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**“Natural and synthetic phenolipids as a strategy to develop healthier foods:
from model to real food systems”**

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Ph.D. thesis

*Natural and synthetic phenolipids as a strategy to
develop healthier foods: from model to real food
systems*



UNIVERSITÀ DEL PIEMONTE ORIENTALE
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ABSTRACT

As food-related health problems become more common (e.g., obesity, cardiovascular diseases, diabetes), there is a growing awareness of the need for a healthy diet that enhances the assimilation of beneficial bioactive compounds like polyphenols and polyunsaturated fatty acids (PUFAs). Several beneficial properties have been indeed demonstrated for these compounds, having proved - both *in vitro* and *in vivo* - to lower the risk of several diseases as a result of their anti-inflammatory and/or antioxidant activities. However, food processing and storage frequently result in lowering/decreasing or transforming these bioactives in foods. Lipid oxidation is one of the main chemical reactions that deteriorates food quality due to the harmfulness of the compounds generated, such as hydroperoxides, aldehydes, and ketones. One of the strategies to increase the shelf-life of health-promoting lipid-containing foods includes the use of antioxidants like phenolic compounds. Nevertheless, most phenolic compounds are hydrophilic, bringing an important limitation to their use in lipid-containing foods. In fact, the way antioxidants are in food has a major impact on how they can take part in lipid oxidation reactions. Despite several different physical structures, most of the foods have three phases: lipid, aqueous, and an interface area between them. Water-soluble prooxidants can easily react with the lipid substrate at the interfacial area. Hence, antioxidants capable of partitioning themselves at the interfacial region are the most efficient in counteracting lipid oxidation, as they are present at the sites where its deleterious reactions occur. Moreover, phenolic compounds are often characterized by a scarce bioavailability in the human body since their hydrophilicity hinders their passage through the phospholipid bilayer of the intestinal epithelium cell membrane. Consequently, even if they are ingested in large amount with the diet, most of them are excreted, rendering their beneficial properties useless to the human body. A solution that could lead to the simultaneous resolution of both issues (lipid oxidation and poor assimilation of polyphenols) may be the so-called lipophenols, which can be from natural or synthetic origin. Lipophenols (or phenolipids) are a class of bioactive compounds derived by the conjugation of phenolic compounds with hydrophobic molecules, including aliphatic

chains, PUFAs, and phytosterols. That conjugation gives lipophenols an amphipathic character, making them of enormous interest to the food industry, as they can be used in oil-based formulas, as well as in emulsion, micellar, and liposomal systems. In the latter systems, lipophenols could act as powerful antioxidants partitioning at the interface area. In addition, in many cases lipophilized phenolic compounds show greater antioxidant activity than the parent compounds. The higher or lower antioxidant activity of lipophenols compared to the corresponding parent molecule depends mainly on the length of the aliphatic chain linked to the phenolic compound (*cut-off* effect), or in general on the size of the conjugated hydrophobic molecule. This is probably due to the different antioxidant mobility in the system, thus being able to reach the sites of oxidation reactions more or less easily. From the conjugation of lipids to phenolic compounds, therefore, two advantages are obtained simultaneously: 1) bioactive lipids are protected from oxidation by the phenolic compound conjugated to them; and 2) phenolic compounds show better stability and bioavailability due to their higher hydrophobicity than the hydrophilic parent compound, which instead undergoes rapid transformation and elimination in humans.

This PhD project is aimed at developing healthy foods using lipophenols, both natural and synthetic, to preserve bioactive compounds (such as phenolic compounds and bioactive lipids) from degradation, increasing their bioaccessibility in the human body.

The first part of this project was focused on the study of synthetic phenolipids. Ferulic derivatives were synthesized through the esterification of ferulic acid with butanol and octanol to obtain alkylferulates with different length of the alkyl chain on its carboxyl group, namely butyl ferulate and octyl ferulate, respectively. The effect of their concentration (5–25 mg/L) and the pH of the system (3.5 and 7.0) on their performance was evaluated in a model system represented by 1.0% oil-in-water emulsions. Both ferulic derivatives were able to successfully counteract lipid oxidation in the emulsions, but butyl ferulate proved to be the most effective antioxidant, with lower levels of primary and secondary oxidation products. The antioxidant activity of both phenolipids was positively correlated with their concentration, and acidic pH resulted in its significant increase.

However, while at pH 3.5 the alkylferulates had no influence on particle size, at pH 7.0 progressively smaller oil droplets were obtained by increasing their concentration.

The second part of the project involved natural phenolipids, specifically the alkylresorcinols, in the context of a circular economy perspective. Alkylresorcinols (C₁₇-C₂₅) were isolated from wheat bran, the main by-products of the milling industry, and tested in 1.0% oil-in-water emulsions. Again, the concentration of the alkylresorcinols (0.15% and 0.30% on the oil weight) and the pH of the system were investigated as factors potentially influencing their antioxidant activity. The emulsions added with the alkylresorcinols exhibited a higher oxidative stability, especially at pH 3.5, with reduced amounts of oxidation products. Next, the same phenolipids extracted from wheat bran were tested in a real food product, namely beef patties, at 0.01% and 0.02% (on the total meat weight), and their efficacy was compared to sodium ascorbate added to the patties at 0.01% (for comparison with alkylresorcinols) and 0.10% (the concentration commonly used in meat products in the Italian market). Although they did not increase color stability, alkylresorcinols significantly reduced lipid oxidation during storage at 4 °C for 9 days. Interestingly, lipophenols at the lowest concentration showed equal or greater efficacy than sodium ascorbate at 0.10%.

Considering that a concentration of both synthetic and natural lipophenols ten times lower than the antioxidants normally used in food products (as reported in literature) was effective in increasing the oxidative shelf-life of the systems tested, the results of this carry important economic implications for industries. At the same time, considering the challenges to increase the intake of bioactive compounds within the human body, the lipophenols could represent an effective, innovative, and sustainable solution for creating foods with beneficial effects on human health.

ABSTRACT

Con il diffondersi dei problemi di salute legati all'alimentazione (come, ad esempio, obesità, malattie cardiovascolari e diabete), sta crescendo la consapevolezza della necessità di una dieta sana che favorisca l'assimilazione di composti bioattivi benefici come i polifenoli e gli acidi grassi polinsaturi. Sono state infatti dimostrate diverse proprietà benefiche di questi composti, che hanno dimostrato - sia *in vitro* che *in vivo* - di ridurre il rischio di diverse patologie grazie alle loro attività antinfiammatorie e/o antiossidanti. Tuttavia, la produzione e la conservazione degli alimenti comportano spesso la riduzione o la trasformazione di questi bioattivi negli alimenti. L'ossidazione dei lipidi è una delle principali reazioni chimiche che deteriorano la qualità degli alimenti a causa della nocività dei composti generati, come gli idroperossidi, le aldeidi e i chetoni. Una delle strategie per aumentare la shelf-life di alimenti contenenti lipidi benefici per la salute prevede l'uso di antiossidanti come i composti fenolici. Nondimeno, la maggior parte dei composti fenolici è idrofila, comportando un'importante limitazione al loro utilizzo negli alimenti contenenti lipidi. L'allocatione fisica degli antiossidanti all'interno della matrice alimentare ha un impatto importante sul modo in cui possono partecipare alle reazioni di ossidazione lipidica. Infatti, nonostante le diverse strutture fisiche, la maggior parte degli alimenti presenta tre fasi: lipidica, acquosa e un'area di interfaccia tra di esse. I composti pro-ossidanti solubili in acqua possono facilmente reagire con il substrato lipidico nell'area interfacciale. Pertanto, gli antiossidanti in grado di partizionarsi nella regione interfacciale sono i più efficienti nel contrastare l'ossidazione lipidica, poiché sono presenti nei siti in cui avvengono le reazioni di degradazione. Inoltre, i composti fenolici sono spesso caratterizzati da una scarsa biodisponibilità nell'organismo umano, poiché la loro idrofilia ne ostacola il passaggio attraverso il doppio strato fosfolipidico della membrana cellulare dell'epitelio intestinale. Di conseguenza, anche se vengono ingeriti in grandi quantità con la dieta, la maggior parte di essi viene escreta, rendendo inutili le loro proprietà benefiche per l'organismo umano. Una soluzione che potrebbe portare alla risoluzione simultanea di entrambi i problemi (ossidazione dei lipidi e scarsa assimilazione dei polifenoli) potrebbe essere rappresentata dai cosiddetti lipofenoli, che possono

essere di origine naturale o sintetica. I lipofenoli (o fenolipidi) sono una classe di composti bioattivi derivati dalla coniugazione di composti fenolici con molecole idrofobiche, tra cui catene alifatiche, acidi grassi polinsaturi e fitosteroli. Questa coniugazione conferisce ai lipofenoli un carattere anfipatico, rendendoli di enorme interesse per l'industria alimentare, in quanto possono essere utilizzati in formulazioni a base olio/grasso, nonché in sistemi emulsionati, micellari e liposomiali. In quest'ultimi, i lipofenoli potrebbero agire come potenti antiossidanti partizionandosi nell'area di interfaccia. Inoltre, in molti casi i composti fenolici lipofilizzati mostrano una maggiore attività antiossidante rispetto agli stessi composti non lipofilizzati (molecole parentali). La maggiore o minore attività antiossidante dei lipofenoli rispetto alla corrispondente molecola parentale dipende principalmente dalla lunghezza della catena alifatica legata al composto fenolico (effetto *cut-off*), o in generale dalla dimensione della molecola idrofobica coniugata. Ciò è probabilmente dovuto alla diversa mobilità dell'antiossidante nel sistema, in grado di raggiungere più o meno facilmente i siti delle reazioni di ossidazione. Dalla coniugazione dei lipidi con i composti fenolici, quindi, si ottengono contemporaneamente due vantaggi: 1) i lipidi bioattivi sono protetti dall'ossidazione dal composto fenolico ad essi coniugato; 2) i composti fenolici mostrano una migliore stabilità e biodisponibilità grazie alla loro maggiore idrofobicità rispetto al composto parentale idrofilo, che invece subisce una rapida trasformazione ed eliminazione nell'uomo.

Questo progetto di dottorato mira a sviluppare alimenti più sani attraverso l'utilizzo di lipofenoli, sia naturali che sintetici, per preservare i composti bioattivi (come i composti fenolici e i lipidi bioattivi) dalla degradazione, aumentandone potenzialmente la bioaccessibilità nel corpo umano.

La prima parte di questo progetto è stata incentrata sullo studio dei lipofenoli sintetici. Sono stati sintetizzati, infatti, derivati ferulici attraverso l'esterificazione dell'acido ferulico con butanolo e ottanolo per ottenere alchilferulati con una diversa lunghezza della catena alchilica sul gruppo carbossilico, rispettivamente il butilferulato e l'ottilferulato. L'effetto della loro concentrazione (5-25 mg/L di emulsione) e del pH del sistema (3,5 e 7,0) sulle loro prestazioni è stato valutato in un sistema

modello rappresentato da emulsioni olio-in-acqua all'1,0%. Entrambi i derivati ferulici sono stati in grado di contrastare con successo l'ossidazione lipidica nelle emulsioni, ma il butilferulato si è rivelato l'antiossidante più efficace, con un minore sviluppo dei prodotti dell'ossidazione primaria (idroperossidi) e secondaria (aldeidi). L'attività antiossidante di entrambi i fenolipidi è risultata positivamente correlata alla loro concentrazione e il pH acido ne ha determinato un aumento significativo. Tuttavia, mentre a pH 3,5 gli alchilferulati non hanno mostrato alcuna influenza sulle dimensioni delle particelle, a pH 7,0 sono state ottenute particelle d'olio progressivamente più piccole aumentando la loro concentrazione.

La seconda parte del progetto ha riguardato i lipofenoli naturali, in particolare gli alchilresorcinoli, in un'ottica di sostenibilità ambientale. Gli alchilresorcinoli (C₁₇-C₂₅) sono stati isolati dalla crusca di frumento, principale sottoprodotto dell'industria molitoria, e testati in emulsioni olio-in-acqua all'1,0%. Anche in questo caso, la concentrazione degli alchilresorcinoli (0,15% e 0,30% sul peso dell'olio) e il pH del sistema sono stati studiati come fattori potenzialmente in grado di modulare la loro attività antiossidante. Le emulsioni addizionate con gli alchilresorcinoli hanno mostrato una maggiore stabilità ossidativa, soprattutto a pH 3,5, con una minore formazione dei prodotti dell'ossidazione. Successivamente, gli stessi lipofenoli estratti dalla crusca di frumento sono stati testati in un prodotto alimentare reale, ovvero carne tritata di manzo, allo 0,01% e allo 0,02% (sul peso totale della carne), e la loro efficacia è stata confrontata con l'ascorbato di sodio aggiunto alla carne allo 0,01% (per il confronto con gli alchilresorcinoli) e allo 0,10% (la concentrazione comunemente impiegata nei prodotti carnei presenti sul mercato italiano). Sebbene non abbiano aumentato la stabilità del colore, gli alchilresorcinoli hanno ridotto significativamente l'ossidazione dei lipidi durante la conservazione a 4 °C per 9 giorni. È interessante notare che i lipofenoli alla concentrazione più bassa hanno mostrato un'efficacia pari o superiore a quella dell'ascorbato di sodio allo 0,10%.

I risultati di questo progetto di ricerca hanno importanti implicazioni economiche per le industrie alimentari. Infatti, una concentrazione di lipofenoli sia sintetici che naturali di dieci volte

inferiore rispetto alla quantità di antiossidanti normalmente utilizzati nei prodotti alimentari (come riportato in letteratura) è stata efficace nel prolungare la shelf-life ossidativa dei sistemi testati. Allo stesso tempo, considerando le sfide per aumentare l'assimilazione di composti bioattivi nel corpo umano, i lipofenoli potrebbero rappresentare soluzioni efficaci, innovative e sostenibili per la formulazione di alimenti con effetti benefici sulla salute umana.

Abbreviations

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ARs, alkylresorcinols

BF, butyl ferulate

BHT, butylated hydroxytoluene

CCL, critical chain length

DLS, dynamic light scattering

DPPH, 2,2'-diphenyl-1-picrylhydrazyl

DVB/CAR/PDMS, divinylbenzene/carboxen/polydimethylsiloxane

EDTA, ethylenediaminetetraacetic acid

FRAP, ferric ion reducing antioxidant power

GC/FID, gas chromatography/flame ionization detector

HS-SPME-GC/MS, head-space-solid phase microextraction-gas chromatography/mass spectrometry

NMR, nuclear magnetic resonance

O/W, oil-in-water

OF, octyl ferulate

ORAC, oxygen radical absorbance capacity

PCA, principal component analysis

PV, peroxide value

TBARS, thiobarbituric acid reactive substances

TLC, thin layer chromatography

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Chapter 1

LITERATURE REVIEW

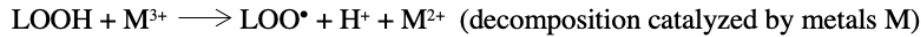
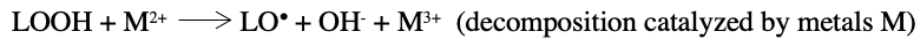
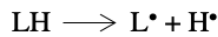
1.1 Degradation of lipids

After decades and decades, lipid oxidation still constitutes a highly active topic and has spread into a wide range of disciplines including food science and technology, nutrition, free radical chemistry, biochemistry, and medicine (Frankel, 2014). However, despite the tremendous progression in the research, lipid oxidation continues to represent a major challenge and several issues still need to be solved. Given the numerous negative consequences lipid oxidation has on food quality, its control is essential. In foods, lipids play a crucial role as they impart odor, appearance, texture properties, mouthfeel and, aroma in addition to their caloric energy role and essential nutrients source such as lipid-soluble vitamins, and essential fatty acids (Frankel, 2014). Food lipids include fatty acids (either saturated, monounsaturated, or polyunsaturated), mono- di- and triacylglycerols (the main constituents of vegetable oils and food lipids), phosphoglycerides (important structural lipids in cell membranes and foods), and minor non-glyceride components such as carotenoids, squalene, sterols, tocopherols and tocotrienols, chlorophyll, retinol (vitamin A), and cholecalciferol (vitamin D) (Frankel, 2014; McClements & Decker, 2018). Lipids can degrade chemically due to two main mechanisms: oxidation and hydrolysis. The cleavage of fatty acids from the glycerol backbone of triacylglycerols by lipase-enzymes results in hydrolytic rancidity. As a result, short-chain, medium-chain and long-chain free fatty acids are formed, which give rancid off-flavors. Oxidation is the result of a complex chain of chemical reactions involving unsaturated lipids and oxygen. Small, volatile carbonyls are produced as a result, and they are the cause of rancid smells and a number of other end products of advanced lipid oxidation that are harmful to human health. Out of these two processes, lipid oxidation is a ubiquitous issue that significantly shortens the shelf-life of foods.

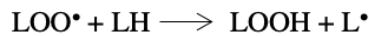
1.1.1 Mechanisms of lipid oxidation

A complex chain of reactions characterizes lipid oxidation. Depending on whether the type of system (e.g., bulk oil or emulsion), different lipid oxidation mechanisms can occur, and several factors can contribute to or minimize the reactions. However, in general lipid oxidation is constituted by free radical chain reactions divided into three steps: initiation, propagation, and termination (**Figure 1.1**) (Frankel, 2014). The first step begins with the abstraction of a hydrogen from an unsaturated lipid (LH) to form an alkyl radical (L^{\bullet}). The formation of free radicals can also be due to the decomposition or thermal dissociation of preformed lipid hydroperoxides (LOOH) (impurities) into alkoxy (LO^{\bullet}) or peroxy radicals (LOO^{\bullet}) (Frankel, 2014). All these reactions are catalyzed by exogenous or endogenous factors, such as heat, light, UV radiation, presence of transition metals. The abstraction of the hydrogen generally occurs from the double bond of unsaturated fatty acids, because of the lower bond energy between the carbon and the hydrogen compared to a regular carbon-hydrogen bond (saturated). All free radicals formed are thermodynamically unstable due to the high energy they possess, and thereby chemically highly reactive, leading to the propagation step. Propagation takes place when atmospheric oxygen combines with the alkyl radical (L^{\bullet}) to create a peroxy radical (LOO^{\bullet}). Lipid hydroperoxide (LOOH) and a new alkyl radical (L^{\bullet}) are formed when peroxy radicals (LOO^{\bullet}) easily extract hydrogen from another unsaturated lipid (LH) (Frankel, 2014). Lastly, termination involves two radical species combining to form a non-radical species (Frankel, 2014).

Initiation:



Propagation:



Termination:

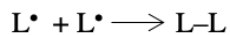
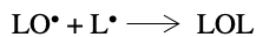


Figure 1.1. Different mechanisms of lipid oxidation (adapted from Frankel, 2014).

Although lipid hydroperoxides are thought to be the main products of lipid oxidation, a wide range of secondary metabolites can be formed by their breakdown. In fact, as already mentioned, heat, UV radiations, visible light, and transition bivalent metals can decompose the hydroperoxides producing alkoxy radicals. Alkoxy radicals are characterized by high energy and abstraction of a hydrogen can occur directly from the adjacent covalent bond. This results in the fatty acid cleavage (β -scission reactions), producing low molecular weight aldehydes that confer the typical off-flavor of oxidized lipids, with an enormous impact on the food quality. Some of the main aldehydes produced from the decomposition of different unsaturated fatty acids include hexanal (from omega-6 fatty acids, such as linoleic acid), propanal (from omega-3 fatty acids, such as linolenic acid), and nonanal (from oleic acid) (Decker et al., 2005). Along with the aforementioned volatile secondary products of oxidation, lipid radicals can react to generate non-volatile compounds including ketones, alcohols, carboxylic acids, dimers, epoxides, bicyclic endoperoxides, and polymers (Frankel, 2014). The placement of the hydroperoxide in the fatty acid's aliphatic chain and the fatty acid's structure -

namely, its length, degree of unsaturation, and double bond location - determine the precise products that result from these reactions. What governs the type of oxidation product that is formed is the position of the hydroperoxide in the aliphatic chain of the fatty acid and its characteristics, such as the chain length, the degree of unsaturation, and the double bond location in the chain. There are two stages in which lipid oxidation produces primary and secondary products: the lag phase (which coincides with the initiation phase) and the exponential phase (which coincides with the propagation phase). Primary oxidation products like hydroperoxides are accumulated throughout the lag phase, while secondary products shows minimal variation over time (McClements & Decker, 2007). The rate at which secondary products are formed increases dramatically throughout the exponential phase. Foods exhibit imperceptible rancidity during the lag phase, but once they enter the exponential phase, off-flavors develop with serious consequences on food quality (McClements & Decker, 2007). During the propagation stage, one alkyl radical typically generates one alkoxy radical; as a result, the auto-oxidation rate is linear. Nonetheless, β -scission processes produce two radicals from a single alkoxy radical, which explains why, once oxidation has started, the rate of lipid oxidation is exponential and happens so quickly. The time required to reach the point where lipid oxidation transition from the lag phase to the exponential phase is commonly denoted as induction period. By prolonging the induction time as much as possible, one can control lipid oxidation, and extend the shelf-life of foods and enhance their quality.

1.1.2 Factors influencing lipid oxidation rate

Numerous factors determine the rate of lipid oxidation, however, the structure of lipids themselves constitutes one of the main factors governing their oxidation. In fact, it is well known that unsaturated fatty acids are more prone to oxidation than saturated ones, due to the lower dissociation energy of the C-H bond located on the allylic carbons of the double bond (McClements & Decker, 2007). Furthermore, the greater is the number of unsaturation and the longer the chain length, the lower is the oxidative stability. Lipid oxidation rate is not significantly influenced by the amount of

lipids present in the system, despite common misconceptions. Even a small quantity of oil in a food product results in a significant production of off-flavors. For instance, it has been demonstrated that rising the fat content from 5% to 20% do not increase the formation of malondialdehyde, a characteristic product of lipid oxidation (Ang & Young, 1992).

Other factors affecting lipid oxidation rate include food composition, storage conditions, temperature, presence of oxygen, water, and exogenous/endogenous prooxidants and antioxidants in contact with lipids. With high temperatures hydroperoxides can decompose propagating lipid oxidation; as a consequence, reducing temperatures during the storage represents a strategy to slow down the rate of oxidation. Oxygen represents one of the key players in lipid oxidation, because it can easily interact with alkyl radicals during their propagation. In addition, oxygen is more soluble in lipids than in water, and remove it from food and packaging can be challenging (McClements & Decker, 2007). Transition metals and prooxidants are soluble in water, which therefore speeds up the oxidation reactions (McClements & Decker, 2018; Waraho et al., 2011). Decreasing the water content in foods can result in slower rates of lipid oxidation. Prooxidants include singlet oxygen, transition metals, and enzymes. As mentioned before, transition metals such as iron and copper can decompose both lipid hydroperoxides and hydrogen peroxide into free radical species. Enzymes like lipoxygenases can remove a hydrogen from unsaturated lipids creating radicals and hydroperoxides, but their activity can be slowed down by denaturation with high temperatures. Lipid hydroperoxides can also be directly decomposed by UV and visible light, accelerating lipid oxidation (Frankel, 2014; McClements & Decker, 2007). Furthermore, in the presence of photosensitizers (e.g., chlorophyll) light induces photooxidation. This process leads to the abstraction of a hydrogen from the double bonds of unsaturated lipids, or the formation of singlet oxygen, which can then react directly with unsaturated lipids (Frankel, 2014). Dark packaging represents a solution to block light exposure.

1.1.3 Impact of lipid oxidation

During lipid oxidation several different compounds are produced, each with unique chemical and physical properties (e.g., chemical reactivity, polarity, partitioning behavior, surface activity, and thermal stability) that can alter the food matrix in terms of texture, color, flavor, and nutritional value (McClements & Decker, 2007). For instance, numerous small volatile compounds are produced during lipid oxidation, causing rancid off-flavors. Among them hexanal has been described as green, tallowy, and leafy and nonanal as soapy-fruity and tallowy (Belitz et al., 2009) therefore formation of volatiles from lipid oxidation should be minimized as it decreases the quality of the food products. The radical species produced as a consequence of the oxidation of lipids are extremely reactive and can promote the co-oxidation of proteins. Primary amino groups on proteins can react with secondary oxidation products, particularly unsaturated aldehydes (Burcham & Kuhan, 1996), affecting protein's hydrophobicity, solubility, water-holding capacity, and aggregation characteristics, which in turn affects the texture of the product (Hematyar et al., 2019). Lipid oxidation can also impact the color of foods since the pigments are usually susceptible to oxidation due to the presence of various double bonds in their structures. The pigments' oxidation can result in color loss or the creation of new color molecules, with a negative impact on consumer acceptability. Pigments prone to oxidation include phenolic compounds, carotenoids, and myoglobin. Free radicals can decompose the double bonds in the structure of the carotenoids, causing color loss. Phenolics can be oxidized by free radicals forming quinones that can later polymerize and form melanin compounds, which are responsible for brown color. Lipid oxidation products, especially aldehydes, can react with myoglobin in meat causing color to darken. Finally, lipid oxidation can cause the loss of essential vitamins and amino acids. Essential vitamins can be oxidized since they act as antioxidants (e.g., tocopherols, ascorbic acid), with a consistent loss of these compounds in foods (Bayram & Decker, 2023), while, as stated before, aldehydes can react with essential amino acids, causing their depletion (Burcham & Kuhan, 1996).

1.1.4 Lipid oxidation in food systems

How lipid oxidation proceeds in foods greatly depends on the physical structure of lipids and on the characteristics of the environment in which they are (McClements & Decker, 2000; Villeneuve et al., 2021). The majority of food products that include lipids are either entirely or partly emulsions. Emulsions consist of two immiscible liquids where one is dispersed in the other as small spherical droplets (McClements & Decker, 2000). Numerous foods, such as milk, cream, dressings, sauces, mayonnaise, etc., exist as oil-in-water emulsions, where the oil droplets are dispersed in an aqueous continuous phase (McClements & Decker, 2000). Since emulsions are thermodynamically unstable systems, the two phases will eventually separate over time (Waraho et al., 2011). Lipid oxidation also produces molecules that are surface active, which can speed up this process. To overcome this separation issue, emulsifiers, such as surface active particles, amphiphilic biopolymers, and small-molecule surfactants, are used to stabilize the oil-water interface of emulsions and prevent their physical desegregation (phenomena like flocculation, aggregation, and coalescence). Emulsifiers enhance the physical stability of emulsions by creating a protective interfacial membrane surrounding oil droplets. They can also act as a barrier to prevent water-soluble prooxidants from starting lipid oxidation processes at the oil-water interface. As a result, the characteristics of the interfacial layer greatly influence its ability to protect the lipid phase from oxidation. Among its characteristics, the main ones are permeability, composition, packing, charge, and thickness (Waraho et al., 2011). Lipid oxidation proceeds more quickly in emulsions than in bulk oils because of the greater surface of oil exposed to the pro-oxidants-containing water. The interface between the oil and the water is thought to be the site of lipid oxidation in emulsions because the aqueous phase can solubilize radicals, prooxidants, and transition metals, which can react quickly with unsaturated lipids due to the large area of contact with the dispersed lipid phase (McClements & Decker, 2000; Villeneuve et al., 2021; Waraho et al., 2011). When surface active lipid hydroperoxides are formed during oxidation, they can partition at the interface and break down into alkoxy and peroxy radicals that further propagate lipid oxidation in the dispersed oil phase (Waraho et al., 2011). The rate of lipid oxidation in

emulsions can be affected by numerous factors including the pH and ionic strength of the aqueous phase, and the properties of the dispersed oil phase, such as viscosity and particle dimension (McClements & Decker, 2000). It is therefore important for food industries to find solutions to retard lipid oxidation in emulsion-based foods that involve the interfacial layer.

1.2 Antioxidants

1.2.1 Classification and mechanisms of action

One of the most popular strategies to retard and control lipid oxidation is certainly the use of antioxidants. By restraining the lipid oxidation, antioxidants can extend the shelf-life of food products and enhance their overall quality (Gulcin, 2020). Based on their origin, they are classified as synthetic or natural (Gulcin, 2020). Industrially produced molecules with chemical structures optimized to achieve a high antioxidant activity are known as synthetic antioxidants, and they include ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), and gallates. Natural antioxidants are usually secondary metabolites naturally produced by plant species, fungi, and microorganisms, and they include phenolic compounds, carotenoids, tocopherols, ascorbic acid (vitamin C), proteins, and amino acids (such as histidine, lysine, and arginine). Industries can also synthesize antioxidants with the exact same chemical structures of the natural ones.

Many different mechanisms are used by the antioxidants to retard lipid oxidation. However, the three main ones are the free radical scavenging activity, the metal chelation activity, and the singlet oxygen quenching (Choe & Min, 2009). In the free radical scavenging mechanism, lower energy radicals are produced when antioxidants donate hydrogen to radical species, impeding the propagation of lipid oxidation (Choe & Min, 2009). The hydrogen bond dissociation energy of antioxidants that scavenge free radicals determines how easily they give hydrogen. Free radical scavengers, because of their low bond dissociation energy, can react with radicals faster than the

radicals can react with unsaturated lipids, preventing the oxidation reactions. As a result of this process, the free radical scavengers are depleted over time, and the induction phase ends. Due to the low bond dissociation energy oxygen-hydrogen bond of the hydroxyl functional group many potent antioxidants exhibit phenolic structures (Gulcin, 2020). The phenols result in radical species with lower energy also due to the resonance delocalization of the radical on the phenolic ring (Choe & Min, 2009). Metal chelators act by trapping transition metals in their structures with various interactions. In this way, lipid oxidation is prevented since the initiators of its reactions and the responsible for the hydroperoxides' decomposition are not active anymore (Choe & Min, 2009). Since metal chelators can solubilize metals or change their redox potential, they can have prooxidant action, hence using them requires extra caution (Frankel, 2014). Lastly, antioxidants that chemically or physically quench singlet oxygen can inhibit the oxidation of lipids (Choe & Min, 2009). We can refer to chemical quenching when singlet oxygen combines with an antioxidant more quickly than it does with unsaturated lipids, producing oxygenated antioxidant molecules and breakdown products (Choe & Min, 2009). The process of physical quenching involves the transfer of energy from singlet oxygen to the antioxidant, resulting in the formation of triplet oxygen and the antioxidant in an excitation state.

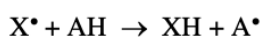
1.2.2 Analytical methods to determine the antioxidant activity

There are various reasons why the antioxidant activity is measured in foods, including *i*) characterization of an ingredient or food, *ii*) comparison of different ingredients or foods, *iii*) evaluation of its variation over time, also in relation to different storage and packaging conditions, and *iv*) meeting legal requirements for health claims. There are numerous methods for the determination of antioxidant activity, which can be grouped into three main categories: spectrophotometric, electrochemical, and chromatographic techniques.

The most widely used are the spectrophotometric techniques, because of their simplicity, speed, and low cost. These techniques are based on two reaction mechanisms, single electron transfer (SET)

and hydrogen atom transfer (HAT) (**Figure 1.2**). Assays based on the HAT mechanisms evaluate an antioxidant's capacity to quench free radicals by donating hydrogen to create stable species (Schaich et al., 2015). An oxidizable fluorometric probe, an antioxidant, and a synthetic free radical generator typically constitute HAT-based procedures (Apak et al., 2016). The majority of HAT-based assays use a competitive strategy where the probe and the antioxidant simultaneously react with the peroxy radicals, which are produced through the thermal breakdown of azocompounds. In the SET mechanism, antioxidant transfer one or more electrons to reduce target compounds, including radicals, carbonyl compounds and metals (Schaich et al., 2015). The assays are based on a redox reaction with an oxidant, which also serves as a probe for reaction monitoring, and examine an antioxidant's capacity to restrain an oxidant that turns color when reduced. The concentration of the antioxidant in the sample correlates with the degree of color change. HAT and SET reactions have the same final product, but different kinetics and dependence on system conditions (Munteanu & Apetrei, 2021; Schaich et al., 2015). Hydrogen atom transfer is pH independent; it is considerably slowed by diffusion, strongly amplified by water and inhibited by hydrogen bonding solvents like alcohols (Schaich et al., 2015). In contrast, SET reactions are not diffusion-controlled and depend on the pH of the system, as ionization increases electron availability (Schaich et al., 2015). Moreover, electron transfer is very fast, as occur in femtoseconds (Schaich et al., 2015).

HAT mechanism (AH = any hydrogen atom donor):



SET mechanism (AH = any electron donor):

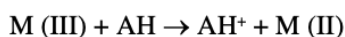
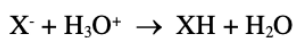
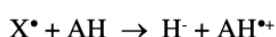


Figure 1.2. HAT and SET mechanisms (adapted from Schaich et al., 2015).

Assays based on hydrogen atom transfer include the Oxygen Radical Absorption Capacity (ORAC) assay, Total Peroxyl Radical Trapping Antioxidant Parameter (TRAP) assay, Total Oxyradical Scavenging Capacity (TOSC) assay, crocin bleaching test, Inhibited Oxygen Uptake (IOU), inhibition of linoleic acid oxidation, and inhibition of LDL oxidation. Assays based on single electron transfer include Ferric Reduction of Antioxidant Power (FRAP), CUPric ion Reducing Antioxidant Capacity (CUPRAC) assay, 2,2'-azinobis-3-ethylbenzthiazolin-6-sulfonic acid (ABTS)/Trolox Equivalent Antioxidant Capacity (TEAC) assay, and 2,2-diphenyl-1-picrylhydrazil (DPPH) radical neutralization assay (Apak et al., 2016; Huang et al., 2005; Munteanu & Apetrei, 2021; Schaich et al., 2015). DPPH and ABTS assays are based on both HAT and SET mechanisms, but SET dominates (Schaich et al., 2015). ORAC, FRAP, ABTS/TEAC, and DPPH are the most frequently used assays, and their characteristics are presented below.

ORAC assay.

The ORAC technique aims to block the oxidation caused by peroxyl radicals. Peroxyl radicals can be either generated by H₂O₂ or NO₂ or by thermal decomposition of azo-compounds, such as 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), and β-phycoerythrin or fluorescein are used as target/probe (Apak et al., 2016; Munteanu & Apetrei, 2021). The peroxyl radicals abstract a hydrogen from the fluorescent probe, causing loss in fluorescence. This reaction can be blocked or delayed as a result of a chain-breaking antioxidant competing with the probe to quench the peroxyl radicals (Munteanu & Apetrei, 2021). The decrease in the probe's fluorescence is proportional to the amount of radicals neutralized by the antioxidant. Advantages of this assay include: *i*) use of biologically relevant radicals, *ii*) its standardization allows data comparison between different laboratories, *iii*) integrates both degree and time of antioxidant reaction (Zulueta et al., 2009). Disadvantages include: *i*) sensible to pH value, *ii*) use of expensive equipment, *iii*) the reagents are water-soluble, thus not allowing to analyze hydrophobic molecules, *iv*) possible large data variability

across equipment, v) β -phycoerythrin can give inconsistency between batches, is photosensitive, and due to nonspecific protein binding can interact with phenolic compounds (Zulueta et al., 2009).

FRAP assay.

This test measures the ability of an antioxidant to reduce trivalent iron (Fe^{3+}) to bivalent iron (Fe^{2+}) under acidic conditions (pH = 3.6) (Munteanu & Apetrei, 2021). Specifically, the reaction involves the presence of a yellow oxidant ferric salt, $\text{Fe(III)(TPTZ)}_2\text{Cl}_3$ (where TPTZ = 2,4,6-tripyridyl-*s*-triazine), which turns deep blue when is reduced (Fe(II)(TPTZ)_2) by an antioxidant (Huang et al. 2005; Munteanu & Apetrei, 2021). Therefore, the antioxidant activity is measured as a function of the increase in color and thus in the absorbance at 593 nm (Munteanu & Apetrei, 2021). Advantages of this assay include: *i*) simplicity and rapidity, *ii*) low-cost, *iii*) the mechanism involved is exclusively SET, thus useful for discriminating the dominant mode of action of an antioxidant. Disadvantages include: *i*) exclusion of biologically relevant radicals, *ii*) side products can be formed and interfere with the analysis by having the same maximum absorption wavelength and extinction coefficient similar to that of the Fe(II)(TPTZ)_2 complex, *iii*) for some antioxidants the reaction may take longer than the time specified in the procedure (4 min), leading to an underestimation of the antioxidant capacity. (Huang et al., 2005).

ABTS/TEAC assay.

The ABTS assay, also known as TEAC assay, is based on the ability of an antioxidant to scavenge the ABTS radical cation ($\text{ABTS}^{\bullet+}$). By reacting the ABTS salt with a strong oxidant, potassium persulfate, the $\text{ABTS}^{\bullet+}$ is produced, characterized by a bluish-green chromophore that reaches maximum wavelengths at 645, 734, and 815 nm. When the antioxidant is present in the reaction medium, it neutralizes the free radical. This results in a decrease in absorbance, which is quantitatively correlated with the concentration of antioxidant present. Advantages of this assay include: *i*) easy to use and low-cost, *ii*) not sensitive to pH changes, therefore can be used to study

the impact of pH on the antioxidant activity, *iii*) the reaction occurs fast (Zulueta et al., 2009). Disadvantages include: *i*) requires an extra step to generate the radical from ABTS salt, *ii*) stability of the radical limited in time, *iii*) the radical is water-soluble, thus only hydrophilic compounds can be analyzed, *iv*) data comparison across laboratories is problematic due to lack of standardization, *v*) since it is an end-point assay, the reaction rate differences between antioxidants and oxidants are excluded, *vi*) the test does not measure radical scavenging activity against biologically relevant radicals (Huang et al., 2005).

DPPH assay.

This method uses the ability of an antioxidant to donate electrons or hydrogen atoms to neutralize a free radical, the DPPH. When solubilized, this stable radical is initially purple-colored with maximum absorbance wavelength at 520 nm, but when is reduced by antioxidants, it turns yellow. The decrease in the absorbance can be measured spectrophotometrically and correlated to the antioxidants' concentration. Advantages of this assay include: *i*) easy to use and low-cost, *ii*) no specific equipment is required, *iii*) the reaction is fast, *iv*) the radical is soluble in organic solvents, thus hydrophobic compounds can be analyzed with this method. Disadvantages include: *i*) sensitivity to solvent and pH, *ii*) the test does not measure radical scavenging activity against biologically relevant radicals, *iii*) access to DPPH is sterically hindered, thus antioxidants that react quickly with peroxy radicals *in vivo* may react slowly or even be inert to this radical.

1.2.3 Antioxidant activity in lipid-containing systems

The ability of an antioxidant to retard lipid oxidation depends not only on its intrinsic properties, but also on the characteristics of the medium in which they act and thus on the phenomena occurring around it. The mechanism of action and reactivity of an antioxidant are defined by its intrinsic properties (e.g., chemical structure). However, based solely on these characteristics, an antioxidant's effectiveness in a food system cannot be predicted (Laguette, Sørensen, et al., 2013). The antioxidant

partitioning in the system mostly affects its physical placement within a food system and, therefore, its capacity to interact with prooxidants and radicals (Laguerre, Sørensen, et al., 2013). In real food systems, physicochemical phenomena might have a greater influence on an antioxidant's efficacy than its intrinsic characteristics, which on the contrary characterize the *in vitro* methods for testing their efficacy (Cruz Figueroa-Espinoza et al., 2013; Laguerre, Bayrasy, et al., 2013; Laguerre, Sørensen, et al., 2013). Indeed, foods contain several phases, such as the lipid phase, aqueous phase, and interface, and they can be polar, non-polar, or intermediate; at the same time, antioxidants share the same heterogeneity in terms of polarity due to the different molecular structures (Laguerre et al., 2017). As a result, an antioxidant's propensity to partition to the phase with comparable polarity has a significant influence on the antioxidant's capacity to take part in lipid oxidation processes (Laguerre, Sørensen, et al., 2013). Based on these considerations, therefore, it is not appropriate to compare the antioxidant activity of a compound from one model system to another model system or to a real food system, because its activity will vary depending on what surrounds it. The most effective antioxidants are those capable of partitioning at the oil-air (bulk oil) or oil-water (emulsion) interface, a capability dictated by their polarity. This mechanism was first proposed by Porter and colleagues in 1989 (Porter et al., 1989) as the “polar paradox”, later rediscussed several times (Laguerre et al., 2015; Shahidi & Zhong, 2011). According to the polar paradox, in fact, less polar antioxidants are most effective in more polar systems, such as O/W emulsions, while polar antioxidants work better in non-polar systems, such as bulk oil. In fact, when Porter and colleagues analyzed sixteen antioxidants with different polarities in emulsions with soy lecithin, they discovered that non-polar antioxidants (e.g., BHA and BHT) had greater action in the emulsions than polar antioxidants (e.g., caffeic acid, chlorogenic acid, gallic acid), while they found in the literature the opposite trend in vegetable oils. When in 1994 Frankel and colleagues tested lipophilic and hydrophilic antioxidants with stripped corn oil in bulk and emulsion systems, they suggested that interfacial phenomena could be the reason that explains the polar paradox (**Figure 1.3**) (Frankel et al., 1994). In other words, they postulated that the differences in the efficacy of the antioxidants (e.g., ascorbic acid was more effective in bulk

oil, ascorbyl palmitate was more effective in emulsion) might be explained by their affinities toward the air-oil interfaces in bulk oil and the oil-water interfaces in emulsions (Frankel et al., 1994). These interfaces constitute indeed the site of lipid oxidation reactions. For example, a polar antioxidant in an oil-in-water emulsion would localize in the aqueous phase, and thus away from the oil droplets and unable to prevent oxidation. In contrast, a less polar antioxidant might locate at the interface, preventing transition metals found in water from reaching unsaturated lipids or hydroperoxides. A polar antioxidant, on the other hand, could give the best performance in bulk oil because it could be located at the interface with air, as opposed to a nonpolar antioxidant that would be solubilized by the lipid phase.

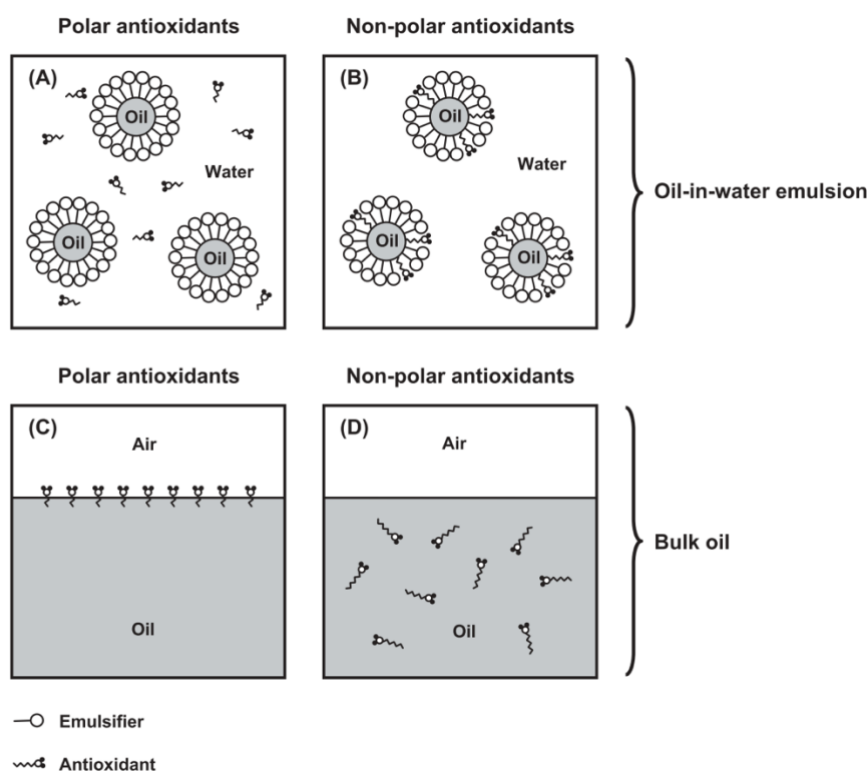


Figure 1.3. Interfacial phenomena as a possible mechanism of action of the polar paradox in oil-in water emulsion (a and b) and in bulk oil (c and d) according to Frankel et al. (1994). From Laguerre et al., 2015.

Since, as mentioned before, the placement of the antioxidant at the interface is of crucial importance for the maximum fulfillment of its antioxidant properties, effective protection from lipid oxidation of, for example, oil-in-water emulsions can be achieved by the use of amphiphilic antioxidants. In fact, the amphiphilic character of the molecule allows its placement at the interface, with the hydrophobic part dispersed in the oil droplet and the hydrophilic part oriented towards the water. Examples of amphiphilic antioxidants are the so-called phenolipids, which are phenolic acid conjugated to a lipid molecule (*Figure 1.4*).

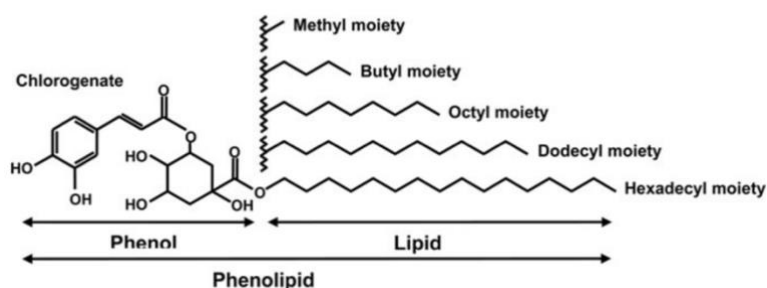


Figure 1.4. Example of a homologous series of phenolipids synthesized through chlorogenic acid transesterification by various alkyl chains. From Laguerre et al., 2015.

However, for these kinds of compounds a limitation of the polar paradox has been elucidated. Indeed, in 2015 Laguerre and colleagues proposed the so-called *cut-off theory* (Laguerre et al., 2015), which rationalizes the non-linear behavior of the synthesized phenolic acid alkyl esters in terms of antioxidant activity as the existence of a critical chain length (CCL) for which maximum activity can be achieved. This *cut-off* effect coincides with different chain lengths depending on the phenolic compound, ranging from two carbon atoms in the case of caffeic acid up to 12 carbon atoms as in the case of chlorogenic acid and coumaric acid (Laguerre et al., 2017). In addition, each compound manifests a different CCL depending on the surrounding system. For caffeate alkyl esters, 8 carbon atoms represent the critical chain length in oil-in-water emulsions and in fish oil-enriched mayonnaise, while in milk enriched with fish oil and in oil-in-water microemulsions the CCL is 2

and 12 carbon atoms, respectively (Laguerre et al., 2017). Again, gallic acid performs best in O/W emulsions when esterified with a 3 carbon atoms alkyl chain, while in liposomes when it has 10 carbon atoms (Laguerre et al., 2017). Regarding ferulate alkyl esters, the CCL in liposomes and in oil-in-water microemulsions where oxidation was induced by AAPH for 6 hours has been found to be 7 and 8 carbon atoms, respectively (Laguerre et al., 2017). To explain this non-linear behavior, Laguerre and colleagues divided the cut-off theory in three parts namely: i) below, ii) at, and iii) above the CCL **Figure 1.5** (Laguerre et al., 2015). Antioxidants with the chain below the CCL are characterized by low hydrophobicity that causes them to partition into the aqueous continuous phase in emulsions. Because they are diluted in the aqueous continuous phase, far from the sites of lipid oxidation, these antioxidants have less potency. At the CCL, antioxidants have intermediate hydrophobicity, resulting in their partitioning at the oil-water interface in emulsions and therefore in their surface-activity. Due to their concentration at the interface, where lipid oxidation takes place and to their phenolic head orientation towards the aqueous phase - where free radicals and transition metals are located-, these antioxidants are very efficient. Above the CCL, antioxidants have been proposed to act in two distinct ways because of their high hydrophobicity. According to the internalization theory, the reason why they are less efficient is due to their dislocation far from the site of lipid oxidation, deep into the core of the oil droplets. While for the self-aggregation theory, these antioxidants are not efficient because they partition into the aqueous phase and form self-aggregates (such as micelles) far from the oxidation sites (Laguerre et al., 2015).

The degree of mobility of the antioxidant in the emulsion can also determine a change in its efficacy. Larger structures, such as phenolic derivatives with very long alkyl chains, result in reduced mobility due to steric hindrance, impairing the diffusion of the antioxidant towards reactive centers, i.e., free radicals and oxidizable substrates. It is also worth mentioning that the alkyl chain can also promote the establishment of hydrophobic interactions between the molecule itself and other hydrophobic molecules in the system (Laguerre et al., 2015).

Hence, if balanced with the right hydrophobicity, phenolipids, by partitioning at the interface, could be a highly effective strategy to counteract lipid oxidation in emulsion-based foods.

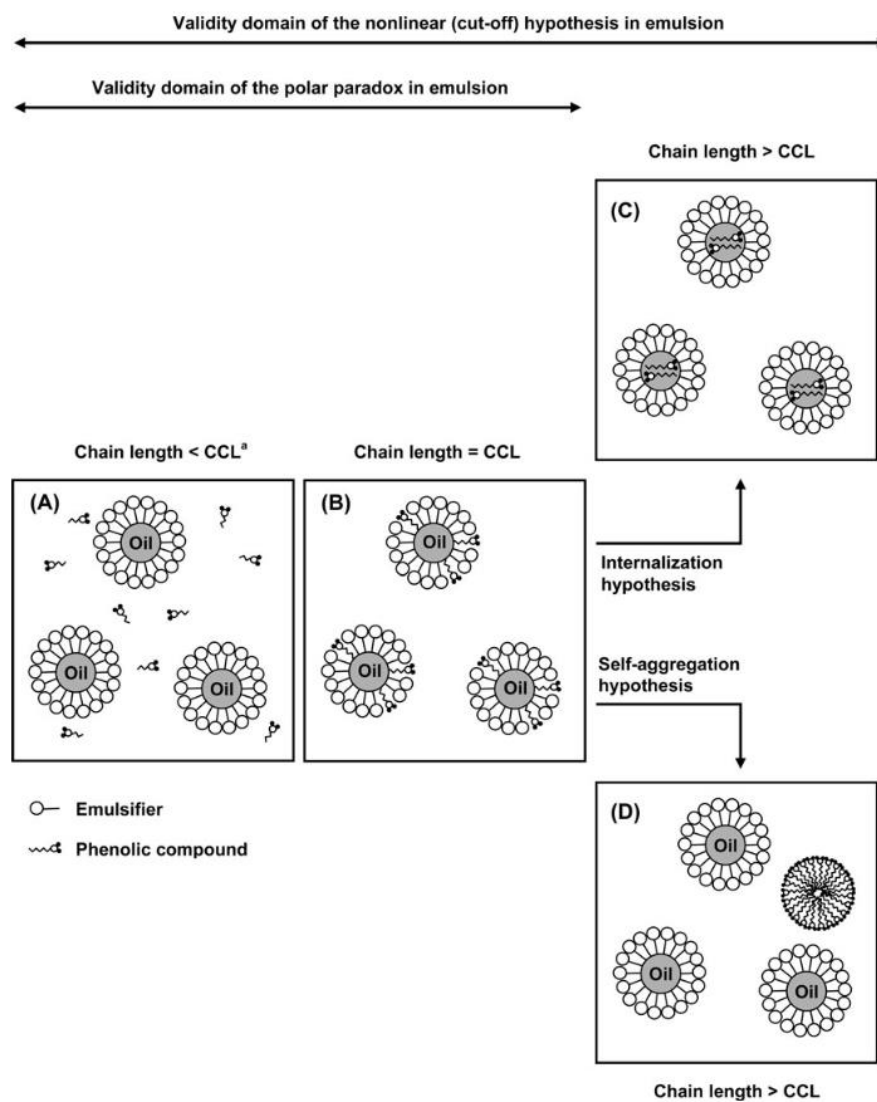


Figure 1.5. Putative scheme of the distribution of antioxidant in emulsified system. From Laguerre et al., 2015.

1.3 Phenolipids

Phenolic compounds are secondary metabolites produced by many plant species for defense against pathogens, growth, and reproduction, with putative potent antioxidant activity. Phenolic acids

are divided in hydroxybenzoic acids and hydroxycinnamic acids based on their structure, but all of them present an aromatic ring bearing at least one hydroxyl group (-OH) and one carboxylic group (-COOH) (Cruz Figueroa-Espinoza et al., 2013). As stated before, the antioxidant activity of these compounds greatly depends on their chemical structure but also on the system. Most of the phenolics share the same hydrophilic character, limiting their use in lipid-containing foods. Increasing their solubility would allow a wider range of applications such as their addition in oils, fats, and emulsions.

Phenolipids (or lipophenols) are a class of bioactive compounds derived by the conjugation of phenolic compounds with hydrophobic molecules, including aliphatic chains, PUFAs, and phytosterols. That conjugation gives lipophenols an amphipathic character, making them molecules of enormous interest to the food industry, as they can be used in oil-based formulas, as well as in emulsified, micellar, and liposomal systems (Arzola-Rodríguez et al., 2022; Berton-Carabin & Villeneuve, 2023). In the latter systems, lipophenols could act as powerful antioxidants due to their amphipathic nature, as mentioned in the previous paragraph. Conjugated phenolic compounds have been demonstrated to possess different antioxidant properties compared to the phenolic parent compound (Arzola-Rodríguez et al., 2022).

Based on their origin, phenolipids can be classified as natural or synthetic.

1.3.1 Synthesis of phenolipids

The lipophilization of phenolic acids can be performed chemically, enzymatically, or chemo-enzymatically (Arzola-Rodríguez et al., 2022; Crauste et al., 2016), usually esterifying the -COOH or -OH residues of the phenolic parent compound with fatty alcohols or fatty acids (respectively) with aliphatic chains of variable length (Arzola-Rodríguez et al., 2022). Grafting a fatty alcohol to the carboxyl group of the phenolic compound preserves its functional group (i.e., the hydroxyl group), responsible for its antioxidant activity (Cruz Figueroa-Espinoza et al., 2013). Typically, chemical esterification is carried out in drastic conditions of temperatures and pH. Since phenolic acids are unstable in alkali (phenolates are considerably more likely to oxidize than phenols), homogeneous or

heterogeneous strongly acidic catalysts are used to carry out the reaction (Cruz Figueroa-Espinoza et al., 2013). Sulfonic resins work well as catalysts in the second scenario, whereas hydrochloric, sulfuric, or *para*-toluene sulfonic acids are employed in the first case. Despite being rapid and simple, chemical processes are typically not selective, leading to undesirable side reactions and requiring many purification procedures to get rid of catalyst residues and by-products, which creates additional waste (Cruz Figueroa-Espinoza et al., 2013). Enzymatic synthesis of phenolipids is usually performed directly esterifying the phenolic acid with enzymes such as lipases, tannin acyl hydrolases, feruloyl esterases, and cutinases (carboxylic ester hydrolase family) in different organic solvents (acetone, cyclohexane, butanol, iso-propanol, diethyl ether, *tert*-butanol, *tert*-butyl methyl ether, and *n*-pentane) or even without solvents, where the alcohol to be esterified plays both the roles of solvent and reagent (Cruz Figueroa-Espinoza et al., 2013). However, the structure of the phenolic may affect the success of the enzymatic process. When the acid function, either directly bound to the aromatic ring or through a double bond, is conjugated to a phenolic with a hydroxyl in *para*-position, esterification has been reported to be partly or even completely impeded (Cruz Figueroa-Espinoza et al., 2013). Therefore, the corresponding esters are only obtained in traces after several days in the lipase-catalyzed esterification of phenolic acids bearing a catechol moiety (benzene ring with two adjacent hydroxyls in *ortho*-position), where one phenolic hydroxyl is in *para*-position respect to the side chain bearing the acid group, such as caffeic or protocatechuic acids (Cruz Figueroa-Espinoza et al., 2013). Lastly, under solvent-free conditions, an intermediate chemo-enzymatic lipophilization method may be accomplished in two phases. To produce the required phenolipid, this procedure first involves the chemical conversion of the phenolic acid into its corresponding methyl ester, which is then transesterified with the fatty alcohol using an enzyme catalyst (Cruz Figueroa-Espinoza et al., 2013). By combining two elements, this technique provides for greater overall yields and shorter reaction times as compared to direct solvent-free esterification. Indeed, compared to its non-esterified precursor, the methyl ester of phenolic acid is far more soluble in fatty alcohols, in particular the long chain ones, which improves the interaction between substrates and enzymes (Cruz Figueroa-Espinoza

et al., 2013). Second, the forward reaction is made easier by the quick evaporation from the medium of the methanol generated during the transesterification (Cruz Figueroa-Espinoza et al., 2013).

1.3.2 Natural phenolipids

Phenolipids can also be isolated from natural sources (Arzola-Rodríguez et al., 2022; Crauste et al., 2016; Domergue & Kosma, 2017). Numerous plant species have been found to contain phenolipids, a summary can be found in **Table 1.1**. Phenolipids can be found in different anatomic part of the plant species, such as cuticles, peel, stem periderm, perisperm, suberized root tissues, and other tissues or cell types. Most of the phenolipids present in nature are composed by ferulic, caffeic, and coumaric acids derivatives, where the hydrophobic part can be either a fatty acid, a phytosterol (campestanoyl, campesteroyl, sitostanoyl, sitosteroyl), or an alkyl chain.

Table 1.1. Occurrence of phenolipids in different plant species.

| Source | Compound | Reference |
|--|--|--------------------------|
| Apple (<i>Malus domestica</i>) cv. Annurca | 12 fatty acid esters of Z- and E-p-coumaryl alcohol: <i>E-p-coumaryl linolenate</i> ; <i>Z-p-coumaryl linolenate</i> ; <i>E-p-coumaryl linoleate</i> ; <i>Z-p-coumaryl oleate</i> ; <i>E-p-Coumaryl Oleate</i> ; <i>Z-p-Coumaryl Stearate</i> ; <i>E-p-Coumaryl Stearate</i> ; <i>Z-p-Coumaryl Arachidate</i> ; <i>E-p-Coumaryl Arachidate</i> ; <i>Z-p-Coumaryl Behenate</i> ; <i>E-p-Coumaryl Behenate</i> ; <i>Z-p-Coumaryl Lignocerate</i> . | (Cefarelli et al., 2005) |
| <u>Plant cuticles</u> | | |
| English Ivy (<i>Hedera helix</i>) | C ₁₆ –C ₃₂ alkyl coumarates | |
| Common beech tree (<i>Fagus sylvatica</i>) | C ₁₈ –C ₂₈ alkyl coumarates | |
| Cattails (<i>Typha domingensis</i> , <i>Typha latifolia</i>) | | (Domergue & Kosma, 2017) |
| Rutabaga (<i>Brassica napus</i> subsp. <i>rapifera</i>) | | |
| Tobacco (<i>Nicotiana tabacum</i>) | alkyl coumarates and alkyl ferulates | |
| Rapeseed (<i>Brassica napus</i> var. <i>napus</i>) | | |
| Arabidopsis (<i>Arabidopsis thaliana</i>) | | |

Salt cress (*Eutrema salsugineum*)

Evergreen tree (*Tamarix aphylla*)

Stem periderm of woody species

| | |
|--|---|
| <i>Pinaceae</i> spp. | alkyl ferulates |
| <i>Fabaceae</i> spp., <i>Myrtaceae</i> spp., <i>Podocarpaceae</i> spp., and <i>Salicaceae</i> spp. | alkyl ferulates |
| Acacia (<i>Acacia dealbata</i> , <i>A. melanoxylon</i> , and <i>A. retinodes</i>) | alkyl caffeates |
| Potato (<i>Solanum tuberosum</i>) | C ₁₆ -C ₃₂ alkyl ferulates, C ₁₆ -C ₁₈ alkyl coumarates |

Suberized Root Tissues

| | |
|---|--|
| Camelina | alkyl caffeates, coumarates, and ferulates |
| Tobacco (<i>Nicotiana tabacum</i>) | alkyl ferulates |
| Tomato (<i>Solanum lycopersicum</i>) | alkyl ferulates |
| Sweet potato (<i>Ipomoea batatas</i>) | alkyl caffeates, coumarates, and ferulates |
| Rice (<i>Oryza sativa</i> subsp. <i>japonica</i>) | alkyl ferulates |
| Corn (<i>Zea mays</i>) | alkyl ferulates |
| <i>Raphanus</i> spp. (<i>Raphanus sativus</i> var. <i>niger</i> and var. <i>longipinnatus</i>) | alkyl coumarates and alkyl caffeates |
| Radish (<i>Raphanus sativus</i> var. <i>sativus</i>) | |
| Rapeseed (<i>Brassica napus</i> var. <i>napus</i>), Arabidopsis, Rutabaga (<i>Brassica napus</i> subsp. <i>rapifera</i>), and salt cress (<i>Eutrema salsugineum</i>) | alkyl caffeates |
| Peas (<i>Pisum sativum</i>) | alkyl coumarates |

Other Tissues or Cell Types

| | |
|---|--|
| Artemisia (<i>Artemisia campestris</i>) | C ₁₈ -C ₂₈ alkyl coumarates |
| Abaca (<i>Musa textilis</i>) | Alkyl coumarates (C ₂₀ -C ₂₈), ω-coumaroyloxy fatty acids (C ₂₂ -C ₂₈), alkyl ferulates (C ₂₀ -C ₂₈), and ω-feruloyloxy fatty acids (C ₂₂ -C ₂₈) |
| Sisál (<i>Agave sisalana</i>) | C ₁₈ -C ₃₀ alkyl ferulates and C ₂₂ -C ₃₀ ω-feruloyloxy fatty acids |
| Sweet potato | alkyl ferulates |

| | | |
|---|---|-------------------------------|
| Cotton (<i>Gossypium hirsutum</i>) | ω -caffeoyloxy fatty acid (ω -caffeoyloxy-docasanoylglycerol) | |
| <u>Others</u> | | |
| Apple (<i>Malus domestica</i>) | Coumaryl alkanoates | |
| Faba bean (<i>Vicia faba</i>) | Cinnamyl alcohol and cinnamyl alkanoates | |
| Apple (<i>Malus domestica</i>) cv. Gala | E- and Z- isomers of <i>p</i> -coumaryl alcohol | (Whitaker et al., 2001) |
| Apple (<i>Malus domestica</i>) cv. Royal Gala | Farnesyl oleate and farnesyl linoleate | (Christeller & Roughan, 2016) |
| Wheat (<i>Triticum aestivum</i>) and rye (<i>Secale cereale</i>) bran | Steryl ferulates | (Nyström et al., 2005) |
| Wheat (<i>Triticum aestivum</i>) and rye (<i>Secale cereale</i>) | Steryl Phenolic Acid Esters | (Hakala et al., 2002) |
| Wheat, corn, rye and triticale | Sitostanyl and campestanil ferulates, sitosteryl and campesteryl ferulates, sitostanyl and campestanil <i>p</i> -coumarates (the latter only in corn) | (Seitz, 1989) |
| Corn (<i>Zea mais</i>) | Steryl cinnamic acid derivatives | (Norton, 1994) |
| Propolis | Caffeic acid esters + caffeic acid amides (synthesized) | (Cos et al., 2002) |
| Wheat (<i>Triticum aestivum</i>), corn (<i>Zea mais</i>), rye (<i>Secale cereale</i>), spelt (<i>Triticum spelta</i>) | Steryl/stanyl ferulates + steryl/stanyl coumarates | (Esche et al., 2012) |
| Wheat (<i>Triticum aestivum</i>), rye (<i>Secale cereale</i>), barley (<i>Hordeum vulgare</i>) | Alkylresorcinols | (Landberg et al., 2014) |
| <i>Anacardiaceae</i> spp. (cashew, mango), <i>Ginkgoaceae</i> (<i>Ginkgo biloba</i>), <i>Gramineae</i> (cereals) | Alkylresorcinols | (Kozubek et al., 2001) |
| Rye (<i>Secale cereale</i>) and cashew nutshell liquid from <i>Anacardium occidentale</i> | Alkylresorcinols - cardol, methylcardol, cardanol (alkylphenol), anacardic acid | (Stasiuk et al., 2008) |

One of the most interesting classes of natural phenolipids is represented by the alkylresorcinols (ARs). ARs (1,3-dihydroxy-5-alkylbenzene derivatives) are a homologue series of phenolipids consisting of a phenolic structure (resorcinol; two hydroxyl groups in the *meta* position) and a hydrophobic chain of different lengths at position 5 of the phenolic ring. Specifically, the hydrophobic domain is an odd-numbered alk(en)yl side chain, whose length in wheat varies mostly from 13 to 27 carbon atoms (Andersson et al., 2008; Chen et al., 2023; Kowalska et al., 2022). The side chain is

mostly saturated but can also be monounsaturated or di-unsaturated. ARs are especially characteristic of cereals, including rye, wheat, and barley, and are thought to be biomarkers of whole grain wheat and rye diet and intake, since they are present almost exclusively in the outer layers of the kernels (Kozubek et al., 2001; Nienartowicz & Kozubek, 1993; Zabolotneva et al., 2022). In wheat bran ARs are present for ~ 0.3% of the total (Chen et al., 2023; Elder et al., 2021; Esche et al., 2012). Although the total ARs content often varies significantly, a relatively stable distribution of the homologues can be found within species, with a C17:0/C21:0 ratio equal to ~0.01 for durum wheat, ~0.1 for common and spelt wheat, and ~1 for rye (Landberg et al., 2014). Beside cereals, ARs are synthesized by some other plant species belonging to different families, such as *Anacardiaceae* (mango, cashew nutshell liquid) and *Amaranthaceae* (quinoa seeds).

1.3.3 Antioxidant activity and properties of phenolipids

As mentioned earlier, antioxidative mechanisms include that of free radicals neutralization converting them into stable species, which can occur either by electron transfer (ET) or hydrogen atom transfer (HAT). The success of both mechanisms greatly depend on the system conditions, such as pH (ET) and solvent (HAT) (Schaich et al., 2015). Phenolipids, possessing a benzene ring and hydroxyl groups, are excellent free radical scavengers. In fact, once donated an electron or hydrogen atom, due to the delocalization on the ring, phenolipid radicals possess low energy, interrupting the propagation of oxidation reactions (Arzola-Rodríguez et al., 2022). Each phenolipidic radical scavenger is capable of inactivating at least two free radicals. Hence, phenolipids exhibit high antioxidant activity, which in many cases, depending on the surrounding environment, is far greater than the parent molecule (non-lipophilized phenolic acid). As mentioned in a previous paragraph, however, this activity is highest for a certain length of the hydrophobic chain conjugated to the phenolic acid (CCL), while it drops considerably, even to the point of becoming pro-oxidant activity when the length is less than or greater than the CCL. In general, and this is true for both natural and synthetic phenolipids, the size of the hydrophobic domain dictates the antioxidant capacity of the

phenolipid, as it determines its partitioning within the system (closer or further from oxidation reactions sites).

Another important aspect of phenolipids is related to the effects *in vivo*. In fact, one of the major limitations of antioxidants is related to their hydrophilicity. Numerous papers highlight enormous potential of many antioxidants as health promoting agents based on successful results of *in vitro* antioxidant assays. However, the antioxidant activity observed *in vitro* probably does not occur *in vivo* (Schaich et al., 2015). There are indeed limits. Phenolic concentrations approach minimal levels in the circulation system since most antioxidants are either quickly conjugated and excreted in the urine or poorly absorbed, making improbable that such minute quantities of antioxidants can exert antioxidant functions in the human body (Schaich et al., 2015). This is due first to their hydrophilicity, which hampers their passage through the hydrophobic phospholipid bilayer of the intestinal epithelium cell membrane (McClements et al., 2015). Secondly, since phenols can react very easily with proteins in foods and cells, they may be activated for other physiological function not examined in the *in vitro* assays or inactivated for classic radical quenching (Schaich et al., 2015). The conjugation of the phenolic compound to a hydrophobic molecule allows its lipophilicity to increase, thus facilitating its absorption at intestinal level. In addition, conjugation should cause phenolic compounds to bind less to other components of the food matrix or other compounds present in the digestive fluids because a functional group is already involved in the bond with the hydrophobic molecule.

From the conjugation of lipids to phenolic compounds, therefore, two advantages are obtained simultaneously: 1) bioactive lipids are protected from oxidation by the phenolic compound conjugated to them; and 2) phenolic compounds show better stability and bioavailability due to their higher hydrophobicity than the hydrophilic parent compound, which instead undergoes rapid transformation and elimination in humans. Hence, the interest in lipophenols lies as much in preserving foods from oxidation as in delivering bioactive molecules undamaged into the human body

in order to be absorbed in sufficient doses to exert their physiological functions, taking advantage of the benefits of both molecules.

1.3.4 Applications of phenolipids

Due to their structure that preserves the putative abilities as radical scavengers, metal chelators, and disruptors of chain oxidation reactions of the phenolic compound, and due to their amphiphilic character, which enables them to interact with biological membranes as well as micelles and drug-transporting vesicles, phenolipids are of great interest for many sectors, such as dermatology, pharmacology, food preservation, and cosmetics (Arzola-Rodríguez et al., 2022). The type of phenolic compound present in the conjugated structure directly influences the biological effects (such as antibacterial, anti-inflammatory, antiparasitic, and anticancer properties) and antioxidant capacity of phenolipids. On the other hand, the hydrophobic part influences the hydrophobic interactions of the phenolipid with the surrounding medium, and therefore its mobility and location in the system.

In the food sector, many lipophenols, both natural and synthetic, have been tested in model systems (e.g., microencapsulated linoleic acid (Fang et al., 2006), fish-oil-enriched emulsions (A. D. M. Sørensen et al., 2012), bulk oil (Lu et al., 2021), and O/W emulsions (Elder et al., 2021; Laguerre et al., 2010; Panya et al., 2012)), in food products (e.g., fish oil enriched milk (A. M. Sørensen et al., 2012), tarallini – a typical Italian bakery product (Marzocchi & Caboni, 2018), frozen French fries (Szydłowska-Czerniak & Rabiej, 2021), and crackers (Elder et al., 2022)). Moreover, esters of gallic acid with different alkyl chains are already employed as antioxidant additives in food products (E310-propyl gallate; E311-octyl gallate; E312-dodecyl gallate) (Figuroa-Espinoza & Villeneuve, 2005).

Phenolipids have also been investigated for their potential inhibition capacity of many foodborne pathogenic and spoilage bacteria activities (Arzola-Rodríguez et al., 2022). Synthetized phenolipids from tyrosol were tested against some Gram-positive and Gram-negative bacteria with pathogenic behavior in humans, and against two strains of the *Leishmania* spp. (Arzola-Rodríguez et al., 2022). Alkyl gallates were found to possess an antimicrobial activity against *Escherichia coli* and

Staphylococcus aureus, while alkyl ferulates were found to be able to disrupt the cell membrane of both *Escherichia coli* and *Listeria monocytogenes* and alter their cell morphology (Arzola-Rodríguez et al., 2022). As amphipathic molecules, phenols have an advantage whenever applied to drug delivery systems, medicines, dermocosmetics, and nutraceuticals since they are more soluble and easier to incorporate into lipid phases and lipocarriers (Arzola-Rodríguez et al., 2022). Many beneficial properties for the human body have been highlighted for many phenolipids, including antioxidant and antcarbonyl stress activity, anticancer activity (cytotoxicity against cell proliferation), neuroprotective effect, interaction with endocannabinoid system, anti-inflammatory activity, enzymatic inhibition capacity, antibacterial and antiparasitic activities (Arzola-Rodríguez et al., 2022; Crauste et al., 2016).

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Chapter 2

HYPOTHESIS, GENERAL AND SPECIFIC AIMS, AND STRUCTURE OF THE DISSERTATION

Lipid oxidation is a major cause of food quality deterioration and thus of food waste. In addition, lipid oxidation causes the loss of health-promoting compounds, such as polyunsaturated fatty acids, lipid-soluble vitamins, and phytosterols. One of the main strategies to address this phenomenon is the addition of antioxidants, both natural and synthetic, to prevent oxidation reactions and thus prolong the shelf-life of foods. However, most antioxidants are hydrophilic, strongly limiting their use in lipid-containing products. A solution to this problem could therefore be phenolipids (or lipophenols). This class of compounds consists of a phenolic compound conjugated to a hydrophobic molecule, such as an alkyl chain. In fact, the increased lipophilicity of phenolic compounds allows their placement at the sites where oxidation reactions occur.

This PhD project is aimed at developing healthy foods using lipophenols, both natural and synthetic and with different molecular structures, to preserve bioactive compounds (such as phenolic compounds and bioactive lipids) from degradation and increase their bioaccessibility in the human body.

Specifically, the project has been divided in two parts: *i*) study of synthetic lipophenols; *ii*) study of natural lipophenols:

- i. In the first part of the project, synthetic lipophenols were investigated (**Chapter 3**). Synthetic phenolipids were considered because, if obtained through green techniques, they could be a powerful tool for companies. They are indeed fine-tuned and highly pure molecules that could lead to high antioxidant activity at low concentrations, considerably extending the shelf-life of foods. Specifically, ferulic derivatives were synthesized using environmentally friendly techniques, esterifying ferulic acid with butanol and octanol to obtain alkylferulates with different length of the alkyl chain on its carboxyl group, butyl ferulate and octyl ferulate,

respectively. The effect of their concentration and the pH of the system on their performance was evaluated in a model system represented by 1.0% oil-in-water emulsions.

ii. The second part of the project has been focused on the evaluation of natural phenolipids, and more specifically of alkylresorcinols. The latter were extracted from wheat bran in a circular economy approach of zero waste. In fact, wheat bran represents one of the main by-products of the milling industry, usually intended to be wasted. The efficacy of alkylresorcinols was first tested in a model system represented by 1.0% oil-in-water emulsions, and again their concentration and the pH of the system were investigated as factors affecting their antioxidant activity (**Chapter 4**). Once established their potentiality as antioxidant agent in lipid-containing systems, alkylresorcinols were studied in different concentrations in a real food product, namely beef patties (**Chapter 5**).

To conclude, final considerations were drawn, highlighting the main innovative points of this PhD project (**Chapter 6**).

Chapter 3

ANTIOXIDANT PROPERTIES OF FERULIC ACID-BASED LIPOPHENOLS IN OIL-IN-WATER (O/W) EMULSIONS

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3.1 Abstract

Butyl ferulate (BF) and octyl ferulate (OF) were investigated to enhance oxidative stability of 1.0% vegetable oil-in-water (O/W) emulsions at pH 3.5 and 7.0 at different concentrations (5, 10, 15, 20, and 25 mg/L of emulsion). Particle size distribution, droplet surface charge, hydroperoxides (PV), hexanal and nonanal were monitored for 14 days at 35 °C. Higher concentrations of BF and OF generated smaller particles at pH 7.0 but not at pH 3.5. Control showed the highest negative surface charge (-58.38 ± 0.31 mV, pH 7.0) compared to BF and OF (-46.71 ± 0.06 mV at levels >0.10 mg/L). Both alkyl ferulates counteracted lipid oxidation, especially at pH 3.5, where PV decreased up to 96% compared to the control and delayed hexanal and nonanal formation. A low content of lipophenols allowed to achieve a tremendous shelf-life extension, demonstrating that BF and OF could be of great interest for food application.

3.2 Introduction

Since fats are predominant ingredients in foods, lipid oxidation is one of the chief chemical reactions that deteriorates food quality, impacting flavor (development of rancid volatiles, like pentanal, hexanal, nonanal and decanal), nutritional value (loss of health-promoting lipids, such as omega-3 fatty acids), protein functionality (co-oxidation), and biological tissues (production of toxic compounds, such as hydroperoxides, aldehydes, and ketones) (Bayram & Decker, 2023; Frankel, 2014). High degree of unsaturation results in a decisive increase in the rate of oxidation, with the result that mono- and polyunsaturated fatty acids are the most unstable, yet also being the most sought after by consumers. Oil-in-water (O/W) emulsions, which make up the majority of foods, are a favorable system for rapid oxidation reactions, as the contact area between lipids and water containing prooxidants (transition metals, enzymes, and photosensitizers) is very large, facilitating their interaction (Li et al., 2020b; Li et al., 2023; McClements & Decker, 2018). One solution to delay lipid oxidation in emulsion systems and increase the shelf-life of health-promoting lipid-containing foods is to use antioxidant compounds (Bravo-Díaz, 2022; Jiang & Charcosset, 2022; Li et al., 2020a; Liu et al., 2022; Zhang, Fan, Liu, & Li, 2023).

In addition, the hydrophilicity of most antioxidants brings an important limitation to their use in lipid-containing foods. According to the polar paradox, less polar antioxidants are most effective in more polar systems such as O/W emulsions (Laguette et al., 2009) and in such multiphase systems the partitioning of antioxidants at the interface region – where oxidation reactions take place – is crucial and can be achieved with amphiphilic antioxidants, such as lipophenols (McClements & Decker, 2018). Lipophenols consist of a phenolic component bound to hydrophobic molecules like alkyl chains, fatty acids, and phytosterols. The lipophilization should enable their positioning at the oil-water interface, with the lipid part in oil droplets and the phenolic part facing the water. Many studies demonstrated the *in vitro* antioxidant capacity of certain lipophenols, involving different phenolic structures conjugated to alkyl chains of different lengths (Liu & Yan, 2019; Sørensen et al.,

2014; Torres de Pinedo, Peñalver, & Morales, 2007; Xu et al., 2022b). Some lipophenols have also been tested in model systems (Elder, Coupland, & Elias, 2021; Lu et al., 2021; Panya et al., 2012; Sørensen, Nielsen, Yang, Xu, & Jacobsen, 2012; Szydłowska-Czerniak, Rabiej, Kyselka, Dragoun, & Filip, 2018), in food products (Elder, Coupland, Hopfer, & Elias, 2022; Marzocchi & Caboni, 2018; Sørensen et al., 2012b), and in biological systems (Alemán-Jiménez et al., 2021; Moine et al., 2021; Totani, Tateishi, Takimoto, Shinohara, & Sasaki, 2012; Xu et al., 2022a). Moreover, esters of gallic acid with different alkyl chains are already employed as antioxidant additives in food products (E310-propyl gallate; E311-octyl gallate; E312-dodecyl gallate) (Figuroa-Espinoza & Villeneuve, 2005). However, each antioxidant behaves differently depending on its molecular structure and the surrounding medium (Villeneuve et al., 2021). Therefore, only the study of the lipophenols in both model and real food systems can make their use by food companies realistic and feasible. The so-called *cut-off* theory (Laguerre et al., 2009) rationalizes the non-linear behavior of the synthesized lipophilic phenolic esters in terms of antioxidant activity as the existence of a critical chain length (CCL) for which maximum activity can be achieved. The CCL depends not only on the type of phenolic compound but also on the system in which it acts (Laguerre et al., 2017). For example, the CCL for alkyl caffeates was found to be 8 in O/W emulsions and in fish oil-enriched mayonnaise, while 2 and 12 in milk enriched with fish oil and in O/W microemulsions, respectively (Laguerre et al., 2017). Gallic acid performed best in O/W emulsions when esterified with a 3 carbon atoms alkyl chain, while in liposomes when it has 10 carbon atoms (Laguerre et al., 2017). Regarding ferulate alkyl esters, the CCL in liposomes and in O/W microemulsions was found to be 7 and 8 carbon atoms, respectively (Laguerre et al., 2017), while methyl and butyl ferulates were the two most effective lipophenols in fish oil enriched milk (Sørensen, Lyneborg, Villeneuve, Jacobsen, 2015). In addition, ferulic acid represents one of the most abundant phenolic acids largely widespread in plant tissues and it is well known for its strong ability to scavenge free radicals due to the presence of substituent in *para* position and a carboxylic group with an adjacent unsaturated C-C double bond (Shahidi et al., 2022).

It is also reported that the potency of the antioxidant activity of phenolics or other antioxidants can change in the presence of a neutral or an acidic environment (Bayram & Decker, 2023; Huang, Frankel, Schwarz, & German, 1996; Li et al., 2020a; Tian et al., 2022), due to different factors, such as the variation of the redox potential based on the pH of the surrounding system, the possible different positioning of molecules in the system, or even the different rate of degradation of molecules (Bayram, Laze, & Decker, 2023; Bayram & Decker, 2023; Jovanovic, Steenken, Hara, & Simic, 1996). To the best of our knowledge, there is a lack of information regarding the ability of ferulate alkyl esters to counteract lipid oxidation as related to different pH conditions in O/W emulsions stored for a prolonged period with no oxidation inducers.

In the present study, therefore, we hypothesized that, based on what reported in the literature, the lipophilization of ferulic acid with a short (C4) and medium (C8) alkyl chain could be a successful strategy to enhance the oxidative resistance toward lipid oxidation during the storage of food emulsions. Two different ferulate alkyl esters (butyl ferulate, BF, and octyl ferulate, OF) were tested at different concentrations in O/W emulsions stored at 35 °C for 14 days. In addition, the effect of pH (3.5 and 7.0) on their activity was also investigated.

3.3 Materials and Methods

3.3.1 Chemicals

Propan-2-ol, *n*-hexane, iso-octane, ethanol absolute anhydrous, 1-butanol, and hydrochloridric acid (37%) were purchased from Carlo Erba (Milan, Italy), while methanol from VWR (Milan, Italy). Ferulic acid, butanol, Dowex 50 X8, polyethylene, ethyl acetate, pyridine, acetic anhydride, toluene, thionyl dichloride, chloroform, 1-octanol, piperidine, Tween 20 (polyethylene glycol sorbitan monolaurate), ferrous sulfate heptahydrate, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, silicic acid (100–200 mesh, 75–150 µm, acid washed), activated charcoal, cumene hydroperoxide, barium chloride dihydrate, butylated hydroxytoluene (BHT), ammonium thiocyanate, and sodium phosphate

monobasic dihydrate were provided by Merk (Darmstadt, Germany). Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA) and sodium phosphate dibasic anhydrous were supplied by Fluka (Milan, Italy). Ultrapure water was prepared in a Milli-Q filter system (Millipore, Milan, Italy).

3.3.2 Synthesis of Butyl and Octyl Ferulates (**1** and **5**)

Both alkyl ferulates were synthesized in the Department of Drug Science and Technology of the University of Turin.

Butyl Ferulate (1)

One hundred mg of ferulic acid (0.5 mmol) were dissolved in 4 mL of butanol and 150 mg of Dowex 50 X8 (previously activated) was added. The reaction was left under reflux overnight. The solution was filtered and dried under vacuum. Then it was purified by column chromatography in petrolether:ethyl acetate (9:1, v/v) to obtain the desired product as a transparent oil in 86% yield.

Octyl Ferulate (5)

Ferulic acid (202 mg, 1 mmol) was dissolved in anhydrous pyridine (3 mL). Acetic anhydride (0.5 mL) was added, and the mixture was stirred at room temperature overnight. The solution was evaporated after addition of toluene and dried to obtain a white solid (*p*-acetoxy-ferulic acid **2**) that was converted without any further purification.

4-*O*-Acetyl ferulic acid (1 mmol) was dissolved in toluene (5 mL) and 0.35 mL of thionyl dichloride were added. The mixture was heated to reflux for 30 min then the solution was evaporated to obtain a white solid (4-*O*-acetoxy-feruloyl chloride **3**) that was used without further purification.

To a solution of 4-*O*-acetoxy-feruloyl chloride **3** (1 mmol) in 5 mL of chloroform, 1-octanol was added, and the reaction was left under shaking at room temperature. Water was added and organic phase extracted twice. The desired 4-*O*-acetoxy-octyl ferulate (**4**) was isolated by column chromatography to obtain 243 mg of desired product (70% yield over three steps).

A solution of compound **4** was dissolved in 95% ethanol, piperidine was added (5 mL). The reaction was monitored by TLC analysis (ethyl acetate:methanol, 9:1; v/v) and when conversion was complete it was partially dried under vacuum. The crude was neutralized with aqueous acetic acid and extracted twice with water and ethyl acetate (1:1; v/v) to obtain pure desired product in quantitative yield.

The quality control of butyl and octyl ferulates was carried out through thin layer chromatography (TLC), nuclear magnetic resonance (NMR; Figures S1–S4 of Supplementary Material) and gas chromatography coupled with mass spectrometry (GC/MS) (see details in the Supplementary Material).

3.3.3 Evaluation of the Antioxidant Properties of Butyl (BF) and Octyl Ferulates (OF)

The antioxidant properties of BF and OF were assessed through *in vitro* assays in terms of radical scavenging activity (DPPH•) and iron chelating activity. For both assays four different concentrations of BF and OF were prepared in ethanol (50, 100, 200, and 400 µmol/L, final concentrations) according to Sørensen et al. (2014) and tested in triplicate ($n=3$).

3.3.3.1 DPPH• Radical Scavenging Assay

Radical scavenging activity was evaluated according to Sørensen et al. (2014) with slight modifications. Briefly, 100 µL of each concentration of the ferulate alkyl esters were added in a 96-well microplate and immediately added with 100 µL of a DPPH• ethanolic solution (0.1 mmol/L). After 30 min of incubation in darkness at 23 °C, the absorbance was read at 517 nm in a BioTek Synergy HT spectrophotometric multi-detection 96-well microplate reader (BioTek Instruments, Milan, Italy) against a negative control in which the sample was replaced by ethanol. BHT was used as positive control at the same concentrations of BF and OF. Results were expressed as percentage of inhibition (%I) according to the following equation:

$$\%I = \left(\frac{Abs_{blk} - Abs_{sample}}{Abs_{blk}} \right) \times 100 \quad [1]$$

where Abs_{blk} is the absorbance of the negative control and Abs_{sample} is the absorbance of the tested sample at 517 nm after 30 min.

3.3.3.2 Iron Chelating Assay

The ability to chelate ferrous ions was determined according to (Yen & Chung, 1999), modified as detailed in (Cantele et al., 2020). EDTA (same concentrations) served as the positive control. Results were expressed as percentage inhibition (%I).

3.3.4 Stripping of the Oil

Polar compounds were removed from 30 g of oil (a mixture of soybean and sunflower oils) according to (Boon et al., 2008) in a chromatographic column (diameter 3 cm; length 35 mm) packed with silicic and activated charcoal. While stripping, collected triacylglycerols were held in an ice bath and protected from light. Then the solvent was removed with a rotary evaporator (Rotavapor, R-210, Buchi, Flawil, Switzerland). Quality control of stripping step was conducted using gas chromatography with flame ionization detection (GC/FID) (Cardenia et al., 2018) (details in Fig. S9 of Supplementary Material) and peroxide value determination (Shantha & Decker, 1994).

3.3.5 Preparation of the Oil-in-Water (O/W) Emulsions and Experimental Conditions

Oil-in-water (O/W) emulsions were prepared as described by Cardenia, Waraho, Rodriguez-Estrada, McClements, & Decker (2011), with stripped oil (1.0% of the total weight of the emulsion; w/w), Tween 20 as non-ionic emulsifier (0.1% of the total weight of the emulsion; w/w), and 10 mmol/L phosphate buffer solution (pH 7.0 and 3.5). After removing the solvent, BF and OF were dissolved into the stripped oil at different concentrations (5, 10, 15, 20, 25 mg/L of emulsion) by

stirring. Tween 20 and buffer solution were then added, mixed with an IKA T25 digital Ultra-Turrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 2 min at maximum speed (20,000 rpm) to obtain a coarse emulsion, and then homogenized by ultrasounds sonication for 3 min at 100 W to obtain a fine emulsion. During the sonication process, emulsions were held in an ice bath, to maintain the temperature ≤ 30 °C. Control samples (without lipophenols) were prepared for each experiment. Subsequently, 1 mL of each emulsion was transferred into 20 mL headspace vials secured with aluminum caps with PTFE/silicone septa and stored in darkness at 35 °C for 14 days. Three independent experiments ($n=3$) were carried out for each sample and each analysis.

3.3.6 Evaluation of the Physical and Oxidative Stability of the Emulsions

3.3.6.1 Particle Size Distribution and Zeta-Potential Measurement

The emulsions were diluted in the corresponding 10 mmol/L buffer solution (pH 7.0 and 3.5) used for their preparation at a ratio of 1:50 (v/v), and then particle size distribution and droplet surface charge (zeta-potential) were measured through dynamic light scattering (DLS) with a Zetasizer Pro (Malvern Instruments, Worcestershire, UK). The measurements were repeated three times for each independent experiment.

3.3.6.2 Primary Oxidation Products

Lipid hydroperoxides were measured in the emulsions as primary oxidation products as reported by Cardenia et al. (2011). Briefly, aliquots of emulsions were dispersed in iso-octane:propan-2-ol and the supernatant was then dissolved in methanol:butanol (2:1; v/v) with 15 μ L of ammonium thiocyanate and 15 μ L of FeCl₂. Absorbance was read at 510 nm and quantification was performed based on a cumene hydroperoxide standard calibration curve in the range of 10–300 μ mol/L ($y = 2.6872x + 0.0135$; $R^2 = 0.9973$). Results were expressed as millimoles of hydroperoxides per kilograms of oil (mmol/kg oil).

3.3.6.3 Secondary Oxidation Products

Headspace solid-phase microextraction gas chromatography coupled with mass spectrometry (HS-SPME-GC/MS) (QP-2010 Plus GC/MS, Shimadzu, Kyoto, Japan) was used to determine hexanal and nonanal content as markers of the secondary oxidation reactions. Vials were heated at 40 °C for 10 min, and a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (df 50/30 µm; 1 cm; Supelco, Bellefonte, PA, USA) coated fiber was exposed in the headspace for 5 min at the same temperature. The fiber was then desorbed in the inlet of the GC at 260 °C in split mode (1:50) and using helium as carrier gas (34.8 m/s linear velocity). The analytes were resolved in a RTX-5 fused silica capillary column (20 m × 0.10 mm, film thickness 0.10 µm; Restek, Bellefonte, PA) with the following oven temperature program: from 40 °C (held for 1 min) to 100 °C with a constant rate of 5 °C/min; then to 240 °C at 30 °C/min and held for 1 min. Transfer line, and ion source temperature were set at 230 °C, and 200 °C, respectively. Ions were acquired in scan mode in a 33–350 m/z range with a scan velocity of 1666 amu/s. Hexanal and nonanal were identified through the mass spectra contained in the NIST08s library and by injection of the pure standards under the same analytical conditions. The quantification was achieved by external standard method (hexanal, 0.212–750 µmol/kg oil, $y = 43.98x + 5100$; nonanal, 0.146–350 µmol/kg oil, $y = 49.67x + 3215$). Results were expressed as µmol/kg oil.

3.3.7 Statistical Analysis

The results of all data were reported as means and standard deviation of three independent experiments ($n=3$). Statistical differences were ascertained by analyzing the results with one-way ANOVA combined with Tukey's post hoc test using IBM SPSS statistical software (version 27; IBM, Chicago, IL, USA). Normality and homoscedasticity were checked before performing the ANOVA test with, respectively, Shapiro–Wilk and Levene's tests with the same software. Differences with a p -value < 0.05 were considered significant. Lag phases of hydroperoxides, hexanal, and nonanal

formation were determined as the first data point significantly different (p -value < 0.05) from day 0 using one-tailed Dunnett's *post hoc* test.

3.4 Results

3.4.1 Synthesis and Characterization of Butyl and Octyl Ferulates

The synthesis of butyl and octyl esters was attempted by esterification of ferulic acid and, because of the different reactivity, availability, and physical state of butanol and octanol, two different synthetic routes were pursued (**Figure 3.1**).

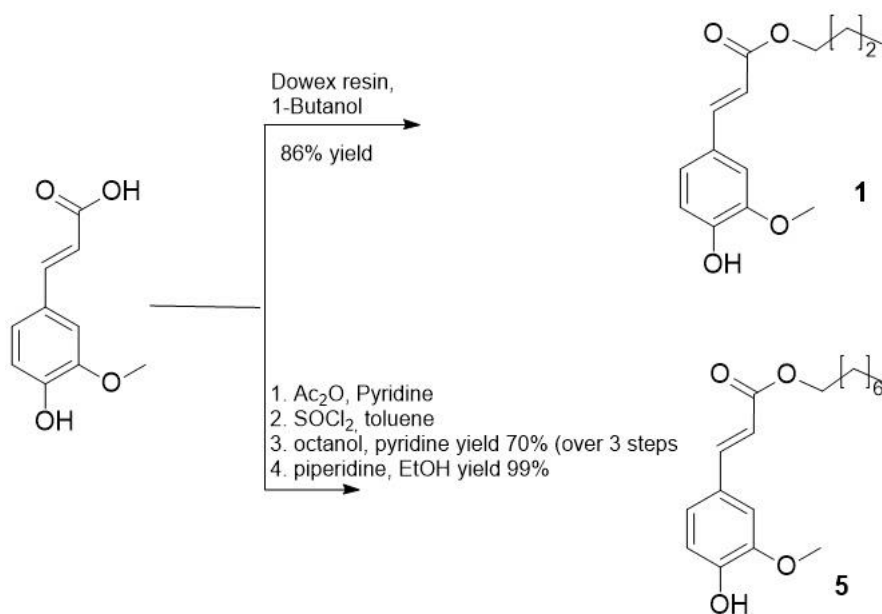


Figure 3.1. Schematic representation of ferulate esters **1** and **5** syntheses.

Butyl ester **1** was obtained by direct acid catalyzed Fischer esterification in presence of solid supported sulfonic acid, while a four steps synthetic scheme was followed for the synthesis of octyl ester **5**. A mild procedure of esterification with butanol in presence of Dowex H⁺ cation-exchange resin was exploited to efficiently obtain butyl ferulate in excellent yield (Turhanen et al., 2019). When synthesis of octyl ester was approached the previous scheme resulted inefficient and the protection

of the phenolic moiety was performed to obtain the ferulic acyl chloride that was further reacted with octanol and deprotected with piperidine in ethanol with an overall yield of 70% (Panda et al., 2012). The purity of synthesized products was fully characterized by TLC, GC/MS and NMR and were delivered to further study only when purity was higher than 99%.

3.4.2 Antioxidant Properties

Both BF and OF did not display iron chelating activity, while antiradical activity was observed (**Figure 3.2**).

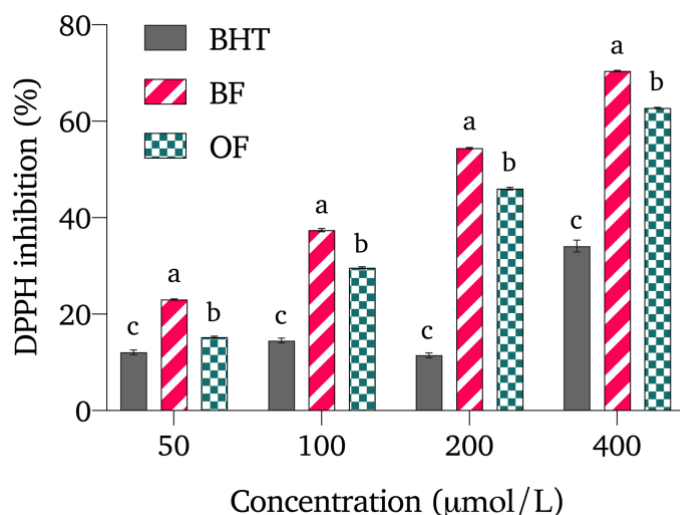


Figure 3.2. Results of DPPH radical scavenging activity of different concentrations (50, 100, 200, and 400 µmol/L) of BF and OF and of positive control (BHT). Each bar represents the mean \pm standard deviation of three independent replicates ($n=3$). Different letters within the same concentration indicate means significantly different at $p < 0.05$.

For each tested compound the scavenging activity was affected by the concentration applied. BHT showed significant increase in its activity only at 400 µmol/L ($p < 0.001$), while lipophilized compounds showed earlier effects at 100 µmol/L ($p < 0.001$) compared to BHT. At 200 µmol/L BF and OF already neutralized 50% of DPPH, while at 400 µmol/L BHT barely reached 35% inhibition. BF displayed slightly superior scavenging efficiency compared to OF. Interestingly, as the

concentration increased the gap between the two narrowed; the OF showed a lower activity of 30%, 20%, 15%, and finally 10% at 50 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$, 200 $\mu\text{mol/L}$, and 400 $\mu\text{mol/L}$, respectively.

3.4.3 Effects of BF and OF on the Physical Stability of the O/W Emulsions

Figure 3.3 shows the particle size distribution of the emulsions with BF and OF at pH 7.0 and 3.5 over 14 days of storage.

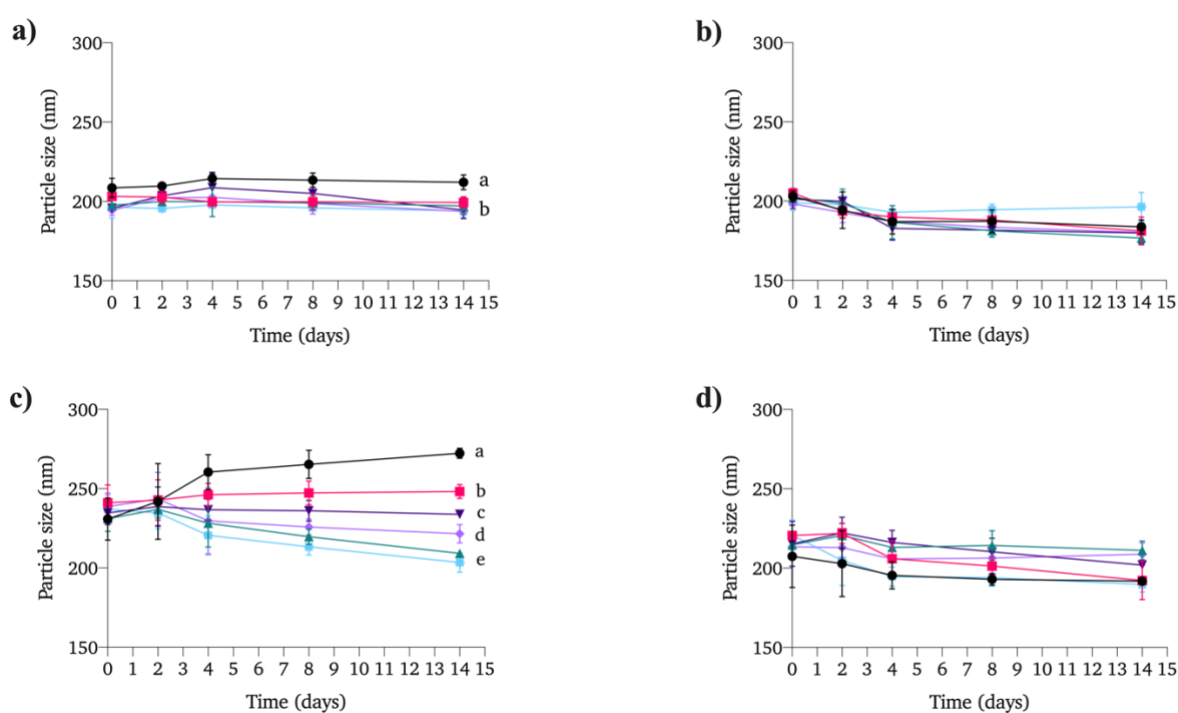


Figure 3.3. Particle size distribution (nm) of 1.0% O/W emulsions without (control) (●) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (■), 10 mg/L (▲), 15 mg/L (▼), 20 mg/L (◆), and 25 mg/L (*) throughout the 14 days of storage. **a)** BF at pH 7.0; **b)** BF at pH 3.5; **c)** OF at pH 7.0; **d)** OF at pH 3.5. Each data point represents the mean \pm standard deviation of three independent replicates ($n=3$). Some error bars lie within the data points. Different letters indicate means significantly different within the day at $p < 0.05$.

In general, particle size ranged 194–242 nm at the beginning of the experiments. BF and OF had similar effects on emulsion droplet size within each environmental condition. At pH 3.5, BF- and OF-enriched emulsions showed no significant differences compared to the control at any concentration on any tested day ($p > 0.05$) (**Figure 3.3b** and **3.3d**). Contrarily, at pH 7.0 the presence

of lipophenols led to a decrease in particle size on the last day (**Figure 3.3a** and **3.3c**). However, while the addition of more than 5 mg/L of BF did not lead to a progressive decrease in droplets dimension (**Figure 3.3a**), in OF particle size decreased in relation to its concentration. In fact, particle size was 272.35 ± 3.18 nm in the control, while with 25 mg/L OF resulted in 203.40 ± 6.22 nm (**Figure 3.3c**). However, all emulsions, including controls, remained physically stable without significant changes in particle size over time in all four experiments ($p > 0.05$).

The droplet surface charge under both acidic and neutral conditions is reported in **Figure 3.4**.

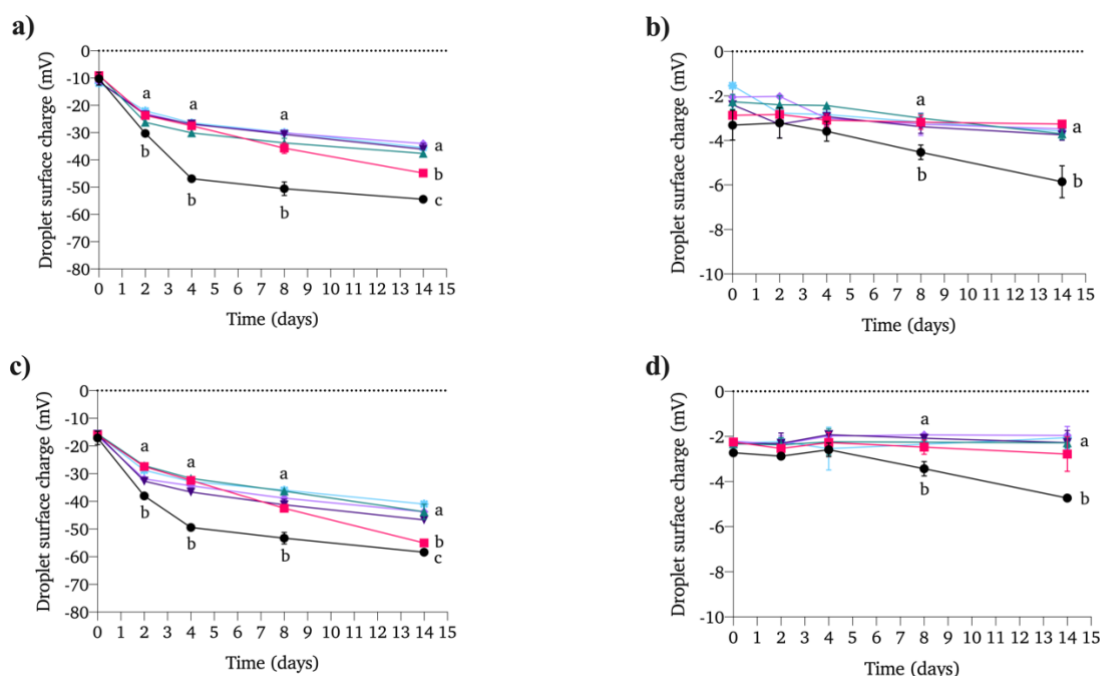


Figure 3.4. Droplet surface charge (mV) of 1.0% O/W emulsions without (control) (●) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (■), 10 mg/L (▲), 15 mg/L (▼), 20 mg/L (◆), and 25 mg/L (*) throughout the 14 days of storage. **a)** BF at pH 7.0; **b)** BF at pH 3.5; **c)** OF at pH 7.0; **d)** OF at pH 3.5. Each data point represents the mean \pm standard deviation of three independent replicates ($n=3$). Some error bars lie within the data points. Different letters indicate means significantly different within the day at $p < 0.05$.

Emulsions with BF and OF were characterized by negative values of zeta-potential at both pH values since day 0. At pH 7.0 (**Figure 3.4a** and **3.4c**), the negative charge drastically increased as the days progressed ($p < 0.001$). The control displayed significantly higher zeta-potential compared to

BF and OF emulsions ($p < 0.001$), as their presence reduced the negative charge as related to their concentration. In fact, the control ranged from -10.22 ± 2.31 mV (day 0) to -54.41 ± 1.02 mV (day 14), while BF and OF at 25 mg/L reached -35.48 ± 2.14 mV and -40.95 ± 0.06 mV, respectively, on the last day. At pH 3.5 (**Figure 3.4b** and **3.4d**) a similar behavior was found, even if the values resulted less negative. At day 14 the control reached a value of -5.85 ± 0.71 mV, whereas the addition of BF reduced the negative charge (ranged from -1.53 ± 0.01 mV to -3.58 ± 0.15 mV). On the other hand, the OF restrained the drop and a steady trend was observed for all concentrations tested ($p > 0.05$).

3.4.4 Effects of BF and OF on the Oxidative Stability of the O/W Emulsions

Increasing the concentrations of lipophenols the lipid hydroperoxides content in emulsions significantly ($p < 0.001$) decreased (**Figure 3.5**).

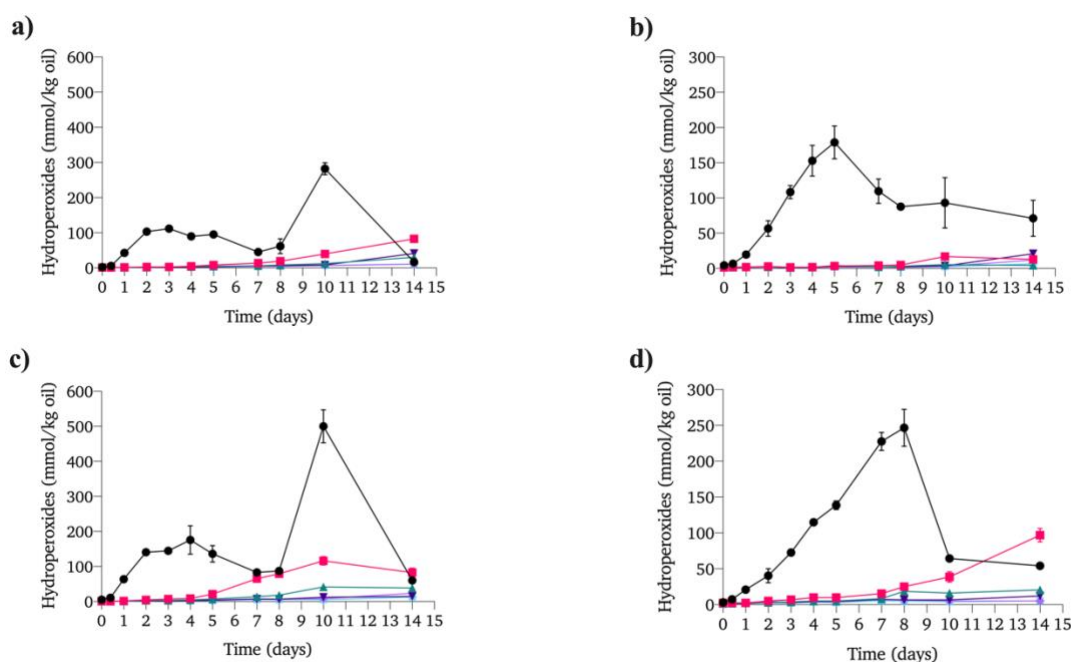


Figure 3.5. Hydroperoxides content (mmol/kg oil) of 1.0% O/W emulsions without (control) (●) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (■), 10 mg/L (▲), 15 mg/L (▼), 20 mg/L (◆), and 25 mg/L (*) throughout the 14 days of storage. **a)** BF at pH 7.0; **b)** BF at pH 3.5; **c)** OF at pH 7.0; **d)** OF at pH 3.5. Each data point represents the mean \pm standard deviation of three independent replicates ($n=3$). Some error bars lie within the data points.

The controls at both pH values showed a significant increase in hydroperoxides already after 4.5h. In contrast, the lag phase was drastically increased when BF and OF were added and, again, this antioxidant activity was related with their concentration. In fact, with the lowest concentration (5 mg/L) of both BF and OF a 5-fold increase in the lag phase of the emulsions at pH 7.0 was achieved, and 10 days of lag phase were reached with BF at 10 mg/L and 15 mg/L and with OF at 10 mg/L, reaching 14 days with the remaining samples (*Figure 3.5a* and *3.5c*). In terms of the maximum concentration observed, the acidic environment favored better oxidative stability than the neutral one, and this concerned all the samples, including the controls, with values up to 282.10 ± 17.30 mmol/kg oil and 500.00 ± 47.29 mmol/kg oil at pH 7.0 in BF- and OF- experiments (*Figure 3.5a* and *3.5c*), respectively, and 178.89 ± 23.34 mmol/kg oil and 246.68 ± 25.85 mmol/kg oil at pH 3.5 (*Figure 3.5b* and *3.5d*). In general, despite the more successful results obtained under acidic conditions, considerably lower levels of hydroperoxides accumulation were found in all the samples added with both lipophenols with respect to the controls (more than 100 times lower).

Similar results were found also for hexanal and nonanal (*Figure 3.6* and *3.7*). The control showed their presence at both pH values from the very beginning of the experiment, and their content rose steeply after only 1 day. On the contrary, when lipophenols were added to the emulsions, the formation of the two aldehydes was significantly delayed in relation to the concentration of the two antioxidants, even to the point of being impeded at the highest concentrations of BF and OF. At pH 7.0, the addition of both BF and OF at 5 mg/L was already able to slow down the decomposition of the hydroperoxides, with the hexanal concentration rising steeply only from day 4 and day 10 for OF and BF, respectively (*Figure 3.6a* and *3.6c*). Nonanal behaved similarly, but with even longer delays in its formation. The greatest inhibition was attained by the two highest concentrations of BF and OF (20 and 25 mg/L), where hexanal and nonanal were found to be extremely low and, in some cases, not present at all for the entire experiment (*Figure 3.7a* and *3.7c*). The acidic environment again promoted greater oxidative stability in all the samples including the controls (*Figure 3.6b* and *3.6d*, *Figure 3.7b* and *3.7d*), where less than half of the values observed in the experiments conducted at

pH 7.0 were found. When lipophenols were added to the emulsions, at pH 3.5 only the 5 mg/L concentrations displayed a slight increase in hexanal and nonanal contents, but with values that were still markedly lower than in the neutral environment.

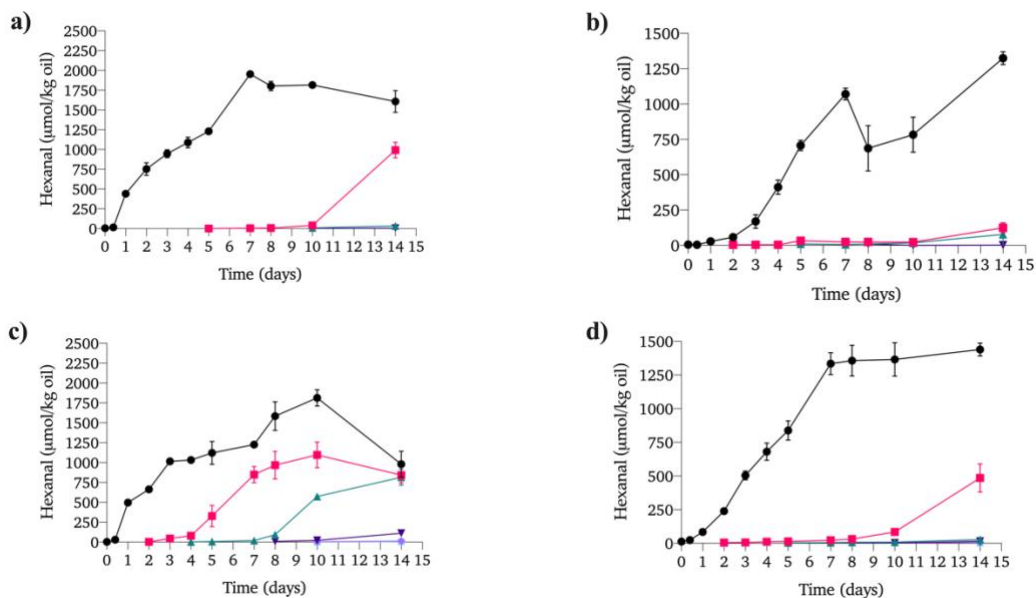


Figure 3.6. Hexanal content ($\mu\text{mol}/\text{kg oil}$) of 1.0% O/W emulsions without (control) (\bullet) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (\blacksquare), 10 mg/L (\blacktriangle), 15 mg/L (\blacktriangledown), 20 mg/L (\blacklozenge), and 25 mg/L ($*$) throughout the 14 days of storage. **a)** BF at pH 7.0; **b)** BF at pH 3.5; **c)** OF at pH 7.0; **d)** OF at pH 3.5. Each data point represents the mean \pm standard deviation of three independent replicates ($n=3$). Some error bars lie within the data points.

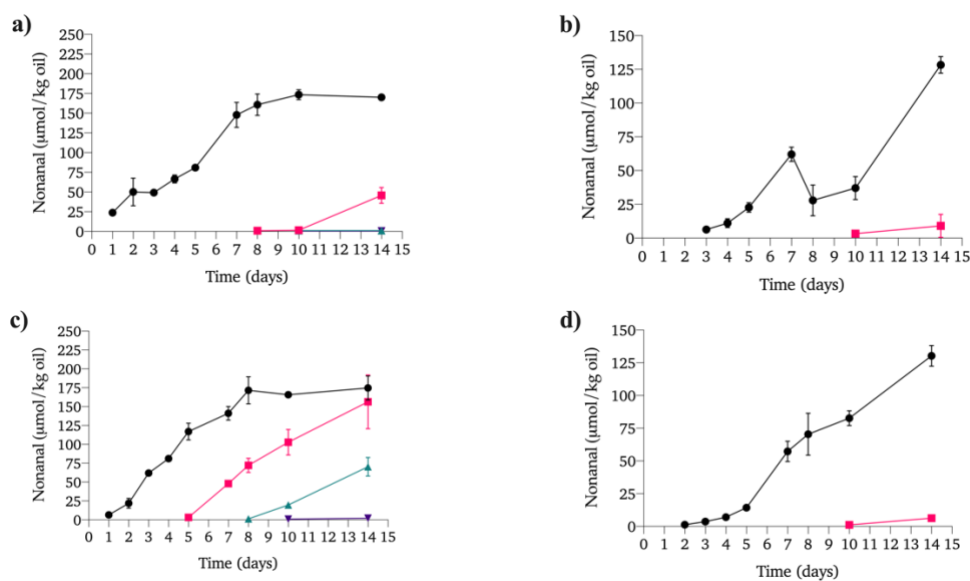


Figure 3.7. Nonanal content ($\mu\text{mol}/\text{kg oil}$) of 1.0% O/W emulsions without (control) (\bullet) and with butyl ferulate (BF) and octyl ferulate (OF) at 5 mg/L (\blacksquare), 10 mg/L (\blacktriangle), 15 mg/L (\blacktriangledown), 20 mg/L (\blacklozenge), and 25 mg/L ($*$) throughout the 14 days of storage. **a)** BF at pH 7.0; **b)** BF at pH 3.5; **c)** OF at pH

7.0; **d**) OF at pH 3.5. Each data point represents the mean \pm standard deviation of three independent replicates ($n=3$). Some error bars lie within the data points.

3.5 Discussion

The aim of this work was to investigate whether the use of two alkyl ferulates could be a worthwhile solution to enhance the oxidative stability of oil-in-water (O/W) emulsions, exploiting the amphipathic nature of butyl ferulate (BF) and octyl ferulate (OF) that would allow their partitioning at the oil-water interface. Moreover, being surface active, they could also affect the chemical and physical properties of the interfacial region, altering the electrostatic repulsion forces between the droplets if they possess a charge, and lowering their interfacial tension, thus impacting the physical stability of the emulsion among other things (Homma, Suzuki, Cui, McClements, & Decker, 2015; McClements & Decker, 2018).

Ultrasounds-assisted sonication successfully resulted in emulsions with a sufficiently small droplet size. Small particles in emulsions are of great scientific interest because significantly more kinetically stable over time, and the bioactive molecules contained in the system are more soluble and with better controlled/prolonged release, digestibility, and functionality in the upper gastrointestinal tract (Amiri-Rigi, Kesavan Pillai, & Naushad Emmambux, 2023). During the experiment, no physical destabilization was observed in any emulsion at both pH evaluated, meaning that all the samples were stable against coalescence, flocculation, or creaming. However, it is interesting to observe how differences were never noted within each day by increasing the concentration of both lipophenols, except for the last day of both BF and OF experiments under neutral conditions, where larger particles were observed as the percentage of lipophenols decreased. McClements & Decker (2018) explained how certain amphiphilic antioxidants with an appreciable amount of nonpolar groups, that allow direct adsorption at the oil–water interface due to the hydrophobic effect, can act as co-emulsifiers, partially replacing the original emulsifier and thus improving the physical stability of emulsions. However, a precondition for this to occur is that the

antioxidant is present in the emulsion in a sufficient concentration relative to the emulsifier. In the present study, it is plausible that the lipophenols located themselves in the interfacial region and filled the available gaps between the polysorbate molecules or placed immediately behind them, helping to lower, if only slightly, the interfacial tension of the droplets and thus to maintain greater stability over time when their concentration is $> 0.5\%$ relative to the emulsifier. Indeed, in the absence of the lipophenols an increasing trend in size over time was quite clear and became statistically different from the other samples at day 14 in both BF- and OF- experiments. In addition, Sørensen et al. (2012a) reported that Tween 20 favors the solubility of some phenolic compounds in the lipid phase of emulsions, contributing to determine their different partitioning into the system. On the other hand, at pH 3.5 no significant differences between samples and no increasing trends were ever noted, and therefore it is assumed that both lipophenols did not affect the interfacial tension of the droplets under acidic conditions. Further studies are necessary to understand why lipophenols affect surface tension depending on pH.

On the other hand, small particle sizes can worsen the oxidative stability by increasing the surface area of the oil droplets exposed to prooxidants-containing water (Jacobsen et al., 2000). Additionally, oxidized lipid species such as hydroperoxides, once are formed, being more polar due to the presence of the oxygen molecule, tend to migrate at the interface, where will encounter a prooxidant agent, causing it to decompose (Laguerre et al., 2017). This decay will produce highly reactive peroxy and alkoxy radicals, which will then spread the oxidation reactions by snatching an electron from a nearby unsaturated lipid. In other words, lipid oxidation in O/W emulsions is not only a matter of chemical reactions, but also of the physical arrangement of the molecules in the overall system and of the speed at which they move (Laguerre et al., 2017). In our study, however, smaller droplets size showed greater oxidative stability. In fact, regarding the emulsions at pH 7.0, when added with both BF and OF, even if they showed smaller particle size compared to the control, they simultaneously exhibited an unequivocally greater oxidative stability. This could be explained by the ability of these two antioxidants to properly partition at the site of oxidation reactions, which likely

occurred at both pH values. Regarding the concentrations, 5 mg/L of BF and OF was already able to increase the oxidative stability of the system, especially at low pH value. In fact, the elongation of the hydroperoxides lag phase was remarkable for both lipophenols in all four experiments compared with the controls, and this would represent an advantage for the food industry, as a very small amount of these antioxidants would suffice to greatly improve the shelf life of emulsion-based food products. Since secondary oxidation products are formed from primary products, it is not surprising to observe a similar behavior in terms of oxidative stability for hexanal and nonanal, the formation of which coincided with the lag phase of the hydroperoxides. In other words, in the control and in all emulsions at pH 7.0, an increased presence of both the aldehydes was observed, reflecting the higher propagation of the hydroperoxides. Contrarily, where lower levels of hydroperoxides were found, lower levels of aldehydes were also detected. Our findings are in line with the actual literature, which shows that other lipophilized phenolic compounds also performed satisfactorily in terms of antioxidant activity in emulsified systems. Methyl and butyl ferulate were found to be highly efficient in fish oil enriched milk; on the other hand, octyl ferulate acted as prooxidant in the same system (Sørensen et al., 2015). This highlights how different systems lead to obtaining different results, thus requiring the evaluation of antioxidants under different conditions. Lipophilization of dihydrocaffeic acid led to excellent results in fish oil-in-water emulsions (Sørensen et al., 2012a). Panya and colleagues (2012) found that C4 and C8 rosmarinate alkyl esters were the most effective in inhibiting the formation of hydroperoxides and hexanal. Finally, lipophilized gallic acid and hydroxytyrosol were able to significantly improve the oxidative stability of olive oil-in-water emulsions (Almeida et al., 2016; Losada Barreiro, Bravo-Díaz, Paiva-Martins, & Romsted, 2013). However, BF and OF proved to be more potent than other lipophilized antioxidants reported in literature and compared to the antioxidants normally used by the industries. In fact, the hydroperoxide values found in emulsions with BF and OF were lower than in O/W emulsions prepared with erythorbyl fatty acid esters, derivatives of lipophilized ascorbic acid (Kim, Yu, Yang, Choi, & Chang, 2023). Huang et al. (1996) found that emulsions added with 1.16 mmol/L α -tocopherol reached in 4 days a hydroperoxides

content of ~100 mmol/kg oil at pH 3, and ~150 mmol/kg oil at pH 7. Similar results were also found with 1.16 mmol/L Trolox-added emulsions, with ~30 mmol/kg oil at pH 3 and >150 mmol/kg oil at pH 7. In the present study, BF and OF at 5 mg/L never exceeded 5 mmol/kg oil and 10 mmol/kg oil, respectively, at both pH values until day 4. Again, hydroperoxides found in the present work were also lower compared to O/W emulsions added with similar concentrations of α - and δ -tocopherol and BHT stored at 37 °C (Chaiyasit, McClements, & Decker, 2005). The strength of BF and OF, though, lies in their exceptional antioxidant efficacy in this type of system. In fact, the concentrations used in this study were in the range of 0.02 mmol/L – 0.1 mmol/L (on the total emulsion volume) and, considering that the typical concentration of an antioxidant is 0.2 mmol/L (McClements & Decker, 2000), BF and OF could be of great interest to the food industry. Indeed, since 20 mg/L of BF and OF (corresponding to 0.08 mmol/L) already drastically prevented the formation of hydroperoxides and volatile compounds, they would enable industries to tremendously reduce the amount of antioxidant to be added to food.

The *in vitro* antioxidant assays revealed that the great oxidative stability given by the presence of BF and OF is due to their ability to neutralize free radicals rather than their ability to chelate transition metals. This is actually not surprising, as it is well known that the ability to chelate iron is given by the presence of a catechol or galloyl group able to form iron-phenolic complexes (Sørensen et al., 2014), which are not present in the molecular structure of ferulic acid. In contrast, it has a hydroxyl group capable of yielding an electron to a free radical. It is therefore plausible that, by positioning themselves at or close to the interface, BF and OF acted as scavengers of the newly formed radicals and prevented the formation of hydroperoxides. BF proved to be slightly more effective than OF in scavenging the DPPH \cdot . Sørensen and colleagues (2014) noted that C4-esterified ferulic acid had the same ability to neutralize DPPH \cdot as C8-esterified ferulic acid, but a slightly higher reducing power. Similar results were found with quercetin, which displayed the highest antiradical activity when esterified with short-chain fatty acids (Oh, Ambigaipalan, & Shahidi, 2019), while in other studies different outcomes were achieved, reporting equal or lower activity than octyl ferulate

(Oh & Shahidi, 2017; Viskupicova, Danihelova, Onderjovic, Liptaj, & Sturdik, 2010). More in-depth analyses are needed to understand why BF is more powerful than OF in this essay. We hypothesize that it is a matter of a greater spatial impediment of OF compared to BF that leads to the lower effectiveness against the radical. In fact, side chains on the aromatic ring of a phenolic compound slow down the reaction with DPPH due to steric hindrance that makes phenol access to the DPPH radical site difficult (Schaich, Tian, & Xie, 2015).

The high antioxidant efficiency of both esters was confirmed by the changes in the droplet surface charge over time. As oxidation proceeded, compounds generated by lipid degradation with pK_a below the operating pH reached the interface, making the surface more negative. Therefore, in the control, where numerous oxidation products were produced, the zeta-potential became drastically more negative as the storage progressed. In contrast, lipophenols led to a steadier surface charge trend over time, probably due to their capability of hampering the oxidation reactions. Additionally, despite being non-ionic, Tween 20 still exhibited a negative zeta-potential, and this can probably be explained by the presence of free fatty acid impurities in Tween 20 itself (Waraho, Cardenia, Rodriguez-Estrada, McClements, & Decker, 2009). The higher negative charge of the droplets at pH 7.0 compared to those at pH 3.5 explains the lower oxidative stability under neutral conditions. Low pH values, despite making iron more soluble in water, promote protonation of the particles, thus repelling the transition metals with the same charge (Li et al., 2023; McClements & Decker, 2000). Conversely, a strong negative charge attracts cations, which can then trigger oxidation reactions. Thus, once again BF and OF prove to be of particular interest to the food industry, since the charge of the droplets partly controls the rate of lipid oxidation in O/W emulsions, as has been widely demonstrated (McClements & Decker, 2000). In this study, pH played a role in the antioxidant performances of the two alkylferulates. It has already been observed that acidic conditions somewhat slow down oxidation reactions, reducing the formation of hydroperoxides and related volatile compounds. For instance, α -tocopherol, myricetin, and taxifolin have been reported to be more effective in O/W emulsions at pH 3.0 and 4.0 than at pH 7.0 (Bayram et al., 2023; Huang et al., 1996). Besides the issue of the droplet

charge, another explanation could be related to antioxidants' redox potential, which varies according to the pH of the system (Jovanovic et al., 1996). The antiradical activity is consequently reliant on the pH of the surrounding environment since the capacity to quench free radicals by transferring an electron or a hydrogen atom depends on the redox potential of the molecule. Furthermore, two other hypotheses include the different partitioning of BF and OF in the system and degradation rate. When pH is lowered, antioxidants can move in the system getting closer to the oxidation sites, with a consequent slower degradation (Huang et al., 1996). Other studies are needed to investigate these hypotheses.

3.6 Conclusions

Physically and oxidatively stable O/W emulsions can be accomplished by using ferulic acid lipophilized with 4 and 8 carbon atoms alkyl chains in both neutral and acidic conditions. Oxidative stability was promoted more at pH 3.5 than at pH 7.0 when the lipophenols were added to the emulsions, significantly limiting the formation of hydroperoxides and their decomposition into hexanal and nonanal. Although the antioxidant capacity was related to the concentration of BF and OF, even their lowest levels led to a significant increase in the emulsions' resistance to oxidation. Their antioxidant activity proved to be due to their ability to scavenge free radicals, while they were unable to chelate ferrous ions. These results suggest that BF and OF could be used, even in small concentrations, as efficient antioxidants in the formulation of emulsion-based foods to successfully preserve bioactive lipids such as polyunsaturated fatty acids and thereby decreasing the presence and intake of harmful compounds originating from oxidation reactions. However, further research is needed to investigate their behavior in real food systems, which are more complex matrices that could influence their antioxidant activity.

3.7 References

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Chapter 4

ANTIOXIDANT PROPERTIES OF ALKYLRESORCINOLS ISOLATED FROM WHEAT BRAN IN OIL-IN-WATER EMULSIONS

4.1 Abstract

Alkylresorcinols (ARs), a homologue series of phenolipids naturally occurring in many by-products, can meet the high demand of the food industry for natural compounds able to counteract lipid oxidation. In this study, ARs (C₁₇–C₂₅) were isolated from wheat bran, and their antioxidant activity was tested in oil-in-water emulsions at pH 3.5 and pH 7.0 at two different concentrations (0.15% and 0.30%; *w/w_{oil}*) during 14 days of storage at 35 °C. Results revealed that lipid oxidation was affected by both ARs concentration and pH of the emulsion. In fact, when ARs were added, a considerable suppression of hydroperoxides and aldehydes (hexanal and nonanal) formation with a consequent extension of their lag phases (5-fold) was observed at pH 3.5 while at a neutral pH, the lag phases were doubled. No influence of ARs on particle size was found. The present work demonstrated how ARs can represent sustainable and innovative natural antioxidants for emulsion-based food products.

4.2 Introduction

As a major cause of food quality deterioration, lipid oxidation is the foremost target on which researchers' efforts are focused. Lipid oxidation is governed by numerous chemical reactions triggered by factors like air, heat, and transition metals, leading to the formation of compounds such as hydroperoxides, aldehydes, and ketones that deteriorate the nutritional and sensory properties of foods and above all are harmful to human health. These reactions happen extensively in the presence of polyunsaturated fatty acids (PUFAs) which, on the other hand, are increasingly attracting consumers -and consequently food industries- for their benefits on human health. Besides, a vast number of foods consist of emulsified systems, where further challenge in the control of lipid oxidation are required, since the presence of water accelerate the oxidative reactions, leading to a more rapid degradation of lipids (Bravo-Díaz, 2022; Laguerre et al., 2017). The latter is especially true for oil-in-water (O/W) emulsions, where the contact area between the dispersed oil droplets and the dispersant water is very large. The interfacial region that separates the aqueous phase from the oil phase and the surfactant indeed represents the crucial site where oxidation reactions take place, as the prooxidants contained in the water (transition metals, photosensitizer, and enzymes) can easily interact with lipids (Bravo-Díaz, 2022).

Hence, new strategies to counteract lipid oxidation are constantly being sought, and one of them is the addition of antioxidants, which quickly react with pro-oxidants through several mechanisms such as radical scavenging, chelating transition metals, or quenching singlet oxygen (Choe & Min, 2009). However, antioxidants often show scarce efficacy in emulsified systems because of their poor solubility in lipids, which causes them to partition into the aqueous phase and thus away from the site of reactions (McClements & Decker, 2018). Additionally, consumer demand for natural antioxidants to replace synthetic ones is constantly growing, being not always an easy task for food industries.

Wheat bran represents 10–14% of the grain and the main co-product produced during the dry-milling of wheat. Every year 150 million tons of wheat bran are produced, most of which is used for

animal feed (Chen et al., 2023). However, bran contains many nutritionally interesting phytochemicals such as β -glucans, vitamins, proteins, polyphenols, lipids, and alkyresorcinols (Cardenia et al., 2018; Chen et al., 2023). Alkylresorcinols (ARs) are a homologue series of phenolipids naturally present in different cereals such as wheat, rye, and barley, consisting of a phenolic structure (resorcinol) and a hydrophobic chain of different lengths at position 5 of the phenolic ring. In wheat bran, ARs are present about $\sim 0.3\%$, *w/w*) with an odd-numbered chain length from 13 to 27 carbon atoms, in both saturated and unsaturated form (Chen et al., 2023; Elder et al., 2021; Esche et al., 2012). Although the total ARs content often varies significantly, the distribution of the homologues is relatively stable within species, with a C17:0/C21:0 ratio equal to ~ 0.01 for durum wheat, ~ 0.1 for common and spelt wheat, and ~ 1 for rye (Landberg et al., 2014). Since ARs are mainly present in the bran part of the kernels, they are, also, considered a biomarkers of whole grain wheat and rye foods and intake (Zabolotneva et al., 2022). Many beneficial biological properties have been reported for ARs, such as antioxidant, antimicrobial, anticancer, antilipidemic and neuroprotective, being also proposed as promising bioregulators of metabolic and immune processes and potential positively regulators of gut microbiome (Zabolotneva et al., 2022). Their antioxidant capacity is due to the presence of two hydroxyl groups on the benzene ring, that can donate protons/electrons to radical molecules, such as lipid radicals. For this reason, ARs can be suggested as natural compounds to prevent lipid oxidation in food products, especially those characterized by emulsified systems, thanks to their amphiphilic structure. While there are numerous papers on the *in vitro* antioxidant activity of ARs, to the best of our knowledge very few studies have been done on the antioxidant activity of ARs in model systems (especially as related to different pH values) and in real foods. Recently, Elder and colleagues (Elder et al., 2021) studied the antioxidant abilities of individual AR homologues (chain length C17:0, C19:0, C21:0, C23:0, C25:0) extracted from rye bran in bulk oil and O/W emulsion, demonstrating that in their presence the formation of primary and secondary oxidation products was delayed. Moreover, AR individual homologues were also studied in low-moisture crackers, where they succeeded to considerably extend their shelf life (Elder et al.,

2022). A winterized, acetonetic rye bran extract of ARs was also tested in O/W emulsions at pH 7, achieving a significant increase in the lag phase of oxidation products (Elder et al., 2019). The use of the entire fraction of ARs naturally present in plants instead of isolating them individually would be a great advantage for companies in both economic and environmental sustainability. Furthermore, an additive effect of individual ARs in terms of antioxidant activity can occur when they are present together within the same system.

Hence, the aim of the present work is to evaluate the antioxidant activity of the ARs naturally present in wheat bran, in 1.0% O/W emulsions. Furthermore, since the antioxidant activity of the molecules varies with, among others, their concentration, and the pH of the environment, ARs were tested in emulsions at two different concentrations (0.15% and 0.30% on the weight of the oil) and pH values.

4.3 Materials and Methods

4.3.1 Chemicals and materials

All solvents and reagents were of analytical grade. Methanol and methyl tert-butyl ether (MTBE) were purchased from VWR (Milan, Italy), *n*-hexane, diethyl ether, ethyl acetate, propan-2-ol, ethanol absolute anhydrous, iso-octane, 1-butanol, and hydrochloridric acid (37%) were obtained from Carlo Erba (Milan, Italy). Anhydrous pyridine, N,O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA:TMCS, 99:1, v/v), 5 α -cholestan-3 β -ol, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA), silicic acid (100–200 mesh, 75–150 μ m), activated charcoal, Tween® 20 (polyethylene glycol sorbitan monolaurate), sodium phosphate monobasic dihydrate, sodium phosphate dibasic anhydrous, ferrous sulfate heptahydrate, barium chloride dihydrate, ammonium thiocyanate, and cumene hydroperoxide were purchased from Merck (Darmstadt, Germany). Milli-Q filter system (Millipore, Milan, Italy) was used to prepare double distilled water. N° 1 filter papers were purchased

from Whatman (Maidstone, England). Solid-phase extraction (SPE) cartridges (Strata NH₂, 55 µm, 70 Å, 1 g/6 mL; Strata SI-1 Silica, 55 µm, 70 Å, 500 mg/3 mL) were provided by Phenomenex (Torrence, CA, USA). Wheat bran was kindly provided by Molini Bongiovanni S.p.A (Cambiano, Italy).

4.3.2 Isolation of alkylresorcinols (ARs) from wheat bran

4.3.2.1 Lipid extraction from wheat bran

ARs were extracted from wheat bran and purified according to Esche et al. (Esche et al., 2012), with slight modifications described as follows. Wheat bran was freeze-dried (Lio 5P, 5 Pascal, Italy) and immediately ground and sieved at 500 µm. Ten grams of the obtained bran were added with 40 mL of a *n*-hexane:chloroform solution (1:1; v/v) and stirred for 1 h in darkness at room temperature to extract the lipid fraction. The mixture was then filtered with a Buchner funnel with Whatman filter paper, and the solvent was removed under vacuum with a Rotavapor (R-210, Buchi, Flawil, Switzerland) at 37 °C. The obtained oil was dissolved in 10 mL of *n*-hexane.

4.3.2.2 Isolation of ARs from lipid fraction

ARs were isolated from the lipid fraction through solid-phase extraction (SPE) following the procedure described by Esche et al. (2012), with slight modifications. Briefly, an aliquot of lipid matter extracted from wheat bran (60 mg) was resuspended in 1 mL of *n*-hexane and loaded on a SPE NH₂ cartridge, previously activated with 10 mL di *n*-hexane. To remove the undesired compounds, different solvents were eluted in the following order: 10 mL *n*-hexane:diethyl ether (98:2; v/v), 20 mL of *n*-hexane:ethyl acetate (96:4; v/v), and 10 mL of *n*-hexane:ethyl acetate (5:95; v/v). ARs were finally eluted with 10 mL of *n*-hexane:ethyl acetate (5:95; v/v) and 5 mL of MTBE. The solvents were then removed with a rotary evaporator (Rotavapor, R-210, Buchi, Flawil, Switzerland) and residues dissolved in 3 mL of *n*-hexane:propan-2-ol (3:2; v/v) (Fraction 1). Since the obtained fraction still

contained other compounds beside ARs (e.g., alcohols, hydrocarbons, esters, phytosterols, fatty acids, diglycerides), the latter were purified through a second SPE. After evaporation of the *n*-hexane:propan-2-ol mixture with nitrogen flush, the residues were dissolved in 500 μ L of *n*-hexane:diethyl ether (80:20; *v/v*) and loaded onto a SPE SI-1 cartridge, previously activated with 3 mL of *n*-hexane. After eluting 5 mL of *n*-hexane:diethyl ether (8:2; *v/v*) and 4 mL of *n*-hexane:diethyl ether (1:1; *v/v*) and discarded both phases, purified ARs were eluted with 3 mL of methanol. Methanol was then evaporated and the ARs dissolved in *n*-hexane:propan-2-ol (3:2; *v/v*) (Fraction 2).

4.3.3 Characterization of ARs

4.3.3.1 Identification and quantification of ARs by GC/MS and GC/FID

The Fraction 2 was dried and ARs were silylated by adding 100 μ L of pyridine and 200 μ L of BSTFA:TMCS (99:1; *v/v*) and slowly stirring the mixture at 40 °C for 20 min in darkness. Solvents were then evaporated with nitrogen flow and 500 μ L of *n*-hexane were added. After centrifugation (3 min at 3,500 \times g), the upper phase was collected, dried, and resuspended in 100 μ L of *n*-hexane.

Identification of ARs was performed in a Shimadzu QP2010 Plus GC/MS (Shimadzu, Kyoto, Japan), using a RXi-5ms fused silica capillary column (10 m, i.d. 0.1 mm, 0.1 μ m film thickness; Restek, Bellefonte, PA) for the chromatographic separation. The AOC-5000 Pal autosampler (Shimadzu, Kyoto, Japan) was used to inject 1 μ L at 345 °C with a split ratio of 1:50. Helium was used as carrier gas with a constant linear velocity of 49.9 cm/s. The oven was programmed as follows: from 100 °C to 310 °C at 7 °C/min; from 310 °C to 320 °C at 1°C/min, from 320 °C to 345 °C at 7 °C/min. Final temperature was held for 14 min. The interface and ion source temperatures were set at 230 and 200 °C, respectively. Acquisition was performed in total ion current (TIC) with a mass range of 33–600 *m/z* and a scan velocity of 1166 amu/s. The NIST08s (National Institute of Standards and Technology, Gaithersburg, USA) library was used to identify the compounds comparing the mass spectra.

Quantification of ARs was achieved by injecting 1 μL of the sample in a GC/FID (GC-2010, Shimadzu, Kyoto, Japan) equipped with the same column of GC/MS and under the same analytical conditions. To quantify, 80 μL of a 5- α -cholestan-3- β -ol solution (1.005 mg/mL; internal standard) were added to ARs before silylation.

4.3.3.2 Determination of antioxidant properties of ARs

The antioxidant properties of ARs were tested in terms of radical scavenging activity (DPPH spectrophotometric assay) and metal-chelating capacity (ferrous ion-chelating spectrophotometric assay), according to the procedures described in Cantele et al. (Cantele et al., 2023). For these assays, the extract was dried and resuspended in methanol, and appropriate dilutions were applied. A BioTek Synergy HT spectrophotometric multi-detection 96-well microplate reader (BioTek Instruments, Milan, Italy) was used to read the absorbance. For quantification, calibration curves were built with Trolox (25–30 μM ; $y = 0.1941x + 0.0483$; $R^2 = 0.9999$) and EDTA (0.001–0.1 mg/mL; $y = 8546.5x - 0.4859$; $R^2 = 0.9947$) for radical scavenging activity and ferrous ion-chelating activity, respectively. Results were expressed as micromoles of Trolox equivalents per gram of sample ($\mu\text{mol TE/g}$) and milligrams of EDTA equivalents per gram of sample (mg EDTAE/g), for radical scavenging and metal chelating activities, respectively.

4.3.4 Stripping of the oil

The oil (mixture of soybean and sunflower seeds oil) was deprived of the polar compounds according to Boon et al. (Boon et al., 2008), using a chromatographic column of 3.0 cm diameter and 35.0 mm length). Three layers were prepared to pack the column: 22.5 g of silicic acid (previously washed with double distilled water three times), 5.6 g of activated charcoal, and again 22.5 g of washed silicic acid. Silicic acid and charcoal were dissolved, respectively, in 100 mL and 70 mL of *n*-hexane. Thirty grams of oil were dissolved in 30 mL of *n*-hexane and eluted with 270 mL of the

same solvent through the packed column. All the procedure was conducted under aluminum foils to avoid light and triacylglycerols were collected in an ice bath. Solvent was removed with rotary evaporator (Rotavapor, R-210, Buchi, Flawil, Switzerland) at 37 °C and the success of the stripping process was evaluated by injecting the obtained oil into GC/FID (Cardenia et al., 2018) and measuring the peroxide value (Shantha & Decker, 1994). The oil was stored at -20 °C until subsequent analyses.

4.3.5 Preparation of the oil-in-water (O/W) emulsions and storage conditions

According to Cardenia et al. (Cardenia et al., 2011), 1.0% of stripped oil, 0.1% Tween 20 (non-ionic emulsifier) and 10 mM phosphate buffer solution at pH 3.5 and pH 7.0 were used to prepare the O/W emulsions (percentages are given for the total weight of the emulsion; *w/w*). ARs were dissolved, after the removal of the solvent, into the oil at 0.15% (AR15) and 0.30% (AR30) on its total weight (*w/w*). Emulsions were prepared according to Cantele et al. (Cantele et al., 2023). Briefly, a coarse emulsion was firstly made using an IKA T25 digital Ultra-Turrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 2 min at maximum speed (20,000 rpm) and then was turned into a fine emulsion with 3 min of ultrasounds sonication at 100 W in ice bath to prevent temperature to increase above 30 °C. Control samples without the addition of ARs were also made for each pH value. One milliliter of each emulsion was transferred into 20 mL headspace vials, sealed with PTFE/silicone septa aluminum caps, and stored at 35 °C in darkness for 14 days. For each sample and each analysis, three separate experiments ($n=3$) were conducted.

4.3.6 Determination of particle size distribution and zeta-potential of the emulsions

Emulsions were diluted at a ratio of 1:50 (*v/v*) in the corresponding pH 7.0 or pH 3.5 10 mM buffer solution, and a Zetasizer Pro (Malvern Instruments, Worcestershire, UK) was used to evaluate the particle size distribution through dynamic light scattering (DLS) and droplet surface charge (zeta-potential). Three measurements were done for each independent experiment.

4.3.7 Determination of primary oxidation products

Lipid hydroperoxide content of the emulsions was measured as primary oxidation markers. The procedure, described by Cardenia et al. (Cardenia et al., 2011), included the addition of 0.3 mL of emulsion to 1.5 mL of iso-octane:propan-2-ol (3:1; v/v), vortex for 30 sec and centrifugation at 3,400 g for 3 min. After that, 0.2 mL of the upper phase were dissolved in 2.8 mL of a 2:1 (v/v) solution of methanol and butanol. Fifteen microliters of ammonium thiocyanate (30%; w/v) and 15 μ L of FeCl₂ were added to the mixture, which was vortexed and incubated at room temperature for 20 min in the dark. Absorbance was read at 510 nm. FeCl₂ was prepared by reacting 0.132 M BaCl₂ and 0.144 M FeSO₄. A cumene hydroperoxide standard calibration curve in the range of 10-300 μ M was used to perform quantification and results were expressed as mmol/kg oil.

4.3.8 Determination of secondary oxidation products

Hexanal and nonanal content were determined as markers of the secondary oxidation reactions by headspace solid-phase microextraction gas chromatography coupled with mass spectrometry (HS-SPME-GC/MS) using a Shimadzu QP2010 Plus GC/MS (Shimadzu, Kyoto, Japan). Vials were conditioned at 40 °C for 10 min to reach the equilibrium of the volatile compounds in the headspace. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated fiber (df 50/30 μ m; 1 cm; Supelco, Bellefonte, PA, USA) was then exposed in the headspace of the vial for 5 min while maintaining the same temperature. To perform the chromatographic separation, the fiber was subsequently desorbed for 3 min in split mode (1:50) using helium as the carrier gas (34.8 m/s linear velocity) in an RTX-5 fused silica capillary column (20 m, i.d. 0.10 mm, film thickness 0.10 μ m; Restek, Bellefonte, PA). Column oven was programmed as 40 °C for 1 minute, then up to 100 °C (5 °C/min), and up to 240 °C (30 °C/min). Final temperature was held for 1 min. Injector, transfer line, and ion source temperatures were, respectively, 260 °C, 230 °C, and 200 °C. Ions were acquired between 33 and 350 *m/z* at a scan speed of 1666 amu/sec. The NIST08s library of compounds mass spectra was used to identify hexanal and nonanal. The external standard method was used to quantify

the two aldehydes with calibration curves of the corresponding pure standard (hexanal, 0.212–750 $\mu\text{mol/kg}$ oil, $y = 43.98x + 5100$; nonanal, 0.146–350 $\mu\text{mol/kg}$ oil, $y = 49.67x + 3215$). Results were expressed as $\mu\text{mol/kg}$ oil.

4.3.9 Statistical analysis

To investigate the impact of wheat bran ARs on the physical and oxidative stabilities of the emulsions, one-way analysis of variance (one-way ANOVA) and Tukey's test was used to determine any statistical difference between the samples at a confidence level of 95%. One-tailed Dunnett's *post hoc* test was used to calculate the hydroperoxides, hexanal, and nonanal lag phases, intended as the first point statistically different from day 0 ($p < 0.05$). IBM SPSS statistical software (version 28; IBM, Chicago, IL, USA) was used.

4.4 Results and discussion

4.4.1 Characterization of ARs extract

GC/MS was used to analyze the wheat bran purified extract, which revealed to contain fifteen different alkyresorcinol homologues. Specifically, five ARs with saturated chain (C17:0, C19:0, C21:0, C23:0, and C25:0), and ten ARs with one unsaturation in the chain (two positional isomers for each abovementioned homologue). Martín-García, Gómez-Caravaca, & Verardo (2021) found the same resorcinolic lipids composition in aleurone fractions of wheat bran. All ARs showed characteristic ions 268 m/z and 281 m/z resulting from McLafferty rearrangement, which were then used as qualifier ions. Molecular ions at m/z 492, 520, 548, 576, and 604 were also used to identify C17:0, C19:0, C21:0, C23:0, and C25:0, respectively (silylated molecules). Molecular ions at m/z 490, 518, 546, 574, and 602 were identified as the corresponding silylated monounsaturated ARs. The spectra of the compounds also found confirmation in the literature (Martín-García et al., 2021). Unsaturated ARs were present in small concentrations ($< 10\%$ of the total ARs) with respect to the

saturated ones. The composition of the homologues (saturated and monounsaturated) was the following: C₁₇, 7.5%; C₁₉, 41.9%; C₂₁, 40.8%; C₂₃, 7.2%; C₂₅, 2.6%, as already observed by other researchers (Andersson et al., 2008). Ratio between C_{17:0} and C_{21:0} was 0.18, in agreement with what has been reported in the literature for common wheat (Landberg et al., 2014). An extract purity of 85% was achieved. Contaminants included 1-monopalmitine, glycerol monostearate, palmitic acid, and stearic acid. The SPE extraction yield was 2.34% ± 0.40.

Several *in vitro* assays widely used to test the antioxidant properties of molecules, reflecting the many ways in which oxidation reactions are triggered. The main initiators of oxidation in lipids, however, are free radicals and bivalent cations such as iron and copper. Hence, in this study the antioxidant properties of ARs were assessed in terms of radical scavenging activity and ferrous ion-chelating activity. A concentration of 1.7 mM of the ARs purified extract was able to effectively inhibit 41.08% of DPPH, resulting in a radical scavenging activity equal to 325.72 ± 5.50 μmol TE/g. This result is comparable or even higher than those reported for individual and mixture of ARs standard (19.2–32.5% of DPPH inhibition respectively) and for wheat bran extracts rich in ARs from different cultivars and regions (Gunenc et al., 2013). On the other hand, the ferrous ions-chelating ability was quantified in 3.35 ± 0.11 mg EDTAE/g (3.4 mM of the extract inhibited 37.32% of the total ions present in the solution). The observed chelating activity was unexpected, since the phenolic part of the ARs does not contain catechol or galloyl groups, but rather just a resorcinol group. The absence of two contiguous hydroxyl groups, in fact, would not allow the iron to replace their two hydrogen atoms. However, the same situation was reported by other authors for ferulic acid and its derivatives, suggesting that there are other types of interactions with iron (Sørensen et al., 2014). Other authors (Hładyszowski et al., 1998) highlighted how resorcinol can prevent liposome Fe²⁺-induced peroxidation, with an IC₅₀ (i.e., the concentration at which inhibition of peroxidation was 50%) of 40 μM, and how this increased markedly in the presence of an alkyl chain on the resorcinol ring, with a considerable decrease in the IC₅₀ from orcinol (1 carbon atom; 35 μM), to olivetol (5 carbon atoms; 19 μM), and finally to pentadecylresorcinol (15 carbon atoms; 14 μM). More recently,

Elder and colleagues (Elder et al., 2019, 2021) observed excellent antiradical activity both in an acetonic rye bran extract containing ARs and when the AR homologs were tested individually.

However, *in vitro* assays for antioxidant activity do not reflect the behavior of the same molecules in more complex systems, being poor predictor in real food products. In fact, the reactions that take place between molecules depend not only on their structures but also on the physico-chemical phenomena occurring in their surroundings. Before hypothesizing the use of new antioxidants in the food industry, their behavior in different environments should be studied first, in order to predict their efficacy in different foods. For this reason, the ARs purified from wheat bran have been tested in O/W emulsions at two different pH values.

4.4.2 Influence of ARs on the physical stability of the emulsions

Droplets dimension was investigated to monitor the physical instability processes that can occur during the storage of emulsified systems. These phenomena include flocculation, coalescence, and creaming, and involve, albeit in different ways, the aggregation of oil particles leading to the separation of the discontinuous phase from the continuous ones and thus loss of stability.

Results of particle sizes are reported in **Figure 4.1**. In general, slightly larger particle sizes were noted in the neutral environment than in the acidic environment. In fact, at day 0 a range of $254.90 \pm 38.69 - 290.37 \pm 34.96$ nm was measured for the emulsions at pH 7.0, while at pH 3.5 dimensions were between $204.47 \pm 17.34 - 218.47 \pm 17.33$ nm. Under the conditions tested in this study, ARs had no effect on the size of the oil droplets, either in relation to their concentration or the pH of the system. In fact, no significant differences with respect to the control were observed throughout the experiment ($p > 0.05$). Thus, we hypothesize that ARs probably did not contribute to decrease the interfacial tension of the droplets. Nevertheless, sufficiently small particles were obtained to ensure kinetically stable emulsions, as no physical instability occurred during the 14 days of storage, evidenced by the constant particle size ($p > 0.05$) and unchanged visual appearance.

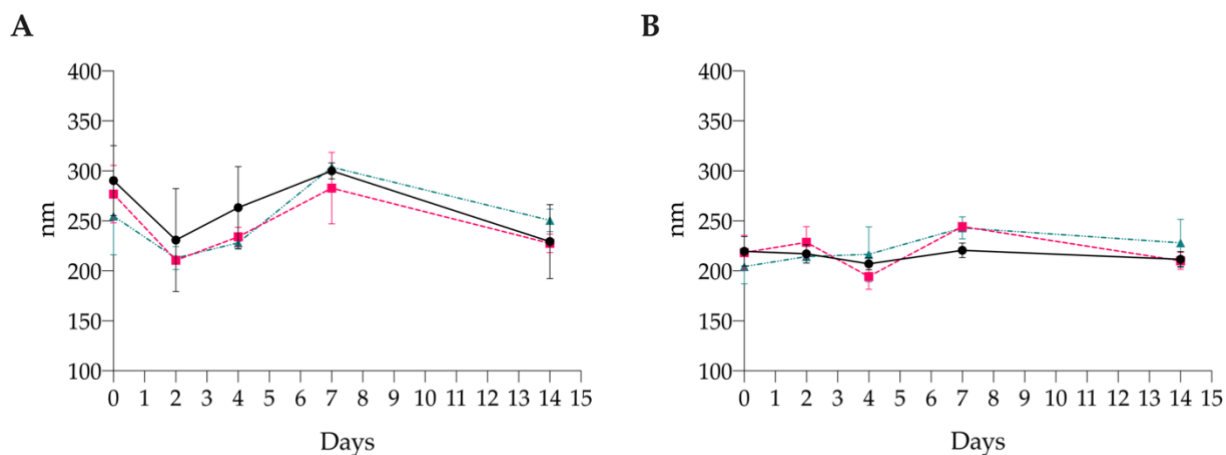


Figure 4.1. Particle size of the O/W emulsions during 14 days of storage at pH 7.0 (A) and pH 3.5 (B): (●) control, (■) with 0.15% alkylresorcinols (AR15), (▲) with 0.30% alkylresorcinols (AR30). Each point represents the mean \pm standard deviation of three independent replicates. Different letters indicate significantly different means ($p < 0.05$) among the samples within the same day; where no letters are displayed, no significant differences were observed ($p > 0.05$).

Along with particle size of the oil droplets, it is also necessary to monitor the electrical charge displayed on the surface (ζ -potential). The latter, in fact, can determine the fate of an emulsion in terms of its physical stability. High values of charge, whether positive or negative, ensure strong repulsion between particles, thus averting their aggregation. Conversely, a weak charge, close to neutrality (0 mV), may not guarantee electrostatic repulsion, thereby leading to instability. In addition, ζ -potential can also affect the oxidative stability of emulsions by changing the rate of lipid oxidation.

The results of droplet surface charge are depicted in **Figure 4.2**. Since day 0, all the emulsions displayed negative zeta-potential values at both pH levels. Although, the Tween 20 used in this work is frequently regarded as a non-ionic emulsifier, observing a negative droplet surface charge was not unexpected since this phenomenon has previously been described by other researchers (Cardenia et al., 2011; Cui et al., 2016). Either free fatty acids in the system or contaminants in the Tween 20 could be the cause of this negative surface charge, or even the preferential adsorption by the oil droplets of OH^- ions (rather than H_3O^+) from water (Hur et al., 2009). However, pH determined a different trend in the surface charge of the emulsions. At pH 7.0, the droplets' negative charge at day

0 was -12.33 ± 1.39 mV (control), -10.28 ± 0.96 mV (AR15), and -8.79 ± 0.63 mV (AR30), which dramatically increased during the experiment, reaching at day 14 values equal to -50.62 ± 3.57 mV (control), -54.03 ± 0.88 mV (AR15), and -54.76 ± 1.62 (AR30). Moreover, no significant differences ($p > 0.05$) were ever noted between the control and AR15 or AR30 during the experiment. On the other side, at pH 3.5 the surface charge was still negative but closer to 0 mV and, although an increase in values was again observed during the experiment, this was not as drastic as in the case of the neutral environment. Indeed, the droplet surface charge of the control went from -2.15 ± 0.5 (day 0) to -5.88 ± 0.07 (day 14) mV, while for AR15 from -2.35 ± 0.22 (day 0) to -5.55 ± 0.55 (day 14) mV, and that of AR30 from -2.21 ± 0.25 (day 0) to -4.43 ± 0.37 (day 14) mV. No differences were noted among the samples except on the last day, where AR30 showed a lower negative charge value than the control and AR15 ($p < 0.05$). This difference in the electrical charge noted on day 14 could be due to the different rate of lipid oxidation, as will be described in section 4.4.5.

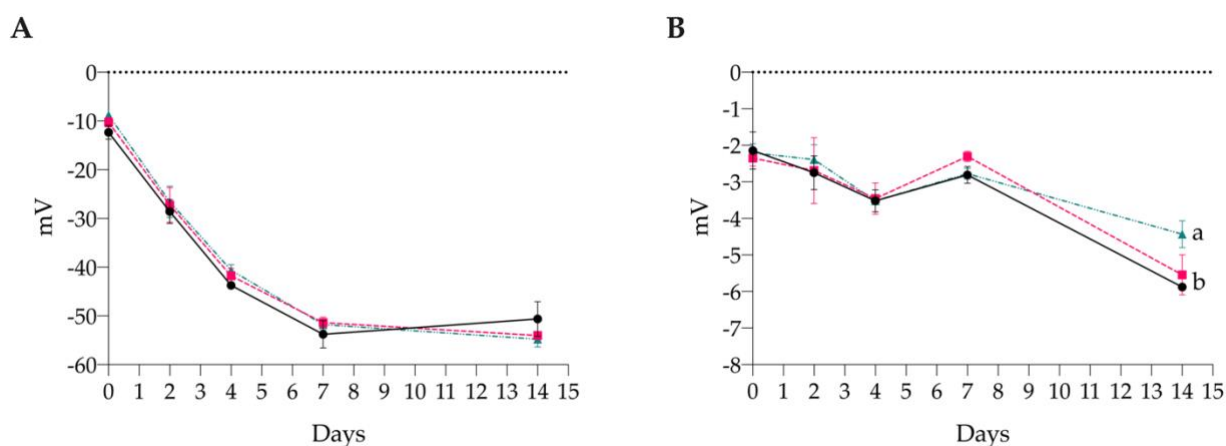


Figure 4.2. Droplet surface charge of the O/W emulsions during 14 days of storage at pH 7.0 (A) and pH 3.5 (B): (●) control, (■) with 0.15% alkylresorcinols (AR15), (▲) with 0.30% alkylresorcinols (AR30). Each point represents the mean \pm standard deviation of three independent replicates. Different letters indicate significantly different means ($p < 0.05$) among the samples within the same day; where no letters are displayed, no significant differences were observed ($p > 0.05$).

The reason why no differences in particle size or surface charge were observed could be due to the lipophilicity of the isolated alkylresorcinols. In fact, the latter possess an alkyl chain long enough to make them too hydrophobic to partition at the oil-water interface.

4.4.5 Influence of ARs on the oxidative stability of the emulsions

Emulsions were formulated with ARs extracted from wheat bran and stored at 35 °C for two weeks in the absence of light while measuring primary and secondary oxidation products, i.e., hydroperoxides, and hexanal and nonanal, respectively. The formation of these products was influenced by both the pH of the emulsion and the concentration of the phenolipids (*Figures 4.3, 4.4, 4.5*).

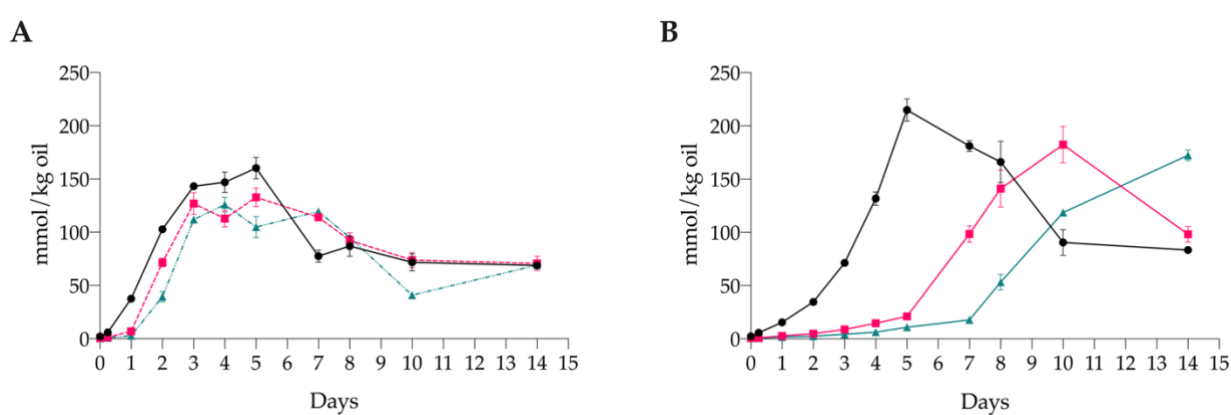


Figure 4.3. Hydroperoxides content in the O/W emulsions during 14 days of storage at pH 7.0 (A) and pH 3.5 (B): (●) control, (■) with 0.15% alkylresorcinols (AR15), (▲) with 0.30% alkylresorcinols (AR30). Each point represents the mean \pm standard deviation of three independent replicates.

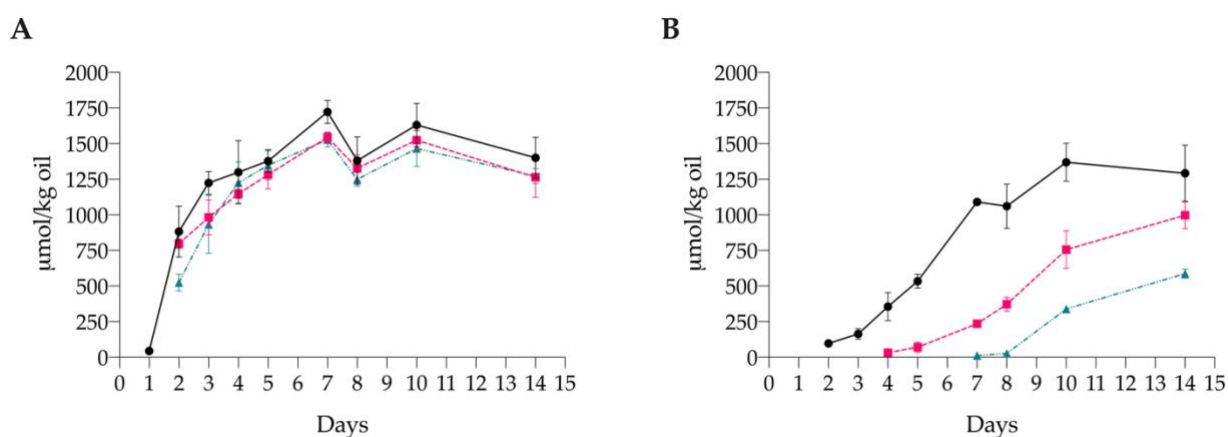


Figure 4.4. Hexanal content in the O/W emulsions during 14 days of storage at pH 7.0 (A) and pH 3.5 (B): (●) control, (■) with 0.15% alkylresorcinols (AR15), (▲) with 0.30% alkylresorcinols (AR30). Each point represents the mean \pm standard deviation of three independent replicates.

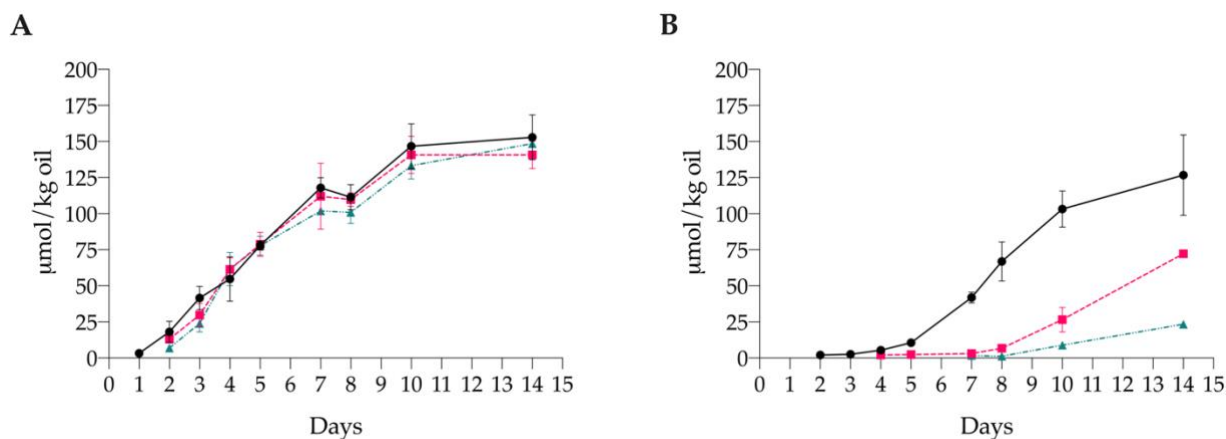


Figure 4.5. Nonanal content in the O/W emulsions during 14 days of storage at pH 7.0 (**A**) and pH 3.5 (**B**): (●) control, (■) with 0.15% alkylresorcinols (AR15), (▲) with 0.30% alkylresorcinols (AR30). Each point represents the mean \pm standard deviation of three independent replicates.

In general, the addition of ARs to the emulsions resulted in a significant delay in the lag phase of both hydroperoxides and aldehydes formation compared to the emulsions without phenolipids (controls). That behavior could be ascribed to the radical scavenging and metal chelating activities of ARs that was observed in the *in vitro* tests. However, the ARs were more effective under the acidic conditions rather than under the neutral one. In fact, the lag phase of hydroperoxides at pH 7.0 was extended from 4.5 hours (control) to 1 day when AR15 and AR30 were added, whereas that of hexanal was increased from 1 day to 2 days. As for nonanal, the lag phase remained the same (3 days), but its generation was delayed by 1 day. In fact, nonanal was found in the control after one day, while in the emulsions with AR15 and AR30 only from the second day of storage. At pH 3.5, the lag phase of hydroperoxides was extended from 1 to 4 and 5 days for AR15 and AR30, respectively. The hexanal lag phase was equal to 5 days for AR15 and 8 days for AR30, compared with the 3-day lag phase showed by the control. Moreover, the addition of the alkylresorcinols in the acid emulsions led to a significant delay in the formation of this aldehyde, being detected only at day 4 (AR15) and day 7 (AR30), compared to the control where it was observed immediately after 2 days. The same scenario was noted for nonanal as well, which first formed at day 2 in the control and then increased

significantly at day 6 (lag phase 5 days), while in AR15 and AR30 it developed similarly to hexanal after 4 and 7 days, respectively, with both a lag phase of 8 days.

In general, the acidic pH favored better oxidative stability of the emulsions, as evidenced by the longer lag phases. This has already been found in other studies (Bayram et al., 2023; Cantele et al., 2023). A first explanation might be found in the charge of the emulsifier, as it can have a major impact on the oxidative state of the system. In fact, although the oil droplets exhibited a negative surface charge either at pH 7.0 or pH 3.5, this was higher under neutral conditions. Strong negative charges accelerate lipid oxidation since they attract more easily the cations responsible for initiating the chain reactions. Among these, iron is one of the most crucial due to its strong prooxidant activity and high solubility and reactivity (Bayram et al., 2023). Transition metals pose a very serious problem for food shelf life. Due to their ubiquitous nature, it is extremely easy to find them in foods, coming from numerous sources such as food ingredients, water, food processing equipment, and packaging (Bayram et al., 2023; Waraho et al., 2009). They greatly speed up lipid oxidation by promoting the breakdown of lipid hydroperoxides into highly reactive alkoxy and peroxy radicals. These radicals can then spread oxidation by easily abstracting a hydrogen from fatty acids (Waraho et al., 2009). For this reason, researchers are always pursuing antioxidants, especially natural ones, that can reduce the catalytic effect of metal ions preventing lipid oxidation and block its propagation by quenching free radicals. In this work, as the experiment proceeded and lipid oxidation progressed, the negative surface charge increased, and this was particularly intense at pH 7.0. The increase in negative charge during oxidation experiments has already been observed several times, and is due to the partitioning of oxidation products, such as free fatty acids, at the interface. Here, if their pK_a value is lower than the pH of the system, some of their functional groups could be deprotonated, thus leading to the increase of the negative charge of the droplets and thus attracting more cationic metals by diffusing the oxidation reactions. Conversely, if the pH is lower than the pK_a of these products, the groups will be protonated, and the surface charge will remain steadier. The second reason why emulsions at pH 3.5 were more oxidatively stable is related to the antioxidant activity of the ARs. In fact, from the

results of the *in vitro* tests already discussed in Section 3.1, these phenolipids seem to be able to act both as metal chelators and free radical quenchers. At pH 7.0, although their efficacy was almost negligible, the ARs still slightly counteracted lipid oxidation. However, the ARs showed their actual efficacy at pH 3.5. The redox potential of antioxidants changes depending on the pH value of the system in which they are located (Jovanovic et al., 1996). Since the ability to quench free radicals by donating an electron or a hydrogen atom depends on the redox potential of the molecule, the antiradical activity is therefore also dependent on the pH of the surrounding environment. The different antioxidant activity of molecules depending on the working pH has already been observed many times. In our previous work (Cantele et al., 2023) the antioxidant activity of two synthetic alkylferulates (C4 and C8) was significantly higher in emulsions at pH 3.5 than in those at pH 7.0. Bayram et al. (Bayram et al., 2023) reached same conclusions where antioxidants such as α -tocopherol, myricetin, and taxifolin resulted in greater oxidative stability of O/W emulsions at acidic pH than at neutral pH. Huang and colleagues (Huang et al., 1996) found that the highest stability of O/W emulsions was obtained when α -tocopherol was added at pH 3.0, while the lowest when α -tocopherol was added at pH 7.0. However, the improved oxidative stability obtained at pH 3.5 could also be due to a different partitioning of ARs in the system and/or slower degradation of ARs. In fact, it has already been observed that by decreasing the pH, the antioxidants may change position by reaching the sites of oxidation reactions, and the rate of their depletion may be significantly decreased (Berton-Carabin & Villeneuve, 2023; Kiokias & Oreopoulou, 2022).

4.5 Conclusions

In this study, we examined the effect of ARs extracted from wheat bran, their concentration, and the pH of the system on the physical and oxidative stability of 1.0% oil-in-water emulsions. Indeed, considering their amphipathic nature that would allow them to position themselves near the sites of oxidation reactions, ARs could be good candidates for increasing the shelf life of emulsion-

based foods. However, it is also well known how both the concentration and the characteristics of the system in which they work are crucial for the molecules to perform their antioxidant activities. The pH value was the main key to the success of these antioxidants in counteracting lipid oxidation in the emulsions. Under neutral conditions, the ARs showed poor efficacy, while at pH 3.5 they were found to greatly extend the lag phases of both hydroperoxides and aldehydes. Moreover, by increasing their concentration in the system, the antioxidant effect was enhanced. Their efficacy appeared to be related to their ability to chelate transition metals such as iron, but more importantly to scavenge free radicals, reactions that in this case could therefore be favored by low pH. ARs, on the other hand, appear not to significantly affect the physical characteristics of emulsions. Therefore, this study demonstrates how wheat bran can be a valuable and innovative source of natural antioxidants to be used in emulsified foods, which can from one hand extend their shelf-life simultaneously upholding their physical properties, and from the other hand can allow to valorize the milling wheat co-product contributing to the reduction of the food waste.

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Chapter 5

EFFECT OF ALKYLRESORCINOLS EXTRACTED FROM WHEAT BRAN ON THE OXIDATIVE STABILITY OF BEEF PATTIES DURING COLD STORAGE

5.1 Abstract

Due to their antioxidant activity, alkylresorcinols (ARs) extracted from by-products could represent promising natural and sustainable antioxidants for the food industry, especially when considering meat products. The purpose of this study was to test the ability of ARs extracted from wheat bran to increase the shelf-life of beef patties during cold storage (4 °C) for 9 days. ARs were solvent extracted and isolated through solid-phase extraction (SPE). GC/MS analysis revealed fifteen alk(en)ylresorcinols (C₁₇–C₂₅), which demonstrated good radical scavenging (200.70 ± 1.33 μmolTE/g extract) and metal chelating (1.38 ± 0.30 mgEDTAE/g extract) activities. The effect of two ARs concentrations (0.01% and 0.02%) was compared to that of sodium ascorbate (0.01% and 0.10%; synthetic antioxidant) on color and oxidative stability of beef patties. Determination of CIELAB values L^* , a^* , b^* , chroma, and hue was conducted to assess the color stability, while lipid hydroperoxides, thiobarbituric acid reactive substances (TBARS) and volatile organic compounds (VOCs) were measured for the determination of oxidative stability. Patties treated with ARs resulted oxidatively more stable compared to both concentrations of sodium ascorbate and the control, with significantly lower contents of hydroperoxides, TBARS, and VOCs (hexanal, 1-hexanol, and 1-octen-3-ol) throughout the experiment ($p < 0.001$). However, no effects on the color stability were observed ($p > 0.05$). Since 0.01% of ARs was equally or more effective than sodium ascorbate at 0.10%, these results carry important implications for the food industry, which could reduce ten times the amount of antioxidants to be used in formulations and replace a synthetic antioxidant with a natural one.

5.2 Introduction

In today's dynamism of the food industry, the search for natural antioxidants has become imperative, driven by consumers' dual demands for clean labels and sustainability considerations. Central to this quest is lipid oxidation, a ubiquitous and potentially harmful chemical reaction within food products, where it represents a pervasive threat to both quality and safety. Formation of off-flavors and potentially toxic oxidized chemical species (i.e., peroxides, aldehydes, ketones), development of rancidity, loss of liposoluble micronutrients and essential fatty acids are all consequences of lipid oxidation (Berton-Carabin & Villeneuve, 2023). Uncontrolled lipid oxidation in foods can have detrimental effects. First, the potential formation of oxidation products can compromise the nutritional value and the sensory attributes of foods, significantly reducing their shelf-life. Second, consumption of oxidized lipids has been associated with adverse health effects caused by the onset of oxidative stress, which leads to the development of chronic diseases (Vieira et al., 2017). In meat and meat products, the simultaneous presence of high amounts of PUFAs in membrane phospholipids, pro-oxidants, salt (NaCl), and free molecular oxygen, and the lack of antioxidants greatly affect oxidation of lipids (Aminzare et al., 2019). Moreover, especially in fresh ground meat preparations, lipid oxidation is promoted by the disruption of cell membranes during processing (e.g., grinding), which exposes unsaturated lipids to high concentrations of heme pigments (about 12% in ground meat), cytochromes, non-heme iron, ferritin, and heavy transition metals (Aminzare et al., 2019; Barbieri et al., 2021). Consumers can perceive oxidation in meat through the changes that occur in its color, texture, flavor, and odor, thus affecting its acceptability (Domínguez et al., 2019). Oxidation reactions can be catalyzed by heme pigments. The free radicals generated by the oxidized lipids can in turn decompose the heme pigments, causing discoloration of the meat. Specifically, when oxymyoglobin (the red pigment typical of fresh meat, where iron is present as Fe^{2+}) comes into contact with a radical, it is oxidized to metmyoglobin, the brownish-gray pigment typical of cooked meat, which contains iron in the form of Fe^{3+} (Greene & Price, 1975). The presence

and concentration of endogenous pro-oxidants like hemoglobin and myoglobin and their accessibility to muscle membrane phospholipids play a key role in lipid oxidation of meat (Wu et al., 2022). In fact, membrane phospholipids are considered the most relevant substrate for the onset of lipid oxidation reactions in muscle foods, being particularly vulnerable due to their large surface area and degrees of unsaturation (Wu et al., 2022).

Principal co-product of the dry-milling of wheat, wheat bran constitutes 10–14% of the grain, with an annual production equal to 150 million tons, generally intended to be discarded, used for animal feed, or burned (Chen et al., 2023). Wheat bran, however, is rich in interesting phytochemical compounds with high nutritional value, such as proteins, lipids, β -glucans, vitamins, and polyphenols (Cardenia et al., 2018; Chen et al., 2023). Straddling the chemical classes of lipids and polyphenols, wheat bran is also rich in alkylresorcinols (ARs), a homologues series of amphiphilic molecules composed of a single phenolic ring and an odd-numbered alk(en)yl side chain at position 5 of the ring, whose length in wheat varies mostly from 17 to 25 carbon atoms (Andersson et al., 2008; Chen et al., 2023; Kowalska et al., 2022). ARs are especially characteristic of cereals, including rye, wheat, and barley, and are thought to be biomarkers of whole grain wheat and rye diet and intake, since they are present almost exclusively in the outer layers of the kernels (Zabolotneva et al., 2022). Antimicrobial, antioxidant, anticancer, neuroprotective, antilipidemic, and gut microbiota regulation properties have been described for ARs (Zabolotneva et al., 2022). However, contradictory results are reported on the antioxidant activity of ARs. In fact, some works report them to be weak hydrogen/electron donor due to the hydroxyl group at *meta* position, failing to inhibit 1,1-diphenyl-2-picrylhydrazyl radical (DPPH \bullet) as effectively as α -tocopherol or other homologues, and failing to hamper the formation of lipid hydroperoxides compared to the negative control in bulk oil (Kamal-Eldin et al., 2000; Korycińska et al., 2009). In other research, ARs retarded the formation of hydroperoxides and thiobarbituric acid reactive substances (TBARs) in vegetable oils greater than butylated hydroxytoluene (BHT) or inhibited the autoxidation of unsaturated fatty acids (linoleic and linolenic acids) (Nienartowicz & Kozubek, 1993; Winata & Lorenz, 1996). More recently, it was

found that the entire spectrum of ARs extracted from rye bran was able to inhibit lipid oxidation of oil-in-water emulsions, where they were found to be mostly partitioned in the oil phase (Elder et al., 2019); and even the individual homologs were effective in delaying the formation of both primary and secondary oxidation products in oil-in water emulsions and bulk oil (Elder et al., 2021). In Chapter 4 it was evidenced how oxidation reactions in oil-in-water emulsions are largely slowed down by the presence of ARs as related to their concentration, especially in an acidic environment (pH 3.5). The efficacy of the ARs has been tested also in low-moisture crackers by Elder and colleagues (Elder et al., 2022), with the surprising result that they inhibited lipid oxidation (measured in terms of hydroperoxides and headspace hexanal) more effectively than α -tocopherol and BHT. Interestingly, it has been claimed by numerous studies that ARs can protect cellular lipid components from oxidative processes, being particularly effective in inhibit Fe^{2+} -induced peroxidation of fatty acids and phospholipids in liposomal bilayer membrane (Kamal-Eldin et al., 2000; Korycińska et al., 2009; Nienartowicz & Kozubek, 1993). Antioxidant effects have been observed for ARs in phospholipid bilayers, and this has been related to their ability to incorporate into membranes thanks to their hydrophobicity. Here they would competitively inhibit the abstraction of hydrogens in the bis-allylic position of phospholipids, thus being more a matter of physicochemical properties and positioning rather than conventional antioxidant properties (Kamal-Eldin et al., 2000).

Based on these considerations, ARs could therefore be considered as effective natural antioxidants for protection of foods, especially muscle foods, thus meeting the need for both a clean and sustainable label. To the best of our knowledge, no research has been conducted on the antioxidant capabilities of ARs to counteract lipid oxidation in meat. Therefore, this work aimed at evaluating the ability of ARs extracted from wheat bran to extend the shelf-life of beef patties by delaying lipid oxidation. Specifically, two concentrations (0.01% and 0.02%) were tested, and the results were compared with those obtained in beef patties added with sodium ascorbate at 0.01% (equal to the lowest concentration used for ARs) and 0.10% (the concentration typically used in commercial patties).

5.3 Materials and Methods

5.3.1 Materials and Chemicals

Wheat bran (*Triticum aestivum* L.) was kindly provided by Molini Bongiovanni S.p.A. (Cambiano, Italy) fresh from the milling process and immediately freeze-dried (Lio 5P, 5Pascal, Italy), ground and sieved at <500 μm . Wheat bran was then stored under vacuum at 4 °C until subsequent analyses. Ground beef meat (*Longissimus dorsi* muscle) was purchased in a local market (Turin, Italy).

Ethyl acetate, *n*-hexane, propan-2-ol, 1-butanol, diethyl ether, chloroform, ethanol absolute anhydrous, iso-octane, and hydrochloridric acid (37%) were purchased from Carlo Erba (Milan, Italy), while methyl tert-butyl ether (MTBE) were from VWR (Milan, Italy). 5 α -Cholestan-3 β -ol, anhydrous pyridine, N,O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA:TMCS, 99:1, *v/v*), ferrozine, ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), Tween® 20, ammonium thiocyanate, barium chloride dihydrate, ferrous sulfate heptahydrate, sodium phosphate monobasic dihydrate, sodium phosphate dibasic anhydrous, thiobarbituric acid, 1,1,3,3-tetramethoxypropane, potassium chloride, 2,6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene, BHT) and trichloroacetic acid were bought from Merck (Darmstadt, Germany). To prepare double distilled water a Milli-Q filter system (Millipore, Milan, Italy) was used. N° 1 filter papers were purchased from Whatman (Maidstone, England). Solid-phase extraction (SPE) cartridges (Strata NH₂, 55 μm , 70 Å, 1 g/6 mL; Strata SI-1 Silica, 55 μm , 70 Å, 500 mg/3 mL) were purchased from Phenomenex (Torrence, CA, USA).

5.3.2 Alkylresorcinols Extraction and Purification

Alkylresorcinols (ARs) were extracted from wheat bran by solvent extraction and purified by solid-phase extraction (SPE) according to a procedure adapted from Esche et al. (Esche et al., 2012).

Briefly, 40 mL of a *n*-hexane:chloroform (1:1; v/v) were added to 10 g of bran and left under stirring at room temperature for 1 h in darkness. After filtration, the solvent was removed with a rotary evaporator (Rotavapor, R-210, Buchi, Switzerland) at 37 °C, and the residue (lipid fraction) dissolved in 10 mL of *n*-hexane. Afterwards, Strata NH₂-SPE cartridge (55 μm, 70 Å, 1 g/6 mL, Phenomenex, Germany) were conditioned with *n*-hexane (10 mL) and then loaded with 1 mL of the oil solution. Different solvents were then eluted to remove major interferents: *n*-hexane:diethyl ether (98:2, v/v; 10 mL), *n*-hexane:ethyl acetate (96:4, v/v; 20 mL), and *n*-hexane:ethyl acetate (5:95, v/v; 10 mL). ARs were finally eluted with 10 mL of *n*-hexane:ethyl acetate (5:95, v/v) and 5 mL of MTBE, which were combined, brought to dryness under vacuum, and dissolved in 500 μL of *n*-hexane:propan-2-ol (3:2; v/v) (Fraction1). A second SPE (Strata SI-1 Silica, 55 μm, 70 Å, 500 mg/3 mL) was then activated with 3 mL of *n*-hexane and the fraction 1 loaded. Five milliliters of *n*-hexane:diethyl ether (8:2; v/v) and 4 mL of *n*-hexane:diethyl ether (1:1; v/v) were eluted to remove the remaining interferents and discarded. Three milliliters of methanol were then eluted to collect the ARs; solvent was then evaporated and the ARs reconstituted with 1.5 mL of *n*-hexane:propan-2-ol (3:2; v/v).

5.3.3 Characterization and Quantification of ARs by GC/MS and GC/FID

The purified ARs were recognized by GC/MS (Shimadzu QP2010 Plus, Kyoto, Japan) and quantified by GC/FID according to the procedure described in Chapter 4. Before injection, the ARs were silylated as described in Chapter 4 using 200 μL of BSTFA:TMCS (99:1; v/v) and 100 μL of pyridine at 40 °C and resuspended in 100 μL of *n*-hexane. Before silylation, 80 μL of 5- α -cholestan-3- β -ol (1.005 mg/mL; internal standard) were added to the sample for quantification. Ions were acquired in scan mode and mass spectra were compared to those present in the NIST08s (National Institute of Standards and Technology, Gaithersburg, USA) library to identify the compounds. ARs were quantified by injecting the same sample in a GC/FID (GC-2010, Shimadzu, Kyoto, Japan) under the same analytical conditions.

5.3.4 Assessment of the Antioxidant Properties of ARs

Metal chelating ability (ferrous ion-chelating spectrophotometric assay) and radical scavenging activity (DPPH• spectrophotometric assay) were assessed to define the antioxidant properties of the ARs extracted and purified from wheat bran, according to the procedures described elsewhere (Cantele et al., 2023). For the tests, the extracted ARs were resuspended in ethanol, and the absorbance was measured using a BioTek Synergy HT spectrophotometric multi-detection 96-well microplate reader (BioTek Instruments, Milan, Italy). To quantify the radical scavenging and ferrous ion-chelating activities, calibration curves were constructed using Trolox (25–30 μM ; $y = 0.1941x + 0.0483$; $R^2 = 0.9999$) and EDTA (0.001–0.1 mg/mL ; $y = 8546.5x - 0.4859$; $R^2 = 0.9947$), respectively. Micromoles of Trolox equivalents per gram of ARs extract ($\mu\text{molTE/g}$) and milligrams of EDTA equivalents per gram of ARs extract (mgEDTAE/g) were used to express the results.

5.3.5 Preparation of Beef Patties and Storage Conditions

To deliver the ARs into the patties, 1.0% oil-in-water (O/W) emulsions were prepared and subsequently mixed with the ground meat. To prepare the emulsions, 1.0% medium chain triglycerides (MCT), 0.1% Tween 20, and 10 mM phosphate buffer solution were used. ARs were dissolved in the oil by stirring at room temperature after the removal of the solvent. The emulsion was roughly homogenized using an IKA T25 digital Ultra-Turrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 2 min at 20,000 rpm, and then refined with a VCX750 ultrasonic processor (Sonics & Materials, Inc., Newton, CT, USA) equipped with a ½ inch diameter tip probe operating at an amplitude of 50%. During sonication, emulsions were kept in an ice bath to prevent temperature to exceed 30 °C. To evaluate the effect of the ARs on their shelf-life, five different patties were prepared: 1) C_NEG, negative control, with emulsion without addition of antioxidants; 2) AR01, with emulsion added with ARs at 0.01%; 3) AR02, with emulsion added with ARs at 0.02%; 4) C_POS+, positive control, with emulsion added with sodium ascorbate at 0.01%; 5) C_POS++, positive control,

emulsion added with sodium ascorbate at 0.10%. Each type of emulsion was added to ground beef at a ratio of 200 mL/kg meat and thoroughly mixed to obtain a homogeneous mixture. Patties were made with 30 ± 1.0 g of the latter and placed in sealed food contact containers in polyethylene terephthalate (PET) on a poly coated (PE paper) paper foil at 4 ± 0.1 °C in darkness. Three independent batches were made for each type of sample ($n=3$).

5.3.6 Color Measurement

The color of the patties was monitored during the experiment to assess if the ARs affects this parameter and its stability during the storage. A CM-2600 spectrophotometer (Konica Minolta Sensing Inc., Osaka, Japan) equipped with an aperture mask of 8 mm, D65 illuminant, and 10° of Standard Observer was used to determine the CIELAB color space indices (L^* , a^* , b^*). Hue angle (h_{ab} , expressed as degrees) and chroma (C^*_{ab}) were calculated according to Cantele et al. (Cantele et al., 2022). Six measurements were carried out for each independent replicate ($n=18$), and the specular component excluded (SCE) values were considered.

5.3.7 Lipid Extraction

A modified version of the method described by Folch and colleagues (Folch et al., 1957) has been used to extract the lipid fraction from the beef patties. Twenty grams of each sample were added to 200 mL of a *n*-hexane:propan-2-ol solution (3:2; *v/v*) with 0.02% BHT and homogenized for 3 min at 20,000 rpm by a T-25 Ultra-Turrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) in a screw capped glass bottle. The bottle was then kept at 60 °C for 15 min, added with 100 mL of *n*-hexane, and mixed again by Ultra-Turrax® for 2 min at the same speed. The solid residue was eliminated by filtration with Buchner funnel and filter paper (Whatman No. 1). The filtrate was added with 100 mL of a 1 M KCl solution, mixed thoroughly, and left overnight at 4 °C to obtain a phase separation. Through a separating funnel the lower phase (organic phase) was collected, added with

sodium sulfate, left in the fridge for 2 h, filtrated, and dried with a rotary evaporator (Rotavapor, R-210, Buchi, Flawil, Switzerland) at 40 °C.

5.3.8 Determination of Lipid Hydroperoxides

The method proposed by Shantha and Decker (Shantha & Decker, 1994) was modified according to Funaro et al. (Funaro et al., 2014) to assess the lipid hydroperoxides content in the beef patties. Twenty milligrams of the extracted lipids were added with 9.8 mL of a chloroform:methanol solution (2:1; v/v) and 50 µL of both ammonium thiocyanate and FeCl₂ solutions, vortexed for 30 sec, and incubated in darkness for 5 min. Absorbance was read at 500 nm with a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) and quantification was achieved by building a calibration curve in the range 1–40 µg/mL of Fe³⁺. Results were expressed as milliequivalents of oxygen per kilogram of fat (meqO₂/kg fat).

5.3.9 Determination of Thiobarbituric Acid Reactive Substances (TBARs)

Thiobarbituric acid reactive substances (TBARs) were measured according to Tarladgis et al. (Tarladgis et al., 1960). Briefly, 2 g of each sample were added to 8 mL of phosphate buffer aqueous solution (pH 7) in a 50 mL Sovirel bottle with a screw cap and homogenized by Ultra-Turrax® for 30 sec at 21,500 rpm. Two milliliters of a trichloroacetic solution (30%; w/v) were added and the mixture homogenized for 30 sec at 17,500 rpm. After filtration (Whatman paper filter No. 41), 5 mL of the filtrate were collected in a 20 mL Sovirel tube and 5 mL of a TBA aqueous solution (0.02 M) were added. The capped tubes were put at 90 °C in a water bath for 20 min, then at 4 °C for 30 min and finally sonicated for 5 min. The absorbance of complex was read at 530 nm and by a calibration curve build with 1,1,3,3-tetramethoxypropane in the range of 0.03–2.26 µg/mL the quantification of malondialdehyde (MDA) was carried out; the results were expressed as milligrams of malondialdehyde (MDA) per kilogram of meat (mgMDA/kg meat).

5.3.10 Determination of Volatile Organic Compounds (VOCs)

Volatile organic compounds (VOCs) were determined through headspace solid-phase microextraction (HS-SPME) and GC/MS (QP-2010 Plus, Shimadzu, Kyoto, Japan) according to Botta et al. (Botta et al., 2022) with slight modifications as follows. Samples (2.0 g) were accurately weighed in 20 mL headspace vials equipped with aluminum caps sealed with a PTFE silicone septum. To isolate the VOCs, a DVB/CAR/PDMS coated fused silica fiber (10 mm length, d_f 50/30 μm ; Supelco, Bellafonte, PA, USA) was exposed to the headspace for 30 min at 40 °C after having equilibrated the vials at the same temperature for 15 min. A Combi Pal system (CTC Analytics AG, Zwingen, Switzerland) was used. The fiber was subsequently desorbed into the GC/MS inlet at 260 °C for 5 min with a split ratio of 1:25. Chromatographic separation of the volatiles was achieved with a RTX-5 fused silica capillary column (20 m \times 0.10 mm \times 0.10 μm ; Restek, Bellafonte, PA, USA) held for 4 min at 40 °C, then rose at 220 °C (4 °C/min) and to 260 °C (20 °C/min). Final temperature was maintained for 3 min. Helium was used as carrier gas with a constant linear velocity of 34.7 cm/sec. Ion source temperature was 200 °C, whilst interface temperature was 230 °C. Ions were acquired in scan mode in the range 33–350 m/z with a scan speed of 1111 amu/sec (0.30 scan/sec). Compounds were identified comparing their spectra with those reported in the NIST08s (National Institute of Standards and Technology, Gaithersburg) library. Environmental contamination was avoided by blank injections of the fibers and vials. Results were expressed as percentage of each class of volatiles on the total area of identified compounds.

5.3.11 Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to ascertain any statistical difference between the samples with a confidence level equal to 95%. Data variability was explored by computing principal component analysis (PCA) on all datasets. All the statistical analyses were performed with IBM SPSS statistical software (version 28; IBM, Chicago, IL, USA).

5.4 Results and Discussion

5.4.1 Characterization of the ARs in the Purified Extract

The purified extract of wheat bran was analyzed using GC/MS, and results were consistent with the ones obtained in our previous work (ARs in oil-in-water emulsions, Chapter 34). The analysis revealed the presence of fifteen distinct resorcinolic lipids: five saturated alkylresorcinols (C17:0, C19:0, C21:0, C23:0, and C25:0), and ten monounsaturated alkenylresorcinols (C17:1, C19:1, C21:1, C23:1, C25:1 two isomers each). Compared to the saturated ARs, monounsaturated ARs were found at lower concentrations (<10% of the total ARs). The characteristic peaks at 268 m/z and 281 m/z , produced by the McLafferty rearrangement during the fragmentation of ARs, were used as qualifier ions for all the alk(en)ylresorcinols. Additionally, C17:0, C19:0, C21:0, C23:0, and C25:0 (silylated molecules) were identified using molecular ions at m/z 492, 520, 548, 576, and 604 respectively. The molecular ions of the corresponding alkenylresorcinols were identified at m/z 490, 518, 546, 574, and 602, respectively. The same composition was found in the work presented in Chapter 4, and in the aleurone fractions of wheat bran by other researchers (Martín-García et al., 2021), whose spectra are consistent with the ones obtained in the present study. In line with the literature (Andersson et al., 2008) and with the results obtained in the previous work (Chapter 4), the percentage composition of ARs was $0.48\% \pm 0.03$ (C₁₅); $7.60\% \pm 0.04$ (C₁₇); $42.38\% \pm 0.15$ (C₁₉); $39.43\% \pm 0.26$ (C₂₁); $7.57\% \pm 0.13$ (C₂₃); $2.10\% \pm 0.20$ (C₂₅); $0.44\% \pm 0.02$ (C₂₇). While the overall content of ARs varies significantly even within the same species, due to genetic, environmental, and agronomic factors, the distribution of the homologs within species is relatively consistent. For example, the C17:0/C21:0 ratio for durum wheat is equal to ~ 0.01 , ~ 0.1 for common and spelt wheat, and ~ 1.0 for rye (Landberg et al., 2014). In this study, C17:0/C21:0 ratio was found to be 0.19, in agreement with the literature and consistent with the previous study. An extract with an 85.91% purity was obtained. The contaminants were stearic acid, palmitic acid, glycerol monostearate, and 1-monopalmitine.

Free radicals and bivalent cations like iron and copper represent the main initiators of lipid oxidation reactions, thus constituting the main concern in lipid-containing foods. Therefore, the purified extract of ARs was tested to evaluate if it was either free radical scavenger and/or metal ion chelator and thus able to retard lipid oxidation. The antiradical activity of the extract was found to be $200.70 \pm 1.33 \mu\text{molTE/g}$ extract, and the ability to chelate ferrous ions was $1.38 \pm 0.30 \text{ mgEDTAE/g}$ extract. Results were again consistent with those obtained in our previous study (Chapter 4). Looking for similarities in the literature for results of *in vitro* antioxidant tests is never easy or obvious due to differences in operating conditions make the comparison often misleading. Indeed, conflicting results are reported in the literature, with works reporting little or no ability of alkylresorcinols to donate a hydrogen/electron (Kamal-Eldin et al., 2000; Korycińska et al., 2009), while others claiming good antiradical activity (Elder et al., 2019, 2022; Nienartowicz & Kozubek, 1993; Winata & Lorenz, 1996). It is true, however, that the chemical structure of resorcinol does not in itself suggest high activity, as the hydroxyl group occurs at *meta* position, which is notoriously deleterious to antioxidant activity (Kamal-Eldin et al., 2000). Despite this, we found good antiradical activity, comparable, if not greater, than that found in the literature for wheat bran extracts rich in ARs, or individual homologues (Gunenc et al., 2013). Finding a positive response in the chelating activity assay was unexpected, given the lack of a catechol or galloyl group on the benzene ring of the resorcinolic lipids. However, this phenomenon has already been found for other molecules (such as ferulic acid), and chelating activity has been attributed to other ways of interacting with iron (Sørensen et al., 2014).

5.4.2 Influence of ARs on the Visual Appearance of the Patties

Color is the first sensory attribute that consumers perceive in meat products and ultimately determines their propensity to purchase or rejection (Tomasevic et al., 2021). It is determined by the amount of myoglobin present at the surface of the meat and its chemical state, the pH-dependent structure of the muscle, and the marbling (Beriaín et al., 2009). Many factors throughout the meat production chain (farmers, processors, consumers) affect meat color. Pre-harvest factors include type

of diet, feeding types, energy intakes, withdrawal times, pre-slaughter stress and slaughter techniques, while post-harvest factors include storage temperature, packaging conditions such as materials and atmosphere compositions, and lipid and protein oxidation (Ijaz et al., 2020; Tomasevic et al., 2021). One of the solutions that can be adopted to keep the color of the meat stable over time is the addition of sodium ascorbate. Ascorbate is a reducing agent that not only inactivates free radicals inhibiting lipid oxidation, but also maintains the reduced ferrous state of myoglobin when added to fresh muscle foods (Lee et al., 2005). Although the European Food Safety Authority (EFSA) established that there is no genotoxicity and safety concerns for this antioxidant and that it can be used as food additives (EFSA Panel on Food additives and Nutrient Sources added to Food (ANS), 2015), sodium ascorbate is, however, synthetic, and thereby disliked by consumers. As they demonstrated to possess antioxidant properties, ARs could also influence the oxidative state of myoglobin and thus on the color stability of the patties.

The effect of storage as well as of the addition of sodium ascorbate and ARs and their concentrations on the development of the CIELAB parameters L^* , a^* , b^* , Hue, and Chroma in the beef patties is shown in **Table 5.1**. Color was measured in all samples immediately after the preparation of the patties, and no differences were recorded between them for L^* , a^* , and Hue ($p > 0.05$), while b^* and Chroma were affected by the presence of sodium ascorbate. In fact, on day 0 color coordinate b^* was higher in C_POS++ ($p < 0.05$), and Chroma was higher in both C_POS+ and C_POS++ ($p < 0.05$). As Chroma defines the color intensity (saturation), the presence of sodium ascorbate in the meat during sample preparation led to less greyish patties. Afterwards, only C_POS++ was able to keep these parameters more stable over time. In contrast, C_POS+ and both concentrations of ARs had no effect on color compared to C_NEG ($p > 0.05$). During the storage, color coordinate L^* significantly increased in all samples except for C_POS++, where it remained constant except that on day 6 it dropped and then rose again on the last day to return to the initial value. Indeed, on each sampling day L^* in C_POS++ was consistently lower than in all other samples ($p < 0.05$), which, in contrast, showed no significant difference from each other ($p > 0.05$). Thus, all

samples except C_POS++ became lighter during storage. It is likely that the increase in L^* values during meat storage is caused by the enzymatic breakdown of proteins, which weakens protein structure and increases light dispersion (Ijaz et al., 2020). Other authors have reported the same increase in lightness (Ahn & Nam, 2004; Ebrahimi et al., 2022; Ijaz et al., 2020; Soriano et al., 2018). Regarding a^* , in all samples this value decreased significantly during the experiment ($p < 0.01$). However, again C_POS++ showed the mildest (from 10.58 ± 0.58 to 8.61 ± 0.96) and most gradual decrease (at day 3 it was still no different from day 0; $p > 0.05$). For each measuring day, C_POS++ showed a significantly higher a^* value than the other treatments, implying the patties were redder. The decrease in a^* value is due to the oxidation of myoglobin (Bekhit et al., 2003; Jacob, 2020). In fact, when exposed to oxygen, the free binding site of heme of myoglobin (deoxymyoglobin, purple-red color) covalently binds molecular oxygen to form oxymyoglobin, which is characterized by a bright cherry-red color. These two redox states of myoglobin have the heme iron in the ferrous state (reduced). When the heme iron of oxymyoglobin is oxidized to the ferric state, metmyoglobin is formed, which is brown in color (Bekhit et al., 2003; Jacob, 2020). Interestingly, in C_NEG, C_POS+, AR01, and AR02 a^* value increased on the last day. This phenomenon can perhaps be explained by the reduction of metmyoglobin to deoxymyoglobin, which was then rapidly converted to oxymyoglobin (Bekhit et al., 2003; Jeremiah & Gibson, 2001; Seideman et al., 1984). That reduction can result from two circumstances. First, brown metmyoglobin can be reduced to purple reduced myoglobin by the activity of some enzymes that are present in the muscle, often called metmyoglobin reducing activity or MRA. The process involves the donation of an electron by the reducing enzymes to the iron molecule that goes from the ferric state (Fe^{3+}) to the ferrous state (Fe^{2+}) (Seideman et al., 1984). Second, it can derive from bacterial growth. Sufficiently high concentrations of bacteria may impede oxygen from getting to the surface of the meat, allowing the brown metmyoglobin to be rapidly reduced enzymatically to purple myoglobin (Seideman et al., 1984). In addition, certain types of bacteria can produce reducing equivalents that might reduce metmyoglobin back to deoxymyoglobin as well (Seideman et al., 1984). The colorimetric value b^* also changed

throughout the cold storage in all samples except for C_POS++. Specifically, yellowness increased from 14.18–15.11 to 16.10–16.68. Those results are consistent with Ebrahimi et al. (Ebrahimi et al., 2022). The saturation of all samples except for C_POS++ again decreased significantly until day 6 and increased on the last day of the experiment. It has already been reported that a diminish in Chroma is correlated to brown color (Beraiain et al., 2009). Finally, during the cold storage the Hue angle increased significantly ($p < 0.001$) in all patties including C_POS++, going from 54.18–55.58 to 61.67–67.07. However, C_POS++ reached the lowest Hue value at the end of the storage ($p < 0.001$). Lee and colleagues (Lee et al., 2005) noticed the same increment in antioxidant treated ground beef patties during a 6-day experiment at 4 °C, as well as Ijaz and co-workers (Ijaz et al., 2020) on a 7-day storage of beef.

Table 5.1. CIELAB values L^* , a^* , b^* , and calculated parameters of Chroma, and Hue angle in beef meat patties stored at 4 °C in darkness for 9 days. C_NEG, negative control; C_POS+, patties added with 0.01% sodium ascorbate; C_POS++, patties added with 0.10% sodium ascorbate; AR01, patties added with 0.01% ARs; AR02, patties added with 0.02% ARs. Results of ANOVA and Tukey's post hoc test are also reported.

| | C_NEG | C_POS+ | C_POS++ | AR01 | AR02 | Sig. |
|------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|------|
| <i>L*</i> | | | | | | |
| Day 0 | 52.05 ± 1.23 ^b | 52.48 ± 0.91 ^b | 52.44 ± 0.90 ^{ab} | 51.68 ± 0.97 ^c | 51.23 ± 0.91 ^b | N.S. |
| 3 | 57.25 ± 1.21 ^{aA} | 57.9 ± 1.63 ^{aA} | 53.66 ± 0.96 ^{aB} | 58.45 ± 0.82 ^{aA} | 56.89 ± 1.73 ^{aA} | *** |
| 6 | 56.43 ± 3.11 ^{aA} | 56.44 ± 1.69 ^{aA} | 49.98 ± 2.00 ^{bB} | 54.53 ± 1.15 ^{bA} | 55.55 ± 1.19 ^{aA} | *** |
| 9 | 57.10 ± 2.30 ^{aA} | 56.47 ± 1.63 ^{aA} | 54.07 ± 1.97 ^{aB} | 56.53 ± 1.68 ^{aA} | 56.88 ± 1.16 ^{aA} | * |
| Sig. | *** | *** | ** | *** | *** | |
| <i>a*</i> | | | | | | |
| Day 0 | 10.29 ± 0.61 ^a | 10.46 ± 0.48 ^a | 10.58 ± 0.58 ^a | 10.24 ± 0.48 ^a | 10.28 ± 0.47 ^a | N.S. |
| 3 | 5.45 ± 0.51 ^{cC} | 7.12 ± 0.95 ^{bB} | 10.05 ± 1.43 ^{abA} | 6.02 ± 0.92 ^{bcBC} | 5.56 ± 0.32 ^{cC} | *** |
| 6 | 5.47 ± 0.49 ^{cB} | 5.63 ± 0.63 ^{cB} | 8.66 ± 0.69 ^{bA} | 5.45 ± 0.36 ^{cB} | 5.91 ± 0.46 ^{cB} | *** |
| 9 | 7.06 ± 0.52 ^{bB} | 7.06 ± 0.41 ^{bB} | 8.61 ± 0.96 ^{bA} | 6.87 ± 0.73 ^{bB} | 7.46 ± 0.32 ^{bB} | *** |
| Sig. | *** | *** | ** | *** | *** | |
| <i>b*</i> | | | | | | |
| Day 0 | 14.25 ± 0.31 ^{bB} | 15.11 ± 0.84 ^{abB} | 15.44 ± 0.79 ^A | 14.38 ± 0.82 ^{bB} | 14.18 ± 0.59 ^{bB} | * |
| 3 | 14.39 ± 0.43 ^{bB} | 15.52 ± 0.93 ^{abA} | 15.61 ± 0.55 ^A | 14.92 ± 0.61 ^{bAB} | 14.10 ± 0.58 ^{bB} | ** |
| 6 | 14.80 ± 0.85 ^b | 14.29 ± 0.94 ^b | 14.64 ± 1.85 | 13.95 ± 0.85 ^b | 14.65 ± 0.29 ^b | N.S. |
| 9 | 16.10 ± 0.68 ^a | 16.11 ± 0.56 ^a | 15.96 ± 1.29 | 16.23 ± 1.01 ^a | 16.68 ± 0.36 ^a | N.S. |
| Sig. | *** | * | n.s. | *** | *** | |
| <i>Chroma</i> | | | | | | |
| Day 0 | 17.58 ± 0.39 ^{aB} | 18.38 ± 0.91 ^{aA} | 18.72 ± 0.92 ^A | 17.67 ± 0.52 ^{aB} | 17.61 ± 0.43 ^{aB} | * |
| 3 | 15.40 ± 0.54 ^{bc} | 17.08 ± 1.20 ^{bAB} | 18.59 ± 1.24 ^A | 16.10 ± 0.87 ^{bBC} | 15.16 ± 0.60 ^{bc} | *** |
| 6 | 15.78 ± 0.85 ^{bAB} | 15.36 ± 1.07 ^{cAB} | 17.03 ± 1.81 ^A | 14.98 ± 0.89 ^{bB} | 15.81 ± 0.38 ^{bAB} | * |
| 9 | 17.58 ± 0.79 ^a | 17.58 ± 0.65 ^{ab} | 18.14 ± 1.56 | 17.63 ± 1.12 ^a | 16.93 ± 1.35 ^a | N.S. |
| Sig. | *** | *** | n.s. | *** | *** | |
| <i>Hue angle</i> | | | | | | |
| Day 0 | 54.18 ± 1.85 ^c | 55.25 ± 1.08 ^c | 55.58 ± 1.11 ^c | 54.50 ± 2.62 ^b | 54.25 ± 2.03 ^c | N.S. |
| 3 | 69.26 ± 1.44 ^{aA} | 65.42 ± 1.96 ^{bB} | 57.36 ± 2.74 ^{bcC} | 68.11 ± 2.37 ^{aAB} | 68.47 ± 1.13 ^{aAB} | *** |
| 6 | 69.69 ± 1.80 ^{aA} | 68.51 ± 1.41 ^{aA} | 59.21 ± 2.83 ^{abB} | 68.65 ± 0.99 ^{aA} | 68.02 ± 1.37 ^{abA} | *** |
| 9 | 66.34 ± 1.09 ^{bA} | 66.31 ± 0.86 ^{abA} | 61.67 ± 1.33 ^{aB} | 67.07 ± 1.79 ^{aA} | 65.91 ± 0.73 ^{bA} | *** |
| Sig. | *** | *** | *** | *** | *** | |

Results are reported as mean ± standard deviation ($n=3$). Different lowercase letters indicate means statistically different at $p < 0.05$ between different days of storage within the same treatment. Different uppercase letters indicate means statistically different at $p < 0.05$ within the same day between the different treatments. Sig. = Significance; n.s. = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

5.4.3 Influence of ARs on the Oxidative Stability of the Patties

One of the main issues that deteriorates meat and meat products decreasing their shelf-life is lipid oxidation, due to their high fat content and low water activity (Aminzare et al., 2019). However, meat represents a great source of essential nutrients such as fat, proteins, and mineral (Aminzare et al., 2019). Antioxidants can be used to delay lipid oxidation and extend their shelf-life because they neutralize free radicals retarding lipid oxidation, prevent the development of off-flavors, and enhances color stability (Aminzare et al., 2019). Potential toxic effects on human health have been evidenced for synthetic antioxidants such as butylated hydroxytoluene (BHT), representing a risk for consumers (Eskandani et al., 2014; Oikawa et al., 1998). Additionally, as consumers' awareness of these risks has grown recently, natural bioactive substances are replacing synthetic antioxidants (Aminzare et al., 2019; Cantele et al., 2020). For these reasons, ARs from wheat bran can represent an effective alternative to synthetic antioxidants. To test whether ARs were able to improve the oxidative stability of the beef patties, both primary oxidation products and secondary oxidation products were monitored during the cold storage. Primary oxidation products were intended as lipid hydroperoxides, while thiobarbituric acid reactive substances (TBARs) and volatile organic compounds (VOCs) were evaluated as secondary oxidation products.

5.4.3.1 Primary Oxidation Products

The assessment of lipid hydroperoxides, whose results are presented in **Figure 5.1** (and detailed in **Table A5.1** of the Appendix) revealed distinctive trends among the tested samples, but in all cases a significant increase was observed ($p < 0.001$).

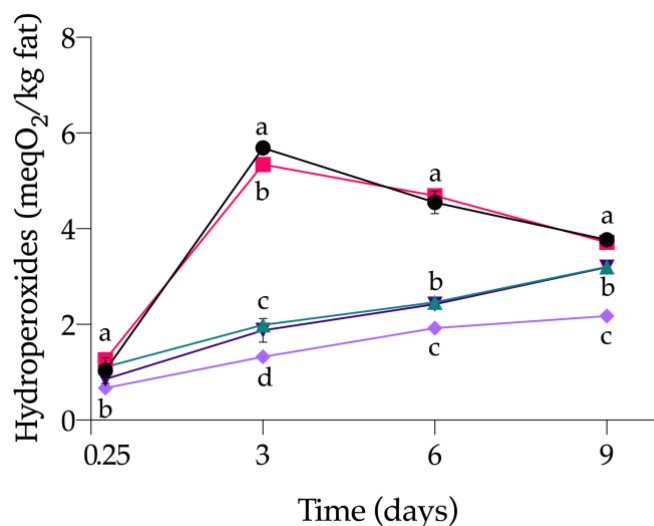


Figure 5.1. Effect of different treatments on the formation of lipid hydroperoxides (meqO₂/kg fat) in beef patties during their storage at 4 °C for 9 days. (●) C_NEG; (■) C_POS+; (▲) C_POS++; (▼) AR01; (◆) AR02. Data are presented as mean ± standard deviation (*n*=3). Some error bars are within data points. Different letters indicate means between the different treatments within the same day significantly different at *p* < 0.05.

During the storage, C_NEG and C_POS+ displayed higher hydroperoxides content than the other samples (*p* < 0.001), indicating a significant influence on oxidation. By 4.5 hours, the level of hydroperoxides was already higher in C_POS+ (1.26 ± 0.08 meqO₂/kg meat) than in the other samples, while AR02 was characterized by the lowest amount (0.67 ± 0.10 meqO₂/kg meat). Higher values in C_NEG and C_POS+ at the first measurement (day 0.25 – 4.5 hours) indicate a more marked triggering of oxidation reactions by lipid contact with oxygen than in the other treatments, where the presence of antioxidants evidently hampered them. As storage progressed, C_POS+ revealed the same trend shown by C_NEG, with overlapping peroxide values (*p* > 0.05). In fact, on day 3 of storage, the maximum level of hydroperoxides observed in the experiment was reached (5.69 ± 0.03 meqO₂/kg meat and 5.35 ± 0.05 meqO₂/kg meat for C_NEG and C_POS+, respectively). A decrease in their values was then observed at day 6 and day 9, corresponding to the degradation of these compounds to form the secondary oxidation compounds, including aldehydes and ketones. Completely different behavior was manifested by the samples added with ARs and 0.20% sodium ascorbate. In fact, significantly lower values were recorded throughout the experiment (*p* < 0.001),

reaching their highest contents only on the last day. Most interestingly, the content of hydroperoxides in AR01 and C_POS++ was the same throughout the experiment ($p > 0.05$), highlighting how ARs at 0.01% retarded the formation of these compounds as effectively as sodium ascorbate at the concentration normally used by industries. The incorporation of ARs at 0.02% surpassed the efficacy of C_POS++ ($p < 0.001$), manifesting a remarkable reduction in hydroperoxide formation during the entire storage. These findings highlight the potential of ARs, particularly at 0.02%, as effective agents in inhibiting the development of primary oxidation products in beef patties.

5.4.3.2 Secondary Oxidation Products

As oxidation progresses, the hydroperoxides decompose, generating secondary oxidation products, one of the main ones being the aldehyde 1,3-propanedial, also called malondialdehyde or MDA, formed from polyunsaturated fatty acids (Ebrahimi et al., 2022). Therefore, the measurement of TBARs provides insight into the evolution of the oxidative stability of a food, more accurately than the measurement of hydroperoxides. Indeed, synthesis and decomposition of hydroperoxides occur simultaneously, and the result is that the measured hydroperoxides are only a snapshot of the compounds that are only present at that time in the matrix, giving no information about the hydroperoxides that have already decomposed (Li et al., 2020). This leads to false judgements and unreliable results.

In the present study, a time-dependent increase in TBARs was observed in all samples until day 6, while on day 9 values decreased (**Figure 5.2**, and detailed in **Table A5.1** of the Appendix). In fact, initial values of 0.24–0.68 mg MDA/kg meat (range of all samples) reached at day 6 0.50–1.07 mg MDA/kg meat and then decreased to 0.42–0.65 mg MDA/kg meat at day 9.

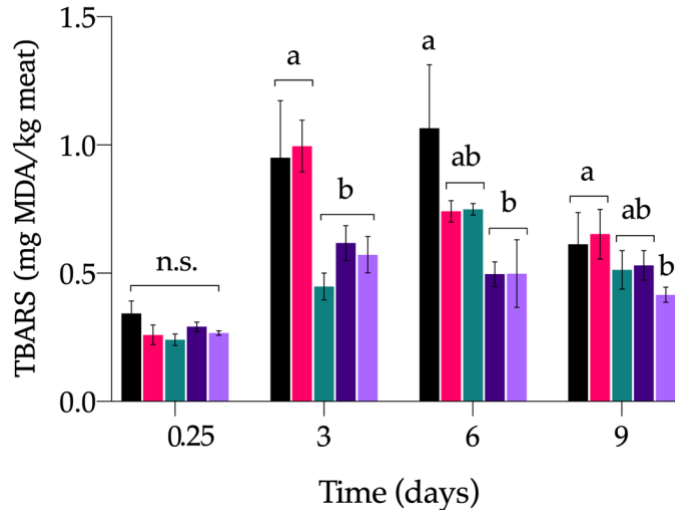


Figure 5.2. Effect of different treatments on TBARs (mg MDA/kg meat) in beef patties during their storage at 4 °C for 9 days. (●) C_NEG; (■) C_POS+; (▲) C_POS++; (▼) AR01; (◆) AR02. Data are presented as mean ± standard deviation ($n=3$). Some error bars are within data points.

C_NEG showed the highest values, which were double or triple of the other treatments, except for C_POS+, that again showed a similar trend to the negative control ($p > 0.05$). Both concentrations of ARs successfully restrained the formation of TBARs formation with respect to C_NEG and demonstrated to be more effective than C_POS+ and C_POS++. Already after 4.5 hours TBARs in C_NEG (0.34 ± 0.05 mg MDA/kg meat) were slightly higher than the other samples (range 0.24-0.29 mg MDA/kg meat), although it was not statistically significant ($p > 0.05$). After 3 days of storage, the content of TBARs in C_NEG and C_POS+ dramatically increased, reaching 0.95 ± 0.22 mg MDA/kg meat and 1.00 ± 0.10 mg MDA/kg meat, respectively, while in the other treatments TBARs doubled with respect to the first measurement (4.5 h). At day 6, TBARs continued to increase in C_NEG and C_POS++, while in AR01 and AR02 they remained constant. On the last day, AR02 showed even a lower content than C_POS++ ($p < 0.001$). It is also worth mentioning that in the negative control and sodium ascorbate-treated patties, TBARs first showed an increase, with a maximum peak at day 3 for C_POS+ and day 6 for C_NEG and C_POS++, followed by a decrease thereafter ($p < 0.001$). In contrast, in AR01 and AR02 only the increase but not the decrease was observed. The decrease in the values of TBARs during storage has already been recorded in other

studies and may be associated with decomposition into tertiary oxidation products or from their reaction with proteins (Ebrahimi et al., 2022; Gumus et al., 2017). In addition, since the decomposition of the primary oxidation products gives rise to the secondary products, it is not surprising to see the trend of TBARs follow that observed for hydroperoxides. Indeed, for C_NEG and C_POS+, a peak in hydroperoxides is seen at day 3, which is then reflected in increased formation of thiobarbituric acid-reactive compounds, and then declines in both parameters. This overlap in the trend of the two oxidative parameters indicates that the formation of TBARs occurred very rapidly following the formation of hydroperoxides. In samples AR01 and AR02, given the limited formation of hydroperoxides, there was no increase in the value of TBARs. Surprisingly, however, although there was no increase in hydroperoxides in C_POS++, the increase and decrease in TBARs was observed. This might be explained by the high instability of the primary oxidation products. Considering that 1 mg MDA/kg meat is reported as the threshold above which rancid flavor begins to develop in meat (Barbieri et al., 2021), we can conclude that ARs successfully exerted a protective effect in inhibit the formation of rancid off-flavors in beef patties during cold storage, especially AR02, which never exceeded 0.57 ± 0.07 mg MDA/kg meat. Similar results were found by other researchers (Šulniūtė et al., 2016) in beef meat hamburgers added with rye and wheat acetone and methanol extracts and stored in modified atmosphere packaging (30% CO₂, 70% O₂) at 4 °C in darkness. The authors concluded that adding bran extracts to meat products could be an appealing approach to improve their oxidative stability while enrich them with functional ingredients with health benefits (Šulniūtė et al., 2016). Natural extracts from byproducts have also shown greater efficacy than ascorbic acid in limiting the formation of TBARs in other studies, such as in that of Andrés and colleagues where aqueous extracts of red grape and olive byproducts were investigated (Andrés et al., 2017), or in that of Soriano and colleagues where oak wood extracts almost completely prevented the formation of TBARs in comparison with the positive control (Soriano et al., 2018).

Lipid oxidation is a complex process that can result in a wide variety of compounds from different pathways. Secondary oxidation products include different volatile organic compounds

(VOCs) with different molecular structures such as aldehydes, ketones, alcohols, carboxylic acids, and hydrocarbons (Domínguez et al., 2019). In the present study, along with TBARs, VOCs were also monitored. Twenty-six different volatile organic molecules were identified in the samples, including: carbon dioxide, ethanol, carbon disulfide, pentane, acetic acid, 3-methyl-butanal, 2-methyl-butanal, 1-penten-3-ol, 3-pentanone, acetoin, 3-methyl-1-butanol, 1-pentanol, 2,3-butanediol, 2,4-dimethyl-hexane, hexanal, 2-octene, 1-hexanol, 1-octen-3-ol, 3-methyl-butanoic acid, 2-heptanone, 2,2-dimethyl-3-heptanone, 2,2,4,6,6-pentamethyl-heptane, 2,3-octanedione, 2-pentyl-furan, 2,6-dimethyl-undecane, dodecane. Only three, among the principal markers of lipid oxidation, exhibited clear and significant differences between the treatments and are thus reported: hexanal, 1-hexanol, and 1-octen-3-ol (**Figure 5.3**) (Casaburi et al., 2015; Domínguez et al., 2019; Kosowska et al., 2017). The type of volatile compound generated during oxidation depends on the fatty acid composition of the meat. The acid composition depends greatly among other things on the species and breed of the animal, the type of muscle, and the diet (Domínguez et al., 2019). It is well known that hexanal constitute one of the main aldehydes generated during lipid oxidation, and it is mainly derived from the decomposition of alkyl hydroperoxides of linolenic acid (Vega & Brewer, 1994). The aldehydes produced can be further decomposed in other molecules by other oxidation products, or by enzymes present in the system. It has been reported that hexanal might be reduced to hexanol by intracellular enzymes like alcohol dehydrogenases (Hashem et al., 2022). Alcohols can also be formed during lipid oxidation by the reaction of alkyl radicals with hydroxyl radicals (Frankel, 2014). From the results obtained for VOCs, the effect of ARs on the development of these secondary products appears evident. In fact, when ARs were present in the patties at both concentrations (0.01% and 0.02%), a significantly lower value respect to the control was recorded for the three molecules from the first sampling ($p < 0.05$). Specifically, hexanal was found already 4.5 hours after the preparation of the patties in C_NEG. In C_POS+ hexanal was found from day 3, with an increasing trend over time, and in C_POS++ only from day 6. AR01, on the other hand, was characterized by the presence of hexanal only from day 6 on, and with significantly lower values than in all other

samples ($p < 0.05$). Interestingly, AR02 never experienced the development of this aldehyde throughout the experiment. High levels of 1-hexanol were observed in both C_NEG and C_POS+ throughout the storage, with an increasing trend over time. In the other treatments, 1-hexanol was found in low amounts, but still significantly lower in AR02 than in AR01 and C_POS++ ($p < 0.05$). However, it might be pointed out that 1-hexanol was found in samples where hexanal was not detected, which suggests that it might also arise from other pathways than the enzymatic and oxidative degradation of hexanal. In any case, the effectiveness of ARs, especially at the highest concentration, to restrain its development is confirmed and aligns with the results obtained for the other oxidative parameters. The 1-octen-3-ol can derive from the intermolecular cyclic rearrangement and cleavage of linoleic acid esters (Hoffmann, 1962), or from the oxidative breakdown of linoleic acid catalyzed by lipoxygenases (Assaf et al., 1997). Again, patties treated with ARs showed significantly lower levels of this alcohol than the other samples, including C_POS++. For all the VOCs reported, a decrease in their levels was found on the last day in all the samples, and this could be due to advanced oxidation reactions, leading to their degradation and transformation into other compounds (Frankel, 2014), or to the interaction of these compounds with other compounds in the matrix, or even to their metabolism by microorganisms. These results confirm the effectiveness of ARs in reducing oxidation already found for hydroperoxides and TBARs.

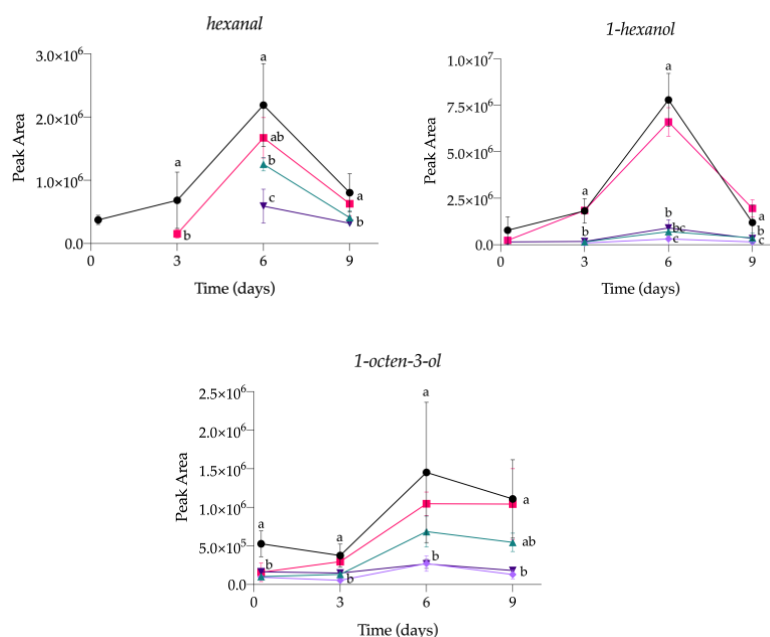


Figure 5.3. Effect of different treatments on the formation of volatile organic compounds (VOCs) from lipid oxidation in beef patties during their storage at 4 °C for 9 days. (●) C_NEG; (■) C_POS+; (▲) C_POS++; (▼) AR01; (◆) AR02. Data are presented as mean \pm standard deviation ($n=3$). Some error bars are within data points. Different letters indicate means within each day (comparison between different treatments) significantly different at $p < 0.05$. Where no letters are displayed, no significant differences were detected ($p > 0.05$).

5.4.4 Principal Component Analysis (PCA)

To highlight the relationships between the primary identified factors and the samples of beef burgers, multivariate analysis was employed. According to PCA, the first two principal components (PC1, 42.97%; PC2, 21.74%) accounted for 64.71% of the variation in the whole. As reported in **Figure 5.4** a clear separation of VOCs from the other measured parameters was observed. Again, components used to describe the color were also completely separated, where a^* was mainly correlated to PC1 and inversely correlated to b^* , which resulted mostly correlated to PC2. On the other hand, Chroma was inversely correlated with VOCs. In addition, L^* and Hue angle were positively correlated with PV and TBARs, since as peroxides increased significant changes in the lightness of the color were observed. Moreover, as reported in Biplot (**Figure 5.4**) different clusters are recognized. As expected, all fresh samples (T0) were clustered together and no effect due to the presence of ARs was observed; moreover, samples were mainly characterized by a^* component, since

the red color was equally present. TBARs and PV as well as L^* largely categorized negative control, ARs at low concentration and positive control at low dosage sample at day 3 of storage (T3). The T6 control samples, both negative and positive at low dosage, were classified by VOCs, which confirms that after 6 days of storage peroxides were degraded leading to an increase in the formation of VOCs. At T9 of storage, only control at a high dosage of sodium ascorbate (C_POS++) together with samples containing ARs were clustered together. These results confirm that ARs could be considered a valuable and natural alternative to ascorbic acid in the formulation of beef burgers, contributing to reducing the environmental impact linked to the use of synthetic preservatives.

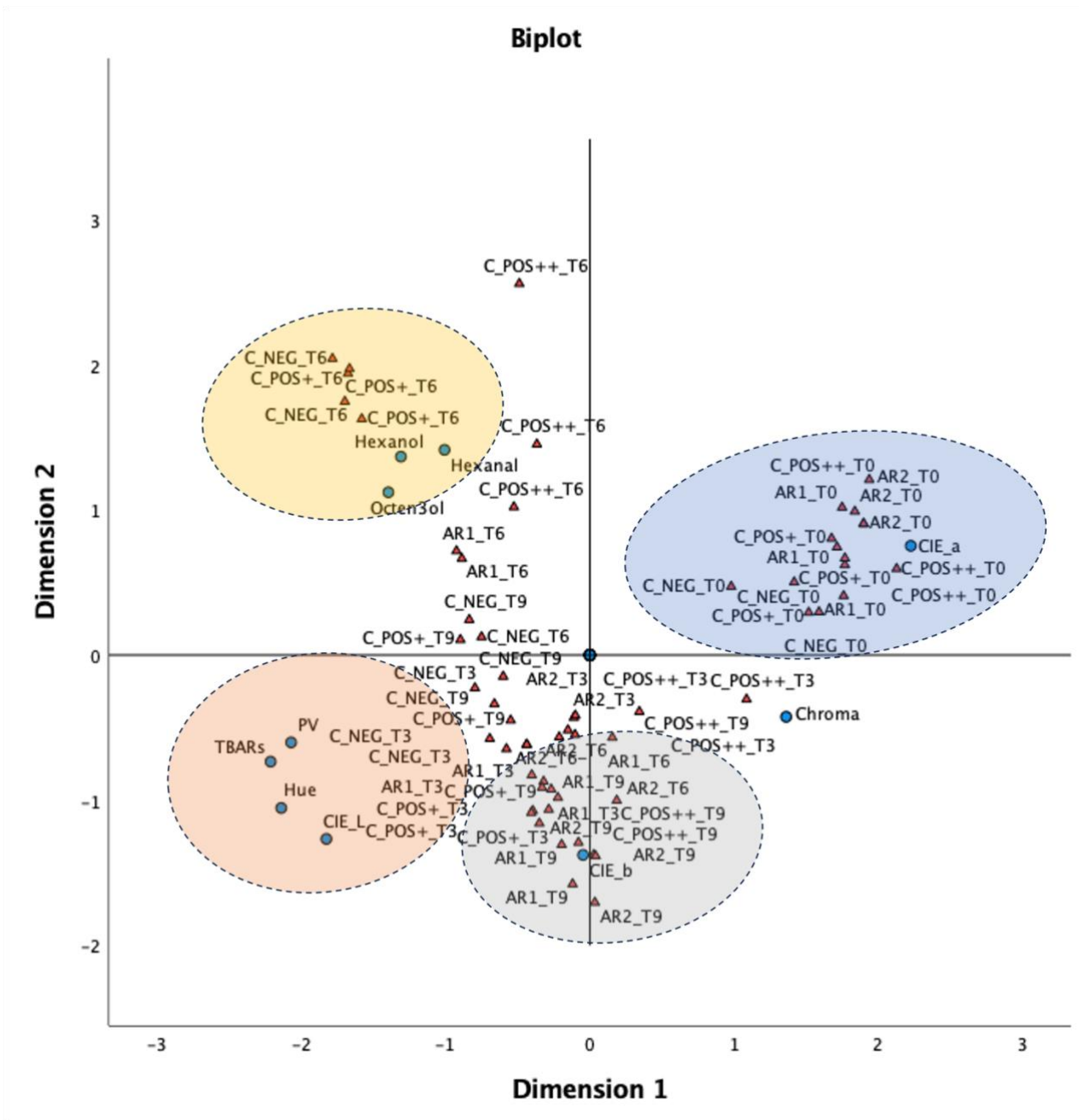


Figure 5.4. Principal component analysis (PCA) Bi-plot of hydroperoxides (PV), thiobarbituric acid reactives (TBARs), volatile organic compounds (VOCs), and CIELAB color values (L^* , a^* , b^* , Chroma, and Hue angle) (blue circles) and treatment time (red triangles) in beef patties (C_NEG, C_POS+, C_POS++, AR01, AR02).

5.5 Conclusions

In the present study the efficacy of natural alkylresorcinols (ARs), isolated from wheat bran, on the increase of beef patties shelf life during cold storage as alternatives to chemical preservatives was investigated. Sodium ascorbate at the lowest concentration (0.01%) was found to be completely ineffective in preserving beef patties from oxidative degradation. In contrast, the superiority of ARs over even the highest concentration of sodium ascorbate (0.10%) was consistent throughout all stages of lipid oxidation, proving to successfully restrain the formation of primary oxidation products, such as hydroperoxides, and their decomposition into secondary products, such as aldehydes. Principal component analysis confirmed these results. The fact that the lowest tested concentration of ARs (0.01%) proved to be equal to or even more effective than sodium ascorbate at 0.10% brings important implications for the food industry. Indeed, not only enables the replacement of a synthetic antioxidant with a natural one making the label “cleaner”, but it also cuts the doses normally used by tenfold, with an important economic implication. However, sodium ascorbate at 0.10% promoted greater color stability protecting the pigments responsible for the red color from degradation, whilst ARs proved ineffective at both concentrations. These results collectively suggest that ARs, at both tested concentrations, exhibit a noteworthy efficacy to delay lipid oxidation in beef patties, ultimately positioning them as promising natural and sustainable candidates for enhancing the oxidative stability of meat and meat products. In light of these results, it will be necessary to investigate not only the microbiological aspect, verifying whether ARs are able to preserve the microbiological safety of the patties and protect them from spoilage, but also their sensory characteristics. In fact, it has already been verified that they do not affect the appearance of the meat before cooking, but it will also be important to assess whether they can alter its flavor and taste after cooking.

5.6 Appendix

Table A5.1. Effect of the different treatments on lipid hydroperoxides (meqO₂/kg fat) and thiobarbituric acid reactives (TBARs) (mgMDA/kg meat) contents in beef patties stored at 4 °C for 9 days. C_NEG, negative control; C_POS+, patties added with 0.01% sodium ascorbate; C_POS++, patties added with 0.10% sodium ascorbate; AR01, patties added with 0.01% ARs; AR02, patties added with 0.02% ARs. Results of ANOVA and Tukey's post hoc test are also reported.

| | C_NEG | C_POS+ | C_POS++ | AR01 | AR02 | Sig. |
|-----------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|------|
| <i>Hydroperoxides</i> | | | | | | |
| Day 0.25 | 1.04 ± 0.27 ^{dAB} | 1.26 ± 0.08 ^{dA} | 1.11 ± 0.22 ^{dAB} | 0.86 ± 0.11 ^{dAB} | 0.67 ± 0.10 ^{dB} | * |
| 3 | 5.69 ± 0.03 ^{aA} | 5.35 ± 0.05 ^{aB} | 1.99 ± 0.02 ^{cC} | 1.88 ± 0.25 ^{cC} | 1.32 ± 0.01 ^{cD} | *** |
| 6 | 4.55 ± 0.23 ^{bA} | 4.69 ± 0.06 ^{bA} | 2.46 ± 0.02 ^{bB} | 2.42 ± 0.06 ^{bB} | 1.92 ± 0.08 ^{bC} | *** |
| 9 | 3.77 ± 0.02 ^{cA} | 3.72 ± 0.06 ^{cA} | 3.20 ± 0.03 ^{aB} | 3.20 ± 0.07 ^{aB} | 2.18 ± 0.03 ^{aC} | *** |
| Sig. | *** | *** | *** | *** | *** | |
| <i>TBARs</i> | | | | | | |
| Day 0.25 | 0.34 ± 0.05 ^b | 0.26 ± 0.04 ^c | 0.24 ± 0.02 ^c | 0.29 ± 0.02 ^b | 0.27 ± 0.01 ^b | N.S. |
| 3 | 0.95 ± 0.22 ^{abA} | 1.00 ± 0.10 ^{aA} | 0.45 ± 0.05 ^{bB} | 0.62 ± 0.07 ^{aB} | 0.57 ± 0.07 ^{aB} | *** |
| 6 | 1.07 ± 0.25 ^{aA} | 0.74 ± 0.04 ^{bAB} | 0.75 ± 0.02 ^{aAB} | 0.50 ± 0.05 ^{aB} | 0.50 ± 0.13 ^{aB} | ** |
| 9 | 0.61 ± 0.12 ^{bAB} | 0.65 ± 0.10 ^{bA} | 0.51 ± 0.08 ^{bAB} | 0.53 ± 0.06 ^{aAB} | 0.42 ± 0.03 ^{abB} | * |
| Sig. | * | *** | *** | *** | ** | |

Results are reported as mean ± standard deviation ($n=3$). Different lowercase letters indicate means statistically different at $p < 0.05$ between different days of storage within the same treatment. Different uppercase letters indicate means statistically different at $p < 0.05$ within the same day between the different treatments. Sig. = Significance; n.s. = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

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Chapter 6

CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 Conclusive remarks

This PhD project is framed in a historical period characterized by the consumer's desire to eat healthy foods rich in beneficial bioactive compounds, such as bioactive lipids and phenolic compounds, due to their beneficial properties for human health. However, bioactive lipids are easily oxidized, while the hydrophilicity of phenolic compounds limits their presence in lipid-based products. Phenolipids, the result of the conjugation of lipid and phenolic molecules, can be an effective solution to deliver these bioactive compounds to the human body through food.

The aim of this PhD project was therefore to study different phenolipids, both natural and synthetic, as a strategy to enhance the oxidative stability of foods, by protecting from oxidation their bioactive compounds. Natural phenolipids were extracted from wheat bran and were found to be alkylresorcinols (conjugation of resorcinol and C₁₅-C₂₇ alkyl chains). On the other hand, two alkylferulates were synthesized, namely butyl ferulate (ferulic acid esterified with butanol), and octyl ferulate (ferulic acid esterified with octanol). The phenolipids were all preliminarily tested in model systems consisting of oil-in-water emulsions. To study their behavior and real efficacy, the effect of their concentration in the system and the effect of the pH of the medium (a neutral pH and an acidic pH) were investigated. The natural phenolipids were then also tested in a real food product, namely beef patties. Both natural and synthetic phenolipids have been shown to be powerful antioxidants, capable of significantly extending the oxidation resistance of both model systems and real food. Three common aspects between synthetic and natural phenolipids were highlighted:

- i. In the range of concentrations tested, all phenolipids showed an increase in antioxidant activity as their concentration in the system increased. This was not predictable or obvious, since antioxidants can show pro-oxidant effects when present in certain

concentrations. In all cases, already the lowest concentration was extremely effective in improving the oxidative stability of the system.

- ii. Both synthetic and natural phenolipids proved most effective in retarding oxidation under acidic conditions of the system. This aspect constitutes the real strength of this PhD project, because, to the best of our knowledge, no one had previously related the efficacy of phenolipids and the pH of the surrounding environment. The increased activity of phenolipids at acidic pH could be due to the fact that antioxidant activities such as neutralization of free radicals (and particularly electron transfer) are highly dependent on pH, among other things. This aspect, however, needs further investigation, as it could also be a matter of the electrical charge of the phenolipid. Another hypothesis could also be the different partitioning of the antioxidant in the system.
- iii. Both synthetic and natural phenolipids have revealed ten or more times the effectiveness of antioxidants normally and currently used by food industries today. This constitutes another salient aspect of this research, as using these compounds would enable industries to tremendously reduce the amount of antioxidant to be added to foods, with important economic implications.

6.2 Future perspectives

In the first instance, it will be important to evaluate other molecular structures of synthetic phenolipids. Currently, experiments on four other alkylferulates, specifically hexyl ferulate, decyl ferulate, dodecyl ferulate, and octadecyl ferulate in 1.0% oil-in-water emulsions, are in progress and nearly completed. In addition, octadecyl ferulate with one unsaturation in the chain and dodecyl ferulate have also been successfully synthesized. It will also be important to evaluate a different synthesis approach, i.e., conjugation of the fatty acid on the hydroxyl group of ferulic acid instead of

the carboxylic group, to test the different impact it might have on its antioxidant activity. It will also be important to evaluate their efficacy in real food systems as done for alkylresorcinols.

As for natural phenolipids, it would be appropriate to test the efficacy of other compounds extracted from other plant matrices.

Finally, it will be important and necessary to assess the fate of these molecules once subjected to the digestive process, to verify their survival under the conditions encountered during the three stages of digestion (oral, gastric, and intestinal), for possible implications for human health. A further step will then be to evaluate their interaction in cellular systems.

LIST OF PUBLICATIONS

- Bonciolini, A.; Cantele, C.; Piochi, M.; Di Lecce, G.; Risso, D.; Cardenia, V. (2024). New Insights on Phytocannabinoids role in Antioxidant Activity of Hemp Inflorescences Extract in High-Oleic Sunflower Oil. *LWT*, 115807, <https://doi.org/10.1016/j.lwt.2024.115807>
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