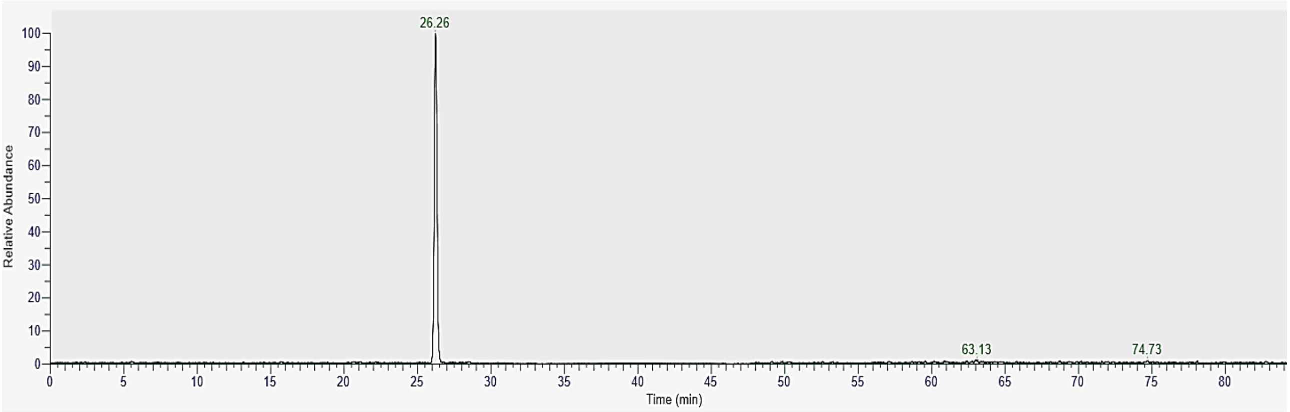


FIG. 63 Full mass chromatogram of 7-ketostigmasterol (R.T. 26.26 min) in LC-Orbitrap-HRMS.

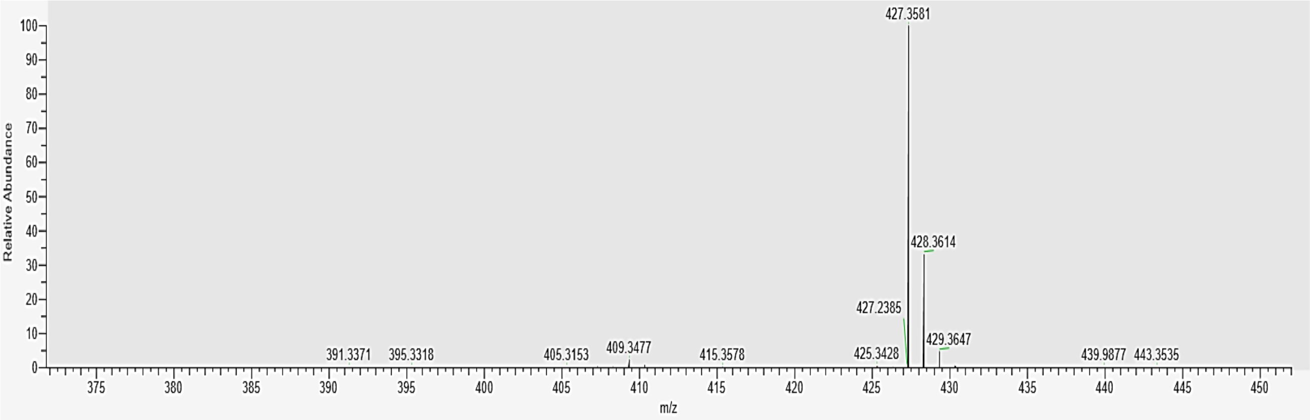


FIG. 64 Mass spectrum Q1 of 7-ketostigmasterol in LC-Orbitrap-HRMS.

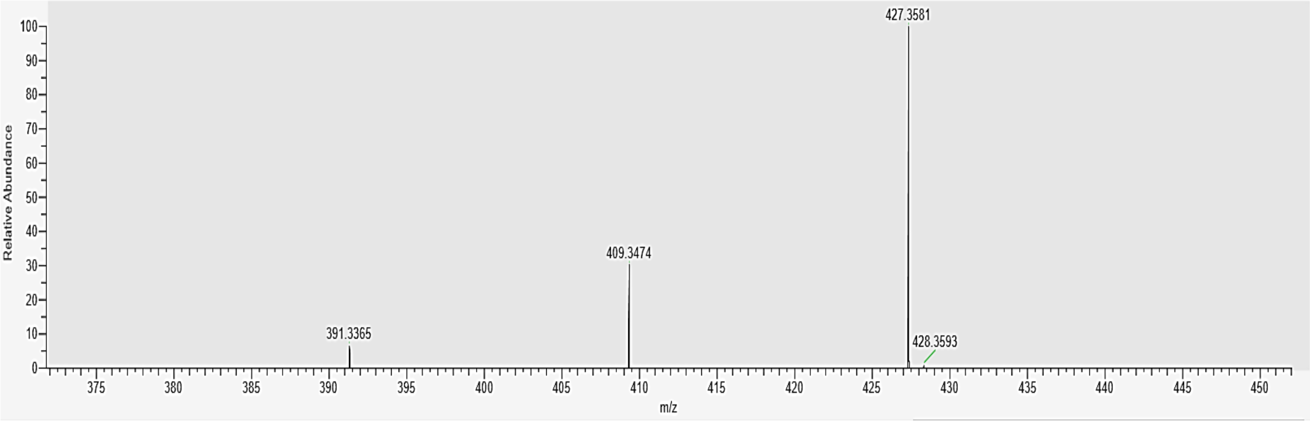


FIG. 65 Mass spectrum of the product ion Q3 of 7-ketostigmasterol in LC-Orbitrap-HRMS.

7α-hydroxystigmasterol

The structure formula for 7α-hydroxystigmasterol is shown in FIG.65. The fragmentation pattern has been reported in FIG.66. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 572, 482, 341,129.

FIG.66 Structure formulas of 7α-hydroxystigmasterol. Image edited by Bortolomeazzi, et al., 2003.

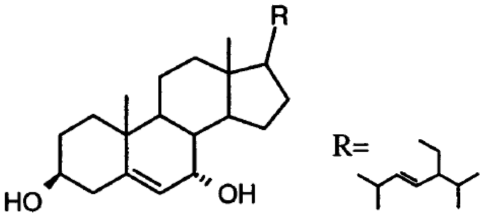
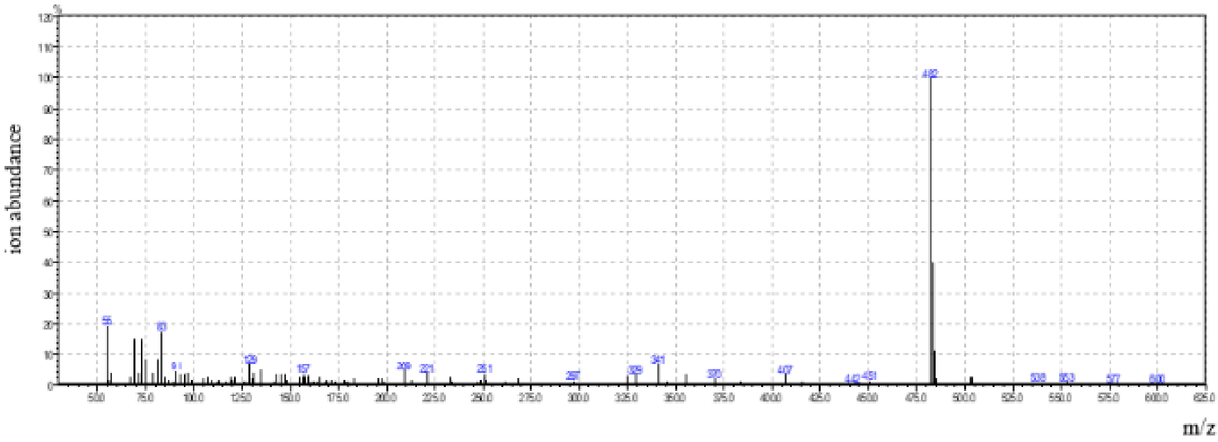


FIG.67 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 7α-hydroxystigmasterol shows the molecular ion at 572.



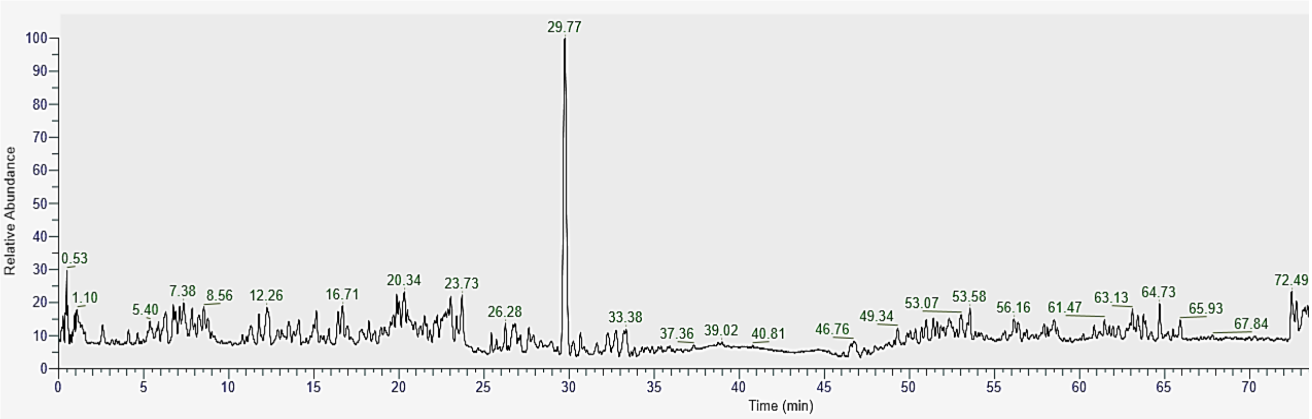


FIG. 68 Full mass chromatogram of 7α-hydroxystigmasterol (R.T. 29.77 min) in LC-Orbitrap-HRMS.

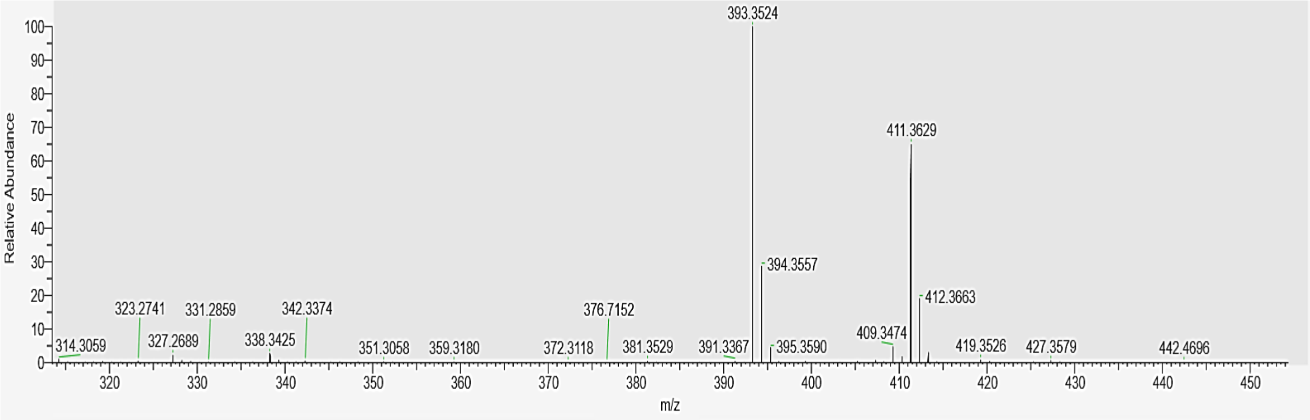


FIG. 69 Mass spectrum Q1 of 7α-hydroxystigmasterol in LC-Orbitrap-HRMS.

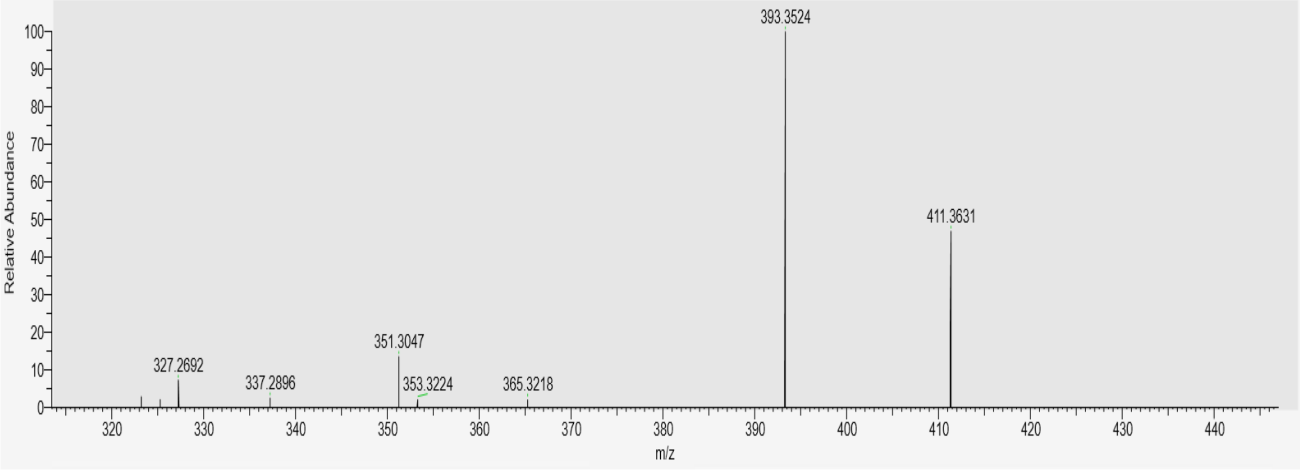


FIG. 70 Mass spectrum of the product ion Q3 of 7α-hydroxystigmasterol in LC-Orbitrap-HRMS.

7β-hydroxystigmasterol

The structure formula for 7β -hydroxystigmasterol is shown in FIG.70. The fragmentation pattern has been reported in FIG.71. The recognition is in agreement with has been reported in the literature Dutta & Appelqvist, 1997. The characteristic ion fragments were observed at m/z 572, 482, 341,129.

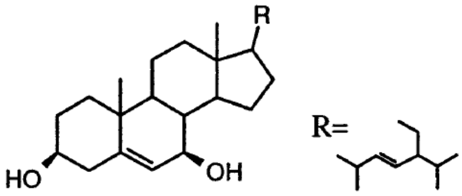


FIG.71 Structure formulas of 7β -hydroxystigmasterol. Image edited by Bortolomeazzi, et al., 2003.

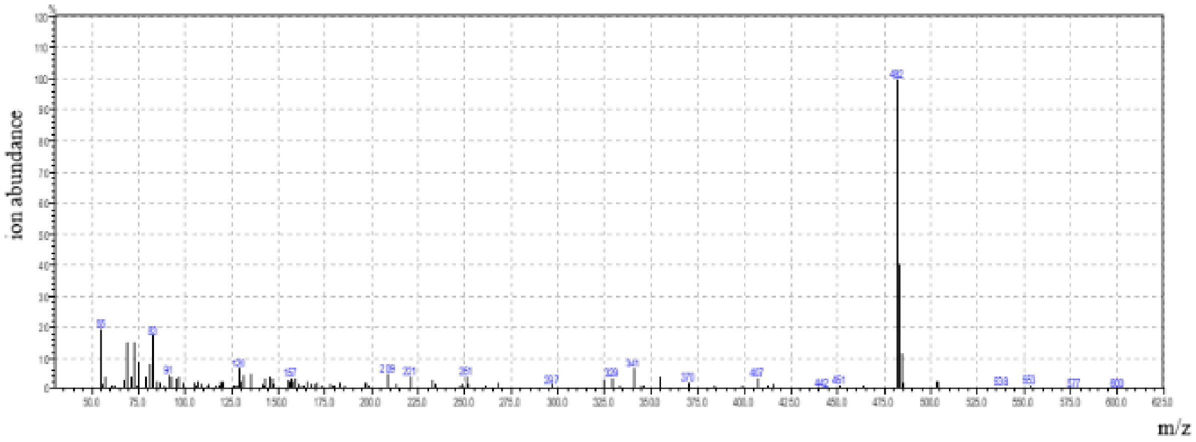


FIG. 72 Mass spectrum of trimethylsilyl (TMS)-ether derivative of 7β -hydroxystigmasterol shows the molecular ion at 572.

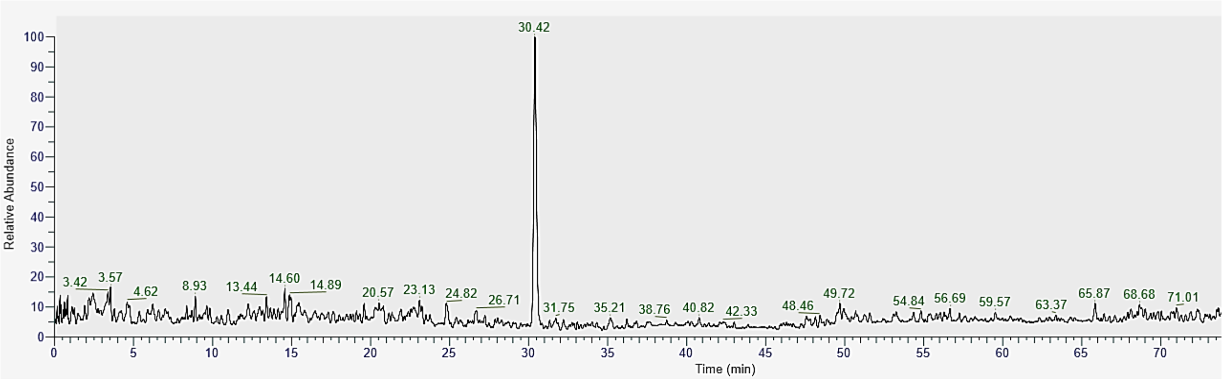


FIG. 73 Full mass chromatogram of 7β-hydroxystigmasterol (R.T. 30.42 min) in LC-Orbitrap-HRMS.

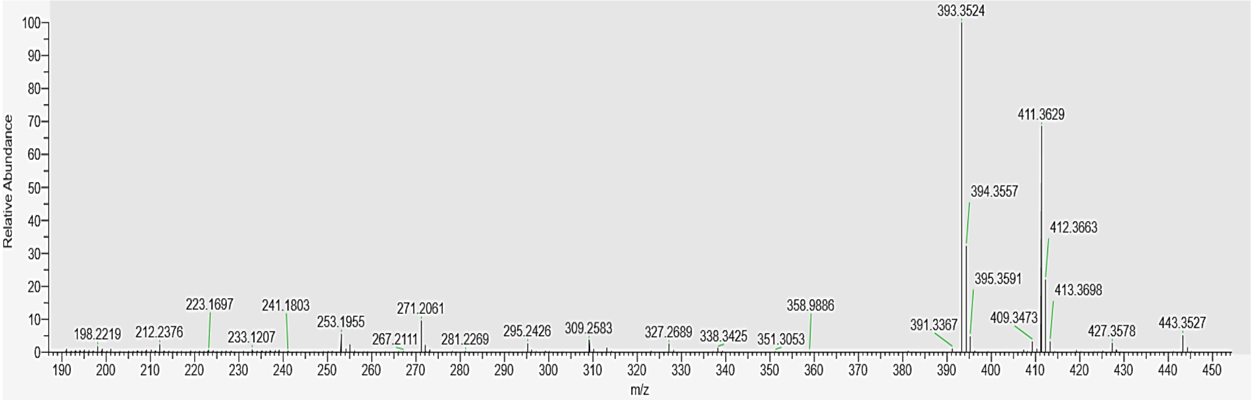


FIG. 74 Mass spectrum Q1 of 7β-hydroxystigmasterol in LC-Orbitrap-HRMS.

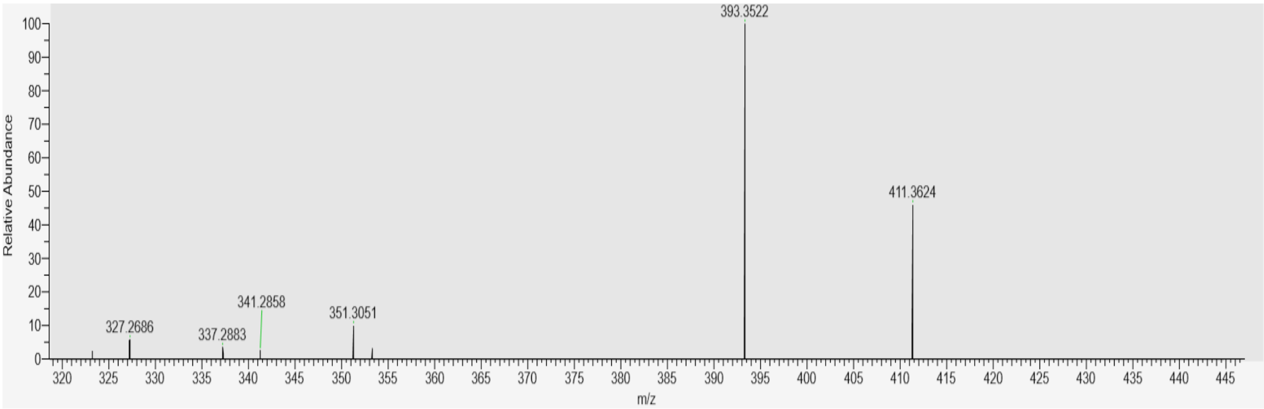


FIG. 75 Mass spectrum of the product ion Q3 of 7β-hydroxystigmasterol in LC-Orbitrap-HRMS.

CHAPTER 5

Preface

Form a technological point of view the world of fatty substance for food purpose had been victim of dualism; there is a great demand from food industry of fats with different degrees of solidity linked to technological role of fats substance in final products responsible of some specific sensory properties such as flavor, palatability, and texture however, most of the fats coming from natural sources are oils so they need to be suitably transformed in order to modulate their hardness and increase the saturation of fatty acids.

At the main time, legislative limitations of the use of trans (from partially hydrogenated fats) and saturated fatty acids and the rising concerns among consumers about negative effects of them on human health drove the innovations in reformulating fat-containing food products with newest high nutritional value fats matters rich in polyunsaturate fatty acids and other antioxidant products.

Oleogelation is one of the most in-trend methods for reducing or replacing the saturated fats in food. Different edible oleogels had been formulated with different liquid oils by conventional sources. Rethinking of life cycle assessment of food supply chain maybe lead a new awareness about new source of high nutritional value fats matters.

Durum wheat oil oleogels: a study on rheological, thermal, and microstructural properties

5.1 Introduction

In a classic semisolid fat product such as ice cream or chocolate, the solid lipids - arranged in a three-dimensional colloidal fat's crystal network - act as promoters of the crystallization representing the first step of the microcrystal network where soft matter is held similar to colloidal gels. For instance, in cake goods, saturated fats play a key role in generating the viscous foam structure, entrapping air bubbles into the fat phase of the cake batters (Kim et al., 2017).

However, the negative health effects of saturated and trans fatty acids consume on human health were intensely discussed (Guo et al., 2023; Kim et al., 2017). Numerous studies have shown that excessive intake of saturated or trans fatty acids can increase the risk of cardiovascular diseases (CVDs) by lowering serum levels of high-density lipoproteins and increasing serum concentrations of low-density lipoproteins (Clifton and Keogh 2017).

In order to reduce the content of saturated fats and trans fats in foods and at the same time to ensure the correct plasticity of fats, many studies have been focused on oleogels, which have similar structural characteristics as saturated hard-fat, although are characterized by higher amounts of unsaturated fatty acids and reduced amounts of saturated fatty acids. The organogelation or oleogelation is emerged as a new method of plasticization oil's technology (Öğütcü and Yilmaz 2015). Oleogels used in foods are usually composed of a liquid vegetable oil and generally recognized as safe (GRAS) additives (da Silva et al., 2018).

From a physical point of view, oleogels are viscoelastic, anhydrous and self-supporting materials; they have rheological characteristics like a solid but are formed in the highest amount (>90%, w/w) of a liquid matter (e.g., vegetable oil). From a thermal point of view, oleogels are structured and thermos-reversible substances. The additives or "oleogelators" lead to the formation of a three-dimensional super-molecular network (Doan et al., 2015). Therefore, based on correct melting and structuring behavior, the oleogels can provide a suitable alternative to fully and partially hydrogenated fats such as margarine or milk fat, in food production (Demirkesen et al., 2019; Jung et al., 2020).

Oleogels were applied to different processed foods, such as chocolate and spreads (Patel et al., 2014), ice cream (Zulim Botega et al., 2013) and biscuits (Hughes et al., 2009).

In that context, the natural waxes, which are long-chain fatty acids esterified into fatty alcohols, are considered promising organogelators; since they showed great performance like texturizing agents. The physicochemical characteristics in terms of thermal and rheological proprieties were in deep investigated combining carnauba wax and candelilla wax with canola oil (Jang et al., 2015), sunflower oil (Mert and Demirkesen 2016) and soybean oil (Rocha et al., 2013). Again, the effect of oleogelation on lipid digestion was recently explored. For instance, in the case of extra virgin olive oil the lipophilic oleogelators led about 40-55% of free fatty acids release during in vitro digestion, while in presence of hydrophilic oleogelators, the free fatty acids release was about 100% (Ciuffarin et al., 2023). On the other hand, Ramírez-Carrasco et al. (2024) demonstrated that in beeswax-based oleogel a reduction of about 10% of free fatty acids released in vitro digestion was observed.

Moreover, oleogelation could be a useful strategy to valorize by-products and co-products, which are an interesting source of unsaturated fatty acids and different bioactive compounds. Recently, durum wheat germ oil (also called wheat oil, WO) received great attention due to its nutraceutical properties and its potential use in the food industry. WO is mainly composed of polyunsaturated fatty acids of which linoleic acid is the most representative (Cardenia et al., 2018).

However, WO due to the presence of antioxidants such as α-tocopherol, β-tocopherol and carotenoids can find application in the pharmacological, cosmetic and nutritional fields (Kumar and Krishna 2015). WO is often used in its crude form obtained by pressing extraction, even if, the solvent extraction usually leads to an increase in yield (about 90%) (Durante et al., 2012). On the other hand, as well reported the solvent extraction and consequent refining process improves the amount, the quality and stability of WO (Li et al., 2016), increasing its applications in the food sector (Vurro et al., 2022a; Vurro et al., 2022b).

However, due to its liquid-like behavior, its application in some food industry sectors such as the confectionery and the baking industry is limited. To overcome that limit, the oleogelation could be a useful strategy to obtain a texturized WO expanding its application. Thus, the aim of the present research was to evaluate the impact of two natural waxes used at different concentrations on the development of wheat oil-based oleogels characterizing their physical and rheological properties in order to better valorize the WO and wheat chain sustainability.

5.2 Material and methods

5.2.1 Materials

Beeswaxes (BW), carnauba waxes (CW) were provided by a local company. Refined sunflower oil (SO) was purchased from local market, while wheat oil (WO) was supplied by an Italian company (Molino Casillo, Corato, Bari).

5.2.2 Oleogels preparations

To assess the stability of oleogel, preliminary trials were performed employing 1.5 and 3 % of BW in the presence of SO or WO, leading to samples characterized by weak elastic moduli and poor stability as shown in Supplementary materials (S1 and S2). On the basis of these preliminary results, new oleogel samples were developed increasing the amount of BW and CW at 4, 7 and 8%. BW oleogels were obtained by stirring waxes and oil in a water bath at 70°C, while CW ones reaching 90°C on the basis of their melting profiles (S3) and according with previous works of Borriello et al., (2021) Sabet et al. (2023) and Wang et al. (2024), by applying a direct method.

This method does not require specific equipment being inexpensive, rapid and scalable; moreover, oleogels obtained by following this method exhibit the higher capacity to mimic the rheological properties of hard-stock fats, as they do not be sheared as a final step unlike ones obtained with indirect ones (Sabet et al., 2023; Valoppi et al., 2023; Wang et al., 2016). After stirring, samples were allowed to cool quiescently in beakers at room temperature overnight, according to (Blake et al., 2014) and gently removed from them by using a spatula before performing analysis. The bees and carnauba wax-based oleogels prepared with different oils and concentrations in triplicate (SO_BW; SO_CW; WO_BW; WO_CW, at 4, 7 and 8% w/w) were used for further measurements.

5.2.3 Analytical determinations

5.2.3.1 Quality control of durum wheat oil

In order to determine the quality and composition of durum wheat oil, fatty acid composition (EEC Regulation no. 2568/91), carotenoids (Makhlouf et al., 2018), tocopherols and tocotrienols (Difonzo et al., 2021), phytosterols (Miazzi et al., 2020) and wax (Milani et al., 2020) were determined.

5.2.3.2 Fundamental rheological analysis

Frequency sweep tests

Fundamental rheological analysis was carried out in dynamic conditions by using a stress-strain rheometer (MCR 52, Physica/Anton Paar, Ostfildern, Germany) equipped with a plate-plate system (PP25) at 20°C. Oleogel samples were transferred from the beaker into the center of the geometry with the help of a spatula, shearing as little as possible in order to avoid excessive structural damage. A strain sweep test was used to identify the linear viscoelastic region (LVR) (Glicerina et al., 2013; Penagos et al., 2022). On the basis of these results, a target strain of 0.046 % (within the linear region) was chosen for measurements.

Oscillatory measurements of storage modulus (G′, Pa) and loss modulus (G″, Pa) were performed within a frequency range from 0 to 100 rad/s.

Analysis was performed with both sunflower and wheat oil-based oleogels realized with bee and carnauba waxes at the different concentrations of 4, 7 and 8% in order to assess their stability.

Three replicates for the sample were carried out.

Temperature sweep tests

Temperature sweeps were also performed to evaluate viscoelastic properties and oleogels stability during the temperature ramp. Temperature was controlled to within 0.1°C by Peltier elements.

Temperature sweep tests were performed from 0°C to 40°C at a linear heating rate (2.5°C$\cdot$min^{-1}). The frequency value was set at 6.28 rad/s. During heating, values of elastic modulus (G′, Pa) and viscous modulus (G″, Pa) were recorded (Tavernier et al., 2017). Three replicates were carried out on oleogels with bee and carnauba waxes, employed at the different concentrations of 4, 7 and 8%.

Microstructural analysis

Microstructure of samples was analyzed by using an optical microscope (BH-2 RFCA, Olympus, Hamburg, Germany) with a 4x of magnification, in brightfield mode, in order to have a correct vision of aggregate distribution in space and not just a detail. One mg of sample was placed on a glass slide and covered with a glass slip carefully placed over the sample, parallel to the plane of the slide and centered to ensure that sample thickness was uniform.

Micrographs were captured using a digital camera (Model 2.1 Rev 1; Polaroid Corporation, NY, USA). The acquired images were subsequently elaborated using the software Image Pro-plus 6.0 (Media Cybernetics Inc., Bethesda, USA) by converting them in grey scale and subsequently thresholded in order to highlight in a more accurate way samples network, especially in terms of empty and void spaces (Glicerina et al., 2015; Wang et al., 2022).

Thresholded images were then processed by using the software Image J (Rasband, W.S Maryland, USA) in order to obtain the box-counting fractal dimension index (Db). These values give an indication of the spatial distribution of the crystal clusters in fat crystal networks, giving a description of their amount and homogeneity of occupied areas (Blake et al., 2014; Bouda et al., 2016).

The reported fractal dimension was an average taken from 10 micrographs. Moreover, in order to better highlight crystal morphology samples were also acquired at 10x and 20x of magnifications.

Oil loss evaluation (OL)

Oil loss was analyzed according to Thomas et al (2023) and Han et al (2022). Briefly, accurately 1.000 ± 0.001 g of oleogel was weighed into a 1.5 mL plastic centrifuge tube and immediately centrifuged at 10.000 g for 15 min. After centrifugation, the 1.5 mL centrifuge tube with 1 g of oleogel was placed upside down on the desk for 24 h to completely remove the released liquid oil.

OL was calculated according to equation 1 below:

$$OL = \frac{(M_1 - M) - (M_2 - M)}{M_1 - M}$$

where M_1 is the mass of the initial sample and centrifuge tube, M_2 is the mass of the sample and centrifuge tube after removing the excess oil, and M is the mass of the centrifuge tube. Higher is the oil loss, lowest will be the gelling properties of oleogel to entrap oil in the matrix. Five replicates for each sample were performed.

5.2.3.3 Thermal Analysis

The melting properties of oleogel samples were evaluated by using a differential scanning calorimeter (Nexta DSC 200, Alfatest, Hitachi High Test, Japan). DSC was calibrated by using indium (melting T 156.60°C, ΔH 28.71 J/g) and tin (melting T 231.93°C, ΔH 60.46 J/g) at a scan rate of 5°C/min using an aluminum pan as reference. Samples (15 mg) were loaded into 40 ml capacity pans and sealed using a sample press. Pans were subjected to the following steps:

- Heating from 20°C to 110°C
- Cooling from 110°C to -20°C
- Heating from -20°C to 110°C

at the scan rate of 5°C/min in an N_2 stream, according to (Aguedo et al. 2009) **Aguedo et al., (2009)** with some modifications. During the thermal ramp, oleogels were heated to above their melt temperature to remove any thermal history (step 1), cooled to impart a controlled thermal history (step 2) and reheated to 110°C to evaluate the correct and stabilized melting profile (step 3).

DSC Nexta software was used to calculate respectively the crystallization and melting onset temperature (T_{onset}), the crystallization and melting maximum value (T_{peak}) and the enthalpy (ΔH) of both crystallization and melting (Gloria and Sievert 2001; Wang et al.. 2022). Each experiment was repeated three times.

5.2.4 Statistical analyses

Analyses of variance (ANOVA) and the test of mean comparison according to Fisher Least Significant Difference (LSD) were conducted on all obtained data. Level of significance was $P<0.05$. The statistical software used was STATISTICA, version 8.0. (StatSoft, Tulsa, Oklahom).

5.3 Results and discussions

The rheological behavior is strictly related to the composition of oil used to generate oleogel. Thus, its composition in terms of fatty acid, tocopherols and tocotrienols, sterols and wax composition was established (Figure S4) and resulted in line with the literature (Squeo et al., 2022).

5.3.1 Fundamental rheological analysis

5.3.1.1 Frequency sweep tests

Figure 1 (a, b) reports result of frequency sweep test in terms of storage (G’) and loss modulus (G”) of all analyzed oleogels. Moreover, in order to better explain the results, elastic and viscous moduli of samples evaluated at 6.25 rad/s are showed in Table 1.

Figure 1 (a,b): Elastic (G’) and viscous (G’’) moduli of sunflower (SF) and wheat (WO) oils based oleogels, realized respectively with beeswax (BW) and carnauba wax (CW) at the different percentages of 4, 7 and 8.

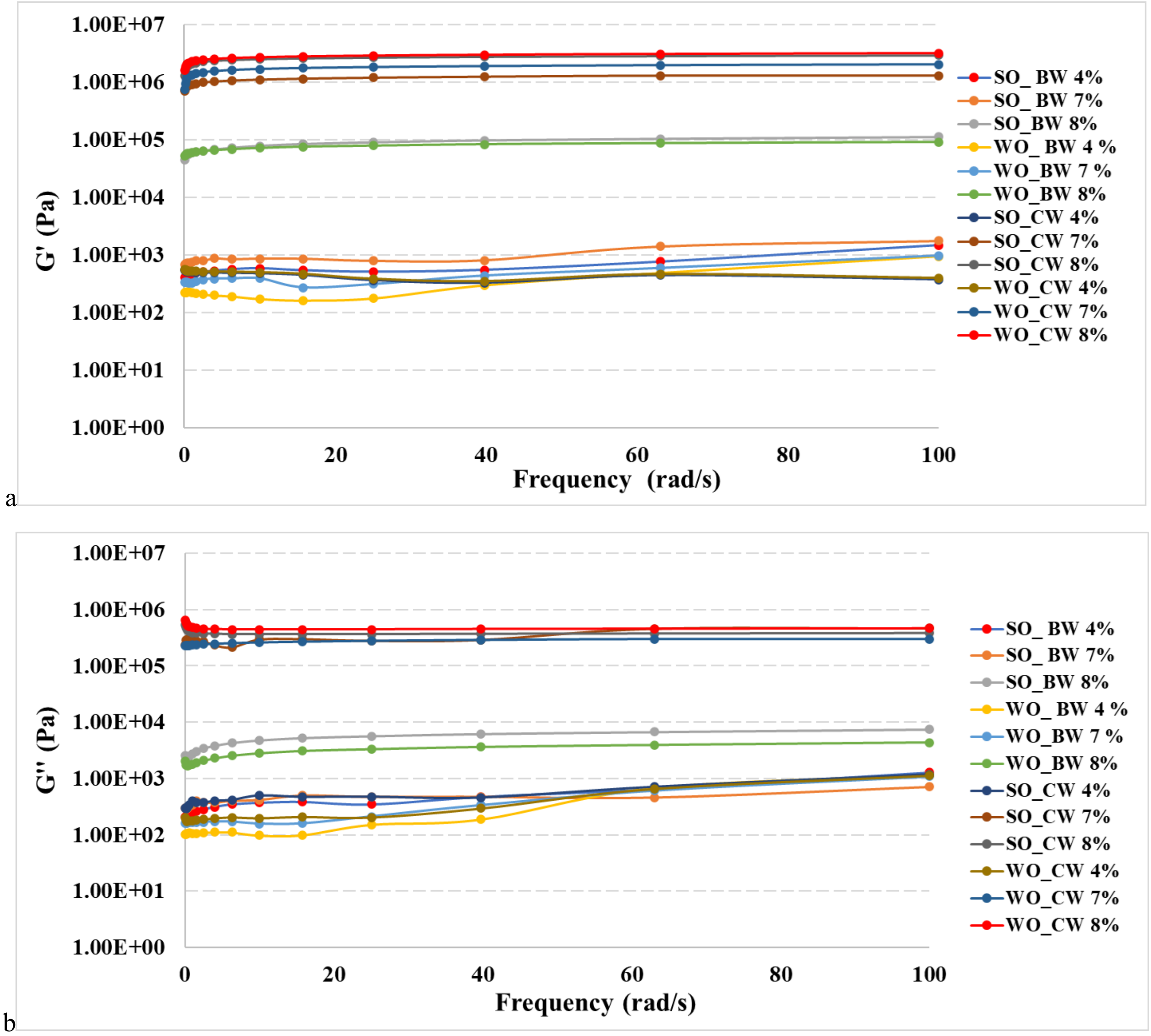


Table 1a. Storage modulus of oleogel samples evaluated at 6.25 rad/s and at 20°C.

- a-b Values followed by different letters differ significantly at $P < 0.05$ level between samples at the same concentration and waxes type.
- A-B Values followed by different letters differ significantly at $P<0.05$ level between samples at the same concentration comparing both waxes.

ELASTIC MODULUS (G’)			
BEEWAXES CONCENTRATION (%)			
	4	**7**	**8**
SUNFLOWER OIL	$5.35*10^{2}\pm2.65*10^{\mathbf{aA}}$	$8.43*10^{2}\pm3.50*10^{\mathbf{aB}}$	$7.32*10^{4}\pm1.23*10^{\mathbf{2aB}}$
WHEAT OIL	$1.92*10^{2}\pm3.18*10^{\mathbf{bB}}$	$3.91*10^{2}\pm2.48*10^{\mathbf{bB}}$	$6.89*10^{4}\pm1.04*10^{\mathbf{2aB}}$
CARNAUBA WAXES CONCENTRATION (%)			
	4	**7**	**8**
SUNFLOWER OIL	$5.78*10^{2}\pm2.18*10^{\mathbf{aA}}$	$1.07*10^{6}\pm2.54^{*}10^{\mathbf{2aA}}$	$2.46*10^{6}\pm1.89*10^{\mathbf{2aA}}$
WHEAT OIL	$5.09*10^{2}\pm4.14*10^{\mathbf{aA}}$	$1.64*10^{6}\pm2.89*10^{\mathbf{2aA}}$	$2.65*10^{6}\pm3.17*10^{\mathbf{2aA}}$

Table 1b: Loss modulus of oleogel samples evaluated at 6.25 rad/s and at 20°C.

- a-b Values followed by different letters differ significantly at $P < 0.05$ level between samples at the same concentration and waxes type.
- A-B Values followed by different letters differ significantly at $P<0.05$ level between samples at the same concentration comparing both waxes.

VISCOUS MODULUS (G’’)			
BEEWAXES CONCENTRATION (%)			
	4	**7**	**8**
SUNFLOWER OIL	$3.47*10^{2}\pm1.72*10$**aA**	$4.00*10^{2}\pm1.94*10$**aB**	$4.28*10^{3}\pm2.16*10^{2}$**aB**
WHEAT OIL	$1.11*10^{2}\pm1.63*10$**bC**	$1.70*10^{2}\pm2.92*10$**bC**	$2.54*10^{3}\pm8.93*10$ **bC**
CARNAUBA WAXES CONCENTRATION (%)			
	4	**7**	**8**
SUNFLOWER OIL	$4.15*10^{2}\pm4.08*10$**aA**	$2.13*10^{5}\pm4.58*10^{2}$**aA**	$3.65*10^{5}\pm2.79*10^{2}$**aA**
WHEAT OIL	$2.05*10^{2}\pm1.18*10$**aB**	$2.55*10^{5}\pm4.47*10^{2}$**aA**	$4.45*10^{5}\pm3.28*10^{2}$**aA**

[a-b] Values followed by different letters differ significantly at $P < 0.05$ level between samples at the same concentration and waxes type.

[A-B] Values followed by different letters differ significantly at $P<0.05$ level between samples at the same concentration comparing both waxes.

[a-b] Values followed by different letters differ significantly at $P < 0.05$ level between samples at the same concentration and waxes type.

[A-B] Values followed by different letters differ significantly at $P<0.05$ level between samples at the same concentration comparing both waxes.

The response of all samples to the imposed oscillatory deformation is the stored potential energy, that give a measure of solid and liquid-like characteristics of viscoelastic materials (Bayod and Tornberg 2011; Glicerina et al., 2015; Morales et al., 2023). As it well reported, in stable gels and oleogels the elastic component (G′) dominates over the viscous one (G'') (Liu et al., 2023; Patel et al., 2020; Thomas et al., 2023). However, in samples realized with BW, both SF and WO at 4 and 7% the elastic modulus values ranged from around 200 to 850 Pa, presenting the lowest values for WO at 4% (1.92 *102) and the highest for SF at 7% (8.43*102). In agree with literature (Kwon and Chang 2022; Pehlivanoğlu et al., 2016) the obtained results highlight the presence of a very weak structure characterized by low stability and a poor network.

Moreover, analyzing the mechanical spectrum of samples composed of WO_BW at 4% and 7% two crossovers were also observed at the frequencies of 50 and 65 rad/s, respectively, which underline an increase of the viscous modulus and their low stability at higher frequencies compared to SO_BW (Figure S5). Raising the BW amount until 8% a clear predominance of G' with respect to G'' was observed in both oils for all the investigated frequencies, showing not significant differences between oil samples. On the other hand, CW at 4% in both SF and WO increased the elastic modulus even if samples did not show optimal rheological values, presenting poor structured networks (Figure 1a; Table 1a).

According to the studies of Gravelle et al., (2017) and Patel et al., (2020), some rheological properties are necessary in oleogels to mimic saturated fats in specific foods such as baked, chocolate, dairy and meat products, that should be characterized by suitable properties such as high hardness, but at the same time plasticity, mouthfeel and scarce brittleness. Those peculiarities were detected in saturated fat replacers characterized by elastic moduli included in the ranges of 1*105 ("soft") – 6*106 ("hard").

The obtained values, in line with values reported in the literature, highlighted the potentiality of these samples to be successfully employed to mimic margarines, butter, cocoa butter, shortenings and spreads in bakery products, ice cream fillings as well as in chocolate and confectionary products (Patel et al., 2014; Zhao et al., 2019; Álvarez et al., 2021; Palla et al., 2021).

However, increasing CW content up to 7% and 8% interesting results were obtained, since G' values of 1.6*106 and 2.6*106 Pa*s were obtained, respectively; the presence of very strong networks and the optimum solid-like gel behavior (Patel et al., 2020; Sivakanthan et al., 2023; Thomas et al., 2023), with no significant differences (p>0.05) was denoted.

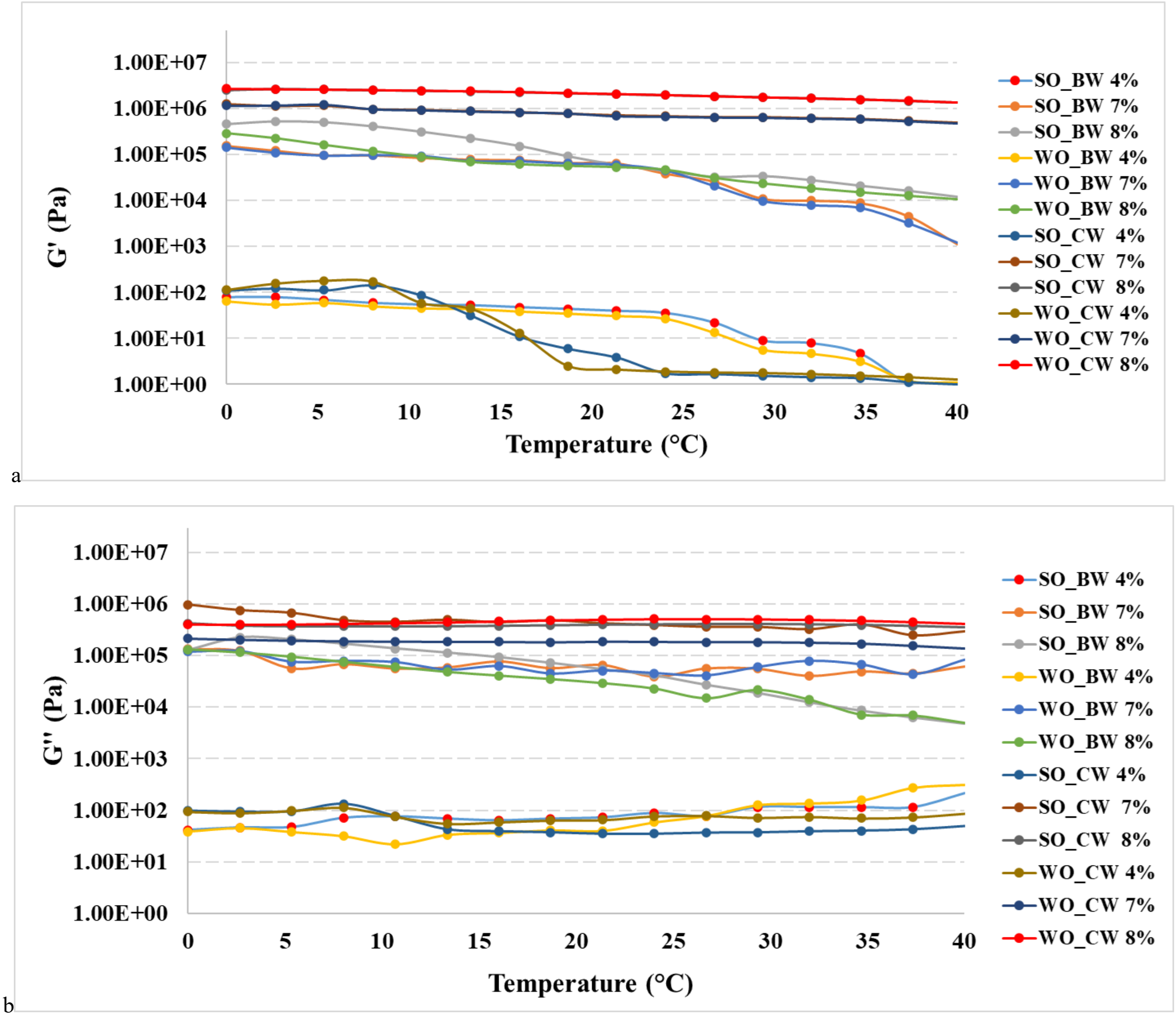
5.3.1.2 Temperature sweep tests

In Figure 2 (a, b) the behavior of elastic (G’) and viscous (G”) moduli evaluated by temperature sweep tests is shown. All oleogel formulations exhibited a drop in G′ as the temperature increased, in agree with Chai et al. (2022) and Wang et al. (2024), with differences related to wax concentration and oleogel composition. However, a similar trend was observed for the frequency sweep test. In specific, CW samples displayed a better gelation behavior than BW, also at higher temperatures, remaining relatively stable with increased gelator concentration, as underlined by elastic moduli (G’) values.

As reported by literature (Blake et al., 2015; Tavernier et al., 2017; Shi et al., 2021; Tanislav et al., 2022) high elastic moduli are related to a higher gelation capacity of gelator compound; in this case, carnauba wax, that is more able to form aggregate structures can entrap and retain oil in more strong way also at high temperatures. About beeswax samples, prevalence of viscous moduli with respect to elastic ones was observed at higher temperatures (within 30 - 40°C) for both oils. In that temperature range, WO presented G” values ranged from 1.27 *10 2 to 3.15 *102 and from 5.98*104 to 8.31 *104 for BW at 4% and 7%, respectively; whereas, SF displayed values from 1.16 *102 to 2.14 *10 2 at 4% and from 5.50 *104 to 6.11*104 at 7 % of BW. However, at BW concentration of 8%, a higher conservative modulus was highlighted than dissipative ones for both SO and WO samples.

As shown in Figure 2, SO BW samples displayed higher stability than wheat oil at lower temperature (from 0 to 20°C). In relation to samples, WO with CW at 7 and 8%, high stability was observed also at higher temperatures, showing no significant differences with respect to SO ones. The higher resistance to melting observed for both WO and SO samples in carnauba waxes compared to bee ones could be attributed to the different chemical compositions of these waxes in terms of esters, fatty acid and hydrocarbons chains that can interact between them giving rise to strong interactions that increase their resistance (Blake et al., 2014). As reported by Hwang et al., (2012) waxes with longer chain wax esters will exhibit superior gelation compared to waxes with shorter chain wax esters. Moreover, despite the wax characteristics, as reported by Sato et al., (2005), Dassanayake et al., (2012) and Choi et al., (2020), the rheological, morphology and stability properties of oleogels drastically changed with the oil used, because of the differences in triacylglycerols (TAGs) composition as well as the number of total polar materials, that involved different interaction between oil and gelator, affecting their physicochemical properties (Table S4) (Kim et al., 2010; Amita Devi & Katar, 2016; Akkaya et al., 2018). This behavior could explain the lowest affinity and structuring ability of WO in BW with respect to SO, but also the highest and most promising stability of wheat oil in carnauba wax, as showed also previously by frequency sweep rheological analysis (section 10.2.3.2).

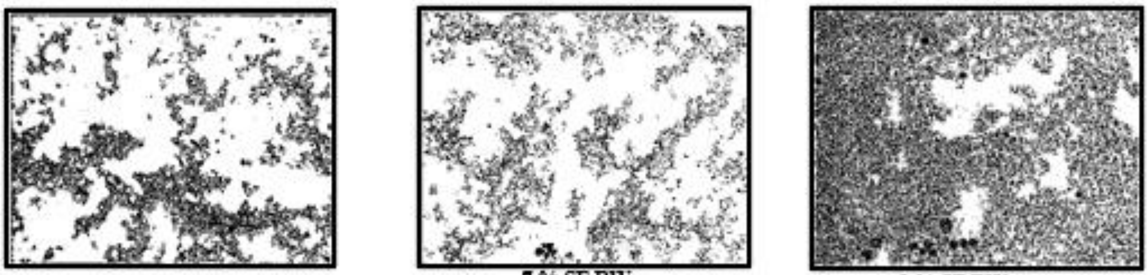
Figure 2 (a,b): Elastic (G’) and viscous (G’’) moduli of sunflower (SF) and wheat (WO) oils based oleogels, realized respectively with beeswax (BW) and carnauba wax (CW) at the different percentages of 4, 7 and 8 evaluated in a temperature range comprised between 0 and 40°C.



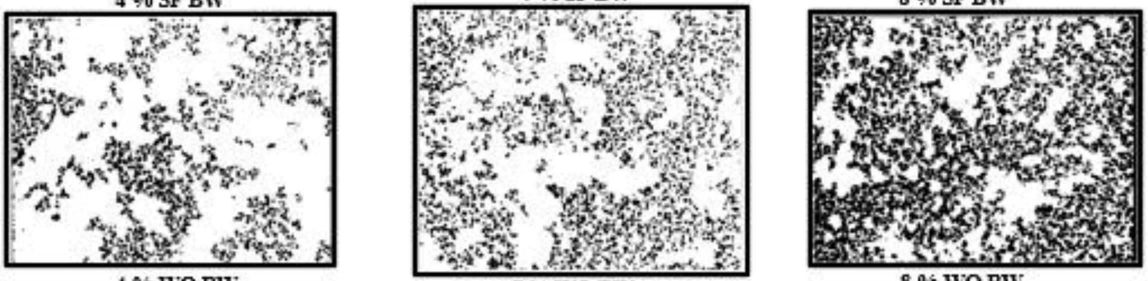
5.3.2 Microstructural Analysis

Crystal morphologies and their arrangement were studied for better understanding the impact of gelator concentrations as well as oil characteristics on the oleogel formation and stability. In Figure 3 the different micrographs of oleogel samples obtained with different oils and waxes at different ratio are displayed. As shown in Figure 3, a progressive increase in the crystal aggregation was observed when the waxes amount for both sunflower (SO) and wheat oil (WO) increased, highlighting as the crystalline structures were also affected by the gelator concentration (Pang et al., 2020). This behavior can be reasonably attributed to the structuring effect exerted by waxes, that entrapped liquid oil into their solid matrix, increased their solid fraction volume, by reducing void spaces, made up from not binding oil, giving rise to a more compact structure, as well as to a denser network (Sagiri et al., 2015). As reported by literature, oleogelation is the process of making oil-based continuous gels in which the gelator, in this case waxes, immobilizes liquid oil within a structured three-dimensional network by oil-wax interaction stabilized through, Van der Waals interactions, hydrogen bonding and electrostatic interaction (Wijarnprecha et al., 2019; Sivakanthan et al., 2022). BW samples showed a more needle-like structure while CW a typical dendritic-spherulitic shape, as highlighted from a detail of micrographs realized with 7 % of waxes at both 10x (S6) and at 20x (S7) of magnification, that according to literature, tend to form clusters of small crystals leading to network formation as a result of aggregate overlapping (Blake et al., 2014; Wang et al., 2016; Dassanayake et al., 2012; Pang et al., 2020; Silva et al., 2021).

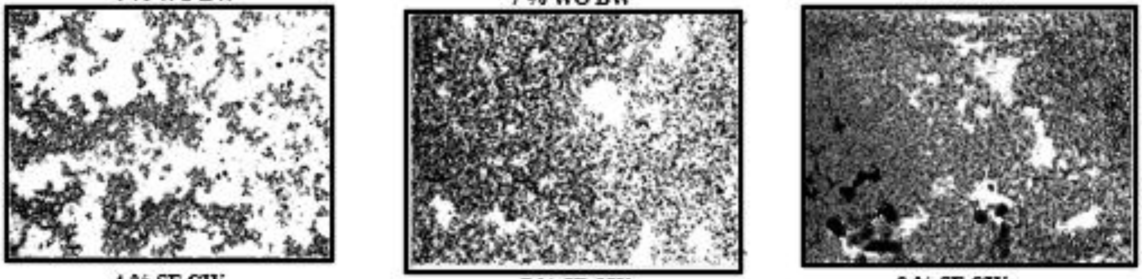
Figure 3. Thresholded micrographs of oleogel samples acquired at 4x of magnification in brightfield mode.



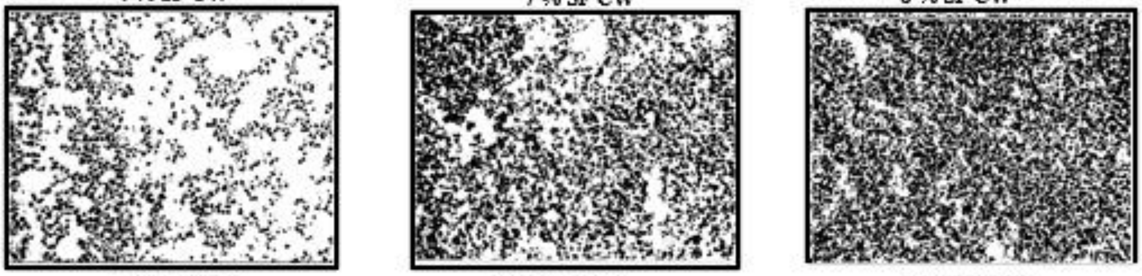
4 % SF BW 7 % SF BW 8 % SF BW



4 % WO BW 7 % WO BW 8 % WO BW



4 % SF CW 7 % SF CW 8 % SF CW



4 % WO CW 7 % WO CW 8 % WO CW

Moreover, samples realized by dispersing oil in beeswax showed a higher amount of void space between crystals, involving the presence of a less strong network with respect to carnauba ones as also confirmed by fractal analysis, even if SO_BW samples presented a more aggregate matrix than wheat oil beeswax. On the other hand, in the presence of carnauba wax (CW) similar structures were highlighted between SO and WO, characterized by the presence of more strong networks between crystals, especially at higher wax concentrations, probably related to an increase in the contact points due to chemical and mechanical interactions between oil and waxes that reduced void spaces, as also showed by box-counting fractal dimension index (Table 2) (Choi et al., 2020). That value is an indicator of the spatial distribution of the solid mass in the oleogels' crystal network; allowing to numerically estimate the uniformity of the solid mass distribution in the oleogels' crystal network; greater fractal dimensions underline more homogeneously distributed mass or more uniformly filled space and so lower empty cavities and so lower pore area fraction (AFp), (Tang & Marangoni, 2006; Blake, 2015; Frolova et al., 2022).

Table 2. Box-counting fractal dimension (Db) of the different oleogels derived from brightfield micrographs.

Samples	**Box- Counting Fractal Dimension (Db)**
4% SF BW	1.38 ± 0.08^{c}
7% SF BW	1.61 ± 0.06^{b}
8% SF BW	1.69 ± 0.07^{b}
4% WO BW	1.35 ± 0.04^{c}
7% WO BW	1.58 ± 0.10^{bc}
8% WO BW	1.67 ± 0.09^{b}
4% SF CW	1.45 ± 0.11^{bc}
7% SF CW	1.81 ± 0.10^{ab}
8% SF CW	1.84 ± 0.10^{ab}
4% WO CW	1.40 ± 0.11^{c}
7% WO CW	1.83 ± 0.03^{ab}
8% WO CW	1.89 ± 0.04^{a}

a-c letters significantly differ at $p< 0.05$ for different samples at the different concentration

Moreover, according to literature (Narine & Marangoni, 1999; Blake & Marangoni 2014, 2015; Shi et al., 2021; Frolova et al., 2022) this parameter can be used to describe some macroscopic properties of the oleogels, such as their oil-binding capacity strictly related with their stability and so the separation of oil from the oleogels during time, as well as to their hardness and viscoelastic properties. Higher fractal numbers values as observed for oleogel samples realized with carnauba wax at both 7 and 8%, underlined the presence of high numbers of homogeneous small crystals evenly distributed through the material, which generated a matrix with high capacity to retain oil; small crystals provide more surface area for oil to adsorb onto compared to the larger ones (Omar et al., 2015; Palla et al., 2019). At the same time, higher fractal numbers are related to lower AFp, that highlighted the presence of a highly structured network with homogenously distributed crystals, able to bind more oil, that reducing the volume of free oil, decrease its migration and so improve structure stability (Blake et al, 2015; Yang et al., 2018). Studies focused on oleogels and fat networks (Vreeker, 1992; Marangoni & Rousseau 1996; Litwinenko et al., 2002; Mellema et al., 2002; Palla et al., 2019; Frolova et al., 2022) highlighted as fractal analysis can be a good indicator for hardness and viscoelastic behavior. Generally, higher fractal dimensions are present in ordered, dense and compact fat networks composed of tightly packed structures with high hardness and higher elastic moduli, whereas, networks characterized by disordered, open and low-density structures, result in lower fractal values, as shown for samples realized with waxes at 4%. In the presence of CW, WO samples displayed similar fractal values as SO ones, not significantly different ($p>0.05$) from them, and even as for 8% WO CW, (1.89) greater than sunflowers oils (1.84), giving further evidence of the strong network and high elastic modulus characterizing this sample. In addition, the fractal approach can detect small changes in rheological properties in terms of hardness and G' of fat networks also in the presence of no significant changes in their solid fat content, as in the case of aging in which post-hardening or post-crystallisation of oils and fats frequently occur (Vreeker et al., 1992 Omar et al., 2015).

However, some deflection from this trend can be observed as in the study of Blake et al., (2015) on different oleogel matrices, because of differences in the particle morphology and particle size of the microstructural elements present in the wax.

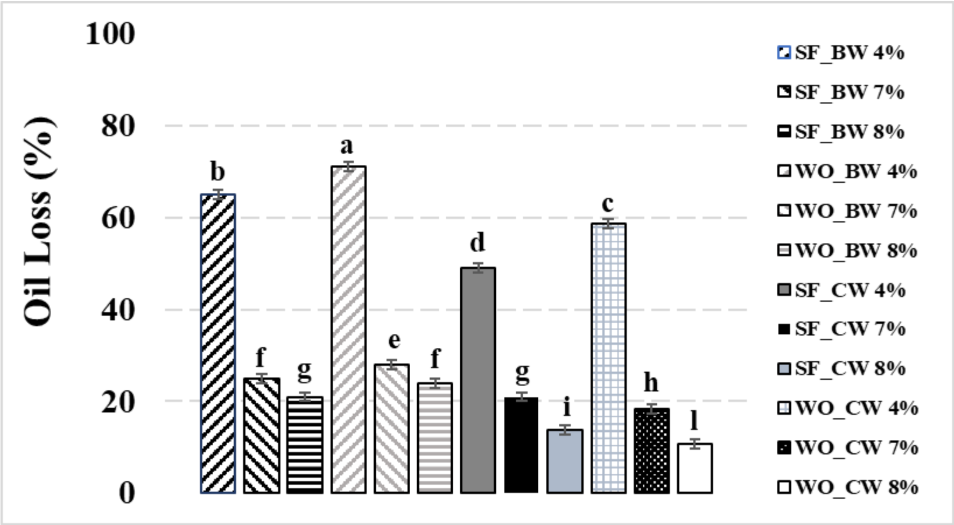
5.3.3 Oil loss evaluation

In Figure 4 is reported the oil loss of different samples after centrifugation and 24h of resting time. According to Blake et al. (2014) and Han et al. (2022) the long-term stability of oleogels was evaluated as the maximum amount of oil that is retained or lost by the material over a specified (usually ‘‘long’’) length of time (in the case of this study, 24 h). This approach is particularly suitable in situations involving products storage, where the long-term stability of a material is of interest. Moreover, the integrity and the maintaining of the oleogels structure according to Zulim et al., 2013; Flöter et al., 2021; Li et al., 2022; Sivakanthan et al., 2022, is mostly expressed as the fraction of oil remaining in the structure after applying external forces, mostly centrifugal force and as oil loss. In our study, samples composed of the lowest percentages of waxes, both BW and CW, presented the highest oil loss percentages, being characterized by poor networks and lowest elastic moduli compared to the other samples, which tend to entrap less liquid, involving a higher oil release and so a poor integrity of the structure (Martins et al., 2016).

On the other hand, oleogels showed a reduction in oil migration as wax concentration increased, presenting better performances in the presence of the carnauba one (Yang et al., 2017).

Very promising results were obtained for WO samples in the presence of 7% and 8% of carnauba waxes, showing the significant lowest oil migration values compared to SO_CW oleogels (Figure 4). Obtained results can be probably attributed to a very stable three-dimensional network formed during oleogelation, characterized by lower cavities and higher surface areas, as stated by microstructural analysis, that was sufficiently strong to entrap the liquid oil and to form a more physically stable gel, also after removing samples from beakers, that doesn't affect their structural characteristics (Yang et al., 2017; Frolova et al., 2022; Kim et al., 2022).

Figure 4. Oil loss (%) of oleogel samples realized with sunflower and wheat oils in presence of respectively 4, 7 and 8 % of bee and carnauba waxes.

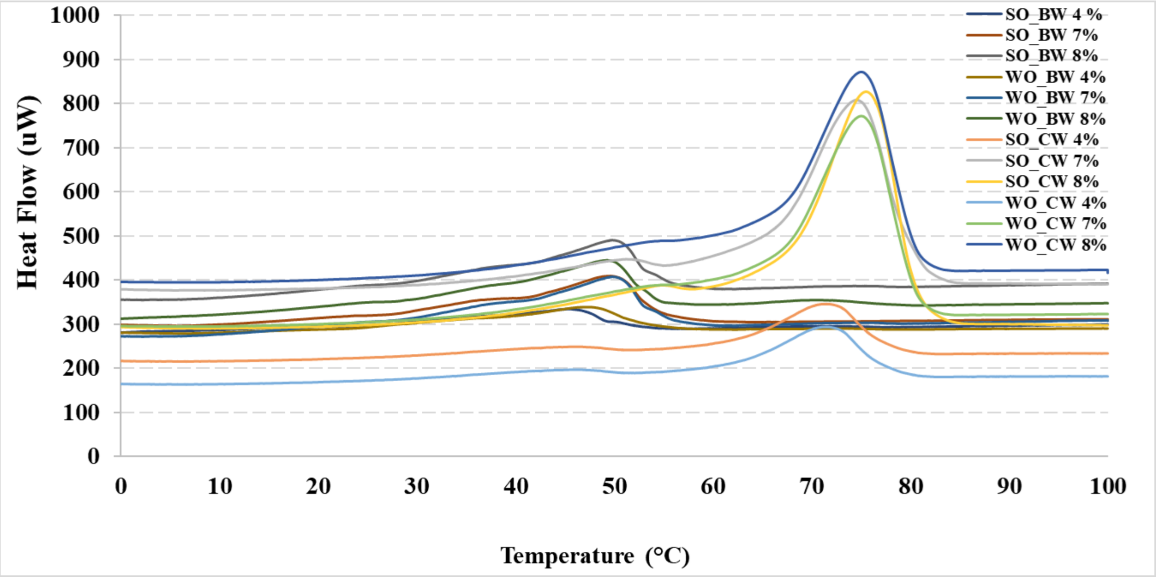


Moreover, According to Palla et al., (2019), the oil loss and so the oil-binding capacity of oleogel samples are strictly correlated with the value of the fractal dimension, which as previously stated was higher for WO_CW.

5.3.4 Thermal analysis

In Figure 5 and Table 3 the melting curve and the parameters related to thermal analysis (T_{onset}), main temperature peak (T_{peak}) and enthalpy (ΔH) of respectively melting (m) and crystallization (c) of sunflower (SO) and wheat (WO) oils based oleogels, realized respectively with beeswax (BW) and carnauba wax (CW) at the different percentages of 4, 7 and 8 are shown.

Figure 5. Melting thermal profiles of sunflower (SO) and wheat (WO) oils based oleogels, realized respectively with beeswax (BW) and carnauba wax (CW) at the different percentages of 4, 7 and 8.



All samples showed an increase in melting temperatures (T_{peak}) and melting enthalpies (ΔHm) with the increase of wax concentration, similar trends regarding the shifting of melting point values versus higher values by increasing wax concentration were reported by several authors (Blake et al., 2014; Martini et al., 2015; Yilmaz et al., 2015).

Carnauba wax samples showed higher onset (T_{onset}) and melting peak (T_{peak}), ranging from 55 to 84°C, compared to beeswaxes ones (35°C to 60°C), in line with previous findings (Basson & Reynhardt, 1988 a,b; Aydeniz Guneser et al., 2021; Qu et al., 2024) probably due to their different chemical compositions. Carnauba wax is mostly constituted by wax esters and free fatty alcohols, while beeswax is high in wax esters and hydrocarbons.

In specific, CW waxes are characterized by higher amounts of long-chained wax ester as reported by Davidovich-Pinhas, (2018) and Brykczynski (2022) and high amount of long chain free fatty alcohols especially C32 and long chain fatty alcohols moieties, that need high temperatures to melt their structures exhibiting also higher gelation ability (Hwang et al., 2012; Doan et al., 2017; Lorenzo et al., 2023).

In addition, the microstructural analysis highlighted the presence of higher dense and aggregate structure when carnauba wax at 7 and 8 % was used, both in the presence of sunflower and wheat oil, with respect to beeswax, as also confirmed by rheological analysis that being able to bind more oil needs more energy to melt, as also highlighted by fractal number and oil binding capacity results.

Table 3. Peak onset (T_{onset}), main temperature peak (T_{peak}) and enthalpy (ΔH) of respectively melting (m) and crystallization (c) of sunflower (SO) and wheat (WO) oils based oleogels, realized respectively with beeswax (BW) and carnauba wax (CW) at the different percentages of 4, 7 and 8.

	Melting			**Crystallization**		
	Tm_{onset} (°C)	Tm_{peak} (°C)	ΔH_m(mJ/mg)	Tc_{onset} (°C)	Tc_{peak} (°C)	ΔHc (mJ/mg)
4% SO_BW	37.89±1.04cd	47.85±0.89cd	0.80±0.04d	46.17±1.18d	43.04±1.78bc	-1.12±0.09c
7% SO_BW	41.30±1.10c	49.55±0.59c	1.17±0.09c	48.91±2.14d	46.19±1.22b	-1.22±0.19c
8% SO_BW	42.19±1.03c	49.83±1.18c	3.97±0.47b	48.43±1.07d	45.22±2.09b	-3.18±0.67b
4%WO_BW	36.48±1.09d	46.05±0.52d	0.94±0.04d	43.28±0.87e	39.09±1.18c	-0.61±0.05d
7%WO_BW	39.20±0.82c	49.30±1.12c	1.47±0.18c	44.21±2.07de	41.81±0.79c	-1.03±0.11c
8%WO_BW	41.52±1.02c	49.55±1.31c	3.74±0.28b	55.05±1.12c	47.28±1.44b	-4.18±0.65b
4% SO_CW	54.74±1.12b	69.50±1.14b	1.09±0.19d	74.92±1.84b	63.44±2.17a	-1.13±0.12c
7% SO_CW	56.71±0.29b	74.71±2.24a	6.11±0.64a	74.37±1.48b	62.40±1.46a	-5.84±0.87a
8% SO_CW	57.87±1.18ab	75.02±1.74a	6.62±0.78a	77.93±1.07a	63.57±1.32a	-6.37±0.93a
4%WO_CW	54.58±1.07b	68.21±1.03b	0.98±0.08d	74.74±1.18b	63.46±1.89a	-1.05±0.08c
7%WO_CW	58.37±1.06a	74.4±2.18a	7.16±0.89a	73.64±1.74b	63.29±2.09a	-6.43±0.89a
8%WO_CW	58.66±1.01a	75.31±1.09a	6.78±0.74a	78.19±1.49a	62.39±2.40a	-7.14±1.11a

$^{a\text{-}e}$ Values followed by different letters differ significantly at $P < 0.05$ level between samples at different concentration and waxes type for each different thermal parameter.

Moreover, in the temperature sweeps part differences in oil's TAGs composition affect the rheological and thermal properties of samples. In WO, the presence of higher amount of saturated fatty acids (**Table S4**) compared to SO (Kim et al., 2010;Akkaya et al., 2018) led to a slightly higher melting point, that positively affected oleogels oxidation stability; in addition, it has to be considered the presence of antioxidants such as α-tocopherol, β-tocopherol.

On the other hand, a higher level of unsaturated fatty acids is correlated to a lower melting point (Amita Devi & Katar, 2016; Perța -Crisan et al., 2023). Moreover, according with literature the choice of structuring agent with high melting points, as highlighted from carnauba wax, then those with lower melting points can provide better oxidative stability to oleogels (Hassim et al., 2022; Perța-Crisan et al., 2023).

In both cases (beeswax and carnauba wax) a shoulder before the main melting peak was highlighted, in relation to the presence of another peak, as showed also for pure wax, but in the former in a less enunciated way probably due to the presence of oils that modify thermal properties of waxes. Double or several peaks can be related to the co-existence of different chemical and molecular compounds, with different thermal properties (Blake et al., 2014; Martini et al., 2015).

Crystallization profiles (Figure S8) underlined the co-existence of several different molecular compounds (several crystallization peak), as well as different crystals populations with different crystallization temperatures in relation to their fractionated degree (Martini et al., 2015).

Moreover, as observed by several authors (Toro-Vazquez et al., 2010; Blake et al., 2014; Dassanayake et al., 2012; Martini et al., 2015; Sivakanthan et al., 2022), the presence of oil with different fatty acid and fatty alcohols compositions dependent on the length of their chemical chains, affect oleogel crystallization, in terms of polymorphic crystal types and its thermal behavior. At last, also for crystallization parameters, a linear relationship between enthalpies and rheological, as well as microstructural parameters was observed, strengthening our previous findings (Table 3).

5.4 Conclusions

Overall results showed the potentiality of wheat durum oil to be employed for developing stable oleogels. In particular, fundamental rheological analysis both frequency and temperature sweeps, highlighted the presence of a very firm structure, with a predominance of elastic modulus also at high temperatures for samples of wheat durum oil-based containing up to 7% and 8 % of carnauba wax, without significant differences with respect to samples obtained with sunflower oil. Again, the lowest stability was observed for both oils (WO and SO) in the presence of beeswax, probably in relation to its lowest gelation ability strictly related to its chemical composition.

Microstructure evaluation also underlined the presence of very strong networks for WO and SO in the presence of carnauba waxes. On the other hand, the fractal analysis dimension also highlighted significant higher values for WO samples than SO, corroborating the high oil binding capacity of WO samples at 7% and 8 % of CW. Obtained results, confirm the suitability of WO-based oleogel to be successfully employed as fat-mimic matter; however, a deeper study focused also on the sensory aspects of the stabilized and developed oleogels is required in order to better define its applications in the food industry. In conclusion, it is possible to highlight the promising ability of wheat oil a by-product obtained from wheat milling processing, rich also in bioactive compounds, to be employed in the food sector, as an alternative to sunflower ones, to develop oleogels with optimal performances and characteristics in terms of rheological and structural stability.

In literature the oleogels formed by different common fats matrix and different oleogeletor had been produced by various techniques and used in spreads, bakeries, confectioneries, and dairy and meat products. The research has been produced acceptable WO-based oleogel with similar technological and rheological properties as the reference SO – based oleogel.

Based on the consideration, most suitable application of this new oleogel developed with durum wheat oil with carnauba wax, are the follow:

- *Ice-creams*: the oleogel should be tested in addition to milk during heating, before adding the solid ingredient. The presence of a little percentage of saturated fatty acids with short chain from other ingredient maybe increase the gelling activity and lead a better texture property compared to a “standard” ice cream.

- *Processed meat*: the oleogel should be tested in processed food like meat products (patty) to reduce the integration of fats matter from pork/caw. Oleogel maybe added directly during mincing stage of the ingredient before the shaping.

Despite other strategies that aim to reduce or replace sutured fats, oleogelation presents a great potential for industrial application in the future due to nutritional and environmental considerations.

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5.6 Supplementary material

Frequency sweep tests (detailed methodology reported in 5.2.3.2 section)

Figure S1a**.** Elastic (G’) moduli of sunflower (SO) and wheat (WO) oil based oleogels, realized respectively with beeswax (BW) and carnauba wax (CW) at the different percentages of 1.5 and 3.

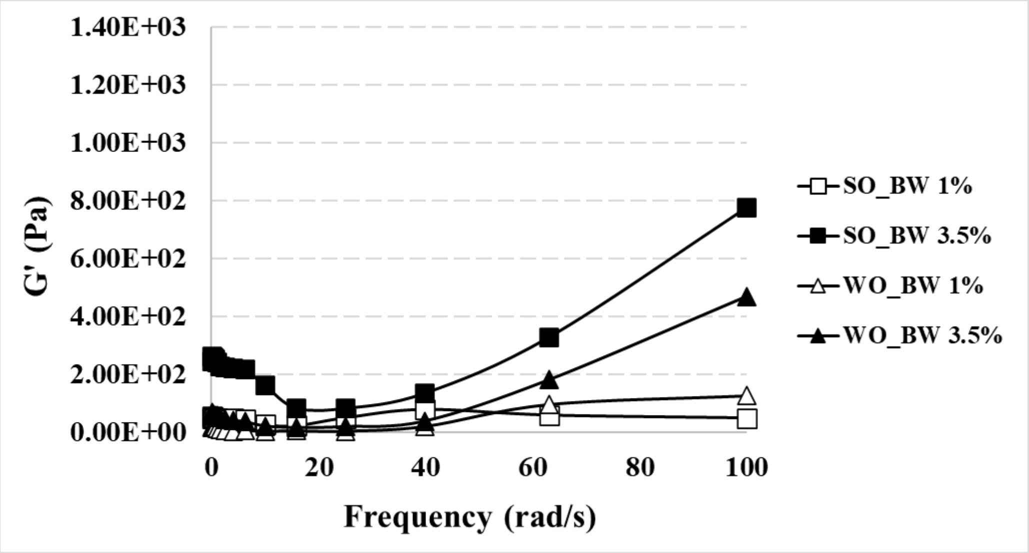
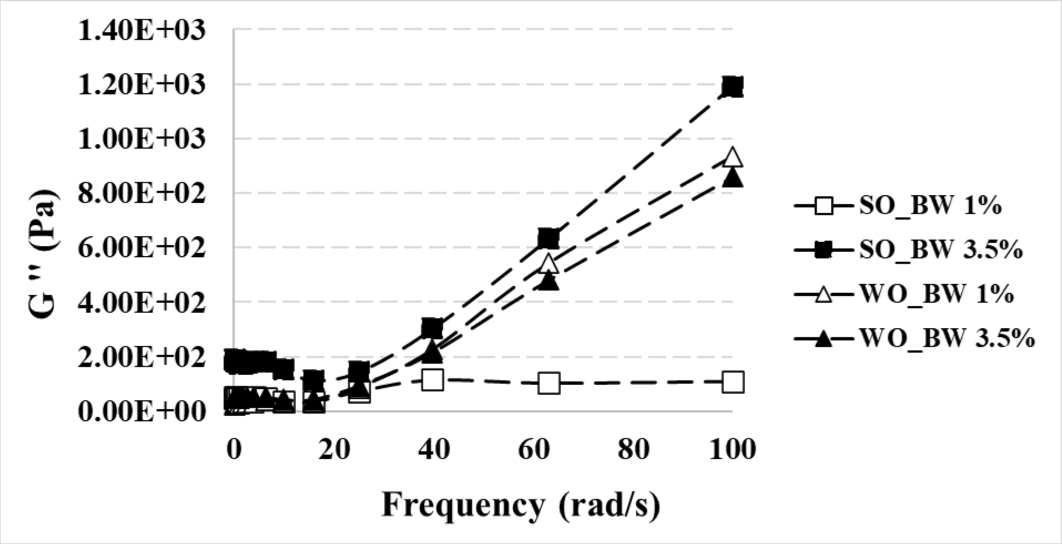


Figure S1b. Viscous (G’’) moduli of sunflower (SO) and wheat (WO) oils based oleogels, realized respectively with beeswax (BW) and carnauba wax (CW) at the different percentages of 1.5 and 3.



Temperature sweeps tests (detailed methodology reported in 5.2.3.2 section)

Figure S2a**.** Elastic (G’) moduli of sunflower (SO) and wheat (WO) oils based oleogels, realized respectively with beeswax (BW) and carnauba wax (CW) at the different percentages of 1.5 and 3 % evaluated in a temperature range comprised between 0 and 40°C.

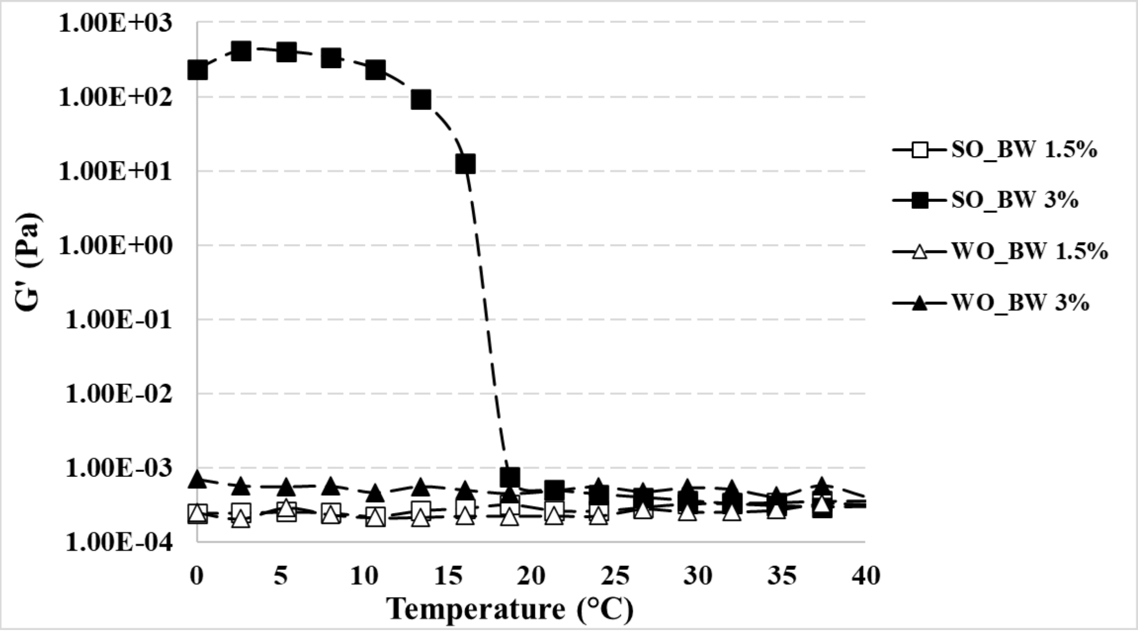
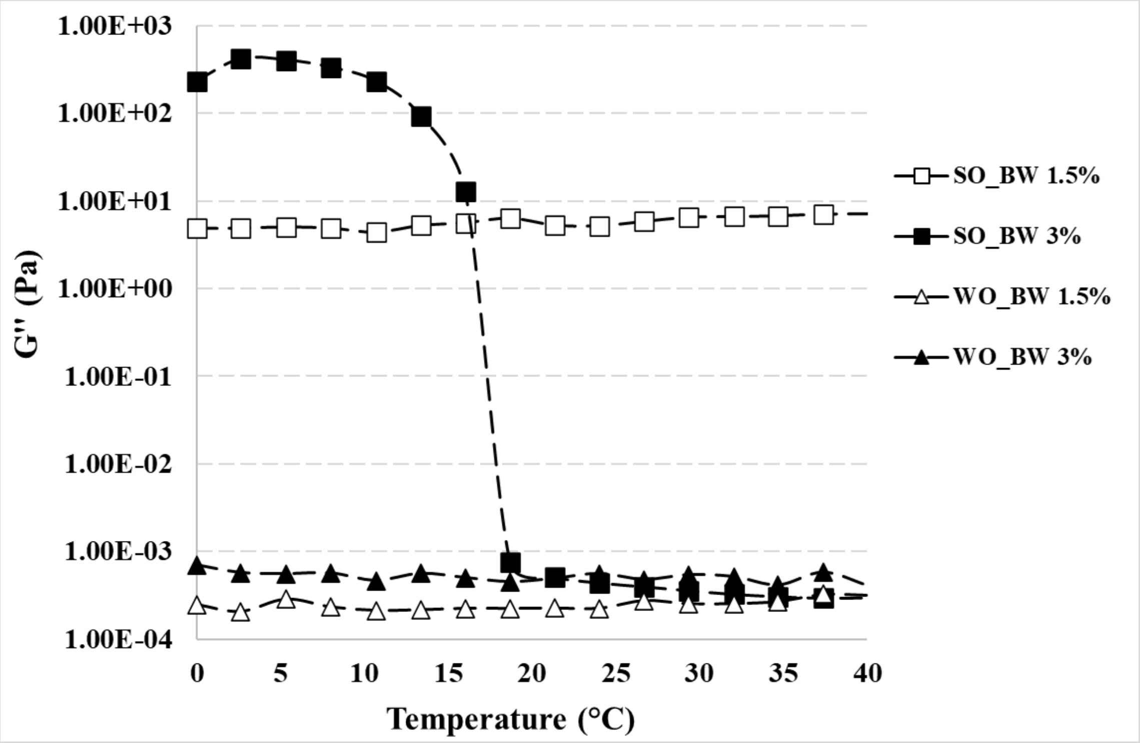
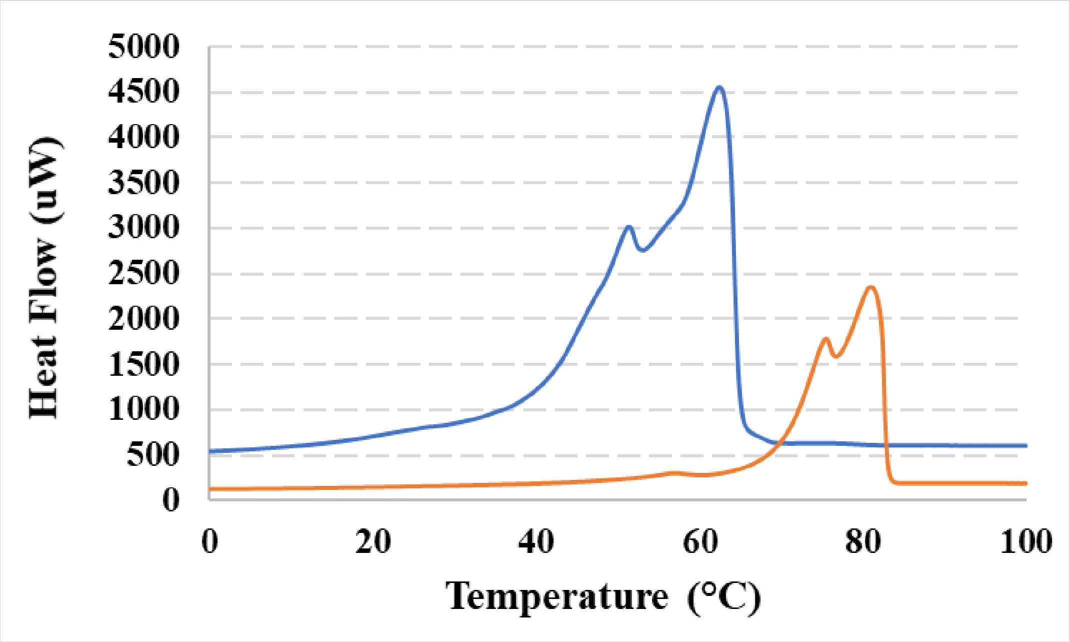


Figure S2b. Viscous (G'') moduli of sunflower (SO) and wheat (WO) oils based oleogels, realized respectively with beeswax (BW) and carnauba wax (CW) at the different percentages of 1.5 and 3 % evaluated in a temperature range comprised between 0 and 40°C.



Thermal Analysis (detailed methodology reported in 5.2.3.3 section)

Figure S3. Thermal profile and melting point of pure beeswax and carnauba wax



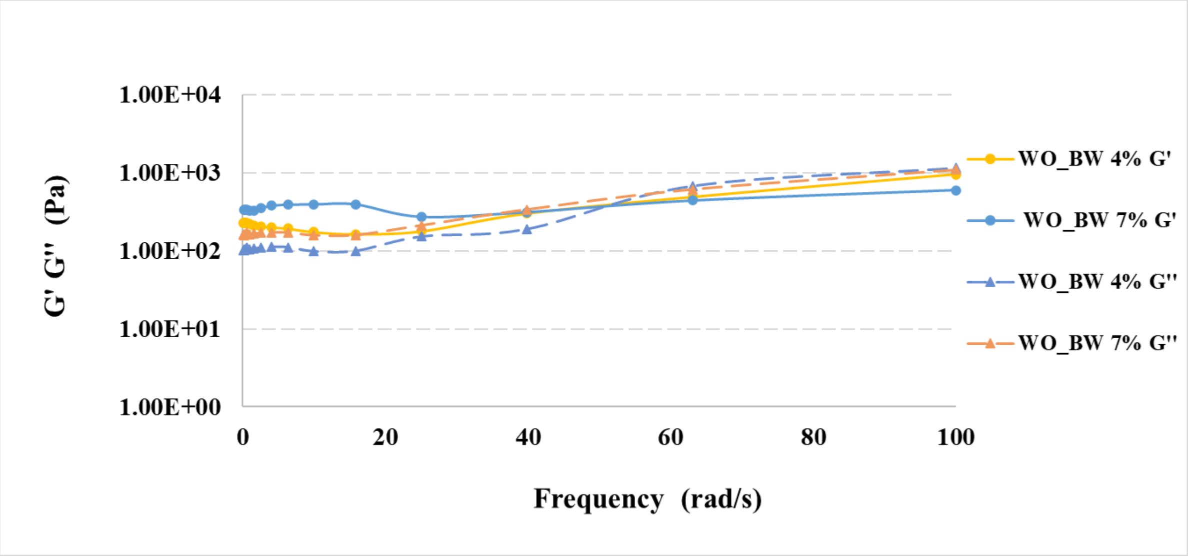
Determination of composition of durum wheat oil

Table S4. Determination of composition of durum wheat oil.

Fatty acids (%)	**Content**
C14:0	0.08
C15:0	0.10
C16:0	14.97
C16:1	0.26
C17:0	0.12
C17:1	0.04
C18:0	1.35
C18:1*c*9	22.01
C18:2 n-6	55.47
C20:0	0.19
C20:1	0.82
C18:3 n-3	3.94
C21:0	0.09
C22:1	0.09
C24:0	0.12
C24:1	0.10
Sterols (% of total sterols)	
Campesterol	13.0
Campestanol	20.3
Stigmasterol	2.0
Δ7-campesterol	1.3
Sitosterol	32.9
Sitostanol	18.5
Δ5-avenasterol	5.9
Δ5,24-stigmastadienol	1.1
Δ7-avenasterol	2.6
Total Sterols (mg/kg oil)	22760
Tocopherols and tocotrienols (mg/kg oil)	
α-Tocopherol	304
β-Tocopherol	42
γ-Tocopherol	106
δ-Tocopherol	<1
α-Tocotrienol	279
β-Tocotrienol	1129
γ-Tocotrienol	29
δ-Tocotrienol	4
Waxes (%)	0.05

Frequency sweep tests (detailed methodology reported in 5.3.1 section)

Figure S5. Detail of G’ and G” crossover of WO_BW at 4% and 7%



Microstructural Analysis (detailed methodology reported in 5.2.3.2 section)

Figure S6. Detailed micrographs of oleogel samples realized with 7 % of both beeswax and carnauba wax ((SO_BW 7% (A); WO_BW 7% (B); SO_CW 7% (C); WO_CW 7% (D)) acquired at 10x of magnification with optical microscope in brightfield mode.

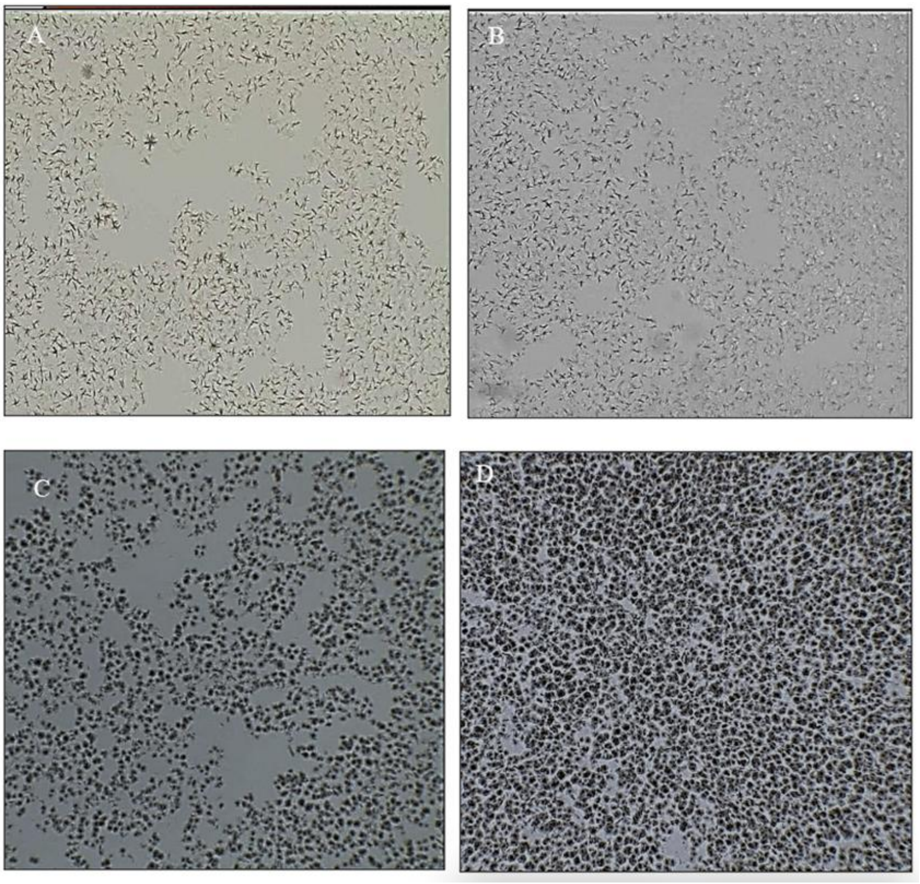
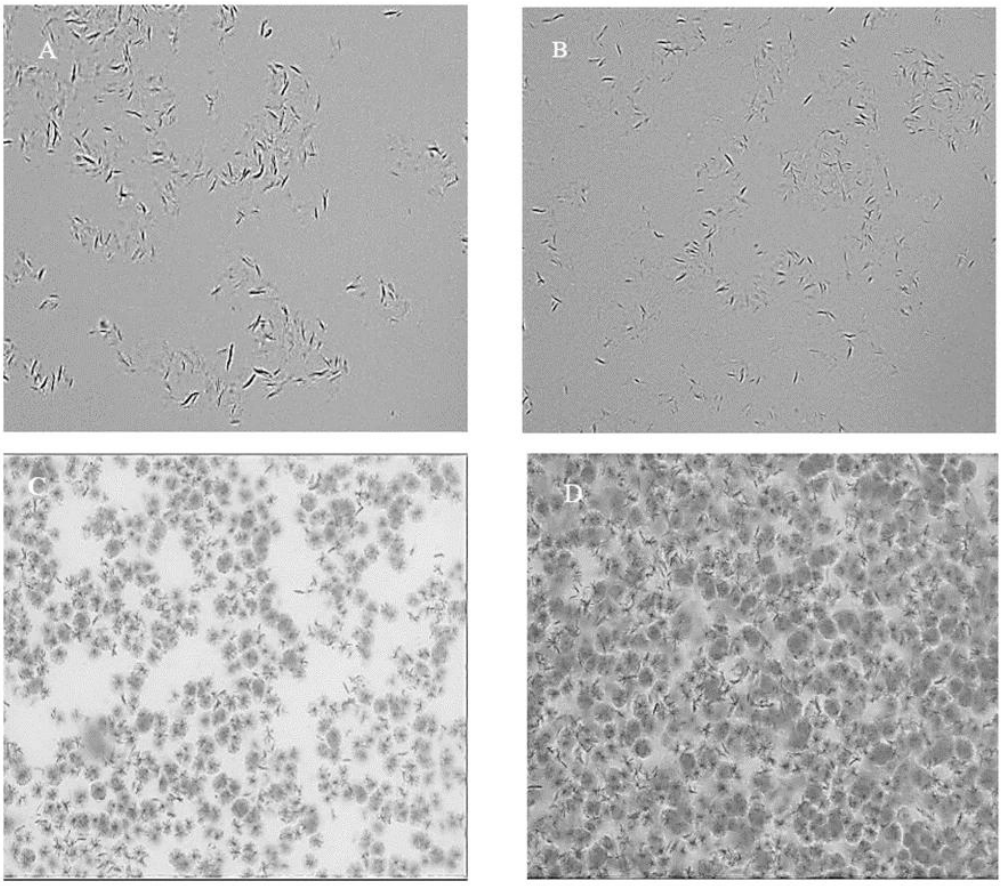
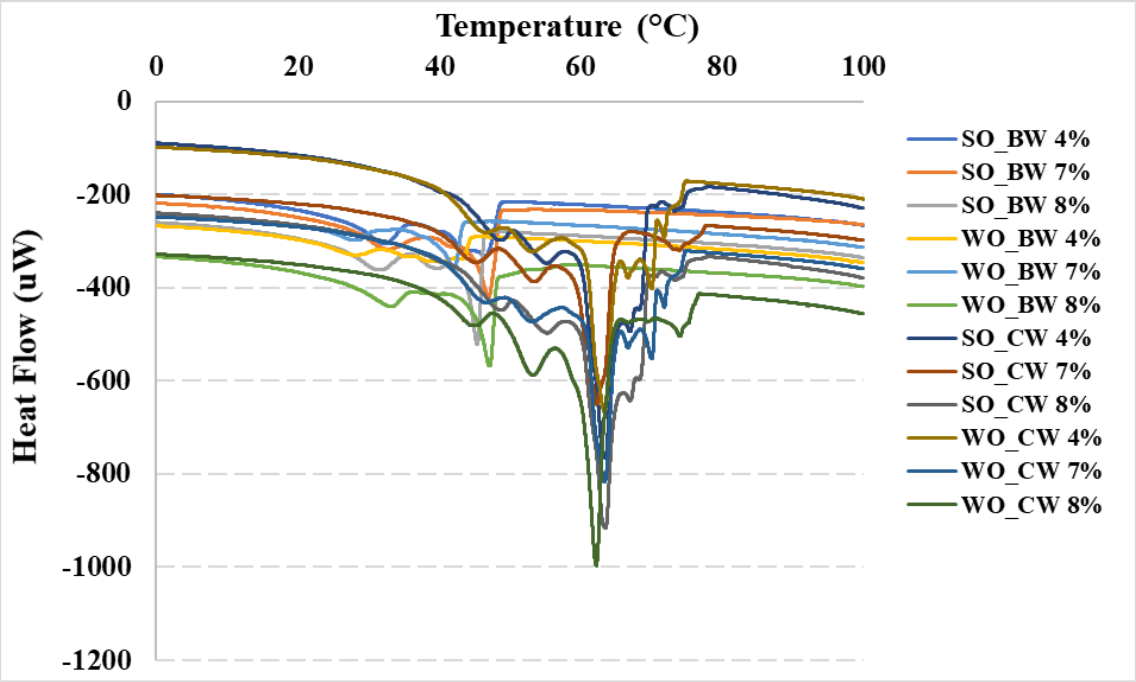


Figure S7. Detailed micrographs of oleogel samples realized with 7 % of both beeswax and carnauba wax ((SO_BW 7% (A); WO_BW 7% (B); SO_CW 7% (C); WO_CW 7% (D)) acquired at 20x of magnification with optical microscope in brightfield mode.



Thermal Analysis (detailed methodology reported in 5.2.3.3 section)

Figure S8. Crystallization profiles of oleogel samples at different beeswax and carnauba concentrations (4%,7% and 8%) in presence of both WO and SO.



CHAPTER 6

Conclusions of PhD research activity and future prospectives

Fats and oils are composed by a wide range of natural components based on fatty acids and other natural molecules, thus representing a heterogeneous group of biological compounds which could be categorized in different classes. The multidisciplinary approach is a key aspect for the investigation of different fats matters and has been exploited all along the present PhD project, reflecting the complexity of world of fatty substances.

Innovation on TAG isomers analysis performed by Ag+-HPLC (ChromSpher Lipids column) with a replacement of mobile phase tested at different column temperatures and different acetonitrile concentrations has been tangible. Results obtained with *n*-heptane and *n*-hexane based mobile phases allowed to conclude on the equivalence of both solvents. These outstanding results allowed the use of *n*-heptane as a less toxic mobile phase without compromising chromatographic performance, paving the way toward a safer working environment. At the same time, the investigation of other two parameters, such as different temperatures (10 and 30°C) and different acetonitrile concentrations (0.1 and 0.3%) provided additional details and considerations around that difficult technique. In particular, the increase of the column temperature and integration of acetonitrile affected both the retention time of analytes related to the saturation level of fatty acids and the resolution and the shape of picks of the analytes. Finally, the best performance for chromatographic separation and better resolution of the isomers considered (SOS/SSO, POS/PSO, POP/PPO, SOO/OSO, POO/OPO) has been found and hexane/heptane switch has been validated.

The investigation of new possible markers to evaluate the oxidation/degradation during manipulation and storage of fats matter in food industry, led to a deep study on the phytosterols oxidation products (POPs), which are potentially harmful for consumers. The study highlighted several lacks on actual scientific literature and stuff availability. For the first time, a preparative LC method able to isolate “homemade” reference material for α-epoxy, β-epoxy, 7α-hydroxy, 7β-hydroxy and 7-K isomers of more represented phytosterols with a high purity (>90 %) level has been developed. Starting from these, a sensitive, reproducible and robust LC-Orbitrap-HRMS analytical method has been successfully validated. Different fats commodities (high oleic sunflower oil, palm oil and palm olein) have been selected to assess the suitability of the analytical method. The evaluation of POPs content has been performed during all the refining process and storage in forced conditions. At the end of the evaluation, it has been observed that, in the refining process, the neutralization led to an increase in the oxidation fats matrix, while the deodorization led to a general decrease for all matrices considered. As far as what concerns the different type of fats matter, it might be highlighted that palm oil displayed the lowest content of POPs, followed by palm olein and high sunflower oil. Considering instead the forced storage of different analyzed fat matrix, this showed a slight evolution of POPs.

That investigation represents the first outcome with this type of structure and instrumental assets. The development of a method for isolation of pure standards laid the foundation for different POPs investigations. Being oriented towards new cutting-edge technologies, new food trends, global health recommendation and sustainability, the third research activity has been focused on a food technological application – the texturization of high nutraceutical value sustainable oil (durum wheat oil). Carnauba wax and bee wax have been chosen and tested. Durum wheat oil obtained from a by-product of wheat processing showed great potential for food application, but with limitation due to its physical and rheological characteristics.

The fundamental rheological analyses of both frequency and temperature highlighted good performances even at high temperatures for durum wheat oil samples with high levels of carnauba wax (7% and 8%). The samples developed with beeswax showed a low stability, potentially linked to its low gelation capacity. The evaluation of the microstructure revealed that, carnauba wax is characterized by the best performances. It was possible to highlight the promising ability of wheat oil to be used in the food sector, as an alternative to sunflower oil, to develop oleogels with excellent workability and rheological and structural stability characteristics.

From a structural point of view, the PhD research activity was therefore composite and dynamic, embracing conceptually complex aspects that required the development of hard and soft skills but at the same time embraced the more practical aspect of food technology. Much has been investigated and much still needs to be done on the projects that have been pursued over the years. The opportunity to interface with the academic world in conjunction with the corporate world has been very inspiring.

Sincerely, thanks to all.

I don’t want to belive. I want to know.

Carl Segan