DEPARTMENT OF AGRICULTURAL, FOOD AND
ENVIRONMENTAL SCIENCES
DEGREE COURSE: Food and Beverage Innovation and management

CHOLESTEROL OXIDATION IN SARDINE (Sardina pilchardus) FILLETS PROCESSED BY COLD ATMOSPHERIC PLASMA (CAP) TECHNOLOGIES

TYPE OF DISSERTATION: research

Student:
Diego Appicciutoli

Supervisor:
PROF. Massimo Mozzon

ACADEMIC YEAR 2022-2023

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACRONYMS AND ABBREVIATIONS

CP *COLD PLASMA*

CAP *COLD ATMOSPHERIC PLASMA*

PAW *PLASMA ACTIVATED WATER*

UV *ULTRAVIOLET*

LTP *LOW TEMPERATURE PLASMA*

Pa *PASCAL*

MW *MEGAWATT*

GAD *GLIDING ARC DISCHARGES*

DBD *DIELETRIC BARRIER DISCHARGE*

mA MILLIAMPERE

kV KILOVOLT

Hz *HERTZ*

MHz MEGAHERTZ

PAW *PLASMA ACTIVATED WATER*

ROS *REACTIVE OXYGEN SPECIES*

RNS *REACTIVE NITROGEN SPECIES*

RONS *REACTIVE OXYGEN AND NITROGEN SPECIES*

eV *ELECTRONVOLT*

PEF *PULSED ELECTRIC FIELD*

STI *SOYBEAN TRYPSIN INHIBITOR*

W *WATT*

nm *NANOMETER*

TBARS *THIOBARBITURIC ACID REACTIVE SUBSTANCES*

SPE *SOLID PHASE EXTRACTION*

COPS *CHOLESTEROL OXIDATION PRODUCTS*

SDW *STERILE DISTILLED WATER*

GC *GAS CHROMATOGRAPHY*

INTRODUCTION AND AIM OF THE THESIS

The world population is expected to grow to 9.7 billion by 2050.

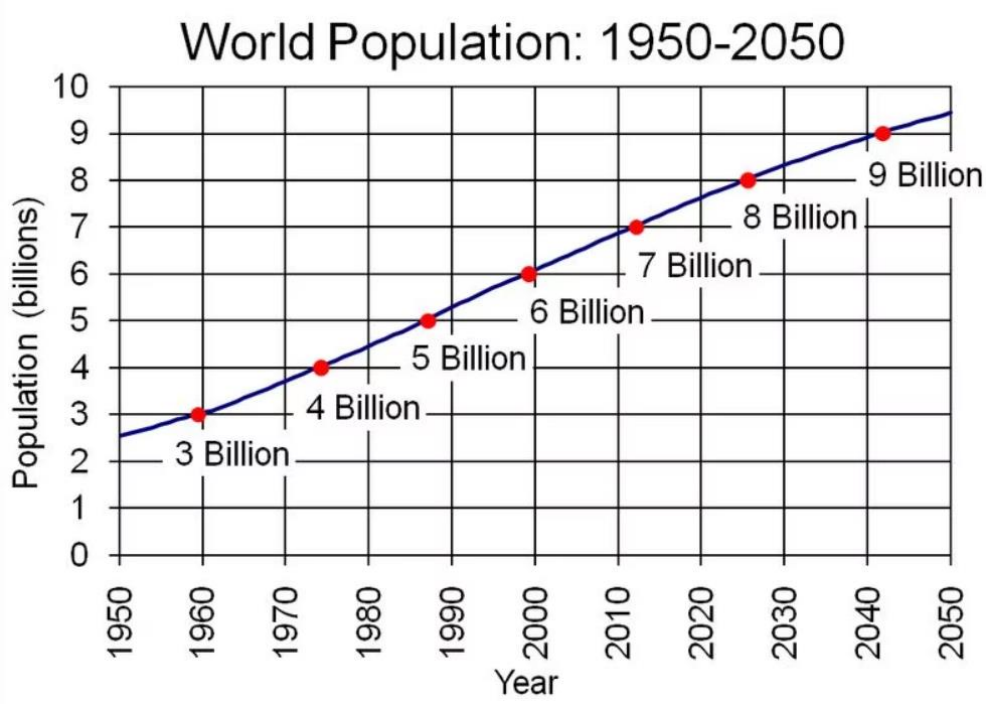


Figure 2-1 Expected population grow (Bureau, June 2011)

The necessity to ensure enough food for a continuous growing population, together with the importance to ensure both quality and safety in the food supply chain pushed under the spotlight new technologies as a keystone for a future-oriented food industry (Juan Antonio Duro et al, 2020). Among emerging technologies, non-thermal one gained a lot attention for their ability to enhance shelf life and food safety. Techniques like supercritical carbon dioxide treatments, high hydrostatic pressure, cold plasma, and ozone technology represent a truly valid alternative to thermal treatment, ensuring freshness without altering nutritionally heat-sensitive compounds in foods (Farhana Mehraj Allai et al, 2023).

From these, cold plasma highlights a broad range of advantages that differentiate him from others, attracting attention of researchers across the globe. Cold plasma is an emerging technology characterized by a broad range of applications. This non-thermal treatment is well adopted in several areas: biological, medical and food industry.

The applications of cold plasma in the food industry includes microbial decontamination of food products, packaging material processing, modification of food components, seed germination performance and degradation of agrochemical residues.

Cold plasma (CP) offers an alternative way for the food industry to achieve surface decontamination of both food products and food packaging. Considering this alternative technology, if in one side we can find all the advantages related to the non-thermal nature of CP and the well-defined preservation-effect of all thermolabile nutritional compounds that are commonly denaturized during common thermal treatments; on the other side, the releasing of highly-reactive molecules such as ozone, UV (ultra violet light) photons , and reactive oxygen and nitrogen species (RONS) during plasmas generation may limits its field of application. (A.R.Ganesan et al, 2020)

The reactive oxygen species from plasma could interact with food lipids and initiate the oxidation process, especially when treating fatty foods. (Mohsen Gavahiana et al, 2018)

Oxidized lipid products represent a relevant issue due to their impact on nutritional value, flavour, and shelf-life of products.

Objective:

Based on this brief introduction of cold plasma side-effects, the main goal of this thesis is to investigate if cold plasma could cause any undesirable effects on food biomolecules. From these, the lipidic fraction, due to its susceptibility to oxidation, was chosen as focus of the analytical activity. The oxidation of lipids and in particular the degradation of cholesterol and the formation of oxidizing compounds were investigated. To do so, we first applied a CP treatment of pure cholesterol samples and then evaluated the lipid oxidation through the quantitative and qualitative analysis of cholesterol oxidation products. Subsequentially, we compared those results with the one obtained by treating and analysing sardine (Sardina pilchardus) fillets with plasma activated water, taking in consideration cholesterol oxidation products.

CHAPTER 1
PLASMA TECNOLOGIES: CONCEPT, DEFINITION AND USES

1.1 What is plasma

Plasma is an emerging, environmental-friendly, and economical technology with a broad range of applications. Considering when the concept of plasma was introduced in the scientific field, the adjectives "innovative" or "emerging" are far from the real situation. CP was invented by Sir William Crookes in the year 1879. He was the first scientist to gain attention to plasma, discovering it as the fourth state of matter with similar properties of gas and activated as an ionized gas. Later the term "plasma" was coined by Irving Langmuir, Nobel Prize winner in 1932. (A.R.Ganesan et al, 2020)

The plasma state, also known as the fourth state of matter, is an ionized gas containing a mixture of electrons, neutral particles, ions, free radicals and other highly reactive particles.

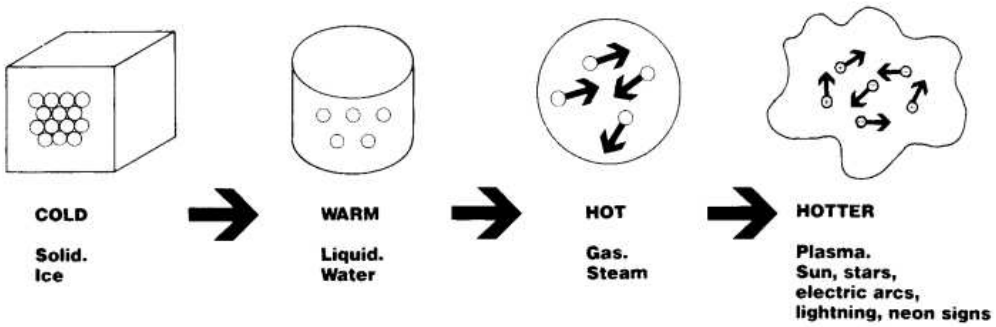


Figure 1-2: Pictorial representations of the four states of matter (Eliezer, 2001)

Another way to describe plasma is to intend it as a physical state of high electrical conductivity with gaseous properties. As shown in figures 1-2 and 1-3, plasma is present also in several circumstances like the stellar plasma or, a more closely example, aurora borealis. The Aurora Borealis, also known as the Northern Lights, is a natural phenomenon that occurs when charged particles from the sun interact with the Earth's magnetic field and atmosphere. The result is a colourful display of light in the sky, most commonly seen in the polar regions (Xiang-Yu Wang et al, 2023).

Focusing on the technological field, a first distinguish should be made based on temperature. Considering the aim of the study and technological boundaries (to achieve a thermodynamic

equilibrium thermonuclear fusion and about 20,000 K are needed), the so called "high temperature plasma" is not considered for food applications and for this thesis work.
For this reason, the industrial technology field is focused on the "low temperature plasma" (LTP) that usually never reach 150°C. (Hao Jiang et al, 2022)
LTP processes use two distinct methods such as thermal plasma also called "hot plasma" (100-150°C) and "non-equilibrium" also known as cold plasma (A.R.Ganesan et al, 2020).

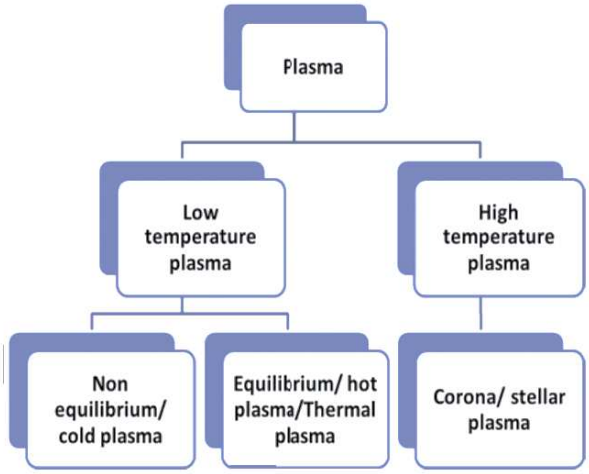


Figure 1-3 Categories of plasma systems (Samal, 2017)

According to how can be produced, plasma may be categorized into two classes: thermal (equilibrium) and non-equilibrium/cold plasma.
Thermal plasma, also known as equilibrium plasma, requires extreme pressure levels (>105Pa) and up to 50 MW of power for its propagation, according to this, thermal plasma is usually adopted in industrial applications where particles such as electrons, ions and neutral molecules act in thermodynamic equilibrium (Flora-Glad Chizoba Ekezie et al, 2017).
High temperature and high reactivity of plasma due to the presence of free ions and radicals makes this a powerful medium to promote a broader range of chemical reactions. Nowadays, applications of thermal plasma technology covers applications in materials processing such as: cleaning technologies i.e. waste treatment, especially toxic waste, coating technologies such as plasma spraying, plasma chemical vapor deposition (CVD), thermal plasma in metallurgy, thermal plasma synthesis of fine/nano powders and thermal plasmas in extractive metallurgy, including recovery of metal values in industrial applications (Samal, 2017).
Considering the high temperature reached by thermal plasma systems (operating at thousands of degrees above ambient), this technology isn't appropriate for the food industry.

This drives the implementation, in the industry, of **cold plasma**, where the operating conditions fits with the food industry requirements where temperature represents a key feature for the defining of processes for heat-sensible components/foods.
CP lacks a local thermodynamic equilibrium, this means that partial ionization results in lower temperatures for the gas molecules and higher temperatures for the electrons, leading to a low global temperature of the system (Hao Jiang et al, 2022).
As expressed before, cold plasma is characterized by a non-equilibrium system, this definition is associated with the behaviours of constituent plasma particles. In the cold plasma treatment, electrons are prone to transfer heat via collisions with heavier particles, however temperature remains constant and never exceeds 35°C. In accordance with the two temperature ranges reached by thermal and non-thermal plasma, if with thermal plasma the effects were related to the hight temperature/pressure, in cold plasma technology, all the consequences of the treatments are based upon the new-formed reactive species. (A.R.Ganesan et al, 2020)
Because in CP ions and uncharged molecules receive only a little amount of energy, maintaining a low temperature, cold plasma is suitable for treating food products that are sensitive to heat (Hao Jiang et al, 2022).

1.2 Cold plasma technologies

According to literature, cold plasma sources can be distinguished considering the pressure where they work into atmospheric pressure and reduced pressure.
Considering plasma generated at atmospheric pressure, different well-distinguished generators have been defined: gliding arc discharge, plasma jet, corona discharge, dielectric barrier discharges (DBD) and radio frequency plasma, while for reduced pressure plasma the generators work through the use of microwaves and radiofrequency.
Starting with **gliding arc discharges** (GAD) (figure 1-4 d), these originates plasma in a reactor where two or more diverging metallic electrodes operates at a high potential difference of 9 kV and 100 mA. Depending on the conditions, GAD produces both thermal and non-thermal plasmas. Usually, an inlet gas made by humid air is pumped into the discharge gap between the electrodes, directing to an arc placed between the narrowest inter-electrode area, subsequentially dissipate away into the diverging area by the inlet gas. This plasma discharge method shows exceptional adaptability for both surface and liquid treatments. Nowadays,

gliding arc discharges have been utilized for experimental analysis of chemical and bacterial decontamination (Flora-Glad Chizoba Ekezie et al, 2017).

Dielectric barrier discharge (DBD**)** (figure 1-4 a) is generated through an alternating current emitted when two metal electrodes are separated by a non-conductor material (dielectric material) such as plastic, quarts or ceramic. The system requires high ignition voltages of 10 kV and requires several precautionary measures; however, it boast of relative simplicity. The insulator is involved in the counteract process against the formation of sparks due to the movement of charges. If at the beginning, this process was initially used to produce ozone gases, nowadays it has a broad range of applications in the food industry (A.R.Ganesan et al, 2020).

Corona discharge (figure 1-4 c) plasma is generated when a space gap filled with air or other gases is exposed to high voltage to create high velocity particle collision with neutral molecules resulting in the generation of more ions. These develops around sharp pointed electrodes containing the electric field. The technique is inexpensive and straightforward to be adopted. Corona discharge have been utilized for surface treatments, microbial decontamination and electro-precipitation (Flora-Glad Chizoba Ekezie et al, 2017).

Plasma jet generators, shown in figure 1-4 b, are made by two coaxial electrodes where the gas, at high flow rates, discharge between them. Inelastic collisions, produced by accelerated free electrons that collide with molecules of background gas, produce various reactive species that exit the nozzle at high velocity (Hao Jiang et al, 2022).

In the end, **radio frequency plasma,** works by placing a gas into an oscillating electromagnetic field. These electromagnetic field can be produced by electrodes kept outside the reactor or by an induction coil, frequencies with which it works are analogous to microwaves, covering Hz and MHz (Flora-Glad Chizoba Ekezie et al, 2017).

From the for kind of plasma generator, DBD are nowadays the most convenient and efficient methods to produce cold plasma. (Hao Jiang et al, 2022)

The cold plasma generator mentioned above are summarized in table 1-1, while a schematic representation of all the mechanical components is shown figure 1-4.

In atmospheric plasma devices, all the materials that must be moved into the treatment zone for the treatment, can be easily managed due to the absence of peculiar mechanical parts that are necessary in low-pressure plasma. One of these parts are the airtight vacuum chambers, which together to other technological challenges regarding treatment speed, the limited volume of production and the impracticability of various foods to withstand vacuum conditions, prevents the alignment of reduced pressure plasma with market needs.

Plasma generated at low pressure, however, ionize more easily and emit UV radiation at large dosages (air present in atmospheric pressure plasma absorbs UV rays) (Hao Jiang et al, 2022). Generators adopted for this category are basically delineated by **microwave powered (MP) plasma (**figure 1-4 e**)**. In contrast to electrode-based methods, microwaves discharges are generated using a magnetron that will produce waves at frequencies over hundreds of MHz. The irradiation is afterwards absorbed by the process gas. MP plasma is considered advantageous because gas requirements are low and the large quantities of reactive species are easily enkindled to air (Flora-Glad Chizoba Ekezie et al, 2017).

Table 1-1 The classification of plasma sources with their respective characteristics and mechanisms (Flora-Glad Chizoba Ekezie et al, 2017)

CP	**Generating mechanism and characteristics**
Dielectric barrier discharges (DBD)	Plasma occurs between two electrodes, and then, an AC high voltage is applied on the electrodes. It is an excellent CP source with 1–10 eV and high density
Gliding arc discharge	Two (or more) metallic electrodes connected to an AC or DC high-voltage transformer. A plasma plume is generated when the high voltage is applied. This arc is then pushed away by a gas flow and glides along the electrodes until it collapses
Plasma jet	Two coaxial electrodes, between which gas flows at high rates. The free electrons are accelerated by the RF field and collide with molecules of background gas. These inelastic collisions can produce various reactive species (excited atoms and molecules, free radicals) that exit the nozzle at high velocity
Corona discharge plasma jet	Containing substantial electric field for expediting the ionization energy of arbitrarily produced electrons to that of milieu gas atoms or molecules
Microwave (MW)/radio frequency (RF) plasma	RF plasma is usually achieved when a gas is placed within an oscillating electromagnetic field, produced by an induction coil or distinct

	electrodes kept outside the reactor. MW/RF CP is usually generated under reduced pressure

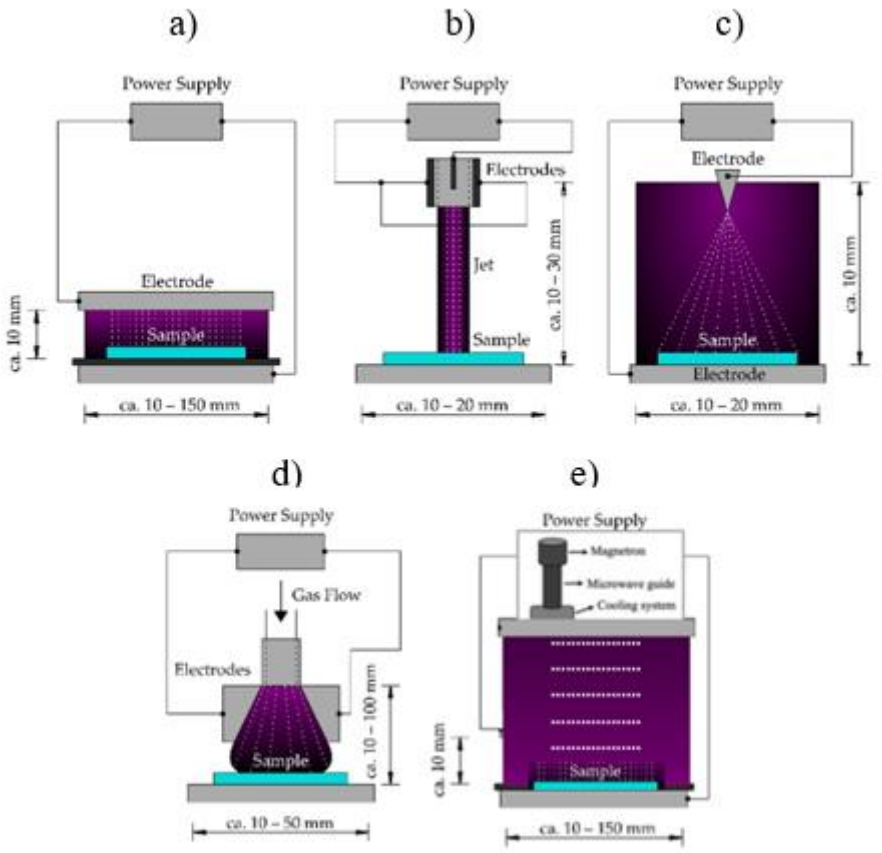


Figure 1-4 Schematic illustration of different plasma sources: a) Dielectric Barrier discharge b) Plasma Jet c) Corona discharge d) Gliding arc discharge e)Microwave discharge (Mária Domonkos et al, 2021)

1.3 Plasma technologies: plasma-activated water

Cold plasma is a source of various gaseous reactive species. When plasma is generated in direct or indirect contact with water, reactive species produced dissolve in water, producing another important technological tool; the so-called plasma activated water (PAW). (Katarína Kucerová et al, 2011)

According to this, cold plasma technologies can be distinguished in two: plasma activated water and direct gaseous cold plasma.

Plasma-activated water is produced by treating water with plasma. Generators usually adopted in PAW synthesis are corona discharge, gliding arc discharge, plasma jet and dielectric barrier discharge. Among these, plasma jet and DBD are the most widely used methods due to the stably deliver of reactive species from gaseous plasma to the aqueous phase (Qian-Yun Han et al, 2022). As shown inf figure 1-5, the source of plasma (plasma plume) can activate the medium by treating above or underneath the water surface.

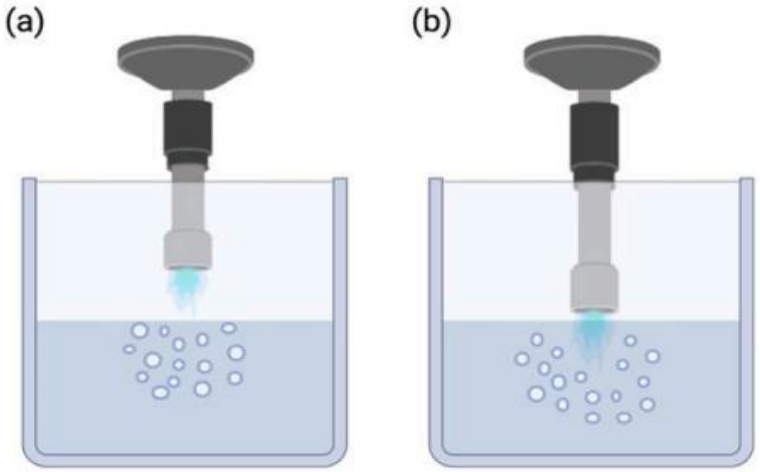


Figure 1-5 Graphic representation of the generation of PAW by (a) plasma discharge over water surface and (b) plasma discharge beneath water (Qian-Yun Han et al, 2022)

The species formed into the gas and, subsequently entrapped in PAW includes both ROS (reactive oxygen species) and reactive nitrogen species (RNS).

Among reactive oxygen species, short-lived species such as hydrated electrons and hydroxyl ions (OH^-) are the first to react again creating stable species including ozone (O_3), superoxides (O^{2-}) and hydrogen peroxide (H_2O_2). Hydroxyl radicals (OH•), are extremely reactive and due to their high redox potential, they interact with other components in water producing new radicals. In the other hand, H_2O_2 is a stable product, characterized to its antibacterial and cytotoxic activity.

PAW is also composed in reactive nitrogen species (RNS). These components are formed when a non-thermal plasma engage with a liquid and from them, we find nitric oxide NO, peroxynitrite ONOO, peroxynitrate O_2NOO- and nitrogen dioxide (NO_2•) radicals. (Mizanur Rahman et al, 2022). A graphic representation of PAW species generation is shown in figure 1-6.

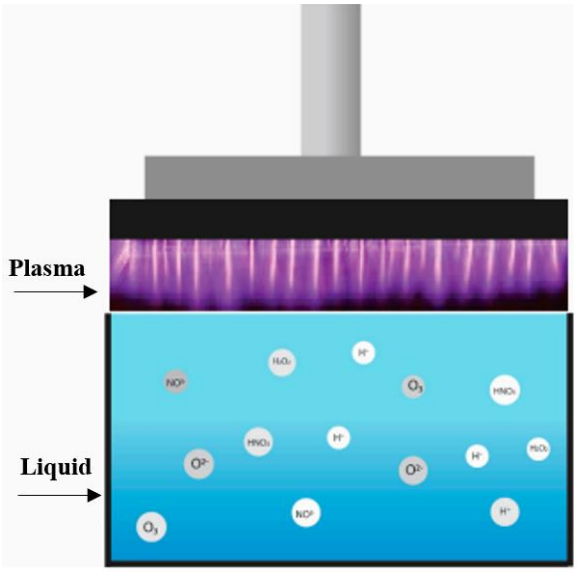


Figure 1-6 Graphic illustrations of plasma-activated water generation (Mizanur Rahman et al, 2022)

Among the various properties of PAW in the food industry, probably the most studied nowadays is its **antibacterial** quality. After being exposed to activated water, RONS present can induce oxidative stress in bacterial cell membrane. As shown in figure 1-7, this will lead to the lipid peroxidation and subsequently to membrane rupture.

Moreover, PAW treatment has shown to generate high-intensity electric fields, increasing membrane permeability by creating temporary pores that will make easier the infiltration of reactive species inside the cell. Once reactive species enter inside the cell, degradation of internal organs, proteins, DNA/RNA, ribosomes and mitochondria occurs as well. Following the cell leakage, the cell will die. (Mizanur Rahman et al, 2022)

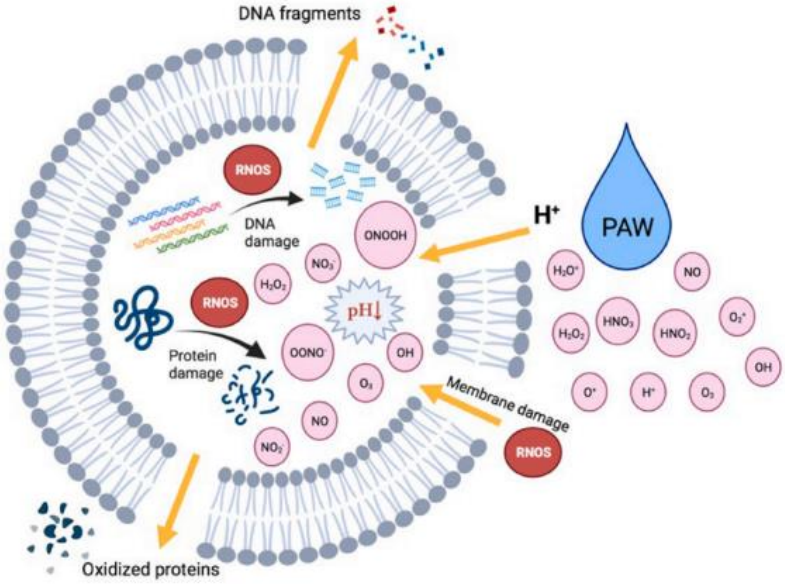


Figure 1-7 Representation of the antimicrobial mechanism of PAW (Qian-Yun Han et al, 2022)

Several studies highlighted the possibility to introduce both PAW and gaseous plasma as key tool for the **decontamination** of fungi-derived **toxins**. In the study of Shi-Qing Wang et al, a deep analysis of degradation of aflatoxins B_1 by radio frequency plasma shown effective positive result. The new-formed products have a theoretical reduced toxicity compared to native aflatoxins, according to the structure-toxicity relationship. (Shi-Qing Wang et al, 2015) Additional to antimicrobial and anti-mycotoxin properties, plasma activated water demonstrates remarkable effectivity against the **reduction** of **pesticides** residue in fresh products. Considering the importance to prevent negative impact on human health, and the considerable role of phytochemical in fruit and vegetables, methods for degradation of pesticides residue represent a highly relevant point for the agrifood industry. Many recent studies have reported extremely satisfactory results showing a decreasing of the concentration of phytochemicals such as phoxim and chlorothalonil of the 85.3-73.6 % after 10 minutes of treatment. The degradation pathway of pesticides with PAW treatment involves the cleavage of different unsaturated double bond of functional group. Active species such as O_3, H_2O_2, NO_2, NO_3, due to their strong oxidation properties, are the responsible of pesticides degradation. (Qian-Yun Han et al, 2022).

A totally different approach to PAW activity were investigated in order to develop new systems of **meat curing**. Research demonstrates that, secondary species formed during plasma generation, can be adopted as a nitrite sources. Nitrite is a commonly used food additive. It's

application in meat products have several functions, colorant, antioxidant bacteriostatic agents. Since nitrite can react with proteins in the meat forming carcinogenic substances-nitroso compounds, the nitrite utilization is limited. On site new-formed components such as nitrites, nitrates, nitric oxide, peroxynitrite, and hydrogen peroxide, shows a general less strong additive activity compared to sodium nitrite. However, meat cured using PAW exhibited no mutagenicity (Qian-Yun Han et al, 2022).

From PAW treatments several **functional properties** can be enhanced. Characteristics of protein gels in meat products such as water-holding capacity and strength of the gel were intensified. The same results were obtained for starches that shows a higher digestibility and better general physicochemical properties related to machinability of dough made by PAW-treated flours (Qian-Yun Han et al, 2022).

Other studies, focused on the agronomic sector, revealed an improving of germination and seedling growth after the watering of soil with plasma activated water. Have been hypnotized that the positive effect on seeds are the result of nitrates and nitrites that act as nitrogen fertilizer (Qian-Yun Han et al, 2022).

Considering quality parameters, due to the presence of RONS in plasma activated water food's biochemical and sensory properties can be affected in both positive/negative way. One possible side effect of PAW treatment, that we find also in gaseous cold plasma, is the changing in pH of samples after the interaction with nitrogen species that turns into nitric acid (Salma Farooq et al, 2023).

More studies on PAW are still needed for fully understand process parameters in order to balance the safety aspect and quality requirements for the industry.

1.4 Plasma technologies: gaseous plasma

The second cold plasma technology is the one that involves a treatment with **gaseous plasma** formed by already mentioned different plasma generators.

These CAP systems work through the exposure of direct plasma to food samples placed into a specific support. According to the model of application of the treatment we can distinguish between direct-contact parallel plate reactor, direct-contact plasma jet, and indirect contact barrier discharge. Direct contact systems represented in figure 1-8 a) and 1-8 b) shows high efficiency with short-lived chemical species. A disadvantage that mitigates its efficiency is the technical challenge caused by the direct contact between plasma and food. Plasma features are inevitably linked to electrical properties of the food product, and this can represent a real

hurdle in terms of repeatability of results. In addition to this, the complexity of the discharge chemistry reaching the product and its impact on the food matrix includes more than 1000 biochemical reactions not yet defined by scientist. The indirect exposure (figure 1-8 c) in the other hand, take advantage of the production of plasma in close proximity to the matrix with the subsequent diffusion and convection of active species from its surface. Far from direct application, in indirect systems, short-lived chemical species react before reaching the food product. Due to this, longer-lived intermediaries like O_3, NO, N_20 and NO_2 are generated. However, the absence of highly reactive chemical species makes this approach less effective for microbial inactivation. *(Peter Paulsen et al, 2022)*.

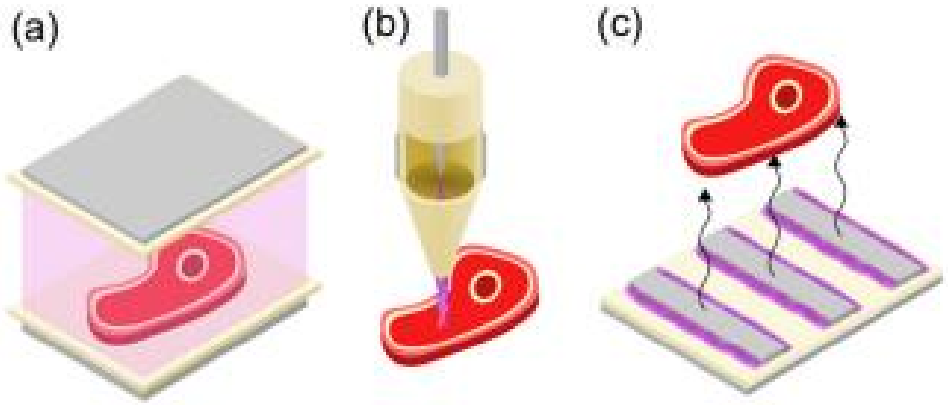


Figure 1-8 Typical DBD systems used for the treatment of food products: (a) direct-contact parallel plate reactor, (b) direct-contact plasma jet, (c) indirect contact surface barrier discharge (Peter Paulsen et al, 2022)

Depending on the method adopted, the space of target material in CAP needs to be observed; it could vary from <1 cm to 20 cm. Similar to this, also treatment time needs to be properly evaluated, it could differ from a minimum of 5 s to a maximum of 60 minutes. These parameters are influenced by physical, chemical, and processing conditions such as pH, texture, active components present in food (A.R.Ganesan et al, 2020).

It is essential to highlight that CAP exerts its antimicrobial action primarily on the surface of the treated food. The chosen method of applying plasma determines the antimicrobial efficacy of exposing microbial populations to plasma (Peter Paulsen et al, 2022).

A variable not yet discussed is the gas **composition** of the **medium**. Changes in the gas composition results in a modification of reactive species profile. Nitrogen and air plasma for example, were observed to increase the antimicrobial property of CP. For practical applications, the cheaper plasma will be the one produced with common atmospheric air. On

the strength of the evidence that ambient air is rich in nitrogen and oxygen, the resulting plasma is primarily filled with reactive elements such as reactive oxygen-nitrogen species (RONS). RONS are responsible of almost all redox reaction in food materials, all positive and negative effects of CP are so strictly connected to their presence. The reactive mixture most commonly found in the atmospheric air plasma is made by O, $^{\cdot}$OH, O_2, O_3, O_2^-, HO_2, H_2O_2, N, N_2^-, NO, NO_2, NO_3, and N_2O. Additionally, reactive species such as H_2O_2, NO_2, NO_3, and O_3, are produced when plasma contacts moisture. These species are stated as long-lived reactive species and their shelf life is more in the liquid phase compared to other short-lived reactive species. Increasing the content of oxygen for the plasma generation involve the formation of higher number of oxygen-reactive species and among them, ozone. Ozone as we will see later is an inorganic molecule that plays a key role in gaseous plasma in reducing microbial load and property modifications. By adopting nitrogen as inducer gas for plasma, reactive species such as excited molecular and atomic nitrogen are formed. Once these react with oxygen present in atmospheric air, reactive nitrogen species will subsequentially synthesized.

Considering the importance of the gas adopted during plasma generation, it is fundamental to deep understand the kind of reaction that occurs during the generation of plasma in order to ultimately dominate the effects on biomolecules present in foods (M. Dharini et al, 2023).

Starting with **reactive oxygen species**, they can be considered the rulers species. Present in almost all kinds of gas plasma, ROS have an extended lifetime compared toother reactive species. This category includes both radical and nonradical oxygen. Radical species are characterized by higher reactivity that turns on chemical instability of the species.

Considering **hydroxyl radical** ($^{\cdot}$OH), the prime functions of them are the abstraction of hydrogen atom, electrophilic addition, and transfer. $^{\cdot}$OH are highly reactive, with a short life of 10^{-9} s and a strong oxidative potential (2.8 eV). There are various reactions through hydroxyl radicals are generated, in all of them, the presence of moisture or water is needed. Some reactions are:

$H_2O + e \rightarrow H + \cdot OH + e^-$

$H_2O + h\nu \rightarrow H + \cdot OH + e^-$

$H_2O + O \rightarrow 2 \cdot OH$

$O^-_2 + H_2O_2 \rightarrow \cdot OH + OH^- - + {}^1O_2$ (*Haber − Weiss reaction*)

Once dissolved, oxygen molecule in liquid react with highly-energy electrons and **superoxide radical** are formed. These can be defined as an anion with an unpaired electron in the antibonding orbital (M. Dharini et al, 2023).

$O_2(H_2O) + e^- \rightarrow O^-_2$

Due to the ionic nature of superoxide radicals, they are less reactive than ·OH and they lifetime is longer (of 5s). However, during its short life, superoxide radical act as an intermediate involving chemical reactions with other species. One important species formed after a reaction with nitric oxide is peroxynitrite ($ONOO^-$), a powerful anti-bacterial agent.

$2O^-_2 + 2H^+ \rightarrow H_2O_2 + O_2$

The last class of radicals are **peroxyl radicals**. They are produced as a result of the protonation of superoxide anion. However, peroxyl radicals can also be generated from the reaction of hydrogen peroxide and hydroxyl radical.

$O^-_2 + H^+ \rightarrow HO_2$
$H_2O_2 + \cdot OH \rightarrow HO_2\cdot + H2O$

Peroxyl radicals are powerful oxidizers and are usually prone to react with other reactive species generating neutral species such as hydrogen peroxide and oxygen.

$HO_2\cdot + O^-_2 + H^+ \rightarrow O_2 + H_2O_2$

Ozone (O_3), also known as triatomic oxygen, is one of the most abundant species in cold plasma. Its generations are the result of the reaction between oxygen molecules with atomic oxygen. According to the reactants involved in the reaction, higher amount of O_2 and atomic oxygen will lead to higher amounts of O_3.

$O_2 + O + M \rightarrow O_3 + M$
M= *any non-reactive species formed during the reaction*

A limitation of O_3 activity is the high instability that easily dissociate ozone into oxygen. However, O_3 demonstrate one of the strongest oxidizing powers with the highest redox potential (~2.07 V) compared to other CP species.

Hydrogen peroxide is the more stable complex among all the ROS. Its generation in gaseous plasma occurs with the presence of moisture in the generation gas. While in PAW, it represents the main actor of its technological activity, in gaseous plasma it has only marginal activity due to the water requirements for its synthesis.

The last reactive oxygen species is **singlet oxygen ($^1O_2$).** This is the product of the photosensitization reaction of oxygen. The result of these UV photons oxidation is an excited state of the triplet ground state of oxygen called singlet oxygen.

$2O \rightarrow {}^1O_2$ (upon radiation − photon)

Additionally, 1O2 is also formed after the electron impact excitation that occurs during the decrease in electron density when compounds are reduced.

$O_2 \rightarrow e^- + {}^1O_2$

Singlet oxygen is characterized by a longer lifetime (75 min) in gaseous plasma while in liquid phase it shows a relatively low (2μs). It shows great oxidizing properties with an excitation energy of 0.98 eV (M. Dharini et al, 2023).

The **RNS** (reactive nitrogen species) group is a family of chemical intermediates all arising from the primary compound formed in the plasma: nitric oxide. Excited species like N and N_2, generated after the energization of nitrogen gas, react successively with oxygen and ROS giving rise to other RNS. Reactive nitrogen species behaviour, and their dependence with other reactive species already present in the neo-formed plasma, makes the characterization of a chemical species profile in gaseous cold plasma arduous.

$N_2 + e- \rightarrow N^{+2} + 2e^-$

$N_2 + e- \rightarrow N + N + e^-$

Nitric oxide (NO) is an antioxidant neutral reactive species with a strong reducing nature. Among the various mechanisms by which nitric oxide can be synthesized in plasma, the

formation of NO from the reaction between nitrogen and atomic oxygen is the most important mechanism (M. Dharini et al, 2023).

$N + O_2 \rightarrow NO + O$

Supplementary reactions involving atomic nitrogen species with OH and O_3 lead to the formation of other nitric oxide.

$N_2 + e^- \rightarrow N + N + e^-$
$N + \cdot OH \rightarrow NO + H$
$N + O_3 \rightarrow NO + O_2$

High moisture levels show to decrease the concentration of NO by generating HNO_2 and, oppositely, the presence of air or O_2 gas results in higher concentration. Nitric oxide is characterized by higher diffusivity that make easier for this reactive species to reach the targeted food. Considering the oxidant properties, NO is not a very effective oxidant, however it is involved in nitrosative damage due to the generation of highly reactive species that will subsequentially oxidize biomolecules.
Nitrogen dioxide is a neutral reactive species that is produced by the reaction of O_3 or O_2 with NO.

$NO + O_2 \rightarrow 2NO_2$
$NO + O_3 \rightarrow NO_2 + O_2$

When NO_2 react with O_3 a highly oxidizing **nitrate** (NO_3) is produced, furthermore, NO_2, by reacting with atomic nitrogen generate **nitrous oxide** (N_2O).

$NO_2 + N \rightarrow N_2O + O$

From the reaction of nitric oxide with superoxide, **peroxynitrite**, an additional nitrogen reactive species is formed.

$O^-_2 + NO \rightarrow ONOO^-$

Without being a radical, peroxynitrite has radical-like activity due to its ability to be decomposed into hydroxyl radicals product. The high diffusivity and its efficient oxidizing nature allow this chemical species to produce single or two-electron oxidations of a broad range of amino acids. According with pH measurement, peroxynitrite decay into O_2^- and NO with basic environment (pH > 6.8), while with acid environment it is reported to decay into NO^3 and H^+ (M. Dharini et al, 2023).

The last group of RNS are **nitric and nitrous acid**. By the interaction of plasma with liquids these acids are generated. Together with hydrogen peroxide, they represent the germicidal protagonists in PAW while their presence and their activity in gaseous plasma is limited.

·OH + NO→HNO2

·OH + NO2→HNO3

H2O2 + NO→HNO2 + ·OH

H2O2 + NO2→HNO3 + ·OH

Another aspect that plays an important role in the several activities of both PAW and CP is the **photon emission**. Photons of different wavelengths are emitted during the molecular and atomic transition of excited species. Depending on the atoms and molecules and on the type of feed gas used during plasma generation, photon emission can be in the range from UV to IR radiation. Ultraviolet radiation below 275 nm, have a germicidal effect on microorganism and compared to other reactive species ability, they can travel several millimetres into the substance treated. Moreover, the higher level of energy of the photons (from 6 to 20 eV), allows radiation to breaking chemical bonds, photolysis of liquid and photoionization. Long-lived species such as ozone, nitric acid, hydrogen peroxide or nitrous acid in the liquid phase can be dissociated into alternative-different reactive species thanks to the catalytic activity of UV radiation (M. Dharini et al, 2023).

$H_2O_2 + h\nu \rightarrow \cdot OH + \cdot OH$

$O_3 + h\nu \rightarrow O_2 + O$

All the properties of the major reactive oxygen species are summarized in figure 1-9.

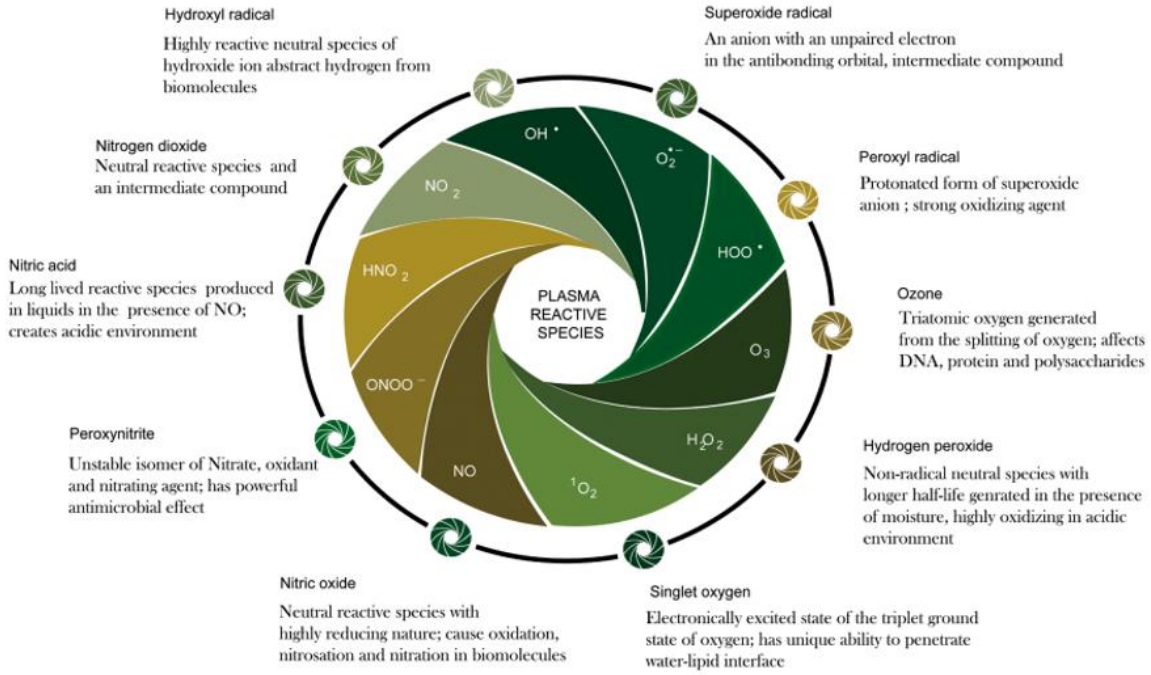


Figure 1-9 Graphic representation of RONS ability (M. Dharini et al, 2023)

1.5 Plasma technologies: current applications in food industry

Cold plasma treatments are finalized into the preservation of food product either in ensuring food safety and security considering the bioactive compound (A.R.Ganesan et al, 2020). Among the several applications, one that shows high interest in terms of food preservation is the ability to **inactivate enzymes**. Reducing undesirable browning reactions, as a result of the broad range of enzymes such as polyphenol oxidases and peroxidases, represent a key point for the preservation of the external physical appearance of foods. Considering the importance of browning reactions in freshly fruits and vegetables, nowadays a plenty of studies focused their attention on CP ability to solve this problem. The enzymatic inhibition could be reached by using both thermal and non-thermal methods. The last include other emerging technologies like PEF (pulsed electric field), ultraviolet light and ultrasound. In this field of application, CP shows a high deactivation rate of enzymes after the treatments. Inactivation data demonstrate how enzymes denaturation is dependent on the molecular structure of the protein, the mass transfer between gas and fluid phase and the enzyme surrounding matrix. (Salma Farooq et al, 2023)

Among emerging technologies, only several shown good results in terms of **degradation of food toxins**, cold plasma proves to be a reasonable instrument against this kind of contaminations. As shown in the study of Shi-Qing Wang et al, an exposure to 300W plasma for 10 minutes resulted in a reduction rate of up to 88.3%. Similarly positive results were shown concerning the **pesticide degradation** in fresh vegetables. Pesticides are mainly adopted in agriculture to reduce crop losses, however many of them are hazardous to humans. From research, it was demonstrated that levels of organophosphorus pesticides for example, were converted into less harmful chemicals (Shi-Qing Wang et al, 2015).

Far away from the agronomic sector, CP technology denoted its advantageous properties for **food packaging material**. The ability to modify material surface properties, were already well discussed in other areas. In the food sector, the arising of interest in this new alternative is due to the promoting of antibacterial and antioxidant effects. Additional to these, pre-treating with cold plasma a broad range of nanofibers results in improving intermolecular capacity and structural arrangement of protein conformation. Other investigated effects are the decreasing of water vapor permeability, this effect play an important role concerning the application on fruit and vegetables with a high respiration rate.

An emerging application of cold plasma technology recognize it as a new tool for **edible oils hydrogenation**, producing trans-free solidified fats. By modulating through plasma, the energy levels of hydrogen species during the catalysis, levels of polyunsaturated-trans isomers drop down thus allowing a reduction of the well-known cardiovascular disease side-effect associated with trans fatty acids (Flora-Glad Chizoba Ekezie et al, 2017).

RONS species present in plasma shown to be an effective aid against the **mitigation of food allergens** and **antinutritional factors**. Since allergens are primary protein, it was shown that by altering the chemical structure also allergic properties were influenced. On the same principle, plant-derived anti-nutritional compounds such as cyanogenic glycosides, lectins, tannins and enzyme inhibitors may be limited by altering the conformational structure through plasma technology. As shown in figure 1-10, the satisfying results of the time-dependant

decline in the soybean trypsin inhibitor (STI) present in soymilk have been reported.

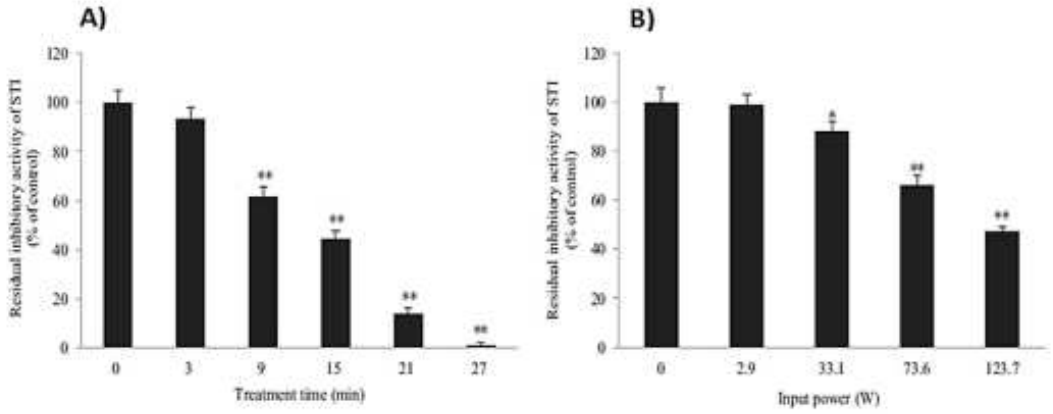


Figure 1-10 Residual STI activities of soymilk after DBD plasma treatment (A) at 51.4 W for different time intervals and (B) or at various input power for 90 s (Flora-Glad Chizoba Ekezie et al, 2017)

Improving seed germination performance is another well studied field of application of CP. Studies shown the effectiveness of plasma treatments to increase water imbibition capacity of seeds and thank to this an improvement of growth parameters and a reduction in the microbial growth follows.

Another recent application involves plasma technologies in the **wastewater management.**

CP activities have been ascribed to diffusion of reactive species, the photolytic activity of UV light and the indirect induction of pyrolytic and chemical reactions through electrohydraulic cavitation (Flora-Glad Chizoba Ekezie et al, 2017).

Among all the possible applications already mentioned, the one nowadays more studied and the one that we are going to analyse with the following thesis work is the **microbial decontamination.**

As we can see in table 1-3, after plasma exposure and so, the bombardment by reactive species, a broad range of microorganism were inhibited.

Table 1-2 Microbial decontamination of food products using cold plasma technology (Flora-Glad Chizoba Ekezie et al, 2017)

Food matrix	Microorganisms	Plasma source	Treatment Conditions	Observations
Mandarin	*Penicillium italicum*	Microwave discharge	P ¼ 900 W, PL ¼ 0.7 kPa, d ¼ 24 cm, ET ¼ 10 min	84% reduction in disease incidence
Chicken eggs	*Salmonella enteritidis*	HVACP	V ¼ 85 kV, f ¼ 60 Hz, ET ¼ 15 min	Up to 5.53 log cfu/egg reduction
Corn	Aflatoxins	HVACP	V ¼ 90 kV, f ¼ 50 Hz, RH ¼ 40%, ET ¼ 10 min	Degradation of aflatoxin reached 82%
Lamb meat	*Brochothrix thermosphacta*	DBD	V ¼ 80 kV, f ¼ 50 Hz, ET ¼ 5 min	2 log cycle reduction
Radish sprout	*Salmonella typhimurium*	Microwave	P ¼ 900 W, PL ¼ 667 Pa, ET ¼ 20 min	Up to 2.6 ± 0.4 log CFU/g reduction
Egg shells	*Salmonella Enteritidis*	APP jet	V ¼ 2e3 kV, f ¼ 1 MHz, ET ¼ 5 min	Reduction factor ranging between 0.22 and 2.27 log CFU/egg
Romaine lettuce	*Escherichia coli* O157:H7	DBD	V ¼ 42.6 kV, RH ¼ 22%, d ¼ 5.0 cm, ET ¼ 10 min	0.4e0.8 log CFU/g decrease in the number of E.coli
Onion Powder	*Bacillus cereus, Aspergillus brasiliensis, Escherichia coli*	Microwave discharge	P ¼ 400 W, d ¼ 24 cm, ET ¼ 40 min	2.1 log spores/cm2 , 1.6 log spores/cm2 , and 1.9 CFU/cm2 reduction, respectively
Groundnuts	*Aspergillus parasiticus, Aspergillus flavus*	Radiofrequency plasma	P=¼ 60 W, RH ¼ 45.3%, f ¼ 13.56 MHz	97.9% and 99.3% reduction, respectively
Dried squid shreds	Aerobic bacteria, marine bacteria, *Staphylococcus aureus*	Corona Discharges	V=¼ 20 kV, f ¼ 58 kHz, d ¼ 25 mm, ET ¼ 3 min	Inactivated by 2.0, 1.6, and 0.9 log units, respectively
Vacuum packaged beef loin	*Staphylococcus aureus, Listeria monocytogenes, Escherichia coli*	DBD	f ¼ 9 kHz, d ¼ 2 cm, P ¼ 29.9 W	>2 log reduction

Note: HVACP ¼ High voltage atmospheric cold plasma, APP ¼ Atmospheric pressure plasma, DBD ¼ Dielectric barrier discharge, P ¼ Power, V ¼ Voltage, RH ¼ Relative humidity, d ¼ distance, f ¼ frequency, ET ¼ exposure time

The primary atmospheric plasma disinfectants are reactive oxygen species that, together with UV emissions and other particles generated, causes the microbial cell death (K.G. Kostov et al, 2010). Relying on a mechanism already described with PAW, cold plasma inactivates gram-positive and gram-negative bacteria in various ways. Gram-negative bacteria i.e., *Escherichia coli* is mainly inhibited by low-level DNA mutations and cell leakage. For gram-positive bacteria such as *Staphylococcus aureus*, the inactivation occurs due to a moderate envelope damage and intracellular distribution (Salma Farooq et al, 2023).

An example of the disinfectant potential of CP against a food matrix is well described by A.R.Ganesan et al research work on meat samples treated with CP. The samples surface shows, after the direct gaseous exposure, a reduction of the total population of *Listeria innocua* to 0.85log10 CFU/g by air plasma treated on jerky. According to litireature, as for thermal-treatment also with cold plasma is possible to define a microbial death kinetic. In Figure 1-11, the death curve of *E.coli* analysed by K.G. Kostov et al shows the survivor curve after a CP treatment.

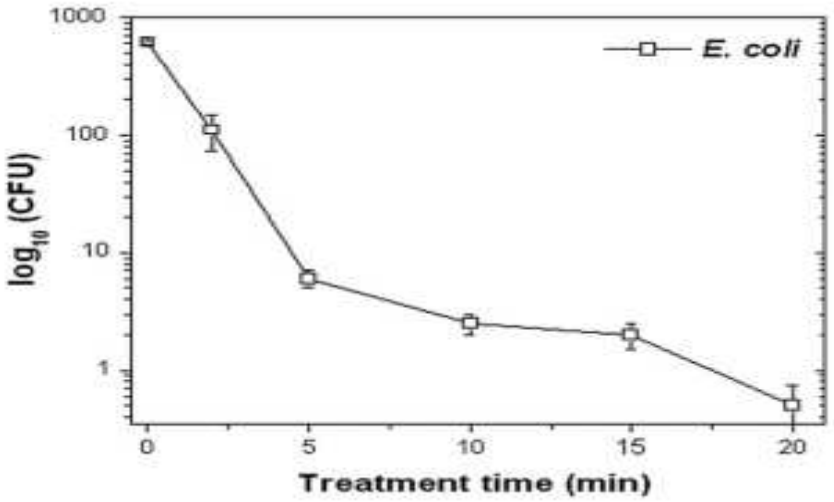


Figure 1-11 E.coli survivor curve after the treatment (K.G. Kostov et al, 2010)

A summary-schematic representation of CAP activity against microorganism is shown in figure 1-12 below. Both photon emission/reactive species production are considered as lethal

agents leading to the cell death after the unwinding of protein secondary structure (A.R.Ganesan et al, 2020).

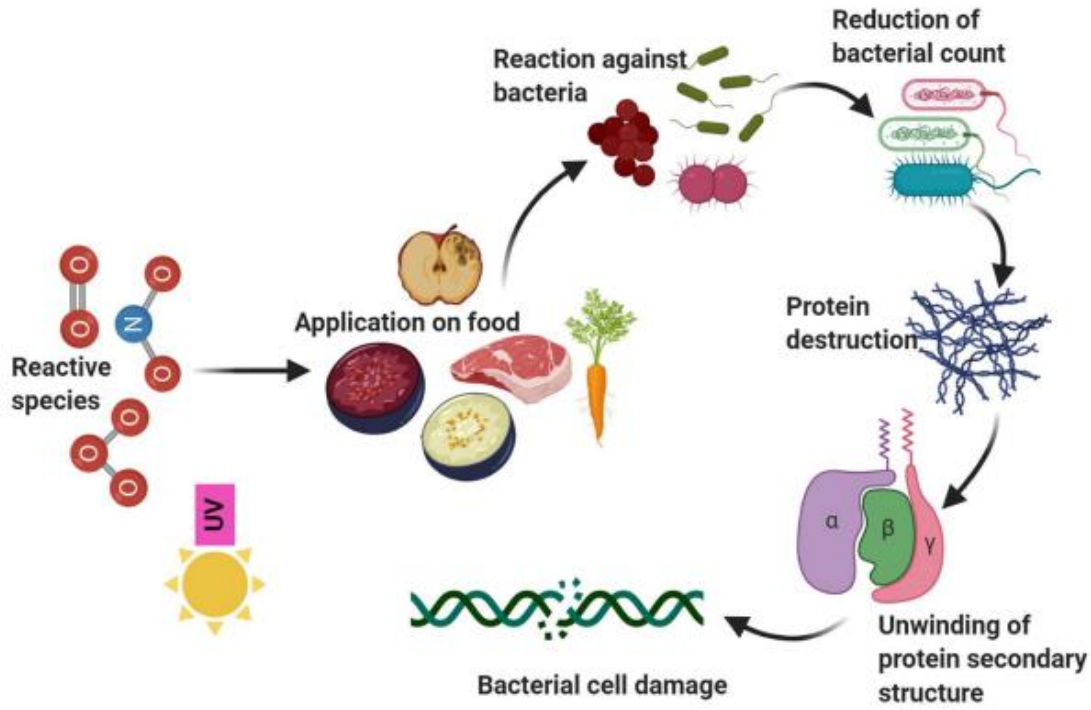


Figure 1-12 The schematic representation of CAP on food matrix resulting in bacterial inactivation and cell damage (A.R.Ganesan et al, 2020)

.

CHAPTER 2
COLD PLASMA:
CHEMICAL BEHAVIOUR AND INTERACTIONS WITH FOOD MATRIX

2.1 Plasma interactions overview

Cold plasma is an emerging and highly reliable technique for decontamination, preservation, and sterilization of food materials, in view of this, let's consider now the main issues related to these emerging tools. The essential limiting factor that must be considered during a gaseous CP treatment are the thickness of the sample (that is also linked to plasma infiltration properties and thus to plasma composition), the distances between electrodes and the samples and the production of tertiary compounds.

Despite the beneficial influence of plasma on biomolecules in terms of functional properties and retaining quality, the interaction of plasma species with food molecules and the subsequent production of tertiary compounds is a less explored phenomena not already well understood. A general overview of plasma reactive species effects in different food matrix is shown in the figure 2-1 below (M. Dharini et al, 2023).

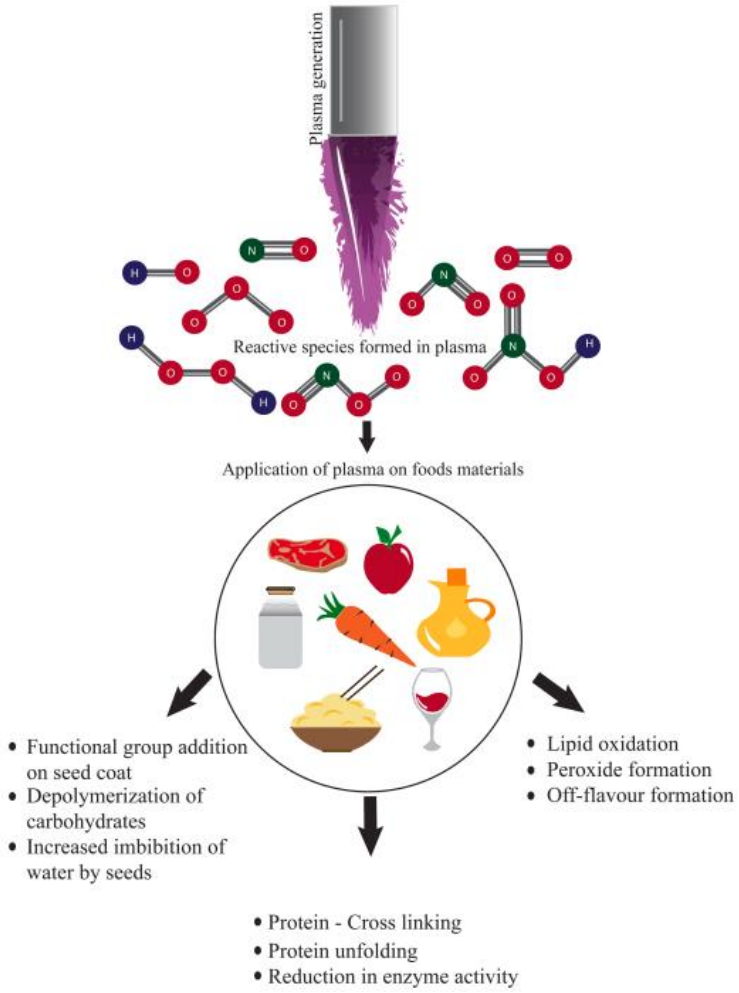


Figure 2-1 Overview on the effect plasma reactive species have on food biomolecules- Proteins, carbohydrates and lipids (M. Dharini et al, 2023)

Starting with **proteins,** the most observable changes include the inactivation of enzymes, enhancement in functional properties, changes in hydrophilicity or hydrophobicity and modification of the cooking properties. From the aminoacidic point of view, aromatic and aliphatic amino acids show to be the more susceptible to the plasma treatment. Plasma alterations on proteins comprise side-chain modification, cross-linking, protein backbone modification, fragmentation, and conformational modification. Both ROS and UV photon (around 250 nm) shows a protein oxidation effect. By altering secondary and tertiary structures, the functional properties of proteins and enzymes are altered in both positive and negative way. Research shows that enzymes with disulphide bonds, after a treatment with cold

plasma, results in an alteration of bonds conformation. These were dismantled to form sulfhydryl groups or their stable acid form (M. Dharini et al, 2023).

Carbohydrates are decisive molecules for the definition and preservation of the quality of many food products. All reducing sugars, including glucose, fructose and non-reducing sucrose are affected in disparate ways to cold plasma processing (Salma Farooq et al, 2023).

When CP is applied to polysaccharides, the main modifications are depolymerization, etching, crosslinking, and the formation of new functional groups. The sum of those reactions leads to the fragmentation of amylose (one of the two starch fractions) into simple sugars. RONS are the main actor involved in the depolymerization of polysaccharides. $^{\cdot}OH$ is the dominant species in this reaction and by generating a carbon-centred radical it causes the cell wall loosening (with the consequentially increasing of the juiciness of the fruit i.e.). The conversion of polymers into monomers (simple sugars) results in decreasing of viscosity, raise of solubility and elevating the water binding capacity. Crosslinking of polymers brings to conformational changes of starch. This modification occurs mainly in two ways, the presence of ROS followed by the subsequent reaction of electrons. That alteration led to the formation of C-O-C linkage between two sidechains (C-OH) with the releasing of water molecules (M. Dharini et al, 2023). The impact of CP treatments on grains and legumes polysaccharide has received greatest attention. The decreasing of boiling time for brown rice, the reduction in pasting and gelatinization temperature, the reduction of the retrogradation propensity and the amylose content are only several of the many applications under the eye of the scientific community (Salma Farooq et al, 2023).

As already well-stated, **vitamin** sensitivity to various processing techniques is crucial for the identification of the best treatment to adopt in order to retain the nutritional properties of food. When applied to plasma, vitamins such as ascorbic acid (vitamin C, one of the less stable vitamins) doesn't considerably decay. Negligible losses have been documented in chopped fruit and vegetables that, after the treatment, shows a deterioration of vitamin C of the 4% (Salma Farooq et al, 2023).

Antioxidants exhibit similar results. According to literature, after CP treatment, no discernible changes in the antioxidant capacity of several fruit and vegetables were observed.

From a case study reviewed by A.R.Ganesan et al, the total anthocyanin, phenol, and tannins were found to be decreased from 424.61 to 356.71 mg/L, 1722.42 to 1670.71 g/L, and 2.2 to 1.86 g/L, respectively, in red wine (Cabernet Sauvignon, and Graševina brand) after application of a high-voltage electric discharge plasma of 60 to 120 Hz for 10 min at 6°C. Likewise to this example, the deterioration of antioxidant compound is clearly limited.

However, CP's effects on food biomolecule depends on a variety of factors including the type of food, the antioxidant under analysis, exposure methods, plasma production source and the already mentioned process parameters; for this reason, further studies are still necessary (Salma Farooq et al, 2023).

2.2 Cold plasma effects on lipids

Lipids are a group of biomolecules formed by a long-chain fatty acids linked by carbons. According to the number of double bonds they are classified as saturated, monounsaturated, and poly-unsaturated fatty acids. With the presence of a catalyst such as light, heat and metals (e.g., Cu, Fe), lipids tend to oxidize in a photo-, thermal-, or auto-oxidation respectively (M. Dharini et al, 2023). Auto-oxidation is the most common pathway of oxidation. It is a spontaneous reaction of food lipids with the presence of oxygen thorough a chain reaction that involves free radicals into a three stages process (Mohsen Gavahiana et al, 2018). The lipid autoxidation pathway is shown in figure 2-2 below. As clearly indicated, the autoxidation pathway occurs in three steps: initiation (due to the presence of radical compounds), propagation and termination.

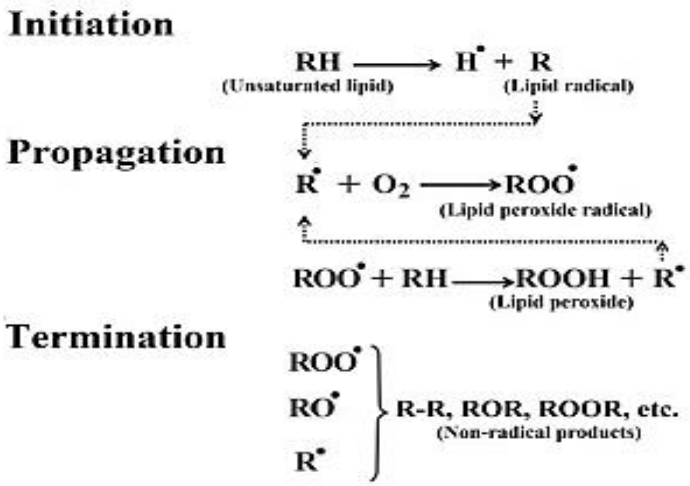


Figure 2-2 A schematic representation of the lipid autoxidation pathway (Mohsen Gavahiana et al, 2018)

Free radicals attack several compounds such as unsaturated fatty acid leading to fats oxidation and subsequentially decreasing of the food quality. By measuring the primary oxidation products (which are usually non-volatile compounds) or the secondary oxidation products

(which are often volatile molecules), the incidence of lipid oxidation in food can be detected. Especially when treating fatty foods, ROS species could interact with food lipids and initiate the oxidation process. The primary targets of ROS are methyl groups, with a greater affinity for those linked by double bonds. The basis of this mechanism lies on the fact that the energy required for abstracting hydrogen atoms is significantly lower than CH- bonds linked elsewhere (272 kJ/mol vs. 422 kJ/mol). As a consequence, the more double bonds a fatty acid contains, the more it will be open to homolytic ROS attacks. The fate of lipid during plasma treatment must be monitored to avoid an alteration of the polyunsaturated fatty acid composition (Mohsen Gavahiana et al, 2018).

As said previously, CP process generates reactive species that are able, among the other applications, to participate actively in microbial decontamination. Unfortunately, species generated (in particular free radicals), by abstracting hydrogen ions from lipids molecules can initiate the lipid oxidation process. In the table 2-1 below, the results of numerous plasma treatments evaluations are summarized, and we can easily denote the oxidative impact of cold plasma on food ingredients.

Table 2-1 Summary of the investigations on the effects of cold plasma on food lipid oxidation (Mohsen Gavahiana et al, 2018)

Food product	Plasma source and condition	Carrier gas	Process time	Oxidation assay	Key findings
Brown and white cooked rice	Direct exposure; atmospheric pressure; Power: 250 W; Frequency: 15 kHz	Air	20	TBAR	• Plasma treatment increased the TBARS values of brown and white rice Lee et al., in press • The TBARS value of brown rice was higher than that of white rice due to higher fat content • Lipid oxidation during plasma process affected the sensory characteristics
Wheat flour	Direct exposure; NTP, Input power: 40, 90 W; Frequency: 9 kHz	Air	2	PV n-hexan	•The oxidation markers increased by increasing process time and applied voltage
Pork butt and beef loin	Indirect exposure; Flexible thin-layer DBD plasma; peak	Air + N_2 +O_2	10	TBARS	• The TBARS values of treated pork and beef samples increased with process time Jayasena et al., 2015 • The TBARS values of beef-loin samples were higher than

	power: 100 W; average power: 2 W; Frequency: 15 kHz				those of pork-butt because of the variations in fat content and fatty-acid composition
Milk	Direct exposure, Encapsulated DBD plasma; Power: 250 W; Frequency: 15 kHz	Air	10	TBARS	• TBARS value was not affected by plasma treatment
Bresaola (a sliced ready-to-eat meat)	Indirect exposure; DBD; Power: 62 W; Frequency: 27.8 kHz	70% N_2 + 30% O_2	1	TBARS	• The TBARS values increased with plasma power, treatment time and storage time
Olive oil	Direct exposure; DBD plasma jet; Input voltage: 6 KV; Frequency: 50 kHz	99.9% Air + 0.1% O_2	60	GC-MS	• Plasma treatment increased the concentration of secondary oxidation products Van Durme & Vandamme, 2016 • plasma- induced oxidation followed a unique mechanism yielding unique oxidation products • Cold plasma was proposed as a technique to evaluate edible oil adulteration
Fresh mackerel fillets	Indirect exposure; DBD plasma; frequency: 50 Hz; Voltage: 70, 80 kV	Air	5	Fatty acid composition PV Dienes	• Plasma treatment modified the fatty acids composition Albertos, Martin-Diana, Cullen, Tiwari, Ojha, Bourke, Rico, et al., in press PV • Increasing the input voltage and process time increased the oxidation rate
Pork loin	Indirect exposure; a commercial cold N2 plasma; Power: 500 W	N_2	2	TBARS	• TBARS value increased by cold plasma treatment Cui et al., 2017 • Addition of antioxidants (BHA, essential oils) reduced the amount of TBARS and compensated the oxidative effects of cold plasma

Note: process time=The maximum plasma exposure time (minutes) to the plasma in the study

PV= peroxidation value, GC-MS=gas chromatography and mass spectrometry,

While for foods such as cereals, fruit and vegetables the oxidation issue is still peripherical and limited by the general low fats content, for other food goods the scenario is completely different. Considering meat products for example, research highlighted as chicken breast is more stable to plasma induced oxidation than red meat. However, by applying atmospheric pressure cold plasma (1 minute at 62W) to bresaola samples directly inside modified atmosphere package (30% O_2 and 70% air), no remarkable oxidation products emerged (TBARS values increased from 0.15 to about 0.35 mg/kg). Other positive results came from milk and dairy products where, as we know, the lipidic profile can be very advantageous for pro-oxidants factors. From the study mentioned by Mohsen Gavahiana et al, 2018, a ten-minute exposure of milk to DBD plasma (250W of power) was sufficient to reduce *Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes* load by 2.4 log CFU</ml without any significant oxidation side effects (Mohsen Gavahiana et al, 2018).

2.3 Cold plasma effects on seafood

According to current publications, it seems that lipid oxidation could be a crucial concern for the treating of **seafood** with plasma due to the presence of a high concentration of polyunsaturated fatty acid. Several authors observed as lipid oxidation during plasma treatment could be related to the subsequent reaction of the primary oxidation products with the reactive species produced with plasma (Mohsen Gavahiana et al, 2018).
However, a recent study focused on CP treatment on mackerel shown that cold atmospheric plasma did not encourage any considerable undesirable oxidation effects in mackerel samples. However, CP could be involved into the ambiguous formation of carbonyls which are related to protein oxidation (figure 2-3) (Juan M. Pérez-Andrés et al, 2020).

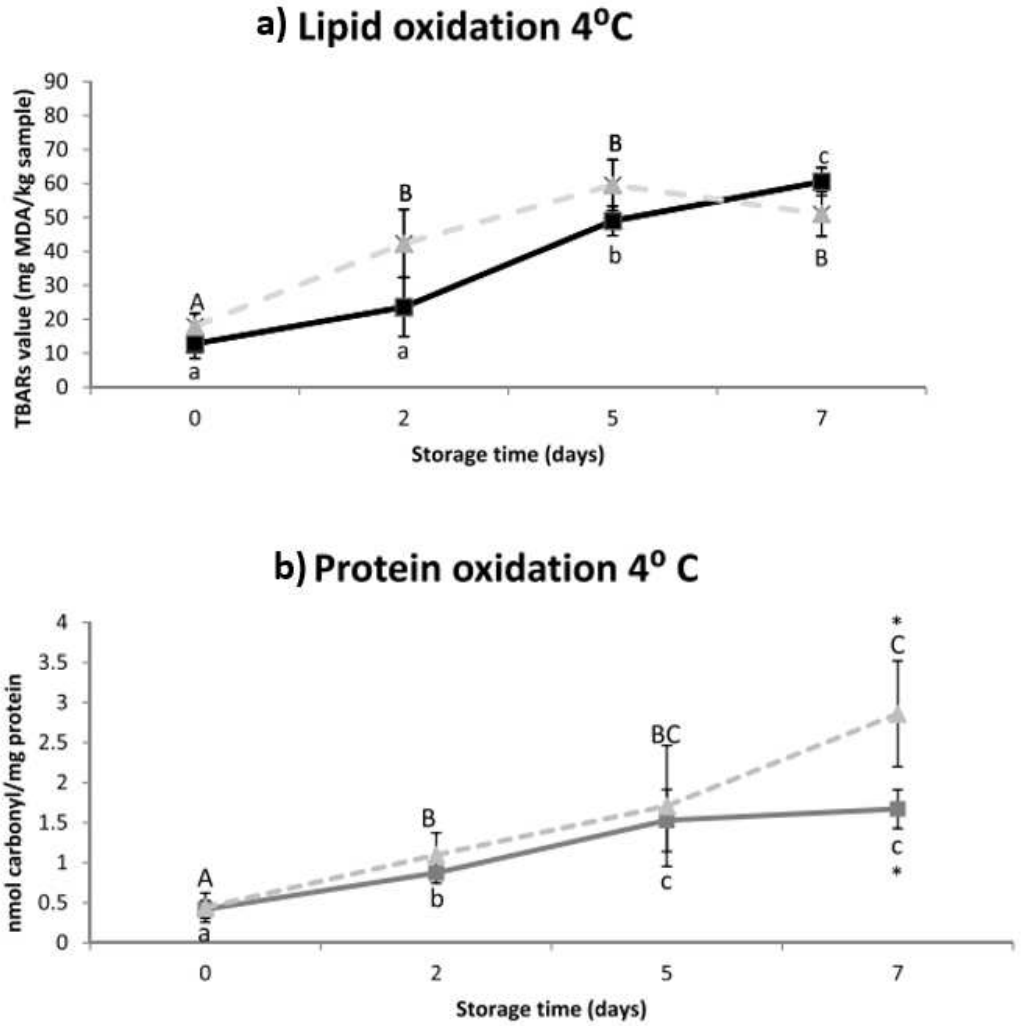


Figure 2-3 a) Lipid oxidation: TBARs values (mg MDA/kg sample) for control (darker grey line) and plasma (dashed line)at 4 °C. Different letters (lowercase for control and uppercase for plasma) indicate significant differences on the TBARs values during days of storage at the same temperature ($p < 0.05$). No significant differences between control and plasma treated samples at the same storage day were found ($p > 0.05$).

b)Protein oxidation; Carbonyl content values (nmol carbonyl/mg protein) for control (darker grey line) and plasma (dashed line) at 4 °C. Different letters (lowercase for control and uppercase for control) indicate significant differences on the carbonyl content value during days of storage at the same temperature ($p < 0.05$). Stars show significant differences between treatments at the same storage day being * ($p < 0.05$) and ** ($p < 0.005$). All the differences are measured separately for 4 °C, 8 °C and −20 °C (Juan M. Pérez-Andrés et al, 2020)

A large number of studies have highlighted that not only the gas composition, but also the spatial-temporal distance from the plasma source to the target and the mode of plasma generation will affect both the microbial inactivating activity and the undesirable side-effects. In another study conducted on Pacific white shrimps, together with the antimicrobial properties, the opportunity to control and reduce "melanosis" (the process that implies the formation of "black spots" due to the enzymatic oxidation and thus, leading to serious economic losses) were furthermore investigated. Results of CP treatments shows a significative lowering in all microorganisms considered, a decreasing of the enzymatic activity, higher water-binding capacity, and a lower cooking loss, additional to this, the shelf life of plasma-treated shrimps was >4 days longer than that of the controls (14.1 vs. 9.8 days) which would help enormously marketability. Both protein oxidation and lipid oxidation can be considered negligible if the treatment were done under certain limits (dielectric barrier discharge, 60 Kv for a maximum of 90 second) that meets the technological requirement for achieving a microbial/enzymatic effect (Peter Paulsen et al, 2022).

Considering that in certain scenarios, seafoods (such as salmon, tuna, mussel, and oysters) are consumed raw, a non-thermal treatment able to ensure safety is highly desired by the industry. Mussels and oysters have been implicated in foodborne poisoning either by contaminant bacteria or virus. Several studies have revealed that by applying CP treatments on these molluscs both antimicrobial and **antiviral** effects ensure the safety of the raw food. Authors observed a higher antiviral effect toward a double-stranded DNA virus (such as Equid Alphaherpesvirus1, EHV) than against a single-stranded RNA virus (Bovine Coronavirus, BCoV or the most frequent food-virus human norovirus). The antiviral effect of CAP exposure preserves the life of molluscs such as oysters that are traded alive and consumed raw (Peter Paulsen et al, 2022).

2.4 Cold plasma treatments and cholesterol oxidation products

Cholesterol (5α-cholesten-3β-ol) is a lipid which belongs to the family of sterols (watch figure 2-4). This lipid is highly present in all meat and fish products where it plays an important role as a structural component of the phospholipid bilayer of the plasma membrane of eukaryotic cells. Cholesterol is fitted into membrane bilayers, here its presence prevents the crystallization of fatty acyl chains and hereby modifying the activity of membrane-bound enzymes. It also has vital functions in the metabolism is an essential precursor for the synthesis of vitamin D (vitamin D3 is a derivative of cholesterol and is generated in the skin starting from 7-

dehydrocholesterol), bile, bile acids salts, steroids, and hormones. As for other lipids, cholesterol oxidation, follows the same pathway marked by free radicals, leading to a chain reaction mechanism and the production of new molecules. C7, C20 ad C25 are the most reactive carbon which are responsible for cholesterol reactive behaviour. From the oxidative reaction of this groups, oxysterols (intermediates of cholesterol oxidation) are produced. They can be described as oxygenated derivatives of cholesterol with a very short half-life relative to cholesterol (Juan M. Pérez-Andrés et al, 2020).

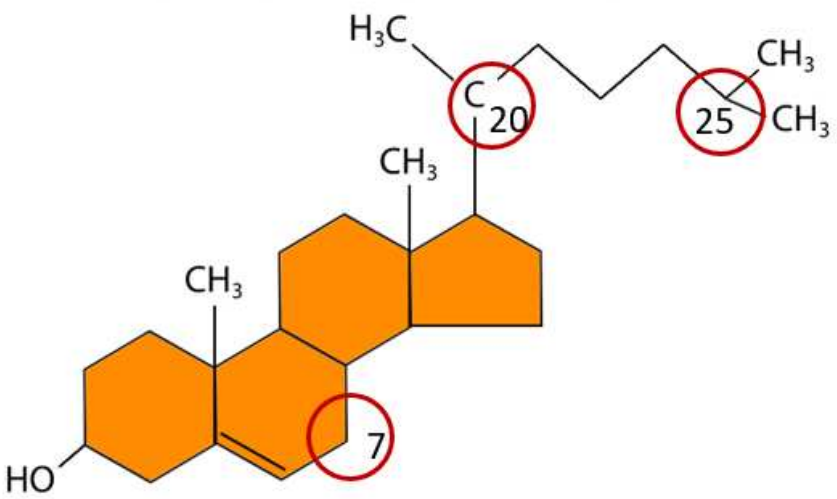


Figure 2-4 Cholesterol structure, red circles indicate the most reactive carbon (Juan M. Pérez-Andrés et al, 2020)

Cholesterol oxidation products also known as COPs, preserve the steroidal motif of the original molecule but with an additional hydroxyl, ketone or epoxy group. Depending on the site of oxidation, COPs can be distinguished as A-ring, B-ring or side-chain oxidation products (COPs structures are shown in the figure below, 2-5). These steroidal compounds can be classified in endogenous (intermediates in biomolecules synthesis and for autoxidation products in tissues) and exogenous that are mainly derived from the diet (dairy products in general are the major source of dietary COPs) (Lisaura Maldonado-Pereiraa et al, 2018).

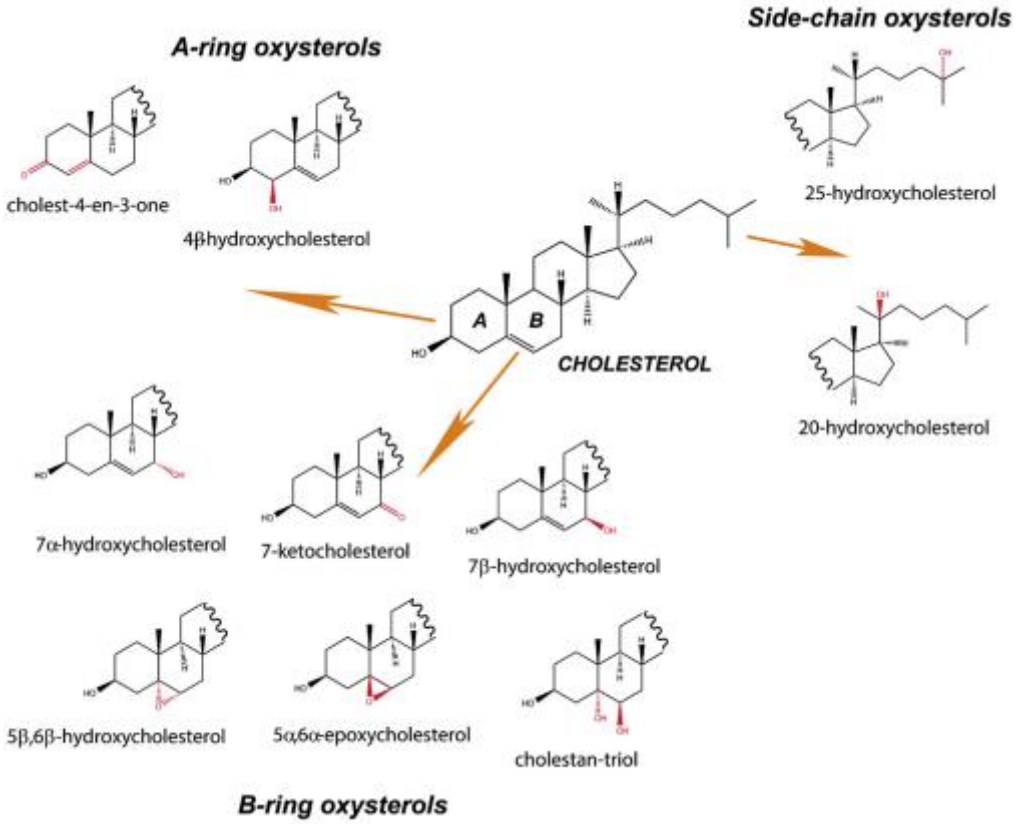


Figure 2-5 Structure of the main COPs classified according to the site of oxidation (Lisaura Maldonado-Pereiraa et al, 2018)

According to the available literature, COPs activity have been studied in order to understand their biological and pathological activity in both in vivo and in vitro systems, with the potential health consequences for humans. A considerable number of studies have demonstrated that COPs can exert pro-inflammatory, pro-fibrogenic, pro-oxidant and pro-apoptotic activities in several cell lines; as well as deleterious properties such as cytotoxicity, mutagenicity, carcinogenicity, age-onset macular degeneration, osteoporosis, colon carcinoma and several neurodegenerative diseases including Huntington's, Parkison's and Alzhaimer's disease (Lisaura Maldonado-Pereiraa et al, 2018).

Nowadays, the possible interactions between CP treatment and cholesterol are still not well defined as other studies focused their attention mainly on the general lipidic profile. In accordance with the requirement for a clear view of those interactions, in the next chapters a deep analysis of this scenario will be demonstrated (Juan M. Pérez-Andrés et al, 2020).

CHAPTER 3

MATERIALS AND METHODS

3.1 **Sampling and cold plasma treatment**

The oxidation of cholesterol induced by plasma reactive species was studied: (i) in a model system (pure cholesterol supported on a glass surface) directly exposed to the plasma atmosphere; (ii) in a solid food (sardine fillets) processed by PAW. Sardine (*Sardina pilchardus* L.) was chosen because of the high sensitivity of tissue lipids to oxidative degradation. Moreover, sardine is the main captured species in the Mediterranean Sea (158,166 tonnes, corresponding to 22.4% of total landings, 2018–2020 average) and the second most important species in term of value (USD 187,606,195), after European anchovy (*Engraulis encrasicolus*) at USD 200,645,882 (FAO, 2022). Sardines have been a staple of Mediterranean people's diet since ancient times, and they still play a significant role in the culinary traditions of the countries overlooking the sea. Besides conventional canned, air-dried, and dry-salted preserves, there is an increasing request of ready-to-use and ready-to-eat products, both minimally processed (fillets, steaks) and lightly preserved (salted, marinated, fermented, cold-smoked), that could be functional to shorter and shorter times available for domestic food preparation.

3.1.1 Direct plasma treatment

A cholesterol solution was selected as the sample for Cold Plasma (CP) treatment for a couple of reasons. First, there is currently limited available literature that thoroughly explores the oxidative products that result from individual cholesterol molecules without any interaction with other food matrices. Second, cholesterol, combined with polyunsaturated fatty acids, is a

significant factor that constrains the application of cold plasma as a non-thermal method for treating fish species due to its oxidative instability.

Experimental procedures related to cold plasma treatment were carried out in the Department of Agricultural and Food Sciences in Cesena.

The CAP system used was previously described and it consisted of a surface dielectric barrier discharge (SDBD) plasma source. The equipment included a box with two gas connections, with one serving as the gas inlet and the other as the gas outlet, which were used for flushing the chamber with the treatment gas. To ensure safe operation and consistent results, the SDBD was air-cooled. The plasma source was connected to a high-voltage microsecond pulsed generator. Figure 3-1 shows a schematic representation of the CAP system.

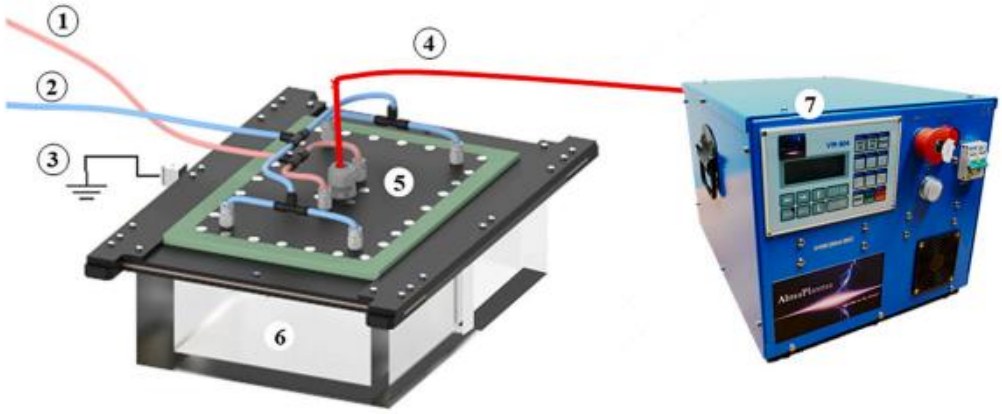


Figure 3-1 Schematic representation of the CAP system. 1) air cooling outlet, 2) air cooling inlet, 3) ground connection, 4) high voltage connection, 5) SDBD, 6) treatment chamber, 7) high voltage generator (Silvia Tappi et al, 2023)

The treatment lasted for 5, 10 and 30 min and two different regimes were investigated: NO_x and O_3. Both of them working with sinusoidal high voltage signal having a peak of voltage of 6.6 kV and a frequency of 23kHz, varying the duty-cycle i.e., the ratio of time in which the signal was turned on over the treatment time. Together with time another variable was considered: the distance from the source; respectively 5cm (near) and 18 cm (far).

Each treatment was repeated in two independent replicates so, from 12 different variables considered (time, distance and regime) 12 samples were prepared.

A pure cholesterol solution was stored in a glass closed recipient avoiding the exposure of oxidative agents such as light, temperature, humidity.

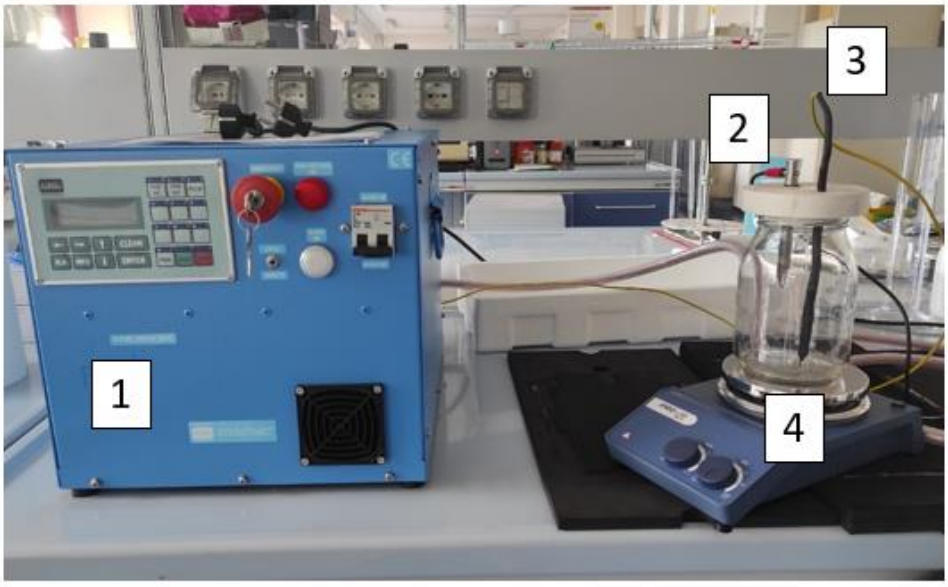
3 ml of the solution of pure cholesterol were spread into an empty-sterilized Petri dish (10 cm diameter, surface area 58 cm^2) and after its completely solidification it was placed into the

application chamber of the CAP system. During the sampling process 2 petri dish containing 3 ml of the cholesterol solution each were considered as the non-treated reference and thus, were marked as controls.
After the treatment, the cholesterol solution was later recovered made by mixing together 3mg of cholesterol hexane diethyl ether 3:1 and placed into a vial sealed with a parafilm layer and stored at -20°C until further steps.

3.1.2. PAW processing of sardine fillets

Fresh sardines were captured in the Adriatic Sea and processed (beheaded, eviscerated and filleted) by a local distributor (Ecopesce S.r.l., Cesenatico, FC, Italy). PAW was prepared by exposing 500 ml of sterile distilled water (SDW) for one minute to a pulsed corona discharge driven by a high voltage power generator (AlmaPulse, AlmaPlasma s.r.l., Bologna, Italy), using peak voltage of 18 kV and pulse repetition frequency of 5 kHz (Laurita et al., 2021). Different dipping times in freshly prepared PAW (10, 20, and 30 minutes) were checked for the degree of fish lipid oxidation, while the solid/liquid ratio was kept constant (1:3 w/v). The water used for PAW production was also used as control dipping medium. Treatments (three replicates each) were carried out in a benchtop oscillating agitator.
Total lipids were extracted from PAW-treated samples and controls by the "Bligh and Dyer" extraction procedure (Zhao et al., 2021). Briefly, each sample (30 ± 2 g) was homogenised with 25 ml of chloroform and 50 ml of methanol by an Ultra-Turrax® T10 disperser (IKA-Werke GmbH & Co. KG, Staufen, Germany). The slurry was filtered through a glass Gooch funnel and the cake was homogenised again with 25 ml of chloroform. Both filtrates were collected in a separatory funnel and a biphasic system was eventually generated by adding 30 ml of aqueous potassium chloride 0.88% w/v. The lower phase, made up of chloroform with small amounts of water and methanol, was collected and the solvent was removed at 40 °C in a BÜCHI Rotavapor® Model R-124 (BÜCHI Labortechnik AG, Flawil, Switzerland). Lipid extracts were stored at -20 °C until they were analysed.
19-hydroxycholesterol (25 µl of 500 mg/l solution in hexane/isopropanol 3:2 v/v) was put into an Erlenmeyer flask with screw cap, as internal standards for COPs quantification. After solvent evaporation under a nitrogen stream, about 250 mg of crude lipids were accurately weighed in the flask and left to react overnight with 10 ml of methanolic potassium hydroxide 1 M, at room temperature and protected from light. The saponified mixture was transferred into a separatory funnel with the aid of 10 ml of water. Three extractions with 10 ml of diethyl

ether each were made, and the organic layers were collected in a clean separatory funnel. The combined extracts were washed three times with 10 ml of water each time, filtered through anhydrous sodium sulphate, and evaporated to dryness in a rotary evaporator at 35 °C (BÜCHI Labortechnik AG, Flawil, Switzerland). The total unsaponifiable matter was finally dissolved in 1 ml of n-hexane for COPs recovery. PAW equipment is shown in figure 3-2.



a)

b)

Figure 3-3 Overview of the equipment used for the production of PAW (a) and enlargement of the reaction chamber (b). 1 - pulsed high-voltage generator; 2 - high-voltage electrode; 3 - ground electrode; 4 - magnetic stirrer; 5 - plasma discharge

3.2 COPs recovery and determination

Solid phase extraction (SPE) (shown in figure 3-3) was used to recover the polar COPs containing fraction from the unsaponifiable matter of fish lipids and oxidised cholesterol recovered from the glass dishes, according to the procedure described in Foligni et al. (2021). COPs fractions were then derivatized for gas chromatography (GC) analysis by a ready-to-use silylating mixture (pyridine/chlorotrimethylsilane/hexamethyldisilazane 10:1:2 v/v/v; Supelco-Bellefonte, PA, USA).

Silylated COPs were analysed using a Trace 1300 gas chromatograph coupled with ISQ 7000 single quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and equipped with a Zebron ZB-5 ms capillary column 30 m × 0.23 µm film thickness (Phenomenex, Torrance; CA, USA). A Dual Detector Microfluidics kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to split 1:1 the injected sample between the mass spectrometer (qualitative analysis) and the FID (quantitative analysis). The mass spectral data and Kovats Retention Indices (RIs) obtained by the analyses were matched with NIST/EPA/NIH Mass spectral Library (Version 2.0a, built 1 July 2002; National Institute of Standards and Technology) The RIs of unknown components was calculated with an automated spreadsheet.

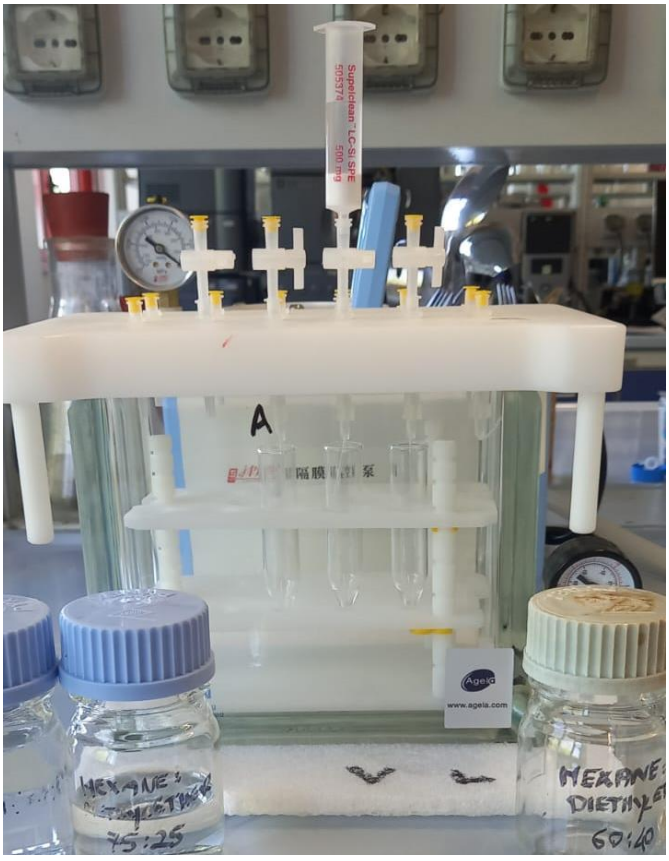


Figure 3-3 SPE column

3.3 Data analysis

Analytical data were analysed by JMP® Version 10 (SAS Institute Inc., Cary, NC, USA) and one-way ANOVA and Tukey-Kramer's honest significant difference (HSD) test were used to compare the experimental variables between treated and control samples. The level of significance was set at $p < 0.05$.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Identification of COPs

The availability of pure analytical standards made the identification of common COPs easy: this was true for peaks at RRT (relative retention time to 5α-cholestane) 1.19, 1.35 (7-OH epimers), 1.41, 1.43 (5,6-epoxy isomers), 1.53 (cholestane triol), and 1.67 (7-keto). Neither the common 25-hydroxycholesterol (pure standard was available) nor other side chain hydroxy derivatives of cholesterol were detected. The presence of less usual COPs was ascertained by careful interpretation of the mass fragmentation patterns and by comparison with MS and chromatographic retention data published in literature. The small amount of these substances made their identification challenging, since the quality of their mass spectra was sensitive to the background noise and the efficiency of the chromatographic separation.

Peaks at RRTs 1.27 and 1.34 were attributed to stereoisomers of cholest-4-ene-3,6-diol. The presence of fragments originated by the loss of methyl group and/or trimethylsilylhydroxy group (TMSOH) at m/z 531 ($[M-15]^+$), 456 ($[M-90]^+$), and 441 ($[M-90-15]^+$) confirmed the molecular ion at m/z 546, corresponding to monohydroxy derivatives of cholesterol. According to literature data (Grandgirard, Martine, Joffre, Juaneda, & Berdeaux, 2004), the ion at m/z 403 is considered characteristic of the 4-ene-6-hydroxy structure. The fragmentation pattern involves the loss of two methyl groups and the side chain (C_8H_{17}), thus confirming the localisation of both hydroxy groups in the ring system. The prominent ion at m/z 147 (whose origin is not known yet) was reported as marker of cholestene-3,4 diols (Breuer, 1995) while fragments at m/z 129 (A-ring fragment), 417 $[M-Aring]^+$, and 327 $[M-90-Aring]^+$ characterizes the A-ring cleavage of TMS derivatives of Δ^5-steroids. Based upon these fragmentation patterns and the chromatographic behaviour reported by Grandgirard et al. (2004), peaks at RRT 1.39 and 1.46 were hence attributed to 4β- and 4α-hydroxycholesterol,

respectively. Mass spectra of component at RRT 1.45 was consistent with monohydroxy derivative of cholesten-3-ol, which was suspected to be 6-cholesten-3,5-diol originated, together with 4-cholesten-3,6-diols (peaks at RRTs 1.27 and 1.34), by a concerted ene addition of singlet oxygen to Δ^5 unsaturation of cholesterol. In fact, back in 1988 Bachowski, Thomas, and Girotti, 1988 observed that the 5α-hydroperoxide was the major oxidation product of cholesterol in photo-oxidized cell membranes. Peak at RRT 1.56 showed a fragmentation pattern similar to pure cholestane-3β,5α,6β-triol and was tentatively attributed to a cholestane triol stereoisomer.

Table 4-1 MS spectral data and chromatographic behaviour (relative retention time (RRT) to 5α-cholestane on DB5-type capillary column) used for COPs identification.

RRT	$[M]^+$ [a]	Major significant ions[a] (base peak in bold)	Name (IUPAC)	Abbreviation
1.18	456 (4)	441 $[M-15]^+$ (2); 366 $[M-90]^+$ (7); 351 $[M-90-15]^+$ (4); 327 $[M-Aring]^+$ (7); 255 $[M-90-SC]^+$ (15); 129 $[Aring]^+$ (58); 111 $[SC]^+$ (70)	Cholesta-5,22-dien-3β-ol	
1.19	n.d.	**456 $[M-90]^+$ (100)**; 441 $[M-90-15]^+$ (4); 129 (23)	Cholest-5-ene-3β,7α-diol	7α-OH
1.20	456 (9)	441 $[M-15]^+$ (2); 366 $[M-90]^+$ (13); 351 $[M-90-15]^+$ (16); 327 $[M-Aring]^+$ (14); 255 $[M-90-SC]^+$ (20); 129 $[Aring]^+$ (75); 111 $[SC]^+$ (80)	Cholesta-5,24-dien-3-ol	
1.27	458 (20)	443 $[M-15]^+$ (7); 368 $[M-90]^+$ (45); 353 $[M-90-15]^+$ (25); 329 $[M-Aring]^+$ (60); 255 $[M-90-SC]^+$ (12); 129 **$[Aring]^+$ (100)**	Cholest-5-en-3β-ol	
1.27	546 (2)	531 $[M-15]^+$ (5); 456 $[M-90]^+$ (37); 441 $[M-90-15]^+$ (30);	Cholest-4-ene-3β,6β-diol	6β-OH

		403 [M-15-15-SC]$^+$ (64); 194 (37)		
1.29	456 (35)	441 [M-15]$^+$ (5); 366 [M-90]$^+$ (2)	Cholesta-4,6-dien-3β-ol	
1.34	546 (2)	531 [M-15]$^+$ (2); 456 [M-90]$^+$ (6); 441 [M-90-15]$^+$ (8); 403 [M-15-15-SC]$^+$ (25); 194 (17)	Cholest-4-ene-3β,6α-diol	6α-OH
1.35	n.d.	**456 [M-90]$^+$ (100)**; 441 [M-90-15]$^+$ (4); 129 (23)	Cholest-5-ene-3β,7β-diol	7β-OH
1.37	470 (2)	455 [M-15]$^+$ (3); 380 [M-90]$^+$ (8); 341 [M-Aring]$^+$ (11); 129 **[Aring]$^+$ (100)**	Ergosta-5,22-dien-3β-ol	
1.38	472 (4)	457 [M-15]$^+$ (4); 382 [M-90]$^+$ (12); 367 [M-90-15]$^+$ (9); 343 [M-Aring]$^+$ (22); 129 [Aring]$^+$ (85)	Ergost-5-en-3β-ol	
1.39	546 (1)	531 [M-15]$^+$ (1); 456 [M-90]$^+$ (9); 441 [M-15-90]$^+$ (4); 417 (6) [M-Aring]$^+$; 366 (14) [M-90-90]$^+$; 327 [M-90-Aring]$^+$ (8); 147 (48); 129 [Aring]$^+$ (25); **73 (100)**	Cholest-5-ene-3β,4β-diol	4β-OH
1.41	474 (7)	459 [M-15]$^+$ (3); 445 [M-29]$^+$ (4); 384 [M-90]$^+$ (19)	5β,6β-Epoxycholestan-3β-ol	5,6β-epoxy
1.43	474 (3)	459 [M-15]$^+$ (2); 445 [M-29]$^+$ (4); 384 [M-90]$^+$ (9)	5α,6α-Epoxycholestan-3β-ol	5,6α-epoxy
1.45	n.d.	456 [M-90]$^+$ (55); 441 [M-15-90]$^+$ (18); 366 [M-90-90]$^+$ (5); 194 (35); 179 (56); 147 (71); **73 (100)**	Cholest-6-ene-3,5-diol	5-OH
1.46	546 (1)	531 [M-15]$^+$ (1); 456 [M-90]$^+$ (8); 441 [M-15-90]$^+$ (4); 417 (6) [M-Aring]$^+$; 366 (19) [M-90-90]$^+$; 327 [M-90-Aring]$^+$	Cholest-5-ene-3β,4α-diol	4α-OH

		(12); 147 (50); 129 $[Aring]^+$ (27); **73 (100)**		
1.53	n.d.	546 $[M-90]^+$ (5); 531 $[M-90-15]^+$ (5); 517 $[M-90-29]^+$ (4); 456 $[M-90-90]^+$ (25); 441 $[M-90-90-15]^+$ (9); 403 $[M-90-15-15-SC]^+$ (43); 321 (25); 129 $[Aring]^+$ (40); **73 (100)**	Cholestane-3β,5α,6β-triol	3,5,6-triol
1.56	n.d.	546 $[M-90]^+$ (4); 531 $[M-90-15]^+$ (2); 456 $[M-90-90]^+$ (47); 441 $[M-90-90-15]^+$ (8); 403 $[M-90-15-15-SC]^+$ (31); **73 (100)**	Cholestane-3β,5,6-triol	triol
1.67	472 (42)	457 $[M-15]^+$ (5); 382 $[M-90]^+$ (18); 367 $[M-90-15]^+$ (52); 129 $[Aring]^+$ (95); **73 (100)**	Cholest-5-en-3β-ol-7-one	7-keto

[a] Relative abundance in parenthesis.
n.d., not detectable; Aring, A ring fragment characteristic of Δ^5 sterols (m/z 129); SC, side chain fragment (C_8H_{17}).

4.2 PAW treatments of fish fillets

Even if a diversity of plasma generation devices and reaction chambers have been designed and developed to produce PAW, most of food-related applications involved batch type systems in which a fixed volume of water is exposed to the plasma discharge. In our experiments, we used pulsed corona discharges generated in the air above the water surface. Atmospheric air has been used extensively as working gas, in the perspective of making the process scale-up feasible and sustainable. Perinban et al. (2019) highlighted that corona discharges are one of the most studied plasma sources for food applications, despite their unstable discharges and electrode corrosion. The reactive species were transferred into the plasma-liquid interface and the bulk liquid region by a diffusion process, which was improved by forced convection through an intense liquid mixing (a magnetic stirrer was set at 500 rpm). The physicochemical properties of water changed significantly during the plasma treatment: pH dropped from 6.12 ± 0.3 to 3.64 ± 0.8, while the accumulation of RONS came with a conductivity increase from

8.87 ± 0.59 to 76.1 ± 0.7 μS/cm, according to the PAW parameters provided by the device designers (Laurita et al., 2021).

The type of water is a critical parameter: sterile distilled, deionized, osmotized, and tap water have been used for the preparation of PAW, but the PAW biocidal effectiveness seems to be strongly dependent on low pH values, which in turn are associated with low hardness. To avoid the introduction of further variables (water physicochemical parameters), we used SDW, free from minerals, organic substances and microorganisms, which has shown optimal efficacy for microbial deactivation in several studies (Zhao, Patange, Sun, & Tiwari, 2020). Anyway, PAW properties are affected by many critical parameters that have a direct impact on the type and concentration of RONS produced: plasma source, energy input (power, voltage, frequency), feeding gas and flow rate, pressure (atmospheric, vacuum chamber), water volume, contact mode of plasma-liquid interface (in-water, above water, aerosol, bubble), activation time. The PAW application mode introduces further variables (dipping time, agitation, temperature), thus making extremely challenging the comparison of the experimental data and the clarification of the links between the reactive species and the chemistry of the lipid oxidation.

Total COPs of sardine fillets ranged from 115.4 to 171.1 μg/g fish lipids, corresponding to 4.96-7.36 1 μg/g fresh matter and 19.5-29.0 μg/g dry matter (average water content of fillets was 74.6 ± 1.1 g/100 g). Those values agreed with COPs levels reported in raw sardines by Barreira et al. (2023) (39.53 ± 2.14 μg/g dry matter), de Carvalho et al. (2021) (11.5 ± 0.1 μg/g dry matter), and Saldanha, Benassi, and Bragagnolo (2008) (19.4 ± 0.4 μg/g dry matter), but were lower than amounts detected by Ferreira et al. (2017) (61.2 ± 2.8 μg/g dry matter) and higher than values observed by Cardenia et al. (2013) (62–371 μg/100 g fresh matter). The calculated percentage of cholesterol oxidation varied from 1.66 to 2.74, which were much higher than values reported by Cardenia et al. (2013) (0.1-0.9%) for sardine fillets stored at 4 °C for 4 h under light exposure and in the dark. Experimental data did not show a significant effect of PAW treatment on cholesterol oxidation. It is noteworthy that storage appears a much more critical factor than processing in managing the COPs formation. In fact, a 6-time increase in total COPs of sardine fillets were observed by Cardenia et al. (2013) after 4 hours of light exposure at 4°C and by Saldanha et al. (2008) after 120 days of storage at -18 °C. To the knowledge of the authors, only Juan M Pérez-Andrés et al. (2020) reported the ability of a cold plasma source (DBD) to degrade pure cholesterol in a model system, but no data were provided about the degradation product. The reaction mechanisms involved in the COPs formation have been largely elucidated (Iuliano, 2011). Hence, the quali-quantitative profile of the COPs could provide useful information about the favourite mechanism of oxidation, thus helping in

the clarification of the relationships between causes (plasma reactive species) and effects (COPs). Particularly, 7-hydroxy and 7-keto derivatives are usually expected to be the major COPs and our data confirmed this general behaviour, thus showing a main contribution of the free-radical mediated oxidation pathway, driven by a chain-reaction mechanism. The dominancy of the β-epimer among 7-hydroxy compounds has been frequently reported and it was confirmed by our experimental data (ratio 7β-OH/7α-OH = 1.5-3.0). The very low amount of the 4-OH, 5-ene isomers confirmed a huge asymmetry in the behaviour of the two allylic positions (C4 and C7) in relation to the Δ5 unsaturation. Epoxidation, too, provided a significant contribution to cholesterol degradation. Both free-radical and non-radical mediated pathways may be involved in the formation of 5,6-epoxides, according to the lipid (FA hydroperoxides and peroxyl radicals) and non-lipid species (hydrogen peroxide, peroxynitrite, and ozone) that can trigger the oxidative cascade. The ratio 5,6β-epoxy/5,6α-epoxy were in the range 1.8-2.4, lower than the values (3-11) reported by Iuliano (2011). 4-ene-6-OH and 6-ene-5-OH structures were consistent with a photosensitized, non-radical mediated oxidation of cholesterol, via singlet oxygen that can reacts directly with the Δ5 double bond by a concerted ene addition involving the shift of the double bond to an allylic position.

Table 4-2 COPs contents (μg/g fat) of crude lipids extracted from sardine fillets soaked in PAW and distilled water. P10-30 are PAW-dipped fillets for 10 to 30 min; C10-30 are water-dipped fillets for 10 to 30 min.

Analyte	C10	C20	C30	P10	P20	P30
7α-OH	25.9 ± 10.5	30.0 ± 4.8	26.8 ± 12.5	19.4 ± 0.2	21.9 ± 2.4	17.1 ± 1.8
6β-OH	7.4 ± 1.0	7.8 ± 0.0	8.0 ± 3.7	2.9 ± 1.8	5.7 ± 3.4	6.6 ± 3.3
6α-OH	3.2 ± 0.5	3.7 ± 0.5	3.3 ± 0.7	3.3 ± 0.3	3.7 ± 0.5	2.3 ± 0.1
7β-OH	37.7 ± 14.5	43.9 ± 7.0	45.1 ± 13.5	57.9 ± 19.0	51.8 ± 9.2	41.4 ± 9.1
4β-OH	1.4 ± 0.5	2.1 ± 0.7	1.3 ± 0.1	2.7 ± 2.4	2.3 ± 0.8	2.0 ± 0.2
5,6β-epoxy	15.3 ± 2.2	20.9 ± 6.1	13.5 ± 5.4	19.9 ± 3.6	21.0 ± 0.4	19.4 ± 1.5
5,6α-epoxy	7.4 ± 4.4	12.2 ± 5.5	5.4 ± 1.1	11.3 ± 5.4	13.0 ± 1.3	10.1 ± 0.4
5-OH	1.6 ± 0.2	1.9 ± 0.1	1.4 ± 0.8	2.7 ± 0.1	1.9 ± 1.1	1.4 ± 0.5
4α-OH	1.8 ± 0.1	1.6 ± 0.4	1.6 ± 0.6	1.1 ± 0.3	1.2 ± 0.0	1.8 ± 0.3
3,5,6-triol	3.2 ± 1.3	4.6 ± 1.5	2.7 ± 0.5	3.9 ± 1.3	4.6 ± 0.7	3.4 ± 2.7
Triol	1.0 ± 1.0	1.0 ± 0.0	1.3 ± 0.5	0.5 ± 0.2	1.1 ± 0.8	2.2 ± 0.8
7-keto	32.7 ± 5.9	41.5 ± 8.2	37.4 ± 4.2	43.0 ± 15.9	38.3 ± 8.3	47.9 ± 1.5
Total COPs	138.5 ± 12.8	171.1 ± 34.0	147.9 ± 43.4	168.7 ± 7.7	166.4 ± 10.0	155.8 ± 18.7

4.3 DBD plasma treatment of pure cholesterol

A broad range of food systems involves the use of gaseous plasma produced by different generators (already described in chapter 1). In our experiments, we adopted the surface dielectric barrier discharge generated in the air above the cholesterol samples inside the application chamber. The DBD systems requires high ignition voltages of 10 kV and requires several precautionary measures; however, it boast of relative simplicity. (A.R.Ganesan et al, 2020)

With a focus on ensuring the scalability and sustainability of the process, the working gas used was atmospheric air. The gaseous CP application mode introduces further variables that are different from PAW. Factors such as moisture, gas ratio, and temperature pose significant challenges in comparing experimental data and elucidating the connections between reactive species and the chemistry of lipid oxidation.

Samples of pure cholesterol were distributed on glass plates and treated with gaseous plasma in different conditions: two power supply regimes (NOx and O3), two different distances between the source and the plates (5 and 15 cm) and three exposure times (5, 10, and 30 min) were tested.

Cholesta-4,6-dien-3-one was the only oxidation derivative found in untreated pure cholesterol, while 6β-OH, 4β-OH, 5,6β-epoxy, Triol, 7-keto and two unidentified compounds were found in the treated samples. 5,6β-epoxy and 3,5,6-cholestane triols, which originate from the epoxy-ring opening, dominated the COPs fraction of pure cholesterol treated with the DBD plasma source. Both free-radical and non-radical mediated pathways may be involved in the formation of 5,6-epoxides. Ozone can also react with the Δ5 unsaturation and yields epoxides, via the unstable intermediate 1,2,3-trioxolane. Unexpectedly, high relative percentages of 4β-OH,5-ene derivative were found while 7-OH derivatives were not detected. Free-radical mediated oxidation pathways start with hydrogen abstraction from allylic positions (C7, predominantly) and evolve through a chain-reaction mechanism, eventually leading to 7-OH and 7-keto derivatives of cholesterol which are usually expected to be the major COPs, while the 4-OH,5-ene isomers are usually present in very low amounts. The presence of 4-ene-6-hydroxycholesterol supported the contribution of the photosensitized, non-radical mediated oxidation of cholesterol. Singlet oxygen reacts directly with the double bonds by a concerted "ene" addition mechanism. Accordingly, oxygen is inserted at either end carbon of a double bond, which is shifted to an allylic position configuration. Photosensitized oxidation of cholesterol produces mainly the 6-ene 5α-hydroperoxide which is converted to the more stable

epimers of 4-ene-6-hydroperoxide. The results of the cholesterol CP analysis are shown in the table 4-3 below.

Table 5-3 Composition (relative %) of the COPs fraction collected from pure cholesterol treated with gaseous plasma in different conditions.

Regime	Source gap	Exp. time	6β-OH	Unid.	4β-OH	5,6β-epoxy	Cholesta-4,6-dien-3-one	Triol	Unid.	7-keto
NOx	Near	5	1.5	0.5	35.0	28.4	0.1	26.7	6.5	1.2
		10	5.1	5.1	19.4	23.6	0.1	35.8	3.5	7.3
		30	6.4	1.2	19.7	15.4	1.8	39.5	6.9	9.1
NOx	Far	5	1.7	0.1	57.7	25.6	0.1	6.6	6.3	1.8
		10	5.5	0.1	47.5	29.1	0.1	9.9	5.7	2.1
		30	9.1	0.1	37.5	32.5	0.1	13.2	5.2	2.2
O3	Near	5	1.1	2.6	73.0	9.6	0.1	4.6	5.3	3.7
		10	1.9	5.7	48.5	12.4	0.1	31.3	0.1	0.1
		30	2.4	7.3	33.5	16.7	1.3	35.3	2.4	1.1
O3	Far	5	2.1	0.5	32.2	55.4	0.1	7.4	1.1	1.2
		10	7.6	0.1	24.5	45.6	0.2	12.7	0.0	9.3
		30	9.3	0.1	25.0	31.3	0.5	29.2	3.4	1.2

CONCLUSIONS

In conclusion, this thesis has explored the intriguing potential of cold plasma treatment in the food industry, with a particular focus on its side effects and its ability to oxidize cholesterol. While in one side the study has shed light on numerous advantages associated with the use of cold plasma technology the oxidation of cholesterol presents a significant limiting factor in its application.

Even if the oxidative pressure exerted by PAW did not increase COPs in a statistically robust way, the levels detected in sardine fillets (115.4-171.1 µg/g fish lipids, corresponding to 4.96-7.36 1 µg/g fresh matter) might represent a risk for human health. As reported by Cardenia et al. (2013), despite the documented wide range of adverse biological effects, no toxicity limit for COPs has been specified yet and the threshold of toxicological concern (TTC) for unclassified compounds (0.15 µg/person/day) has been suggested as reference. Therefore, it would be highly desirable that processing and storage do not increase further the levels of COPs in cholesterol containing foods.

The pattern of the oxidation products could provide useful info about the preferred mechanism of oxidation, thus helping in the clarification of the relationships between causes (plasma reactive species) and effects (sterols oxidation products). The high number of variables affecting the quali-quantitative composition of the RONS mixture make extremely challenging the comparison of the experimental data and the clarification of the links between the reactive species and the chemistry of the lipid oxidation. Further studies are needed to clarify the actual mechanisms of generation of reactive species and the mechanisms of their interactions with sensitive food constituent, to increase the knowledge of critical parameters and safety aspects of the technology and support the scale-up of PAW applications in industrial food processing.

BIBLIOGRAPHY

A.R.Ganesan et al, U. T. P. E. a. G. R., 2020. Application of cold plasma on food matrices: A review on current and future prospects. *WILEY Journal of Food Processing and Preservation,* p. 16.

Bureau, U. C., June 2011. *United States Census, International Data Base.* s.l.:s.n.

Cardenia, Vladimiro, Rodriguez-Estrada, Maria Teresa, Baldacci, Elena; Lercker, Giovanni. 2013. Health-related lipids components of sardine muscle as affected by photooxidation. Food and Chemical Toxycology. Voloume 57 pages 32-38.

Eliezer, S. E. a. Y., 2001. *The Fourth State of Matter An Introduction to Plasma Science.* Second a cura di s.l.:IoP.

Farhana Mehraj Allai et al, Z. A. A. A. N. A. M. k. G., 2023. Recent advances in non-thermal processing technologies for enhancing shelf life and improving food safety. *Applied Food Research,* Volume 3, p. 19.

Flora-Glad Chizoba Ekezie et al, D.-W. S. J.-H. C., 2017. A review on recent advances in cold plasma technology for the food industry: Current applications and future trends. *Trends in Food Science & Technology,* Volume 69, pp. 46-58.

André GRANDGIRARD*, Lucy MARTINE, Pierre JUANÉDA, Catherine CORDELET. Sitostanetriol is not formed in vivo from sitosterol in the rat. Unité de Nutrition Lipidique, INRA, 17 rue Sully, BP 86510, 21065 Dijon Cedex, France. Reprod. Nutr. Dev. 44 (2004) 609–616 609 © INRA, EDP Sciences, 2005

Hao Jiang et al, Q. L. W. S. X. Y. a. s. W., 2022. Food preservation by cold plasma from dielectric barrier discharges in agri-food industries. *Frontiers in Nutrition,* p. 14.

Juan Antonio Duro et al, C. L. T. K. K.-H. E. K.-H. E., 2020. Global inequalities in food consumption, cropland demand and land-use efficiency: A decomposition analysis. *Global Enviroment Change,* Volume 64, p. 11.

\

Juan M. Pérez-Andrés et al, J. C. S. M. H. N. P. B. P. J. C. T. R. a. B. K. T., 2020. Effect of Cold Plasma on Meat Cholesterol and lipid oxidation. *Foods,* Volume 9, p. 13.

Juan M. Pérez-Andrés et al, M. d. A. S. M. H. N. P. B. P. C. B. K. T., 2020. Effects of cold atmospheric plasma on mackerel lipid and protein oxidation during storage. *LWT - Food Science and Technology,* Volume 118, p. 10.

K.G. Kostov et al, V. R. C. K.-I. B. M. M. A. R. H. M. K. a. R. M., 2010. Bacterial sterilization by a dielectric barrier discharge (DBD) in air. *Surface & Coatings Technology ,* Volume 204, pp. 2954-2959.

Katarína Kucerová et al, M. H. L. S. M. B. a. k. H., 2011. Effect of Plasma Activated Water, Hydrogen Peroxide, and nitrates on Lettuce Growth and Its Physiological Parameters. *Applied sciences,* Volume 11, p. 13.

Lisaura Maldonado-Pereiraa et al, M. S. C. B. I. G. M.-M., 2018. The role of cholesterol oxidation products in food toxicity. *Food and Chemical Toxicity,* Volume 118, pp. 908-939.

M. Dharini et al, S. J. a. R. M., 2023. Cold plasma reactive species: Generation, properties, and interaction with food biomolecules. *Food Chemistry,* Volume 405, p. 15.

Mária Domonkos et al, P. T. J. T. a. P. D., 2021. Applications of Cold Atmospheric Pressure Plasma Technology in Medicine, Agriculture and Food Industry. *Applied sciences,* Volume 11, p. 19.

Mizanur Rahman et al, M. S. H. R. I. R. R. A. S. M. A. A. S. A. M. A. R. R. P. Z. H. H. A. A.-M. A. V.-M. a. A. R. S., 2022. Plasma-Activated Water for Food Safety and Quality: A Review of Recent Developments. *International Journal of Enviromenal Research and Public Health,* Volume 19, p. 18.

Mohsen Gavahiana et al, Y.-H. C. A. M. K. F. J. B. N. M., 2018. A critical analysis of the cold plasma induced lipid oxidation in foods. *Trends in Food Science & Technology,* Issue 77, pp. 32-41.

Peter Paulsen et al, I. C. A. B. K. H. B. P. W. K. S. N. N. J. W. E. M. F. J. M. S., 2022. Treatment of Fresh Meat, Fish and Products Thereof with Cold Atmospheric Plasma to Inactivate Microbial Pathogens and Extend Shelf Life. *MDPI,* Volume 11, p. 21.

Qian-Yun Han et al, X. W. J.-Y. g. C.-S. Z. a. Y.-Y. N., 2022. Application of plasma-activated water in the food industry: A review of recent research developments. *Food Chemistry,* Volume 405, p. 15.

Salma Farooq et al, A. H. D. K. K. D. S. S. V. K. P. W. S. A. R. P. S. M. M. K., 2023. Cold plasma treatment advancements in food processing and impact on the physiochemical characteristics of food products. *Food Science and Biotechnology,* Volume 32, pp. 621-638.

Samal, S., 2017. Thermal plasma technology: The prospective future in material processing. *Journal of Cleaner Production,* Volume 142, pp. 3131-3150.

Shi-Qing Wang et al, G. H. Y. L. J. X. Y. Z. a. W. J., 2015. Degradation of aflatoxin B1 by low-temperature radio frequency plasma and degradation product elucidation. *Eur Food Res Technol,* Volume 241, pp. 103-113.

Xiang-Yu Wang et al, Q.-H. Z. C. W. Y.-L. Z. B.-B. T.-Y. X. K. O. L. R. L. M. L. Q.-G. Z. G.-J. L. J. L. Y.-Z. M. &. Y. W., 2023. Unusual shrinkage and reshaping of Earth's magnetosphere under a strong northward interplanetary magnetic field. *Communications earth & enviroment,* p. 8.

Zhao, Y. M., Oliveira, M., Burgess, C. M., Cropotova, J., Rustad, T., Sun, D. W., & Tiwari, B. K. (2021). Combined effects of ultrasound, plasma-activated water, and peracetic acid on decontamination of mackerel fillets. Lwt, 150(September 2020), 111957.