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**EFFECT OF DIFFERENT COOKING METHODS ON
THE FORMATION OF CHOLESTEROL OXIDATION
PRODUCTS (COP_s) IN PORK LOIN**

**EFFETTO DI DIVERSI METODI DI COTTURA SULLA FORMAZIONE
DI PRODOTTI DI OSSIDAZIONE DEL COLESTEROLO (COP_s) NELLA
LONZA DI MAIALE**

TIPO TESI: Sperimentale

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Questo importante traguardo lo dedico a voi,
al mio amato Nonno Remo e alla cara Nonna Anna.
So che ci tenevate ad esserci, ma il destino, nonna a te in particolar modo,
te ne ha privato per poco, spero che da lassù siate orgogliosi di me.

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RIASSUNTO

La lonza è una carne ottenuta dalla macellazione del maiale, il cui consumo prevede un trattamento di cottura che influisce sul contenuto di colesterolo, migliorando la sua estrazione e/o degradazione. Questa matrice alimentare contiene colesterolo che in seguito a trattamenti termici può ossidarsi, formando ossisteroli (COPs). È stato dimostrato che gli ossisteroli sono anche in minime quantità, dannosi per la salute, essendo potenzialmente citotossici, mutageni, cancerogeni ed aterogeni. Il cibo è un sistema complesso dove la formazione di ossisteroli dipende dalla composizione chimica: presenza di acidi grassi polinsaturi e antiossidanti (tocoferoli e carotenoidi).

Per queste ragioni, lo scopo di questa tesi è stato quello di valutare l'effetto del trattamento termico sulla formazione di questi composti (COPs), presenti nella lonza di maiale. La termoossidazione è stata indotta usando un forno tradizionale, un forno combinato e un forno a microonde.

L'intero pezzo di lonza è stato sezionato in tranci di circa 700g, i quali sono stati sottoposti a tre diversi metodi di cottura, tradizionali ed emergenti: cottura al forno tradizionale (180 °C), cottura al forno combinato (forno tradizionale a 170 °C + vapore) e cottura in forno a microonde (180 °C + 200W), fino a raggiungere la temperatura al cuore della carne di 75°C. Una volta raffreddato, il campione è stato tritato e liofilizzato, per poi essere conservato sottovuoto a -18 °C. In seguito, è stata effettuata l'estrazione dei lipidi che sono stati saponificati a freddo per determinare colesterolo e ossisteroli (COPs). Per purificare i COPs dal colesterolo è stata applicata la SPE (Solid phase extraction).

Colesterolo e COPs sono stati analizzati con il sistema GC-FID.

In tutti i campioni (crudi e cotti), il COP presente in maggior quantità è stato il 7K, seguito dal 7 β -HC, 7 α -HC, 5 β -CE, 5 α -CE e dal CT che risultavano in quantità minori.

Tutti i campioni crudi hanno all'incirca 13 mg/kg grasso di COPs totali e tutti i trattamenti termici abitualmente utilizzati dai consumatori incrementano la quantità di COPs totali.

Per concludere il nostro studio ha messo in luce che il forno tradizionale e il combinato hanno un effetto simile sull'ossidazione del colesterolo, producendo un basso livello di COPs totali, all'incirca 20 mg per kg di grasso; mentre la quantità maggiore di COPs è stata raggiunta nella cottura al microonde a 180 °C più 200 W, con un valore che si aggira sui 124.2 mg per kg di grasso.

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ACRONYMS AND ABBREVIATIONS

C	Carbon
ROS	Reactive oxygen species
7-HC	7-hydroxycholesterol
7K	7-ketocholesterol
5 α -CE	5 α -cholestane
25-OH	25-hydroxycholesterol
ACAT	Acyl-CoA:cholesterol acyltransferase
CT	Cholestane triol
LDL	Low density lipoproteins
HDL	High density lipoproteins
FAO	Food and agriculture organization
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
HPLC	High Performance Liquid Chromatography
SPE	Solid phase extraction
EI	Electric ionization
PCA	Principal Component Analysis

1 INTRODUCTION

1.1 Cholesterol: General information

Cholesterol (Etymology: 19th Century: from chole- + Greek *stereos* hard, solid, so called because first observed in gallstones) was discovered for the first time in 1815 by Michel Eugène Chevreu and is an important minor food constituent. It has several vital functions in a living organism, including the biosynthesis of steroid hormones and bile acids, as well as essential components of lipoproteins and lipid membranes in animal tissues and vitamin D (Sabolová et al., 2017). It is synthesized in animals but not in plants, where phytosterol is present.

In animals, thus in human body too, cholesterol is produced in liver. Apart from endogenous cholesterol, there is also an exogenous source deriving from the diet: in foods rich in animal fats, such as meat, butter, cold cuts, cheeses, egg yolk, liver. Instead, it is absent in fruit, vegetables and cereals.

1.1.1 Chemical structure

Cholesterol is synthesized from squalene that is an intermediate in the biosynthetic pathway to produce human steroid hormones. Like all sterols, cholesterol is characterized by a steroid nucleus, almost planar and relatively rigid, which is constituted of four rings with shared carbon-carbon bonds (three rings with six carbon atoms and one to five atoms) (**Fig. 1**). Cholesterol is an amphipathic molecule with a polar head (the hydroxyl group on the C-3 atom) and a non-polar hydrocarbon body (the core steroid, with two methyl groups, and the aliphatic side chain on the C-17 atom).

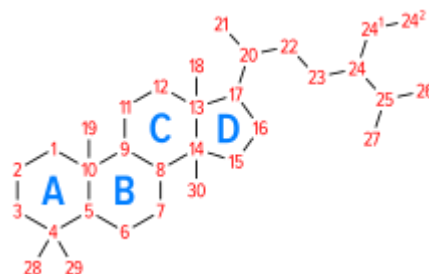


Figure 1. A typical cholesterol's structure.

1.1.2 Role in the cell membrane

Cholesterol, a fundamental component in the structure of all biological membranes, plays important roles in maintaining cellular homeostasis, regulating the communication between cells and external environment and by modulating structure and function of integral membrane proteins. For its structure, cholesterol fits into the lipid double layer with the polar head close of the polar heads of the phospholipids, and with the apolar body intercalated between the apolar tails of the phospholipids (**Fig. 2**), ensuring mechanical resistance and resistance to the lipid bilayer fluidity necessary for normal membrane functions.

Cell in a body is surrounded by a cell membrane, which essentially is a functional interface separating the cell from its surroundings. Moreover, most internal cell organelles and structures are also surrounded by a membrane. Cholesterol is particularly abundant in the plasma membranes, representing typically 25–40 mol% of the total lipid content. In internal cell membranes the cholesterol content is typically lower than in the plasma membrane.

Cholesterol regulates a plethora of biological processes, either by directly interacting with proteins embedded in the membranes or by regulating the biophysical properties of lipid bilayers and, hence, indirectly modulating protein function. The flat and stiff steroid moiety present in the cholesterol molecule implies that the conformational order of phospholipids in the vicinity of cholesterol is promoted extensively, thereby increasing lipid packing, decreasing membrane elasticity, and rendering the membrane less accessible to small water-soluble molecules (Kulig et al. 2016).

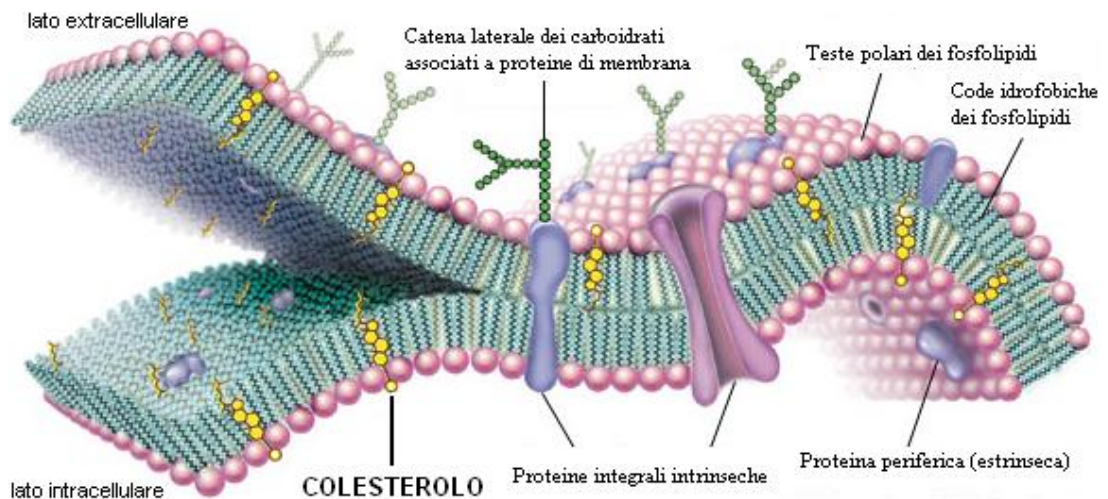


Figure 2. A cellular membrane (www.alamy.it)

1.2 Cholesterol oxidation products (COPs)

Cholesterol molecules present in lipid bilayers are largely susceptible to oxidation. Cholesterol oxidation products or oxysterols differ from cholesterol by the presence of one or more oxygen-containing groups not found in cholesterol (**Fig. 3**). Such a relatively minor change in the chemical structure of cholesterol leads to significant changes in the biophysical properties of oxysterols, which in turn may significantly modulate the properties and the dynamics of the lipid bilayers.

In food and biological systems, the oxidation of cholesterol occurs via numerous chemical reactions. These reactions are either nonenzymatic or enzymatic. Nonenzymatic cholesterol oxidation leads mainly to the generation of products in which the sterol ring system is oxidized. On the other hand, enzymatic processes usually end up in products with an oxidized side chain. However, there are a few exceptions to this rule. In general oxysterols differ from cholesterol by additional polar groups (one or several): hydroxyl, keto, hydroperoxyl, epoxy or carboxyl. Thus, in human body the presence of oxysterols is related to an endogenous source of cholesterol, which in a stressing oxidative physiological status may undergo to oxidation, and the diet exogenous source of oxysterols, already formed in food (Dantas et al., 2015).

Nonenzymatic cholesterol oxidation occurs due to reactions with reactive oxygen species (ROS). In this respect, the oxidation of cholesterol is like that of other lipids that are also prone to attacks by ROS. The interaction of cholesterol with ROS leads to the abstraction of hydrogen from the C-7 position and the formation of a radical carbon. This long-living radical can react with oxygen, thus forming a cholesterol peroxy radical (COO₂·). This radical can further abstract hydrogen from other lipid molecules, leading to the formation of a relatively stable cholesterol hydroperoxide (7 α - or 7 β -Hydroxycholesterol) with the —OOH moiety at the C-7 position. They are further transformed into hydroxy- and keto cholesterol (7 α - and 7 β -HC, and 7-ketocholesterol, respectively), which are the most abundant non-enzymatically generated oxysterols present in most tissues (Kulig et al., 2016).

In food, oxysterols are formed via non enzymatic, mostly by thermo-oxidation, an autocatalytic reaction and may continue via enzymatic. Another group of COPs are the 5, 6 α - and 5, 6 β -epoxycholesterols were identified as products of cholesterol oxidation by air. They may also be hydrolysed into 3,5,6-cholestanetriol, which is considered particularly toxic. In food, oxidation of carbons C20 and C25 on the cholesterol side chain may also occur, producing 20-hydroxycholesterol (20-HC) and 25-hydroxycholesterol (25-HC). Also 20, 24 and 26-hydroperoxides can be formed when cholesterol is strongly oxidized by air. These oxides are derived almost exclusively from enzymes, such as cholesterol 24-hydroxylase,

cholesterol 25-hydroxylase, and sterol 27-hydroxylase. In human body, enzymatic oxidation of cholesterol occurs due to the action of several enzymes, mostly related to the cytochrome P450 family.

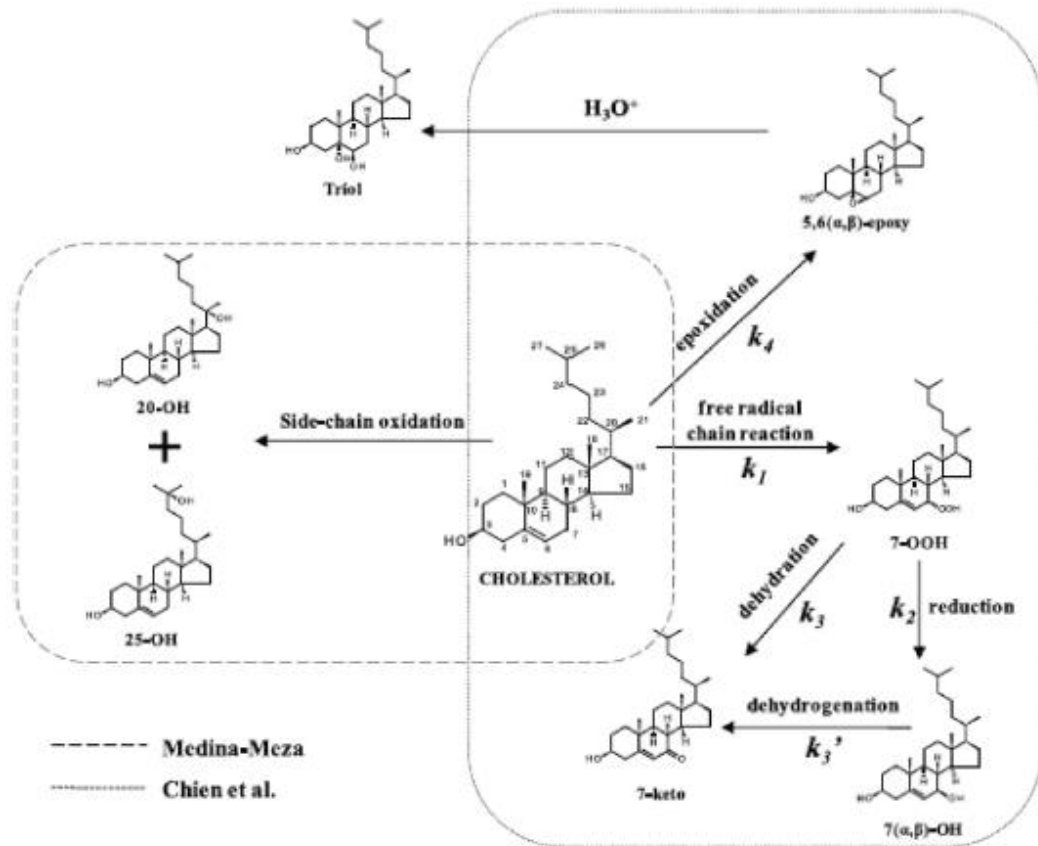


Figure 3. The formation pathways of some COPs during thermo-oxidation (Dantas et al., 2015).

Oxidized sterols occur in elevated levels in cholesterol-rich food due to oxidative conditions connected with food production, storage, and preparation. It has been demonstrated that oxysterols absorbed from food can enter circulation. However, an exact quantitative estimation of the levels of endogenous vs. exogenous oxysterols is missing. As oxysterols originate from cholesterol, their generation depends, to a certain extent, on the presence of cholesterol. It can be roughly estimated that under physiological conditions the concentration of oxysterols is three orders of magnitude lower than that of cholesterol. Under oxidative stress in the presence of ROS cholesterol is less prone to nonenzymatic oxidation than unsaturated acyl chains of phospholipids, the latter usually being in abundance with respect to cholesterol. Nevertheless, *in vitro* studies have shown that oxysterols are more abundant than other oxidized lipids in the cell membranes of oxidatively stressed cells, which is probably due to

less efficient mechanisms of oxysterol clearance. Regarding the clearance mechanisms, oxysterols are eliminated by either metabolic processes or direct elimination. Metabolism leads to the formation of chemically modified species that differ in their physicochemical properties (e.g., solubility or lipophilicity) and/or physiological action (e.g., cytotoxicity) from parent oxysterols (Kulig et al., 2016).

1.2.1 Whereabouts and concentrations of oxysterols

The whereabouts and levels of oxysterols in the human body are topics of great importance considering their potential physiological role. Like cholesterol, oxysterols are typically very hydrophobic and confined to cell membranes. However, small chemical differences can considerably affect how they interact with other membrane components, and this in turn can have a significant effect on membrane structure. Under normal conditions, oxysterol concentration is maintained at very low and strictly regulated levels, usually in the presence of a large excess of cholesterol. In vivo, the usually low oxysterol to cholesterol ratio means that oxysterols have a relatively small impact on the membrane structure. However, in some pathophysiological (and experimental) conditions, oxysterol concentration may rise to much higher levels (>20 mol% of total sterol concentration). In these situations, the effect of oxysterols on membrane properties may be significant (Kulig et al., 2016).

Another often neglected issue in the discussion of oxysterol levels is the amount and the state of oxysterol esterification. Cholesterol esterification (mediated by acyl CoA cholesterol acyl transferases) in cells is the natural cellular process for limiting the amount of free (unesterified) cholesterol in cell membranes, in order to maintain normal membrane structure (an excess of cholesteryl ester, as metabolically inert, is stored in cytoplasmic lipid droplets). Acyl CoA cholesterol acyl transferases can also esterify oxidized forms of cholesterol. In fact, many oxysterols exist as esters in vivo (Kulig et al., 2016).

1.2.2 Biological and pathological role of oxysterols

It has been shown that some oxysterols are cytotoxic in vitro and considered to cause injury to endothelial cells, leading to their dysfunction and, ultimately, triggering programmed cell death. The mechanism of oxysterol-triggered cell death has not yet been fully elucidated, but the ability of oxysterols (as well as lipid peroxides) to alter metabolic pathways, induce modification of proteins, and increase the intercellular concentration of reactive oxygen species may contribute to the toxic effects and trigger the intense calcium efflux that causes cell apoptosis. Several cytotoxic routes have been proposed recently. These include intracellular ROS overproduction, mitochondrial membrane modification, polyamine

metabolic alterations, and perturbations in intracellular calcium levels. An imbalance in calcium homeostasis is known to be crucial in toxic cell injury by activating calcium-dependent caspase enzymes, which irreversibly damage cell organelles and lead to cell death. Several *in vitro* studies have shown the potential apoptotic effect of many oxysterols. 7K (7-ketocholesterol), 7 β -HC, 25-Hydroxycholesterol (25-HC), and Cholestane-triol (CT) derivatives have been shown to cause apoptotic effects in different cell lines. Oxysterols can be also used as biomarkers in neurodegenerative diseases. Since the blood– brain barrier prevents cholesterol uptake, *de novo* synthesis is responsible for all cholesterol content in the central nervous system. Brain neurons regulate cholesterol synthesis and maintain its homeostasis. An excess of cholesterol is converted into 24S-Hydroxycholesterol (24S-HC), making the brain the main source of this oxysterol in circulation. Plasma levels of 24S-HC reflect the number of metabolically active neurons in the brain, thus they are proportional to the degree of brain damage in neurodegenerative conditions such as multiple sclerosis, Alzheimer's, and Hunting-ton's diseases (Kulig et al., 2016).

1.2.3 7 α - and 7 β -Hydroxycholesterol

7 α -Hydroxycholesterol (7 α -HC) has been found to be the major oxysterol in advanced atherosclerotic lesions, with a concentration that is almost comparable to cholesterol levels. Plasma oxysterol concentrations have been shown to be higher in smokers (as compared to non-smokers), and long-term vitamin E supplementation seems to be effective in reducing the plasma levels of 7 β -HC.

It has been demonstrated that 7 α -HC, 7 β -HC, and 7K cause inflammatory phenotype in endothelium cells and 7K induce creation of foam cells (Kulig et al., 2016).

1.2.4 7-Ketocholesterol

7-Ketocholesterol (7K) is a toxic oxysterol that is associated with many diseases and disabilities of aging, as well as several orphan diseases. 7K is the most common product of a reaction between cholesterol and oxygen radicals and is the most concentrated oxysterol found in the blood and arterial plaques of coronary artery disease patients as well as various other disease tissues and cell types. Unlike cholesterol, 7K consistently shows cytotoxicity to cells and its physiological function in humans or other complex organisms is unknown. Oxysterols, particularly 7K, have also been shown to diffuse through membranes where they affect receptor and enzymatic function. 7K is highly implicated in many different diseases with several pathologies: Cardiovascular and arterial diseases, atherosclerotic plaque calcification,

hepatic disorders, neurodegenerative diseases, ocular disorders, pulmonary diseases, gastrointestinal diseases and congenital disorders ([Anderson et al., 2020](#)).

1.2.5 Safety issue

The content of COPs in food sources has been largely overlooked, despite a consistent body of evidences on their biological and pathological activities in humans. COPs are unavoidable and unintentionally formed during food processing, storage, handling and even household preparations, making human exposure a tangible risk. There is the gap between the scientific evidence of COPs formation in biological systems, and the chemical exposure and toxicological significance through dietary intake.

From studies published decades ago, the toxicity and the potential hazard activity of COPs have been demonstrated. As an “ensemble” of molecules rather than a single compound, performing an assessment of COPs exposure is difficult. Ideally, occurrence data with concentrations and frequency should be available in exhaustive, consistent lists. In addition, individuals and subpopulations (i.e. infants, children and elder people) may be exposed or respond differently to these chemicals. A combination of exposure (total dietary intake) and kinetic modelling (dose-depending activity) could give the initial hints in the estimation of human exposure to COPs.

Currently, there are no federal regulations for food processing and storage conditions considering the content of COPs and the consequent human risk exposure, even considering the broad body of evidence demonstrating the direct relationship between COPs and several chronic diseases ([Pereira et al., 2018](#)).

1.3 COPs occurrence in food

Endogenous COPs have been considered as key intermediates in bile acid and steroid hormone biosynthesis or as autoxidation products in tissues, especially under conditions of oxidative stress. Exogenous COPs, on the other hand, are mainly derived from diet by consumption of high cholesterol containing foods. This intake results in an accumulation of COPs in several organs and tissues. Among foods, animal food products including meat and meat products, fish, eggs and egg products, cheese, milk and other dairy products are the major source of dietary COPs (**Tab. 1**) ([Pereira et al., 2018](#)) and the most common oxysterols found in food are reported in **Fig. 4**.

Table 1. Content of COPs in different types of meat, adaptation of *Current Knowledge about Oxysterols: A Review (Brzeska et al., 2016)*

	Beef	Beef hamburger	Chicken hamburger	Pork chops
Raw	8.6 µg/g fat	2.3 µg/g fat	4.0 µg/g fat	8.7 µg/g fat
Thermally processed	30.0 µg/g fat			19.3 µg/g fat
Microwave		12.3 µg/g fat	24.6 µg/g fat	
Fried in olive oil		3.4 µg/g fat	10.8 µg/g fat	

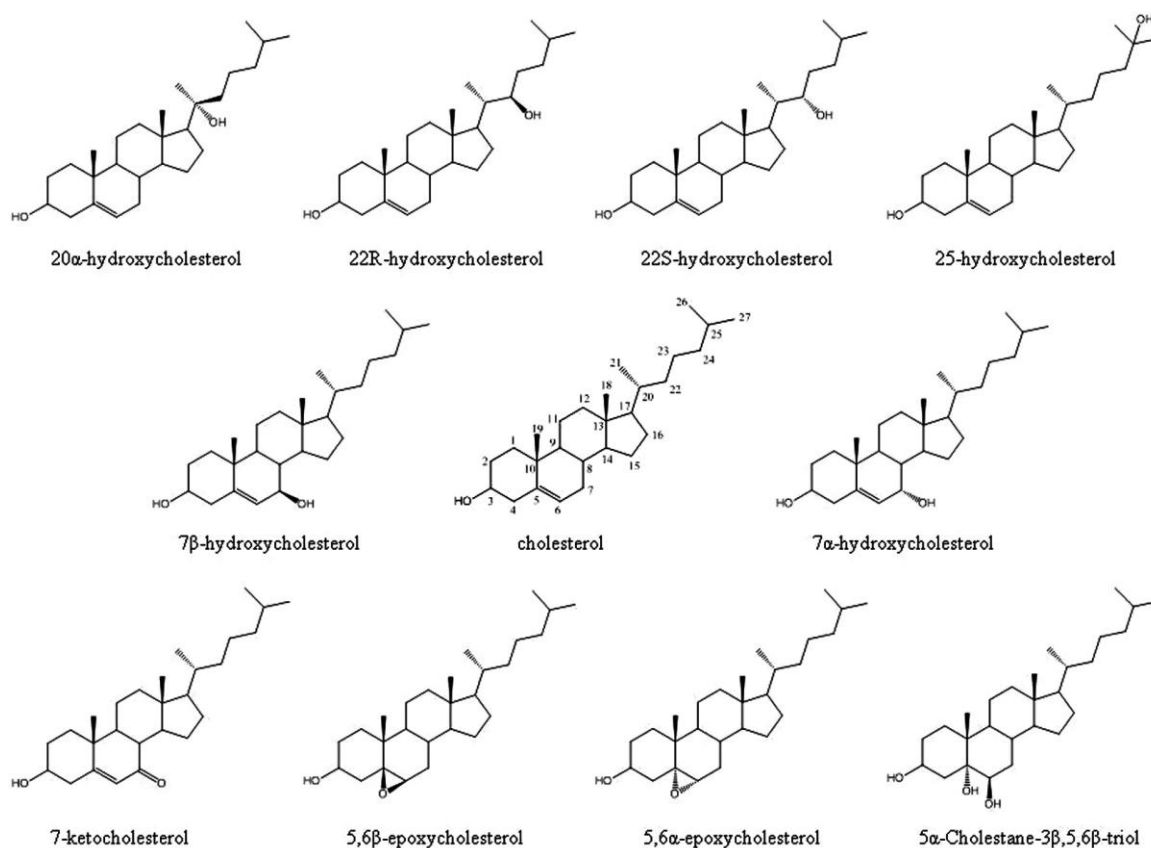


Figure 4. Structural formulas of the most important COPs in food and cholesterol (Georgiou et al., 2017).

1.3.1 7-ketocholesterol

To follow the cholesterol oxidation process, 7K has been often used as an oxidation marker since it is easily formed and is one of the most representative ring COPs. However, 7K does not always rise with increasing time/temperature conditions, especially in complex systems and high-protein or extensively processed foods.

COPs in raw meats are usually present in low amounts, but their concentrations tend to increase dramatically after exposure to prooxidant agents such as light. 7K was usually the most abundant oxysterol (about 30% of total COPs) in photooxidized meat. During storage of fresh meat and meat products, 7K tends to increase and usually becomes the most relevant COP, regardless of dietary supplementation and packaging conditions. Cholesterol oxidation is critical in sliced meat products, due to the very large surface-to-volume ratio.

Cooking usually favours the formation of COPs, but their relative composition and accumulation greatly depend on the initial oxidative status of the meat, the type of meat, and the cooking conditions. Generally, 7K is not the most abundant oxysterol, since during cooking its breakdown rate seems to be greater than its formation rate, thus giving rise to undetected compounds. In turn, 7K could also interact with other compounds (such as proteins, peptides, or free amino acids), and consequently form Schiff bases (Rodriguez-Estrada et al., 2014). The content of 7K varied depending on the type of matrix. Most food products showed a 7K level lower than 2.0 ppm (expressed on sample basis), except for the beef meat (3.5 ppm). On the other hand, beef meat and the meat products showed a higher percent ratio of cholesterol oxidation ($\leq 0.5\%$) as compared to the rest of the samples which are biscuits, snacks, grated cheese, egg noodles, whole egg powder, whole milk powder, salami, raw and cooked meat ($\leq 0.2\%$). Attention should be focused on the quality of the raw materials and the overall product technology, in order to reduce cholesterol oxidation in beef meat and the meat products (Lercker and Rodriguez-Estrada, 2000).

1.3.2 Pork meat production and consumption

Pork is the meat obtained from the slaughter of the pig. It is eaten cooked or subjected to salting or smoking to produce cured meats.

Pork meat is the most consumed in the world and is 37% of world meat consumption, preceding chicken and beef. The largest pork producer is China, followed by the European Union and the United States of America. The countries where pork is mainly consumed in the order of China, the United States of America, Brazil, Germany and France. According to the

FAO (2017), pork is the most consumed globally: 37% of total consumption. Next comes 35% chicken and 21.6% beef.

It is mainly consumed in China, then followed by the United States and then by Brazil, Germany and France. According to the latest Food Outlook, world meat production for the second year in a row is not expected to increase (+ 0.3%) reaching 322 million products. In terms of supply, it is the production of beef that grows the most while the pork that is the most consumed shows a slight drop. However, the pork trade is expected to increase by 4.1 %. Pork meat is composed mainly of protein, followed by fat, with a low content of vitamins and carbohydrates (**Tab 2**).

*Table 2. Different components of pork meat (valori-alimenti.com),
U.I.: Unità internazionali.*

Type of meat	Low fat	Medium fat	High fat
Proteins (g)	19,91	17,23	14,54
Fat (g)	6,81	22,07	37,34
Carbohydrates (g)	1,1	0,55	0
Calories (kcal)	148	274	399
Vitamin A <u>U.I.</u>	0	0	0
Vitamin B ₁ (mcg)	490	420	340
Vitamin B ₂ (mcg)	140	120	100
Vitamin C (mg)	0	0	0
Ca (mg)	8	7	6
P (mg)	156	156	156
Fe (mg)	1,7	1,4	1,2

1.4 Heat treatments

Cooking is essential prior to the consumption of meat as it improves taste, flavour, and digestibility, kills microorganisms, and extends shelf life. However, the negative effects of cooking include lipid oxidation and the generation of aromatic polycyclic hydrocarbons. Lipid oxidation affects the taste, flavour, appearance, nutritional value, and safety of food, resulting in the deterioration of consumable products and an undesirable odour. The oxidation reaction in meat depends on the methods of cooking, temperature, and time. More significant qualitative changes are observed when food is cooked at a higher temperature, and these changes also depend on the preparation time at different temperatures. Excessive oxidation of meat lipids produces potential precursors of highly reactive aldehydes in food, which is a source of oxidative stress that has been linked to atherosclerosis, inflammation, arthritis, Alzheimer's, and Parkinson's diseases. Lipid oxidation in meat during cooking involves cholesterol, and the formation of cholesterol oxidation products (COPs) is related to cooking temperature and time. Cholesterol is a compound of biological importance, but its oxidation products have been proven to be cytotoxic, mutagenic, and carcinogenic and COPs are also considered as a primary factor in causing atherosclerosis (Lee et al., 2006).

Cooking, dehydration, and deep frying are some of the main causes of cholesterol oxidation in foodstuff of animal origin. The extent of cholesterol oxidation in food is influenced by the food matrix composition, presence of pro and antioxidants, as well as food processing and storage conditions (Min et al., 2016).

The different heating methods are a key factor in the cholesterol oxidation process. Greater quantities of COPs are formed when the food is subjected to direct heat. High temperatures produce large quantities of free radicals due to the acceleration of propagation reactions and the decomposition of lipid hydroperoxides. Free radicals are one of the main promoters of cholesterol degradation. It was concluded that cholesterol oxides are produced after a heat treatment of 120 °C in a short time, and that the composition of the products formed is directly related to temperature and heating time. Moreover, the production of cholesterol oxides reached a maximum when they were heated to 150 °C (Dantas et al., 2015).

During the heating process, oxysterols can not only be formed from cholesterol, but also decomposed mainly in the presence of oxygen (Sabolová et al., 2017).

1.4.1 Cooking techniques for pork meat

The process of cooking is a critical stage in meat preparation for consumption. Three main factors differ among the various cooking techniques: the temperature on the surface of the meat, the temperature profile through the meat and the method of heat transfer, contact, air or steam. The temperature on the surface is important for the odour, flavour and colour of the meat. The temperature gradient influences the rate and extent of the changes in protein structures in the meat, whereas the method of heat transfer – especially the humidity – influences the odour, flavour and colour of the meat. Therefore, during cooking, sensory properties of meat are formed. The texture becomes soft and tender; flavour and colour change and become more attractive for consumers. On the other hand, unfavourable processes in heated meat can also be observed. Cooking loss of meat reaches 40% depending on the preparation and cooking method applied; oxidative processes in nutrients, mainly lipids and vitamins, take place; and loss of other nutritional components is observed (Danowska-Oziewicz 2008).

Convection oven

In convection ovens or traditional oven, cooking requires hot air circulating around food. Heat is transferred from the hot air to the surface of the loin by convection and radiation, and from the baking plate by conduction. The heat is transported from the surface into the core of the loin by conduction and convection. Mass transport through the loin is driven by diffusion and convection. At the surface, mass is lost through evaporation and as exudate (Blikra et al., 2019).

Steaming

Steaming is a technique to cook foods by exposing them directly to steam in a closed pan or pot. Steam at normal temperature is 100° C the same as boiling water. However, it carries much more heat than boiling water and cooks very rapidly.

Combi oven

A combi oven is a three-in-one oven which allows to cook with steam, hot air (convection) or a combination of both. Products cooked in the combi oven were characterized by the smallest cooking loss, the highest retention of water and the lowest content of fat compared to other heating methods.

Steam – convection ovens or “combi” ovens are commonly used in restaurants and various catering points. According to the oven producers, that equipment is a useful tool for cooking

healthy and tasty dishes with minimum energy and time required. It allows to create number of recipes of consistent quality. It gives possibility to cook with hot steam, grill, bake, roast or braise. Thanks to such an oven it is possible to save fat, water, and to decrease the cook losses. It saves time and space in the restaurant as there is only one piece of equipment necessary for multiple cooking procedures. In the modern convection-steam ovens it is possible to cook or heat various types of food like meat, vegetables or cakes at the same time, without any smell transfer (Zajac et al., 2015; Zhuang et al., 2008).

Sous vide

Sous vide is a method of cooking in vacuumed plastic pouches at precisely controlled temperatures. Precise temperature control gives more choice over doneness and texture than traditional cooking methods. Cooking in heat-stable, vacuumed pouches improves shelf-life and can enhance taste and nutrition.

Vacuum-sealing has several benefits: it allows heat to be efficiently transferred from the water (or steam) to the food; it increases the food's shelf-life by eliminating the risk of recontamination during storage; it inhibits off-flavours from oxidation and prevents evaporative losses of flavour volatiles and moisture during cooking; and reduces aerobic bacterial growth this results in especially flavourful and nutritious food.

Precise temperature control has more benefits for chefs than vacuumed packaging does: it allows almost-perfect reproducibility; it allows greater control over doneness than traditional cooking methods; food can be pasteurized and made safe at lower temperatures, so that it does not have to be cooked well-done to be safe; and tough cuts of meat (which were traditionally braised to make them tender) can be made tender and still be a medium or a medium-rare doneness (Baldwin, 2012).

Three basic principles govern *sous vide* cooking: pressure, temperature, and time.

- Pressure is determined by the power of the vacuum packer.
- Temperatures used in *sous vide* cooking are always below that of simmering water, which is about 87° to 93°C.
- Time.

Microwave

Food heating in microwave ovens is the result of both bipolar rotation and ionic conduction mechanisms, which involve interactions between polar molecules or charged ions present in food and the microwave electric field. These molecules try to orient themselves in the direction of the electric field and, since the latter changes its direction depending on the frequency, such

molecules modify their orientation and collide with adjacent particles, thus causing a rapid temperature increase. Foods with significant contents of water, fat, or salts display high heating rates when they are microwaved. Lipids are particularly sensitive to microwave heating, because they have a low specific heat and are rapidly heated (Leal-Castañeda et al., 2017).

Microwave cooking might have disadvantages such as affecting meat proteins that significantly change the quality parameters of the product, causing cooking defects and some negative effects on the structural properties (Barbosa-Cánovas et al., 2014).

Cooking Principle:

- a). The microwaves generated by the magnetron reflected cavity and are distributed uniformly as the food rotates on the turntable. The food is thus cooked evenly.
- b). The microwaves are absorbed by the food up to a depth of about 1 inch (2.5 cm).

Cooking then continues as the heat is dissipated within the food.

- c). Cooking time varies according to the container used and the properties of the food like:
 - i) Quantity and Density
 - ii) Water Content
 - iii) Temperature

1.4.2 Effect of cooking on COPs in pork meat

Pork is not rich in antioxidants that protect it from oxidation and therefore storage, cooking and heating processes as well as light and oxygen cause an increase in the levels of oxysterols.

The major COPs found in the whole piece of pork loin were CT, 20-HC, and 25-HC (**Tab. 3**), whose concentrations varied according to the different cooking and reheating methods used. Moreover, the aerobic storage of cooked pork loin under a refrigerated condition also increased the formation of cholesterol oxides on reheating. Convection oven (180 °C) produced the least amount of COPs compared to other cooking methods (steaming and microwaving). 20 α -HC, 25-HC, and CT were the major COPs produced when cooking pork loin cut in slices of 1 cm thickness, while 7 β -HC, α -epoxide, and 7K were not detected in all cooked samples (Min et al., 2016).

Echarte et al. (2004) found that the most toxic COPs, 25-HC and CT, were rarely observed in the pork liver patè. 7K, considered to be a good indicator of oxidation, was present in all analysed samples.

Table 3. Cholesterol oxidation products ($\mu\text{g}/100\text{ g}$) in pork loin. Values are means \pm standard error with different superscript in the same column differ significantly (Min *et al.*, 2016)

Heating treatment	7 β -HC	20 α -HC	25-HC	CT	7K	α -epoxide	Total COPs /Cholesterol (%)
Raw	n.d.	16,43 \pm 6,56 ^B	61,28 \pm 30,07	103,54 \pm 52,21 ^C	n.d.	n.d.	0,67 \pm 0,26 ^B
Steaming	n.d.	19,88 \pm 9,76 ^A	73,59 \pm 21,20	193,53 \pm 14,73 ^A	n.d.	n.d.	1,15 \pm 0,25 ^{AB}
Convection oven	n.d.	32,43 \pm 5,29 ^B	83,86 \pm 36,48	284,86 \pm 8,13 ^{BC}	n.d.	n.d.	0,94 \pm 0,20 ^B
Microwaving	n.d.	20,38 \pm 3,40 ^B	89,47 \pm 4,73	186,91 \pm 29,63 ^B	n.d.	n.d.	1,12 \pm 0,21 ^A

2 THE AIM OF THE THESIS

The aim of this thesis consists in evaluating the effect of thermal treatment on cholesterol and cholesterol oxidation products (COPs) in a susceptible matrix to oxidation such as pork loin.

The investigated cooking techniques were convection oven, combi oven and microwave oven, as they represent some of most common cooking techniques (traditional and emerging) for meat in consumers nowadays habits.

3 MATERIALS AND METHODS

3.1 Standards and reagent

Standards: cholesterol, 5 α -CE, 24-HC, 7K (>99%); KOH (85%); NaCl; Na₂SO₄; BSTFA (*N,O*-bis-trimethylsilyl-trifluoroacetamide with 1% trimethylchlorosilane) and solvents HPLC grade used for standards, sample preparation were purchased from Sigma Aldrich (Milan, Italy). Ultrapure water was prepared using a milli-Q system (Millipore, Millford, USA). Supelclean LC-Si SPE tube (500 mg, volume 6 mL) were purchased from Sigma Aldrich (Milan, Italy).

3.2 Sampling

Fresh pork loin (White Landrace) (**Fig 5**) was purchased from a local distributor. Three sampling (a loin for each sampling of 2.7-3.5 kg) were performed during the period ranged from October to December 2019. Meat was cut as reported in **Fig 6** in four or three pieces of around 670 g as replicates for the same cooking technique and the control sample, raw (n=1), was sampled as total of slices of 100 g of 1 cm thick, collected in different points of the entire loin. Meat pieces (670 g each) were then submitted to different thermal treatments.



Figure 5. Illustration of sampling of pork loin.

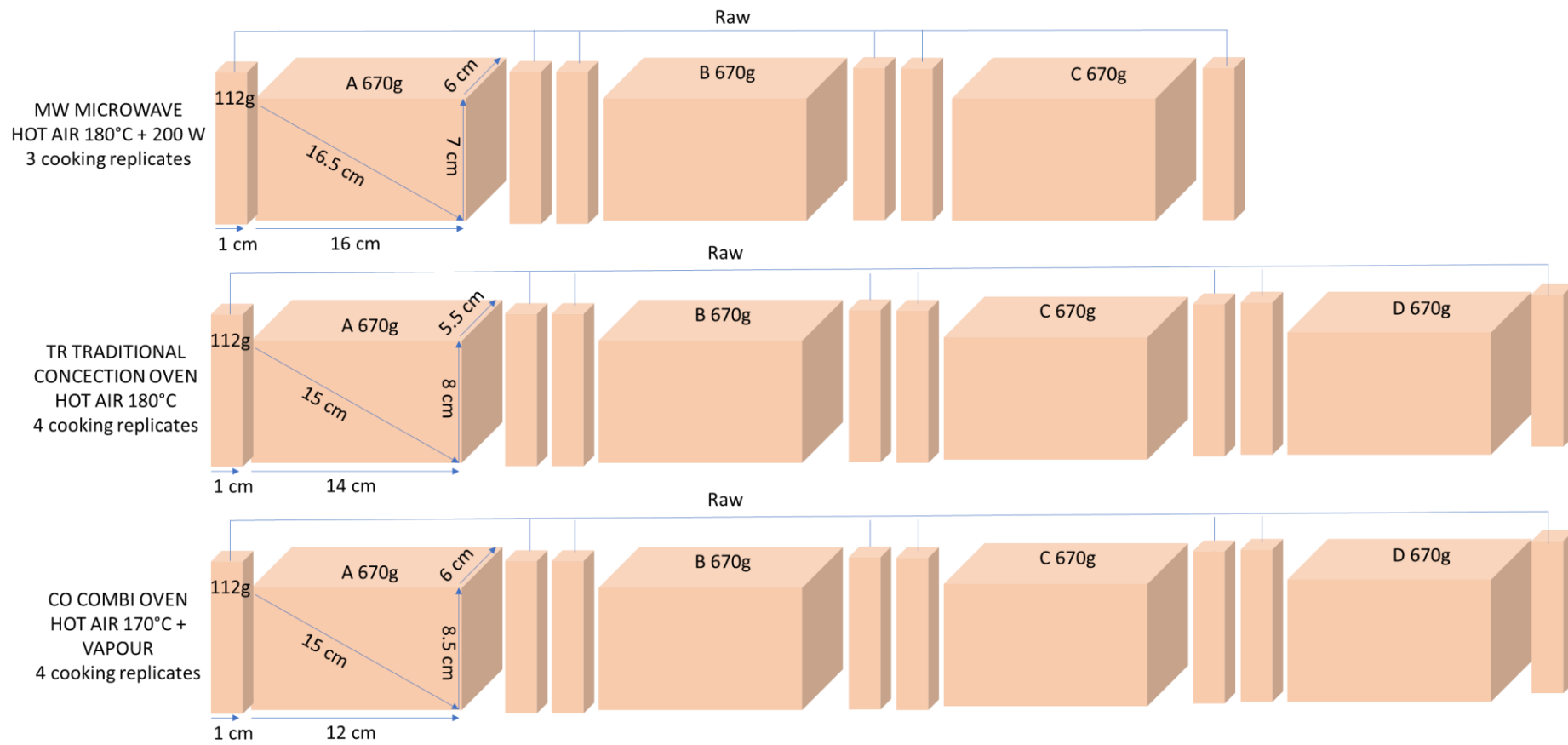


Figure 6 Pieces of meat cooked in different ways

3.3 Cooking treatments

Four techniques of oven baking were taken into consideration for cooking pork loin: convection-oven (TR), microwave-oven (MW) and combi-oven (CO) (**Tab. 4**). In detail, TR was performed in a pre-heated oven at 180°C. CO was achieved by steam injection (around 30%) in the oven chamber and the pork loin were cooked at 170°C. For MW, samples were cooked with 200 W and hot air at 180°C. Immediately after cooking, the core temperatures of the samples were checked, and each sample was cooled, minced in a grinder, freeze-dried, stored at -18°C and subsequently used for analysis. For each meat sampling, each thermal treatment was performed in at least three replicates and a raw sample (CR, n=1) was kept as reference. Cooking times and temperatures of the three cooking methods were decided in line with real household conditions.

Table 4. Specification of cooking conditions (microwaving (MW), convection oven (TR) and combi oven mode (CO) applied to pork loin sliced in pieces of around 670 g. The experimental design included 3 sampling (L1, L2 and L3) for each a raw control slice was sampled.

Sample	g piece of meat before cooking g	g piece of meat after cooking g	cooking description	oven temperature °C	core T °C	time min	time of cooking
L1 CR	675						
L1 MW A	673	435	hot air + 200 W	180	85	35	
L1 MW B	675	430	hot air + 200 W	180	80	36	36,3 ± 1,5
L1 MW B	673	429	hot air + 200 W	180	78	38	
L1 loin weight g	2021						
L2 CR	855						
L2 TR A	674	464	hot air	180	75	70	
L2 TR B	673	459	hot air	180	75	71	74,8 ± 5,2
L2 TR C	667	452	hot air	180	78	77	
L2 TR D	675	465	hot air	180	78	81	
L2 loin weight g	3544						

L3 CR	518						
L3 CO A	664	432	hot air + steam	170	78	58	
L3 CO B	646	401	hot air + steam	170	78	61	
L3 CO C	624	432	hot air + steam	170	79	68	62,0 ± 4,2
L3 CO D	649	397	hot air + steam	170	80	61	
L3 loin weight g	3100						

3.4 Water determination

Raw and cooked samples freeze-dried entirely (670 g) and by difference in weight, % of water was calculated.

3.5 Cholesterol and COPs determination

3.5.1 Lipid extraction

Total lipids were isolated as described by Folch method (Folch et al., 1957). Minced freeze-dried pork meat (2 g) were added of a solution of chloroform: methanol (2:1, v/v, 40 mL) and agitated (5 min). Successively, the mixture was transferred in a 50 mL tube and centrifuged (3000 rpm x 10 min). The upper part (organic) was transferred to a second 50 mL tube and was added with distilled water (5 mL). The mixture was vortexed and again centrifuged (2500 rpm x 10 min at 4 °C). The upper part was transferred with a Pasteur pipette and water was eliminated. The remaining water was eliminated from the organic solvent with anhydrous sodium sulphate (3 g) and then the solvent (containing the lipid fraction) was evaporated with rotary evaporator (30°C) and the fat yield was calculated.

3.5.2 Cold saponification

A cold saponification (Sander et al., 1989) was applied to the extracted fat to determine cholesterol and COPs. The fat (250 mg) were added with 5 α -cholestane solution (100 μ l, 10 mg/L toluene) and 24-hydroxycholesterol solution (25 μ l, 0.5 mg/L in hexane/isopropanol (3:2 v/v)) as internal standards for cholesterol and COPs, respectively. The sample was dried under nitrogen flow and added with KOH 1 M in methanol (10 mL), wrapped with aluminium foil and shaken (18 h) for saponification. It was neutralized with water (10 mL) and extracted with diethyl ether (10 ml), shaken vigorously and separated (3 times). The pooled diethyl fractions

were washed with 0.5 M KOH (5 mL) and distilled water (5 mL). The extracts were dried over anhydrous sodium sulphate and taken to dryness with a rotary evaporator (40°C). The saponified matter was added with *n*-hexane/diethyl ether (1 mL, 75:25 v/v) from which 100 and 900 µL were used for the determination of cholesterol and COPs, respectively.

For cholesterol, 100 µL of unsaponifiable matter was taken to dryness under nitrogen flow, derivatized (20 min at room temperature) with BSTFA (200 µL, *N,O*-bis-trimethylsilyl-trifluoroacetamide), dried and added with *n*-hexane (20 µL).

3.5.3 COPs determination after saponification by SPE

For COPs, a purification on SPE along a tailored Larkeson (Larkeson et al., 2000) method was performed on a silica cartridge (Supelclean LC-Si), conditioned with *n*-hexane (10 mL), deposited with the unsaponifiable extract (900 µL), washed with *n*-hexane:diethyl ether 75:25, v/v (2 mL), *n*-hexane:diethyl ether 60:40, v/v (6 mL) and COPs eluted with (4 mL) of acetone. The purified fraction was taken to dryness and derivatized as for cholesterol and added with *n*-hexane (100 µL).

3.5.4 GC-FID analysis

The COPs were analysed by injecting 1 µL of the derivatized solution into a Varian 430-GC (HTA s.r.l., Brescia, IT) gas chromatograph that was equipped with a split/splitless injector and a flame ionization detector (FID). Separation of the COPs was accomplished using a capillary SLB-5ms column (30 m x 0.25 mm i.d., 0.25 µm film thickness, Supelco, (St. Louis, Mi, USA)) using helium as carrier gas (1.2 mL/min). The programming sequence for the GC oven temperature was as follows: an initial temperature of 90 °C held for 0.5 min and increased to 290 °C at a rate of 30.0 °C min⁻¹. Once the column reached 290 °C, the temperature was increased to 300 °C at a rate of 1.0 °C min⁻¹ and held for a total running time of 25 min. The injector was operating in splitless mode at 350 °C. The detector temperature was 360 °C, and air and hydrogen had flow rates of 300 and 30 mL min⁻¹ to the detector respectively.

The identification was performed by comparison with pure standards of retention time. Quantification was performed by internal standard, 5α-cholestane for cholesterol and 24-hydroxycholesterol for COPs. Limit of detection (LOD) and quantification (LOQ) were as follows: 0.4 and 1.2 (cholesterol), respectively, and 0.7 and 2.0 (COPs).

3.6 Statistical analysis

PCA Principal component analysis was performed with the software R Project for Statistical Computing.

4 RESULTS AND DISCUSSION

4.1 Effect of cooking on water, fat and cholesterol

Table 5 shows the results of % water, % fat and cholesterol content. % water ranged from 47,3 % to 69,4 %. As result of heat treatments, the percentage of water varies in a decreasing significant way with respect to raw meat (CR) for microwave (MW) and convention oven (TR), while it remained constant for the combi oven cooking (CO) (**Fig. 7**). The % of fat varied from 11,9% to 19,9% in raw samples and 11,9% to 19,3% in cooked samples. % fat decreased significantly in the MW oven when compared to the respective raw reference sample, while it remained constant in the TR and CO oven.

The highest retention of water was observed in samples that were cooked in the CO oven in accordance with [Danowska \(2009\)](#). Probably the presence of steam in the oven chamber slowed the water evaporation from the sample. The lowest amount of water was found in meat cooked with TR oven, in discordance with [Danowska \(2009\)](#) who, conducted the study on patties, and found the lowest amount of water in patties which were subjected to the MW heating. The water is probably lost due to heat-induced protein denaturation during cooking of the meat, which causes less water to be entrapped within the protein structures held by capillary forces.

In accord with our outcomes, pork loin cooked in the MW oven contained the smallest amount of fat among all the investigated products, in according with [Khan et al. \(2015\)](#). In disaccord, [Danowska \(2009\)](#) found the smallest amount of fat after CO cooking in patties. As the cooking loss is a combination of liquid and soluble matters lost from the meat during cooking, it may suggest that both water and fat loss during cooking are affected by the cooking temperature.

Table 5. Water and fat percentage after freeze-drying, and cholesterol in raw CR and cooked (microwaving MW, hot air TR and combi mode cooking CO) in pork loin in three samplings (1, 2 and 3). Data are expressed as values of n replicates \pm standard deviation (SD).

Sample	n	% water	n	% fat	n	cholesterol mg/kg fat
L1 CR	1	69,4	1	15,9 \pm 0,3	1	3426,6
L1 MW	3	57,6 \pm 1,0	2	11,9 \pm 1,2	2	5898,4 \pm 1107,2
L2 CR	1	68,0	2	19,9 \pm 0,5	2	3442,6 \pm 346,4
L2 TR	4	53,5 \pm 4,9	4	19,3 \pm 1,8	4	3971,2 \pm 336,6
L3 CR	1	47,3	1	11,9	1	6524,3
L3 CO	4	49,7 \pm 5,2	3	11,9 \pm 1,4	3	7046,8 \pm 1575,7

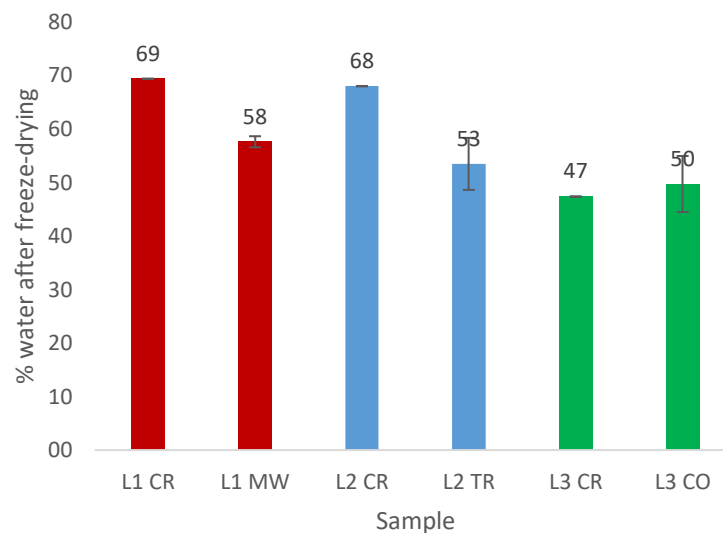


Figure 7. Water percentage after freeze-drying in raw CR and cooked (microwaving MW, hot air TR and combi mode cooking CO) in pork loin in three samplings (1, 2 and 3).

Cholesterol ranged from 3426,6 to 6524,3 mg/kg fat in raw samples and from 3971,2 to 7046,8 mg/kg in cooked samples, showing an increase in MW samples when compared to raw samples. No variation in TR and CO samples was recorded (**Table 5**). In **Fig. 8**, it is possible to notice an inverse interplay between % fat and cholesterol. Fat could change its composition during cooking by losing short chain fatty acids, which could result in a concentration of cholesterol, higher for MW treatment.

In partial accord, different cooking methods applied to muscle meat from Iberian pigs (grilled, fried, microwave and roasted) did not produce changes in total lipid content in meat, but MW applied was less intensive (80 °C) than our treatment (Broncano et al., 2009).

In disaccord with [Khan et al. \(2015\)](#) as the author stated cooking of meat produced an increase of lipid and total cholesterol, related to water loss. However, the matrix and its slicing were different from our pork loin of 670 g, 14 cm around against 1 cm.

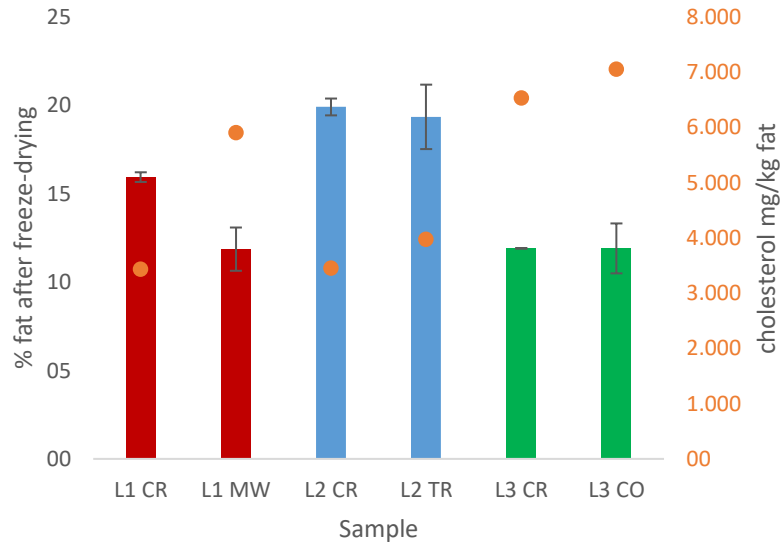


Figure 8. Interplay between % fat and cholesterol.

4.2 Effect of heat treatment on COPs

In raw and cooked samples, the following COPs were identified: 7α -HC, 7β -HC, 5β -CE, 5α -CE, CT and 7K (**Table 6**). In all samples, the most predominant COP was, 7K, that ranged from 7,0 to 68,7 mg/kg fat, followed by 7β -HC that varied from 1,4 to 36,3 mg/kg fat, while 7α -HC and 5β -CE, 5α -CE and CT were present in lesser amount. [Min et al. \(2016\)](#) identified only 20α -HC, 25 -HC and CT in pork loin in raw and cooked pork loin (slices). Raw materials differences may explain this variation in COPs. However, thermo-oxidation tends to form COPs oxidized in C7 position, as in our results.

In general, all cooking treatment caused an increment in COPs content, as suggested in literature for pourer in antioxidant matrices ([Dantas et al., 2015](#)). The major effect was recorded for MW treatment as it produced the highest concentration in all COPs identified. Focusing on the major COPs present such as 7K and 7β -HC (**Fig. 9**), it emerged TR (traditional, convection oven, hot air) and CO (hot air plus steam) had the same effect on cholesterol oxidation, which is less important than 200 W of microwave. All cooking methods

caused an increase of 7 β -HC in particular MW method, in disaccord with Khan et al. (2015) that said only microwave cooking produces 7 β -OH in loin ham.

Table 6. Cholesterol oxidation products (COPs) expressed as mg/kg fat in raw CR and cooked (microwaving MW, hot air TR and combi mode cooking CO) in pork loin in three samplings (1, 2 and 3). Data are expressed as values of n replicates \pm standard deviation (SD).

Sample	n	7 α -HC	7 β -HC	5 β -CE	5 α -CE	CT	7K
L1 CR	1	5,6 \pm 0,0	2,7 \pm 0,0	1,7 \pm 0,0	0,8 \pm 0,0	0,5 \pm 0,0	7,0 \pm 0,0
L1 MW	2	16,0 \pm 5,9	36,3 \pm 13,6	10,3 \pm 0,3	4,8 \pm 0,9	4,1 \pm 2,2	68,7 \pm 5,5
L2 CR	1	4,7 \pm 0,0	1,4 \pm 0,0	2,4 \pm 0,0	1,0 \pm 0,0	1,5 \pm 0,0	7,7 \pm 0,0
L2 TR	3	5,1 \pm 2,7	4,0 \pm 1,8	3,7 \pm 2,0	1,5 \pm 0,7	1,2 \pm 0,6	10,5 \pm 3,6
L3 CR	1	3,5 \pm 0,0	2,9 \pm 0,0	1,7 \pm 0,0	0,8 \pm 0,0	0,5 \pm 0,0	7,9 \pm 0,0
L3 CO	2	4,4 \pm 0,9	4,3 \pm 0,5	3,6 \pm 0,6	1,9 \pm 0,3	1,3 \pm 0,3	10,0 \pm 1,5

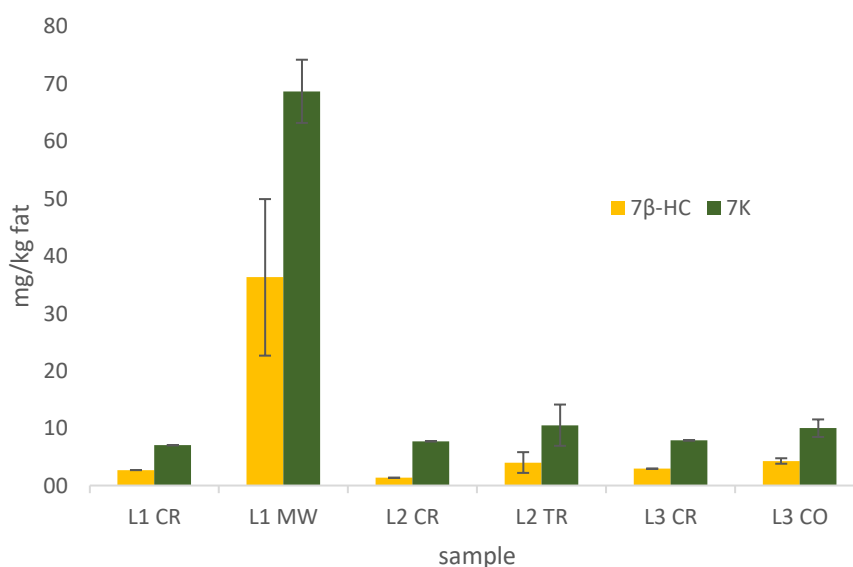


Figure 9. Major COPs (7 β -HC and 7K) in raw and cooked pork loin.

Values of total COPs in **Table 7** varied from 12,8 to 124,2 mg/kg fat corresponding to a range of 0.2 to 2.2 % COPs/cholesterol. All raw samples had around 13 mg/kg fat of total, while TR and CO samples were around 20 mg/kg fat, meaning these cooking techniques had a similar effect on COPs formation. The highest value was registered for MW samples (124.2 mg/kg fat), meaning 200 W enhanced the production of COPs more than CO and TR treatments, till 2.2 % COPs/cholesterol (**Fig. 10**). In accord with Khan et al. (2015) cooking process increase the COPs formation and MW led to the

production of higher amounts of COPs as compared with other methods in loin ham. In disaccord, [Broncano et al. \(2009\)](#) found in Iberian pig meat cooked in slices of 1 cm that there were no significant differences among different cooking methods (TR convection oven, MW, frying and roasting) on COPs values, but MW treatment was less intense than the one performed in our tests. Moreover, [Min et al. \(2015\)](#) found that convection oven produced the least amount of COPs compared to other cooking methods (pan roasting, MW and steaming). The authors applied steam cooking to pork loin, that is in part similar to CO treatment performed in our tests, which uses hot air and steam to heat. CO and TR produced total COPs similar and low levels of COPs around 20 mg/kg fat.

CO and TR cooking led to increment of COPs of 8.1 and 9.2 mg/kg cooked meat (**Table 7**), with a lower impact on cholesterol oxidation when compared to MW cooking, increasing almost 6-fold total COPs in meat after cooking (51.6 mg/kg cooked meat). Thus, eating 100 g of meat cooked with MW correspond to an intake of 5.2 mg of total COPs, which with 2.2 % COPs/cholesterol could be more harmful than CO and TR for the consumer.

Table 7. Cholesterol, total COPs and their ratio in percentage to cholesterol in raw CR and cooked (microwaving MW, hot air TR and combi mode cooking CO) in pork loin in three samplings (1, 2 and 3). Data are expressed as values of n replicates ± standard deviation (SD).

Sample	n	cholesterol mg/kg fat	n	total COPs mg/kg fat	% COPs/cholesterol	total COPs mg/kg meat
L1 CR	1	3426,6	1,0	12,8	0,4	7,6
L1 MW	2	5898,4 ± 1107,2	2,0	124,2 ± 16,4	2,2 ± 0,9	51,6 ± 8,2
L2 CR	2	3442,6 ± 346,4	1,0	13,9	0,4	7,5
L2 TR	4	3971,2 ± 336,6	3,0	20,9 ± 7,7	0,5 ± 0,1	9,2 ± 4,2
L3 CR	1	6524,3	1,0	13,9	0,2	5,6
L3 CO	3	7046,8 ± 1575,7	2,0	21,0 ± 2,3	0,3 ± 0,1	8,1 ± 1,3

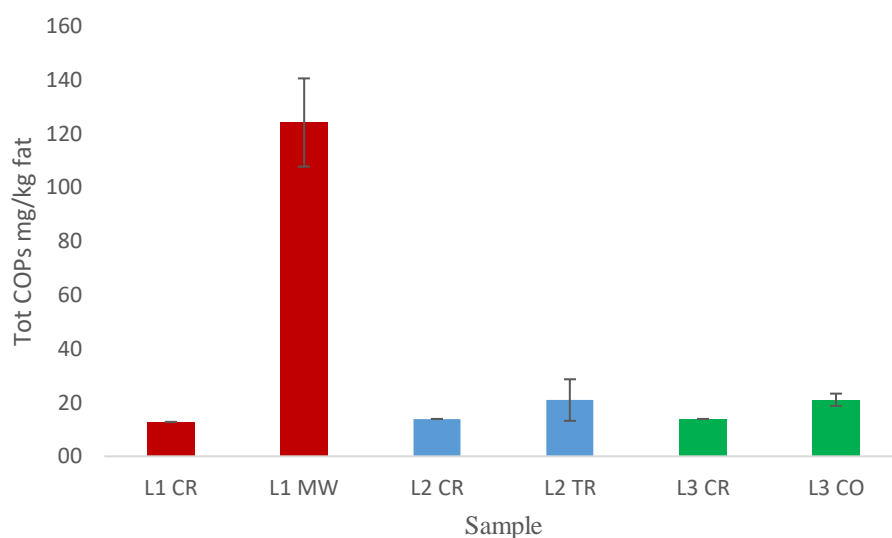


Figure 10. Total COPs (mg/kg fat) in raw and cooked pork loin (microwaving MW, hot air TR and combi mode cooking CO).

4.3 Principal Component Analysis (PCA)

Principal component analysis (PCA) was performed on data obtained for raw and processed pork loin applying twelve variables (% fat, % water, % dry matter, cholesterol, total COPs, % COPs/cholesterol, 7α -HC, 7β -HC, 5β -CE, 5α -CE, CT and 7K) reported in **Table 5, 6, 7**. **Fig. 12** reports the loading plot along the two first principal components (PC1 and PC2), explaining 62.15% and 22.35% of total variance of pork loin model, respectively. As score plot (**Fig. 11**) shows, samples were clustered in three zones, not close to the origin of the plot. It emerged raw samples of TR and MW were similar in fat and cholesterol content, while CR of CO treatment was richer in cholesterol. However, when focusing on the effect of heat treatment, it emerged TR and CO enhanced COPs formation, but with a low impact as in the score plot, raw (CR) and correspondent cooked samples are close. On the other hand, MW had a greater effect on COPs formation, as samples are far from the respective raw sample (L4 CR). MW samples were characterized by higher contents of all COPs

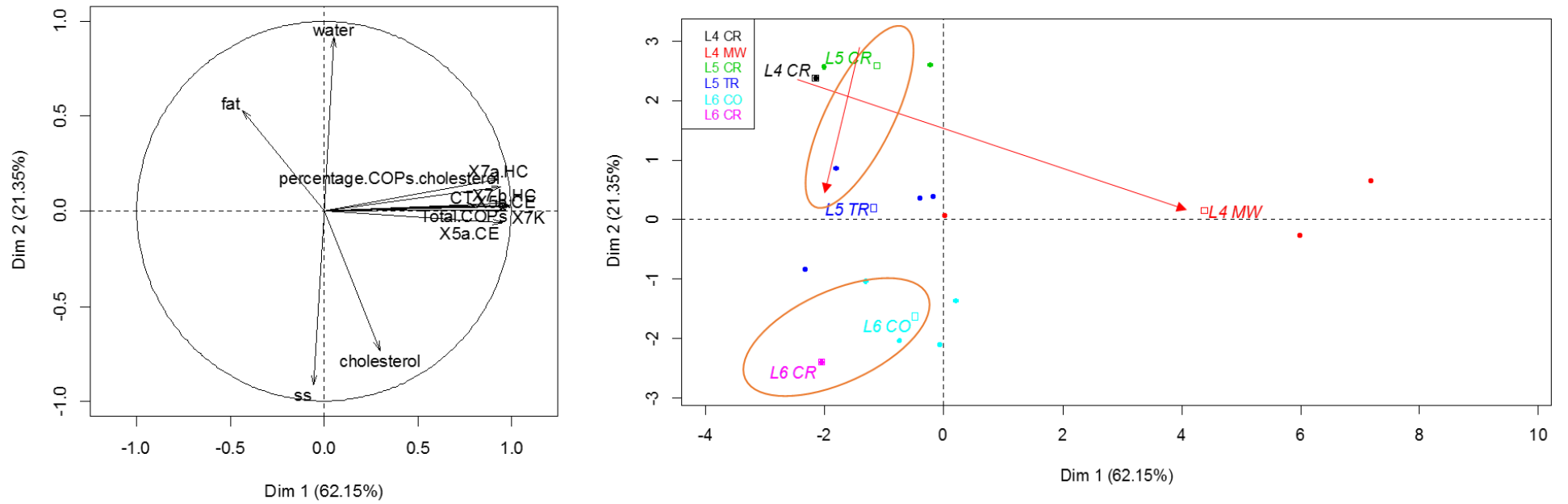


Figure 11. PCA: loading plot (left) and score plot (right). Raw CR and cooked (microwaving MW, hot air TR and combi mode cooking CO) in pork loin in three samplings (4, 5 and 6).

CONCLUSIONS

COPs are widespread, in all products rich in cholesterol especially in those products that have a low content of antioxidants such as pork meat. In all samples (raw and cooked), the most predominant COP was 7K, followed by 7 β -HC and 7 α -HC, 5 β -CE, 5 α -CE and CT were present in lesser amount. All raw samples had around 13 mg/kg fat of total COPs and all thermal treatment performed along consumer habits (convection oven, combi oven and microwave oven) increased the COPs content. Convection oven and combi oven heating had a similar effect on cholesterol oxidation producing low levels of total COPs (around 20 mg/kg fat). The highest value was registered for microwave cooked samples (124.2 mg/kg fat), meaning 200 W enhanced the production of COPs more than convection oven and combi oven treatments, till 2.2 % COPs/cholesterol, that could be harmful for the consumer, even if the temperature of cooking was around 180°C for all treatments.

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