CHEMICAL EFFECTS OF COLD ATMOSPHERIC PLASMA ON FOOD NUTRIENTS

Juan Manuel Pérez Andrés
Technological University Dublin

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CHEMICAL EFFECTS OF COLD ATMOSPHERIC PLASMA ON FOOD NUTRIENTS

Juan Manuel Pérez Andrés

Thesis submitted to Technological University Dublin in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

School of Food Science and Environmental Health

Technological University Dublin

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Prof. Patrick Joseph Cullen

Prof. Brijesh Tiwari

November 2020
Sense el teu esforç, el teu sacrifici i la teua lluita tot això no haguera sigut possible. Segur que papà estaria molt orgullós.

*Enhorabona Dra. Carmen Andrés Bort*
Abstract

A range of nonthermal techniques have demonstrated process efficacy in ensuring food product safety, extension of shelf-life and in general a retention of key quality attributes. However, various physical, chemical and biochemical effects of nonthermal techniques on both macro and micronutrients are evident, leading to both desirable and undesirable changes in food products. It is important to outline the effects of non-thermal techniques on food chemistry and the associated degradation mechanisms with the treatment of foods. Oxidation is one of the key mechanisms responsible for undesirable effects induced by non-thermal techniques. Degradation of key macromolecules largely depends on the processing conditions employed. Various extrinsic and intrinsic control parameters of high pressure processing, pulsed electric field, ultrasound processing and cold atmospheric plasma on chemistry of processed food is outlined.

Currently, cold atmospheric plasma (CAP) is a novel processing technology, which has demonstrated its ability for food product decontamination, gaining the interest of the food industry. Mackerel is a highly consumed fish due to its rich content of fatty acids and high nutritional values. The effect of cold atmospheric plasma on the shelf-life stability of lipids and proteins of commercially packaged mackerel fillets was studied. The results showed no significant effects on lipid oxidation between control samples and those treated at 80 kV for extended treatment times of 5 minutes using a dielectric barrier discharge system. In addition, no significant modification (p>0.05) was found for the fatty acid composition and nutritional values after treatment. Finally, protein oxidation was not encouraged by the plasma treatment between treated and untreated samples (p>0.05). These results suggest that cold atmospheric plasma could be employed as a microbial decontamination tool for mackerel fillets without impacting key quality parameters.
Proteins, as food ingredients, are employed in the food industry, not only for their high nutritional value, but also because of their techno-functional properties. Modifications of their native structure, from the action of external factors such as pH, temperature or processing by emerging technologies, can lead to a change of their functionality; and consequently, their applicability. It has been reported that CAP could lead to modification of food components such as proteins. The effect of CAP on the techno-functional properties of two common food ingredients (haemoglobin and gelatine from pork), and a novel source of functional proteins extracted from a meat co-product (bovine lung protein) was investigated, where significant effects were found for their functional, rheological and gelling properties.

Cholesterol is an important component in meat products, but is susceptible to oxidation leading to the possible formation of toxic compounds. The oxidation of cholesterol can be caused by auto-oxidation, photo-oxidation and thermo-oxidation, as well as, oxidation of other food components in the same matrix such as polyunsaturated fatty acids (PUFAs). The impact of in-package cold plasma technology on the cholesterol and other lipid stability of four different types of meat (beef, pork, lamb and chicken breast) was investigated. CAP was not found to have any impact on the samples’ cholesterol content; however, it did accelerate the oxidation of other lipids.

The effects of CAP on the content of two fat-soluble vitamins (K and E) and biogenic amines formation in two different fish products, with high (Atlantic mackerel) and low (haddock) fat content was studied over storage period of 7 days at 4°C. Plasma treatment resulted in a significant reduction in the α-tocopherol content of the mackerel but not in haddock after 7 days of storage. Moreover, the treatment caused a reduction in δ-tocopherol in mackerel after 5 min treatment and haddock after 10 min treatment. The vitamin K content was not significantly affected by the CAP treatment. The CAP treatment resulted in accelerated growth
of most biogenic amines, including histamine and tyramine. The CAP treatment can lead to reduction in vitamin E content and accelerated biogenic amines formation of fish fillets.
Declaration page

I certify that this thesis which I now submit for examination for the award of Doctor of Philosophy (PhD), is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for graduate study by research of the Technological University Dublin and has not been submitted in whole or in part for another award in any other third level institution.

The work reported on in this thesis conforms to the principles and requirements of the TU Dublin's guidelines for ethics in research.

TU Dublin has permission to keep, lend or copy this thesis in whole or in part, on condition that any such use of the material of the thesis be duly acknowledged.

Juan Manuel Pérez Andrés

10 November 2020
Acknowledgment

Thank you very much to everyone who helped me not to give up and to finish this endless journey.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>AAS</td>
<td>$\alpha$-aminoadipic</td>
</tr>
<tr>
<td>AI</td>
<td>Atherogenicity index</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAP</td>
<td>Cold Atmospheric Plasma</td>
</tr>
<tr>
<td>CCP</td>
<td>Capacitively Coupled Plasma</td>
</tr>
<tr>
<td>CCBG</td>
<td>Coomassie Brilliant Blue G-250 (CCBG)</td>
</tr>
<tr>
<td>CLA</td>
<td>Conjugated Linoleic Acid</td>
</tr>
<tr>
<td>DBD</td>
<td>Dielectric Barrier Discharge</td>
</tr>
<tr>
<td>DNPH</td>
<td>Dinitrophenylhydrazine</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic Acid</td>
</tr>
<tr>
<td>ECR</td>
<td>Electron Cyclotron Resonance</td>
</tr>
<tr>
<td>EGTA</td>
<td>Egtazic Acid</td>
</tr>
<tr>
<td>ELP</td>
<td>Extracted Lung Proteins</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>FID</td>
<td>Flame Ionisation Detector</td>
</tr>
<tr>
<td>FOS</td>
<td>Fructooligosaccharides</td>
</tr>
<tr>
<td></td>
<td>elastic/storage ($G'$) modulus and the viscous/loss modulus ($G''$)</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GGS</td>
<td>$\gamma$-glutamic</td>
</tr>
<tr>
<td>GLM</td>
<td>General Linear Model</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HMF</td>
<td>Hydroxymethylfurfural</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxy-2-nonenal</td>
</tr>
<tr>
<td>HPP</td>
<td>High Pressure Processing</td>
</tr>
<tr>
<td>HVCAP</td>
<td>High Voltage Cold Atmospheric Plasma</td>
</tr>
<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma</td>
</tr>
<tr>
<td>ISTD</td>
<td>Internal Standard</td>
</tr>
<tr>
<td>LED</td>
<td>Light Emitting Diodes</td>
</tr>
<tr>
<td>LDP</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LGC</td>
<td>Lowest Gelation Concentration</td>
</tr>
<tr>
<td>Mb</td>
<td>Myoglobin</td>
</tr>
<tr>
<td>MD</td>
<td>Microwave Discharge</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MW</td>
<td>Microwave Heating</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated Fatty Acid</td>
</tr>
<tr>
<td>NACMCF</td>
<td>US National Advisory Committee on Microbiological Criteria for Foods</td>
</tr>
<tr>
<td>OHC</td>
<td>Oil Holding Capacity</td>
</tr>
<tr>
<td>PEF</td>
<td>Pulse Electric Fields</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>PP</td>
<td>Polypropylene</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PPO</td>
<td>Polyphenoloxidase</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RF</td>
<td>Radio Frequency</td>
</tr>
<tr>
<td>RMS</td>
<td>Root mean square</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated Fatty Acids</td>
</tr>
<tr>
<td>SI</td>
<td>Saturation index</td>
</tr>
<tr>
<td>TBARs</td>
<td>Thiobarbituric Acid Reactive Substances</td>
</tr>
<tr>
<td>Tg</td>
<td>Gelation Temperature</td>
</tr>
<tr>
<td>TI</td>
<td>Thrombogenicity Index</td>
</tr>
<tr>
<td>Tmel</td>
<td>Melting Temperature</td>
</tr>
<tr>
<td>TMCS</td>
<td>Trimethylchlorosilane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WHC</td>
<td>Water-Holding Capacity</td>
</tr>
</tbody>
</table>
Chapter 1: Cold atmospheric plasma

1.1- Plasma physics

1.1.1 Introduction

In 1922, the American scientist Irving Langmuir proposed that the electrons, ions and neutrons in an ionized gas could be considered as corpuscular material entrained in some kind of fluid medium and termed this medium “plasma”. Plasma is considered the fourth state of the matter. The term “plasma” refers to a partially or wholly ionized gas composed essentially of photons, ions and free electrons as well as atoms in their fundamental or excited states, which when considered in total possess a net neutral charge. Evidence of plasma can be found in the nature such as lightening, aurora or northern lights and stars (de los Arcos, 2011).

When a solid (1st state) is heated, bonds between molecules weakened and as consequence it melts forming a liquid. If temperature keeps increasing the liquid (2nd state) starts evaporating until a gas is formed (3rd state). Finally, plasma (4th state) is produced when a neutral gas is heated until some charged particles such as electrons and ions are formed.

Focusing on the atomic structure, an atom is composed by a nucleus, where neutrons and protons are agglomerated; and by different orbitals, where electrons are placed around the nucleus (similar to the planets around the Sun). Depending on the atomic weight of a given atom, the numbers of electrons and protons are different, and consequently, the number of orbitals (“layers”) will vary (Figure 1.1) (Atkins, De Paula, & Keeler, 2018).
From an electromagnetic point of view, nature tries to ensure a net force equal to 0, i.e. $\Delta G = 0$ (where $G$ is free energy of Gibbs). At this state it is said that atom is at its ground state, where atoms are stable and the total charge inside the atomic structure is zero. When a specific energy is applied, electrons from a lower layer can move to an upper one. This phenomenon is called excitation, and the electron will remain in this excited state during a certain time (life time ranging from ns to min) or until the source of energy is not applied anymore.

At that stage, the electron will return to its previous level (i.e. to its ground state), emitting this excess of energy as light with a specific wavelength (Atkins et al., 2018).

The energy needed to excite an electron depends on the properties of the atom and generally, it is an exclusive and specific quantity for each type of atom. For instance, the energy required to excite a hydrogen atom is different than the one required to have the same effect on an oxygen atom. When an atom is on its excited state is not stable, so its natural tendency is to make itself stable interacting with another atom in the proximity. If the energy supplied to the atom is too high, it may happen that the electron is released from the atom; in this case, the atom became ionised (in this example with positive charge, because one electron is missing). The fact that there are different ionic species with positive and negative charges coexist, having a strong electrostatic coupling, means that they try to electrically neutralize each other. This phenomenon is known as recombination. As a result, it has to be highlighted that to ensure a stable plasma formation, sufficient energy to be continuously applied to the system, resulting
in a positive balance between the population of excited atom and ionised particles, while counteracting the ground state and recombined ones (Atkins et al., 2018).

At atmospheric pressure gases are lowly ionised (around $10^3$ particles per cm$^3$) and they cannot be considered as plasma. To be considered as such the concentration of radical species has to be over $10^9$ charged particles per cm$^3$ (Dendy, 1995). Therefore, to produce plasma it is necessary to find a way to generate the required density of ionised ions, i.e., energy is required. From the basics, a gas is composed of free molecules that move freely around the available bulk volume. When energy is applied to these molecules they will start moving quicker and helicoidally, so the chances of a collision occurs increase as the energy increases. It is necessary to mention that there are two different kinds of collisions: elastic and inelastic. With the first one, when two particles collide, energy is transferred between the two of them and they move together as a unique entity. On the other hand, in the case that a collision happens in an inelastic way, an electron will be released from the atomic/molecular structure of one of the particles, leading to the formation of two new charged species: the free electron and the ionised particle, which both could interact with other particles of the environment (Atkins et al., 2018).

According with these assumptions, to generate plasma high amounts of energy are required. For instance, ten electron volts (eV) per cm$^3$ of gas have to be applied to create ionised species and free electrons, forcing atoms and molecules to collide each other to have over $10^9$ ionised particles in such a volume (F. F. Chen, 1984). Since achieving such temperatures is not practical, other energy sources need to be used. Consequently, the most common method to reach enough concentration of ionised particles is using an electric field to increase the formation of radical species and electrons, to be considered a plasma. Once a plasma has been generated, regardless of any further increases in the electric field intensity, and in spite of increased probability of more charged particles being created, it would will not change to another state of matter.
From a physics point of view, plasma must satisfy some of the following criteria. Even though, plasma is a state where positive, negative and neutrals particles are coexisting; plasma maintains almost perfect neutral charge balance. The fact that charged particles, like electrons and ions, are not static means that plasma is in a quasi-neutral state (Dendy, 1995). So, when evaluating the charges in a plasma a negligible difference in the charges might be observed, which is represented by “$\Delta$”.

$$-q_e n_e = q_i n_i \pm \Delta$$  

*(eq. 1.1)*

Where $\Delta$ is negligible, $q_e$ = electron charge; $n_e$ = number of electrons; $q_i$ = ion charge; $n_i$ = number of ions.

Any disturbance from the quasi-neutral equilibrium (i.e. increased values of $\Delta$) will set up electric fields in a plasma, due to the creation of zones with electrical potential difference. The electrons will move in response to these fields; but as they accelerate, they tend to overshoot the zero field position.

Collisions between particles are of the most relevance to create and maintain the plasma state. Once a particle is ionised, a process of interaction with the surrounding particles of opposite charge will commence. It has to be mentioned that, as a second condition that plasma must satisfy, interactions between individual charged particles must be insignificant compared to collective effects. This introduces the concept of Debye screening or shielding, which is the ability of a plasma to shield out electric potentials that are applied to it, avoiding the presence of any electric field in the body of the plasma forming an sphere called Debye Sphere (Figure 1.2), (F. F. Chen, 1984).
This condition of no electric field requires that the number of particles, also called plasma parameter ($\Lambda$) has to be much higher than 1 inside the Debye sphere, i.e, $\Lambda \gg 1$. This plasma parameter can be calculated as follows.

$$\Lambda = n\lambda_D^3, \quad (eq. \ 1.2)$$

Where, $\lambda_D$ is the Debye length, which is the radius where a particle can be affected by another one, and it is also a measure of the shielding distance or thickness of the sheath, which follows the next equation (F. F. Chen, 1984).

$$\lambda_D = \frac{v_{th}}{\sqrt{ne^2/m\epsilon_0}} \quad (eq. 1.3)$$

Where, $\lambda_D$ (Debye length), $ne$ = number of electron; $m$ – mass; $\epsilon_0$ – dielectric constant for vacuum; $v_{TH}$ – thermal velocity

Debye length is the radius of the Debye sphere corresponding to the limit where the phenomenon of quasi neutrality in plasma exists. Inside these sphere, a charged particle can feel attraction or repulsion for another particle (depending of its charge) following Coulomb forces (Atkins et al., 2018). Once one particle gets inside the Debye sphere of another one, a deviation of the trajectory could be observed on one or both of them. This deviation is considered as a collision too. Consequently, a collision does not have to be directly like two pool balls colliding together. The deviation of the trajectory will depend on the energy of each particle. It may also happen that one of them is moving too fast and it could penetrate the other’s Debye sphere without being perturbed following its own pathway (F. F. Chen, 1984).
However, when a collision happens ionisation is not guarantee. There is a minimum energy \( (E_{\text{min}}) \) necessary to get an extra charged particle, leading to a new atom being ionised.

\[
E_{\text{min}} = E_i \left( \frac{m+M}{M} \right) = E_i \left( 1 + \frac{m}{M} \right)
\]  
(eq.1.4)

Where \( E_{\text{min}} \) (minimum energy), \( E_i \) (ionization energy), \( m \) (mass of projectile) and \( M \) (mass of target)

If particle masses are very different (\( m \ll M \)), like electrons compared with ions, the energy required to have a collision is a slightly greater than the ionization energy \( (E_i) \), i.e, \( E_{\text{min}} \approx E_i \).

On the other hand, in the event of a collision between two particles with similar mass, i.e., \( m \approx M \), the minimum energy would be double of the ionization energy \( E_{\text{min}} \approx 2E_i \). This is the reason why it is necessary to have more electrons to guarantee more collisions and consequently more stable plasma.

As mentioned, the frequency of collision between particles within the plasma is a very important factor, since collisions are the only way to maintain the plasma within a feasible range of temperatures. Maxwell-Boltzmann distribution gives an idea about the speed of a specific kind of particles (electrons, ions, neutrals) of a plasma (F. F. Chen, 1984).

\[
f(u) = A \exp \left( -\frac{1}{2} \frac{mu^2}{KT} \right)
\]  
(eq.1.5)

where \( f \ du \) is the number of particles per \( m^3 \) with velocity between \( u \) and \( u + du \), \( \frac{1}{2} mu^2 \) is the kinetic energy, and \( K \) is Boltzmann’s constant, \( K = 1.38 \times 10^{-23} \text{ J/}^\circ\text{K} \).
In addition, the collision frequency is defined as: τ, which is the mean time between collisions, λ is mean free path, n₀ is the neutral density, <v> is the average velocity (Maxwell-Boltzman distribution, eq.5), and σ is the cross-section for electron-neutral collisions.

\[ f_c = \frac{1}{\tau} = \frac{\langle v \rangle}{\lambda} = n_0 \sigma \langle v \rangle \quad \text{(eq.1.6)} \]

Where τ (mean time between collisions), λ (mean free path), no (neutral density), <v> (average velocity), σ (cross-section for electron-neutral collisions).

Another important parameter in a plasma is the density of these particles: electrons (ne), ions (nix) and neutrals (nx). Density is a function of position and distance. For example, in a plasma jet (which is one of the available designs of plasma sources) when a plasma bullet is released from the nozzle it starts moving like a hard ball with nothing at all or just a minimum loss of particle density of plasma on its trajectory (d'Agostino et al., 2008). A completely different example could be a meteorite which loses plasma during its pathway through atmosphere, emitting luminosity. To develop a more efficient plasma system the conservation of plasma density needs to be observed, and it will depend on the gas nature, nozzle geometry and frequency of the energy source (d'Agostino et al., 2008). Also, temperature of each individual particle should be characterised in the plasma. Based on the temperature there are two kinds of plasma: thermal and cold plasma (de los Arcos, 2011). The first ones are more ionised and they are produced at high pressure, then collisions between heavy particles such as neutrals and ions and electrons are very frequent, and consequently their temperatures are the same (te = tix = tx). Cold plasma are characterised as not being in equilibrium between the temperatures of the components of plasma (te ≠ tix ≠ tx). In general, these are at atmospheric pressure and just a small percentage of the whole gas is ionised. In this kind of plasma the temperature of the electrons is extremely high (form 5,000 to 100,000 °C); but there are other heavier particles,
which are the main components, having a temperature near room temperature, therefore the temperature of the plasma is the same as the heavier particles.

There are three different zones inside the discharged (i.e. ionised) area that have to be distinguished:

- Bulk: where plasma is present. Central part of the plasma.
- Sheath: any area next to any surface where the number of ions and electrons are not the same, generating an electric field.
- Presheath: is where the different between particles density start occurring. It is between the bulk and the sheath.

Following all this information above, it is importance to consider the plasma sheath (Dendy, 1995). Imagine a system with two electrodes (one on the top and one at the bottom) and two non-conductive materials on the sides. Once the power supply has been switched on, at some stage, the ionisation of the gas will happen and later on the plasma will be created. In the bulk gas the amount of positive and negatives particles would be the same so there would be no electric field.

![Common plasma system configuration.](image)

However, when these charged particles interact with a surface (any of the edges on the system) they could create an electric field, because this area close to the surface would have a greater quantity of positive or negative ions, creating a “charged area” called a sheath. (Figure 1.3) (Dendy, 1995).

Because of electrons are much lighter than ions and neutrals, they will reach the surface of the non-conductive materials before the heavier particles, consequently the electrons will collide
with this surface that will become negatively charged. The phenomenon when an ion is attached to a surface is called etching (d'Agostino et al., 2008). Then, this new negative surface would attract positive charged particles according with Coulomb law and at the same time, it would repeal all the electrons from this area. Therefore, a higher density of electrons would be present in the middle of the chamber and more positive charges would be found in the edges (Figure 1.4). By the time, when positive charges would attach to the surfaces, neutralizing them, electrons would be able to start moving again to the surface and charging it.

![Figure 1.4: Plasma-sheath species.](image)

This process makes kind of a dynamic cycle of particles movement, and as a result, the width of the sheath varies during the time (repeating increasing and decreasing cycles) depending on the frequency of the source (N. Misra, O. Schlüter, & P. J. Cullen, 2016).

The unavoidable existence of the sheath, from a theoretical point of view, may have implications on how the plasma affects the surfaces of the material which are in contact with. The dimension of the volume where the gas is included governs the formation of the plasma along with the properties of the edge surface, for instance, if they are conductive or non-conductive material. In the event that another external object is introduced inside the chamber, a sheath would be also created on its surface and theoretically, plasma would not be interacting directly with it because the quasi-neutral conditions would not be satisfied. However, in spite
of the new sheath being around the introduced object, it does not mean that all the radical species generated in the plasma body have an interaction with this object.

The size of the sheath, along with the frequency of the energy source, also depends on the voltage. Once plasma is generated by increasing the voltage it does not mean that more radical particles are generated in the bulk because there is not more available ionisable particles in the gas volume, and also there is not enough room to separate the negative and positive particles to reach a quasi-neutrally plasma (Dendy, 1995). For instance, if 40 kV of energy are applied to an specific system and plasma is created, the act of increasing the voltage to 80 kV it does not imply that more charged particles are generated, because only the energy of the particles is higher and also because of the limitation in space of the system to allow more collisions. In addition, there is the possibility that the impact of the ions to a specific surface creates ions inside the structure of this surface that can remain there, an etching effect, creating an electric field until such ions are neutralised. The formation of ions is related with the release of electrons. Then, the density of electron in the bulk will increase and it can be measured. Ions arrive at the sheath edge with a finite drift velocity (Bohm velocity) which can be calculated:

$$v_b \geq \sqrt{\frac{k_B \cdot T_e}{m_i}}$$

(eq.1.7)

Where \(v_b\) (Bohm velocity), \(T_e\) (electron temperature), \(m_i\) (ion mass) and \(k_B\) (Boltzmann constant)

According with the Bohm sheath criterion ions must enter the sheath region with a velocity greater than the acoustic velocity (F. F. Chen, 1984).

Plasma is usually generated using a power source and two electrodes. In standard conditions and at atmospheric pressure, gases are a good dielectric material, and it is necessary to apply a large potential difference to break the gas molecules down (d'Agostino et al., 2008). The
electrical breakdown creates several discharges which subsequently generate more in avalanches if there is still a power source. The generation of the plasma depends on the nature of the precursor gas, and characteristics of the energy source (X. Lu et al., 2016). Paschen’s law (eq.6) states that the breakdown voltage depends on the product of the pressure (p), the distance between the two electrodes (d), the constant related to the excitation and ionization (B), as well as type of the gas (F. F. Chen, 1984).

\[
V_b = \frac{B \cdot p \cdot d}{\ln\left[\frac{A \cdot p \cdot d}{\ln(1 + (1/\gamma))}\right]} \quad (eq.1.8)
\]

Where \(V_b\) (breakdown voltage), \(p\) (pressure), \(d\) (distance between the electrode)\(\gamma\) (second-electron emission), \(A\) (constant related to saturation ionization in the gas), \(B\) (constant related to the excitation and ionization).

When “p\(\cdot\)d” is small, the electrons’ mean free paths are longer than d, and large voltages are required to accelerate ions to energies that can release secondary electrons from the surface. When pd is large, electrons lose energy in collisions with the neutral gas, and the voltage rises again. The value of the “p\(\cdot\)d” product at the minimum of \(V_b\) of the Panchen curves are dependent on the gas type (F. F. Chen, 1984).

### 1.2-Plasma sources

In this section, the most common designs of plasma sources are explained:

#### 1.2.1-Capacitively coupled plasma (CCP):

CCP is one of the most common types of industrial plasma sources. It consists of two metal electrodes (cylindrical or flat) in parallel. One of two electrodes is connected to the power supply, and the other one is grounded. The source works in radio frequency (RF) in the range of MHz. The main advantage of this configuration is that the plasma generated is homogenous
and the temperature is lower than 50 °C. There are some devices that a coolant is installed to avoid high temperatures (usually it is a back flushing helium system). However, it is very difficult to control the number of ions generated (flux) and their velocity (energy), which can cause a problem. Another issue is the possibility of the radio frequencies to cause the phenomenon called “self-bias”. In CCP systems the frequency is high and if a sample is treated for a long period it can be charged and some of the molecules inside the matrix could be polarised.

1.2.2-Inductively coupled plasma (ICP):
ICP is a type of plasma source in which the energy is supplied by electrical currents which are produced by electromagnetic induction, that is, by time-varying magnetic fields. Imagine heating up a bottle of water without using a heating device only shaking. After a long period of shaking, it would reach a moment when the water would start boiling. This example is similar to how ICP works. These systems are characterised because they do not have electrodes, which is beneficial in terms of avoiding possible contaminations. There are two types of configurations for ICP systems: planar and cylindrical. In planar geometry, the electrode is a coil of flat metal wound like a spiral. In cylindrical geometry, it is like a helical spring.

1.2.3-Electron cyclotron resonance (ECR):
An ECR is similar to ICP, with a fast changing magnetic field but in this case the frequency is very high (2.45 GHz). This frequency is not a random value; it is the value of the frequency of oscillations of electrons. Plasma is generated using a microwaves source. The direct current magnetic field compress the plasma, pushing it to the centre of the gas container to avoid any contact with the surface.
1.2.4-Dielectric barrier discharge (DBD):
These devices can be made in many configurations, typically planar, using parallel plates separated by a dielectric material; or cylindrical, using coaxial plates with a dielectric tube between them. The two electrodes create a potential difference and align radical species. The process uses a high voltage alternating current, often at lower frequencies, but recently even at microwave levels. The main characteristic of this configuration is the fact that a dielectric material is added between the two electrodes. When observing an electrode at a microscopic level it is not completely flat and polished. In areas where there are deformations more free electrons will accumulate and when an electrical current is applied all the energy will go straight to this area creating arching, similar to a lightening during a storm. Adding a dielectric material to its surface, electrons can be orientated so all the surface will be charged homogenously generating a uniform plasma. Common dielectric materials include glass, quartz, ceramics and polymers.

1.3. Plasma applications

1.3.1-Current applications
Cold plasma has several applications in different industries. The first application of plasma was carried out by the material processing industries. Cold plasma is used for etching of semiconductors as well as the plasma chemical vapour deposition (Ostrikov, Neyts, & Meyyappan, 2013). Nowadays, due to cold plasma generates a broad of species (atoms, molecules and radicals), in a large number of different energetic states (metastable, excited, ionised and ground states) cold atmospheric plasma (CAP) is used for the synthesis of nanomaterials. All these species can be applied on an existing nanomaterial or during the synthesis of a new ones, causing an assembly of solid objects with quite different atomic
arrangements, and consequently, this in turn will lead to the different properties of these nano-solids (Ostrikov et al., 2013).

Cold plasma is used in the textile industry to alter the surface characteristics of fabrics to improve their properties, including surface cleaning, adhesion, modification of surface topography and surface energy (Abd Jelil, 2015). For instance, plasma can sterilise textile which are susceptible of hosting pathogenic bacteria, which can lead to several problems such as odour generation, strength deterioration as well as health issues (Szulc et al., 2018). In addition, depending on the type of textile treated, plasma can modify their surface improving their wettability or water-repellent ability, wickability, UV-protection, anti-felting, shrink and wrinkle resistance properties and flame retardancy. Moreover, it can influence some physical properties of the fabrics such as pilling resistance, yellowness and whiteness index, loss of whiteness, water-vapour and air permeability, thermal properties and fabric hand properties, and this without the use of water or chemicals (Abd Jelil, 2015).

Plasma has been also used in medical treatments. It can be applied directly to the skin, organs, tissues and living cells, for therapeutics purposes in oncology, dentistry, dermatology and endoscopy (Von Woedtke, Reuter, Masur, & Weltmann, 2013). CAP treatment can help tissue regeneration and wound healing due to a stimulation of cell proliferation and angiogenesis, and moreover, it can initiate the death of cancer cells (Weltmann & Von Woedtke, 2016). Different plasma sources such as surface dielectric barrier discharge (DBD) and plasma jet configurations had been tested depending on the application required (Kaushik et al., 2019). In addition, plasma can be used for the decontamination of surfaces and in the biomedical industry for different applications (Kaushik et al., 2019).
1.3.2-Meat and fish industry

Food poisoning events are widespread globally for a wide variety of food types. There are more than 72 million people experiencing a high level of food insecurity globally (Dury, Bendjebbar, Hainzelin, Giordano, & Bricas, 2019). Food safety is one of the most important concerns of the food industry, whose objective is to offer their customers a product which is safe without affecting the nutritional value or organoleptic quality, i.e. safe and high quality products. In the last few years, cold atmospheric plasma (CAP) has gained interest within the food sector because of its potential applications for decontamination of food products (Mandal, Singh, & Singh, 2018). Cold plasma treatment helps the preservation of the treated food working at ambient or sub-lethal temperatures (i.e. temperatures below the limit of pathogen survival), thereby minimizing negative effects on nutritional and quality parameters associated with thermal treatments (Awuah, Ramaswamy, & Economides, 2007). Moreover, it is environmentally friendly, with a low energy consumption when compared to traditional processing thermal technologies (Rodriguez-Gonzalez, Buckow, Koutchma, & Balasubramaniam, 2015).

CAP has potential for the meat industry, due to its remarkable characteristic that it can efficiently inactivate bacteria, moulds, biofilms, yeasts, spores, and other hazardous microorganisms, including potential bio-terrorism agents in meat products (N. Misra & Jo, 2017). For instance, Noriega, Shama, Laca, Díaz, and Kong (2011) studied the efficiency of a plasma jet to decontaminate *Listeria innocua* on chicken muscle and chicken skin, reporting 3 log reductions for muscle after 4 min of treatment and 1 log reduction for skin after 8 min of treatment. The amount of *Listeria innocua* was also reduced on dry-cured beef using a dielectric barrier discharge (Rød, Hansen, Leipold, & Knøchel, 2012). The effect of plasma in chicken fillets was also studied by JM Wang, Zhuang, Lawrence, and Zhang (2018) using a dielectric barrier discharge system. They reported a significant reduction in both mesophile and
psychrophile microorganisms, by applying 3 minutes of treatment at 80 kV, after 3 days of storage at 4 °C. Lis et al. (2018) investigated the inactivation of Salmonella typhimurium and Listeria monocytogenes using a circular plasma system on a ready to eat meal. The authors applied the plasma to rolled fillets of ham which were previously inoculated with these two bacteria strains, finding a significant reduction after treatment. In another experiment, pork sides were packaged under three different atmospheres (I: 20% O₂ + 60% N₂ + 20% CO₂, II: 40% O₂ + 40% N₂ + 20% CO₂ and III: 60% O₂ + 20% N₂ + 20% CO₂) and treated at 85 kV for 1 minute using a dielectric barrier discharge system (M. Huang et al., 2019). Samples were stored and the total viable aerobic counts were analysed at 0, 4, 8 and 12 days. They reported that there was a significant reduction between treated and control samples for all the conditions and storage times. The content of another pathogen commonly present on meat, Campylobacter jejuni, was significantly reduced on chicken breast and skin after plasma treatment using a plasma jet device after just 30 seconds (Rossow, Ludewig, & Braun, 2018). The population of Aspergillus flavus, Escherichia coli O157:H7, Salmonella typhimurium, and Listeria monocytogenes on beef jerky was significantly reduced after plasma treatment using a dielectric barrier after 2.5 minutes and it kept decreasing with increasing treatment times of 5 and 10 minutes (Yong et al., 2017). Chicken breasts packaged in trays under normal (air) and a modified atmosphere (5% N₂, 30% CO₂ and 65% O₂) were treated at 80 kV during 180 seconds using a dielectric barrier discharge device (Jiamei Wang, Zhuang, Hinton Jr, & Zhang, 2016). Significant reduction on the content of Pseudomonas Spp, psychrophiles and mesophiles was found only for the treated samples under modified atmosphere. It is suggested that the head space composition had an impact on the efficiency of the cold plasma technology due to the formation of different species that could lead to different reactions.

However, any processing technology for food decontamination could potentially affect one or more of the food components and associated quality parameters. For instance, some other
non-thermal technologies such as pulsed electric field (PEF), high processing pressure (HPP) and ultrasound (US), can lead to undesirable effects, such as lipid and protein oxidation (Tokuşoğlu & Swanson, 2014). In the specific case of cold atmospheric plasma, the different radical species present in its atmosphere could also cause some negatives changes on the quality of food products (H.-J. Kim, D. Jayasena, H. Yong, & C. Jo, 2016). For instance, in some of the studies previously mentioned, such as M. Huang et al. (2019) microbiological reduction was found after treatment on pork, however protein and lipid oxidation was accelerated. The levels of lipid oxidation was also higher after plasma treatment in beef jerky (Yong et al., 2017), dry-cured beef (Rød et al., 2012) and bacon (B. Kim et al., 2011) after their respective CAP treatments described above, in spite of all of them reporting an extension of the shelf-life for the treated samples. In another experiment, two antioxidant such as ethanolic coconut husk extract and ascorbic acids were added separately at two different concentrations (100 ppm and 200 ppm) to some packaged Asian sea bass (90% argon and 10% oxygen) before plasma treatment to avoid possible oxidation (Olatunde, Benjakul, & Vongkamjan, 2019a). After 5 minutes treatment at 16 kV, they reported a reduction of the population of total viable count (TVC) and psychrophilic bacteria count (PBC), though, they found that CAP induced significantly lipid and protein oxidation, regardless the addition of antioxidant.

1.4. Aim and objective of the thesis

Before scaling up cold atmospheric plasma processing technology, it is necessary to optimise this technology to find a balance between safety and quality. Hence, it is important to understand the possible detrimental effects of this technology on the different food components (lipids, proteins and vitamins) to avoid significant negative effects as much as possible.
The objective of this thesis is to investigate how cold atmospheric plasma may affect some of the main macro and micronutrients in fish and meat products. To reach these objectives different studies were carried out:

1. Study 1: Review on chemical modifications of lipids and proteins by non-thermal food processing technologies
2. Study 2: Effects of cold atmospheric plasma on mackerel lipid and protein oxidation during storage.
3. Study 3: Effect of cold atmospheric plasma on the techno-functional properties of model animal proteins used as ingredients
4. Study 4: Effect of cold plasma on meat cholesterol and other lipid fractions.
5. Study 5: Stability of mackerel and haddock fish micronutrients with cold plasma treatments.
Chapter 2: Chemical modifications of lipids and proteins by non-thermal food processing technologies

2.1-Introduction

Classical thermal technologies are based on the use of heat to extend shelf-life and ensure product safety by inactivating spoilage enzymes and microorganisms. Techniques such as thermal sterilization and pasteurization are a cornerstone of food processing. In these cases, heat is generated by electrical resistance or combustion which is transferred to the product. These technologies require relatively high energy which results in high costs and consequently are not environmentally friendly. Use of novel thermal technologies are rapidly emerging, offering greater efficiency and process control, including; ohmic heating and dielectric heating, which includes radio frequency (RF) and microwave heating (MW). Such techniques have demonstrated process efficacy in ensuring product safety, extension of shelf-life and good retention of critical quality attributes along with providing a more sustainable food processing sector (Sakr & Liu, 2014). The main difference from the traditional techniques is that the heat is generated directly inside the product, allowing a reduction of heat/energy loss, leading to lower costs and greener solutions (Pereira & Vicente, 2010). However when a product is heated, even to moderate temperatures, flavours, essential nutrients and vitamins can be modified (Awuah et al., 2007; Butz & Tauscher, 2002).

Alternatives to classical and novel thermal techniques are a range of technologies collectively called “non-thermal technologies”. These technologies are effective at ambient or sub-lethal temperatures, thereby minimising negative thermal consequences. High pressure processing, pulsed electric field, cold plasma and ultrasound processing are the leading non-thermal...
technologies (Jermann, Koutchma, Margas, Leadley, & Ros-Polski, 2015; Knorr et al., 2011). They can inactivate both pathogenic and spoilage microorganisms associated with food, resulting in extensions of shelf-life with microbiological safety profiles. The potential and adoption of such non-thermal treatments has been further expanded by regulatory agencies increasingly acknowledging their demonstrated efficacies (P. J. Cullen et al., 2018). Of note here is the expansion of the definition of pasteurization beyond solely a thermal treatment by the NACMCF (the US National Advisory Committee on Microbiological Criteria for Foods Adopted August 27, 2004 Washington, DC) to include any treatments which can “reduce the most resistant microorganism(s) of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage”. Apart from their use as a single intervention technology, several studies have shown that such technologies used along with conventional techniques can assure food safety with limited impacts on the food quality. For instance, ultrasound-assisted hot air drying can reduce the drying time of strawberries in the range of 13 – 44 %, thus moderating the damage on food quality (Gamboa-Santos, Montilla, Carchel, Villamiel, & Garcia-Perez, 2014). In the context of sterilisation, using high pressure together with mild or high temperatures treatments to inactivate bacterial spores have also shown benefits (Reineke et al., 2012). Comparably, a combination of non-thermal technologies is also proposed (hurdle approach), to achieve effective microbial inactivation whilst mitigating negative effects on product quality. In order to meet growing consumer demand for high quality food, it is necessary to understand the mechanisms of action driving these potential technologies and the response of food chemistry to such processes. Applications of novel thermal and non-thermal technologies have been reviewed extensively covering various aspects of food quality and safety (Ling, Tang, Kong, Mitcham, & Wang, 2015; Pinela & Ferreira, 2017).
However, the effects of non-thermal techniques on food chemistry and the associated degradation mechanisms have not been reviewed to date. The objective of this work is to review the effects of four of the leading non-thermal technologies namely; high pressure processing, pulse electric fields, ultrasound and cold plasma, on biomolecules associated with food quality, focusing on lipids and proteins.

2.2-High pressure processing

High-pressure processing (HPP) is a method of food processing where food is subjected to elevated pressures (up to 900 MPa). HPP is the leading non-thermal technology in terms of research to date, consumer and regulatory acceptance and industrial adoption with a wide range of food products on the global market. HPP technology has been reviewed extensively highlighting the range of applications it can offer in the food industry, assessed alone or in combination with conventional techniques (Balasubramaniam, Martinez-Monteagudo, & Gupta, 2015; Barba, Terefe, Buckow, Knorr, & Orlien, 2015; Reineke et al., 2012). HPP is an efficient non-thermal technology to inactivate a wide variety of pathogenic and spoilage vegetative cells, yeasts, mould, spores and viruses associated with food products (Daryaei, Yousef, & Balasubramaniam, 2016; Kingsley, 2013). Intrinsic food parameters governing process efficacy include water activity, pH and composition of food such as fats and oils (Georget et al., 2015). It is known that compression increases the temperature of the food by approximately 3 °C/100 MPa (Butz & Tauscher, 2002) and potentially up to 8.7 °C/100 MPa if the samples have high levels of fats and oils (Rasanayagam et al., 2003). The rapid increase in temperature during compression and subsequent cooling upon decompression is a unique benefit of high pressure-based technologies to reduce product thermal exposure during treatment (S. Martinez-Monteagudo & Balasubramaniam, 2016).
Pressure can affect the physical properties of the food matrix such as the superficial tension, density, viscosity, dipolar moment, dielectric constant, and thermal properties; as well as equilibrium processes including ionization, dissociation of weak acids, and acid-base equilibrium (S. I. Martinez-Monteagudo & Saldana, 2014). Moreover, high pressures can impact the rate of these reactions by delaying or accelerating them. In addition, HPP can modify the pH of the environment as it enhances the formation of ions from ionisable substances. A change in pH can affect protein denaturation, growth of microorganisms and the kinetics of chemical reactions (S. I. Martinez-Monteagudo & Saldana, 2014). Even if the temperatures applied are considered as low, high pressure processing technology can affect various nutrients and bioactive molecules. For example, high weight molecules such as proteins are formed by Van der Waals forces, hydrogen and hydrostatic bonds which are weak, which can be affected by HPP. However, lower molecular weight molecules like vitamins are basically formed by covalent bonds, are typically sufficiently strong to withstand HPP conditions.

One of the most common reactions associated with food is the oxidation of lipids. It results in a modification of colour, flavours, functional properties, nutritional values and may lead to the formation of toxic sub products (Schaich, 2005). First, a free alkyl is formed by removing a hydrogen atom from the α-methylene group of a fatty acid. This initiation step is strongly encouraged by heat, light or by the presence of metal ions and enzymes initially present in the food. The second step is called propagation. The free radical formed being highly reactive reacts with molecular oxygen to form a lipid peroxyl radical. This in turn can react with other fatty acids and generate hydroperoxide and further free radicals. Finally, this new free radical can reinitiate this process with other fatty acids. This chain reaction mechanism stops when two free fatty acids radicals react and create a non-radical, which can happen after 10 to 100 cycles. The termination step can also occur under the presence of antioxidant molecules (vitamin E, vitamin C, catalase etc.) which can neutralize free radicals (Morrissey, Kerry, &
Lipid oxidation is commonly measured using the TBARs method (Thiobarbituric Acid Reactive Substances), expressed in milligrams of malonaldehyde (MDA) per kilogram of sample (Ghani, Barril, Bedgood, & Prenzler, 2017). According to Connell (1990), TBAR values of 1-2 mg MDA/ kg sample is the range of acceptability of odour/taste in fish.

The effects of high pressure on lipid oxidation have been investigated (L. G. Medina-Meza, Barnaba, & Barbosa-Canovas, 2014). High pressure should not initiate lipid oxidation, as the heterolytic cleavage to form the free radical is not favoured by increases in pressure. However, the formation of covalent bounds during the propagation steps could be encouraged by pressure. Cheftel (1995) observed that at values above 350 MPa, sarcoplasmic and myofibrilar proteins were denatured and myoglobin and oxymyoglobin converted to the denatured ferric form. As a consequence of these transformations, lipid oxidation was catalysed. Orlien, Hansen, and Skibsted (2000) found that lipid oxidation levels depend more on the applied pressure than on the processing time, and suggested that lipid oxidation is due to damage of the cell membrane which could lead to the release of free radicals or their precursors. Bolumar, Skibsted, and Orlien (2012) applied a range of pressures for different treatment times and temperatures (5, 25, and 40 °C) and observed that increasing these parameters raised the production of free radicals, thus encouraging lipid oxidation which may be due to synergistic effects of high pressure and temperature. In addition, they established thresholds for radical formation of 400 MPa at 25°C and at 500 MPa at 5 °C. Bolumar, Andersen, and Orlien (2014) suggested that HPP induced the formation of free radicals either by an iron-catalysed Fenton’s reaction mechanism, or by the formation of protein-derived radicals. Reddy, Jayathilakan, Chauhan, Pandey, and Radhakrishna (2015) applied 300 and 600 MPa over 5 and 10 minutes on raw chevon samples followed by storage at 4 °C for a month and reported a significant increase of lipid oxidation at 600 MPa during the storage period. In a similar study, Q. Wang...
et al. (2013) stored yak fat at 4 °C and 15 °C during 20 days after being HHP treated at 0.1, 100, 200, 400 and 600 MPa. Lipid oxidation was observed to increase with a rise in pressure, storage temperature and treatment time. Indeed, the TBARS values obtained were much higher at 400 and 600 MPa compared with 200 MPa, revealing higher rates of lipid oxidation. These results match with the findings of the researchers cited previously, suggesting that lipid oxidation is encouraged after a pressure of 300-400 MPa. These results also agree with the Kaur, Rao, and Nema (2016) study on black tiger shrimps, where a significant increase of lipid oxidation was observed after high pressure treatments, however the MDA values remained acceptable for treatments above 300 MPa. Fuentes, Utrera, Estevez, Ventanas, and Ventanas (2014) studied the influence of intramuscular fat content on lipid oxidation after high pressure treatment. They applied 600 MPa on two different parts of a dry-cured ham, namely the flank (lower fat content) and the hip (higher fat content), under subsequent storage at 2 °C over 120 days. The TBARs values obtained were higher for the samples analysed immediately after treatment in the flank samples. This could be due to the fact that most of the fat content in the flank samples were unsaturated which are more reactive and easier to oxidise. However, at the end of the storage period, the hip samples were more susceptible to oxidisation as the lipid concentration was higher. Conversely, several studies report no significant effects of HPP on lipid oxidation, for example a storage study of 30 days on dried fermented sausages after different pressure treatments (Alfaia et al., 2015). Similarly, Chouhan, Kaur, and Rao (2015) did not detect any significant effects of pressure on lipid oxidation immediately after applying 250 and 350 MPa for 10 min with hilsa fish (Tenualosa ilisha), but noted an increase of lipid oxidation during storage. No alteration was observed on the lipid compounds or fatty acids composition of cow milk after high pressure treatments from 250 to 900 MPa (Rodriguez-Alcala, Castro-Gomez, Felipe, Noriega, & Fontecha, 2015). A decrease of TBARs values were observed for treatments of 10 min at 300 MPa at 5 and 40 °C on salmon fillets (Ojagh, Nunez-
Flores, Lopez-Caballero, Montero, & Gomez-Guillen, 2011). Moreover, Lerasle et al. (2014) studied the influence of modified atmosphere packaging and high pressure treatment. They prepared two batches of raw poultry sausage, one packaged with atmospheric air and one with a modified atmosphere composed of 50 % CO$_2$ and 50 % N$_2$, with both pressurised at 500 MPa for 5 minutes. After treatment the samples were stored at 4 °C over 22 days. They found that lipid oxidation was significantly encouraged by the storage time and package atmosphere, but not by the pressure, with the treated samples having higher TBARs values.

Protein structure is composed of covalent and hydrophilic bonds, electrostatic and Van der Waals interactions, which make its native structure stable over a specific range of pressures and temperatures. Outside these conditions protein can be denatured (Smeller, 2002). In addition, there is an intermediate state between the native and the denatured state which is called the molten globule state, where proteins can have specific functional properties that do not exist in any of the other two states (Elms, Chodera, Bustamante, & Marqusee, 2012).

High pressure causes physical compression of the sample, and consequently can modify the native structure of a protein, affecting solubility, gelation, emulsion, foaming and water holding capacities. A reduction in the volume results in a reduction or elimination of the voids initially present in the protein structure as the protein unfolds. Thus, as globular proteins have more cavities than fibrous proteins, they are less stable when a high pressure is applied (Damodaran, 2008). In addition, the hydration effect on a protein is related to a reduction of the volume caused by electrostriction around the ionic groups, hydrogen-bonded hydration around the polar groups and hydrophobic hydration (Gekko & Hasegawa, 1986). Proteins are usually denatured after applying a pressure from 100 to 1,200 MPa and the midpoint of pressure-induced transition occurs at 400 – 800 MPa (Heremans, 1982). It is known that high temperatures can irreversibly denature proteins by transferring nonpolar hydrocarbons from the hydrophobic core toward water. However, in the case of high pressure, protein denaturation
is initiated by the intrusion of water into the inner part of the protein matrix, which depends on the level of pressure applied (Knorr, Heinz, & Buckow, 2006). In general, below 200 MPa there are only conformational changes on the tertiary and quaternary structure of the proteins, which are constituted by weak bonds such as hydrogen and electrostatic bonds, hydrophobic and Van der Waals interactions. These modifications are reversible after treatment and the protein can recover its native structure. However, depending on the type of protein, pressures above 300 MPa can also affect the secondary structure, and consequently cause irreversible denaturation (Oey, 2016). All these changes cited on protein conformational structure can initiate various reactions, causing modifications of the food quality. In addition to the pressure and treatment time, these reactions could be encouraged by temperature, pH, ionic strength and the presence of other components in the food matrix such as metal ions and other free radicals.

One of the most common reactions where proteins are involved is oxidation. They can be oxidised following the same chain reactions as lipids. A free radical can interact with a protein, initiating the oxidation of the latter (Lund, Heinonen, Baron, & Estevez, 2011). Protein oxidation can be measured by the loss of sulphydryl groups (S-H) or by the formation of protein carbonyls. Sulphydryl or thiol groups are very reactive and sensitive to oxidation, leading to the formation of disulphide groups. In meat for instance, these disulphide bonds can form a gel-like network, thus changing the texture of the product (Omana, Plastow, & Betti, 2011). Protein carbonyls are the common product of protein oxidation. Carbonyls (aldehydes and ketones) can be formed in proteins through four different pathways, namely; i) direct oxidation of the side chains from lysine, threonine, arginine and proline, ii) non enzymatic glycation in the presence of reducing sugars, iii) oxidative cleavage of the peptide backbone via the α-amidation pathway or via oxidation of glutamyl side chains, and iv) covalent binding to non-protein carbonyl compounds such as 4-hydroxy-2-nonenal (HNE) or malondialdehyde (MDA) (Estevez, 2011). Some researchers have suggested that there could be a correlation between
lipid oxidation and protein oxidation, as both elements are present in the food matrix. The oxidation of both proteins and lipids are initiated by a free radical which can be derived from a lipid or protein, causing the oxidation of other lipids or proteins (C. Guyon, Meynier, & de Lamballerie, 2016; Lund et al., 2011). As HPP can encourage lipid oxidation, protein oxidation could also occur. For example, metmyoglobin Fe$^{3+}$ is formed after a pressure treatment is applied beyond 350 MPa, as a result of the oxidation of oxymyoglobin Fe$^{2+}$ (Cheftel, 1995). In addition, these researchers observed that a pressure of 200 MPa often causes protein gelation, giving a denser structure, which leads to an increase in texture. Similar results were obtained by Reddy et al. (2015) after applying 300 MPa and 600 MPa for fresh chevon. They studied the effect of high pressure processing on myoglobin, oxymyoglobin and metmyoglobin, which are proteins related to the colour of some food products. No changes were observed in myoglobin content. However, there was a correlation between the decrease of oxymyoglobin content and the increase of metmyoglobin content. As a result, a decolouration of the samples was more intense for higher pressures and longer times. Fuentes, Ventanas, Morcuende, Estevez, and Ventanas (2010) applied 600 MPa for vacuum packaged dry-cured ham using three different presentations: non-sliced vacuum-packaged dry-cured ham, dry-cured ham slices stretched out in the package and dry-cured ham slices piled horizontally. To study the protein oxidation, they analysed the formation of α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively) which are the results of different amino acids oxidation, such as proline, lysine and arginine. There was a significant increase of the content of these two protein carbonyls after pressurization. Moreover, they found that lipid oxidation was highly encouraged for the treated samples which depended on the type of packaging used. Ojagh et al. (2011) attempted to mitigate the negative effects of high pressure by covering salmon samples with a fish gelatin–lignin film. In this experiment, they compared the formation of protein carbonyls in samples with and without the film at 300 MPa during 5 minutes treatment.
at 5 °C and 40 °C. They found that protein oxidation increased significantly for all the raw samples. However, the values for the samples covered by the film were significantly lower than the uncovered samples. On the other hand, Cava, Ladero, Gonzalez, Carrasco, and Ramirez (2009) found that HPP does not affect protein oxidation in dry-cured ham and dry-cured loin. However, lipid oxidation increased in the dry-cured loin samples and decreased for the dry-cured ham after this treatment. High pressure did not have any effect on the concentration of thiol groups after 600 MPa of pressure treatment during 5 minutes on sarcoplasmic and myofibrillar proteins of brine enhanced pork semitendinosus (Grossi, Bolumar, Soltoft-Jensen, & Orlien, 2014). However, these values decreased during storage for sarcoplasmatic proteins, but not for myofibrilla proteins, where there was a formation of S-H groups. The explanation of this increase was based on Omana et al. (2011) experiments, who found an increase of S-H groups after a pressure treatment for chicken breast meat. They suggested that this increase was related to the unfolding state of the protein, where the majority of the available sulfhydryl groups were exposed to the environment and that most of the sulfhydryl groups in the inner core were already oxidised to form disulphide bonds. However, Grossi et al. (2014) observed in a similar study the formation of protein carbonyl immediately after treatment for sarcoplasmic proteins, but with degradation during storage. The results suggest that high pressure (above 600 MPa) could accelerate the decomposition of the carbonyl group formed after oxidation.

Another common reaction of food proteins is the Maillard reaction, where an amino group reacts with a carbonyl group which is initially present in the food matrix such as reducing sugars, osones, furfural, hydroxymethylfurfural (HMF) and pyrrole derivatives. As a result, the protein suffers a modification and brown polymers called melanoidins are formed. It is common in the food industry because it generates pleasant flavours and desired brown colour compounds in cooked foods. However, it can lead to the production of acrylamide, a potential
human carcinogen element, a result of the interaction between the amino acid asparagine and a reducing sugar (Poulsen et al., 2013). The Maillard reaction involves complex sequences of reactions, including condensation, cyclization, dehydration, rearrangement, isomerization and polymerization (S. I. Martinez-Monteagudo & Saldana, 2014). These reactions are influenced by temperature, metal ions, water activity and pH. Studies regarding the use of novel technologies to control the Maillard reaction are being currently carried out (Jaeger, Janositz, & Knorr, 2010). It is known that pH is sensitive to pressure, as a dissociation of ionisable substances can occur under high pressure conditions. Consequently, HPP could have an effect on the Maillard reaction (Hill, Ledward, & Ames, 1996). S. I. Martinez-Monteagudo and Saldana (2014) suggested that due to the complexity of the Maillard reaction, the effect of pressure should be evaluated for individual reaction steps to understand if pressure encourages or delays the overall reaction. Santos et al. (2015) proposed that high pressure accelerates the Maillard reaction for wine after a pressure treatment between 400-500 MPa for 5 minutes. The authors measured volatile compounds which are the products of the Maillard reaction, namely 2-furfural, benzaldehyde and phenylacetaldehyde, and they found an increase of their content after treatment. On the other hand, Campus, Flores, Martinez, and Toldra (2008) found a reduction of several flavour compounds from the Maillard reaction after pressure treatment. However, their content increased during storage, and they suggested that the presence of their precursors such as the substrates, free amino acids can continue reacting, leading to their formation. Figure 2.1 schematically outlines some of the key chemistries induced due to HHP.
Finally, carboxylation is another common reaction in food chemistry which is based on the removal of the α-carboxylic acid group of a free amino acid. The resultant amines are known as biogenic amines. The presence of these amines in food is of interest from a toxicological aspect and for their role as possible quality indicators (C. Ruiz-Capillas & Jimenez-Colmenero, 2004). This reaction consists of two mechanisms. The first is performed by a pyridoxal phosphate coenzyme, and the second is mediated by microorganisms which contain a covalently bound pyruvoyl residue on their active site (Eitenmiller & Desouza, 1984). There are few reported studies on the effect of high pressure on biogenic amines (Naila, Flint, Fletcher, Bremer, & Meerdink, 2010). For examples, biogenic amines content was reduced on Caciotta and Pecorino cheeses when raw milk was treated at 100 MPa before cheese production (Lanciotti et al., 2007). Reduction of these amines after pressurization was found as well for vacuum-packed trout flesh (Matejkova, Krizek, Vacha, & Dadakova, 2013). Simon-Sarkadi, Pasztor-Huszar, Istvan, and Kisko (2012) applied 500 MPa to sausage finding an inhibition of
the formation of two biogenic amines namely; cadaverine and putrescine, whereas, this
treatment encouraged the formation of tyramine and spermine. Spermidine and spermine were
found in soya milk and they reamined stable after 200MPa and 300 MPa pressurarization at
55,65 and 75 °C (Toro-Funes, Bosch-Fuste, Veciana-Nogues, & Vidal-Carou, 2014). However,
C. Ruiz-Capillas, Carballo, and Jimenez-Colmenero (2007) applied 400 MPa at 30° C for 10
minutes to frankfurters finding an increase of the biogenic amines content. Table 2.1
summerises some key factors and effects of HHP on food products reported in the literature.

Table 2.1: Effect of high pressure processing on chemical changes in food products

<table>
<thead>
<tr>
<th>Food product</th>
<th>Treatment</th>
<th>Changes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef loin and chicken</td>
<td>0.1, 200, 400, 600, and 800 MPa at 5, 25, and 40 °C for 5 and 10 min</td>
<td>Lipid oxidation: ↑400 MPa at 25 °C, 10’ ↑500 MPa at 5 °C, 10’</td>
<td>(Bolumar et al., 2012)</td>
</tr>
<tr>
<td>Fresh chevon</td>
<td>300 and 600 MPa at 28°C for 5 and 10 min</td>
<td>From 300 MPa at 28 °C, 5’ ↑ Lipid oxidation ↑Protein oxidation</td>
<td>(Reddy et al., 2015)</td>
</tr>
<tr>
<td>Yak</td>
<td>0.1, 100, 200, 400 and 600 MPa at 4 and 15 °C for 5 for 30 min</td>
<td>Lipid oxidation: ↑400 MPa at 4 and 15 °C, 30’</td>
<td>(Q. Wang et al., 2013)</td>
</tr>
<tr>
<td>Black tiger shrimp</td>
<td>0.1, 300, 400, 500 and 600 MPa at 27 °C for 3, 6, 9, 12 and 15 min</td>
<td>Lipid oxidation: ↑300 MPa at 27 °C, 12’ ↑400 MPa at 5 °C, 3’</td>
<td>(Kaur et al., 2016)</td>
</tr>
<tr>
<td>Dry-cured hams</td>
<td>600 MPa at 12 °C for 6 min</td>
<td>↑ Lipid oxidation</td>
<td>(Fuentes et al., 2014)</td>
</tr>
<tr>
<td>Dry fermented sausage</td>
<td>202 MPa at 10 °C for 960 s 260 MPa at 10 °C for 390 and 1530 s 400 MPa at 10 °C for 154, 960 and 1800 s 540 MPa at 10 °C for 390 and 1530 s 600 MPa at 10 °C for 960 s</td>
<td>No effect</td>
<td>(Alfaia et al., 2015)</td>
</tr>
<tr>
<td>Product</td>
<td>Conditions</td>
<td>Result</td>
<td>Source</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------------------------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Hilsa</td>
<td>250 and 300 MPa at 27°C for 10 min</td>
<td>= lipid oxidation</td>
<td>(Chouhan et al., 2015)</td>
</tr>
<tr>
<td>Salmon</td>
<td>300 at 5 and 40°C for 10 min</td>
<td>↓ Lipid oxidation ↑ Protein oxidation</td>
<td>(Ojagh et al., 2011)</td>
</tr>
<tr>
<td>Dry cured ham</td>
<td>600 MPa at 12 °C for 6 min</td>
<td>↑ Protein oxidation</td>
<td>(Fuentes et al., 2010)</td>
</tr>
<tr>
<td>Dry-cured ham and dry-cured loin</td>
<td>200 MPa and 300 MPa at &lt; 14°C for 15 and 30 min</td>
<td>↑ Lipid oxidation = Protein oxidation</td>
<td>(Cava et al., 2009)</td>
</tr>
<tr>
<td>Milk</td>
<td>100 MPa</td>
<td>↓ Biogenic amines content</td>
<td>(Lanciotti et al., 2007)</td>
</tr>
<tr>
<td>Trout flesh</td>
<td>300 and 500 MPa for 10 min at 20 °C</td>
<td>↓ Biogenic amines content</td>
<td>(Matejkova et al., 2013)</td>
</tr>
<tr>
<td>Soya milk</td>
<td>200MPa and 300 MPa at 55.65 and 75 °C</td>
<td>= Biogenic amines</td>
<td>(Toro-Funes et al., 2014)</td>
</tr>
<tr>
<td>Frankfurters</td>
<td>400 MPa at 30°C for 10 min</td>
<td>↑ Biogenic amines</td>
<td>(C. Ruiz-Capillas et al., 2007)</td>
</tr>
</tbody>
</table>

↑: Increase, ↓: Decrease, =: No effect

2.3-Pulsed Electric Fields

Pulse electric fields (PEF) are a group of non-thermal food process technology which have made the transition from the lab to the food industry (Mahnic-Kalamiza, Vorobiev, & Miklavcic, 2014; Puertolas, Alvarez, Raso, & de Maranon, 2013). This technology consists of the application of short electric pulses (1–100 μs) of high and low intensity electric fields. The food product is located between two electrodes inside a chamber and the pulse electric field is applied over a short period. Different PEF systems have been created and optimised for efficacy and scale depending on the application, and can be employed alone or in combination with thermal technology (K. Huang & Wang, 2009; Y. F. Liu, Oey, Bremer, Carne, & Silcock, 2017; Wu, Zhao, Yang, Yan, & Sun, 2016). The main two functions of this technology are inactivation of microorganisms and extraction of intracellular components. PEF is primarily based on a phenomenon called electroporation, which consists of the formation of pores on
cellular membranes. As a result, there is a transitory or permanent modification of the permeability of the cell membrane and typically limited increases in the bulk temperature. The effectiveness of the electro-permeability process depends on various parameters including; the characteristics of the PEF system (holding time, energy, temperature, frequency, electric strength, pulse shape and width), the food product (pH and conductivity) and the features of the bacteria cell (membrane, shape, size and envelope structure) (Puertolas, Luengo, Alvarez, & Raso, 2012). The process can damage the membrane leading to cell inactivation, as well as modification of the inner part of the cell and extraction of different substances. This process technology can effectively inactivate microorganisms which are related to the deterioration of food such as vegetative forms of bacteria, yeast, and moulds (Saldana, Alvarez, Condon, & Raso, 2014). The application of high-voltage pulsed electric fields can lead to some electrochemical reactions, affecting the quality of specific food products (Roodenburg, 2007). It is known that many chemically active species can be produced by an electric discharge in a food and also by electrode reactions with the product, such as decomposition of the chemical structure of liquids close to the electrode surfaces (electrolysis), eventually producing toxic chemical species, such as oxygen peroxide, hydroxyl radicals, or chloride ions (Mañas & Vercet, 2006; Matser, Schuten, Mastwijk, & Lommen, 2007).

Arroyo et al. (2015) studied the lipid oxidation of fresh and frozen turkey breast treated by PEF. In this experiment, three different; frequencies (10, 55 and 110 Hz), pulses (100, 200 and 300 pulses) each with a pulse width of 20 μs, and voltages (7.5, 10, 12.5 kV – fresh samples, 14, 20 and 25 kV – frozen samples) were tested. No significant differences were observed for the MDA values in both batches. Cortes, Esteve, Frigola, and Torregrosa (2005) noted a partial inactivation of peroxidise activity and no modification of the TBARs values after PEF treatment of horchata samples. These findings are in agreement with those of Suwandy, Carne, van de Ven, Bekhit, and Hopkins (2015), who concluded that PEF does not induce lipid
oxidation of beef. Peanut oil was treated by PEF using a square-wave pulse generated with a pulse duration (τ) of 40 μs and pulse frequency (f) of 1008 Hz (Zeng, Han, & Zi, 2010). The authors suggest that this technology could restrain the rate of the lipid oxidation reaction thus extending the shelf-life of lipid rich products.

Additionally, Ma et al. (2016) found that PEF did not affect lipid oxidation immediately after treatment on three different parts of chilled and frozen–thawed cooked lamb (shoulder, rib and loin). However, after 7 days of storage the MDA values of the treated samples were higher than the control, but still acceptable from a quality point of view (lower than 2 mg MDA/kg sample). No significant changes were found in the concentration of saturated and unsaturated fatty acids after PEF treatment using bipolar square-wave pulses of 4 μs, at 35 kV/cm field strength and a frequency of 200 Hz for a fruit juice-soy milk beverage during 800 and 1400 μs treatments (Morales-de la Pena, Salvia-Trujillo, Rojas-Grau, & Martin-Belloso, 2011). The authors reported that the treatment led to a greater reduction of the total fatty acid content. Faridnia et al. (2015) found a significant increase in lipid oxidation both immediately after treatment and during storage for frozen-thawed beef semitendinosus muscles using process conditions of; constant pulse width of 20 μs, electric field strength of 1.4 kV/cm, constant frequency of 50 Hz, pulse number of 1032, and total specific energy input of 250 kJ/kg. Volatiles which resulted from lipid oxidation and are responsible for off-flavours, were significantly higher for the treated samples. These results could be related to the significant decrease in the concentrations of; myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n7), oleic acid (C18:1n9c) and linoleic acid (C18:2n6c). The consequences of pulsed electric field treatment on food proteins has been reviewed by Zhao, Tang, Lu, Chen, and Li (2014), who suggest that PEF has less impact on the food proteins than thermal technologies. Although, they mention that PEF can inactivate some proteins and change their structure and properties. Zhao, Yang, Tang, Zhang, and Hua (2009) demonstrated that PEF induces sulfhydryl-disulfide interchange
reactions leading the formation of protein aggregates and a decrease in the solubility of the protein. Deactivation of peroxidase (POD) and polyphenoloxidase (PPO) for apple juice, which can cause undesirable browning reactions, were achieved using PEF (Schilling et al., 2008). Similarly, Bi et al. (2013) showed that the inactivation efficacy for these two enzymes with the same matrix increases as a function of electric field strength, resulting in product lightening. Protein oxidation was not detected for egg white protein solution after treatment at 200, 400, 600 and 800 s under a constant electric field intensity of 25 kV/cm (Wu, Zhao, Yang, & Chen, 2014). The only observed change was a slight increase in the content of sulfhydryl groups, which could be related to a gentle modification of the structure of the protein. Some effects of pulsed electric field on food products are summarised on Table 2.2.

Table. 2.2: Effect of pulsed electric field on chemical changes in food products.

<table>
<thead>
<tr>
<th>Food product</th>
<th>Treatment</th>
<th>Changes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh and frozen turkey breast</td>
<td>Frequencies: 10, 55 and 110 Hz Pulses: 100, 200 and 300 pulses Voltages for fresh samples: 7.5, 10, 12.5 kV Voltage for frozen samples: 14, 20 and 25 kV Pulse width: 20 μs</td>
<td>No lipid oxidation:</td>
<td>(Arroyo et al., 2015)</td>
</tr>
<tr>
<td>Horchata</td>
<td>Electric field intensity 20-35 kV/cm Treatment times 100 - 475 μs</td>
<td>No lipid oxidation:</td>
<td>(Cortes et al., 2005)</td>
</tr>
<tr>
<td>Cold-Boned Beef Loins and Topsides</td>
<td>Voltage: 10 kV Frequency: 90 Hz Pulse width: 20 μs</td>
<td>No lipid oxidation:</td>
<td>(Suwandy et al., 2015)</td>
</tr>
<tr>
<td>Peanut oil</td>
<td>Voltage: 20, 30, 40 and 50 kV Pulse frequency: 1008 Hz Pulse width: 40 μs</td>
<td>Restrain lipid oxidation:</td>
<td>(Zeng et al., 2010)</td>
</tr>
<tr>
<td>Product</td>
<td>Electric Field Strength</td>
<td>Specific Energy Input</td>
<td>Pulse Width</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------</td>
<td>-----------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Chilled and frozen-thawed cooked lamb</td>
<td>1–1.4 kV·cm⁻¹</td>
<td>88–109 kJ·kg⁻¹</td>
<td>20 μs</td>
</tr>
<tr>
<td>Fruit juice-soymilk beverage</td>
<td>35 kV/cm</td>
<td>250 kJ/kg</td>
<td>20 μs</td>
</tr>
<tr>
<td>Frozen-thawed beef semitendinosus muscles</td>
<td>1.4 kV/cm</td>
<td>250 kJ/kg</td>
<td>20 μs</td>
</tr>
<tr>
<td>Egg white</td>
<td>25, 30, 35 kV/cm</td>
<td>6.5 and 65.5 kJ/kg</td>
<td>2 μs</td>
</tr>
<tr>
<td>Apple juice</td>
<td>5 L/h</td>
<td>Deactivation of peroxidase (POD) and polyphenoloxidase (PPO) (Schilling et al., 2008)</td>
<td></td>
</tr>
</tbody>
</table>

For Egg white, electric field intensity: 25 kV/cm, no protein oxidation (Wu et al., 2014).
2.4-Ultrasound processing

Acoustic energy has also been investigated as a novel technology for food processing. High frequency ultrasound (low intensity or low power) is commonly used as a non-destructive quality assessment technique. Whereas low frequency (high power) employs high intensity sound waves which can have significant impact on food properties, offering a technologically driven solution for various food processing operations including sterilization, extraction, emulsification, freezing, sonocrystallization, drying, defoaming (Awad, Moharram, Shaltout, Asker, & Youssef, 2012). Cavitation is the main mechanism of ultrasound when applied to liquid foods using contact type systems such as ultrasound baths and probe-based systems. Here, ultrasound is propagated via a series of compression and rarefaction waves through the liquid, which at sufficient power can produce cavitation. These bubbles can reach an unstable size and collapse, generating physical and chemical effects such as localised high temperatures and pressures, radiation forces, microstreaming, shock waves, microjets and free radicals (Mason, 2002; O’Brien, 2007). A second approach is to use airborne acoustic ultrasound aimed primarily at treating solid foods. Although there is a lack of knowledge regarding the mechanisms involved; the following factors can be considered: high acoustic pressures, standing waves, radiation pressure and microstreaming. This technology has been used for drying, defoaming and decontamination (Charoux, Ojha, O'Donnell, Cardoni, & Tiwari, 2017). As for the other technologies, anti-microbiological efficacy (log reduction), sensory parameters (colour, flavour) and physico-chemical characteristics (gelation, viscosity) appear to be the
most studied factors following a sonication treatment. There are relatively few studies focusing on the effect of ultrasound processing on biomolecules. A summary of the modifications of this technology on different food products is summarised in table 2.3.

Table 2.3: Effect of ultrasound processing on chemical changes in food products.

<table>
<thead>
<tr>
<th>Food products</th>
<th>Conditions</th>
<th>Main findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheddar cheese whey</td>
<td>Frequencies between 20 to 2,000 kHz and specific energies between 8.0 to 390 kJ/kg for 10 and 30 min at 37 ₀C</td>
<td>Lipid oxidation occurred for both treated and untreated samples</td>
<td>(Torkamani, Juliano, Ajlouni, &amp; Singh, 2014)</td>
</tr>
<tr>
<td>Mackerel, cod, hake and salmon</td>
<td>Ultrasonic bath at 30 kHz for 5, 15, 25, 35 and 45 min</td>
<td>No effect of ultrasound on lipid oxidation for mackerel, cod and hake</td>
<td>(S Pedrós-Garrido et al., 2017)</td>
</tr>
<tr>
<td>Pasteurised milk</td>
<td>Probe-based system at 24 kHz for 2.5, 5, 10, 15 and 20 min</td>
<td>Increase in secondary volatile products derived from lipid oxidation</td>
<td>(Riener, Noci, Cronin, Morgan, &amp; Lyng, 2009)</td>
</tr>
<tr>
<td>Raw, thermized and pasteurized milk</td>
<td>Probe-based system at 24 kHz for 2, 4, 8 and 16 min at 15-25 ₀C, and 0, 1, 2, 4, 6 and 8 days of storage before sampling</td>
<td>Volatile compounds derived from lipid oxidation increased in concentration with sonication and storage time</td>
<td>(Chouliara, Georgogianni, Kanellopoulou, &amp; Kontominas, 2010)</td>
</tr>
<tr>
<td>Raw and pasteurised skim milk</td>
<td>Frequencies at 20, 400, 1,000, 1,600, 2,000 kHz at 4, 20, 45 and 63 ₀C up to 20 min</td>
<td>Highest amount of volatiles detected at 400 and 1,000 kHz, where the cavitation yield and hydroxyl radical production were greatest</td>
<td>(Juliano et al., 2014)</td>
</tr>
</tbody>
</table>
Refined sunflower oil  Ultrasound probe at 20 kHz; power at 150 W for 2 min at 20 °C  Increase of peroxide value after sonication treatment  (Chemat et al., 2004)

Beef  Probe-based system at 20 kHz at power intensity of 2.39, 6.23, 11.32 and 20.96 W.cm<sup>-2</sup> for 30, 60, 90 and 120 min  Greater lipid oxidation during ultrasound–assisted brining compared to static brining  (Kang et al., 2016)

Soy protein isolate  Probe-based system at 20 kHz at 200, 400 and 600 W for 15 and 30 min  Increase of sulphydryl content upon sonication  (Hu et al., 2013)

Bovine serum albumin solutions  Probe-based system at 20 W.cm<sup>-2</sup> for 7 to 90 min  Reduction of sulphydryl groups  (Gülseren, Güzey, Bruce, & Weiss, 2007)

Soy protein isolate, egg white protein and whey protein  Ultrasonic processor at 20 kHz, 4.27 and 0.71 Q for 20 min  Increase of the protein surface hydrophobicity  (Arzeni et al., 2012)

Soy protein isolates  Probe-based system at 25 kHz at 200, 400, and 600 W for 15 min  Increase of the protein surface hydrophobicity  (L. Chen, Chen, Ren, & Zhao, 2011)

Black bean protein isolates  Probe-based system at 20 kHz at 150, 300 and 400 W for 12 and 24 min  Increase of the protein surface hydrophobicity  (Jiang et al., 2014)

Torkamani et al. (2014) studied the effect of ultrasound on different quality parameters of Cheddar cheese whey including polar lipids, free and bound fatty acids and lipid oxidation derived compounds. Lipid oxidation occurred for both untreated and treated samples.
notwithstanding the ultrasound treatment over time. S Pedrós-Garrido et al. (2017) did not report any effects of ultrasound on lipid oxidation for mackerel, cod and hake, whereas treated salmon showed a slight decrease in TBARS values. A probe-based system at 24 kHz was used by Riener et al. (2009) to treat pasteurised milk for 2.5, 5, 10, 15 and 20 min. Secondary volatile products derived from lipid oxidation including carbonyl compounds; pentanal, hexanal and heptanal were detected. According to the researchers, the increase in these compounds was caused by the radical species formed under the high temperatures and pressures conditions created by cavitation. Similar results were obtained by Chouliara et al. (2010) for ultrasound-treated raw, thermized and pasteurised milk. In another study, various types of milk (raw milk pasteurised skim milk) were submitted to sound waves at different frequencies (20, 400, 1,000, 1,600 and 2,000 kHz) and temperatures (4, 20, 45 and 63 °C) up to 409 kJ/kg, and the volatile compounds generated by lipid oxidation analysed. The highest amount of volatiles was detected at 400 and 1,000 kHz, at the same frequencies where the cavitation yield and the hydroxyl radical production were the greatest (Juliano et al., 2014). It was observed that the production of radicals did not increase linearly with the acoustic frequency. The sonochemical yield depends on the number of active bubbles, the average temperature within these bubbles and the mass transfer effects. The first two parameters are themselves dependent on the acoustic power and frequency. An increase in the acoustic power raises the number of active cavitation bubbles, the size of these bubbles and the temperature during collapse. Greater negative pressures can be reached during the rarefaction phases and greater positive pressures during the compression phases (Kanthale, Ashokkumar, & Grieser, 2008). An increase in the frequency also raises the number of active cavitation bubbles, but reduces the time for these bubbles to grow during rarefaction phases, and to collapse during compression phases (Leighton, 1995). Consequently, fewer acoustic cycles are required for bubbles to reach their active resonance size, and transient cavitation occurs at faster rates (Beckett & Hua, 2001). As
the maximum potential energy of the bubble is reached at its maximum size, at high frequencies
the collapse is occurring with low energy. However, a greater number of active bubbles
facilitates a high production of free radical species. Moreover, the effects of mass transfer
during cavitation should be taken into account. During the rarefaction phase the pressure inside
the bubble decreases, inducing evaporation of the solutes from the bubble/liquid interface, thus
increasing the amount of water vapour. During the compression phase the pressure inside the
bubble increases, inducing condensation at the bubble wall, thus reducing the amount of water
vapour. The shorter the acoustic cycle; the lower the amount of water vapour within the
cavitation bubble (Ashokkumar et al., 2008). Hence, in the case of high frequencies the amount
of evaporated water decreases. This could explain the reduction in hydroxyl radical generation
at the higher frequencies. It has also been demonstrated by the authors that low temperatures
and sonication times help to reduce the volatiles compounds derived from lipid oxidation. This
underlines the role of temperatures in the production of hydroxyl radicals, as the dissociation
of water into OH radicals and H atoms is encouraged by high temperatures (Riesz & Kondo,
1992). In another study on refined sunflower oil samples, free acidity, total polar compounds,
peroxide value, conjugated dienes concentration, and fatty acid composition were identified
and quantified before and after ultrasound treatment. The peroxide value was the only
parameter with a significant difference between non-sonicated and sonicated samples (Chemat
et al., 2004). The oxidation of beef proteins and lipids during an ultrasound-assisted brining
process has been investigated by Kang et al. (2016). The TBARS analysis showed that
ultrasound promoted greater lipid oxidation compared to static brining (0.2 MDA (mg/kg meat)
at 120 min for static brining, 1.2 MDA for the same processing time at 20.96 Wcm$^{-2}$). This
result could be explained by the same consequence of cavitation described previously, namely
sonolysis. When the transient cavities undergo violent collapse, high temperatures and
pressures are generated (4,000 K and 1000 atm respectively), and the dissociation of water
produces hydroxyl free radicals and hydrogen atoms. In addition to temperature, ultrasound intensity plays a governing role in the production of free radical species. Indeed, Jana and Chatterjee (1995) found a linear correlation between ultrasound intensity and the generation of \( \cdot \text{OH} \) radicals, up to a threshold of 3.5 W cm\(^{-2} \), which is likely due to saturation with cavitation bubbles. These free radicals could be responsible of lipid oxidation.

Besides lipid oxidation, protein reactions upon sonication treatment have also been studied. In a study on pea protein, a rise in sulfhydryl groups was observed after ultrasonic treatment. The results reported by Hu et al. (2013) on soy protein isolate are similar. The increase of SH groups content could be related to the conformation changes of the proteins upon sonication treatment. Under the effect of acoustic cavitation, the thiol groups are more exposed as the protein unfolds, conversely, the cleavage of the disulphide bonds S-S of the native proteins leads to increases in the content of SH groups. A few studies have shown that sonication does not have any effect on the content of sulfhydryl groups (Arzeni et al., 2012; Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011). However different conclusions have been reached regarding the effect of ultrasound on the sulfhydryl group content in other studies. Indeed, the increase in carbonyl group content and decrease in sulfhydryl group content in ultrasound treated beef proteins indicate that protein oxidation increases with high ultrasound intensities and treatment times (Kang et al., 2016). Lipid oxidation has also been observed in this study, and it has been hypothesized that the products of this reaction (i.e. malondialdehyde, carbonyl compounds) can react with proteins and form carbonyl groups. Gülseren et al. (2007) treated bovine serum albumin solutions (BSA) with an ultrasound probe at 20 W·cm\(^{-2} \) for 7 to 90 min. They observed a reduction in sulfhydryl groups by 31 % after 90 min of sonication. The reactive species formed during sonication (i.e. \( \cdot \text{OH} \) and \( \cdot \text{H} \)) cross-react and produce hydrogen peroxide (H\(_2\)O\(_2\))
which can alter the chemical structure of the molecules. Consequently, upon a sonication treatment, the SH groups localised in the inner structure of the molecules are more exposed to the interface, thus are more susceptible to oxidation. This could explain the decrease of SH groups after sonication treatment. Protein surface hydrophobicity is another commonly analysed parameter which is related to the stability, conformation and functional properties of proteins. Ultrasound treatment increases the protein surface hydrophobicity, as demonstrated in several studies including; soy protein isolates (L. Chen et al., 2011), black bean protein isolates (Jiang et al., 2014), soy protein isolate, egg white protein and whey protein concentrate (Arzeni et al., 2012). The hydrophobic groups of the proteins initially localised in the interior of the molecule are exposed to the more polar surrounding environment due to the mechanical effects of cavitation. Several studies have analysed the protein structural changes (Jiang et al., 2014; Kang et al., 2016). The results point towards a common conclusion, namely that the α-helix content decreases as oxidation occurs (upon sonication), and the β-sheets content increases. As a consequence of the unfolding of the proteins, intern hydrophobic regions are unmasked, and sulfhydryl group content decreases as S-S bonds are created. These disulfide bonds influence the functional properties of the proteins, and play an important role in the gel network structure and mechanical strength. A significant reduction in particle size treated by ultrasound was noticed for soy protein isolate and whey protein concentrate (Arzeni et al., 2012; Gordon & Pilosof, 2010; Jambrak, Lelas, Mason, Krešić, & Badanjak, 2009). This reduction appeared to be greater during the first minute of treatment while the sample size is bigger. Cavitation, micro-streaming and turbulent forces occurring during a sonication treatment violently agitate the aggregates, thus reducing the size distribution. Unlike in a homogeneous solution, cavitation bubbles collapse asymmetrically in the
presence of solid particles. High speed jets of liquid are expelled from the imploded bubble toward the solid surface. This physical mechanism, called microjets, together with shockwaves can lead to pitting, erosion and corrosion of the solid surface, as well as increasing sonochemical production following a breakage of the chemical bonds within the molecule (Y. Lu, Riyanto, & Weavers, 2002). Microstreaming is another consequence of cavitation which is characterised by microscopic turbulences at the surface of the solid, increasing the rate of mass transfer (Hagenson & Doraiswamy, 1998). The probability of larger particles to be subject to cavitational energy is higher than for smaller particles, therefore the rate of the size reduction for high weight molecules is faster than for smaller molecular weight species (Gordon & Pilosof, 2010). However, different behaviours were observed for egg whites proteins (Arzeni et al., 2012), bovine serum albumin solutions (Gülseren et al., 2007) and hydroxypropylmethylcellulose (Camino, Pérez, & Pilosof, 2009), where an increase in the particle size after ultrasound treatment was noticed. In these cases, any partial degradation could be overcome by the formation of small aggregates due to non-covalent bonds, such as electrostatic and hydrophobic interactions. This would be one consequence of the high hydrophobic index rise upon sonication.

5-Cold atmospheric plasma

In 1928, the American scientist Irving Langmuir proposed that the electrons, ions and neutrons in an ionized gas could be considered as a corpuscular material entrained in a fluid medium and termed this medium “plasma” (Langmuir, 1928). Nowadays, the term plasma refers to a partially or wholly ionized gas composed essentially of photons, ions and free electrons as well as atoms in a fundamental or excited state possessing a net neutral charge. A new technology coined cold atmospheric plasma has been developed for sterilizing and modifying material with
application in the food, agriculture, textile, electronic, biotechnology and medicine sectors (Kramer et al., 2015). Depending on the application, different plasma sources have been built such as corona discharge, dielectric barrier discharge (DBD), microwave discharge (MD), gliding arc and plasma jet (V. Scholtz, J. Pazlarova, H. Souskova, J. Khun, & J. Julak, 2015). Recently, cold plasma has emerged as a non-thermal technology with potential applications for food decontamination (N. Misra, O. Schlüter, & P. Cullen, 2016). The main interest in this technology is that it can efficiently inactivate bacteria, yeasts, moulds, spores, biofilms and other hazardous microorganisms, including potential bio-terrorism agents at low/ambient temperatures and at atmospheric pressure (Liao et al., 2017; Thirumdas, Sarangapani, & Annapure, 2015). The efficiency of the treatment depends of different parameters including the flow of the electric charge (direct or alternating), voltage, plasma source, distance between the source and the product, treatment time, packaging material and atmosphere. Plasma can be a tool to maintain the microbial safety of fresh food products, thus increasing the self-life of the products, with limited impact on food quality (N. N. Misra, Tiwari, Raghavarao, & Cullen, 2011). Besides plasma being a potential technology to ensure food safety, it is necessary to study how it is affecting food quality. During cold atmospheric plasma treatment negative and positive ions, free radical molecules, electrons, UV-photons, nitrogen and carbon oxides and ozone are formed depending of the gas used (Graves, 2012; X. Lu et al., 2016). The production of reactive species (Figure 2.2) in cold atmospheric plasma can promote hundreds of reactions involving several components: He, Ar, N, O, and H atoms and molecular species composed of these atoms (in ground and excited states), various atomic and molecular ion species and electrons (Gorbanev, Stehling, O’Connell, & Chechik, 2016; Graves, 2012; X. Lu et al., 2016). Such reactions can affect the components of the food product, representing a risk for the key quality parameters of the food product (H.-J. Kim, 2016; N. Misra, 2016).
However, plasma treatment does not have the same effect on solid or liquid matrices. In the first case, plasma is not able to penetrate significantly inside the product, and is generally classified as a surface treatment, usefully where most of the microorganisms reside. Thus, the process will have limited effects on the bulk components of many solid foods. Obviously, the degree of penetration of the plasma varies depending on the food product. Whereas when a liquid food is treated, plasma can be diffused or infused in the liquid, potentially leading to more significant impacts (Surowsky, Schluter, & Knorr, 2015).

It is necessary to highlight the formation of ozone by atmospheric plasma devices. Ozone is a triatomic oxygen molecule formed by the interaction of a diatomic oxygen molecule with free oxygen radical. The breakdown of the bond O-O to form this radical requires an energy that could be provided by the plasma system. Ozone is very reactive and unstable specie,
decomposing into hydroxyl, hydroperoxy and superoxide radicals which have high oxidation potentials. The use of ozone in the food industry has been investigated (O'Donnell, Tiwari, Cullen, & Rice, 2012). Ozone can reduce mycotoxins and microbial contamination of food products and has also been shown to be efficient in removing pesticide residues from different food matrices. However, due to the high reactivity of ozone many reactions can occur, which can potentially affect the quality of the food product (Glowacz, Colgan, & Rees, 2015; Greene, Guzel-Seydim, & Seydim, 2012; Perry & Yousef, 2011). Table 2.4 lists some modifications on food nutrients by cold atmospheric plasma.

Table 2.4: Effect of cold atmospheric plasma on chemical changes in food products.

<table>
<thead>
<tr>
<th>Food product</th>
<th>Plasma source</th>
<th>Treatment</th>
<th>Changes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat flour</td>
<td></td>
<td>Frequency: 9 kHz</td>
<td>↑ Lipid oxidation</td>
<td>(Bahrami et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Power 40 W and voltage 15 V</td>
<td>Fatty acid degradation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Power 90 W and voltage: 20 V</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time: 1 and 2 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walnuts and</td>
<td></td>
<td>Frequency: 13.56 MHz</td>
<td>↑ Lipid oxidation</td>
<td>(Thirumdas et al., 2015)</td>
</tr>
<tr>
<td>peanuts</td>
<td></td>
<td>Pressure: 0.5 mbar</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Power: 40, 50, 60 W</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time: 5, 10 and 15 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw milk</td>
<td>Corona discharge</td>
<td>Power: 9 kV</td>
<td>↑ Lipid oxidation</td>
<td>(Korachi et al., 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Current: 90 mA</td>
<td>= Fatty acids content</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time: 3, 6, 9, 12, 15 or 20 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>Radio-frequency driven plasma jet</td>
<td>Power: 25 KV</td>
<td>↑ Lipid oxidation</td>
<td>(Van Durme, Nikiforov, Vandamme, Leys, &amp; De Winne, 2014)</td>
</tr>
</tbody>
</table>
The formation of radical species could suggest the hypothesis that plasma increases lipid oxidation. Wheat flour was treated by atmospheric plasma during 1 and 2 min at 15 and 20 kV by Bahrami et al. (2016), who analysed the level of lipid oxidation by measuring the hydroperoxides and volatile compounds produced after plasma treatment, and found a significant increase of their content for all the treated samples. The difference was greater for longer treatment times and higher voltages. Moreover, they found an important reduction in the fatty acid profile, particularly linoleic acid, which is an essential fatty acid for humans, with a complete reduction after 120 s of treatment at 20 kV. Thirumdas et al. (2015) observed an increase of 20 % of peroxides content for plasma treated peanuts and walnuts at 60 kV, which can lead to the oxidation of lipids. Joshi et al. (2011) demonstrated that plasma encourages lipid oxidation on the cellular membrane of *Escherichia coli* using a dielectric barrier discharge (DBD) system. From these studies, it can be concluded that the longer the treatment time the higher the radical species concentration resulting in higher levels of lipid oxidation, and more pronounced impacts on food quality. In another study, a corona discharge plasma system was used for milk samples at intervals of 0, 3, 6, 9, 12, 15 and 20 minutes (Korachi et al., 2015). The researchers did not notice noteworthy differences in the fatty acid profile among all the samples. They suggested that long chain fatty acids such as C18:00 could suffer a hydrolysis process, as their content was slightly reduced after treatment, while C10:00 and C12:00 showed...
a low increase. However, a significant increase was observed for the volatile compounds content, which could be related to lipid oxidation. In similar studies, Van Durme et al. (2014) and Vandamme et al. (2015) used a plasma jet device and a DBD system for vegetable and fish oils respectively, and report that plasma accelerates lipid oxidation. However, Korachi and Aslan (2011) did not find any evidence of lipid oxidation nor changes of the fatty acids profile on the cellular membrane of *Escherichia coli* and *Staphylococcus aureus* treated by an atmospheric plasma corona discharge device. In addition, no significant changes in the fatty acid composition of beef jerky were observed after radio-frequency (RF) atmospheric pressure plasma with 5 min of treatment (J. S. Kim et al., 2014).

Apart from lipids, the effect of plasma on proteins has also been studied (N. N. Misra, Pankaj, Segat, & Ishikawa, 2016). Takai et al. (2014b) investigated the effect of a low frequency plasma jet on an aqueous solution of 14 amino acids. They noticed that these biomolecules suffered some modifications by using high resolution mass spectroscopy. After treatment, new molecules were observed, resulting from different reactions such as oxidation, sulfonation, amidation, sulfoxidation, hydroxylation, dehydrogenation, nitration and dimerization depending on each specific amino acid. Segat, Misra, Cullen, and Innocente (2016) studied the inactivation kinetics of an alkaline phosphatase solution at different treatment times between 5 and 300 s and at three different voltages: 40, 50 and 60 kV using a DBD. The inactivation of this enzyme was detected after a few seconds of treatment and it was attributed to the loss of α-helical and β-sheet secondary structures of the protein. Tappi et al. (2014) found that polyphenol oxidase activity significantly decreased for apples after plasma treatment. In another study, polyphenoloxidase (PPO) and peroxidase (POD) were denaturalised by losing the α-helical structure using a plasma jet (Surowsky, Fischer, Schluetter, & Knorr, 2013). These enzymes are part of the undesirable browning reactions related to a reduction of the nutritional and sensorial quality of vegetables and fruits. In another experiment, Park et al. (2016a) studied
the effect of plasma on haemoglobin and myoglobin (Mb) using different gases, observing that plasma treatment leads to the modification of the secondary structure of both proteins, from α-helical to β-sheet, and a degradation of the heme group. Conversely, E. G. Alves et al. (2016) studied the effect of plasma on fructooligosaccharides (FOS) and found no significant changes in FOS concentrations after applying 70 kV using a DBD system. These carbohydrates are used in the food industry as sweeteners and are interesting from a health perspective point of view due to their prebiotic activity.

Cold plasma is an early stage technology with regards to food applications and data on the induced chemistries from the array of plasma technologies and process conditions available are only emerging. A challenge here is the complexity of the ‘cocktail’ of active species generated, the widely different time-scales over which they may act and diagnostics of the interaction with the target product. Of course, it is this complexity with provides the advantages observed in terms of process efficacy and lack of apparent resistance to the approach by microbial populations. A priory area of research will be unravelling both the mechanisms of antimicrobial action along with induced chemistry for treated foods and linking them to the key reactive species generated.

The following paper has been published from this study:

Chapter 3: Effects of cold atmospheric plasma on mackerel lipid and protein oxidation during storage

3.1. Introduction

Fish and seafood products are some of the most common foods in our diet. Mackerel is a popular fish in Europe with more than 800 million tonnes consumed per year (FAO, 2018). It is considered a healthy diet option due to its high content of vitamins, high-quality protein as well as several other essential nutrients such as polyunsaturated fatty acids (Domingo, 2016; Swanson, Block, & Mousa, 2012). In addition, fatty acids present in fish are known to be an important source of essential omega-3 polyunsaturated fatty acids (n-3 PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are necessary for functions in our metabolism and their intake through the diet is important because humans cannot synthesise them. Numerous studies have highlighted the benefits of EPA and DHA (F. Shahidi & Ambigaipalan, 2018; Swanson et al., 2012). For example, they are essential for fetal development, and supplementation during pregnancy has also been linked to decreased immune responses in infants including decreased incidence of allergies. The supplementation of both, EPA and DHA, has been reported to lead to optimal pregnancy length (Swanson et al., 2012). Kumar, Mastana, and Lindley (2016) suggested a diet rich in these fatty acids for patients with various chronic inflammatory diseases, like asthma, due to their anti-inflammatory properties. In addition, they have been reported to play a role in avoiding coronary heart diseases such as atherosclerosis and peripheral arterial disease (Lee, O'keefe, Lavie, & Harris, 2009), as well as Alzheimer’s and dementia (Kröger & Laforce, 2016).
Despite all the benefits associated with the intake of fish products, there are challenges facing the industry. Fish quality starts to deteriorate post catch and during storage with spoilage microorganisms the primary factor causing fish spoilage (Gram & Dalgaard, 2002). In this regard, it is paramount to find effective means to ensure the safety and quality of fish products. The food industry is increasingly seeking new processing technologies capable of inactivating microorganisms whilst maintaining food quality. A particular focus has been on non-thermal processing technologies, which are effective at ambient or sub-lethal temperatures (i.e. temperatures below 50 °C) such as high hydrostatic pressure, pulsed electric field, ultrasound processing and more recently cold plasma (Jermann et al., 2015). These technologies have been applied to inactivate microorganisms such as viruses, spores, yeasts, bacteria and biofilms (Arvanitoyannis, Kotsanopoulos, & Savva, 2017; Daryaei et al., 2016; Saldana et al., 2014). Numerous studies have examined their potential for the decontamination of fish products, including high pressure treatment of mackerel, cod, and salmon (De Alba et al., 2019; Rode & Hovda, 2016), sea bass (Teixeira et al., 2014), herring and haddock (Karim et al., 2011) and smoked cod (Montiel, De Alba, Bravo, Gaya, & Medina, 2012). Ultrasound has been used to reduce the microbiological counts in mackerel, cod, hake and salmon fillets (S. Pedrós-Garrido et al., 2017).

The principal advantage of such technologies is their operation at low temperatures, thus minimising the negative effects often found with thermal processing (Awuah et al., 2007). Nevertheless, they too can also induce chemical/biochemical changes in food constituents, affecting the quality of the food product (Pérez-Andrés, Charoux, Cullen, & Tiwari, 2018).

Cold atmospheric plasma treatment can induce a wide array of active species including ozone, carbon and nitrogen oxides, free radicals, as well as negative and positive ions depending on the different gases used (X. Lu et al., 2016). Plasma for food applications is still in its infancy but has demonstrated significant potential for food decontamination and associated increases
of shelf-life (Bourke, Ziuzina, Boehm, Cullen, & Keener, 2018). A growing literature is addressing the unknowns surrounding the efficacy and mechanisms of action for microbial inactivation (Liao et al., 2017). The effect of plasma on microbial inactivation has been investigated in different food substrates, including orange juice (L. Xu, Garner, Tao, & Keener, 2017), fresh and dried nut samples (Juglans regia L.) (Amini & Ghoranneviss, 2016), ground nuts (Devi, Thirumdas, Sarangapani, Deshmukh, & Annapure, 2017), strawberries (Misra et al., 2014) and blueberries (Lacombe et al., 2017).

Cold atmospheric plasma has also been shown to increase the shelf-life of meat products (Xiang et al., 2018), including chicken breasts (Jiamei Wang, Zhuang, Hinton, & Zhang, 2016), beef jerky (Yong et al., 2017) and pork loin (Yong et al., 2017). Moreover, fish products, like dried filefish fillets (S. Y. Park & S. D. Ha, 2015) and mackerel (Albertos et al., 2017) have been also tested.

The radical species present in the plasma are responsible for microbial inactivation although they may also lead to chemical reactions affecting the quality of the food product (Kim, D. Jayasena, H. Yong, & C. Jo, 2016; Misra, 2016; Pérez-Andrés et al., 2018). The challenge for all effective preservation techniques is to retain the sensory and quality attributes of the food while maximising shelf-life and safety profiles. Scale-up of plasma technology is another challenge which has recently been discussed by P. J. Cullen et al. (2018). Given the array of radical species found with atmospheric plasma and the wide range of time-scales of their reactions, there are many unknowns surrounding the chemical reactions induced from plasma treatment of foods (Vladimir Scholtz, Jarmila Pazlarova, Hana Souskova, Josef Khun, & Jaroslav Julak, 2015).

The goal of this study was to contribute to the understanding of the effects of cold plasma on the lipids and proteins of mackerel during storage and to support scale-up approaches for this
technology. For that reason, mackerel fillets were packaged and plasma treated in food packaging trays typically employed by the industry. Key quality markers were monitored for different storage temperatures and time. The impact of cold atmospheric plasma on the stability of mackerel fatty acids, such as EPA, DHA, PUFA, monounsaturated fatty acids (MUFA), saturated fatty acids (SFA), as well as nutritional quality indices and lipid and protein oxidation were investigated.

3.2. Material and methods

3.2.1. Chemicals and reagents

Trichloroacetic acid, HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)), EGTA (Egtazic acid), disodium sulphate, hydrochloric acid, potassium hydroxide, 1,1,3,3-tetraethoxypropane , 2-thiobarbituric acid , methanol, heptane, Supelco-37 FAME standard, tricosanoic acid methyl ester, as well as acetyl chloride, sucrose, mannitol and potassium chloride were purchased from Sigma-Aldrich (Arklow, Co. Wicklow, Ireland). N-pentane as well as sodium chloride was purchased from Fisher Scientific (Dublin, Ireland). Ultra-pure water (18.2 MΩ cm−1) was generated in-house using a Millipore water purification system (Millipore, Cork, Ireland). All chemical were GC grade.

3.2.2 Sample preparation

Fresh mackerel was purchased at a local fish monger in Dublin in September 2016. Each mackerel was divided into two fillets and each fillet, with skin attached facing down, was packaged individually (Ilpra Foodpack VG 400 Packaging Machine, Italy) in a black amorphous polyethylene terephthalate (APET/PE) pack (195x155x37 mm), and sealed under atmospheric air conditions with a low oxygen permeable barrier polyvinyl-chloride film
to mirror common commercial practice.

3.2.3 Plasma treatment

An in-house dielectric barrier discharge atmospheric plasma system was employed, which was described previously (S. K. Pankaj, Misra, & Cullen, 2013). Each packaged tray was located between two circular aluminium electrodes (outer diameter = 158 mm) separated by two polypropylene (PP) dielectric layers (2 mm thickness). The distance between the dielectric layers was the height of the tray employed (37 mm). Five minutes of treatment was performed at a discharge voltage of 80 kV Root Mean Square (RMS). The choice of these conditions was based on a recent study in the area carried out by our group (Albertos et al., 2017) which showed this treatment as the most effective one to reduce total aerobic psychrotrophic bacteria and Pseudomonas counts in in-packaged plasma treated mackerel fillets with no impact on colour or TBARs values. Both the control and plasma samples were kept at 4 °C and held for 24 hours to allow the radical species formed to interact with the food following the method of Ziuzina, Patil, Cullen, Keener, and Bourke (2013). After that, samples were randomly divided into three different batches and stored at three different temperatures: 4 °C, 8 °C and -20 °C. Samples from each temperature were collected after 2, 5 and 7 days and in the case of the -20 °C batch also after 2 weeks. For each day, samples were homogenised using a blender (Robot Coupe-R301 Ultra ;Nisbets, Cork, Ireland) and were stored under vacuum at -80 °C until the day of the analysis. All the treatments were carried out in duplicate.

3.2.4 Lipid oxidation

Lipid oxidation was quantified using the TBARs method according to Maraschiello, Sárraga, and Garcia Regueiro (1999), which is based on the detection of malondialdehyde (MDA), a by-product of peroxidation of polyunsaturated fatty acids and esters, associated with off-
flavours and off-aromas in meat products (Ghani et al., 2017). Briefly, 1 g of blended mackerel sample was suspended in 20 mL of MilliQ water and homogenised with an Ultraturrax homogeniser (Labortechnik, Staufen, Germany) at 13,500 rpm for 30 seconds. 5 mL of 25% trichloroacetic acid solution was added and the solution which was kept at 4 °C for 15 min. After centrifugation at 3,500 rpm for 30 minutes at 4 °C using a Hettich Rotanta 460R centrifuge (Fisher Scientific Ireland, Dublin, Ireland), the solution was filtered using a Whatman number 51 filter paper (Sparks Lab Supplies Ltd., Dublin, Ireland). A 1.5 mL aliquot of the filtrate was collected and mixed with 1.5 mL 0.6% of 2-thiobarbituric acid with the reaction performed in a water bath at 70 °C for 30 minutes. A standard curve was prepared using a 1,1,3,3-tetraethoxypropane solution. Absorbance of samples and standards were measured spectrophotometrically on a UV–Vis Spectrophotometer (Shimadzu UV-1700, Columbia, USA) at a wavelength of 532 nm. Results were expressed as milligrams of malondialdehyde per kilogram of mackerel (mg MDA/ kg mackerel).

3.2.5 Fatty acid profile

3.2.5.1 Microwave-assisted preparation of fatty acid methyl esters (FAMEs)

Microwave assisted FAME preparation was carried out using a MARS 6 Express 40 position Microwave Reaction System (CEM Corporation, Matthews, NC, USA) according to Brunton, Mason, and Collins (2015). Briefly, approximately 0.8 g of chilled blended mackerel was accurately weighed out into a reaction Xpress vessel. 100 µL of internal standard (C23:0 methyl ester, achieving a final concentration following extraction of 0.1 mg/mL) as well as 10 mL of potassium hydroxide (2.5%, w/v) in methanol (MeOH) solution were added to the vessel. The reaction vessels containing a 10 mm stir bar each were closed well and introduced into the MARS 6. The saponification programme consisted of bringing the temperature up to 130 °C over 4 minutes and holding at this temperature for another 4 minutes. Then, tubes were cooled
down in an ice bath. For esterification, 15 mL of 5% (v/v) acetyl chloride in MeOH solution were added to the vessels. Esterification was carried out by heating the vessels to 120 °C over 4 minutes and holding at this temperature for 2 minutes. The reaction tubes were removed and cooled on ice for 5 minutes. To the cooled vessels, 10 mL of pentane was added and vessels were inverted to achieve extraction. After that, about 20 mL of saturated NaCl solution was added, and the inversion process was repeated. The separation of the organic and aqueous layer was achieved, and an aliquot of the upper pentane layer, where the FAMEs were located was transferred into GC vials (1.5 mL) containing about 0.2 g anhydrous sodium sulphate and analysed using gas chromatography.

2.5.2 Gas chromatography-flame ionisation detector analysis

Separation was carried out using a Clarus 580 Gas Chromatograph (Perkin Elmer, Massachusetts, USA) fitted with a flame ionisation detector (GC-FID). The separation of 37 FAMEs was carried out in 35 minutes employing a CP-Sil 88 capillary column (Agilent, Santa Clara, California, USA) with a length of 100 m x 0.25 mm ID and 0.2 μm film. The inlet temperature was set to 250 °C and the injection volume was 0.5 μL. The carrier gas was hydrogen at a constant flow of 1.25 mL/min, and the split ratio was set at 10:1. The oven was set to 80 °C with an initial temperature ramp of 6.2 °C/min to 220 °C which was held for 3.2 minutes. A second temperature ramp of 6.3 °C/min to 240 °C followed which was held for 6.5 minutes. The FID was set at 270 °C. Compounds were identified by comparing their retention times with those of authentic standard of FAMEs from the Supelco 37 FAME mix. The content of each fatty acid was calculated using following equation (Eq. 3.1).

\[
\text{FA content} = \frac{\text{Peak Area(FAME)}}{\text{Peak Area(ISTD)}} \times \frac{\text{Weight(ISTD)}}{\text{Weight(Sample)}} \times \text{ISTD purity} \times 10 \times 0.96 \quad (eq. 3.1)
\]
Where, FA content is the amount of a given fatty acid in the sample (mg/g), 10 is the dilution factor and 0.96 is the conversion factor for the internal standard to account for the internal standard already being presented as a FAME.

Due to the variability of the fat content, fatty acid content was normalised and results were expressed in percentages:

\[
\text{FA content (\%) = } \frac{\text{FA content (mg/g)}}{\text{Total fat content (mg/g)}} \times 100 \quad (\text{eq. 3.2})
\]

A full list of all calculated fatty acids in g/kg fish is provided as supplementary material. (Sup 2).

2.5.3 Nutritional quality indices

Nutritional quality indices of plasma treated mackerel were assessed from fatty acids data. Saturation index (SI) is a carcinogenesis marker while atherogenicity (AI) and thrombogenicity (TI) are related to the incidence of coronary heart disease. They were calculated using the following equations (Ulbricht & Southgate, 1991):

\[
\text{SI} = \frac{[\text{C14:0} + \text{C16:0} + \text{C18:0}]}{[\sum \text{MUFA} + \sum \text{PUFA}]} \quad (\text{eq. 3.3})
\]

\[
\text{AI} = \frac{[\text{C12:0} + 4 \times (\text{C14:0} + \text{C16:0})]}{[\sum \text{MUFA} + \sum \text{PUFA}]} \quad (\text{eq. 3.4})
\]

\[
\text{TI} = \frac{[\text{C14:0} + \text{C16:0} + \text{C18:0}]}{0.5 \times (\sum \text{MUFA} + \sum \text{n6}) + 3 \times \sum \text{n3} + \frac{\sum \text{n3}}{\sum \text{n6}}} \quad (\text{eq. 3.5})
\]
2.6 Protein oxidation

Protein carbonyls are biomarkers of protein oxidation. They are generated through several different mechanisms induced during oxidative stress. The content of carbonyl groups was determined by the dinitrophenylhydrazine (DNPH) method using a Biocell protein carbonyl enzyme Immuno-assay kit (BioCell Corporation Ltd, Auckland, New Zealand) (Buss, Chan, Sluis, Domigan, & Winterbourn, 1997). All required reagents were provided by the test kit. Protein extraction was performed according to the procedure by Devries et al. (2008). Briefly, 1 g of mackerel sample was added to 25 mL protein extraction buffer (70 mmol/L sucrose, 220 mmol/L mannitol, 5 mmol/L Hepes, pH 7.2, 1 mmol/L EGTA, pH 7.2, and 150 mmol/L KCl) and homogenised for 30 seconds using an Ultraturrax homogeniser (Labortechnik, Staufen, Germany) set to 13,500 rpm. Each homogenate was adjusted to pH 7.2 using either 1 mol/L HCl or 1 mol/L NaOH and then centrifuged for 10 min at 3,500 rpm at 4 °C using a Hettich Rotina 460R centrifuge (Fisher Scientific Ireland, Dublin, Ireland). The amount of soluble protein in the supernatant was quantified by measuring its absorbance at 280 nm using an ND-100 Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Protein carbonyl content determination was then carried out according to the Biocell protein carbonyl enzyme Immuno-assay kit manufacturer's guidelines and measured using a plate reader (FLUOstar Omega Microplate Reader, BMG Labtech GmbH, Offenburg, Germany). Protein carbonyl content was calculated using a standard curve and results expressed as nmol DNPH/ mg protein.

2.7 Statistical analysis

Analysis of variance (ANOVA) of the dependent variables was carried out using Minitab 17.1.0 (Minitab Inc, Stage College, Pennsylvania, USA). Statistics were performed using a general linear model (GLM) where the treatment and temperature were considered as fixed and
days as random factors. Tukey’s multiple comparison was used for post-hoc analysis on the significant factors.

3.3 Results and discussion

3.1 Lipid oxidation

One of the most common undesirable reactions found in food is lipid oxidation, which can affect both product taste and odour. Lipid oxidation processes occur via a free radical chain mechanism. Consequently, all radical species forming the plasma bulk could encourage these reactions (Gavahian, Chu, Khaneghah, Barba, & Misra, 2018). One of the most common products of lipid oxidation is malondialdehyde, which can be measured by TBARs analysis.

Plasma treatment was found to have no impact on lipid oxidation, as no significant differences (p>0.05) were found for the TBARs values between untreated and treated samples (Figure 3.1).
Figure 3.1: TBARs values (mg MDA/kg sample) for control (black line) and plasma (grey line) A) at -20°C, B) at 4°C and C) at 8°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). All the differences are measured separately for 4°C, 8°C and -20°C.
However, the TBARs values significantly increased (p<0.05) during storage for both the control and plasma samples (Figure 3.1), which is in line with previous research. For example, Romotowska, Karlsdottir, Gudjonsdottir, Kristinsson, and Arason (2016) investigated lipid deterioration of mackerel during frozen storage at -18 °C and -25 °C, with TBARs formation observed over time. These results agree with the findings of Albertos et al. (2017) for mackerel treated with a dielectric barrier discharge at 70 and 80 kV for treatment times of up to 5 minutes. These authors observed no significant effect on TBARs values between the treated and untreated samples for any of the applied voltages or treatment times. No significant differences in TBARs values were also reported in Atlantic herring treated at 70 kV for 5 min compared to the control samples 1 day after treatment but a significant increase of those values was observed during the storage period (Albertos et al., 2019). When Lee et al. (2016) employed a plasma treatment for chicken breasts using a flexible thin-layer dielectric barrier discharge (FTDBD) plasma, no significant differences in the TBARs values was detected. Similar results were found after plasma treatment using a dielectric barrier discharge on milk samples during a 5 and 10 minutes treatment process at 250 W (Kim et al., 2015). Contrary to these findings, several articles report that plasma induces lipid oxidation. For example, the TBARs values for plasma treated sushi products (Nigiri and Hosomaki) were found to increase after being treated at 70 kV or 80 kV for 5 min (Piotr Kulawik, Alvarez, et al., 2018). A higher surface area and a higher concentration of unsaturated fatty acids resulted in higher levels of TBARs in Hosomaki sushi (Dawczynski, Schubert, & Jahreis, 2007; Piotr Kulawik, Alvarez, et al., 2018). A significant increase in TBARs values has been reported by (Albertos et al., 2019) in Atlantic herring treated at 80 KV for 5 min. When dried Alaska pollock shreds, semi dried squid and Gwamegi (semi-dried raw Pacific saury) were treated by corona discharge plasma jet (CDPJ), a significant increase (P<0.05) in TBARs values were detected (Choi, Puligundla, & Mok, 2016, 2017; Puligundla, Choi, & Mok, 2018). According to these authors, that increase during
the CDPJ treatment may be attributed to the further oxidation of primary lipid oxidation products by reactive species generated by the jet plasma. Similar results were obtained by Olatunde, Benjakul, and Vongkamjan (2019b) who found an increase in TBARs values in plasma treated Asian sea bass slices with increasing treatment times that could be as a result of the ozone generated. In their study, the samples were packed in heat sealed bags filled with argon/oxygen (90:10), treated with high voltage cold atmospheric plasma (HVCAP) at 80 kV RMS for 2.5, 5.0, 7.5 and 10 min and stored at 4 °C for 1 h as post-treatment time. TBARs values were also significantly higher for plasma treated brown and white rice for all treatment conditions with significant increases found for with increasing treatment times of up to 20 minutes (Lee et al., 2018). It has been reported that the nature of the food matrix and the way the product is handled prior to plasma exposure can affect the oxidation rate in plasma treated samples (Gavahian et al., 2018). The optimization of the process parameters is also needed in order to reduce the rate of lipid oxidation in plasma treated products.

The limit above which TBARs levels would indicate that secondary oxidation products could be detected by consumers have been reported to be 8 mg malondialdehyde/ kg of fish tissue (Schormüller, 1968) while Connell (1990) suggested TBARs values of 1–2 mg MDA/kg of fish flesh as the limit beyond which fish will normally develop an objectionable odour. The results obtained in the present work were much higher than these proposed limits. However, it is important to highlight that the aim of the present study was to compare non-treated and plasma treated samples rather than a quantification of MDA during storage, with no significant differences being found. Such values could be explained by the overheating that might be caused by a blender used to homogenize the samples and the penetration of oxygen and other species that could have happen while mincing. However, the results obtained in the present work are in line with those reported in mackerel by Romotowska et al. (2016). According to these authors, an increase of oxidation products may be related to the total lipid content
seasonal variation, due to differences in accessibility of the feed source and also due to the geographical location of the catch seemed to exert an influence on the lipid stability. It has also been suggested that higher values of lipid oxidation products detected in mackerel could be generated during storage and transportation of the fillets (Cropotova, Mozuraityte, Standal, & Rustad, 2019)

3.2 Fatty acids profile

The fatty acid profiles for both the control and plasma treated samples during storage were analysed. The study focused on two fatty acids present in the fish which offer high nutritional values namely eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3). The effects of cold atmospheric plasma on the fatty acid profiles for the mackerel fillets are summarised in Table 3.1.
Table 3.1: Fatty acid profile during storage study. No significant differences (p> 0.05) were found for any lipid class. The differences are measured separately for 4°C, 8°C and -20°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Treatment</th>
<th>Time (days)</th>
<th>SFA (%)</th>
<th>MUFA (%)</th>
<th>PUFA (%)</th>
<th>EPA (%)</th>
<th>DHA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Control</td>
<td>0</td>
<td>34.3 ± 0.2</td>
<td>30.7 ± 2.7</td>
<td>35.0 ± 2.8</td>
<td>9.9 ± 1.5</td>
<td>16.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>37.5 ± 5.9</td>
<td>30.2 ± 3.4</td>
<td>32.3 ± 2.5</td>
<td>8.4 ± 0.7</td>
<td>14.6 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>0</td>
<td>35.1 ± 0.3</td>
<td>27.8 ± 1.4</td>
<td>37.1 ± 1.5</td>
<td>10.2 ± 0.6</td>
<td>18.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>36.4 ± 2.4</td>
<td>14.3 ± 8.3</td>
<td>39.3 ± 6.6</td>
<td>11.5 ± 0.7</td>
<td>20.9 ± 5.8</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>0</td>
<td>34.3 ± 0.2</td>
<td>30.7 ± 2.7</td>
<td>35.0 ± 2.8</td>
<td>9.9 ± 1.5</td>
<td>16.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>36.2 ± 6.4</td>
<td>30.0 ± 5.4</td>
<td>33.8 ± 11.8</td>
<td>9.3 ± 3.5</td>
<td>17.7 ± 7.3</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
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<td>35.1 ± 0.3</td>
<td>27.8 ± 1.4</td>
<td>37.1 ± 1.5</td>
<td>10.2 ± 0.6</td>
<td>18.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>34.7 ± 1.1</td>
<td>30.3 ± 2.3</td>
<td>35.0 ± 3.2</td>
<td>9.63 ± 2.4</td>
<td>15.4 ± 0.9</td>
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<td></td>
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<td>5</td>
<td>33.1 ± 2.6</td>
<td>34.1 ± 2.1</td>
<td>32.8 ± 0.6</td>
<td>9.5 ± 0.7</td>
<td>15.9 ± 0.8</td>
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<tr>
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<td>7</td>
<td>33.9 ± 0.2</td>
<td>29.7 ± 0.5</td>
<td>36.4 ± 0.3</td>
<td>10.3 ± 0.6</td>
<td>18.3 ± 0.9</td>
</tr>
<tr>
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<td>35.0 ± 0.3</td>
<td>29.3 ± 4.1</td>
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<td>9.8 ± 1.1</td>
<td>16.7 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>36.7 ± 0.5</td>
<td>30.1 ± 0.2</td>
<td>33.2 ± 0.5</td>
<td>8.1 ± 0.2</td>
<td>16.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>0</td>
<td>35.1 ± 0.3</td>
<td>27.8 ± 1.4</td>
<td>37.1 ± 1.5</td>
<td>10.2 ± 0.6</td>
<td>18.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>36.4 ± 2.4</td>
<td>14.3 ± 8.3</td>
<td>39.3 ± 6.6</td>
<td>11.5 ± 0.7</td>
<td>20.9 ± 5.8</td>
</tr>
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<td>5</td>
<td>34.7 ± 1.4</td>
<td>28.9 ± 0.5</td>
<td>36.4 ± 0.9</td>
<td>10.3 ± 0.6</td>
<td>17.7 ± 0.6</td>
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<td>34.2 ± 0.6</td>
<td>27.3 ± 1.1</td>
<td>38.6 ± 0.5</td>
<td>11.7 ± 1.0</td>
<td>17.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>34.1 ± 0.9</td>
<td>31.3 ± 0.9</td>
<td>34.7 ± 1.4</td>
<td>9.5 ± 0.2</td>
<td>17.2 ± 1.0</td>
</tr>
</tbody>
</table>

- SFA: Saturated Fatty Acid, MUFA: Monounsaturated Fatty Acid, PUFA: Polyunsaturated Fatty Acid, EPA: Eicosapentaenoic Acid, DHA: Docosahexaenoic Acid.
No significant differences (p>0.05) were found for the fatty acid profiles between the control
and treated samples for the same temperature and storage day. These results agree with those
reported by Piotr Kulawik, Alvarez, et al. (2018), who described no significant differences in
the content of each fatty acid studied for sushi products. In addition, there was no significant
change in the fatty acid composition of beef jerky treated with a radio-frequency atmospheric
pressure plasma discharge (J. S. Kim et al., 2014).

However, in another study on mackerel, significant differences between the control and treated
samples were observed for the content of palmitic acid (C16:0), oleic acid (C18:1, n-9),
eicosapentaenoic acid (C20:5, n-3) and docosahexaenoic acid (C22:6 n-3) after treatment with
a DBD operating at a discharge voltage of 70 kV and 80 kV for 1, 3 and 5 minutes (Albertos
et al., 2017). This disagreement with our study could be due to different sample preparation
methods employed. In the present work, a whole fillet with the skin attached and facing down
was treated. This skin likely provided extra protection against the action of the plasma as only
the upper side of the mackerel muscle was exposed and hence allowed to interact with the
plasma atmosphere, possibly lessening any effect of the plasma. In addition, in our experiment,
the fillet was blended after the treatment and stored. This homogenisation could mask possible
localised negative effects induced by plasma.

The effect of cold atmospheric plasma on the fatty acid profile has also been studied on
different matrices including soy bean oil (Yepez & Keener, 2016). A significant change of the
fatty acid composition was reported after applying a plasma discharge at 90 kV for extended
treatment times of 1, 1.5, 2, 4, 6 and 12 hours. They reported that α-linolenic (18:3, n-6) and
linoleic (18:2, n-6) acid significantly decreased as a function of treatment time, while oleic
(C18:1, n-9), stearic (C18:0), and palmitic (C16:0) acid significantly increased. Kim et al.
(2015) found a significant change of butyric (C4:0) and caprylic acid (C8:0) content in milk
for plasma treatments of 5 and 10 minutes with an input power of 250 W. Surowsky et al.
(2015) reported that the effect of plasma was more evident in liquid samples than solid samples due to their ability to interact with the bulk of the liquid matrix; this could explain why a significant effect of plasma treatment for oil and milk was previously reported. On the other hand, the effect of the temperature on the oxidative stability of fatty acid methyl esters has also been studied. Giua, Blasi, Simonetti, and Cossignani (2013) found that the methyl forms of conjugated linoleic acid (Me-CLA) showed the highest isomer profile modifications during a heating treatment at 180 °C for 15, 30, 45 and 60 min in order to simulate a frying process. In fact, the percentage of cis-9,trans-11 and trans-10,cis-12 CLA isomers decreased during heating time and after 60 min, 13.6% of trans,trans isomers and 4.9% of cis,cis isomers were detected. In another study, Cossignani, Giua, Simonetti, and Blasi (2014) found that methyl octanoate was the main methyl ester compound detected both for Me-CLA and for Me-LA oxidized samples after being treated at 180 °C for 15, 30, 45 and 60 min. Other studies focused on the effect of cold plasma on the fatty acid profile of chocolate milk drink and guava-flavored whey beverage when compared to conventional pasteurization has been published (Coutinho et al., 2019; Silveira et al., 2019). The chocolate milk drink subjected to intermediate cold plasma treatment conditions presented an improved fatty acid profile when compared to the pasteurized product, with a significant reduction in stearic acid and an increase in myristoleic acid, linoleic, and PUFA levels (Coutinho et al., 2019). However, when mild and severe conditions were applied, there was an increase in SFA and a decrease in both MUFA and PUFA. The changes in fatty acids were attributed to the oxygen radicals produced during plasma treatment, including ozone, that react with the unsaturated fatty acids and break down the double bonds, leading to an increase in SFA (Gavahian et al., 2018). No significant differences were found by Silveira et al. (2019) between the plasma-treated guava-flavored whey beverage and the pasteurized product for butanoic, octanoic, decanoic, dodecanolic, myristic, and stearic acids levels. However, an increase in palmitic acid and/or a decrease in
hexanoic acid, oleic acid, and MUFA were observed probably due to the reactivity of the plasma reactive species against the unsaturated fatty acids.

3.3 Nutritional indices

Nutritional indices were calculated from the fatty acid profiles of both treated and untreated samples using the equations outlined in section 2.5.3. The results are summarised in Table 3.2. No significant differences (p>0.05) were found for any index, which is to be expected given no significant differences were found for fatty acid profiles between treatments. To the best of the authors’ knowledge, this is the first time that these indices have been calculated for cold plasma treated mackerel. AI relates the risk of atherosclerosis and is based on fatty acids which can increase (C12:0, C14:0 and C16:0) or decrease (ΣMUFA, ΣPUFA) the level of blood cholesterol. TI values relate to the tendency to form clots in the blood vessels, defined as the relationship between the pro-thrombogenic (saturated) and the anti-thrombogenic fatty acids (MUFAs, n-6 PUFAs and n-3 PUFAs) (Ulbricht & Southgate, 1991). The smaller the AI and TI values, the greater health benefits derived from the product (Ulbricht & Southgate, 1991). In the present work, no significant differences (p>0.05) were found in the AI values in the control and plasma treated samples along the storage at the temperatures studied. Both treated and untreated samples had an AI value of 0.7 on day 0. The AI values were 0.6 and 0.7 in plasma treated samples along the 14 days at -20 °C, while the control had an AI value which varied from 0.6 to 0.9. At 4 °C, plasma treated samples showed AI values of 0.7 and 0.8 during the 7 days of storage which were in a similar range than the untreated samples. When samples were stored at 8 °C, the AI values in plasma treated and untreated samples varied from 0.6 to 0.8.
Table 3.2: Nutritional indices values during storage study. No significant differences (p > 0.05) were found for any nutritional class. The differences are measured separately for 4°C, 8°C and -20°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Treatment</th>
<th>Time (days)</th>
<th>Saturation index (SI)</th>
<th>Atherogenic index (AI)</th>
<th>Thrombogenic index (TI)</th>
<th>n-6/n-3</th>
<th>PUFA/SFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>Control</td>
<td>0</td>
<td>0.5 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.6 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>0.1 ± 0.0</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>5</td>
<td>0.5 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0.5 ± 0.0</td>
<td>0.9 ± 0.0</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>0.5 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Control</td>
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<td>0.7 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>1.0 ± 0.1</td>
</tr>
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<td>0.7 ± 0.1</td>
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<td>0.1 ± 0.0</td>
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<td>0.7 ± 0.1</td>
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<td>0.1 ± 0.0</td>
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<td>0.5 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>0.5 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>8°C</td>
<td>Control</td>
<td>0</td>
<td>0.5 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.1</td>
<td>0.9 ± 0.4</td>
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<tr>
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<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>1.0 ± 0.1</td>
</tr>
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<td>0.6 ± 0.0</td>
<td>0.3 ± 0.0</td>
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</tr>
<tr>
<td>-20°C</td>
<td>Control</td>
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<td>0.7 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
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</tbody>
</table>
On the other hand, the TI values obtained on day 0 in plasma treated samples were 0.3 which did not change (p>0.05) along the investigated storage period for any of the temperatures studied. This index showed values between 0.2 and 0.4 in the control samples during the storage. AI values of 0.48 and TI values of 0.24 have been reported for mackerel by Fernandes et al. (2014) who indicated the range of the expected values for AI and TI indices to be up to 1 and 0.5, respectively. According to these authors, AI and TI values detected in the present work would be within the expected range both in plasma treated and untreated mackerel fillets and during the storage period.

Mackerel is considered a healthy food because the ratio PUFA/SFA is above 0.45 and the plasma treatment did not decrease the ratio below that recommended value (Hmso, 1994). Moreover, it can be observed that this ratio did not decrease during the storage for any of the temperatures studied. SI indicates the relationship between the sum of saturated fatty acids (C14:0, C16:0 and C18:0) (pro-thrombogenic) and unsaturated fatty acids (anti-thrombogenic). It has been reported that myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) are associated with an increased risk of coronary heart disease (Zong et al., 2016). Although to author’s knowledge, there is no numerical values assigned to SI, a food with lower values of these SFA compared to unsaturated fatty acids would be considered a healthier food. In the present work, the SI value in untreated mackerel varied from 0.5 to 0.6 and it was at the same level (p>0.05) in plasma treated samples during the storage period for all the temperatures studied. The n-6/n-3 ratio is considered a good indicator of nutritional value of dietary fat. Diets containing higher amount of n–6 PUFA or high n–6/n–3 ratio have been reported to promote the pathogenesis of cancer, inflammatory and cardiovascular diseases (Simopoulos, 2002, 2008). According to the nutritional recommendations, the n-6/n-3 ratio should not be higher than 4.0 (Hmso, 1994). In our study, the values of this ratio were within this range for plasma treated mackerel, both immediately after the treatment, and along the storage period for all the
temperatures studied. Two studies focused on the effect of cold plasma on nutritional indices of chocolate milk drink and guava-flavored whey beverage compared to the pasteurised product has been published (Coutinho et al., 2019; Silveira et al., 2019). According to Coutinho et al. (2019), the chocolate milk drink submitted to mild plasma conditions had similar AI and TI indices (p>0.05) when compared to the pasteurized product. However, when mild and severe plasma treatment conditions were applied, higher AI and TI indices were obtained, highlighting the importance of optimizing the process conditions to minimize negative impacts. In another study, a plasma treated guava-flavored whey beverage showed higher AI and TI values than the pasteurised product (Silveira et al., 2019).

3.4 Protein oxidation

Like lipid oxidation, protein oxidation is also initiated by free radicals and can be measured by the loss of sulfhydryl groups (S–H) or by the formation of protein carbonyls which are both formed during this oxidation process. Radical species can lead to the scission of the peptide backbone, the conversion of one amino acid to a different one, the formation of crosslinking as well as oxidative changes of the amino acid side chains. For instance, an increase in the carbonyl content could lead to the formation of crosslinking, resulting in a decrease of the juiciness, tenderness and quality traits of meat products (Estevez, 2011). Hence, it is important to study if plasma treatments encourage this undesirable reaction.

The carbonyl content in mackerel ranged from 0.5-1.5 nmol/mg protein for the control samples, to 0.5-2.5 nmol/mg of protein for the plasma treated samples. The results indicated that the plasma treatment significantly accelerated the formation of carbonyl groups (p<0.05) for all storage temperatures studied (Figure 3.2); i.e. -20°C (Figure 3.2A), 4°C (Figure 3.2B) and 8°C (Figure 3.2C). In addition, the carbonyl content increased significantly for treated and untreated samples during storage independent of the temperature. Moreover, in the present study the
storage temperature had a significant impact (p<0.05) on carbonyl formation, resulting in greater formation at higher temperatures.

A formation of protein carbonyls in mackerel mince during a storage study at 5 °C has been also reported by Eymard, Baron, and Jacobsen (2009) and at -18 °C Ozen and Soyer (2018). Eymard et al. (2009) found values much higher than the ones presented in this manuscript (from 2 nmol of carbonyl per mg of protein up to 10 nmol/mg after 92 hours). This increase reported by Eymard et al. (2009) could be due to the mincing of the mackerel (before the treatment), making it possibly easier for oxygen to penetrate the matrix, possibly resulting in accelerated oxidation. However, in another study, Babakhani, Farvin, and Jacobsen (2016) reported that the initial content of carbonyl groups in mackerel mince was 1 nmol/mg protein and it increased up to 1.4 nmol/mg protein after 192 hours of storage at 5 °C. The reason of this low values can be explained because the mackerel mince was packaged under vacuum. This may have slowed down the oxidation due to the lack of potential gases such as oxygen which can facilitate the oxidation of the food components.
Figure 3.2: Carbonyl content values (nmol carbonyl/mg protein) for control (black line) and plasma (grey line) A) at -20°C, B) at 4°C and C) at 8°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.
The effect of high voltage cold atmospheric plasma (HVCAP) generated with the mixture of argon and oxygen (90:10) for 5 min at 28 °C with the addition of two different antioxidants such as ascorbic acid and ethanolic coconut husk extract on the quality of sea bass, has been investigated by Olatunde et al. (2019a). In this study, heat-sealed bags containing sea bass slices with and without antioxidants, filled with 90% argon and 10% oxygen using a vacuum-packaging machine, were treated at 16 kV RMS for 5 minutes using a dielectric barrier discharge system. A significantly higher (p<0.05) carbonyl content of HVCAP treated samples compared to the control ones was reported, regardless the addition of the antioxidants, concluding that HVCAP could lead to the oxidation of proteins.

There is a lack of data in the literature to compare with and to explain in more detail the effects of cold atmospheric plasma on protein oxidation in food matrices. To date, most studies have examined the effects of plasma on protein solutions. A whey protein isolate solution (2% (w/v) in 50 mmol/L phosphate buffer, pH 6.8) was treated by cold atmospheric plasma at 70 kV for periods of 1, 5, 10, 15, 30 and 60 minutes and reported a significant increase in the carbonyl content in plasma treated samples compared to the untreated ones (Segat, Misra, Cullen, & Innocente, 2015). They suggested that the formation of carbonyls could be attributed to the modifications of a number of amino acid side chain groups, especially with NH or NH2 or by peptide bond cleavages. On the other hand, K. H. Lee et al. (2018) found a decrease in the total free sulphhydryl groups (–SH) for peanut protein isolate solutions treated by cold plasma which is related to the oxidation of these groups to form disulphide bonds. Moreover, a decreased in the content of sulphhydryl groups was also reported all the treatment times (1, 5, 10, 15, 30, and 60 minutes) after applying a voltage of 70 kV on whey protein isolate solution (Segat, Misra, Cullen, & Innocente, 2015). A significant processing time dependant increase (p<0.05) in carbonyl content has been detected in the crude protease extract from squid mantle treated by a dielectric barrier discharge system at 60 kV for different treatment times (Nyaisaba et al.,
These authors also found a decrease in the total sulfhydryl group (p<0.05) (up to about 40%) as exposure time extended which was attributed to the formation of disulfide through cross-linking of sulfhydryl groups influenced by reactive species generated by cold plasma (Segat et al., 2015). Similar results were reported by Sharifian, Soltanizadeh, and Abbaszadeh (2019) who found an increase (p<0.05) in the carbonyl content of beef myofibrillar proteins after the DBD plasma treatment which was higher at increased processing times. The carbonyl formation has been attributed to the modification of certain amino acid side chains with –NH2 or –NH, or to the cleavage of peptide bonds (Segat et al., 2015). Sharifian et al. (2019) reported a significant increase (p<0.05) in free sulfhydryl groups in beef myofibrillar proteins after 10 min of atmospheric cold plasma (ACP) treatment compared to the untreated samples and those treated for 5 min. According to these authors, the alteration of the tertiary structure of the myofibrillar proteins caused by 10 min plasma treatment, could have facilitated the hidden sulfhydryl groups to become exposed at the protein surface and consequently, be more vulnerable to the treatment. ACP has been reported to induce the loss of –SH groups from amino acid cysteine present in the protein structure (Segat et al., 2015).

The data obtained showed that cold atmospheric plasma did not encourage observable undesirable reactions such as lipid oxidation to the bulk of the treated samples. In addition, the stability of the fatty acid composition of mackerel was not affected by the treatment, along with their nutritional quality indices. However, cold atmospheric plasma could accelerate the formation of carbonyls which are related to protein oxidation. Further research is required on the impact of cold plasma technology on the quality of seafood products in order to be implemented by the fish industry.
The following paper has been published from this study:

Chapter 4: Effect of cold atmospheric plasma on the techno-functional properties of model animal proteins used as ingredients

4.1-Introduction

In recent years emerging non-thermal technologies, which work under sub lethal temperature conditions, have been used for food decontamination such as: high hydrostatic pressure (H.-W. Huang, Wu, Lu, Shyu, & Wang, 2017), cold atmospheric plasma (Bourke et al., 2018), ultrasound (Arvanitoyannis et al., 2017), pulsed electric field (Francisco J Barba et al., 2015), ozone (P. Cullen & Norton, 2012), and light emitting diodes (LED) and ultraviolet (UV)(X. Li & Farid, 2016). The main advantages of these technologies is that they operate at mild temperatures, which avoid some of the losses in quality associated with high temperatures such as degradation of colour (pigments), vitamins loss, texture modification, off-flavours and protein structure modifications (Awuah et al., 2007); as well as, reduction of energy consumption when compared to traditional processing techniques (Rodriguez-Gonzalez et al., 2015). However, apart from their efficacy in reducing the microbial loads of food products, it has been found that these processing technologies may affect negatively some food components (Perez-Andres, Charoux, Cullen, & Tiwari, 2018). For example, Xuan et al. (2018) found that high pressure promoted lipid oxidation, as well as a decrease in the content of myofibrillar proteins related to damage on the tertiary structure of fresh razor clam. Similar results were reported by Claire Guyon, Le Vessel, Meynier, and de Lamballerie (2018), who described a modification of the amount of amino acids in beef mince after pressurization, and an increase of protein oxidation. In the case of ultrasound, it was observed that accelerated
significantly both lipid and protein oxidation. For instance, in Italian Salami oxidation of lipids and proteins were reported just after 3 minutes in an ultrasound bath at 25 kHz (L. D. Alves et al., 2018). Moreover, oxidation of these food components in beef were also reported at all intensities (ranging from 2.39, to 20.96 W cm$^{-2}$) and time studied (ranging from 30 to120 min) (Kang et al., 2016).

Proteins, as food ingredients, are employed in the food industry, not only for their high nutritional value, but also because of their techno-functional properties. They are an important structural component of many foods, imparting key properties to the final products such as gelling properties, emulsifying ability or water and oil holding capacity. Modifications of their native structure, due to the action of external factors such as pH, temperature or processing by emerging technologies, can lead to a change of their functionality; and consequently, the features of the food product where they are used as ingredients (Kristo, 2015). In this sense, emerging technologies can lead to protein modifications which, potentially, can modify protein functionality, as for example better solubility or enhanced emulsifying ability.

A review dealing with the effect of these novel technologies on the functional properties of food proteins has been published (Mirmoghtadaie, Aliabadi, & Hosseini, 2016). Specific effects have been reported for each of the most promising emerging technologies. For example, high hydrostatic pressure has been already investigated to modify the functional properties of food proteins (Jian Yang & Powers, 2016). A faba protein solution (1% w/v in ultrapure water) was treated at 103 and 207 MPa during 6 cycles, with a significant improvement in solubility and foaming capacity found post treatment, however, the emulsifying capacity decreased. (Jingqi Yang, Liu, Zeng, & Chen, 2018). In addition, sarcoplasmatic proteins from hake were treated at 200, 400 and 600 MPa for 6 minutes. Its solubility decreased significantly with increasing pressure; in contrast, an increment in the hydrophobicity was found as the pressure applied increased (Villamonte, Pottier, & de Lamballerie, 2016). In another study, an
improvement of solubility, foaming, emulsifying capacities and emulsifying stability of a hazelnut protein solution (2% w/v in ultrapure water) after treatment at 25, 50, 75, 100 and 150 MPa was also reported (Saricaoglu, Gul, Besir, & Atalar, 2018).

The impact of ultrasound on protein techno-functional properties has been reviewed (O’sullivan, Park, Beevers, Greenwood, & Norton, 2017). For example, a myosin extract from silver carp was treated by an ultrasound probe at 20 kHz at different intensities (from 100 to 250 W) for different times (3, 6, 9 and 12 minutes). After treatment, significant changes in the structure and physicochemical properties of myosin were reported, such as an increase in its solubility and hydrophobicity (R. Liu, Liu, Xiong, Fu, & Chen, 2017). The solubility and emulsifying capacity of a millet protein concentrate solution (10% w/w in ultrapure water) was also improved after 20, 60 and 100 W treatment, when treatments longer than 5 minutes were applied, using a sonotrode probe (Nazari, Mohammadifar, Shojaei-Aliabadi, Feizollahi, & Mirmoghtadaie, 2018). Solubility, emulsifying and water holding capacity in bull myofibrillar proteins increased significantly with increasing power and treatment time (Amiri, Sharifian, & Soltanizadeh, 2018).

The use of pulsed electric fields (PEF), to modify food proteins has been reviewed by Giteru, Oey, and Ali (2017). Soy protein was treated using PEF, at different intensity (10 to 40 kV/cm) applying pulses for times between 100 and 600 microseconds. Significant increases in the solubility and hydrophobicity of the treated samples compared with controls were reported. (Y. Li, Chen, & Mo, 2007). However, a solubility decrease of egg white proteins was found with a constant electric field intensity of 25 kV/cm regardless of treatment time (Wu et al., 2014).

Cold atmospheric plasma (CAP) is a partially ionised gas composed of reactive species such as photons, ions and electrons, which can interact with food components including food proteins. This interaction can lead to several types of reactions such as dimerization, oxidation,
deamidation, nitration, sulfoxidation, dehydrogenation and/or hydroxylation of amino acids (Takai et al., 2014a). It is well known that CAP can lead to oxidation and degradation of food components (Gavahian et al., 2018; Shashi K Pankaj, Wan, & Keener, 2018). However, scarce research has been reported on whether this technology can affect, either positively or negatively, the functionality of food proteins. A few studies on this area, which have been reviewed by Tolouie, Mohammadifar, Ghomi, and Hashemi (2017). For instance, peanut isolated protein was treated using plasma generated by dielectric barrier discharge (DBD) system at 35 V over 1, 2, 3 and 4 minutes; with a modification of its secondary structure found, leading to an improvement of its emulsion stability, water holding capacity and solubility (H. Ji et al., 2018).

The objective of this work is to assess the impact of CAP on key functional properties of three model protein powders: gelatin, as an example of partially hydrolysed protein; haemoglobin as an isolated and native protein; and lung protein extract, as an example of an isolated protein blend. In addition, conformational changes in the protein structure were monitored by means of surface hydrophobicity.

4.2-Material and methods

2.1. Chemicals and reagents

Hydrochloric acid, sodium chloride, sodium hydroxide, sodium phosphate monobasic sodium phosphate dibasic, Coomassie brilliant blue G and pork gelatine, were obtained from Sigma-Aldrich (Arklow, Co. Wicklow, Ireland). Rapeseed oil was purchased from a local market. Ultra-pure water (18.2 MΩ cm−1) was generated in-house using a Millipore water purification system (Millipore, Cork, Ireland). Haemoglobin was obtained following the methodology described by Álvarez, Drummond, and Mullen (2018), while extraction of the
lung protein was conducted as described by Lynch, Álvarez, O'Neill, Keenan, and Mullen (2018).

2.2. Plasma treatment

Forty grams of each protein were weighed and spread homogenously into polystyrene petri dishes (150 mm x 15 mm). Each petri dish was placed between two aluminium plate electrodes (outer diameter = 158 mm) separated by two polypropylene(PP) dielectric layers (2 mm thickness) forming a dielectric plasma barrier (DBD) reactor as previously described by S. K. Pankaj et al. (2013). Discharge voltage was set at 80 kV (RMS) with a treatment time of 15 minutes. During the treatment a dynamic powder cloud was observed inside the reactor, homogenising each treatment. After treatment, control and plasma samples were kept at 4°C for 24 hours prior to analysis.

2.3. Functional properties

2.3.1. Solubility

The method reported by Penteado, Lajolo, and Pereiradossantos (1979) was used to study protein solubility. 0.5 grams of the protein sample were added to 10 mL of milliQ water. Different pH solutions were prepared over the range of 3-8. After that, all solutions were centrifuged at 2400 g for 30 minutes at 4°C in a Hettich Rotanta 460R centrifuge (Fisher Scientific Ireland, Dublin, Ireland). The amount of soluble protein in the supernatant was quantified by measuring absorbance at 280 nm using a using a ND-100 Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). All the experiments were carried out in triplicate. The solubility was calculated as:

\[
%S = \frac{P_d}{P_t} \times 10
\]  

(eq. 4.1)
Where %S is the percentage of solubility, Pd is the amount of soluble protein (g) and Pt the amount (g) the total protein used in the assay.

2.3.2 Emulsifying capacity

Emulsifying capacity was determined by the Inklaar and Fortuin (1969) method. Solutions of different concentrations of protein sample in ultrapure water (containing 0.075 of NaCl w/v) were prepared ranging from 2 mg/mL to 10 mg/mL NaCl was added to increase the protein-emulsifying capacity because protein folding is induced. In the case of extracted lung proteins (ELP) the pH after dissolving in water was around 10, usually too high for emulsification to occur; consequently the pH was adjusted to a value of 6, using 1 M hydrochloric acid (HCl) solution. After that, 13 mL of rapeseed oil was added to each sample and mixed at 13500 rpm for 2 minutes using a using a Omni Prep Multi-Sample Homogenizer (Omni International). Subsequently, samples were centrifuge at 1200 g for 10 minutes at 4⁰C in a Hettich Rotanta 460R centrifuge. The leftover oil not consumed during the emulsification process was weighed. Triplicates of all experimental results were obtained. The percentage of emulsification was calculated as:

\[
%E = \left(1 - \frac{g_e \cdot \rho_e}{V_a}\right) \times 100
\]  

(eq. 4.2)

where %E is the percentage of emulsification, ge the weight of the leftover oil (mL), \(\rho_e\) the density of rapeseed oil and \(V_a\) the volume of the added oil (mL).

2.3.3 Rheological and gelation properties

Lowest gelation concentration (LGC) and gelation temperature (Tg) were studied for the three proteins. Melting temperature (Tmel) was also obtained for the gels formed using pork gelatine. LGC was analysed according to the approach reported by Coffmann and Garciaj (1977).
Protein samples were prepared in water in a range of concentrations between 2 and 12% each stirred with a vortex and heated to 85°C over 30 minutes. Subsequently, the samples were left to cool to 4°C and stored overnight in a fridge to allow the formation of a gel. The lowest gelation concentration was selected where a consistent gel was formed by examining their resistance to flow under gravity.

Rheological measurements were performed using a controlled stress rheometer (Anton Paar MCR 301, Anton Paar Gmbh, Graz, Austria) employing a parallel plate geometry (diameter 50 mm) and a 1 mm gap. A solution containing a 10% (w/v) of each protein was employed for analysis. To prevent evaporation, water was added to the top of the upper plate. Ten minutes were allowed to stabilise the sample at 20 °C after the 1 mm gap was reached. Then, a gradient of temperature was applied, consisting of: 0-2.5 min at 20°C, 2.5-15 min covering a temperature ramp of 20-80°C, 15-20 min at a constant temperature of 80°C and 20-40 min over covering the temperature ramp of 80°C to 20°C. Measurements were taken at a strain of 1% and a frequency of 1 Hz. In the specific case of the pork gelatin, the solution was heated at 50°C in a water bath during 10 minutes before the sample was loaded onto the rheometer.

Depending of the nature of the protein the Tg could be determined by three methods. Firstly, the intersection point between the elastic/storage (G’) modulus and the viscous/loss modulus (G’’) (Lamsal, Jung, & Johnson, 2007). If there is no line crossing between the parameters, the gelling temperature is obtained once the G’ modulus reaches a value of 1 Pa. Finally, if neither of these two conditions occurs, the gelling temperature is determined as the temperature of the inflection point. Tmel was obtained where a second crossover occurred between the viscous/loss (G’’) and the elastic/storage moduli (G’).
2.3.4. Water and Oil holding capacity

The water and oil holding capacities were determined according to the method of (Beuchat, 1977). The oil holding capacity was measured by adding 10 grams of oil to 0.5 g of the protein sample and gently homogenised using a vortex stirrer. After resting for 30 minutes, the mixture was centrifuged at 2200 g for 10 min at 4°C in a Hettich Rotanta 460R centrifuge (Fisher Scientific Ireland, Dublin, Ireland). The same method was employed to measure the water holding capacity, except for replacing 10 grams of oil with 10 g of ultra-pure water.

2.3.5. Surface Hydrophobicity

The surface hydrophobicity capacity was evaluated using the method of Cao, Zhao, and Xiong (2016). This method is based on the hydrophobic interactions between basic and aromatic amino acid residues of proteins and the anionic form of the well-known stain Coomassie Brilliant Blue G-250 (CCBG) which forms a complex which can be measured at 585 nm. Briefly, 1.2 ml of a solution of 5 mg/ml of protein in 20 mM phosphate buffer at pH 6.0 were mixed with 300 µl of a 0.1 mg/ml solution CBBG in ultra-pure water. A control was prepared by mixing 1.2 ml of the phosphate buffer with 300 µl of the CCBG solution. Samples were stirred at 2000 rpm for 3 minutes using a multi shaker (Heidolph Multi Reax, Heidolph Instruments GmbH & CO, Schwabach, Germany) and after, centrifuged at 2000 g for 10 min at 4 °C in a Hettich Rotanta 460R centrifuge. The supernatant was taken and centrifuged again under the same conditions. Finally, the absorbance of the supernatant was measured at 585 nm using a ND-100 Nanodrop Spectrophotometer. The amount of CBBG bound was calculated using the following formula:

\[
\text{CCBG bound} \ (\mu g) = 30 \ \mu g \times \frac{(Abs_{\text{control}} - Abs_{\text{sample}})}{Abs_{\text{control}}} 
\]
2.4 Statistical analysis

Analysis of variance (ANOVA) of dependent variables was carried out using Minitab 17.1.0 (Minitab Inc). Statistics were calculated using a general lineal model (GLM) considering the entire variable as fixed factors. Tukey’s multiple comparison was calculated to study the effect of the plasma treatment on the functional properties. Experiments were performed in duplicate and analyses were carried out in triplicate.

3-Results and discussion

3.1. Solubility

Protein solubility depends on both protein characteristics (amino acid sequence, crosslinking, aggregate state and protein structure) and external factors (pH, temperature, ionic strength). For example, proteins are characterised to be zwitterion, i.e., they have net charge at a specific pH, which is called the isoelectric point (pI), but outside of this pH value, their functional groups could change their individual charge leading to a modification of the net charge of the whole protein and consequently, this phenomenon can affect its solubility. Proteins with an open structure, presenting a large charged surface area, would have better solvation ability and thus, improved solubility. Solubility assessment is very important in determining the potential applications of proteins as food ingredients.

The solubility of the both haemoglobin and ELP after treatment is compared with control at different pH values, ranging from 3 to 8. The results can be observed in Figure 4.1.
It was found that there is a significant (p<0.05) solubility decrease of haemoglobin at pH 4, 5 and 6 after plasma treatment (Figure 4.1A). However, the treatment increases the solubility significantly at pH 3 (p>0.05). Finally, no significant differences were found between the control and the treated samples at pH 7 and 8. Regarding the solubility of ELP, it was affected by the treatment, with a decrease in solubility observed (p<0.05) at pH 3, 4 and 6. On the other hand, no effect was found for the other pH values studied: 5, 7 and 8. Such analysis could not be performed for the pork gelatin, as the powder could not be dispersed and a gel formed.
From these results it could be observed that plasma tends to decrease the solubility of proteins. This could be due to a modification of the protein surface, exposing more hydrophobic groups which hinder the interaction with water. It was suggested that cold atmospheric plasma could cause crosslinking of proteins (S. K. Pankaj et al., 2014); which is one of the factors which modifies protein solubility. This is in agreement with the results observed here; due to formation of aggregates after CAP treatment. Other researchers have reported modifications on the secondary structure of haemoglobin and myoglobin after treatment using a jet plasma (Park et al. (2016b). These researchers found that there was a modification of the secondary structures, resulting in a decrease of the α-helical conformation and an increase of β-sheet structures; which may have an effect on protein solubility. The effect of plasma on the solubility of other proteins has already been reported. For instance, (Bußler et al., 2016) reported than the solubility of proteins extracted from Tenebrio molitor flour decreased after plasma treatment. It is know that plasma can induce oxidation mechanisms (Perez-Andres et al., 2018), and moreover, it is suggested that high levels of oxidation can result in protein denaturation and precipitation, which is associated with decreased protein solubility (Zhang, Xiao, & Ahn, 2013). Conversely, it was observed that the solubility of pea protein isolate, just after being treated (frequency of 3.0 kHz and voltage of 8.8 kV) was increased when compared to the control; however, over storage time, the solubility decreased faster in treated samples (Bußler, Steins, Ehlbeck, & Schlüter, 2015). Similar results were found by (H. Ji et al., 2018), who observed that CAP improved the solubility of peanut protein after 1 minute treatment at 35 kV using a similar source system.

3.2. Emulsifying capacity

The emulsification capacity of a specific protein depends basically on its structure, and the location of the hydrophobic and hydrophilic amino acid groups, i.e, their amphipathic character. An amphipathic molecule is one where the hydrophobic groups predominant in a
part of its structure and hydrophilic ones on the other side. The amphipathic nature of protein, which is essential for emulsifying capacity, originates from the well-balanced distribution of the hydrophobic and hydrophilic regions of the protein molecule. An emulsion, a mixture of two immiscible liquids (for example water and oil), is not stable by nature and trends to separate spontaneously into two phases. A protein with a good emulsifying capacity is able to stabilize the mixture, impeding phase separation. Some proteins are used as emulsifiers to stabilize foods such as salad dressings, sauces and mayonnaise.

It was observed that CAP treatment reduced significantly (p<0.05) the emulsifying capacity of the three proteins studied (Figure 4.2).
Figure 4.2: Emulsifying capacity and stability results for haemoglobin (A), pork gelatin (B) ELP protein (C).
Firstly, focusing on haemoglobin, the statistical analysis indicated that the emulsifying ability decreased (p<0.05) at haemoglobin concentrations of 2 mg/mL and 4 mg/mL after treatment; this effect was not observed at higher concentrations. On the other hand, the emulsification stability showed no differences between the control and the treated samples (p<0.05), for any of the concentrations employed. In the case of pork gelatin, the emulsifying capacity was lower for the untreated samples at 2, 6 and 8 mg/mL (p<0.05). It was found that stability for both treated and control samples, decreased over time; however, only from day 5 and 7, where treated samples performed significantly poorer than the control samples (p<0.05). Finally, the modification of this functional property was studied in ELP. The trend of this protein was similar to those described previously. The emulsifying capacity was significantly lower (p<0.05) for protein concentrations of 2 and 6 mg/mL. In addition, the emulsion was not stable during the experimental period, and stability was significantly lower for the plasma treated samples after 3, 5 and 7 days.

As mentioned in the previous section, CAP may cause protein aggregation and changes on the protein structure. Both factors together may have an effect on the emulsifying properties. An increase in the particle size, due to aggregation of the protein, generates a new organisation of the different protein groups. The hydrophilic groups would be exposed on the surface, covering the hydrophobic groups located in the inner part, which could not further interact with the apolar phase of the emulsion. As consequence, the amphipathic character of the protein decreases and its emulsifying capacity and stability are negatively affected. However, at higher concentrations the effect of the treatment was not that obvious due to the presence of sufficient hydrophobic groups to form an emulsion. To the best of our knowledge, no other studies regarding emulsifying ability of proteins after CAP treatment have been reported.
3.3. Rheology gelling properties

The impact of CAP on the gelling and rheological properties of the proteins investigated is summarised in Table 4.1.

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<th>LGC (%)</th>
<th>T gel (°C)</th>
<th>T mel (°C)</th>
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<tbody>
<tr>
<td>Haemoglobin</td>
<td>4</td>
<td>65.30 ± 0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Haemoglobin Plasma</td>
<td>4</td>
<td>63.23 ± 1.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Pork gelatin</td>
<td>2</td>
<td>79.33 ± 1.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.68 ± 1.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pork gelatin plasma</td>
<td>3</td>
<td>68.5 ± 4.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.90 ± 1.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ELP</td>
<td>12</td>
<td>60.53 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>ELP plasma</td>
<td>12</td>
<td>51.83 ± 3.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
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</tbody>
</table>

CAP significantly decreased (p<0.05) the gelation temperature of pork gelatine and ELP. This could be due to the treatment leading to a partial denaturalisation of these proteins, and subsequently lower temperatures are required to initially unfold the proteins. This modification could help the formation of gels at lower temperatures, because less energy is needed to initiate the protein denaturation, which might be beneficial for specific food products. In contrast, the melting temperature of pork gelatin was not affected by the treatment, since proteins forming the gel are already denaturised and the effect of CAP is masked by the previous thermal treatment to from the gel. In another study, cold atmospheric plasma changed the elastic and viscous modulus of dough, suggesting that the radical species formed during the treatment could modify the protein structure, leading to the modification of the rheological properties observed (Segat et al., 2015).

3.4. Water & oil holding capacity

Water and oil holding capacity is the ability of a protein to adsorb such compounds inside its structure. For food products, such capacity prevents fluid leakage during food processing or storage. For instance, the water-holding capacity (WHC) affects the tenderness and juiciness of meat and fish products. Higher water and oil capacity leads to better cooking yields, since
lower amounts of water or oil are lost. For this reason, both parameters are important factors in the characterization of the functional properties of food proteins, even more when intended to be used in meat products. The effect of CAP on the water and oil capacity of these proteins is studied (Table 4.2 and 4.3).

Table 4.2: Results of water holding capacity. N/D: it was not determined because haemoglobin was completely soluble under the experimental conditions.

<table>
<thead>
<tr>
<th></th>
<th>Haemoglobin</th>
<th>Pork gelatin</th>
<th>Extracted lung proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g water/g protein)</td>
<td>(g water/g protein)</td>
<td>(g water/g protein)</td>
</tr>
<tr>
<td>Control</td>
<td>N/D</td>
<td>5.36 ± 0.11(^a)</td>
<td>4.41 ± 0.03(^a)</td>
</tr>
<tr>
<td>Plasma</td>
<td>N/D</td>
<td>6.10 ± 0.44(^b)</td>
<td>4.56 ± 0.05(^b)</td>
</tr>
</tbody>
</table>

Table 4.3: Results of oil holding capacity.

<table>
<thead>
<tr>
<th></th>
<th>Haemoglobin</th>
<th>Pork gelatin</th>
<th>Extracted lung proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g oil/g protein)</td>
<td>(g oil/g protein)</td>
<td>(g oil/g protein)</td>
</tr>
<tr>
<td>Control</td>
<td>2.98 ± 0.02(^a)</td>
<td>1.75 ± 0.13(^a)</td>
<td>2.82 ± 0.09(^a)</td>
</tr>
<tr>
<td>Plasma</td>
<td>3.03 ± 0.06(^a)</td>
<td>1.55 ± 0.07(^a)</td>
<td>4.57 ± 0.06(^b)</td>
</tr>
</tbody>
</table>

CAP treatment improved significantly (p<0.05) the water holding capacity of both pork gelatin and ELP, suggesting a partially denaturalisation of the proteins present in these samples. CAP, as previously discussed, can lead to protein unfolding effects, allowing the water molecules to interact with more hydrophilic groups buried in the structure, increasing their availability for interaction. On the other hand, there was no impact on the oil holding capacity of haemoglobin or pork gelatin after 80 kV treatment, for 15 minutes. However, in the specific case of ELP, the OHC was significantly improved after the treatment compared to the control. ELP is composed by a blend of different types of proteins, and CAP may affect differently each one; thus, some may increase the exposure of hydrophilic groups, while others could expose more hydrophobic groups. This could explain why both WHC and OHC has been increased for this particular sample.
The water and oil holding capacity of pea testa flour was reported to be improved after 10 minutes treatment at a frequency of 3.0 kHz and a voltage of 8.8 kV, using a DBD system (Bußler et al., 2015). These authors reported that plasma induced modifications of the protein composition and structure observed with changes in the fluorescence emission spectra. In contrast, when the same plasma system and conditions was applied to an insect flour, it was found that the water holding capacity significantly decreased after treatment, regardless of processing time (from 1 to 15 minutes) (Bußler et al., 2016). The OHC in these samples was affected by the treatment; however, treatment time was not significantly correlated.

3.5. Surface hydrophobicity

To correlate the changes observed in the functional properties with modifications of the protein structure, the hydrophobicity was determined. An increment in this parameter is indicative of protein unfolding and a subsequent exposure of hydrophobic residues.

Statistical analysis shows that there were not any significant differences (p>0.05) between control and treated samples for both pork gelatin and ELP. Results are reported in Figure 4.3.

![Figure 4.3: Surface hydrophobicity results of control and treated samples for haemoglobin, pork gelatin and extracted lung proteins. Different letters indicate a significant difference (p<0.05) for hydrophobic surface between control and treated for a specific protein](image-url)
However, in the case of haemoglobin the hydrophobicity surface increased significantly (p<0.05) with plasma treatment. The radical species generated by CAP treatment may disrupt the native haemoglobin conformation, unfolding the protein and exposing these non-polar groups, leading to an increase of the surface hydrophobicity. Haemoglobin has a high content of hydrophobic amino acids, and minor changes in their structure will lead to changes in hydrophobicity. However, gelatin is already a hydrolysed protein, and its structure is already compromised, so oxidation and crosslinking effects will be more relevant after CAP treatment. Finally, ELP powder, as previously described, is a blend of many different proteins, so changes in its structure may not lead to an appreciable modification on the hydrophobicity, because a balancing effect may occur.

These results agree with those reported by (Segat et al., 2015). These authors applied CAP on solutions of whey protein isolate at 2% (w/v) in phosphate buffer (50 Mm, pH 6.8), finding a modification of its structure, leading to an increase of the surface hydrophobicity. A modification of the secondary structure and hydrophobicity surface of peanut protein by cold plasma was also reported (H. Ji et al., 2018). It was observed than the surface hydrophobicity decreased linearly after 1, 2, 3 and 4 minutes. Similar trends were found for the –SH groups but an increase was highlighted after 4 minutes treatment. Plasma discharges can lead to etching, cross-linking of proteins and oxidative reactions that can change the structure of proteins. One such modification is the exposition of the inner –SH groups, which can be oxidised to form disulphide bonds, which subsequently can be disrupt to free –SH. (Takai et al., 2014a) suggested that the formation of disulphide bonds and cleavage can occur simultaneously.
The following paper has been published from this study:

Chapter 5: Effect of cold plasma on meat cholesterol and other lipid fractions.

5.1. Introduction

Globally, meat consumption continues to increase. According to the latest report from the Food and Agriculture Organisation (F. Food, 2018), the production of meat has increased by 1.7% from 2017 to 2018, reaching a value of 335 millions tonnes (36.9% poultry, 36.6% pig, 21.9% bovine and 4.6% ovine). Cholesterol is present in all these meat products and 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 with its long axis preventing the crystallization of fatty acyl chains modifying the activity of membrane-bound enzymes (Singh et al., 2016). It also has vital functions in the metabolism and function of body tissue (Andersen, Doerr, Murphy, & McNeely, 2016). Cholesterol is an essential precursor for the synthesis of vitamin D, bile, bile acids salts, steroids and hormones. For instance, vitamin D3 is a derivative of cholesterol and is formed in the skin from 7-dehydrocholesterol. Moreover, deficiencies in cholesterol during embryogenesis and organogenesis cause severe abnormalities in the foetus (Roux et al., 2000).

In contrast, high concentrations of cholesterol in cells can be cytotoxic and pro-inflammatory (Andersen et al., 2016). Furthermore, high levels in plasma can lead to the development of diseases through atherogenesis, the agglomeration of low density lipoprotein (LDP) cholesterol on the arterial wall, creating plaques which can obstruct blood flow leading to cardiovascular diseases (Orekhov, Ivanova, & Bobryshev, 2016). In addition, a disturbance of the cholesterol metabolism can also cause numerous chronic diseases, including cancer, as well as disorders of metabolic and neurological tissues (Orekhov et al., 2016). However, some authors suggest
that only oxidised cholesterol, can contribute to the pathophysiology of human diseases such as carcinogenic, cytotoxic, mutagenic, atherogenic and neurodegenerative diseases (Singh et al., 2016). The formation of cholesterol oxidation products in food is initiated by free radicals, which can be generated by auto-oxidation, photo-oxidation and thermo-oxidation (I. G. Medina-Meza & Barnaba, 2013), leading to a chain reaction mechanisms (L Xu & Porter, 2015).

Cholesterol (5α-cholesten-3β-ol) is a lipid which belongs to the family of sterols (Figure 5.1). It is characterised by its reactive behaviour, especially on C7, C20 and C25, leading to the formation of oxysterols which have similar structures but with the addition of other functional groups such as hydroxyl, hydroperoxide, ketone and epoxide (Maldonado-Pereira, Schweiss, Barnaba, & Medina-Meza, 2018; Vicente, Sampaio, Ferrari, & Torres, 2012). The most commons oxidation products from C7 are: 7-α-hydroxycholesterol, 7-ketocholesterol and 7-β-hydroxycholesterol, while 20-hydroxycholesterol and 25-hydroxicholesterol are formed when the oxidation occurs on C20 and C25 respectively (Maldonado-Pereira et al., 2018). These compounds belong to the group of oxysterols, a class of compounds reported to be involved in several neurodegenerative diseases including Huntington’s, Parkinson’s and Alzheimer’s disease (Griffiths & Wang, 2019).

![Figure 5.1: Cholesterol structure. Circles indicate the most reactive carbon.](image-url)
Cold atmospheric plasma (CAP) is a technology with several uses in various industries. A plasma atmosphere is made up of different free radical molecules, electrons, UV-photons, positive and negative ions, ozone as well as carbon and nitrogen oxides, which varies according to the gas applied (X. Lu et al., 2016). Recently, the technology has been proposed as a decontamination step for the preservation and safety assurance of foods (Mandal et al., 2018). Jiamei Wang, Zhuang, Hinton Jr, et al. (2016) described a dielectric barrier discharge operating at 80 kV for 180 s which was applied to chicken breasts packaged in food trays in both atmospheric air and modified atmosphere gas (65% O₂, 30% CO₂ and 5% N₂). While no significant reduction of microbial populations was found for samples packaged using air atmosphere, they reported that the treatment was effective for chicken packaged under modified atmosphere, suggesting that CAP treatment could increase shelf-life from 7 days to at least 14 days. Furthermore, Yong et al. (2017) treated beef jerky at 15 kV for 2.5, 5, and 10 min using a dielectric barrier discharge system which resulted in a reduction of Listeria monocytogenes, Escherichia coli, Salmonella typhimurium and Aspergillus flavus for all treatment times. Significant reductions of Listeria monocytogenes and Escherichia coli were also found for pork loin treated by a dielectric barrier discharge (DBD) plasma operating at 3 kV with a 30 kHz bipolar square wave for treatment times of 5 and 10 minutes (H. J. Kim, Yong, Park, Choe, & Jo, 2013).

However, it is shown that these radical species present in the plasma may modify food components leading to their oxidation (Pérez-Andrés, Charoux, Cullen, & Tiwari, 2018). Lipid oxidation has long been considered a radical chain reaction triggered by hydrogen abstractions with hydrogen peroxides being considered the first stable products. However, following their decomposition, hydrogen peroxides may generate secondary lipid oxidation products (Irwin, Hedges, & Sharnbrook, 2004). When investigating lipid oxidation, simultaneous pathways for generation of secondary lipid oxidation products should be considered; various reactions of
addition, rearrangement or dismutation of lipid peroxyl radicals (LOO-) can lead to further formation of dimers, epoxides, aldehydes or ketones in parallel to hydroperoxides. Therefore, to fully assess lipid oxidation along with the determination of lipid peroxides, it is important to analyze a complex mix of secondary lipid oxidation products (Irwin et al., 2004; Tsikas, 2017). The thiobarbituric acid reactive substances (TBARS) assay is a commonly used method to assess secondary lipid oxidation by measuring the content of a secondary degradation product, namely malonaldehyde. While heated in an acidic medium, malonaldehyde reacts with thiobarbituric acid (TBA) to form a pink Schiff base adduct with an absorption maximum in the region of 532-535 nm (Guzmán-Chozas, Vicario-Romero, & Guillén-Sans, 1998). However, the TBARS method also determines a complex mixture of various other secondary oxidation products including alkanals, alkenals, alkadienals and others which react with TBA. Nevertheless, it is widely used as an indicator of lipid oxidation, particularly in meat products [16].

Using TBARS to detect lipid oxidation, it has been reported that cold atmospheric plasma can accelerate the production of peroxides, as well as lipid and protein oxidation in pork during storage (M. Huang et al., 2019). A flexible thin-layer dielectric barrier discharge plasma also oxidised lipids in beef jerky (Yong et al., 2017), as well as pork butt and beef loin (Jayasena et al., 2015). In another study, it was reported that TBARs values, related to lipid oxidation, were significantly higher in dry-cured beef, “bresaola” after plasma treatment (Rød et al., 2012). Finally, B. Kim et al. (2011) also found an increase in lipid oxidation products in bacon after cold atmospheric plasma treatment. On the other hand, CAP may not only have an impact on the quality of meat products, it can also accelerates the oxidation of proteins (Albertos et al., 2017).
There is a need for more research on the effects of plasma on meat chemistry before adoption of this technology by the industry. It is important to evaluate how plasma may affect all the components present in the matrix, and to determine if CAP could increase the shelf-life of the products without affecting their quality or safety or exposing the consumers to any health risk. The objective of this study is to study if CAP could cause any undesirable effects on lipids such as the oxidation or degradation of cholesterol leading to the formation of oxidised compounds. To investigate the effects, we first applied CAP directly to a cholesterol standard. The results were then compared to the effects observed for four different meat minces, namely; beef, lamb, pork and chicken, where the complexity of the matrix may display protective properties.

5.2. Material and methods

5.2.1. Chemicals and reagents
Cholesterol standard (purity 98 %), the internal standard 5α-cholestan-3β-ol (purity 98 %), pyridine, bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1 % of trimethylchlorosilane (TMCS) and potassium hydroxide were purchased from Sigma-Aldrich (Arklow, Co. Wicklow, Ireland). Chloroform was purchased from Sigma-Aldrich (Oslo, Norway). Methanol, hexane and dichloromethane were purchased from Fisher Scientific (Dublin, Ireland). Ultra-pure water (18.2 MΩ cm$^{-1}$) was generated in-house using a Millipore water purification system (Millipore, Cork, Ireland). All chemicals were GC grade.

2.2. Cholesterol standard preparation
A 1 mg/ml solution of cholesterol standard solution in methanol was prepared. An aliquot of 200 µl of this solution was dispensed into a glass petri dish (2.5 cm radius x 1.5 cm height), and the solvent was let to evaporate. Then, the petri dish was sealed using Parafilm.
2.3. Cholesterol standard plasma treatment

A petri dish containing cholesterol standard as previously described was treated using an in-house dielectric barrier discharge atmospheric plasma system, which was described previously [16]. This petri dish was placed between two circular aluminium electrodes (outer diameter = 158 mm) separated by two polypropylene (PP) dielectric layers (2 mm thickness). The distance between the dielectric layers was the height of the petri dish (1.5 cm). Two different voltages, 60 kV and 80 kV RMS (root mean square) were applied for two different durations, 5 and 10 minutes in triplicate; these conditions were previously shown to control microbial growth in mackerel (Albertos et al., 2017). After the treatment, control (n=3) and treated samples (n=3) were kept at 4°C over 24 hours to ensure the induced plasma components interact with the cholesterol as suggested by Ziuzina, et al. [17]. Following this storage, 200 ul of internal standard (5α-cholestan-3β-ol, 50 mg/ml in dichloromethane) was added to the petri dish and left to stand until full evaporation of the dichloromethane. Once the dichloromethane was evaporated, the residues of both, the cholesterol and internal, standards were reconstituted in 10 ml of dichloromethane and kept at -80 °C until the day of analysis.

2.4. Meat sample preparation

Fresh meat minces from four different species (beef, lamb, pork and chicken breast) were purchased at a local butcher in Dublin in November 2018. Neither the type of muscle used to produce the mince nor the origin of the meat were identified at this stage. Using a spoon, a homogeneous A portion of 100 g of each was packaged individually (Ilpra Foodpack VG 400 Packaging Machine, Italy) in a black amorphous polyethylene terephthalate (APET/PE) tray (195x155x30 mm), and sealed under atmospheric air conditions using a low oxygen permeable barrier polyvinyl-chloride film (3 cm3/m2/24 h at Standard Temperature and Pressure (STP); Versatile Packaging, Ireland), to mirror common commercial practice. Samples of each meat were divided in four different batches: control and treated with 24 hours of storage post-
treatment as well as control and treated with 7 days of storage post-treatment. All conditions were prepared in triplicate.

### 2.5. Meat sample plasma treatment

The same dielectric barrier discharge system as described in Section 2.3 was used for this study. The samples were treated with an in-package mode, where the plasma was induced inside the gas contained inside the sealed package. Each packed sample was placed between the two electrodes separated by 3 cm, i.e., the height of the tray. Ten minutes of treatment was performed at a discharge voltage of 80 kV RMS, these settings have previously been shown to control microbial growth in mackerel (Albertos et al., 2017). Similar to the cholesterol standard study, once the CAP treatment was finished both the control and plasma samples were kept at 4°C for either 24 hours or 7 days. Following this storage, i.e. either 24 hours or 7 days, all samples were individually vacuum-packed and stored at -80 °C until the day of the analysis. All conditions were prepared in triplicate.

### 2.6. Cholesterol analysis

#### 2.6.1. Cholesterol extraction

Cholesterol was quantified according to (Grasso, Harrison, Monahan, & Brunton, 2019). This method has previously been fully validated for the determination of cholesterol in turkey meat products. Briefly, approximately 0.5 g of chilled meat mince sample, 200 µL of internal standard (5α-cholestan-3β-ol, concentration: 50 mg/ml) and 30 ml of 4 M KOH in methanol were mixed and homogenized using an Ultraturrax homogenizer (Labortechnik, Staufen, Germany) at 13,500 rpm for 30 seconds in a 50 ml tube. Saponification was carried out by placing the sample into a water bath at 60 °C for one hour. After cooling down, a liquid-liquid extraction was performed by adding 5 ml of Milli Q water and 5 ml of heptane; phase separation was achieved by centrifuging the sample at 4,000 rpm (2,500 g) for 10 minutes. The organic
phase was transferred to another tube and the extraction was repeated twice more by adding 5 ml of heptane each time. All organic layers were combined and dried using a sample concentrator and reconstituted into 10 ml of dichloromethane. Derivatization of cholesterol was performed by mixing 0.5 ml of this extract, 200 µL of BSTFA-TMCS, 100 µL of pyridine and leaving them in a water bath for 15 minutes at 70 °C. After cooling down, the solution was diluted to 10 ml with dichloromethane and an aliquot was transfer to the vial to be injected into the gas chromatography system.

2.6.2. Gas chromatography-flame ionisation detector analysis

Separation was carried out using a Clarus 580 Gas Chromatograph (Perkin Elmer, Massachusetts, USA) fitted with a flame ionisation detector (GC-FID) set at 260 °C according to Grasso et al. (2019). The separation and quantification of cholesterol was carried out employing a ZB-5 capillary column (Phenomenex, Torrance, CA, 90 USA) with a thickness of film thickness of 0.25 µm and a length of 30 m x 0.25 mm. The injection volume was 0.5 µL and the inlet temperature was set to 200 °C. Hydrogen was flushed at a constant flow of 2.0 mL/min, and the split ratio was set at 5:1. The oven temperature started at 180 °C with an initial temperature ramp of 8.0 °C/min to 260 °C followed be a second temperature ramp of 2 °C/min to 280 °C which was held for 10 minutes.

The content of cholesterol was calculated using following equation (Eq. (5.1)) according to (Grasso et al., 2019).

\[
\text{Content (mg/g)} = \frac{\text{Peak Area(FAME)}}{\text{Peak Area(ISTD)}} \times \frac{\text{Weight ISTD}}{\text{Weight Sample}} \times \frac{\text{IS Purity}}{\text{RRF}} \times 20 \quad (eq. \ 5.1)
\]

Where, IS Purity is the purity of the internal standard as given on the certificate of analysis, RRF is the relative response factor for cholesterol, and 20 is the dilution factor.
2.7. Lipid content

The total lipid content was determined by the Bligh & Dyer (B&D) method which applies a mixture of chloroform, methanol and Milli-Q water for the extraction of lipids from the muscle tissue (Bligh & Dyer, 1959). The extraction was performed in duplicate, as follows. Experimental meat samples were minced with a kitchen blender (Bosch MSM87140, Frankfurt, Germany) and 10 g of the obtained mince was transferred into centrifuge tubes. The centrifuge tubes were kept on ice during the whole procedure. Then, distilled water (10 mL), chloroform (20 mL) and cold methanol (40 mL) were added to each tube. The mixture was homogenized using Ultra Turrax (IKA T18, Staufen, Germany) for 2 minutes at 9,000 rpm. Additional amount of chloroform (20 mL) and distilled water (20 mL) was added separately, and the mixture was homogenized again using Ultra Turrax for 30 seconds after each addition. After the homogenization, the tubes were centrifuged (Hettich Universal 16A Centrifuge, Berlin, Germany) for 10 minutes at 11,800 g. Chloroform phase (2 mL) was collected from the bottom of each of the tubes and transferred into a pre-weighed Kimax glass tube (10 mL). The Kimax glass tube with chloroform phase was placed in an evaporation unit and kept at 60°C with N2-stream for 1 hour. After the evaporation, the tubes were corked, cooled down to room temperature and weighed again. The results are expressed as total lipid (average ± standard deviation) in percentage of wet weight meat sample (Bligh & Dyer, 1959).

The remaining chloroform phase in the centrifuge tubes was collected and transferred to plastic tubes resistant to chloroform, flushed with N2-gas and stored at -80°C prior to analysis of peroxide values (PV) and thiobarbituric reactive substances (TBARS).

2.8. Peroxide value

PV was determined by using the iodometric titration method described by Society (2003). The end point of titration was assessed potentiometrically with an automatic titrator (TitroLine
7800, Xylem Analytics, Mainz, Germany) fitted with a platinum electrode (Pt 62). The analysis was performed in duplicate and the results were expressed in meq active oxygen/kg lipids as a mean value ±SD.

### 2.9. TBARS

The TBARS assay assesses secondary lipid oxidation products through a reaction between malondialdehyde and thiobarbituric acid (TBA). It is a good indicator of the general oxidative status in fish products because TBA reacts with a wide range of aldehydes and oxidized molecules derived from lipids and proteins. In complex food matrices such as meat and fish, lipid oxidation may take place via complex pathways due to co-oxidation reactions between lipid radicals, secondary oxidation products, pro-oxidants such as transition metals, blood or myoglobin, as well as other system components such as proteins (Tsikas, 2017). Co-oxidation reactions result in the oxidation of other food molecules such as proteins with involvement of lipid oxidation intermediates and products (Guzmán-Chozas et al., 1998; Irwin et al., 2004). Therefore, it is very important to apply a method such as the TBARS assay that can assess the general status of secondary lipid oxidation.

For this study, secondary lipid oxidation was investigated using TBARS determined in the chloroform phase according to Ke and Woyewoda (1979). As a standard, 1.1.3.3-tetraethoxypropane (T 9889) was used. The analysis was performed in triplicate and the results were expressed as average ± standard deviation in μMol TBARS/g lipid.

### 2.10 Colour

Colour characteristics of plasma-treated and untreated meat samples were measured instrumentally using a Minolta Chroma meter CR-400 (Konica-Minolta, Osaka, Japan). Before starting the analysis, the instrument was calibrated with a standard white plate. The data were recorded in color coordinates of L* (lightness, black = 0, white =100), a* (redness >0, greenness <0), and b* (yellowness, b* >0, blue <0) according to the (DUVAL, 2001). Three
measurements were performed on each of the meat samples, and the average with standard deviation determined.

### 2.11 Statistical analysis

Analysis of variance (ANOVA) of dependent variables was carried out using Minitab 17.1.0 (Minitab Inc). Statistics were calculated using a general lineal model (GLM) considering the entire variable as fixed factors. When differences were observed (p<0.05) a Tukey’s multiple comparison was calculated to study the effect of the plasma treatment on the cholesterol. Experiments were performed in triplicate and analyses were carried out in triplicate.

### 5.3. Results and discussion

#### 3.1. Cholesterol content

Cholesterol is an important food nutrient due to its role in the biosynthesis of vitamin D, bile acids and steroid hormones such as gonadal (testosterone, estrogens, progesterone) and adrenal (aldosterone, cortisol) (Cornelissen, Singh, Singh, Singh, & De Meester, 2016). Moreover, it is an important constituent of the cell membrane. For this reason, it is important to study if a novel non-thermal technology such as CAP could have any impact on this micronutrient despite previous research showing that CAP may not be able to significantly penetrate solids (Pérez-Andrés et al., 2018).

##### 3.1.1. Cholesterol standard

Cholesterol standard was exposed to CAP at two voltages (60 and 80 kV) for two different durations, namely 5 or 10 min. Overall, results show that CAP has a significant effect (p<0.05) on the cholesterol content of the treated standard with CAP treatment significantly reducing the amount of cholesterol recovered from the petri-dishes. As seen in Figure 5.2, the cholesterol
content decreased by around 35% for all the different treatments, suggesting that this could be because plasma can lead to oxidation of cholesterol. As it was mentioned previously oxidation of cholesterol can be initiated by free radical species, consequently, many of the different radical species which are presented in the plasma bulk could cause this reaction to happen (Zerbinati & Iuliano, 2017). However, there was no significant differences between the different treatments, i.e. oxidation and/or degradation was not dependent on voltage or duration of treatment.

![Graph showing cholesterol content of control and plasma samples](image)

Figure 5. 2: Cholesterol content of control and plasma samples. Different letters indicate a significant difference (p<0.05) for cholesterol at specific treatment condition.

Although the effect of cold plasma on isolated nutrients has already been reported, to the best of our knowledge, this is the first time that the effect of CAP on cholesterol is reported. However, as the present study used GC-FID for the quantification of cholesterol, it was not possible to identify the breakdown products produced by the plasma. Further research using e.g. a GC coupled to a (tandem) mass spectrometer may give further insight into the processes involved and may help in identifying which carbons in the cholesterol molecule are more prone to oxidation/degradation than others.
Previously, several food protein powders (gelatin, hemoglobin and lung protein extract) were treated at 80 kV (RMS) for 15 minutes using a dielectric barrier discharge plasma system (Pérez-Andrés, Álvarez, Cullen, & Tiwari, 2019). Authors observed that the direct treatment of cold plasma on these three protein powders affected their native structure, leading to a significant change in their functional, rheological and gelling properties. Similar results were observed by Hui Ji et al. (2018) after treating peanut isolated protein with a different dielectric barrier discharge at 35 kV for 1, 2, 3 or 4 minutes.

3.1.2 Cholesterol in meat products.

CAP did not have a significant impact on the cholesterol content in any of the four different meat mince samples (p>0.05). This may be because plasma only affects the surface of the meat and hence cannot penetrate significantly into the meat product (Pérez-Andrés et al., 2018). On the other hand, the complexity of the matrix and components inside the food could protect the cholesterol from the potential impact of cold plasma. For instance, other lipids present in the meat such as triglycerides in general and polyunsaturated fatty acids more specifically, could be more susceptible to oxidation and/or reaction with the plasma thus protecting cholesterol from interaction with the radical species resulting in cholesterol oxidation and/or degradation. In addition, the presence of naturally occurring antioxidants such as carnosine, anserine, carnitine or taurine, in meat could not only prevent the formation of undesirable oxidation products in general (Carrillo, Barrio, del Mar Cavia, & Alonso-Torre, 2017) but more specifically the oxidation of cholesterol. Moreover, there are also some antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase), vitamins with antioxidant properties (ascorbic acid and α-tocopherol) and minerals like zinc and selenium (Descalzo & Sancho, 2008), which could give an extra protection against the potential impact of the reactive species present in the plasma atmosphere. Nevertheless, given that the samples were minced offering a large exposure area, were exposed to extended treatment times and retained in the
induced plasma species for up to 7 days due to the use of the in-package technology the data points to no observable effects on the cholesterol contents of the tested meats.

Figure 5.3: Cholesterol content of control and plasma samples for: A) Beef, B) Lamb, C) Pork, D) Chicken. No significant differences between control and treated sample (p>0.05). All the differences are measured separately for each individual kind of meat.
3.2. Lipid content and oxidation

3.2.1. Lipid content

Total lipid content in meat samples varied from 1.7±0.7 for chicken (white meat) to 30.6±1.9% for lamb (red meat) (Figure 5.4). However, no significant variation in the total lipid content was found between plasma-treated and untreated samples during the storage period for any of the meats investigated.

Figure 5.4: Lipid content in control and plasma-treated meat samples.

3.2.2. Peroxide value

As the official method used (Society, 2003), has a detection limit of 0.05 mL lipid per 12 mL chloroform (equivalent to a lipid content of about 5-6%), it was not possible to measure peroxide value in chicken samples due to their very low lipid content (<2% w/w). However, novel methods such as the one recently published by Cropotova and Rustad (2020) may give further information on peroxide values of low-lipid samples.

The rest of the samples with higher lipid content in the tissue, i.e. those with more than 5% lipid content, showed increased peroxide values in all plasma-treated samples compared to non-treated (control) samples (Figure 5.5). At the same time, only plasma-treated pork and beef samples
exceeded the limit for PV established by Alimentarius (1999) (10 meq O2/kg lipid) on day 7 of chilled storage (Figure 5.5).

Plasma-treatment could result in the accumulation of reactive species accelerating lipid oxidation (Pérez-Andrés et al., 2018). This suggestion is supported by the study of Albertos et al. (2017) who previously used a non-thermal plasma (NTP) system to treat fresh mackerel fillets. They observed a similar trend for PV results after the treatment of mackerel samples with NTP. The treatment resulted in an over 5-fold increase in peroxide values compared to control samples, with both exposure time and voltage affecting the rate of oxidation. Furthermore, these observations are in line with those made by Yong et al. (2017) who reported a significant increase in the PV of beef jerky following flexible thin-layer plasma treatment for 10 min. However, this group only found a significant increase after 10 min while beef jerky treated for 2.5 or 5 min was not significantly different from the control.

3.2.3. TBARS

Lipid oxidation leads to the formation of a very wide range of different oxidation products, making the determination of lipid oxidation challenging. The determination of TBARS is one of the oldest
and the most commonly used methods for assessing secondary lipid oxidation status by measuring one of the end product of polyunsaturated fatty acid peroxidation – malondialdehyde (Fereidoon Shahidi & Zhong, 2005) (Barriuso, Astiasarán, & Ansorena, 2013; Dasgupta & Klein, 2014; Fernández, Pérez-Álvarez, & Fernández-López, 1997; Frankel, 2012; Mendes, Cardoso, & Pestana, 2009; Peiretti et al., 2011; Fereidoon Shahidi & Zhong, 2005), but it will also determine other aldehydes (Barriuso et al., 2013).

Except for beef samples after 7 days of storage and all the chicken samples, plasma-treated meat samples were characterized with significantly higher TBARS-values compared to the untreated (Figure 5.6). This effect can be explained by the radical-initiating mechanism of plasma treatment as mentioned previously (Albertos et al., 2017). However, the highest TBARS-values were found in chicken samples with the lowest lipid content. This is in contrast to the findings reported by Jayasena et al. (2015) who reported a significant increase of TBARS values in beef loin following treatment with flexible thin-layer dielectric barrier discharge plasma for 10 min. Furthermore, these authors only report a significant increase after 10 min treatment while samples treated after 2.5 or 5 min were not significantly different from the control. Furthermore, the same authors also reported TBARS for pork butt treated for the same durations as the beef loin (i.e. 2.5, 5 and 10 min). However, unlike the beef loin, pork butt samples did not display significant increases in TBARS when compared to the control. More studies should be performed in this direction to investigate these opposing findings reported thus far.
3.3. Colour

No significant difference between plasma-treated and untreated meat samples (for each category of meat) was observed, suggesting that plasma treatment does not have notable effects on the colour of meat tissue. These findings are in line with those for beef and pork reported by Jayasena et al. (2015). This group also reported no significant changes in L values of beef loin and pork butt while also no significant changes were observed for b* for pork butt. In contrast, however, the same group reports significantly lower a* values for plasma-treated beef loin and pork butt along with significantly higher b* values for beef loin. Finally, (Yong et al., 2017) reported significantly lower L* values for plasma-treated beef jerky treated for 10 min when compared to the control sample while reporting significantly higher b* values. These authors did not find any significant effect on a* values caused by plasma treatment, in line with the observations made in this study.
Chapter 6: Stability of mackerel and haddock fish micronutrients with cold plasma treatments.

6.1. Introduction

Cold plasma is a new processing technology which has gained interest from the fish and sea food industry as a potential application to decontaminate these food products, leading to increased self-life (P. Kulawik & Tiwari, 2019). For example, microbiological reductions of mackerel samples using a Dielectric Barrier Discharge (DBD) plasma system was reported by Albertos et al. (2017). These results showed that Lactic acid bacteria (LAB), Psychrotrophic bacteria and Pseudomonas were reduced at operational conditions of 70 and 80 kV for 1, 3 and 5 minutes. The microbiological quality of dried filefish fillets was reported to be improved after 3, 5, 10, and 20 min of cold plasma treatment (Shin Young Park & Sang-Do Ha, 2015). Noteworthy reductions in Penicillium citrinum and Cladosporium cladosporioides were found and the counts were significantly (P < 0.05) reduced with increases in treatment time. The efficacy of cold atmospheric plasma was also reported for herring, with significant inactivation of Pseudomonas, total aerobic psychrotrophic bacteria, total aerobic mesophilic bacteria as well as LAB and Enterobacteriaceae after 5 minutes treatment at 70 kV using a DBD system (Albertos et al., 2019).

One of the problems associated with microbiological spoilage of fish products is the formation of high biogenic amine levels. Their formation, due to the decarboxylation of free amino acids, can be mediated by certain microorganisms which are associated with food poisoning. Consequently, biogenic amines are considered one of the indicators of food quality (Özogul & Özogul, 2019). The most common biogenic amine related to food poisoning is histamine,
which is formed from the amino acid histidine after the cleavage of the acid group. The intake of high biogenic amine concentrations can be toxic, leading to headaches, skin irritation, sweating, low blood pressure and other symptoms typical for allergic reactions (Claudia Ruiz-Capillas & Herrero, 2019). The reduction of microbiological contamination together with refrigerated or frozen storage are one of the main methods to inhibit biogenic amine formation (Özogul & Özogul, 2019).

Although, cold plasma can effectively kill microorganisms it may also initiate undesirable reactions, leading to a modification of nutrients (Pérez-Andrés et al., 2018). Protein oxidation was reported to be accelerated for mackerel during storage after plasma treatment using a DBD system (Pérez-Andrés et al., 2020). In addition, lipid oxidation was also higher after cold plasma treatment of mackerel (Albertos et al., 2017), herring (Albertos et al., 2019), dried filefish (Shin Young Park & Sang-Do Ha, 2015), sushi (Piotr Kulawik, Alvarez, et al., 2018) and dried squid (Choi et al., 2017).

Vitamins are essential nutrients consumed through the diet and are essential for prevention against a number of chronic diseases. Depending of their structure, they are classified into two general groups: fat-soluble vitamins: A, D, E, K and water-soluble vitamins; B (B1, B2, B3, B5, B6, B7, B9 and B12) and C vitamins , (Belitz, Grosch, & Schieberle, 2009). Vitamin E is an antioxidant, the main function of which is to protect the phospholipid layer from oxidative damage (Nelson, Lehninger, & Cox, 2008). Vitamin E is an exogenous compound for fish, who need to acquire it from their diet. The exact requirement and thus, content in fish flesh, depends on a number of factors such as fish species, polyunsaturated fatty acids content or environmental factors within the fish habitat (Hamre, 2011), and can vary significantly from specimen to specimen. Phylloquinone (vitamin K1) and menaquinone-4 (MK-4) are the main forms of vitamin K in fish products (Schurgers & Vermeer, 2000). The intake of vitamin K has several benefits such as bone and vascular health, metabolism, reproduction, and is used in
inhibiting cancer progression (Fusaro, Gallieni, Porta, Nickolas, & Khairallah, 2020). In addition, vitamin K is a key factor in the synthesis of blood clotting and plays an important role in chronic low-grade inflammatory diseases such as mobility disability, cardiovascular disease, cognitive impairment, osteoarthritis, dementia and frailty syndrome (Simes, Viegas, Araújo, & Marreiros, 2020).

However, vitamins are very sensitive to changes in their environment such as pH, ionic strength, water activity, photo and thermally induced isomerization and to the presence of enzymatic and trace metallic catalyst and other reactants (free radical, reducing sugars, proteins, active oxygen species, etc.) (Damodaran, Parkin, & Fennema, 2007). Consequently, their preservation during processing and storage is paramount for good nutrition. Vitamin losses can occur through chemical reactions which lead to inactive products, or by extraction or leaching, as in the case of water-soluble vitamins during blanching and cooking (Belitz et al., 2009).

Due to its antimicrobial properties, there is a possibility to use CAP in the reduction of biogenic amine formation, however, there is few data supporting this. Moreover, despite the promising potential to extend the shelf-life of food products using CAP technology, there is no data which would investigate the effect of CAP treatment on vitamin K and vitamin E content in fish fillets. Therefore, the aim of this study was to investigate the potential impact of CAP on vitamin E and vitamin K content as well as the biogenic amine formation of two different fish products, one with high fat content and related to biogenic amine poisoning (Atlantic mackerel) and the another with low fat content and not related to increased biogenic amine formation (haddock).

6.2. Material and methods
2.1. Chemicals and reagents

Phylloquinone (K1) European Pharmacopoeia (EP) Reference Standard, menaquinone-4 (MK-4) Supelco analytical standard were purchased from Sigma-Aldrich (Wicklow, Ireland). Phylloquinone-d7 [2-methyl-3-phytyl-1,4-naphthoquinone- d7 (5,6,7,8-d4, 2-methyl-d3)] (K1-d7), and menaquinone-4-d7 [2-methyl-3-geranylgeranyl-1,4-naphthoquinone-d7 (5,6,7,8-d4, 2-methyl-d3)] (MK-4-d7) were purchased from Toronto Research Chemicals (Toronto, Canada).

Romil SpS (super purity solvent) methanol (MeOH), 2- propanol (IPA), and cyclohexane were purchased from Lennox (Dublin, Ireland). Absolute ethanol and diethyl ether for EMSURE® ACS analysis were purchased from Merck (Dublin, Ireland). Ultrapure water (UPW) (18.2 MWcm) was generated in house using a Millipore water purification system. Potassium hydroxide (KOH) (ACS reagent, ≥85%, pellets), potassium carbonate (K2CO3) (anhydrous, free-flowing, Redi-Dri™, ACS reagent, ≥99%), potassium phosphate monobasic (KH2PO4) (anhydrous, free-flowing, Redi-Dri™, ACS reagent, ≥99%), lipase from Candida rugosa (Type VII, ≥700 unit/mg solid) were purchased from Sigma-Aldrich (Wicklow, Ireland). Bond elute SI cartridge (500 mg, 3mL), Captiva Econo PTFE membrane filters (13 mm, 0.2 µm), Vials (screw, 2 mL), Caps (screw, 9mm, PTFE/S/PTFE), flat bottom glass insert were purchased from Agilent Technologies Ltd (Cork, Ireland). Polypropylene tubes (15 mL, conical; and 50 mL, flat) were obtained from Sarstedt Ltd. (Wexford, Ireland).

A Dispensette® III solvent dispenser (Brand GMBH + Co KG; Wertheim Germany), TopMix multi-vortexer (Fisher Scientific; Dublin, Ireland), Grant GLS400 water bath (Grant instruments Ltd; Royston, UK), Turbomav LV evaporator (Biotage GB Limited; Hengoed, UK), Merris Minimix Vibrational Shaker (Galway, Ireland), and Hettich Zentrifugen Rotanta 460 R centrifuge (Tuttlingen, Germany) were used during sample preparation for vitamin K analysis.
A ME36S microbalance and an A200S digital electronic analytical balance, both from Sartorius (Dublin, Ireland), were used for standard preparation.

N-hexane and HPLC grade water, methanol and acetonitrile were purchased from Sigma-Aldrich (Poznań, Poland). The Supelco analytical standards of alpha and sigma-tocopherol and biogenic amines were purchased from Sigma-Aldrich (Poland).

2.2 Fish sample preparation

Fresh mackerel and haddock filets were purchased at a local fishmonger in Dublin in November 2018. All fillets were minced and homogenised for each species, 100 g portions packaged individually (Ilpra Foodpack VG 400 Packaging Machine, Italy) in a black amorphous polyethylene terephthalate (APET/PE) skin pack (195×155×30 mm), and sealed under normal air conditions with a low oxygen permeable barrier polyvinyl-chloride film (3 cm3/m2/24 h at standard temperature and pressure (STP); Versatile Packaging, Ireland) to mirror common commercial practice. The samples were divided into six different batches: controls after 1 and 7 days, plasma treated for 5 min after 1 and 7 days, and plasma treated for 10 min after 1 and 7 days.

2.3 Fish sample plasma treatment

The same dielectric barrier discharge system as described by Pankaj, Misra, and Cullen (2013) was used for this study. In this case, each tray containing 100 grams of each fish sample was placed between the electrodes separated by the height of the tray, i.e. 3 cm. Treatment lasted 5 and 10 min, at a discharge voltage of 80 kV RMS. Both the control and plasma samples were kept at 4 °C for either 1 or 7 days and afterwards, all samples were individually vacuum-packed and stored at -80 °C until the day of analysis.
2.4 Vitamin K analysis

2.4.1 Sample preparation

The procedure was performed under subdued lighting to prevent photo-oxidation and decomposition of the vitamin K vitamers. Briefly, 1.5 g of fish was weighed directly into a 50 mL polypropylene tube and spiked with 50 µL of a composite solution of K1-d7 (0.15 µg/mL) and MK-4-d7 (0.4 µg/mL). After a 15-minute period of equilibration at room temperature, 10 mL of potassium phosphate buffer (0.8M; pH 8.0) was added, and the mixture was vortexed for 1 min. Lipase (0.8 g) was added (to release the bound analytes), and the mixture was then vortexed for 1 minute, followed by incubation in a water bath at 37°C for 120 min under continuous shaking. During the incubation the mixture was vortexed for 1 min at 30 min intervals. After the incubation period, the digest was cooled to ambient temperature. To denature proteins, 20 mL of ethanol was added, and the mixture was vortexed for 1 minute. To deactivate the lipase enzyme, 0.8 g K2CO3 was added, and the mixture was vortexed for 1 min. The lipase enzyme was subsequently deactivated by adding 0.8 g of K2CO3 and the mixture was vortexed for 1 min. Cyclohexane (10 mL) was added (to extract the analytes), and the mixture was shaken for 8 min in a Merris Minimix vibrational shaker. Phase separation was achieved by centrifugation at 3500 rpm for 10 min. The upper (cyclohexane) phase was transferred into 15 mL polypropylene tubes and evaporated to dryness under a gentle stream of nitrogen at 40°C using a TurboVap LV evaporator. The sample extract was reconstituted in 500 µL of cyclohexane and vortexed for 1 min.

The sample extract was purified by solid phase extraction (SPE) using Bond Elute 500 mg silica SPE cartridges. Cartridges were conditioned by washing with 5 mL of cyclohexane, followed by addition of the sample extract, and the analytes were eluted using 4 mL of cyclohexane: diethyl ether (98:2 v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen at 40°C using a TurboVap LV evaporator. The sample extract was then
reconstituted in cyclohexane:IPA (50:50 v/v), vortexed for 1 minute, and then filtered using a 0.2 \( \mu \)m PTFE membrane filter into an Agilent autosampler vial.

2.4.2 UHPLC-MS/MS instrumentation and conditions

The UHPLC-MS/MS system consisted of an Agilent 1290 infinity II UHPLC (Agilent Technologies Ltd, Cork, Ireland) coupled to a Sciex QTRAP 6500+ mass spectrometer equipped with an APCI interface (A.B. Sciex, Warrington, UK). Separations were completed using a Cosmocore Cholesterol (2.1 × 150 mm, 2.6 \( \mu \)m; Nacalai Tesque, Apex Scientific Ltd, Maynooth, Ireland). The column temperature was maintained at 50°C. The mobile phase consisted of methanol (solvent A) and cyclohexane:IPA (50:50 v/v) solution (solvent B). The mobile phase conditions were as follows: 0-1 min 1% B, 1-10 min 40% B, 10-12.3 min 99% B, 12.3-14.3 min 1% B. The flow rate was maintained at 0.45 mL/min.

The mass spectrometer conditions were as follows: ion source, positive APCI; needle current, 3 \( \mu \)A; probe temperature, 350°C; curtain gas (35 psi) and ion source gas (35 psi), air; collision gas, nitrogen. The quantitative analysis of the target analytes was performed in multiple reaction monitoring (MRM) mode. Table 6.1 summarises the MRM conditions and parameters of the analytes and their internal standards (ISs).

<table>
<thead>
<tr>
<th>Table 6.1: MRM parameters of K1, MK-4, and ISs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRM transitions (m/z)</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>MK-4</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>PK-1</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
2.5 α- and σ-tocopherols

As in case of vitamin K, the analysis was performed under subdued lighting to inhibit the possible photo-oxidation. The analysis was performed according to the method described by Sanchez-Machado, Lopez-Hernandez, and Paseiro-Losada (2002) with modifications. Fish samples were homogenized and 5 g of the sample were mixed with 20 mL of 0.5M KOH in methanol and 0.5 mL solution of 0.01% BHT in ethanol. The solutions were homogenized for 1 min and flushed with nitrogen prior to sealing. Afterwards the solutions were incubated in a water bath at 80 °C for 15 min and shaken with shaking after every 5 min. After saponification the samples were cooled in an ice water bath for 10 min and mixed with 5 mL of n-hexane for extraction. The mixtures were vortexed for 20 s and centrifuged at 373 RPM for 2 min. The n-hexane layer was collected and the extraction procedure repeated an additional two times. The combined extracts with n-hexane were washed with 10 mL of HPLC grade water and shaken for 10 min and transferred to 15 mL tubes. The washing procedure was repeated three times. After washing the extracts were evaporated to dryness under a constant stream of nitrogen and the residues dissolved in 2 mL of HPLC mobile phase, consisting of 70:30 acetonitrile and methanol (v/v).

The separations were performed on a Dionex Ultimate 3000 UHPLC unit (Thermo Scientific, Waltham, USA) equipped with a VWD-3400 UV/VIS detector (Thermo Scientific, USA) and a low-pressure gradient pump with a four channel mixer. During the analysis the samples awaiting in the autosampler were protected from light exposure and temperature of the sampler was maintained at 4°C. The separation was carried on a Reprosil 100 C18, 5µm, 250 x 4.6 mm column (Dr Masich Gmbh, Ammerbuch, Germany) at 30°C. The mobile phase was set at
a flow rate of 1.0 ml/min. The detection was carried out at 205 nm wavelength. The detection and quantification limits for α-tocopherol were 0.2 and 0.4 mg/kg respectively.

2.6 Biogenic amines

The sample preparation protocol was performed according to the method described by Piotr Kulawik, Dordevic, Gamberuś, Szczurowska, and Zając (2018). A mixture of amine standard base solution was prepared by addition of 1 mL of each free base standard solution (1µg/mL) into 0.5 mL of 10 mg/mL dansyl chloride in acetone solution. The standard amine solution was subjected to the same extraction and derivatization procedure as the sample. The 3-point calibration curve has been created using the amine concentration of 5, 2.5 and 1.25 mg/kg.

HPLC analysis was performed on a Dionex Ultimate 3000 HPLC (Thermo Scientific, USA) equipped with fluorescent detector (FLD 3400 RS, Thermo Scientific), autosampler (ACCT-3000T) and a low-pressure gradient pump with four channel mixer. The detector setting for excitation was 340 nm and for emission 540 nm. The separation was carried on a Kromasil 100-5-C18 4.6x250 mm column (Akzo Nobel, Amsterdam, Netherlands) at 30°C. The flow rate was 0.8 mL/min with elution program set as described previously (Moret, Smela, Populin, & Conte, 2005). The detection and quantification limit for each biogenic amine was 0.001 mg/kg. Chromeleon 7.0 software (Thermo Scientific, USA) have been used to calculate the obtained results.

2.7 Statistical analysis

Analysis of variance (ANOVA) for dependent variables was carried out using Minitab 17.1.0 (Minitab Inc). Statistics were calculated using a general linear model (GLM) considering all the variables as fixed factors. Tukey’s multiple comparison was calculated to study the effect of the plasma treatment on the vitamins and biogenic amines. Experiments and analyses were performed using 3 independent samples and extractions.
6.3-Results and discussion

6.3.1 Vitamin K

The concentration of vitamin K1 and vitamin MK-4 for haddock were below the limit of quantification. In the case, of mackerel the content of K1 could not be detected for the same reason, and consequently, only the results for mackerel were reported (Table 6.2). These results show that the cold atmospheric plasma treatment did have any impact on the content of phylloquinone and menaquinone-4. Results showed that after 7 days storage the content of MK-4 after 5 minutes treatment was significantly lower than the control. However, this effect was not observed after 10 minutes treatment, which more effect was expected. The reason explaining this inconcurrence could be due to the fact of the values are very low (ug/kg) and the variations are high. In addition, a reduction of their content was observed during storage, independent of the treatment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Day</th>
<th>MK-4 (ug/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>C</td>
<td>1</td>
<td>3.39±0.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>3.87±0.36</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>1</td>
<td>3.44±0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>2.70±0.30</td>
</tr>
<tr>
<td></td>
<td>P10</td>
<td>1</td>
<td>2.63±0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>3.17±0.39</td>
</tr>
</tbody>
</table>


The content of menaquinone-4 obtained range between 1.81-4.23 ug/kg, which are slightly higher than those reported by Ostermeyer and Schmidt (2001) in mackerel, 0.7-1.1 ug/kg. In a
different study the concentration of vitamin MK-4 in mackerel was much higher between 18-26 ug/kg. Vitamin K are fat soluble vitamins, the difference between the content of these vitamins in the same specie could be caused by the different content of fat in the samples.

### 3.2 α- and σ-tocopherols

According to Rani (2012) the vitamin E content in Atlantic mackerel and haddock is approximately 0.43 and 0.39 mg/100 g respectively. Similar results have been obtained in this study (Table 6.3).

**Table 6.3: α- and σ-tocopherol content of plasma treated fish (mg/100g).**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Day</th>
<th>α-tocopherol</th>
<th>σ-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1</td>
<td>0.439&lt;sup&gt;c&lt;/sup&gt; ± 0.040</td>
<td>0.086&lt;sup&gt;b&lt;/sup&gt; ± 0.041</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.435&lt;sup&gt;bc&lt;/sup&gt; ± 0.027</td>
<td>0.090&lt;sup&gt;ab&lt;/sup&gt; ± 0.021</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>P5</td>
<td>1</td>
<td>0.361&lt;sup&gt;a&lt;/sup&gt; ± 0.026</td>
<td>0.079&lt;sup&gt;ab&lt;/sup&gt; ± 0.042</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0.407&lt;sup&gt;abc&lt;/sup&gt; ± 0.027</td>
<td>0.050&lt;sup&gt;a&lt;/sup&gt; ± 0.013</td>
</tr>
<tr>
<td></td>
<td>P10</td>
<td>1</td>
<td>0.377&lt;sup&gt;ab&lt;/sup&gt; ± 0.114</td>
<td>0.125&lt;sup&gt;b&lt;/sup&gt; ± 0.027</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0.392&lt;sup&gt;abc&lt;/sup&gt; ± 0.035</td>
<td>0.101&lt;sup&gt;b&lt;/sup&gt; ± 0.014</td>
</tr>
<tr>
<td>H</td>
<td>C</td>
<td>1</td>
<td>0.212&lt;sup&gt;a&lt;/sup&gt; ± 0.032</td>
<td>0.099&lt;sup&gt;bc&lt;/sup&gt; ± 0.012</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.408&lt;sup&gt;b&lt;/sup&gt; ± 0.072</td>
<td>0.110&lt;sup&gt;c&lt;/sup&gt; ± 0.010</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>1</td>
<td>0.348&lt;sup&gt;b&lt;/sup&gt; ± 0.057</td>
<td>0.094&lt;sup&gt;ab&lt;/sup&gt; ± 0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0.538&lt;sup&gt;c&lt;/sup&gt; ± 0.046</td>
<td>0.099&lt;sup&gt;bc&lt;/sup&gt; ± 0.012</td>
</tr>
</tbody>
</table>
The plasma treatment significantly reduced the content of alpha-tocopherol in mackerel muscle, although no significant differences were observed between 5 and 10 min plasma treatments. The observed reduction is probably due to the increased oxidation caused by the plasma treatment (Vandamme et al., 2015). In case of plasma treated samples, a small increase in alpha-tocopherol content can be observed after 7 days of storage, although the levels were still lower than of the control. On day 7 of storage, there were no significant differences between the control and plasma-treated samples.

The alpha-tocopherol level of the haddock control samples after 1 day was surprisingly low. Similar unexpected results were observed for the plasma treated samples, in which no clear trend can be observed.

The reason for the observed increase in alpha-tocopherol levels of plasma treated samples after 7 days is not yet known. However plasma treatment has been shown to increase the content of various bioactive compounds, including flavonols, anthocyanins or vitamin C (Almeida et al., 2017; Kovačević, Kljusurić, et al., 2016; Kovačević, Putnik, et al., 2016; Sarangapani, O'Toole, Cullen, & Bourke, 2017). Such increase in bioactive compounds observed in plasma treated food products is due to membrane disruption of cells which results in leakage of intracellular compounds and their easier extraction by solvents (Muhammad et al., 2018). Although no studies on the levels of vitamin E in plasma treated food samples have been performed so far, a similar mechanism could be responsible in case of the results observed in this study. Such
results were observed by Seybold, Fröhlich, Bitsch, Otto, and Böhm (2004), who observed an increase in vitamin E in processed tomato, after mild heat treatment, which was attributed to the release of alpha-tocopherol from its intracellular binding sites, which allowed for their easier extraction and resulted in higher levels observed in analysis.

3.3 Biogenic amines

The biogenic amine levels were significantly affected by both plasma treatment and storage time (Table 6.4 and Table 6.5). The most important biogenic amines in terms of food safety are histamine, tyramine, both responsible for biogenic amine associated food poisoning (Özogul & Özogul, 2019). The level of histamine, which is considered hazardous, varies in the existing literature. The US FDA recommends a critical limit of 50 mg/kg, even though most of the poisoning incidents occur in fish with histamine levels above 200 or even 500 mg/kg (Food & Administration, 2011). The European Union has set the critical limit for histamine levels at 200 mg/kg (Commission, 2005). On the other hand the results of the meta-analysis performed by Colombo, Cattaneo, Confalonieri, and Bernardi (2018) have found that poisoning usually occurs at histamine levels in fish products of 1000 mg/kg. In this study, plasma treatment significantly reduced the histamine content in mackerel after 1 day. However, after 7 days of storage the histamine levels of all mackerel samples were close to the limit of 200 mg/kg, with a significantly higher content observed in samples with 10 min plasma treatment. In the haddock samples the increase in histamine was relatively small, however as in the case of the plasma treated samples they contained higher levels of histamine after 7 days of storage. Histamine is formed through decarboxylation of histidine by the activity of histamine-forming bacteria, such as Morganella morganii, Enterobacter aerogenes, Raoultella planticola, Raoultella ornithinolytica or Photobacterium damselae, which produce histidine decarboxylase. Once this enzyme is present in fish flesh, the histamine formation will continue even though the bacterial contamination is inhibited (Björnsdóttir-Butler, Bolton, Jaykus,
McClellan-Green, & Green, 2010; Food & Administration, 2011). Therefore, the treatment applied at the beginning of the storage period could prevent the formation of histidine decarboxylase resulting in a lower histamine content. This has been observed at the beginning of the storage of the mackerel samples, however not after 7 days. A possible explanation could be low susceptibility or minimal reductions of histamine-forming bacteria by the cold plasma treatment. Chiper, Chen, Mejlholm, Dalgaard, and Stamate (2011) used cold-plasma treatment on cold-smoked salmon samples inoculated with *Photobacterium phosphoreum*, which is a histamine-forming bacteria (Phuvasate & Su, 2010), and observed reductions of no more than 1 log cfu/g after 13 days of storage. The plasma treatment could reduce the counts of other native bacteria, allowing the histamine-producing bacteria to grow at a faster rate. However, to verify this hypothesis more research related to the effect of cold plasma treatment on specific histamine-producing bacteria should be performed. Moreover, the future research should also take into the account that the cold plasma treatment effectiveness of fish samples is much lower than the effectiveness of the same treatment applied on agar slabs or plain surfaces (P. Kulawik & Tiwari, 2019).

As in case of histamine, plasma treatment inhibited the formation of tyramine at day 1, but resulted in even higher levels of this biogenic amines after 7 days. In case of putrescine, tryptamine, spermine and spermidine, the plasma treatment resulted in higher levels of those biogenic amines both at day 1 and 7. The only significant differences throughout the storage period were observed in the content of 2-phenylethylamine and, to some extent, cadaverine. Aside from histamine and tyramine, other biogenic amines can also be potentially hazardous. Putrescine, cadaverine and 2-phenylethylamine can act as histamine potentiators or form cancerogenic nitrosamines, while spermine and spermidine can result in nephrotoxicity, acute blood pressure decrease or increased carcinogenesis (Özogul & Özogul, 2019).
The results of biogenic amines analysis show that cold-plasma treatment in the studied conditions cannot be used as an effective method to reduce biogenic amines formation in fish products.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Day</th>
<th>TRP</th>
<th>2-PHE</th>
<th>PUT</th>
<th>CAD</th>
<th>HIS</th>
<th>TYR</th>
<th>SPD</th>
<th>SPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>P5</td>
<td>1</td>
<td>0.50(^a) ± 0.09</td>
<td>1.73(^b) ± 0.36</td>
<td>0.46(^a) ± 0.02</td>
<td>20.62(^c) ± 1.50</td>
<td>28.26(^b) ± 7.29</td>
<td>6.92(^b) ± 1.32</td>
<td>0.32(^a) ± 0.07</td>
<td>0.12(^a) ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>2.46(^{cd}) ± 0.48</td>
<td>19.75(^c) ± 7.27</td>
<td>8.27(^c) ± 2.02</td>
<td>31.36(^c) ± 1.20</td>
<td>149.34(^c) ± 13.80</td>
<td>18.42(^c) ± 2.57</td>
<td>0.33(^a) ± 0.15</td>
<td>0.16(^a) ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.89(^c) ± 0.38</td>
<td>3.63(^c) ± 0.26</td>
<td>0.89(^b) ± 0.14</td>
<td>15.27(^b) ± 1.39</td>
<td>17.32(^c) ± 1.73</td>
<td>1.28(^a) ± 0.23</td>
<td>1.04(^b) ± 0.19</td>
<td>0.55(^b) ± 0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>2.69(^{cd}) ± 0.70</td>
<td>4.10(^c) ± 0.46</td>
<td>15.21(^d) ± 2.07</td>
<td>24.77(^d) ± 2.32</td>
<td>155.80(^c) ± 7.49</td>
<td>28.05(^d) ± 6.15</td>
<td>1.10(^b) ± 0.14</td>
<td>0.93(^d) ± 0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.56(^b) ± 2.52</td>
<td>1.00(^b) ± 0.12</td>
<td>0.89(^b) ± 0.13</td>
<td>13.84(^a) ± 1.45</td>
<td>16.85(^d) ± 1.06</td>
<td>1.22(^a) ± 0.08</td>
<td>1.32(^c) ± 0.15</td>
<td>0.72(^c) ± 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>3.39(^d) ± 0.38</td>
<td>6.19(^d) ± 0.30</td>
<td>21.27(^c) ± 1.06</td>
<td>34.22(^d) ± 0.90</td>
<td>185.91(^d) ± 8.96</td>
<td>41.19(^d) ± 4.77</td>
<td>1.35(^c) ± 0.12</td>
<td>1.01(^d) ± 0.15</td>
</tr>
</tbody>
</table>


M – mackerel, C – control, P5 – plasma treatment for 5 min, P10 – plasma treatment for 10 min.
Table 6.5: Biogenic amines content of plasma treated haddock (mg/kg).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Day</th>
<th>TRP</th>
<th>2-PHE</th>
<th>PUT</th>
<th>CAD</th>
<th>HIS</th>
<th>TYR</th>
<th>SPD</th>
<th>SPR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.37(\text{a} \pm 0.05)</td>
<td>0.14(\text{a} \pm 0.01)</td>
<td>1.94(\text{b} \pm 0.24)</td>
<td>8.55(\text{a} \pm 0.14)</td>
<td>2.44(\text{a} \pm 0.63)</td>
<td>0.76(\text{a} \pm 0.07)</td>
<td>0.29(\text{a} \pm 0.04)</td>
<td>0.15(\text{a} \pm 0.01)</td>
</tr>
<tr>
<td>H</td>
<td>P5</td>
<td>7</td>
<td>0.38(\text{a} \pm 0.08)</td>
<td>0.19(\text{b} \pm 0.02)</td>
<td>9.14(\text{c} \pm 0.94)</td>
<td>34.99(\text{d} \pm 4.49)</td>
<td>29.38(\text{c} \pm 4.84)</td>
<td>2.15(\text{bc} \pm 0.65)</td>
<td>0.39(\text{b} \pm 0.10)</td>
<td>0.34(\text{b} \pm 0.06)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.33(\text{a} \pm 0.06)</td>
<td>0.21(\text{bc} \pm 0.02)</td>
<td>2.00(\text{b} \pm 0.30)</td>
<td>28.81(\text{b} \pm 0.80)</td>
<td>3.75(\text{b} \pm 0.78)</td>
<td>2.20(\text{bc} \pm 0.38)</td>
<td>0.64(\text{b} \pm 0.05)</td>
<td>0.81(\text{d} \pm 0.08)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0.56(\text{c} \pm 0.06)</td>
<td>0.31(\text{d} \pm 0.06)</td>
<td>12.40(\text{d} \pm 2.50)</td>
<td>30.10(\text{bc} \pm 2.93)</td>
<td>48.45(\text{d} \pm 5.25)</td>
<td>5.06(\text{d} \pm 1.38)</td>
<td>0.92(\text{d} \pm 0.10)</td>
<td>1.18(\text{d} \pm 0.18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.33(\text{a} \pm 0.04)</td>
<td>0.24(\text{c} \pm 0.04)</td>
<td>1.36(\text{a} \pm 0.37)</td>
<td>28.92(\text{b} \pm 1.31)</td>
<td>2.56(\text{a} \pm 0.76)</td>
<td>1.71(\text{bc} \pm 0.31)</td>
<td>0.40(\text{b} \pm 0.13)</td>
<td>0.52(\text{c} \pm 0.06)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0.46(\text{b} \pm 0.06)</td>
<td>0.36(\text{b} \pm 0.05)</td>
<td>13.51(\text{d} \pm 1.53)</td>
<td>32.06(\text{c} \pm 1.94)</td>
<td>31.13(\text{d} \pm 6.65)</td>
<td>3.64(\text{d} \pm 1.15)</td>
<td>0.39(\text{bc} \pm 0.12)</td>
<td>0.50(\text{d} \pm 0.07)</td>
</tr>
</tbody>
</table>


H – haddock, C – control, P5 – plasma treatment for 5 min, P10 – plasma treatment for 10 min.
Chapter 7: Conclusions and recommendations

7.1 General discussion and conclusions

Non-thermal food processing technologies have shown great promise as microbial decontamination tools, with a large body of work found in the literature. However, the effects of these novel technologies on food chemistry have not been studied deeply (Table 7.1). These promising technologies can damage some nutrients, such as proteins and lipids. Consequently, more research is required into the effects and mechanisms of action of each technology on food chemistry. Such insights are key to ensure that these emerging technologies are accepted by industry, regulatory agencies and consumers alike.

Table 7.1: Effect of novel technologies on chemical changes in food products.

<table>
<thead>
<tr>
<th>Effects</th>
<th>High Pressure</th>
<th>Pulse electric field</th>
<th>Ultrasound</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidation</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Denaturation</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Aggregation</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Lipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidation</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Lipolysis</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

+++: Significant effect, ++: Moderate effect, +: Low effect, -: No reported

The data obtained in the mackerel fillet studied showed that cold atmospheric plasma did not induce observable undesirable reactions such as lipid oxidation to the bulk of the treated samples. In addition, the stability of the fatty acid composition of mackerel was not affect by the treatment, along with their nutritional quality indexes. However, cold atmospheric plasma could accelerate the formation of carbonyls which are related to protein oxidation.
Depending of the native structure and nature of the protein, CAP treatment affected the functional properties in different ways. The findings point to the specific nature of plasma-protein interactions and the need for individual proteins to be studied as a function of plasma conditions. These changes could be beneficial or detrimental depending on the features desired. For instance, the solubility of the proteins extracted from bovine lung decreased significantly after treatment, while its oil holding capacity had a significant improvement. Thus, treated lung proteins could be employed in meat products, where oil holding capacity is more important than solubility. Changes in the functional properties could not be related with the observed changes in hydrophobicity, meaning that other effects such as oxidation or crosslinking might have a more important role than protein unfolding. From this research, it can be concluded that CAP has a potential application in modifying protein-based food ingredients, increasing the applicability of novel protein sources along with those currently used. Depending on the requirements of a specific product, a food protein could be treated to achieve the desired properties. However, further research should be carried out to optimise CAP’s different parameters for specific food proteins.

CAP induced reactive species can degrade cholesterol in its pure form. However, the cholesterol content in meat samples was not affected by the treatment, suggesting that a matrix effect plays an important role in protecting this food component from degradation. In addition, cold plasma did not have any impact on the lipid content, but higher values of peroxide value and TBARS were found for the treated samples, indicating that plasma can induce the acceleration of primary and secondary lipid oxidation. Finally, colour was not affected by the treatment supporting the suitability of the technology for meat products.

Cold plasma treatment did not appear to have significant impact on vitamin MK-4 content of mackerel and neither, on the sigma-tocopherol content of mackerel and haddock. However, the treatment resulted in a significant decrease of the concentration of alpha-tocopherol in both
fish. On the other hand, cold atmospheric plasma treatment increased the level of biogenic amines which is another undesirable effect of this technology and the exact mechanism of this result should be further investigated.

The general conclusions derived from this work are listed as follows:

- Non-thermal technologies have potential as decontamination technologies however, its application on food products should be considered because of their negative impact on freshness and quality.
- No effect was found on the fatty acid profile, nutritional quality indexes and oxidation of lipids after the treatment, however, CAP could accelerate the formation of carbonyls which are related to protein oxidation.
- Cold plasma can be used to modify the functionality of food ingredients to achieve the desired properties of a specific food product.
- Cold plasma induces primary and secondary oxidation of meat products.
- Cold plasma reduces the content of alpha-tocopherol in mackerel and haddock.
- Cold plasma accelerates the formation of biogenic amines, which can be toxic at high levels.

7.2 Future recommendations

Plasma is identified as a potential technology for food decontamination and associated shelf-life extension. Nevertheless, more research is needed to find a better balance between safety and quality. It is required to optimise this technology to avoid any undesirable effects caused by the chemical reactions initiated by the radical species presented in the plasma atmosphere. However, further research is required on the impact of this technology on the quality of food products prior to its approval and adoption by regulators and industry respectively. There is a need to understand the chemical reactions associated with plasma species to avoid quality
deterioration. Plasma studies also need to be performed on real food products and not only model solutions to determine which operational conditions suit the vast array of potential food products which could be treated. Optimisation of plasma control parameters such as power, current and voltage, in particular the inducer gas employed, need to be investigated in depth to identify the conditions which can provide the required product safety and yet retain key quality attributes.

Throughout this thesis focus was paid to the use of a non-contact CAP prototype system if such a system was to be used in a factory in a continuous manner it would be worth examining its robust in an industrial setting (e.g. electrode abrasion). Optical emission spectroscopy (OES) analysis would provide a real-time monitoring method in such a setting as part of a quality control framework.
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